



Hagfish feeding habits along a depth gradient inferred from stable isotopes

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ABSTRACT: Feeding habits of 3 hagfish species were investigated along a depth gradient (~50 to 900 m) in New Zealand using nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) stable isotopes. *Neomyxine biniplicata* had the lowest mean $\delta^{15}\text{N}$ value (14.2‰), followed by *Eptatretus cirrhatus* (14.9‰) and *Eptatretus* sp. 1 (15.8‰). *Neomyxine biniplicata* (~50 m depth) was characterized by (1) relative low lipid content in muscles and (2) consistent body condition index which together with its trophic position indicated that this species probably acquires its food by active predation, supplemented by opportunistic scavenging. *Eptatretus cirrhatus* (48 to 912 m) and *Eptatretus* sp. 1 (290 to 922 m) had similar morphology, but their $\delta^{15}\text{N}$ signature indicated that they were feeding on slightly different trophic levels. For *Eptatretus* sp. 1, the combination of (1) variable lipid content, indicating phases of feeding and fasting, (2) decreasing body condition index with depth, indicating less regular feeding at depth, (3) increasing $\delta^{15}\text{N}$ with depth and (4) decreasing $\delta^{13}\text{C}$ signature with depth, pointed towards a feeding behaviour specialized in scavenging on large but rare falls of high-level predators such as whales, sharks or bony fishes. On the other hand, *E. cirrhatus* was characterized by (1) less variable lipid content, (2) a body condition index not influenced by depth, (3) $\delta^{15}\text{N}$ values decreasing with depth and $\delta^{13}\text{C}$ values constant across its depth range, which is likely to indicate a more opportunistic and mobile feeding behaviour on a range of prey.

KEY WORDS: Fish · Ecology · Nitrogen · Carbon · Body condition index · Lipid content · *Eptatretus* · *Neomyxine*

INTRODUCTION

Investigations of foraging strategies of top predators within ecosystems are an important step towards understanding ecological structure and function for fisheries management. Hagfish (Myxiniidae) are an important group of demersal fishes because they are often found in high abundance, and have a growing commercial interest for fisheries and pharmaceutical resources (Downing et al. 1984, Powell et al. 2005, Knapp et al. 2011). Knowledge of their feeding and population ecology will be critical to regulation and sustainable fisheries management.

Hagfish have elongated eel-like bodies with a leathery skin and secrete significant amounts of slime that they use as a defence mechanism against predation (Zintzen et al. 2011). They are commonly considered to be scavengers, but acquisition of food by predation has been suspected (Shelton 1978, Martini 1998) and recently observed (Zintzen et al. 2011). Hagfish often burrow in sediments and create bioturbation searching for invertebrate prey (Martini 1998). Stomach content analysis of several species of hagfish has revealed the presence of a large variety of prey, including polychaetes, shrimps, hermit crabs, cephalopods, brittlestars, bony fishes, sharks,

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birds and whale flesh (Strahan 1963, Shelton 1978, Johnson 1994, Martini 1998). It is assumed from stomach contents that hagfish food resources are of mesopelagic origin in deeper-living species, but consist primarily of benthic invertebrates for shallow-living species (Martini 1998). This suggests that hagfish from deep waters could preferentially feed on carrion falls, such as carcasses of marine mammals. For example, at 1310 m depth, *Eptatretus deani* was observed feeding to satiation on bait fall (Smith 1985). Fishes with scavenging behaviour in the deep-sea are often considered to be opportunistic feeders because of the presumed rarity of potential feeding events. However, it is unclear to what extent hagfish are truly non-discriminatory in their food selection. For any given species, the potential degree of prey specificity has not been explored. Variation in prey or feeding strategies within a species and along important environmental gradients, such as depth, has not previously been explored.

Fasting is an important aspect of hagfish feeding strategy. It has been suggested that hagfishes often stay immobile between feeding events, which may be separated by long periods of time, as the probability of nekton fall can be low at depth (Smith & Baco 2003). A 'sit and wait' behaviour coupled with a low basal energy requirement make them good candidates for prolonged periods of fasting (Martini 1998, Cox et al. 2011, Drazen et al. 2011). One species, *Eptatretus stoutii*, has reportedly been kept in an aquarium for 9 to 11 mo without food (Tamburri & Barry 1999). Fasting would be a particularly useful adaptive strategy for species specializing in nekton falls, but would be less important for more opportunistic species, which could also feed on benthic fauna. Fasting fishes tend to use their stored lipids first and to conserve proteins (Weatherley & Gill 1981, Rueda et al. 1998). As hagfishes stock lipids in their muscles (Flood 1998), it is likely that the lipids are used as a source of energy during fasting periods. Muscle of *E. deani*, a deep-living species, comprised an average of 34% lipids (dry weight) but demonstrated quite variable values in percent lipids between individuals (Drazen et al. 2011), indicating that some individuals were fasting while others had recently eaten. The shallower species *E. stoutii* had much lower and less variable lipid content in their muscle (8%, Drazen et al. 2011), probably as a result of food being more frequently available at shallower depths.

Carbon and nitrogen stable isotopes are commonly used to study marine trophic relationships and, more recently, feeding patterns and habitats of mobile

marine species (Logan & Lutcavage 2008, Hückstädt et al. 2012). By measuring carbon and nitrogen isotopes, one can gain insights into dietary variation and feeding strategies among species, and among populations from different locations.

Carbon isotopes ($^{13}\text{C}/^{12}\text{C}$ or $\delta^{13}\text{C}$) integrate information on the origin of resources, from primary production (pelagic vs. benthic) to higher trophic levels (Pinnegar & Polunin 2000). Heavier isotopes (^{13}C) increase as trophic level increases, with tissues tending to be weakly enriched in the heavier (^{13}C) carbon isotope at a rate of approximately $\delta^{13}\text{C} < 1\text{‰}$ per trophic level (DeNiro & Epstein 1981).

Nitrogen isotopes ($^{15}\text{N}/^{14}\text{N}$ or $\delta^{15}\text{N}$) increase with trophic transfer at a rate of 2 to 4 ‰ per trophic level (DeNiro & Epstein 1981, Peterson & Fry 1987), generally allowing a clearer assessment of trophic position and feeding niche width than carbon isotopes. In addition, when long periods of fasting occur, individuals will catabolise endogenous amino acids to meet the demands of protein synthesis (Deschner et al. 2012), and consume their own body tissue. The result is ^{15}N enrichment in the remaining muscle tissue (Hatch et al. 2006).

