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# Using stable isotope chronologies within squid gladii (*Doryteuthis gahi*) to evaluate dietary differences by fishing region and season

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Abstract: Stable isotopes are a useful tool for understanding diet and migration in squid, which often play an important role in marine food webs. The Patagonian long-finned squid fishery around Falkland Islands waters is managed separately for each of its biannual fishing seasons and 2 sub-areas of the fishing zone. However, factors driving differences in the timing of recruitment and squid size between sub-areas have never been resolved. To investigate whether seasonal and spatial variation in life history parameters of the Patagonian long-finned squid Doryteuthis gahi is driven by differences in diet, stable isotope profiles ( $\delta^{13}$ C and  $\delta^{15}$ N) were measured along the gladius— the internal chitinous shell-to reconstruct a chronological record of habitat and feeding variations and to compare fishing seasons and sub-areas using a mixed modelling approach. Here we show that isotopic niche was distinct between the fishing seasons, but negligible differences in isotopic niche were found between sub-areas of the fishing zone. This suggests that spatial differences in life history parameters are not a result of dietary variation. Substantial variation in  $\delta^{13}$ C and  $\delta^{15}$ N values along the gladius indicated versatility in feeding behaviours and dietary habits over time. Squid in the second fishing season occupied a lower trophic level (mean = 3.2) than the first season (mean = 3.5). Patterns in  $\delta^{13}$ C values indicated more complex and substantial migration patterns in the second fishing season. This coincides with the current knowledge of this species' spawning migrations. We anticipate that these data may be used for future studies investigating the role of squid within the Patagonian Shelf marine ecosystem. Analysis of the squid gladius has applications for other loliginid squid species in future studies.

**Key Words:** Loliginidae, Gladius, Trophic Ecology, Squid, Feeding Ecology, Migration, South Atlantic

#### 1. INTRODUCTION

Loliginid squid play a significant role in coastal marine ecosystems worldwide, representing important links in food webs as both voracious predators and prey to higher trophic level organisms (Clarke 1996, Rodhouse & Nigmatullin 1996, Smale 1996, Gasalla et al. 2010, Coll et al. 2013, de la Chesnais et al. 2019). They provide an ecological support service, having indirect value as prey for other harvested species (Hunsicker et al. 2010), and in some ecosystems have been identified as a keystone species (Gasalla et al. 2010, Coll et al. 2013). The Patagonian shelf in the southwest Atlantic is a unique ecosystem where squids are the dominant group (Laptikovsky et al. 2010), constituting 79.6% of the total annual catch in Falkland Islands waters in 2021 (Falkland Islands Government 2022). The Patagonian long-finned squid *Doryteuthis gahi* (which constituted 28.4% of the total annual catch in Falkland Islands waters in 2021; Falkland Islands Government 2022) provides substantial top-down control on its euphausiid and amphipod prey (Guerra et al. 1991, Arkhipkin et al. 2012) and is

a major nutrient vector, transferring around 60 000 t of biomass to oligotrophic inshore waters (Arkhipkin 2013). *D. gahi* are an important prey source for marine mammals (Schiavini et al. 1997), seabirds (Piatkowski et al. 2001, Clausen et al. 2005) and commercially important finfish (Laptikhovsky et al. 2010, Arkhipkin 2013), and play a central role in the Patagonian shelf marine food web, but information on ontogenetic changes in their feeding ecology is limited.

During its annual lifecycle, *D. gahi* migrates from shallow inshore spawning and nursery grounds (<100 m depth) to offshore feeding grounds around the shelf edge (150–300 m), forming dense feeding schools which are targeted by a large, economically valuable commercial fishery (Arkhipkin et al. 2008). Upon maturation, they return to shallow inshore waters to spawn, typically attaining an adult size of 13–17 cm dorsal mantle length (DML)

(Arkhipkin et al. 2013). The timing of the biannual fishing seasons roughly corresponds to the appearance of the 2 spawning cohorts within the designated fishing zone, known as the 'Loligo box' (Fig. 1). The autumn spawning cohort (ASC) and spring spawning cohort (SSC) differ in the duration of embryogenesis, the extent of offshore migration and timing of ontogenetic events (Arkhipkin et al. 2013). Fishing seasons are assessed separately and in real-time using a depletion model, and separately for north and south sub-areas, which are separated at latitude 52° S (Rosenberg et al. 1990, Winter & Arkhipkin 2015) (Fig 1). Although the sub-areas are considered genetically homogenous (Shaw et al. 2004), they are currently managed as separate units due to a significant disparity in squid adult size and timing of recruitment, which was discovered during the early stages of development of the fishery (Agnew et al. 1998). Variability in size at maturity and adult size in cephalopods is largely attributed to diet and temperature (Pecl et al. 2004). The difference in adult size found between the 2 sub-areas of the Loligo box is considered to be a result either of recruitment from 2 separate spawning areas (Arkhipkin & Middleton 2002), where optimal environmental conditions have resulted in faster growth of juvenile squid from one of the spawning areas (Forsythe 2004), or of differences in the diet at undetermined periods of their life history (Agnew et al. 1998).

