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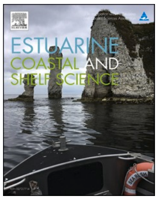

Trophic ecology of the squid *Doryteuthis gahi* in the Southwest Atlantic inferred from stable isotope analysis

Tobias Büring [a,d,*], Jessica B. Jones [b], Graham Pierce [c], Francisco Rocha [d], Paco Bustamante [e,f], Maud Brault-Favrou [e], Alexander I. Arkhipkin [a]

[a] *Falkland Islands Fisheries Department, United Kingdom*
[b] *Scottish Wildlife Trust, Harbourside House 110 Commercial St, Edinburgh, Scotland, United Kingdom*
[c] *Instituto de Investigacións Mariñas, Spain*
[d] *Universidade de Vigo, Departamento de Ecología y Biología Animal, Campus de Vigo As Lagoas-Marcosende, 36310, Vigo, Spain*
[e] *LIttoral, Environnement and Societés (LIENSs), UMR 7266 CNRS – La Rochelle Université, 17000, La Rochelle, France*
[f] *Institut Universitaire de France (IUF), 1 rue Descartes, 75005, Paris, France*

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ABSTRACT

The Patagonian longfin squid *Doryteuthis gahi* has an annual life cycle with two seasonal cohorts (autumn and spring spawners). Previous studies estimated the trophic level of adult squid by stable isotope analysis of their muscle or gladius tissues, but few studies compared different sizes and spawning cohorts. In this study, mixed models were used to compare carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotope signatures of muscle and gladius tissues of the autumn and spring spawning cohorts and squid of different sizes. A published isoscape model provided δ^{15}N baselines to calculate the trophic level of *D. gahi*. Sampled herbivorous prey such as Euphausiacea, planktonic juveniles *Munida gregaria* and salps *Ihlea* supported the trophic level estimates. The autumn spawning cohort had higher δ^{13}C values than the spring spawning cohort. δ^{15}N values increased with increasing dorsal mantle length, suggesting an ontogenetic change towards larger prey. Muscle tissue was enriched in ^{15}N but slightly depleted in ^{13}C compared to gladius tissue. Adult squid had an estimated trophic level of ~3.2, with no significant differences between spawning cohorts. Stable isotope values followed a seasonal trend, with lower δ^{15}N values and lower δ^{13}C value between July and September. Large individuals of the spring spawning cohort were found to have the least niche overlap with other groups of *D. gahi*. Our study of the trophic levels and ecology of *D. gahi* can provide important information on its role in the food chain and can build the basis of studies on interaction with other species, and the impact of environmental changes on their populations. This information can inform the development of management strategies aimed at preserving the balance of the ecosystem and ensuring its sustainable use.

1. Introduction

Squid are one of the main components of marine food webs linking primary consumers and marine top predators (Coll et al., 2013; Gasalla et al., 2010; Navarro et al., 2013). They are active predators with their diet ranging from small zooplankton to cephalopods and fish (Guerra et al., 1991; Markaida and Sosa-Nishizaki, 2003; Pierce et al., 1994; Rodhouse and Nigmatullin, 1996). Identification of prey items in stomach contents of squid is difficult as squid macerate their food before ingestion and in some cases might reject identifiable parts such as fish heads (Porteiro et al., 1995), complicating classical stomach content analysis (Boyle and Rodhouse, 2005). As a result, alternative methods such as serological analysis (Boyle and Rodhouse, 2005; Grisley and Boyle, 1985, 1988) or stable isotope analysis (SIA) have been used to support stomach content analysis to determine the diet and trophic levels of squid (Navarro et al., 2013; Ruiz-Cooley et al., 2004, 2006). Specifically, nitrogen (^{15}N/^{14}N) and carbon (^{13}C/^{12}C) ratios denoted as δ, can be used to study the ecotrophic niche of a species (Bearhop et al., 2004). Carbon isotope ratios change relatively little per trophic level in marine ecosystems, on average between 0.4 and 1.3‰ (McCutchan et al., 2003). Different sources of carbon fixation in primary producers can lead to larger differences in δ^{13}C within the ecosystem (Fry, 2006),

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* Corresponding author. Falkland Islands Fisheries Department, United Kingdom.
E-mail address: TBuring@naturalresources.gov.fk (T. Büring).