This study used stable isotopes to investigate the feeding habits of 3 species of Myxinidae found in New Zealand waters (one species of the genus *Neomyxine* and 2 species of *Eptatretus*) along a depth gradient of 50 to ~900 m. The 2 *Eptatretus* species are morphologically very similar, *E. cirrhatus* being only differentiated from *Eptatretus* sp. 1 (a recognized but still undescribed species) by having 1 or 2 extra pre-branchial slime pores and a white rim around some of its slime pores. We tested the following hypotheses: (1) different but morphologically similar species of hagfish occupy different niches by feeding at different trophic levels, (2) hagfishes at different depths feed on different types of food resources, and (3) hagfishes will show signs of food deprivation in some specimens, as measured by lipid contents and body condition, indicating that they can fast, waiting for the next feeding opportunity

METHODS

Hagfish collection

Three species of hagfish, *Eptatretus cirrhatus* (n = 34), *Eptatretus* sp. 1 (n = 39) and *Neomyxine binipliata* (n = 11), were sampled using fish traps deployed at 50, 100, 300, 500, 700 and 900 m depths at 2 locations in New Zealand waters (Table 1). White Island

Table 1. Number of hagfish specimens collected per location

| | White Island | Kaikoura |
|------------------------------|--------------|----------|
| <i>Neomyxine biniplicata</i> | 0 | 11 |
| <i>Eptatretus cirrhatus</i> | 30 | 4 |
| <i>Eptatretus</i> sp. 1 | 22 | 17 |
| Total | 52 | 32 |

(37.5211° S, 177.1821° E) was sampled in March 2009 and Kaikoura (42.4315° S, 173.7087° E) was sampled in November 2010. The fish traps were deployed along 5 (White Island) and 3 (Kaikoura) transects running from 50 to 900 m. The fish trap design was circular (diameter: 1.6 m, height: 0.8 m, mesh size: 2 cm) and fitted with 3 funnelling entrances tapering from 0.4 to 0.2 m (square entrance). The bait consisted of 4 kg of *Sardinops sagax* that were thawed and chopped prior to being placed into 2 bait bags installed inside the fish traps. Each fish trap was equipped with a sensor (Star Oddi DST centi TD) that measured depth to a precision of 0.4 to 0.6% of the depth range (e.g. ± 1 m at 50 m depth and ± 9 m at 1200 m depth). The fish traps were retrieved after 2 to 4 h, and the specimens of hagfish were immediately bagged and frozen at -20°C .

Hagfish measurements

Hagfishes were identified after thawing and cleaning of excess slime. Their length was measured to the nearest mm as total length (TL, excluding barbels). Specimens were then weighed to the nearest gram. Dorsal muscle thickness was measured using calipers at mid-body length.

Stable isotope sample preparation

Muscle tissue samples were taken from under the skin at the mid-body dorsal region of each hagfish, freeze dried and ground finely. Due to variable inter- and intra-species lipid contents in hagfish muscle, chemical lipid extraction of samples is done prior to analysis or values must be corrected using normalisation factors which deduct the lipid contribution from the base tissue value (Logan et al. 2008). Trophic changes in carbon isotopes can be altered or masked by lipids stored in tissues because they incorporate less ^{13}C than the protein fraction of the tissue, due to a kinetic isotope effect (DeNiro & Epstein 1977). Chemical lipid extraction methods are found

to affect the $\delta^{15}\text{N}$ values of fish tissue (Sweeting et al. 2006, Kolasinski et al. 2009), so samples should be analysed twice to determine accurate $\delta^{13}\text{C}$ (lipid free tissue) and $\delta^{15}\text{N}$ (non delipidized tissue) values. Chemical lipid extraction of ground muscle samples was done prior to analysis, following the method of Bligh & Dyer (1959), modified by Hobson et al. (2000), Kolasinski et al. (2009) and Rogers (2009). A portion (~ 1 g) of ground muscle was agitated for 1 h with 30 ml of a 2:1 solution of chloroform:methanol. The delipidized hagfish tissue was separated from the lipids by centrifuging at 3500 rpm for 5 min. The extracted lipids were decanted and the solids resuspended with another 30 ml of a 2:1 mixture of chloroform:methanol, agitated briefly and the centrifuge procedure repeated. This rinsing step was performed another 3 times until the solvent ran clear. The delipidized muscle was oven-dried overnight at 30°C to remove any residual solvent. Untreated samples containing lipids are referred to as 'untreated' samples (noted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and delipidized samples as 'extracted' (noted $\delta^{13}\text{C}'$ and $\delta^{15}\text{N}'$).

Stable isotope analysis

Carbon and nitrogen content and isotopic composition of the untreated and extracted hagfish were analyzed according to methods described in Kolasinski et al. (2009) and Rogers (2009) at the Stable Isotope Laboratory, GNS Science, New Zealand. Analytical precision of the measurements is $\pm 0.2\%$, and reproducibility of the results is within $\pm 0.2\%$ for carbon and $\pm 0.3\%$ for nitrogen (1 SD).

Lipid content and body condition of hagfishes

The atomic C:N ratio ($\% \text{C} \times 14 / \% \text{N} \times 12$) or $\Delta\delta^{13}\text{C}$ values (with $\Delta\delta^{13}\text{C} = \delta^{13}\text{C}' - \delta^{13}\text{C}$) can serve as proxies for lipid content in animal tissues because muscle tissues with higher lipid content will have greater negative $\delta^{13}\text{C}$ values (DeNiro & Epstein 1977). They can also be used to indicate nutritional stress and loss of lipids from tissues due to fasting (Graves et al. 2012). Several equations are available in the literature to evaluate the percentage of lipid in different tissues from $\Delta\delta^{13}\text{C}$ values or bulk C:N ratios (McConnaughey & McRoy 1979, Kiljunen et al. 2006, Sweeting et al. 2006, Post et al. 2007, Logan et al. 2008). The results we obtained using those equations can be found in Supplement 1 available at

res.com/articles/suppl/m485p223_supp.pdf. Due to the inconsistency of those results, it was decided to use $\Delta\delta^{13}\text{C}$ values as a proxy for the lipid content of hagfish muscles in this study.

Lipids in muscles are used as energy storage and their state can give an indication of whether specimens have been feeding recently, or have been in a period of food deprivation. Within any species, specimens with high lipid levels have fed recently (within the last month), whereas specimens with low lipid levels have been using their lipid as an energy source (Tocher 2003).