Until recently, stomach content analysis was the main method used to study diet, but this only represents the most recent feeding, with no indication of long-term feeding habits (Jackson et al. 2007, Cherel et al. 2009), and biases from net feeding can arise (Rodhouse & Nigmatullin 1996, Lorrain et al. 2011). Prey identification from stomach contents can also be challenging; squid beaks tear prey into small pieces that can be difficult to identify (Cherel & Hobson 2005, Cherel et al. 2009), and the rapid digestive process (Semmens 2002) leaves only hard parts that resist digestion such as exoskeletons, bones, and otoliths (Jackson et al. 2007). Stable isotope analysis is an alternative tool used in ecological studies to examine trophic positions and migratory patterns of numerous marine organisms, including cephalopods (Hobson 1999,

Cherel & Hobson 2005, Rosas-Luis et al. 2016, Meath et al. 2019). Due to trophic fractionation, consumers are stepwise enriched in nitrogen stable isotopes ( $\delta^{15}$ N) relative to their food by 2.5 to 3.4 ‰ with each trophic level (Minagawa & Wada 1984, McCutchan et al. 2003, Vanderklift & Ponsard 2003, Cherel & Hobson 2005). Carbon stable isotopes ( $\delta^{13}$ C) are only slightly enriched along the food chain, by 0.5 to 0.8 ‰ (Zanden & Rasmussen 2001, McCutchan et al. 2003), but are often used to determine primary sources in a trophic network; here in the marine environment, i.e. lower vs. higher latitude plankton (Cherel & Hobson 2007), inshore vs. offshore, or phytoplankton vs. benthic contribution to intake (Hobson et al. 1994, Cherel & Hobson 2005, Lorrain et al. 2011).

High metabolic turnover in most animal tissues makes it difficult to observe isotopic variation throughout ontogeny. Although it is possible to compare tissue samples among squid at different life history stages or sizes (Martínez-Baena et al. 2016, Rosas-Luis et al. 2016), there are alternatives which could provide time-series data at high resolution. Four recording structures in a squid's body grow continuously with no metabolic turnover after synthesis; the gladius, beak, eye lens, and statolith (Xavier et al. 2015). Containing continuous growth increments, these structures have been used in age and growth studies of squid (Arkhipkin et al. 2018), and have also been used to obtain a chronological record of feeding ecology using stable isotope analysis, e.g. gladius (Kato et al. 2016), beak (Hu et al. 2022), eye lens (Meath et al. 2019, Liu et al. 2020).

The gladius is a hard-internal body part, a remnant of the ancestral molluscan shell. It is a mainly chitinous supporting structure that lies on or is partially embedded in the muscles along the dorsal midline of the mantle (Bizikov 1991, Bizikov 2008). As the isotopic signal is preserved along the growth axis, and the gladius grows in tandem with the mantle, sequential sampling along the gladius provides a chronological record of diet and habitat (Lorrain et al. 2011). Stable isotope analysis has been applied to gladii of several squid species, but these

studies have been almost exclusively focused on pelagic ommastrephid squid (Cherel et al. 2009, Ruiz-Cooley et al. 2010, Lorrain et al. 2011, Ruiz-Cooley et al. 2013, Kato et al. 2016, Li et al. 2017, Merten et al. 2017). The exception to date is Rosas-Luis et al. (2017), who investigated changes in the trophic ecology of *D. gahi* and the ommastrephid squid *Illex argentines* north of the Falkland Islands Conservation Zone. Gladius  $\delta^{15}$ N and  $\delta^{13}$ C values indicated substantial interspecific and ontogenetic differences in the trophic niche width that corresponded to their established migration patterns.

This paper aims to investigate ontogenetic variation in sequential isotopic values ( $\delta^{15}$ N and  $\delta^{13}$ C) along the gladius of *D. gahi* in Falkland Islands waters. We examine whether diet and migration route differed between fishing seasons, sub-areas, and ontogenetically, to help inform whether subareas should further be treated as separate management units within the fishery.

#### 2. MATERIALS & METHODS

#### 2.1. Biological Sampling

Samples of 'mixed' squid (unsorted by size) were collected and frozen by scientific observers onboard commercial trawling vessels every 2 weeks throughout 2019. Bottom trawls operated at a trawling speed of 4–4.5 knots using a 40 mm mesh liner in the cod end. Collection took place during pre-season scientific surveys and during the 2 sequential commercial fishing seasons (Season 1: February–May; Season 2: July–October) within the Loligo box; Fig. 1). Depth of collection ranged from 125–225 m in Season 1 and 109–288 m in Season 2, to target areas of high squid densities.

From the fortnightly samples of mixed squid, 53 squid were selected from the whole year of sampling for processing in the Falklands Islands Fisheries Department laboratory to incorporate both fishing seasons, sub-areas (north and south of  $52^{\circ}$  S), and sexes to obtain an overall impression of the population (Table 1). Samples were measured (DML ± 1 mm), weighed (total weight ± 0.1 g), and visually assessed for sex and maturity stage according to Lipinski (1979). Gladii were extracted for further processing.

