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ORIGINAL PAPER

Tissue, ontogenic and sex-related differences in δ^{13}C and δ^{15}N values of the oceanic squid *Todarodes filippovae* (Cephalopoda: Ommastrephidae)

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Abstract Stable isotopes are increasingly used in the study of trophic interactions of many aquatic animals and most recently cephalopods. To evaluate the application of the method to squids, it is important to assess isotopic differences among and within consumer tissues that may confound the resolution of ecological relationships. Inter- and intra-tissue isotopic variation was examined in 55 individuals of the oceanic squid *Todarodes filippovae* that were collected at the beginning of April 2000 in the southwestern Indian Ocean (between 44°S, 76°E, and Saint Paul and Amsterdam islands, 38°S, 78°E). Delipidated soft tissues (mantle, arm, buccal mass, gill and reproductive organs) showed small δ^{13}C and δ^{15}N differences, which were probably tissue-specific. A lower carbon value was observed in the digestive gland as a consequence of incomplete lipid removal. Hard tissues, such as beaks and gladii, had lower ^{15}N values than soft tissues, which can be explained by the presence of chitin, a ^{15}N-depleted molecule. Females ($n = 38$) and males ($n = 17$) had identical δ^{13}C values, but females showed higher δ^{15}N values than males. The difference was size-related rather than sex-related, however, as females were generally larger than males. A comparison of similar-sized females and males produced identical nitrogen values. These data suggest dietary shifts from lower to higher trophic levels during growth, because δ^{15}N values of large *T. filippovae* were much higher than that of small specimens. As expected, nitrogen values of lower beaks and gladii of large squids increased from the oldest to the most recently formed region, reflecting the progressive growth of chitinized tissues in parallel with dietary changes. Sequential sampling along the growth increments of squid beaks and gladii can likely be used to produce a chronological record of dietary information throughout an individual's history.

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Introduction

Oceanic squids are a key, but poorly studied group of marine organisms. Their role in the pelagic ecosystem of the world's oceans is underlined by their importance in the diet of marine predators (Clarke 1996; Cherel and Klages 1998). In turn, their diet has been determined in a few species, with mesopelagic fish being the main prey of larger squids (Rodhouse and Nigmatullin 1996). To date, stomach content analysis has been the principal way of studying cephalopod diets, although this is hampered by the maceration of food during feeding. The hard parts of prey, which are usually needed for identification, are often rejected causing potential errors in estimating diet composition. Cephalopods may also feed unnaturally in the presence of sampling gear (Rodhouse and Nigmatullin 1996). Stomach contents furthermore represent only the most recent feeding, with no indication of long-term feeding habits (Jackson

et al. 2007a). Dietary studies using the direct method of stomach content analysis are best complemented by new indirect methods, such as signature lipid and stable isotope analysis, which are not hindered by the lack of temporal integration (Jackson et al. 2007a).

Stable isotope ratios of carbon ($^{13}C/^{12}C$, $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$, $\delta^{15}N$) in proteins have been used extensively to trace pathways of organic matter among organisms, because the stable isotopic signature of a consumer reflects that of its diet (McCutchan et al. 2003). Consumer tissues are stepwise-enriched in ^{15}N relative to their food and consequently $\delta^{15}N$ measurements serve as indicators of a consumer's trophic position (McCutchan et al. 2003; Vanderklift and Ponsard 2003). In contrast, $\delta^{13}C$ values vary little (no or slight increase) along the food chain and are mainly used to determine primary sources in a trophic network (McCutchan et al. 2003). In the marine environment, $\delta^{13}C$ values indicate the lower- versus higher-latitude plankton, and inshore versus offshore, or pelagic versus benthic contribution to food intake (Hobson et al. 1994; Cherel and Hobson 2007).

Scant information is available on the stable isotopic signatures of cephalopods, but a few preliminary works have underlined their potential for investigating migration and trophic level (Takai et al. 2000; Ruiz-Cooley et al. 2004, 2006; Cherel and Hobson 2005). Likewise, very few studies have validated the application of the method to cephalopods. Controlled feeding experiments showed that stable isotope signatures of muscle of the squid *Lolliguncula brevis* distinguished between individuals feeding on different diets (Stowasser et al. 2006). They also quantified differential diet-tissue isotopic discrimination factors for muscle tissues and chitinized beaks of the cuttlefish *Sepia officinalis* (Hobson and Cherel 2006). These controlled feeding experiments were conducted with neritic species. Unfortunately, oceanic squids have not been raised in captivity, thus precluding accurate measurements of their tissue-related protein turn-over rates and discrimination factors.

Controlled feeding experiments on various organisms have shown that $\delta^{13}C$ and $\delta^{15}N$ values are tissue dependent, with differences being related to tissue metabolic characteristics, e.g. relative turnover rates, types of biochemical reactions, and biochemical composition and components (DeNiro and Epstein 1978, 1981). It is thus important to assess isotopic differences among and within tissues that may confound the resolution of ecological relationships. Within this context, our objective was to evaluate the application of the stable isotope method to oceanic squid by quantifying inter- and intra-tissue variation in $\delta^{13}C$ and $\delta^{15}N$ values within the same individuals. We investigated the isotopic signature of various soft tissues (muscle tissues, gill, reproductive organs, and digestive gland) and hard tissues (beaks and gladii) of the ommastrephid *Todarodes filippovae*. We expected little variation in $\delta^{13}C$ and $\delta^{15}N$ values among soft tissues, because they do not have large accumulations of structural compounds, including proteins with unusual amino acid composition. On the other hand, the main constituents of the beak and gladius are structural molecules, i.e. chitin and proteins (Hunt and Nixon 1981; Miserez et al. 2007), with chitin being depleted in ^{15}N, but not in ^{13}C, when compared to proteins and diet (DeNiro and Epstein 1978, 1981; Schimmelmann and DeNiro 1986; Webb et al. 1998). We therefore expected lower $\delta^{15}N$ values, but not $\delta^{13}C$ values, in hard tissues than in soft tissues of *T. filippovae*.