A body condition index (B_c) was calculated for each specimen after fixation in formalin and preservation in 70% isopropanol using:

$$B_c = \frac{W}{L} \times 1000$$

where W is the width (in mm) of the dorsal muscle taken at mid-body length and L is the length of the specimen (in mm). This index was chosen rather than a mass body index, based on length and weight, due to potential bias introduced when recording weight of specimens with food in their stomach and intestine. The dorsal muscle is the most important one in hagfishes and is responsible for most of their movements and swimming activity. We anticipate that well-fed specimens over an extended period of time (at the scale of a year) will have greater muscle mass which will be mostly accumulated in the dorsal muscle. For each species, B_c was scaled from 0 (specimen with the lowest B_c value) to 100 (specimen with the highest B_c value) to allow meaningful comparisons of values between species.

Data analysis

The extracted (lipid free) samples for stable carbon ($\delta^{13}\text{C}'$) isotopes and untreated samples for stable nitrogen ($\delta^{15}\text{N}$) isotopes were used for the analysis. An initial permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was fitted on the 2 isotopic variables to test for the effect of location (Lo, a fixed factor with 2 levels), species (Sp, a fixed factor with 3 levels) and depth (De, a continuous variable) as a covariate. The PERMANOVA was done using a Type I (sequential) sum of squares which meant that each term was fitted taking into account all previous terms in the model. Normalised Euclidean distances between hagfish individuals, based on their $\delta^{13}\text{C}'$ and $\delta^{15}\text{N}$ signature, was used as a dissimilarity measure.

Hagfish specimens from the 2 locations (White Island and Kaikoura) were pooled together for the analysis (see Supplement 2). To further investigate the *a priori* grouping by species based on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}'$ values, a canonical analysis of principle coordinates (CAP) was performed on the data using normalised Euclidean distances between specimens (Anderson & Willis 2003). In this form, CAP is equivalent to a classical discriminant analysis. Leave-one-out mis-classification error (Lachenbruch & Mickey 1968) was used to obtain a direct measure of the ability of the CAP discriminant model to identify species on the basis of these 2 stable isotope measures alone. This cross-validation approach provided a rigorous assessment of the distinctiveness of the isotopic signatures for the 3 species.

The effects of species, depth of capture and specimen lengths (a continuous variable) were investigated separately on both $\delta^{13}\text{C}'$ and $\delta^{15}\text{N}$ values using ANCOVA. For each species, the effect of depth on $\delta^{15}\text{N}$, $\delta^{13}\text{C}'$, lipid content ($\Delta\delta^{13}\text{C}$) and body condition (B_c) was then evaluated using linear regression. Multiple comparisons between species were done using Tukey's honestly significant differences (HSD). The effect of $\delta^{15}\text{N}$ values on lipid content and body condition were also inspected for each species using linear regressions. The distributions of the residuals were inspected to assess the adequacy of linear regression models and did not show any violation of assumptions. Prior to ANCOVA and regressions, data were checked for homogeneity of variances using the Fligner test and approximate normality using Q-Q plots. Finally, correlation between the 3 variables of $\delta^{15}\text{N}$, lipid content and body condition were investigated to identify any potential signs of food deprivation.

CAP and PERMANOVA analyses were done using PERMANOVA+ (Anderson et al. 2008), the add-on for PRIMER v6 (Clarke & Gorley 2006) and all other statistical analyses were done using R (R Development Core Team 2012).

RESULTS

The 3 hagfish species were identifiable as 3 separate groups on the basis of stable carbon and nitrogen values (Fig. 1, Table 2). The PERMANOVA partitioning showed a significant effect of species on $\delta^{13}\text{C}'$ and $\delta^{15}\text{N}$, even after taking into account the effect of location (Table 3). However, a significant interaction between depth and species was still detected, even after fitting the depth by location

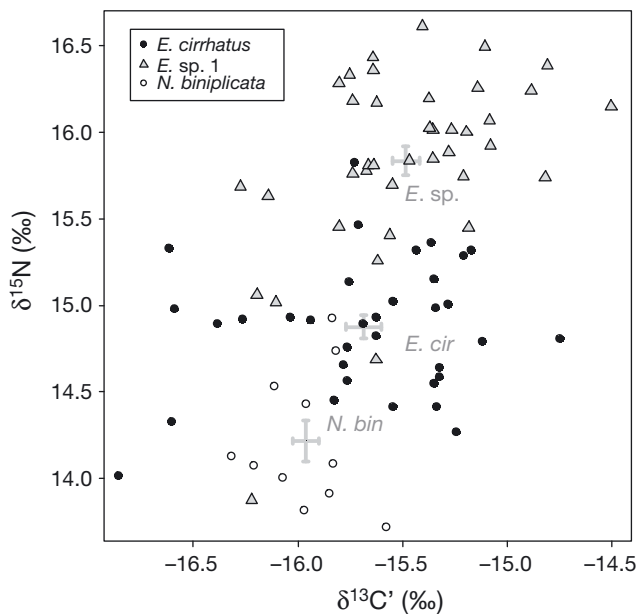


Fig. 1. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Stable carbon ($\delta^{13}C'$) and nitrogen ($\delta^{15}N$) isotope values for 3 species of hagfish. $\delta^{15}N$ values are for untreated muscle samples and $\delta^{13}C'$ values are for extracted (lipid-free) samples. Also given are the mean (± 1 SE) values for each species

interaction. This means that, even though the depth effects on isotopic signatures depended on the factor location, the depth by species interaction was still significant over and above any variation in the effects of depth from one location to the next. The diagnostics of the CAP analysis showed that, overall, 77% of the specimens were correctly classified into their respective species using $\delta^{13}C'$ and $\delta^{15}N$ information. By species, the percentage of 'left-out' specimens correctly classified by the CAP discriminant model was 73% for *Neomyxine biniplicata*, 87% for *Eptatretus* sp. 1 and 68% for *E. cirrhatus*. Mean $\delta^{13}C'$ values (\pm SD) were broadly similar but *N. biniplicata* differed significantly from *Eptatretus* sp. 1 (Tukey's HSD test, $p < 0.01$). *N. biniplicata* had the lowest mean $\delta^{13}C'$ value ($-16.0 \pm 0.2\text{‰}$), followed by *E. cir-*