#### 2.2. Stable Isotope Analysis

Each gladius was cleaned with distilled water and cut into 1 cm sections from the posterior end (i.e. the oldest material representing the pre-hatching and paralarval stage) to the anterior end (i.e. the youngest material representing the adult stage) and wing sections were removed (Fig. 2). Sections were cut in a V-shape using fine scissors to follow the direction of the growth increments following Lorrain et al. (2011). Each sample was oven-dried for 48 hours at 60°C. The number of sections per individual ranged from 9 to 23, depending on squid size, with a total of 755 sections processed. As the gladius represents the somatic growth of each individual and grows in concert with the mantle, DML has been used in place of section to aid in the interpretation of results.

Stable isotope analysis (C and N) was conducted at the Littoral, Environnement and Sociétés (LIENSs) Joint Research Unit stable isotope facility (CNRS – La Rochelle Université, France). Similar to bird feathers (e.g. Mills et al. 2021), each 1 cm section was cut into small fragments using stainless steel scissors and put into 1.5 ml Eppendorf tubes, then ground into a fine homogenous powder with a plastic pestle adapted for use in Eppendorf tubes and weighed in a tin container using an XPR6UD5 Mettler Toledo Micro balance (precision  $\pm 0.5 \mu g$ ).

The ratios of  ${}^{13}C/{}^{12}C$ ,  ${}^{15}N/{}^{14}N$  and C/N were measured using a Flash 2000 elemental analyser (Thermo Scientific, Milan, Italy) coupled with a Delta V Plus isotope ratio mass spectrometer with a Conflo IV interface (Thermo Scientific, Bremen, Germany). Isotope ratios of samples are reported as  $\delta^{13}C$  or  $\delta^{15}N$  values:

$$\delta R_{sample} = [(R_{sample}/R_{standard}) - 1] \times 10^3$$

where *R* is <sup>13</sup>C/<sup>12</sup>C or <sup>15</sup>N/<sup>14</sup>N, respectively. Results are expressed in the  $\delta$  unit notation as permil (‰) deviation from the international standards Vienna-Pee Dee Belemnite ( $\delta^{13}$ C) and atmospheric N<sub>2</sub> ( $\delta^{15}$ N). Based on international standards routinely inserted within the sampling sequence (USGS-61 and USGS-62), the analytical precision was estimated as ±0.10 ‰ and ±0.15 ‰ for  $\delta^{13}$ C and  $\delta^{15}$ N, respectively.

#### 2.3. Statistical Analysis

Bi-plots, in which the isotopic values of animal tissues are represented in  $\delta$ -space (Newsome et al. 2007), were presented (using data from all gladius sections) to delineate isotopic niches for each combination of fishing season and area. To compare niche width among groups, the standard ellipse area (SEA) was calculated for each combination using the package 'SIBER' in the R environment (Jackson et al. 2011, R Core Team 2020). SEA is suggested to be a more unbiased metric compared to convex hull area, and the dataset does not warrant any correction for small sample size (Jackson et al. 2011). The trophic level was calculated according to Cherel et al. (2008) using the following equation:

Trophic Level = 
$$[(\delta^{15}N - 3.4)/3.2] + 2.0$$

where 3.4 represents the mean  $\delta^{15}N$  (‰) of *Salpa thompsoni* with a trophic level of 2 (Perissinotto & Pakhomov 1998) and 3.2 is the average trophic enrichment factor (Sweeting et al. 2007).

To investigate ontogenetic patterns in isotopic values, response variables  $C(\delta^{13}C)$  and  $N(\delta^{15}N)$  were modelled using a Generalized Additive Mixed Modelling (GAMM) approach (with a gaussian distribution and an identity link) to allow for non-linear relationships. Data exploration for outliers, collinearity and relationships between response variables and covariates was undertaken according to Zuur et al. (2010). No extreme outliers were observed prior to analysis and potential collinearity was considered during model construction. Though it was possible to investigate temporal differences using collection *Month* as a co-variable, *Season* (fishing season, categorical, 2 levels) was adopted as a fixed covariate instead to reduce degrees of freedom and to reflect the underlying questions of the study itself. Other fixed co-variates were *Sex* (categorical, 2 levels) and *Lat* (Latitude, continuous). *Depth* and *Lat* were found to be collinear prior to analysis and therefore only *Lat* was investigated as a potential fixed co-variate given the aim of investigating spatial differences in diet. *DML* (continuous), which is analogous to section of the gladius, was used to describe the temporal effect within each gladius. In this case, the first section (0–1 cm) is defined as 1 cm *DML*. To model dependency, the addition of *DML* nested within Season was explored.

A preliminary analysis indicated non-linear effects for *DML* and *Lat*. Smoothing techniques were therefore employed using thin plate regression splines (Wood 2003). As the dataset consisted of multiple observations for each squid, dependency was incorporated by including the random effect ID (a unique identification number for each squid) to allow intercepts to vary by individual. Mixed effects models were applied according to the 10-step protocol described in Zuur et al. (2009) using restricted maximum likelihood (REML) estimation for the final models. Given that autocorrelation plots indicated violation of independence during model

selection (temporal autocorrelation was expected between data points given the serially sampled nature of the data), a lag–1 autocorrelation structure (AR1) was investigated for the random component. Model selection occurred based on the lowest value of the Akaike information criterion (AIC), providing that there were no serious patterns in residuals and all remaining explanatory variables had a significant effect. Mixed modelling was performed in the R package 'mgcv' (Wood 2017).