Beaks and gladii grow continuously by accretion of new molecules of chitin and proteins with no turnover after synthesis. Consequently, they retain molecules laid down throughout the lives of cephalopods, and their $\delta^{13}C$ and $\delta^{15}N$ values integrate the feeding ecology of the individual over its lifetime. Indeed, various parts of the lower beak have different isotopic signatures, with the tip of the wings, the most recently synthesized part, being a good index of the feeding ecology just prior to its capture (Cherel and Hobson 2005; Hobson and Cherel 2006). To the best of our knowledge, no information is available on the stable isotopic signature of gladii. Due to the continuous and linear growth of the gladius with dorsal mantle length (ML) (Bizikov 1991; Perez et al. 1996), we hypothesized that various parts of the gladius, like the beak, retain different $\delta^{13}C$ and $\delta^{15}N$ values, with the anterior tip, which includes the most recent increments, having an isotopic signature close to that of the lower beak wing.

Material and methods

Individuals of *T. filippovae* were collected from the research vessel *La Curieuse* during a cruise undertaken in the southwestern Indian Ocean at the beginning of April 2000. Squids were taken from 22 pelagic trawls (13–380 m) during five consecutive nights in oceanic waters between 44°S, 76°E, and Saint Paul and Amsterdam islands (38°S, 78°E). The area was located in the vicinity of the Subtropical Front and in southern subtropical waters (Park et al. 1993), where the holotype of *T. filippovae* was initially collected (Adam 1974). Overall, *T. filippovae* has a circumpolar distribution in the Southern Ocean, from south of the Polar Front to north of the Subtropical Front (Rodhouse 1998).

All the collected individuals ($n = 60$) were frozen on board and returned to the laboratory in France for analysis. Three specimens were badly damaged and two of them were fixed in formaldehyde, thus precluding their use in the present work. Once defrosted, data collected for each remaining individual ($n = 55$) included body mass, ML,

lower rostral length (LRL), and sex. Each specimen was also assigned a maturity stage (after Lipinski 1979), based on the size and colour of the reproductive organs. Squids were dissected and samples of the main tissues were subsequently stored in 70% ethanol. Ethanol preservation increases $\delta^{13}C$ values due to partial removal of isotopically lighter lipids present in the samples (Kaehler and Pakhomov 2001). This was not a problem here because we subsequently removed lipids from all soft tissues (see below). Beaks and buccal masses were sampled from very small ommastrephids (identified as belonging to *T. filippovae*) that were found in stomach contents of rockhopper penguins sampled at Amsterdam Island in October–November 1996.

Isotopic analysis was performed on muscle tissues (mantle and buccal mass) of all 55 specimens (Table 1), on a larger number of tissues, including reproductive organs, of 10 females and 10 males (Table 2), and on various parts of chitinized tissues (lower beak and gladius) of six other females (Table 3). Lower beaks, like gladii, grow by accretion of new molecules of proteins and chitin. It is secreted by an epithelium which adds new material on the posterior side of the wings and on the lateral side of the lateral wall, forming successive "growth rings" (Clarke 1965). We sampled three different parts of lower beaks for isotopic analyses, (1) the tip of the wing (most recently formed region), (2) a piece of the lateral wall (which integrates over a longer time period), and (3) the rostrum (the central and oldest part of the beak, which accretes material throughout the individual's life). In ommastrephids, the gladius consists of two morphological parts: anteriorly, an elongated proostracum plate and, posteriorly, a much shorter terminal spoon-shaped conus. Longitudinal growth occurs mainly by addition of chitinous material to the anterior side of the proostracum, but also, to a lesser extent, by addition of newly formed material to the posterior side of the conus. This means that the gladius grows asymmetrically both anteriorward and posteriorward at different rates. Growth increments of the proostracum plate are clearly distinguishable in its anterior part; they gradually become fainter as the gladius narrows posteriorly and cannot be distinguished near the conus region (Bizikov 1991; Perez et al. 1996). For isotopic analysis, we cut three small parts of the gladius, (1) the anterior tip (most recently formed region of the proostracum), (2) the narrower (older) part of the proostracum plate, near the conus, and (3) the posterior tip of the gladius (posterior tip of the conus that, due to lower conus growth rates, integrate a recent, but longer time period than the anterior tip of the proostracum).

Before isotopic analysis, tissue samples were dried in an oven at +50°C. They were ground to a fine powder and lipids of soft tissues were removed using cyclohexane (Kojadinovic et al. 2008). The extent of lipid extraction was subsequently checked through the C/N mass ratio of the samples, because C/N in soft tissues is positively related to their lipid content (Post et al. 2007). In hard tissues that contain no fat, C/N mass ratio was used to compare their relative contents in chitin, which has a higher C/N ratio than proteins (see below). Relative abundance of ^{13}C and ^{15}N were determined with an elemental analyzer connected on-line to an isotope-ratio mass spectrometer (Micromass, Manchester, UK). Results are presented in the usual δ notation relative to PDB belemnite and atmospheric N_2 (Air) for $\delta^{13}C$ and $\delta^{15}N$, respectively. Replicate measurements of internal laboratory standards (acetanilide) indicate measurement errors <0.15‰ and <0.20‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Data were statistically analysed using SYSTAT 9.0 for WINDOWS. Values are means ± SD.