Table 3. PERMANOVA results for the effect of location (fixed, 2 levels), species (fixed, 3 levels) and depth as a covariate on isotope signature ($\delta^{13}C'$ and $\delta^{15}N$) of hagfishes. Euclidean distances were used on normalised variables, partitioning was done using Type I (sequential) sum of squares and p-values were obtained using 9999 permutations

| Source | df | MS | Pseudo-F | p |
|-------------------------|----|--------|----------|--------|
| Location | 1 | 88.067 | 87.905 | 0.0008 |
| Species | 2 | 30.252 | 30.196 | 0.0001 |
| Depth | 1 | 29.772 | 29.717 | 0.0652 |
| Depth \times Location | 1 | 10.458 | 10.438 | 0.0005 |
| Depth \times Species | 1 | 61.125 | 61.013 | 0.0062 |
| Residual | 77 | 10.018 | | |
| Total | 83 | | | |

rhatius ($-15.7 \pm 0.5\text{‰}$) and *Eptatretus* sp. 1 ($-15.5 \pm 0.4\text{‰}$). *N. biniplicata* also had the lowest mean $\delta^{15}N$ value ($14.2 \pm 0.4\text{‰}$), followed by *E. cirrhatus* ($14.9 \pm 0.4\text{‰}$) and *Eptatretus* sp. 1 ($15.8 \pm 0.5\text{‰}$). Stable nitrogen values also differed significantly between every pair of species (Tukey's HSD tests, $p < 0.001$).

Although hagfish length did not explain significant variation in either of the stable isotopes, a significant interaction between depth and species was detected for $\delta^{15}N$ and also for $\delta^{13}C'$ values (Table 4). *Neomyxine biniplicata* was restricted to 50 m depth, while *Eptatretus cirrhatus* was sampled along the entire depth range (48 to 912 m) and *Eptatretus* sp. 1 occurred from 290 to 922 m. $\delta^{15}N$ values significantly decreased with depth for *E. cirrhatus* (linear regression, $p < 0.05$) and significantly increased with depth for *Eptatretus* sp. 1 ($p < 0.01$, Fig. 2A). This suggested that captured specimens of *E. cirrhatus* were feeding on lower trophic levels at deeper depths, while specimens of *Eptatretus* sp. 1 were either feeding on higher trophic levels or fasting at deeper depths. The variation around the mean $\delta^{15}N$ value was slightly higher for *Eptatretus* sp. 1 than for *E. cirrhatus*, indicating that *Eptatretus* sp. 1 was feeding on a wider range of prey than *E. cirrhatus*.

Carbon isotopes indicated that *Neomyxine biniplicata* was feeding on prey whose carbon was of a less

Table 2. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Mean (\pm SD) stable carbon and nitrogen isotope values from muscles of each of 3 species of hagfish, either before (untreated) or after chemical lipid extraction. C:N indicates the atomic C:N ratio ($\%C \times 14 / \%N \times 12$). $\Delta\delta^{13}C$ is the difference between $\delta^{13}C'$ and $\delta^{13}C$ values

| Species | n | Depth (m) | Length (mm) | Untreated | | | Lipid-extracted | | | $\Delta\delta^{13}C$ (‰) |
|-------------------------|----|-----------|-------------|--------------------|--------------------|----------------|---------------------|--------------------|---------------|--------------------------|
| | | | | $\delta^{13}C$ (‰) | $\delta^{15}N$ (‰) | C:N | $\delta^{13}C'$ (‰) | $\delta^{15}N$ (‰) | C:N | |
| <i>N. biniplicata</i> | 11 | 63 | 278–410 | -17.7 ± 0.3 | 14.2 ± 0.4 | 5.6 ± 0.4 | -16.0 ± 0.2 | 15.1 ± 0.4 | 3.7 ± 0.1 | 1.8 ± 0.3 |
| <i>E. cirrhatus</i> | 34 | 48–912 | 538–788 | -20.0 ± 0.7 | 14.9 ± 0.4 | 11.1 ± 0.7 | -15.7 ± 0.5 | 16.0 ± 0.4 | 3.8 ± 0.1 | 4.3 ± 0.7 |
| <i>Eptatretus</i> sp. 1 | 39 | 290–922 | 379–716 | -20.4 ± 0.7 | 15.8 ± 0.5 | 13.0 ± 0.7 | -15.5 ± 0.4 | 16.9 ± 0.5 | 3.8 ± 0.1 | 4.9 ± 0.8 |

Table 4. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Analysis of covariance showing the effect of hagfish species (Sp; categorical with 3 levels), depth (De; as a continuous variable) and length (Le; also as a continuous variable) of specimens on each of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}'$, as indicated. p-values <0.05 are shown in **bold**

| Source | $\delta^{15}\text{N}$ | | | | $\delta^{13}\text{C}'$ | | | |
|----------------------------|-----------------------|--------|---------|------------------|------------------------|-------|---------|--------------|
| | df | MS | F-value | p | df | MS | F-value | p |
| Species | 2 | 14.916 | 80.663 | <0.001 | 2 | 1.068 | 6.388 | 0.003 |
| Length | 1 | 0.097 | 0.523 | 0.471 | 1 | 0.265 | 1.585 | 0.212 |
| Depth | 1 | 0.001 | 0.006 | 0.94 | 1 | 0.001 | 0.001 | 0.977 |
| Sp \times Le | 2 | 0.103 | 0.557 | 0.575 | 2 | 0.236 | 1.412 | 0.25 |
| Sp \times De | 1 | 2.776 | 15.012 | <0.001 | 1 | 0.927 | 5.544 | 0.021 |
| Le \times De | 1 | 0.105 | 0.566 | 0.454 | 1 | 0.263 | 1.575 | 0.213 |
| Sp \times Le \times De | 1 | 0.326 | 1.762 | 0.188 | 1 | 0.401 | 2.399 | 0.126 |
| Residuals | 74 | 13.684 | 0.185 | 0.185 | 74 | 0.167 | | |

benthic (or more pelagic) origin (lower mean $\delta^{13}\text{C}'$ values), although the differences between the 2 *Eptatretus* species were small (less than 0.5‰ on average). The $\delta^{13}\text{C}'$ values for *E. cirrhatus* did not change with depth (Fig. 2B, linear regression, $p = 0.23$). However, $\delta^{13}\text{C}'$ values of *Eptatretus* sp. 1 showed a weak but significant positive relationship with depth ($p < 0.05$). Thus, specimens of *Eptatretus* sp. 1 caught at deeper stations were sourcing their carbon from a more benthic origin than those caught at shallower depths.