#### 3. RESULTS

#### 3.1. Seasonal and Geographical Differences

Values of  $\delta^{13}$ C and  $\delta^{15}$ N varied from -21.06 to -16.21 ‰ (mean -18.66 ‰) and from 3.78 to 13.30 ‰ (mean 7.69 ‰), respectively. Little overlap in trophic niche was found among individuals from different fishing seasons (Fig. 3). Those from Season 2 had a wider niche width, and individuals caught north (SEA = 2.71) and south (SEA = 2.70) of the Loligo box in this season had largely overlapping niches. In Season 1, squid to the north had a much wider isotopic niche (SEA = 2.47) than those in the south (SEA = 1.49). Individuals collected in Season 1 had higher  $\delta^{13}$ C and  $\delta^{15}$ N values than those collected in Season 2. The mean trophic level was 3.33, ranging from 2.12–5.06, with those from Season 1 occupying a higher trophic level (3.53) than those from Season 2 (3.20) (Table 1).

#### 3.2. Ontogenetic trends

Trophic level oscillated substantially throughout ontogeny and was higher within each ontogenetic stage for squid collected during Season 1 (Fig. 4). The largest individuals (collected during Season 2) displayed a steady increase in trophic level from 14–15 cm DML onwards. Seasonal differences in the total number of sections for each squid (representing the

size of individuals) were evident, indicating differences in growth rate or age between the 2 fishing seasons (as the gladius length represents the somatic growth of each squid). There was a clear ontogenetic pattern in both isotopes along the gladius from paralarval (i.e. section 1) to adult material (Figs. 5 & 6).

The oscillating trends in both isotopes with increasing size were similar for both fishing seasons and subareas, showing the same peaks and troughs (Figs. 5 & 6). These trends were less pronounced in Season 1, particularly in the southern sub-area. The northern sub-area of the Loligo box showed particularly high variability in  $\delta^{15}N$  values throughout ontogeny. Ontogenetic trends in isotopic profiles were largely similar between both sexes. The error associated with the LOESS smoothers increased towards the latter stages of ontogeny, with fewer data that were only available from the largest individuals.

The candidate models that explored the effects of spatial and temporal variables on stable isotope ratios (models for C and N were fitted separately) are given in Table 2. Prior to the addition of the random effects structure, the beyond optimal models (Diggle et al. 2002) that contained all fixed components and interactions that made biological sense were run for each isotope.

For C, all covariates were significant at the 5% level for the beyond optimal model (Model 1, Table 2). The inclusion of the random component ID substantially reduced the AIC (Model 2) and significantly improved model fit ( $\chi^2$ [1]=187.0, p < 0.001). Fixed co-variates were then optimised. Expected degrees of freedom for the effect of the smoother Lat were 1 once the random effects were added, suggesting a linear trend. Lat was re-fitted without a smoother and this linear trend was non-significant at the 5% level (F = 0.003, p = 0.95). Model 4 was the optimum model, in which the smoothing term DML (representing the ontogenetic effect) was modelled separately for each fishing Season (regression parameters for this model can be

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found in Table 3). An autocorrelation function applied to the residuals of Model 4 suggested some residual correlation with a lag of 1, therefore an AR1 structure for DML nested within ID was included, which substantially reduced the AIC to 1043.9 and significantly improved model fit ( $\chi^2[1] = 358.6$ , p < 0.001).

For N, all steps were similar to the carbon model, except that in the beyond optimum model Sex was not significant at the 5% level (Model 5, Table 2) and was removed prior to inclusion of the random component. Both stable isotopes were expressed using the same covariates, random structure, and AR1 structure in their final models (for N, Model 8; Table 2). Plotting of residual values against all potential covariates suggested no residual patterns were evident in either model. More variation was explained in the model for carbon (62%) than for nitrogen (46%).

Fitted curves are shown in Fig. 7 and indicate that values of both  $\delta^{13}$ C and  $\delta^{15}$ N were higher during the first fishing season, but that the trends observed are largely similar and reflect the trends found in the raw data for both isotopes. The complexity of the smoothing curve is given by the estimated degrees of freedom (edf), where higher values of edf indicate a more complex curve. The smoothing term applied to the first fishing season had lower edf than the second fishing season for both stable isotopes, indicating a less complex trend, closer to linear. Model validation for both stable isotopes found no obvious trends upon inspection of residuals (see the Supplement Material).

#### 4. DISCUSSION

Studies have demonstrated the viability of using the stable isotope analysis approach to assess aspects of a cephalopod's ecology such as spatial distribution, trophic level, and ontogenetic changes in diet (Takai et al. 2000, Cherel & Hobson 2007, Guerra et al. 2010, Kato et al. 2016, Li et al. 2017, Liu et al. 2019, Hu et al. 2022). In the present study, we used a stable isotope analysis approach on sections of the gladius of *Doryteuthis gahi* to explore onto gene tic, geographic, and seasonal shifts in diet.