Table 1 Body size and stable isotope values of *Todarodes filippovae*. Values are means ± SD

	All specimens (n = 55)	Females (n = 38)	Males (n = 17)	Statistics (t tests)	
				t	p
Mantle length (mm)	346 ± 47	364 ± 42	305 ± 28	5.30	<0.0001
Lower rostral length (mm)	8.6 ± 1.1	9.0 ± 1.0	7.6 ± 0.7	5.61	<0.0001
Body mass (g)	845 ± 377	991 ± 354	520 ± 160	5.24	<0.0001
Mantle					
$\delta^{13}C$ (‰)	−18.2 ± 0.4	−18.2 ± 0.3	−18.3 ± 0.6	0.82	0.417
$\delta^{15}N$ (‰)	11.4 ± 1.0	11.6 ± 0.9	10.7 ± 1.1	3.30	0.002
C/N mass ratio	3.31 ± 0.07	3.31 ± 0.05	3.30 ± 0.10	0.66	0.515
Buccal mass					
$\delta^{13}C$ (‰)	−18.3 ± 0.3	−18.3 ± 0.3	−18.4 ± 0.4	0.66	0.510
$\delta^{15}N$ (‰)	10.4 ± 1.1	10.7 ± 1.0	9.6 ± 0.9	4.16	<0.0001
C/N mass ratio	3.35 ± 0.05	3.34 ± 0.05	3.36 ± 0.05	1.26	0.215

Table 2 Stable carbon and nitrogen isotope values of various tissues of *Todarodes filippovae*. Values in the same column with different superscript letters are significantly different ($p < 0.05$). Values are means ± SD

Tissue	All specimens ($n = 20$)			Females ($n = 10$)		Males ($n = 10$)		Statistics (t tests)	
	δ^{13}C (‰)	δ^{15}N (‰)	C/N mass ratio	δ^{13}C (‰)	δ^{15}N (‰)	δ^{13}C (‰)	δ^{15}N (‰)	δ^{13}C	δ^{15}N
Mantle	-18.2 ± 0.6^{a}	11.3 ± 1.2^{a}	$3.29 \pm 0.10^{a,b}$	$-18.0 \pm 0.4^{a,b}$	$11.9 \pm 0.9^{a,b}$	-18.3 ± 0.7^{a}	10.7 ± 1.1^{a}	$t = 1.05$	$t = 2.66$
								$p = 0.307$	$p = 0.016$
Arm	-18.0 ± 0.4^{a}	10.9 ± 1.2^{a}	3.25 ± 0.03^{a}	$-18.0 \pm 0.4^{a,b}$	$11.8 \pm 0.8^{a,b}$	$-18.1 \pm 0.4^{a,b}$	$10.0 \pm 0.8^{a,b}$	$t = 0.63$	$t = 4.83$
								$p = 0.537$	$p < 0.0001$
Buccal mass	-18.3 ± 0.3^{a}	$10.4 \pm 1.2^{a,b}$	$3.33 \pm 0.05^{a,b}$	-18.2 ± 0.3^{a}	$11.2 \pm 0.9^{a,b,c}$	-18.3 ± 0.4^{a}	$9.5 \pm 0.8^{a,b,c}$	$t = 1.00$	$t = 4.20$
								$p = 0.330$	$p = 0.001$
Lower beak (wing)	-17.6 ± 0.3^{b}	7.6 ± 1.5^{c}	3.74 ± 0.21^{c}	-17.6 ± 0.3^{b}	8.7 ± 1.0^{d}	-17.5 ± 0.4^{b}	6.5 ± 1.1^{d}	$t = 0.81$	$t = 4.73$
								$p = 0.428$	$p < 0.0001$
Gill	-18.4 ± 0.3^{a}	9.4 ± 1.2^{b}	3.49 ± 0.05^{b}	-18.4 ± 0.2^{a}	10.2 ± 0.7^{c}	-18.3 ± 0.3^{a}	$8.5 \pm 0.9^{b,c}$	$t = 0.63$	$t = 4.71$
								$p = 0.535$	$p < 0.0001$
Digestive gland	-20.2 ± 0.7^{c}	$10.2 \pm 1.9^{a,b}$	4.96 ± 0.49^{d}	-20.3 ± 0.5^{c}	$11.0 \pm 1.5^{a,c}$	-20.1 ± 0.9^{c}	$9.4 \pm 2.0^{a,b,c}$	$t = 0.61$	$t = 1.89$
								$p = 0.548$	$p = 0.075$
Ovary/testis				-18.1 ± 0.2^{a}	$10.7 \pm 0.8^{a,b,c}$	$-17.7 \pm 0.4^{a,b}$	8.4 ± 1.0^{c}		
Nidamental gland/ Spermatophore complex				-18.3 ± 0.2^{a}	$10.1 \pm 0.9^{c,d}$	-18.4 ± 0.3^{a}	$8.9 \pm 0.9^{b,c}$		
Statistics (ANOVA)	$F_{5,114} = 80.38$	$F_{5,114} = 17.57$	$F_{5,114} = 172.05$	$F_{7,71} = 63.93$	$F_{7,71} = 10.68$	$F_{7,72} = 23.87$	$F_{7,72} = 12.08$		
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$		