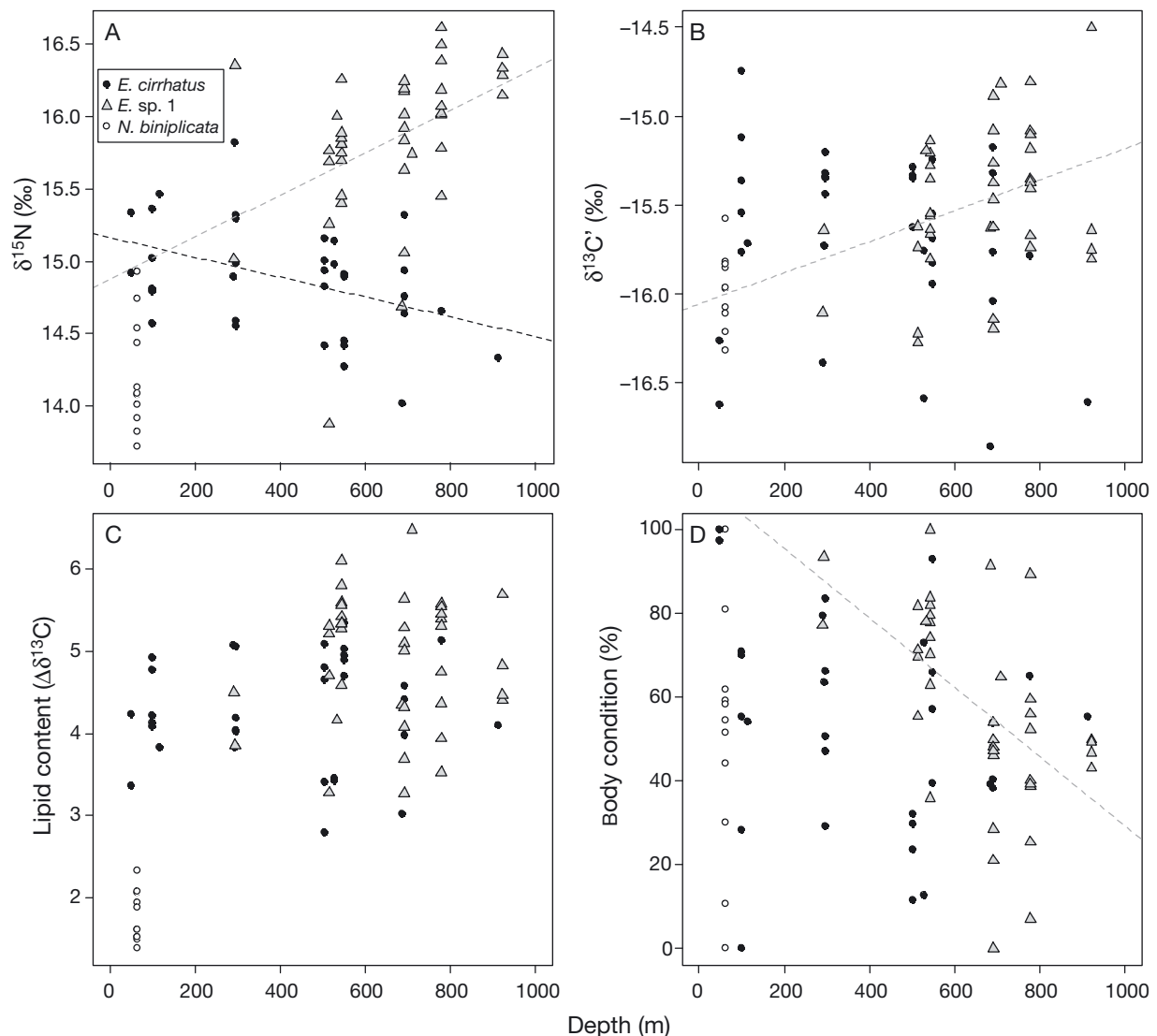


Fig. 2. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Effect of depth on (A) stable nitrogen isotope values ($\delta^{15}\text{N}$), (B) stable carbon isotope values ($\delta^{13}\text{C}'$), (C) lipid content in muscles ($\Delta\delta^{13}\text{C}$) and (D) body condition index in 3 hagfish species. Only significant regression lines ($p < 0.05$) are shown: black lines are for *E. cirrhatus* and gray lines for *Eptatretus* sp. 1

The mean estimate for lipid content ($\Delta\delta^{13}\text{C}$) of *Eptatretus cirrhatus* and *Eptatretus* sp. 1 did not change overall with depth (linear regression, $p = 0.48$ and $p = 0.91$, respectively, Fig. 2C). However, the variation in lipid content around the mean was lower at shallow depths (50 to 300 m), indicating that those specimens were feeding regularly (i.e. no part of this population was either overfed or starving). At greater depth, the variation in lipid content of *E. cirrhatus* increased, indicating that some specimens had recently fed while others were consuming their stored lipids. Variation in the mean lipid content of muscles for *Eptatretus* sp. 1 was larger than for *E. cirrhatus*.

There was no effect of depth on the body condition index for *Eptatretus cirrhatus*, but variation around the mean was higher at shallow than at deeper depths (Fig. 2D). The body condition index significantly decreased with depth for *Eptatretus* sp. 1 (linear regression, $p < 0.001$). *Eptatretus* sp. 1 had less muscle tissue at depth than in shallow water. However, there was large variation in body condition values at all depths except for 300 and 900 m.

The range in the untreated C:N ratio varied considerably between individuals: 4.2 to 7.6 (mean = 5.6) for *Neomyxine biniplicata*, 6.1 to 24.9 (mean = 11.1) for *Eptatretus cirrhatus* and 6.7 to 24.6 (mean = 13.0) for *Eptatretus* sp. 1 (Table 2). After lipid extraction, C:N ratios of the 3 studied species were all comparable to each other with mean (\pm SD) values of 3.7 ± 0.1 for *N. biniplicata*, 3.8 ± 0.1 for *E. cirrhatus* and 3.8 ± 0.1 for *Eptatretus* sp. 1 (Table 2). There was a strong relationship between bulk C:N ratios and $\Delta\delta^{13}\text{C}$ (Fig. 3). Estimates for the lipid content of hagfish muscle ($\Delta\delta^{13}\text{C}$) ranged from 1.38 to 6.48. *N. biniplicata* had significantly lower muscle lipid content (mean \pm SD $\Delta\delta^{13}\text{C} = 1.76 \pm 0.09\%$, t -test $p < 0.001$) than the 2 *Eptatretus* species (*E. cirrhatus*: $\Delta\delta^{13}\text{C} = 4.31 \pm 0.12\%$; *Eptatretus* sp. 1: $\Delta\delta^{13}\text{C} = 4.89 \pm 0.13\%$), which were also significantly different from each other for that parameter (Fig. 2C, 2-sample t -test, $p < 0.001$).