Values of  $\delta^{13}$ C and  $\delta^{15}$ N were within a similar range to the gladii of other squid species such as Dosidicus gigas (Ruiz-Cooley et al. 2010, Li et al. 2017) and Sthenoteuthis pteropus (Merten et al. 2017), but  $\delta^{13}$ C was higher than for D. gahi in international waters to the north of Falkland Islands waters (Rosas-Luis et al. 2017). In the marine environment, offshore waters are normally more depleted in <sup>13</sup>C than coastal waters. Depletion in <sup>13</sup>C of samples collected in international waters (in Rosas-Luis et al. 2017) compared to Loligo box samples (present study) is reasonable given that the former samples are located further offshore. It is important to note that direct comparisons of  $\delta^{15}N$  measurements (and trophic level) between ecosystems and species should be considered with caution, as in this study the baseline used to calculate trophic level was based on literature values (and in Rosas-Luis et al. 2017) rather than a calculation of the isotopic baseline based on  $\delta^{15}$ N values of primary consumers collected in situ at the same time and location as squid samples. The isotopic composition of ambient water and/or the base of the food chain varies with time and space, complicating the interpretation of diet (Trueman et al. 2012). Future studies should take this baseline effect into consideration and calculate baseline values using primary consumers collected across different seasons and geographic areas.

#### 4.1. Seasonal and Geographical Differences

Stomach content analysis of other loliginid squid species has shown substantial differences in diet between fishing zones and seasons (Gasalla et al. 2010). In Scottish and Irish waters,

season was shown to significantly influence predator-prey size relationships in *Loligo forbesii* (Collins & Pierce 1996, Wangvoralak et al. 2011). The present study has revealed seasonal differences in isotopic values but no evidence of substantial isotopic differences between the subareas of the Loligo box. These conclusions were supported by the 2 GAMM models, with the inclusion of separate smoothers for each fishing season substantially improving model fit, but with latitude (to investigate geographic differences between sub-areas) and sex having no significant effect.

The large range of  $\delta^{13}$ C values in Season 2 suggests that geographic movement may be more pronounced than in Season 1. This is reflected in the higher edf in the smoothing curve applied to Season 2 in the carbon mixed model, indicating a more complex trend and more pronounced movements. This is consistent with the existing ecological information on this species. Squid of different cohorts occur on their feeding grounds in the warmest available near-bottom water layers of the Transient Zone; a mixing of sub-Antarctic superficial waters, which represents the upper layer of the Falkland Current, with continental shelf waters. The 5.5°C isotherm is considered to mark the limitation of squid distribution, and the position of this isotherm within the water column changes seasonally (Arkhipkin et al. 2004). Because the timing of their migration patterns coincides with different seasons (and therefore temperatures), squid from the SSC can penetrate deeper waters than those from the ASC. Season 1 operates at a shallow depth and primarily targets maturing individuals from the ASC and then SSC individuals emigrating from the nursery grounds towards the end of the season. Season 2 operates in deeper water targeting maturing and mature individuals from the SSC.

SEA is a proxy for trophic niche width and can vary with structure, relative extent, and quality of different habitats, and with higher prey diversity integrated into the diet (Quevedo et al. 2009). Bi-plots using SEAs showed little overlap in the isotopic niche between fishing seasons, with squid from Season 1 occupying a higher isotopic niche and squid from Season 2 having a

lower and wider isotopic niche. As individuals from the SSC (primarily recruiting to the fishery during Season 2) have a more extensive offshore migration (Arkhipkin et al. 2013), they are likely to have a more diverse spectrum of prey available for feeding, which is a likely explanation for why they occupy a wider isotopic niche. The Patagonian shelf is one of the most productive areas in the world (Csirke 1987). The eastern branch of the Falkland Current forms a long front that runs along the Patagonian shelf break and slope and frames the Patagonian large marine ecosystem from the east (Acha et al. 2004, Franco et al. 2008, Arkhipkin 2013). Mixing of these waters with inshore waters results in high productivity in both summer and winter (Agnew 2002). The species composition of phytoplankton within this region is not well understood and would produce a different isotopic signature de pending on the composition, which may contribute to seasonal differences. The position and seasonal timing of productive fronts are likely responsible for the differences in trophic levels of squids between the 2 fishing seasons, with squid from Season 1 having a higher trophic level. As productivity is high in both summer and winter, seasonal differences are likely a result of squid feeding on different prey in combination with more complex (and substantial) patterns of migration in Season 2. A decrease in the variation of stable isotope patterns was found in the gladius of D. gigas during El Niño events and is considered a result of this environmental phenomenon compressing the space and time for foraging and migrations (Li et al. 2017). A similar environmentally driven effect may be occurring during Season 1 when the extent of offshore migration is restricted by oceanographic boundaries. A recent stomach content analysis study of D. gahi confirmed that there are seasonal differences in dietary composition in Falkland Islands waters, with Euphausiacea consumed in higher amounts by the ASC squid and fish consumed more frequently by the SSC squid (Büring et al. 2022). In addition, individuals from Season 2 were larger than those from Season 1. The wider trophic niche in Season 2 may also represent a size dependent diet change in D. gahi, from Euphausiacea in small sizes to fish and squid in larger sizes (Büring et al. 2022), with the larger individuals having a much wider array of potential prey items, resulting in a wider isotopic niche. The lack of obvious differences in stable isotope values between the sub-areas does not support the hypothesis that geographic differences in squid size are a result of differences in food availability. These differences may instead be related to differences in environmental variables during early ontogeny, resulting in accelerated growth in those individuals that are fished as adults in the northern sub-area of the Loligo box (Arkhipkin & Middleton 2002, Forsythe 2004).