Table 3 Stable carbon and nitrogen isotope values of muscle and chitinized tissues of *Todarodes filippovae* ($n = 6$). Values are means ± SD

Tissue	δ^{13}C (‰)	δ^{15}N (‰)	C/N mass ratio
Muscle tissue			
Mantle	-18.0 ± 0.2	12.0 ± 0.5	3.27 ± 0.06
Buccal mass	-18.1 ± 0.2	11.2 ± 0.6	3.29 ± 0.03
Statistics (Mann–Whitney)	$U = 13.0$	$U = 6.0$	$U = 18.0$
	$p = 0.423$	$p = 0.054$	$p = 1.000$
Lower beak			
Rostrum	-18.4 ± 0.6	6.1 ± 2.2	3.45 ± 0.06
Lateral wall	-17.4 ± 0.3	8.4 ± 0.8	3.41 ± 0.34
Wing	-17.5 ± 0.2	9.3 ± 0.3	3.60 ± 0.16
Statistics (Kruskal–Wallis)	$H = 11.47$	$H = 11.63$	$H = 5.19$
	$p = 0.003$	$p = 0.003$	$p = 0.075$
Gladius			
Middle	-18.0 ± 0.1	5.3 ± 0.7	3.78 ± 0.04
Posterior	-18.0 ± 0.3	7.2 ± 1.1	3.68 ± 0.07
Anterior	-17.5 ± 0.3	10.7 ± 0.4	3.77 ± 0.04
Statistics (Kruskal–Wallis)	$H = 8.50$	$H = 14.75$	$H = 5.30$
	$p = 0.014$	$p = 0.001$	$p = 0.071$
Overall statistics (Kruskal–Wallis)	$H = 30.73$	$H = 42.61$	$H = 33.68$
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

Results

General characteristics

The sample consisted of 55 *T. filippovae*, 38 females and 17 males, which were collected from throughout the study area. Overall, body size of females was larger than that of males (Table 1, Fig. 1). There were a variety of maturity-stages in the sample: immature females, stage 2 ($n = 4$) and 3 ($n = 34$); immature males stage 3 ($n = 8$), maturing males stage 4 ($n = 1$), and mature males stage 5 ($n = 8$). Immature (stage 2) females and (stage 3) males were of similar size (mean ML and body mass: 280 ± 21 and 287 ± 25 mm, and 406 ± 95 and 417 ± 125 g, respectively), but stage 3

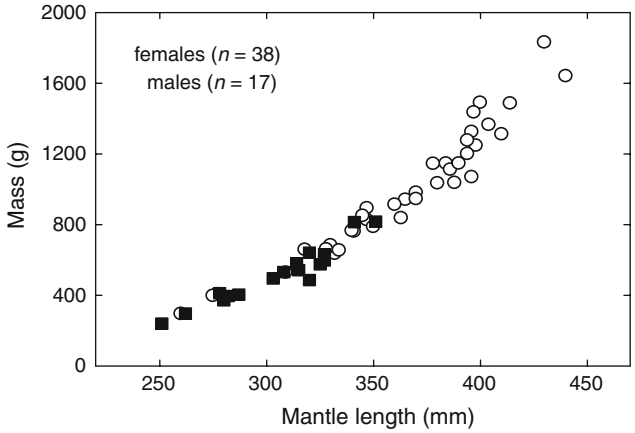

Fig. 1 Relationship between body mass and mantle length in female ($n = 38$ *white circles*) and male ($n = 17$ *black squares*) *Todarodes filippovae*

females were larger (374 ± 31 mm and 1,060 ± 306 g, reaching 440 mm ML and 1,830 g in our data set).

No sex-related differences in δ^{13}C values and C/N mass ratios were found in mantle and buccal mass muscle tissues, but δ^{15}N values were higher in females than in males (Table 1). δ^{15}N values of mantle and buccal mass were positively and linearly related to both ML and LRL (Fig. 2), indicating that the observed sex-related differences in δ^{15}N values were in fact due to differences in size between females and males. Indeed, comparable-sized stage 2 females and stage 3 males had similar δ^{15}N values (10.3 ± 0.8 and 10.5 ± 1.3‰, and 9.4 ± 1.0 and 9.4 ± 1.0‰ for mantle and buccal mass, respectively), and the highest δ^{15}N values were found for the larger stage 3 females (11.8 ± 0.8 and 10.9 ± 0.8‰ for mantle and buccal mass, respectively). Unlike δ^{15}N values, δ^{13}C values of mantle and buccal mass were not linearly related to ML or LRL (Fig. 3).

Tissue variations

Overall mean values of δ^{13}C, δ^{15}N and C/N mass ratio ($n = 20$) were different among tissues (Table 2). The wings of the lower beaks and digestive glands had higher and much higher C/N mass ratio values than the other tissues, respectively, and they also had different δ^{13}C values than gill and muscle tissues (mantle, arm and buccal mass) (post hoc Tukey HSD multiple comparison tests). δ^{15}N values of lower beak wings were significantly lower than those from all other tissues, and gill values differed from those of the arms and mantle.