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and it is thus essential to know the "baseline" carbon isotope ratio. The main differences in $\delta^{13}C$ have been found between coastal and offshore waters, with the latter normally being more depleted in ^{13}C (France, 1995; Miller et al., 2008), between benthic and pelagic ecosystems due to different food carbon sources (Fry, 2006) and between lower and higher latitudes (Jaeger et al., 2010). A retention of the heavier nitrogen isotope (^{15}N) in biological processes compared to the lighter form (^{14}N) leads to an average enrichment of around 3.4‰ $\delta^{15}N$ per trophic level (Minagawa and Wada, 1984), although higher trophic levels might experience greater variations (Ruiz-Cooley et al., 2021). As the range of $\delta^{15}N$ values in seawater can vary between 0 and 12‰ (i.e., higher than the enrichment factor per trophic level; Somes et al., 2010), it is also necessary to adjust the estimate of the trophic level based on the $\delta^{15}N$ values in primary producers or consumers (Cabana and Rasmussen, 1996; Navarro et al., 2013). Instead of taking the of $\delta^{15}N$ values in primary producers in their region into account, some studies have relied upon literature values to estimate trophic level of primary producers, which could lead to unreliable estimates (Rosas-Luis et al., 2017). Furthermore, the use of SIA does not allow to identify single prey species and is dependent on mixing models including reference species from the same area (Phillips et al., 2014), which makes it advisable to use a combination of SIA and classical stomach content analysis.

Within an organism, each tissue type has a different rate of turnover. Muscle and liver tissue have a turnover rate of days to weeks, whereas hard structures such as calcified tissues metabolise much slower and therefore have a lower turnover rate of months to years (Sweeting et al., 2005). The trophic position of a species can therefore be studied over a longer time period by using SIA compared to stomach content analysis, which reflects the diet just prior to capture (Post, 2002; Rosas-luis et al., 2016). In squid, the gladius can be used to study the species diet over a longer time span, but the youngest piece (anterior end) of the gladius can give information about the recent trophic level of the squid (Rosas-Luis et al., 2017; Ruiz-Cooley et al., 2010).

The Patagonian longfin squid *Doryteuthis gahi* (Loliginidae) is an abundant medium-sized squid distributed along the Patagonian Shelf of the Southwest Atlantic and in the Southeast Pacific (Jereb and Roper, 2010). The population structure of *D. gahi* in Falkland Islands waters consists of two main annual cohorts characterized by different seasons of spawning – the autumn-spawning cohort (ASC) and the spring-spawning cohort (SSC). Hence, the same ontogenetic phases of squid from each cohort lives in different seasons and in different habitats, experiencing different environmental conditions, such as different temperature regimes and depth distributions (Arkhipkin et al., 2004; Jones et al., 2018). These environmental conditions also apply to the prey species of *D. gahi* and therefore the diet differs between the two cohorts (Büring et al., 2022).

D. gahi is an active predator, feeding on planktonic crustaceans, chaetognaths, fish and squid (Büring et al., 2022; Guerra et al., 1991; Rosas-Luis et al., 2014). The most important prey species throughout the whole lifecycle of *D. gahi* are Euphausiacea, such as *Euphausia lucens* (Büring et al., 2022; Guerra et al., 1991; Rosas-Luis et al., 2014). *E. lucens* is typically herbivorous but can also switch to a diet consisting of copepods when the concentration of algae decreases (Gibbons et al., 1991). Another important prey species of *D. gahi* is the planktonic stages of the lobster krill *Munida gregaria* (Brickle et al., 2002; Büring et al., 2022).

Previous studies found $\delta^{15}N$ values in the gladius of *D. gahi* ranged from 6 to 12‰ and $\delta^{13}C$ values between −23‰ and −19‰, which were comparable with isotopic values of *Illex argentinus* (Rosas-Luis et al., 2016, 2017). According to Rosas-Luis et al. (2016) the trophic level of *D. gahi* was estimated based on *Salpa thompsoni* values (Perissinotto and Pakhomov, 1998), resulting in an unrealistic trophic level of ~5. This is higher than the trophic levels of much larger squid species, such as the giant squid *Architeuthis dux* (trophic level 4.6) and the colossal squid *Mesonychoteuthis hamiltoni* (trophic level 6.1) (Cherel et al., 2008; Guerreiro et al., 2015). However, the studies by Rosas-Luis et al. (2016, 2017) were based on limited samples and a lack of baseline information, making it impossible to conduct a comprehensive analysis of ontogeny or comparisons between spawning cohorts.

The present study aims to investigate the trophic niche of different size groups of both cohorts of *D. gahi* by analysing $\delta^{13}C$ and $\delta^{15}N$ values of muscle tissue for a short-term signal and the anterior end of the gladius for a medium-term signal based on recent nitrogen baseline values. Furthermore, we compared muscle with gladius tissues and modelled the relationship between the tissue types. The aim is to differentiate between the trophic niches and trophic levels of the two spawning cohorts of *D. gahi* and to gain deeper insights into differences of their feeding ecology.