#### 4.2. Ontogenetic trends

LOESS smoothers fitted to each fishing season suggested squid in Season 1 occupied a higher trophic level (approximately 0.5 levels higher) than Season 2, but that oscillating trends in trophic level throughout ontogeny were highly variable and were largely consistent between the 2 fishing seasons. Substantial ontogenetic variation in gladius  $\delta^{13}$ C and  $\delta^{15}$ N values has been detected in other squid species such as *D. gigas* (Ruiz-Cooley et al. 2010, Li et al. 2017). Substantial ontogenetic variation in isotopic signals was apparent in this study, with oscillating trends found for both stable isotopes. This suggests that feeding dynamics are variable, and that squid have versatile feeding behaviours throughout their life history, reflecting their role as active, opportunistic predators. Squid are often found to exhibit a generalized diet and wide trophic niche (Otogo et al. 2016, Ibáñez et al. 2021). This is consistent with a stomach content and parasite study by Brickle et al. (2001), which revealed that *D. gahi* within Falkland Islands waters had a highly varied diet, predominantly consisting of euphausiids, amphipods, and chaetognaths. Ontogenetic diet change from ~70% frequency of occurrence (FO) of Euphausiacea in small squid to more than 60% FO of fish and cephalopods at larger sizes has recently been reported for *D. gahi* in a stomach content study within Falkland Islands waters (Büring et al. 2022). Such ontogenetic shifts have been found in other squid species (e.g. *D. gigas*), with juvenile diets largely consisting of zooplankton and krill, abruptly changing to a diet dominated by fish and squid in later ontogeny (Ibáñez & Keyl 2010). Cannibalism has also been found to be more prevalent in larger individuals (Ibáñez & Keyl 2010).

Results of the present study suggest that there are indeed dietary shifts, with fitted curves (particularly from the  $\delta^{15}$ N GAMM model) showing substantial variation during early and juvenile stages (from 1–8 cm DML) and rapid shifts from increasing to decreasing trends in  $\delta^{15}$ N at reported sizes that maturation occurs. For individuals >15 cm DML (which were only recovered in Season 2), ontogenetic trends exceeding 15 cm DML indicated a progressive increase of 1 trophic level. Individual size has been shown to strongly influence the trophic role of marine organisms (Murphy et al. 2020), including the loliginid squid *L. forbesii*, which was found to consume a large range of prey sizes. In this case, prey size was always less than the predator size (Collins & Pierce 1996). Morphological traits used as a proxy for activity level (such as fin-to-mantle length ratio or fin musculature) are strong predictors of cephalopod trophic allometries (Murphy et al. 2020). This is likely to be true for *D. gahi* with substantial morphometric differences found between males of the population, where the largest individuals are suggested to have enhanced swimming performance (Jones et al. 2019).

Due to trophic fractionation, consumers can be expected to become enriched in  $\delta^{15}$ N by 2.0–3.4 ‰ with each trophic step (Minagawa & Wada 1984, McCutchan et al. 2003). Squid are carnivorous throughout ontogeny and therefore start life at a trophic level of at least 3 (Meath et al. 2019). In this study, *D. gahi* started life at a trophic level of approximately 3.5–3.75, with high levels of fluctuation throughout ontogeny. The first 3 sections of gladius (0–3 cm DML), representing early ontogeny, indicate a decrease in trophic level. The first section is likely to represent the pre-hatching stage as well as the post-hatching paralarvae utilizing yolk reserves

(with the yolk reserves likely to be closely linked to the diet of the spawning females). During early development, food availability may be limited in squid by the efficiency of their preycapture skills (Vidal et al. 2002). Early juvenile stages (0.4–3 cm DML) have been collected in shallow coastal waters between 30 and 65 m depth (Hatfield & Rodhouse 1994), and low trophic level is reflected in the isotope values for this size-range.

Young and juvenile *D. gahi* of both sexes (<8 cm DML) have been found to aggregate in dense near bottom feeding schools on nursery grounds, at depths of less than 100 m (Patterson 1988, Arkhipkin et al. 2013). Once they have attained sizes of 9–10 cm DML, immature squid migrate offshore to depths of 100–200 m to feed (Arkhipkin et al. 2013). These sizes correspond with the observed changes in trophic level, which the present study suggests may reflect changes in types of prey available for foraging in different locations on the shelf. This pattern is also reflected in the fitted curves for the nitrogen and carbon GAMMs, which indicate a shift in trends of  $\delta^{15}$ N at 7 cm DML in Season 1 and 8 cm DML in Season 2. It is likely that there are differences in growth rates and size-at maturity between fishing seasons. Specific feeding strategies at different stages of ontogeny have also been found when analyzing  $\delta^{15}$ N and  $\delta^{13}$ C values from muscle tissue in 5 species of cephalopod in the Bay of Biscay (Chouvelon et al. 2011).