Analysing the data by sex reduced the variance in tissue δ^{15}N—but not δ^{13}C—values (Table 2). For a given tissue, the mean δ^{15}N values were higher in females than in males, with no significant differences in δ^{13}C values. Overall, δ^{13}C values of various tissues (excluding the digestive gland) encompassed a narrow range (0.8 and 0.9‰ for females and males, respectively), and the digestive gland had consistently lower δ^{13}C values than other tissues for both females and males (post hoc Tukey HSD multiple comparison tests). Within each sex, δ^{15}N values of various tissues (excluding the lower beak) encompassed a wider range (1.8 and 2.3‰ for females and males, respectively) than δ^{13}C values, with the wing of the lower beak having consistently

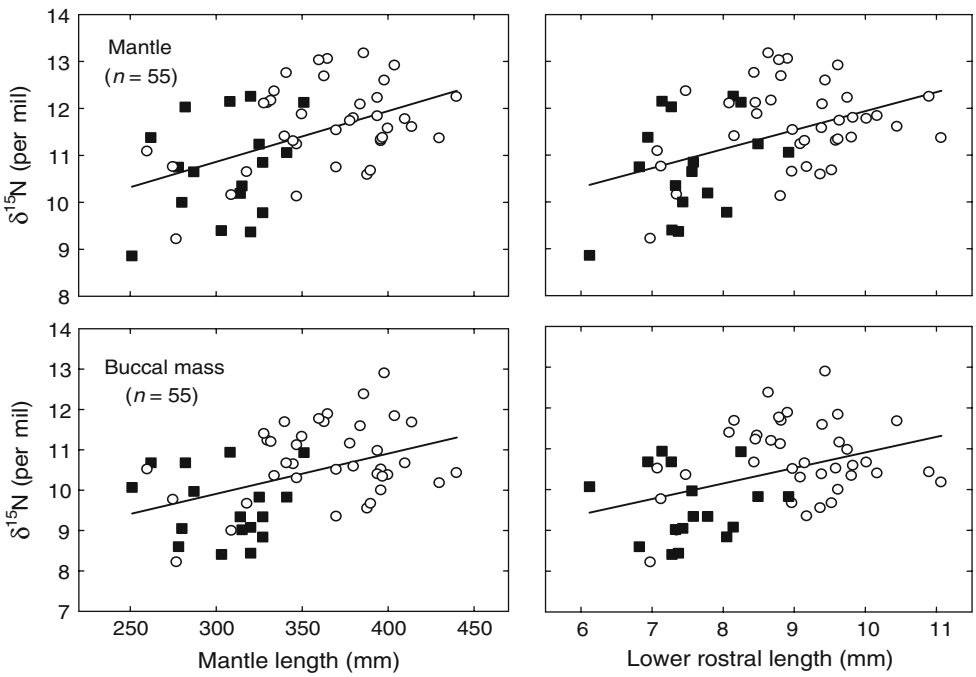

Fig. 2 Relationships between δ^{15}N values of mantle and buccal mass, and mantle length and lower rostral length in female ($n = 38$ *white circles*) and male ($n = 17$ *black squares*) *Todarodes filippovae*. Equations of regression lines are: $y = 0.01x + 7.62$ ($r = 0.49$, $F = 16.86$, $p < 0.0001$) and $y = 0.01x + 6.92$ ($r = 0.44$, $F = 12.66$, $p = 0.001$) for δ^{15}N values of mantle and buccal mass versus mantle length, respectively, and $y = 0.41x + 7.87$ ($r = 0.45$, $F = 13.19$, $p = 0.001$) and $y = 0.38x + 7.10$ ($r = 0.41$, $F = 10.47$, $p = 0.002$) for δ^{15}N values of mantle and buccal mass versus lower rostral length, respectively

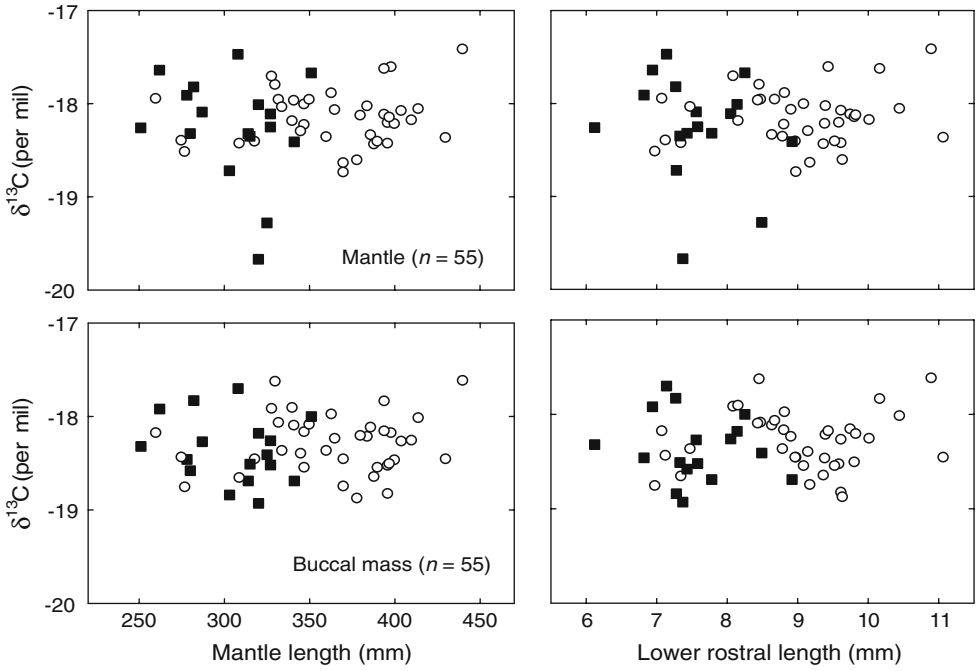

Fig. 3 Relationships between δ^{13}C values of mantle and buccal mass, and mantle length and lower rostral length in female (n = 38 *white circles*) and male (n = 17 *black squares*) *Todarodes filippovae*