2. Material and methods

2.1. Sample collection and processing

D. gahi specimens (n = 269) were collected throughout 2020 in waters around the Falkland Islands as well as from international waters at latitude 45°S aboard bottom trawling fishing vessels and during two research cruises, which used also bottom trawling gear (Fig. 1). In addition, zooplankton samples were collected at three stations during the research cruises and onboard a small vessel near Stanley Harbour (see section 'Zooplankton samples').

The depth range of sampling stations for *D. gahi* was 100–400 m with a median of 170 m. The mean sampling depth of the ASC was 165 m, while the mean sampling depth of the SSC was 199 m. Samples were divided into five areas: North (<52° South), East (<58° West), South (>52° South), West (>61° West) and High Seas (North of the FICZ; the spatial distribution of samples is addressed in the statistics section). Areas accounting for the different current regimes, such as the South and East are influenced by the Falkland/Malvinas Current, whereas West, North and 'Out of Zone' are influenced by the Argentine drift (Acha et al., 2004; Thorpe, 2009). Further subdivisions were made to account for stock assessment models of *D. gahi* from the Fisheries Department (Winter, 2021a, 2021b). Individuals were frozen and brought to the Falkland Islands Fisheries Department laboratory for further analysis.

Once defrosted, dorsal mantle length (DML ±0.5 cm) and total body mass (TW ±0.1 g) were measured and individuals were visually assessed for sex and maturity stages 1–5 (Lipinski, 1979, Table 1). The individuals for the study were selected from random samples of 100 individuals, with the aim to collect equal amounts of each size class, sex and from various regions throughout the year. Therefore, 2 small, 2 medium and 2 large individuals belonging to each sex were taken if

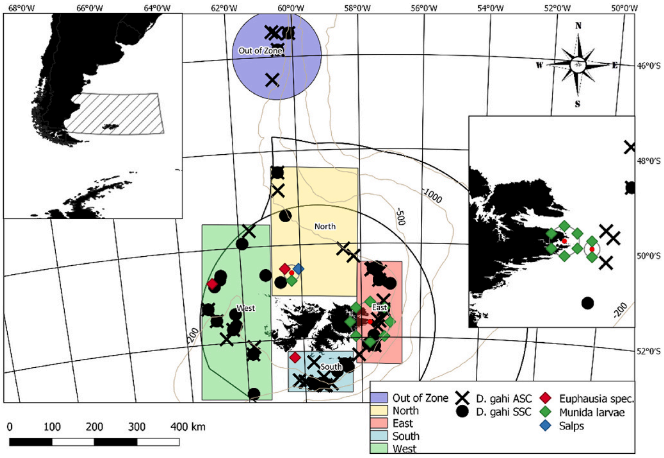

Fig. 1. Overview map (top left) and sampling area of *Doryteuthis gahi* samples (autumn spawning (ASC) and spring spawning cohort (SSC)) and zooplankton samples around the Falkland Islands, with Falkland Islands inner (FICZ) and outer (FOCZ) conservation zones; sampling areas shaded.

Table 1
Number of individuals sampled, mean DML in cm and standard deviation per tissue type summarised by spawning cohorts (autumn spawning (ASC) and spring spawning cohort (SSC)) and sex (males and females); *gladius tissue was taken from the same individuals which were sampled for muscle tissue.

Type	Cohort	Sex	Length	n*
Gladius	ASC	F	10.63 ± 2.54	34
		M	13.35 ± 6.38	37
	SSC	F	10.97 ± 3.53	25
		M	15.15 ± 6.85	28
Muscle	ASC	F	11.07 ± 3.09	78
		M	13.10 ± 5.75	79
	SSC	F	11.32 ± 3.86	49
		M	14.54 ± 6.48	63

available. Individuals were assigned to each spawning cohort using their DML, maturity and month of collection. For individuals sampled in February, the depth in which they were caught was also taken into account (large ASC individuals occur in deeper waters, whereas small immature SSC individuals occur in shallower waters), based on findings by Arkhipkin et al. (2004; 2013).

A 1 cm^2 piece of muscle tissue was dissected from the mantle at the dorsal anterior part of the body (Fig. 2). The skin was removed, the tissue was flushed with distilled water and stored dry in an Eppendorf tube. For the gladius, a subsample from the whole sample (n = 124) was taken, which was chosen to ensure a range of DML and that both sexes were included. These samples were cleaned with distilled water, measured for total gladius length (±0.1 cm) and then cut into 5 pieces of equal length from the posterior part (i.e., the oldest material representing the juvenile stage) to the anterior part (i.e., the youngest material representing the adult stage) (Arkhipkin et al., 2012); and the wing sections were removed (Fig. 2).

Only the anterior part of the gladius was used for further analysis. To follow the direction of the growth increments, sections were cut in a V-shape (Lorrain et al., 2011) and stored dry in Eppendorf tubes.