Along continental shelves,  $\delta^{13}$ C gradients commonly exist in which values are higher in shallow inshore waters compared to deeper offshore waters due to the presence of benthic algae, which is about 5 ‰ more enriched than phytoplankton (Fry 1981). During early ontogeny,  $\delta^{13}$ C values were high, and a decreasing ontogenetic trend can be observed from approximately 8 cm DML onwards. This coincides with the approximate size at which squid migrate offshore.

Differences in migratory behaviour between males and females have been confirmed using  $\delta^{13}$ C values of serially sampled gladius sections in the ommastrephid squid *Ommastrephes bartramii* (Kato et al. 2016), but this is not evident in our study. Though females undertake more substantial offshore migrations than their male counterparts (Arkhipkin & Middleton 2002), they are present within the same water mass, and the basis of the food web is likely the same for both sexes, regardless of spatial segregation.

Previous studies to the north of the Falkland Islands Conservation Zone (in international waters) conducted stable isotope analysis, dividing gladii into 3 sections representing juvenile, maturing, and adult stages of ontogenesis (Rosas-Luis et al. 2017). These authors concluded that the more positive values of the maturing section of  $\delta^{13}$ C compared to adult and juvenile sections could be related to migrations of squid from inshore spawning grounds to offshore feeding grounds. However, such individuals are hypothesized to represent a part of the Falklands population which have been displaced northwards by the Falkland Current into the high seas, without the possibility of return (Arkhipkin et al. 2006). This would suggest that the inshore return migration would not be observed in the isotopic signal of these individuals, and there is perhaps something else driving the observed pattern. No return migration is evident in the present data set, as samples were collected on adult feeding grounds before the subsequent inshore migration for spawning.

#### 5. CONCLUSIONS

Stable isotope analysis of serially sampled sections of the gladius of *Doryteuthis gahi* indicated that this technique is a powerful tool for depicting high-resolution ontogenetic variations and individual foraging strategies of squids, complementing traditional stomach content analysis methods. The findings of this study do not support the hypothesis that differences in the size

and timing of appearance of squid to the north and south of the commercial fishing area are due to differences in diet. No differences in foraging were found between sub-areas of the commercial fishing zone. Substantial variation in  $\delta^{13}$ C and  $\delta^{15}$ N values were found between fishing seasons and onto genetically, demonstrating versatility in feeding behaviours and dietary habits over time. This indicates that this species has the potential to adapt and change food source, which may be useful if changes in habitat composition and environmental change occur. Squid from different fishing seasons occupied different isotopic niches, likely due to seasonal differences in the availability of different prey types. Squid from Season 2 occupied a lower trophic level, and patterns in  $\delta^{13}$ C values indicated more complex and substantial migration than in Season 1. As no substantial dietary differences were found between subareas, environmental-based differences (i.e. temperature) should be investigated to determine whether sub-areas should further be treated as separate management units. Future studies should focus on the importance of cephalopods within the Patagonian shelf marine ecosystem and the impacts that fluctuations in abundance of important cephalopod species will have on the food web. Though several studies have analysed the gladius of squid, analyses are often limited to ommastrephid squid species, which develop a gladius with a very different structure to loliginid squid gladii. In a wider context, this study has confirmed the viability of stable isotope analysis of the loliginid squid gladius in particular and demonstrates how these results can provide evidence to aid in fisheries management decisions.

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Table 1. Summary statistics of the stable isotopic dataset for *Doryteuthis gahi*. Season 1: February–May; Season 2: July–October; sub-area: region of the designated fishing area (N = north, S = south); n: number of individuals; DML: dorsal mantle length; trophic level: position the organism occupies within the food web, calculated using Eq. (2)

Fishing Season	Sub-Area	Sex	Ν	Mean DML $\pm$ SD	Trophic Level $\pm$ SD
1	Ν	F	4	$13.7 \pm 1.8$	$3.85 \pm 1.41$
		М	9	$12.9 \pm 1.3$	
	S	F	2	$13.1 \pm 1.5$	$3.62 \pm 1.16$
		Μ	8	$12.8\pm1.2$	
2	Ν	F	4	$14.5\pm1.8$	$3.23 \pm 1.42$
		М	10	$15.4 \pm 1.3$	
	S	F	2	$14.6 \pm 1.3$	$3.51 \pm 3.03$
		Μ	14	$16.6 \pm 3.1$	

Table 2. Models applied to isotope data from *Doryteuthis gahi* gladius sections, numbered for easy reference and with expressions included.  $f_2(DML_i)$ : Season<sub>i</sub>: one smoother is used for each fishing season and season is fitted as a mean term;  $C_{ij}$ : carbon values of DML *j* within squid *i* (random effect);  $a_j$ , random intercept for each DMLj;  $\varepsilon_i$ : ~ N(0, $\sigma^2$ ). DML: dorsal mantle length. AIC = Akaike's Information Criterion.