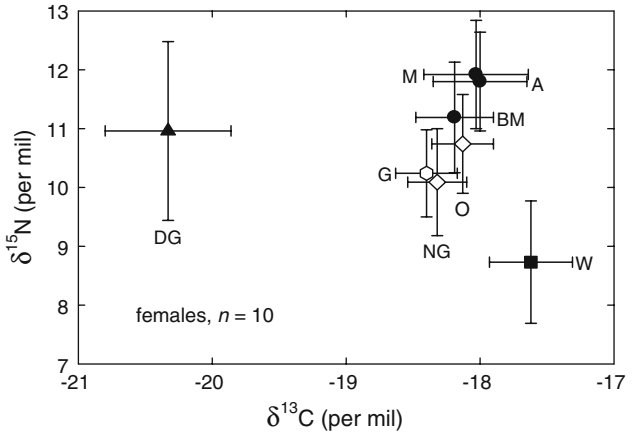

Fig. 4 Relationship between δ^{15}N and δ^{13}C values in various tissues of female *Todarodes filippovae*, including muscle tissues (*black circles*) and sexual organs (*white diamonds*). *A* arm, *BM* buccal mass, *DG* digestive gland (*black triangle*), *G* gill (*white hexagon*), *M* mantle, *NG* nidamental gland, *O* ovary, *W* wing of lower beak (*black square*). Values are means ± SD

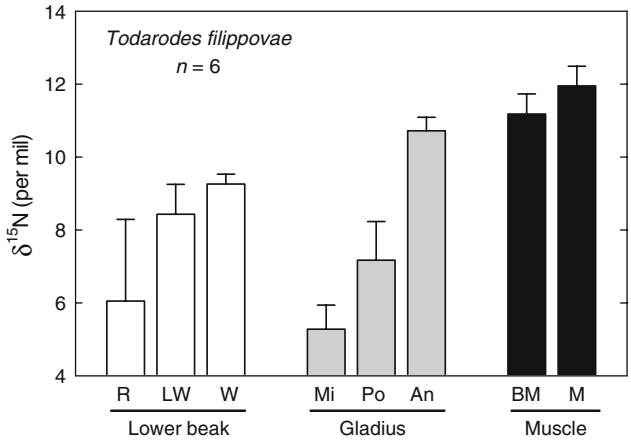

Fig. 5 δ^{15}N values in various parts of lower beak and gladius, and in different muscle tissues of *Todarodes filippovae*. Lower beak, *R* rostrum, *LW* lateral wall, *W* wing, gladius, *Mi* middle, *Po* posterior part, *An* anterior part, muscle tissues, *BM* buccal mass, *M* mantle. Values are means ± SD

lower δ^{15}N values than other tissues for both sexes (Table 2, Fig. 4).

Ontogenic variations

Within each chitinized tissue (lower beak and gladius), there were variations in δ^{13}C values, with the most recently formed regions (wing and anterior tip, respectively) being enriched in ^{13}C when compared to the older regions (Table 3). As expected, δ^{15}N values increased from the oldest to the more recent regions in the order: rostrum < lateral wall < wing, and middle < posterior tip < anterior tip, within lower beak and gladius, respectively (Fig. 5). As also expected, the variance in δ^{15}N values decreased in the same order, with the most recently formed regions of the lower beak and gladius having the lowest variances. A comparison of the two tissues showed firstly, that their older regions had similar δ^{13}C and δ^{15}N values, and secondly, that δ^{15}N—but not δ^{13}C—values of the anterior tip of the gladius were higher than those from the wing of the lower beak (Mann–Whitney, U = 36.0, p = 0.004). Consequently, the progressive increase in δ^{15}N values was overall larger in the gladius than in the lower beak (5.4 vs. 3.2‰, respectively).

As previously shown in various cephalopod species (Cherel and Hobson 2005; Hobson and Cherel 2006), little variation was found in $\delta^{13}C$ values of the chitinized lower beak and muscle tissues, but $\delta^{15}N$ values of the lower beak were lower than those of mantle and buccal mass tissues (Table 3). Accordingly, $\delta^{15}N$ values of the mid-region and posterior tip of the gladius were generally lower than muscle values, but the anterior tip and buccal mass had similar nitrogen signatures (Mann–Whitney, $U = 9.0$ and $p = 0.149$).

Finally, both the buccal mass and whole lower beak of small *T. filippovae* ($n = 9$, LRL = 1.4 ± 0.2 mm) eaten by rockhopper penguins at Amsterdam Island had low $\delta^{13}C$ (−20.1 ± 0.2 and −20.0 ± 0.3‰, respectively) and $\delta^{15}N$ values (6.2 ± 0.7 and 1.8 ± 0.8‰). The beak C/N mass ratio was noticeably higher (4.2 ± 0.3) than that of hard tissues of larger individuals (Table 3). As previously found in larger specimens, $\delta^{13}C$ values were similar for both tissues, although $\delta^{15}N$ values were higher in the buccal mass than in the lower beak (paired t test, $t = 26.73$, $p < 0.0001$), with an average difference of 4.4 ± 0.5‰. Lower beaks of small squids and the older parts of the lower beaks and gladii of larger specimens had different isotopic signatures (Kruskal-Wallis, $H = 16.20$ and 15.69 for $\delta^{13}C$ and $\delta^{15}N$ values, respectively, both $p < 0.0001$). Both $\delta^{13}C$ and $\delta^{15}N$ values were lower in the small lower beaks compared to the beak rostrum and middle region of the gladii. Accordingly, buccal mass $\delta^{13}C$ and $\delta^{15}N$ values in the small specimens were also lower than in the larger specimens (Mann-Whitney, both $U = 54.0$ and $p = 0.001$ for $\delta^{13}C$ and $\delta^{15}N$ values).