There was a clear and positive relationship between lipid content in hagfish muscles and $\delta^{15}\text{N}$ values (Fig. 4A, linear regression, $p = 0.001$). Species feeding at higher trophic levels accumulated more lipids in their muscle than did those feeding at lower trophic levels. Although this pattern held when combining all species of hagfish together, it was not observed when looking at species individually and there was even an opposite trend for *Eptatretus cirrhatus*, which had increasing $\delta^{15}\text{N}$ values with decreasing lipid content (linear regression, $p < 0.001$ —

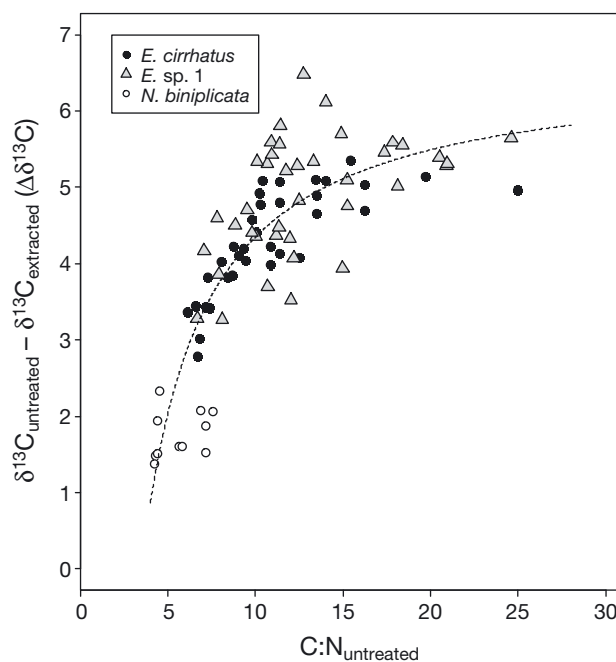


Fig. 3. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Relationship between C:N ratios of untreated samples and stable carbon isotopic values of the lipid fraction from hagfish muscles. $\delta^{13}\text{C}$ and $\delta^{13}\text{C}'$ values are for the untreated and extracted (lipid-free) samples, respectively. C:N ratios are from untreated samples. The regression line is from the lipid-normalization model of McConnaughey & McRoy (1979), where the constants have been estimated by iterative non-linear least square regression to be $D = 7.692$ and $I = -0.093$

omitting one outlier which was the only individual having both a relatively low lipid content and a low $\delta^{15}\text{N}$ value).

The body condition index had no clear relationship with either $\delta^{15}\text{N}$ values (Fig. 4B) or the lipid content (Fig. 5). For *Neomyxine biniplicata*, there was a trend for specimens with high body condition index to feed at a higher trophic level (Fig. 5A). There was no significant relationship detected between lipid content and either body condition or trophic level, although this could be due to the low number of *N. biniplicata* specimens available. For *Eptatretus cirrhatus*, 26, 50 and 24% of the specimens had a body condition index ranging from 0–33, 34–66 and 67–100%, respectively (Fig. 5B, as indicated by the proportion of specimens between the different dotted lines). The specimens with low lipid content and a low body condition index ('skinny') had intermediate values for $\delta^{15}\text{N}$, as did the lipid-rich specimens with a high body condition index ('fat'). Specimens with intermediate lipid content generally fed on organisms from a higher trophic level, and this was particularly visible for specimens having also an intermediate body con-

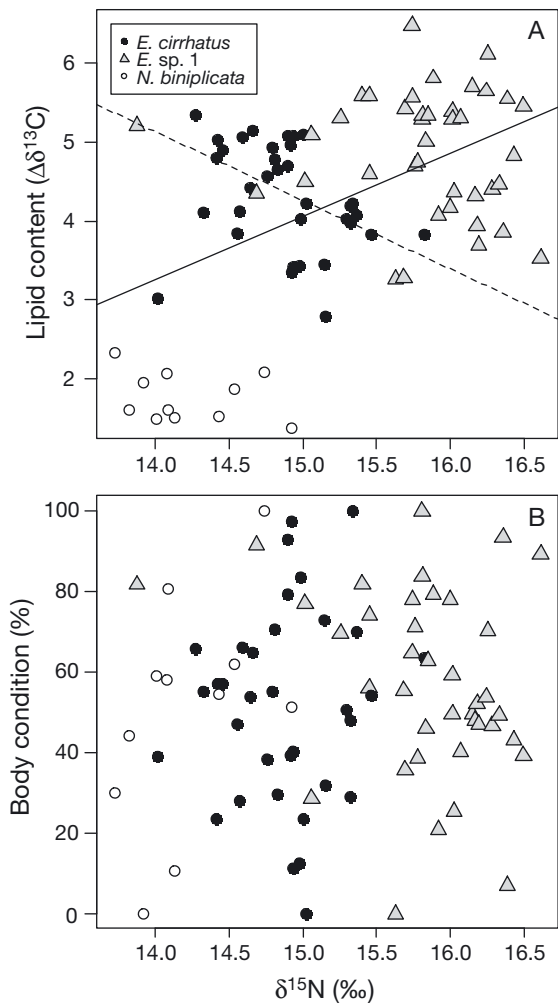


Fig. 4. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Effect of stable nitrogen isotope values ($\delta^{15}\text{N}$) on (A) lipid content in muscles ($\Delta\delta^{13}\text{C}$) and (B) body condition index in 3 hagfish species. Only significant regression lines ($p < 0.05$) are shown. The dotted regression line is for *E. cirrhatus* and the plain line is for all species combined