Response	MN	Expression	df	<b>R</b> <sup>2</sup>	AIC
				(adj.)	
С	1	$C_i \sim f_1(Lat_i) + f_2(DML_i)$ : Season <sub>i</sub> + Sex <sub>i</sub> + $\varepsilon_i$	10	0.67	1595.7
	2	$C_i \sim f_1(Lat_i) + f_2(DML_i)$ : Season <sub>i</sub> + Sex <sub>i</sub>	11	0.63	1410.7
		$+ a_j + \varepsilon_i$			
	3	$C_{ij} \sim f_1(Lat_i) + f_2(DML_i)$ : Season <sub>i</sub> + $a_j + \varepsilon_{ij}$	10	0.63	1408.1
	4	$C_{ij} \sim f_1(DML_{i_i})$ : Season <sub>i</sub> + $a_j$ + $\varepsilon_{ij}$	8	0.63	1400.4
N	5	$N_i \sim f_1(Lat_i) + f_2(DML_i)$ : Season <sub>i</sub> + Sex <sub>i</sub>	10	0.51	2070.8
		$+ \varepsilon_i$			
	6	$N_{ij} \sim f_1(Lat_i) + f_2(DML_i)$ : Season <sub>i</sub> + $\varepsilon_i$	9	0.51	2065.4
	7	$N_{ij} \sim f_1(Lat_i) + f_2(DML_i)$ : Season <sub>i</sub> + $a_j + \varepsilon_{ij}$	10	0.46	1828.8
	8	$N_{ij} \sim f_1(DML_i)$ : Season <sub>i</sub> + $a_j$ + $\varepsilon_{ij}$	8	0.46	1822.9

Table 3. Estimated regression parameters for mixed additive models for the response variables  $\delta^{13}$ C and  $\delta^{15}$ N. F and p-values are included, as well as expected degrees of freedom (edf) for smooth terms. Both models were corrected for temporal autocorrelation using an AR1 structure

Response	Fixed Covariate	edf	F-value	P-value
С	Season	-	60.77	< 0.001
	DML:Season1	5.58	14.83	< 0.001
	DML:Season2	8.11	42.04	< 0.001
Ν	Season	-	25.08	< 0.001
	DML:Season1	5.81	30.46	< 0.001
	DML:Season2	8.33	40.70	< 0.001



Figure 1. Falkland Islands Conservation and Management Zone (FICZ, thick black line) with shaded grid squares indicating the designated fishing area - the 'Loligo box' - and its northern (dark grey) and southern (light grey) sub-areas. White points are sampling sites.



Figure 2. Schematic dorsal view of *Doryteuthis gahi* gladius and its position relative to the mantle. The grid represents the 1 cm sections collected from the posterior end (oldest material representing juvenile stages) to the anterior end (region of most recent growth representing adult stages).



Figure 3. Bi-plot of  $\delta^{13}$ C and  $\delta^{15}$ N of all *Doryteuthis gahi* gladii sections. Standard ellipse areas (SEAs) indicate the isotopic niche of *D. gahi* by fishing season and sub-area. S1: Season 1 (February–May); S2: Season 2 (July–October), N (S): are the north (south) sub-area of the designated fishing zone.



Figure 4. Correlation of trophic level with dorsal mantle length (DML, which is analogous to section of gladius) taken from *Doryteuthis gahi*, with LOESS smoothers (span = 0.6) fitted for each fishing season (for seasons see Fig. 3).



Figure 5. Ontogenetic trajectories of  $\delta^{13}$ C (‰) by DML for fishing season and sub-area for each individual squid. LOESS smoothers for each combination of fishing season (see Fig. 3) and sub-area (see Fig. 1) are included (black line). Shaded envelopes: 95% CI. Span = 0.4



Figure. 6. Ontogenetic trajectories of  $\delta^{15}N$  (‰) by dorsal mantle length (DML, which is analogous to section of gladius) for each individual squid. LOESS smoothers for each combination of fishing season and sub-area are included. Span = 0.4.



Figure 7. Fitted curves based on model predictions for values of (a)  $\delta^{13}$ C and (b)  $\delta^{15}$ N by DML, with individual smoothing curves for each fishing season and 95% confidence intervals represented by shaded envelopes

#### Supplementary Material

Model validation for the optimum generalized additive mixed models applied to the response variables C ( $\delta^{13}$ C) and N ( $\delta^{15}$ N). Fitted values were plotted against residuals to ensure no residual patterns were evident.



Figure S1. Model validation for the optimum generalized additive mixed models applied to the response variable C ( $\delta^{13}$ C). Fitted values were plotted against residuals to ensure no residual patterns were evident.



Figure S2. Model validation for the optimum generalized additive mixed models applied to the response variables N ( $\delta^{15}$ N). Fitted values were plotted against residuals to ensure no residual patterns were evident.