Discussion

In Tasmanian waters, *T. filippovae* has a life cycle of about a year with the sexually dimorphic females reaching their larger size by predominantly growing faster than males (Jackson et al. 2007b). Accordingly, females caught in the southwestern Indian Ocean were on average larger and heavier than males. $\delta^{13}C$ values of females and males were not statistically different, but $\delta^{15}N$ values of all tissues (except the digestive gland) were higher in females than in males. A closer examination of the data, however, showed that the difference was not related to sex but, instead, to squid size. Indeed, there was a linear increase in $\delta^{15}N$ values of muscle tissues for all individuals with ML and the length of the rostrum of the lower beaks (LRL). The lack of a sex-related effect was emphasized by indistinguishable $\delta^{15}N$ values of similar-sized stage 2 females and stage 3 males. This is in accordance with the nitrogen signatures of the mantle in two other species of squids, *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* (Parry 2008). On the other hand, a progressive increase in $\delta^{15}N$ values with increasing size corresponds with the known dietary shift from lower to higher trophic levels during cephalopod growth (Rodhouse and Nigmatullin 1996; Cherel and Hobson 2005).

The ~3‰ range in $\delta^{15}N$ values within maturity stages of *T. filippovae* is quite large, and corresponds to one trophic level (Minagawa and Wada 1984; Hobson and Cherel 2006). Such large ranges were also found in both *O. bartramii* and *S. oualaniensis* (Parry 2008); they likely reflect the trophic plasticity and opportunism of the voracious and fast-growing ommastrephid squids, which prey on various proportions of crustaceans, mesopelagic fish, and on their own species (Rodhouse and Nigmatullin 1996). In contrast, $\delta^{13}C$ values fell with a narrow range within maturity stages (~1.3‰) and were unrelated to body size. However, $\delta^{13}C$ values were positively and linearly related to $\delta^{15}N$ values of mantle and buccal mass (data not shown), which is in agreement with a small but significant shift to more positive $\delta^{13}C$ values of consumers as their trophic position increases (Rau et al. 1983).

Inter-tissue isotopic variations have been studied in a few invertebrates, including one molluscan species (Lorrain et al. 2002). Significant variation in isotopic signatures occurred among tissues, with a maximum range of ~2.5‰ in $\delta^{13}C$ (delipidated tissues) and $\delta^{15}N$ values (Hobson and Clark 1992; Pinnegar and Polunin 1999; Arneson and MacAvoy 2005; Vollaire et al. 2007). Our data on most soft tissues of *T. filippovae* are in agreement with this, with $\delta^{13}C$ and $\delta^{15}N$ values encompassing <1‰ and <2.5‰, respectively. The high C/N mass ratio of the digestive gland suggests incomplete lipid extraction during sample preparation resulting in a lower $\delta^{13}C$ value. In invertebrates, digestive gland and hepatopancreas contain large amounts of dietary lipids, and, because lipids are depleted in ^{13}C when compared to proteins and carbohydrates, they induce lower $\delta^{13}C$ values in fatty compared to lean tissues (Lorrain et al. 2002; Bodin et al. 2007). Finally, and as expected due to their different biochemical compositions, beaks and gladii of *T. filippovae* had different $\delta^{15}N$ values, but not $\delta^{13}C$ values, than soft tissues. Both hard tissues contain large amounts of chitin (Hunt and Nixon 1981; Miserez et al. 2007), which is depleted in ^{15}N when compared to muscle and diet (DeNiro and Epstein 1978; Webb et al. 1998), thus resulting in lower nitrogen isotopic signatures in chitinized tissues.

Previous research has been predominantly undertaken on captive animals fed on controlled diets. Hence, individuals reached an isotopic equilibrium, and inter-tissue differences in $\delta^{13}C$ and $\delta^{15}N$ values were a consequence of metabolic differences among tissues (e.g. types of biochemical reactions, biochemical composition and components). Since *T. filippovae* fed naturally before being collected in the wild, its inter-tissue isotopic differences are likely due

not only to metabolic properties but also to tissue-specific turnover rates. Temporal integration is attributed to physiological processes of protein accretion and metabolic tissue replacement (Miller 2006). For fast-living and growing animals such as cephalopods, rapid growth rate is brought about by high rates of protein synthesis and high retention efficiencies of synthesised protein and, therefore, little protein degradation (Houlihan et al. 1990). Hence, temporal integration of isotopes in cephalopod tissues is almost exclusively due to protein accretion and little to protein turnover. Consequently, it is likely that most variations among fast-growing soft tissues in *T. filippovae* were due to differential metabolic properties rather than different temporal integrations.