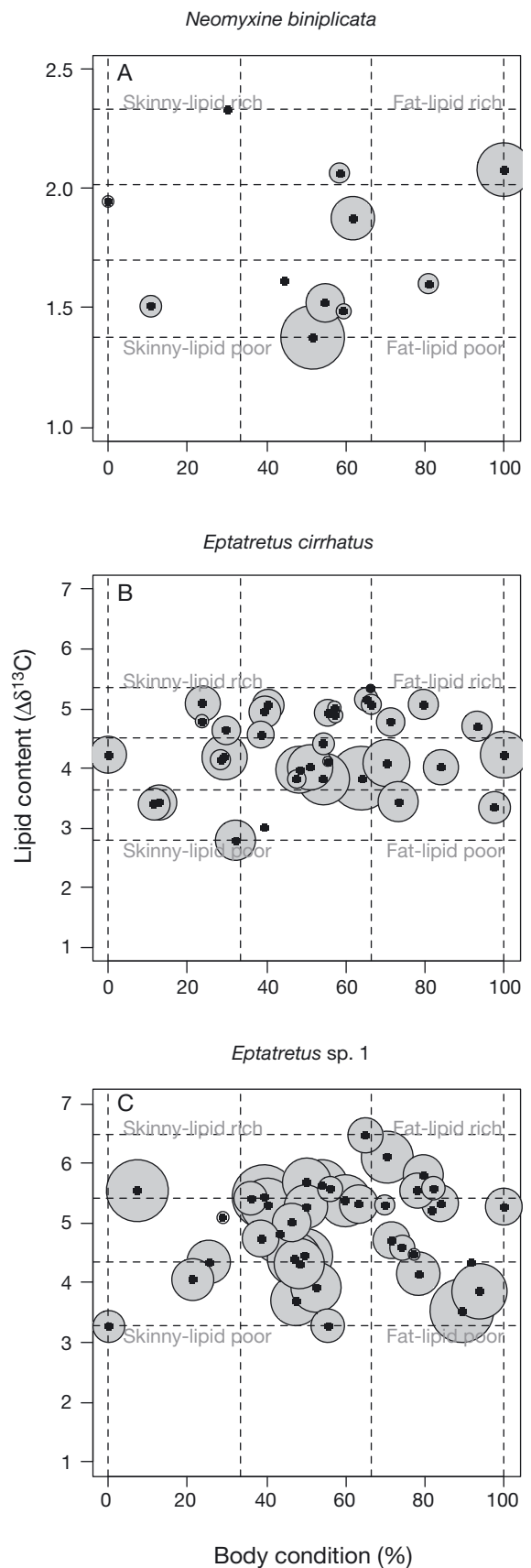


Fig. 5. *Eptatretus cirrhatus*, *Eptatretus* sp. 1 and *Neomyxine biniplicata*. Relationship between lipid content in muscles and body condition index for each of 3 species of hagfishes. Superimposed as bubbles on the plot is the trophic level for each individual (as indicated by their $\delta^{15}\text{N}$ values); bubbles are scaled for each species from 0 (specimen feeding on the lowest trophic level, low $\delta^{15}\text{N}$ values; no bubble) to 100 (specimen feeding on the highest trophic level, high $\delta^{15}\text{N}$ values; largest bubbles). The dotted lines arbitrarily divide the ordered data in each scatter-plot into 9 equal-area zones based on the level of lipid content and body condition values (low, medium and high, respectively). Certain zones are qualified as comprising 'skinny' or 'fat' individuals (by reference to their relative body condition index), and comprising 'lipid rich' or 'lipid poor' individuals (by reference to their relative lipid content). (A) *N. biniplicata*, (B) *E. cirrhatus*, (C) *Eptatretus* sp. 1

dition index. In general, specimens which had a higher lipid content fed at a relatively low trophic level but this had no apparent impact on the body condition index. For *Eptatretus* sp. 1, 13, 49 and 38% of the specimens had a body condition index ranging from 0–33, 34–66 and 67–100%, respectively (Fig. 5C). The largest fraction of the specimens had an intermediate lipid content and body condition index. There were a few specimens that were both lipid-rich and had a low body condition index. For this species, there were no clear patterns in the distribution of $\delta^{15}\text{N}$ values in relation to the body condition index or lipid content.

DISCUSSION

Assessment of the stable isotopes, body condition and lipid content of tissues of New Zealand hagfishes *Neomyxine biniplicata*, *Eptatretus cirrhatus* and *Eptatretus* sp. 1 indicate that they feed consistently on higher trophic levels ($\delta^{15}\text{N}$ values: 13.7 to 16.6‰) when compared to other marine species found in New Zealand (Table 5); it is likely that the 3 hagfish species from this study generally feed 1 to 3 trophic levels above primary consumers (i.e. TL3 to TL5). *N. biniplicata* had the lowest $\delta^{15}\text{N}$ values. This species is relatively small with a slender body (Richardson & Jowett 1951) and is very active when observed using baited underwater video (Zintzen et al. 2012). Stable isotopes suggest that this species employs an active feeding strategy, consuming small carrion and also actively preying on invertebrates from soft sediments. A closely associated species, *Neomyxine* sp. 1, was also observed actively hunting live *Cepola haastii*, a benthic fish species (Zintzen et al. 2011).

The stable nitrogen isotope values confirm that *Neomyxine biniplicata* feeds relatively high up the trophic chain, although maybe not so high as to scavenge preferentially on carrion from apex predators. The lipid fraction in its muscle was low compared to the other 2 species which could indicate that this species has low nutritional stress and regularly feeds, actively preying on lipid-poor benthic prey such as invertebrates. *Eptatretus cirrhatus* was feeding at a slightly higher trophic level than *N. biniplicata*, but at a lower level than *Eptatretus* sp. 1. The 2 *Eptatretus* species are morphologically very similar and sympatric, having been recognized as separate species only recently (V. Zintzen pers. obs.). Nonetheless, evidence here suggests that they may occupy a slightly different niche by specializing on different types of prey. The known depth range for *E. cirrhatus* in New Zealand waters is 1 to 922 m although it is more common at 90 to 700 m. *Eptatretus* sp. 1 is found from 290 to 922 m, but is more regularly caught deeper than 500 m (Te Papa Museum database). $\delta^{15}\text{N}$ values decrease with depth for *E. cirrhatus*; perhaps *Eptatretus* sp. 1 has a competitive advantage over *E. cirrhatus* when feeding at deeper sites, where a larger fraction of the food resource is likely to come from carrion falls. It is also possible that *E. cirrhatus* tends to consume a greater proportion of benthic prey from lower trophic levels, leading to lower $\delta^{15}\text{N}$ values for this species. This hypothesis is supported by the values of $\delta^{13}\text{C}$ remaining fairly constant with depth for *E. cirrhatus*, indicating similarity of carbon sources for its prey across this depth range. If *E. cirrhatus* mostly feeds on carrion from large megafauna at deeper depths, this would be reflected in its $\delta^{13}\text{C}$ values because these animals would presumably have a range of

Table 5. Stable nitrogen and carbon isotope results from present and past studies in New Zealand marine waters

| Species | Trophic position | $\delta^{15}\text{N}$ (‰) | $\delta^{13}\text{C}$ (‰) | Source |
|--|---------------------------|---------------------------|-----------------------------|-------------------------|
| <i>Zostera capricorni</i> (Seagrass) | Primary producer | 5.4 | –16.0 to –12.2 | Alfaro et al. (2006) |
| <i>Pecten novaezelandiae</i> (Scallop) | Filter feeder | 8.1 to 9.1 | –19.2 to –16.6 | Forrest et al. (2007) |
| <i>Perna canaliculus</i> (Mussel) | Filter feeder | 9.2 to 11.0 | –18.9 to –18.0 | Forrest et al. (2007) |
| <i>Atrina zelandica</i> (Mussel) | Filter feeder | 9.2 to 10.5 | –18.2 to –17.5 | Forrest et al. (2007) |
| <i>Eptatretus cirrhatus</i> (Hagfish) | Scavenger | –2.8 to 15.5 | –29.2 to –16.7 | McLeod & Wing (2007) |
| <i>Forsterygion</i> sp. (Fish) | Predator on invertebrates | 12.5 | –16.5 | Alfaro et al. (2006) |
| <i>Parapercis colias</i> (Fish) | Benthic predator | 7.5 to 14.7 | –25.7 to –16.6 ^a | Rodgers & Wing (2008) |
| <i>Carcharodon carcharias</i> (Shark) ^b | Top predator | 15.6 | n.a. | K. Rogers (pers. comm.) |
| <i>Tursiops</i> sp. (Dolphin) | Top predator | 12.8 to 15.9 | –16.6 to –13.7 | Lusseau & Wing (2006) |
| <i>Neomyxine biniplicata</i> (Fish) | Opportunistic feeder | 13.7 to 14.9 | –16.3 to –15.6 ^a | Present study |
| <i>Eptatretus cirrhatus</i> (Fish) | Opportunistic feeder | 14.0 to 15.8 | –16.9 to –14.8 ^a | Present study |
| <i>Eptatretus</i> sp. 1 (Fish) | Opportunistic feeder | 13.9 to 16.6 | –16.3 to –14.5 ^a | Present study |

^aDelipidized samples; ^bjuvenile (size: 2.85 m), Wellington harbour (NZ), 4 Oct 2010

carbon isotopic signatures due to their higher mobility. Our results did not show this. *Eptatretus* sp. 1 showed increasing $\delta^{13}\text{C}'$ values with depth. This could indicate a higher reliance on prey which had obtained their carbon from a different, more benthic, pathway. Sperm whales *Physeter macrocephalus* are common in the canyons off Kaikoura and could represent a source of food, primarily for *Eptatretus* sp. 1. These cetaceans are apex predators, their diet consisting mostly of cephalopods and fishes (Gaskin & Cawthorn 1967, Evans & Hindell 2004, Gómez-Villota 2007) and they have mostly been sighted in water deeper than 500 m and never shallower than 200 m (Jaquet et al. 2000). Considering the importance of scavenging on epipelagic prey versus benthic prey, Drazen et al. (2008) found that 2 deep-sea macrourids (4100 m depth) bypassed the benthic food web by consuming relatively large amounts of epipelagic taxa, supplemented by benthic prey. As a result, the macrourids displayed intermediate $\delta^{15}\text{N}$ values because benthic prey had high stable isotope values for nitrogen compared to epipelagic fishes. If benthic prey display a general increase in $\delta^{15}\text{N}$ values with depth, then the interpretation of our results is not so straightforward. In this case, the increase of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}'$ values with depth for *Eptatretus* sp. 1 could simply be explained by a diet containing an increased amount of benthic prey. The analysis of $\delta^{15}\text{N}$ from additional taxa in this food web or the use of compound-specific nitrogen isotope analysis of individual amino acids (Choy et al. 2012) could improve our understanding of these patterns.

For both *Eptatretus* species, the lipid content in muscle was high compared to other values for fishes from the literature (e.g. Hoffman & Sutton 2010) and did not change with depth, but *Eptatretus* sp. 1 had slightly more variable values, suggesting some nutritional stress. High but variable lipid content in muscle tissue has previously been observed in *E. deani*, a deep-living species (Drazen et al. 2011). A possible explanation for this variability is that deep-living hagfish individuals are using their lipid reserve between feeding opportunities, so that at any one time, intraspecific variability in lipid content will reflect time lapsed since the last meal. This strategy is again in line with the hypothesis that *Eptatretus* sp. 1 would be a deeper living species relying on less frequent meals. Another explanation is that lipid content varies with the type of prey the hagfish recently fed on. Our results clearly show a positive relationship between trophic level and lipid content which supports the idea that diet affects lipid metabolism

(Bailey & Robison 1986). This relationship only holds at the interspecific level, however, not within a species. For *E. cirrhatus*, there is an increase in $\delta^{15}\text{N}$ values with decreasing lipid content which was not observed with *Eptatretus* sp. 1. Previously it has been shown that fasting or nutritional stress can cause elevated $\delta^{15}\text{N}$ values in tissue (Hobson et al. 1993, Gaye-Siessegger et al. 2004), due to a faster recycling of the lighter ^{14}N isotope. It suggests that *E. cirrhatus* has a reduced capacity compared to *Eptatretus* sp. 1 to sustain periods of fasting and more quickly enters a starvation mode where proteins are preferentially used as an energy source.

Several authors have linked $\Delta\delta^{13}\text{C}$ and C:N ratios to lipid content (see Supplement 1). The maximum hagfish C:N ratio in this study was 24.9 (mean value of 11.2) which is extremely high compared to what has been observed in other fishes (Kiljunen et al. 2006, Hoffman & Sutton 2010). Glycogen granules have been identified as 'abundant' within the ultrastructure of muscle fibres in hagfish (Mellgren & Mathisen 1966, Korneliussen & Nicolaysen 1973). Glycogen comprises very little nitrogen, so it is possible that the high C:N ratios observed in this study could also be due to high levels of this compound. Lipids could be used to add buoyancy or as an energy source between periods of feeding for scavengers (Drazen 2007). However, hagfish starved for 1 mo consumed more than 90% of their glycogen in the liver and skeletal muscle, whereas protein and triglyceride contents appeared less affected (Emdin 1982). These results suggest that fasting for more than 1 mo is necessary for the hagfish to start using its lipid reserve. In light of the high variability in lipid content observed in this study, it is possible that several specimens had been fasting for an extended period of time, possibly several months or more.

This study reports for the first time stable carbon and nitrogen isotope results for hagfishes along a depth gradient. We have found that sympatric and morphologically similar species apparently feed at slightly different trophic levels, and that certain species are actively preying on alternative resources. Although they were first described as opportunistic scavengers, hagfish present some degree of specialization in their foraging and feeding strategies which may provide important information for fisheries management. Phylogenetic research might provide further insights regarding causes of speciation for hagfishes and the potential role of niche differentiation in feeding preferences and strategies along depth gradients in the evolution of the Myxiniidae.

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