Small but significant differences in δ^{13}C values occurred between and within the two chitinized tissues (lower beak and gladius) of *T. filippovae*, with the most recently formed regions (wing and anterior tip, respectively) being enriched in ^{13}C when compared to the older regions (rostrum and middle section). The most parsimonious explanation of this enrichment is the small, incremental shift to more positive δ^{13}C values of consumers as their trophic position increases (Rau et al. 1983). Indeed, the small ^{13}C enrichment was linked to increases in δ^{15}N values from the oldest to the most recently formed regions of both lower beaks and gladii. The nitrogen signatures therefore verified our hypothesis of ^{15}N enrichment in beaks and gladii with growth, which may reflect ontogenic dietary shifts. Such ontogenic δ^{15}N increases were recently described within and between beaks of different sizes (*Benthoctopus thielei*, *Kondakovia longimana* and *Moroteuthis ingens*: Cherel and Hobson 2005; *S. officinalis*: Hobson and Cherel 2006; *Dosidicus gigas*: Ruiz-Cooley et al. 2006), but not within gladii. A comparison of the two tissues showed that the nitrogen signatures of their oldest regions were similar; they were nevertheless much higher than the δ^{15}N value of whole lower beaks of small juvenile *T. filippovae* eaten by penguins. The most likely explanation is that while the isotopic signature of small beaks truly reflects the feeding ecology at the beginning of life, the beak rostrum and the mid-gladius region integrate a much longer time-period. On the other hand, the isotopic signature of the beak wing and anterior tip of the gladius, which represent the most recent growth periods of these structures, are likely to be good indices of the diet during the most recent period of the squid's life. Within that context, it is noticeable that the anterior tip of the gladius was enriched in ^{15}N when compared to the lower beak wing. Sizes and shapes of the two hard structures are markedly different, with the length of the elongated gladius being roughly equal to mantle length (Perez et al. 1996). Consequently, growth increments are larger, better defined and easier to sample along the gladius axis (Bizikov 1991; Perez et al. 1996). Hence, similar-sized pieces of proostracum plate and beak wings integrate shorter and longer time periods, respectively. The anterior tip of the gladius therefore more effectively reflects the very recent trophic ecology of the squid.

Interpretation of isotopic values of hard structures could be confused by differences in biochemical composition between and within beaks and gladii. Different ratios of chitin to protein were recently found in undarkened, darkening and darkened parts of squid beaks, with much more chitin in undarkened than in darkened parts (Miserez et al. 2008). Since chitin is impoverished in ^{15}N relative to diet (−9.5‰ on average) and has a higher C/N ratio than protein (6.9 vs. 3–4) (Schimmelmann and DeNiro 1986; Webb et al. 1998), the higher the chitin content is, the lower δ^{15}N value and the higher C/N ratio should be. We are confident in our interpretation of δ^{15}N values within hard tissues, because C/N mass ratios were not statistically different within (Table 3). On the other hand, C/N mass ratios were slightly higher in gladii than in beaks, and significantly higher in the undarkened beaks of very small individuals, thus potentially affecting our trophic interpretation of δ^{15}N values. Since the gladius is richer in chitin than darkened beaks (Hunt and Nixon 1981; Miserez et al. 2008), the highest δ^{15}N value of the anterior tip of the gladius is all the more remarkable, underlining the usefulness of the gladius in tracking recent feeding. Comparison of isotopic values of small undarkened beaks with those from larger darkened beaks is more problematic, because the low δ^{15}N values of the former not only reflect the fact small squids feed at lower trophic levels but also have a higher chitin content in their beaks.

As expected, δ^{15}N values of the buccal masses of small specimens of *T. filippovae* were much lower than those of large squids. Ontogenic differences in nitrogen signatures of buccal masses were ~5.0‰, close to that in gladii (5.4‰). Such an ontogenic difference is within the range of those recently found in the mantle of *O. bartramii* and *S. oualaniensis* (6.7‰ and 3.7‰, respectively; Parry 2008). Taking into account a discrimination factor of ~3‰ between two consecutive trophic levels (Minagawa and Wada 1984; Hobson and Cherel 2006), a 5.4‰ difference corresponds to slightly less than a two-level difference between the trophic position of small and large *T. filippovae*. This increase in trophic position emphasizes the importance of oceanic squids as consumers of marine resources at various trophic levels throughout their life.

This study is the first, to our knowledge, to examine isotopic variations among the main tissues of cephalopods. *T. filippovae* belongs to the group of fast-swimming muscular pelagic squids. However, the community of oceanic cephalopods also includes ammoniacal and gelatinous species. Clearly, more isotopic information is needed on other species that are representative of the large diversity of

open-sea cephalopods to better investigate their trophic relationships and role in the pelagic ecosystem of the world's oceans. The present investigation nevertheless highlights practical considerations regarding the usefulness of the method for depicting the feeding ecology of squids.

Firstly, the isotopic signature within hard tissues of large squids depicts ontogenic shifts in their feeding ecology. Hence, sequential sampling and analysis of isotopes along the growth axis of beaks (Clarke 1965; Raya and Hernandez-Gonzalez 1998) and gladii (Bizikov 1991) can be used to produce a chronological record of dietary information, allowing for the reconstruction of an individual's trophic history. Due to its elongated shape and daily growth rings, the proostracum plate of the gladius is potentially the best tissue to record temporal variation in squid diet.

Secondly, a consistent isotopic feature of cephalopods is the much higher δ^{15}N values in soft than in chitinized tissues (Cherel and Hobson 2005; Hobson and Cherel 2006; Ruiz-Cooley et al. 2006; this study). Consequently, correction factors must be applied before comparing the δ^{15}N values of hard tissues with those from other tissues and organisms. The difference between δ^{15}N values of lower beak wings, and the mantle and the buccal mass of *T. filippovae* amounted to 3.5 and 2.6‰, respectively. While, again, more isotopic information is needed on other squid species, these correction factors can be used for estimating the nitrogen signature of cephalopods whose beaks were identified from predators' stomachs, and hence for investigating the trophic structure and dynamics of poorly known oceanic and deep-sea ecosystems.

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