2.2. Zooplankton samples

To allow for comparisons of $\delta^{15}N$ values from this study with other ecosystems and studies, and to incorporate seasonal and spatial variability in the $\delta^{15}N$ of small organisms responsible for the ecosystem N baseline in the year of sampling 2020, the approach by Cabana and Rasmussen (1996) was followed.

1. Larval and early juvenile planktonic *Munida gregaria* were collected out of zooplankton samples taken on board the *PV Protegat* during February 2020 with a 500 µm mesh size Bongo net from depths between 50 and 200 m and a tow time of approximately 30 min.
2. *M. gregaria* planktonic juveniles were sampled onboard the SAERI (South Atlantic Environmental Research Institute) RV *Jack Sollis* near Stanley Harbour with a 350 µm mesh Bongo net during February, September and November 2020 in a depth of around 5 m with a tow time of 10 min.
3. In February and June 2020, salps of the genus *Ihlea*, Euphausiacea, Chaetognatha and Amphipoda were collected on board the *FV Castelo* with a 500 µm Isaacs-Kidd plankton net by horizontal trawling (20–150 m depth). Single plankton species were identified using an identification key (Boltovskoy, 1999), but salps couldn't be identified to species level due to the sample condition (damaged).

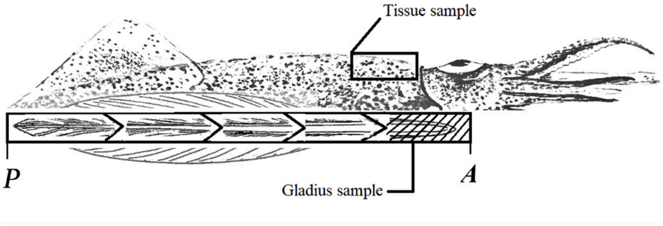

Fig. 2. Schematic view of *Doryteuthis gahi* with position of the gladius. P = Posterior part (oldest part representing juvenile stage), A = Anterior part (youngest part representing adult stage); locations of the tissue sample and the gladius sample are included.

Individuals of each species were pooled and species were stored separately to obtain average $\delta^{15}N$ baseline data from the ecosystem of the Falkland Islands.

2.3. Stable isotope measurements

Zooplankton samples, muscle tissue and gladius fragments of *D. gahi* were oven dried for 24 h at 80 °C. Samples were then ground into a fine homogenous powder with a porcelain pestle and mortar prior to sending them to LIttoral, ENvironnement and Societés (LIENSs) Joint Research Unit stable isotope facility (CNRS – La Rochelle Université, France) for further processing. Each sample was then weighed into a tin container using a Sartorius M5 microbalance (±1 µg). The ratios of the heavier ^{13}C and ^{15}N to the lighter ^{12}C and ^{14}N, respectively, as well as the C/N ratio 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). Results are expressed in the δ unit notation as per mil (‰) deviation from the international standards Vienna-Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$). Isotope values are reported as $\delta^{13}C$ or $\delta^{15}N$ values:

$$\delta R_{sample} = \left[\left(R_{sample} \big/ R_{standard}\right) - 1\right] \times 10^3 \qquad \text{(eq.1)}$$

where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively.

The analytical precision was ±0.10‰ for $\delta^{13}C$ and ±0.15‰ $\delta^{15}N$ based on internal standards USGS-61 and USGS-62 inserted every ten measurements.

The trophic level was calculated with two methods:

1. With the equation provided by different studies (Cabana and Rasmussen, 1996; Stowasser et al., 2012; Sweeting et al., 2005) trophic level was calculated using the formula:

$$TL_{consumer} = \left[\left(\delta^{15}N_{consumer} - \delta^{15}N\ \begin{matrix} primary \\ consumer \end{matrix}\right) \Big/ 3.4\right] + 2 \qquad \text{(eq.2a)}$$

where 3.4 represents an average enrichment in ^{15}N per trophic level (Minagawa and Wada, 1984) and 2 is the reference value for trophic position 2 (Cabana and Rasmussen, 1996; McCutchan et al., 2003; Montecinos et al., 2016; Stowasser et al., 2012).

2. We used the isoscape data provided by Glew et al. (2021), where a $\delta^{15}N$ baseline was modelled in R-INLA (integrated nested Laplace approximation) for the Southern Ocean, originally based on POM (particulate organic matter) measurements. Data was extracted from their first interaction model for each season and location of our samples and applied to our samples. With the modified eq. 2

$$TL_{consumer} = \left[\left(\delta^{15}N_{consumer} - \delta^{15}N\ \begin{matrix} primary \\ consumer \end{matrix}\right) \Big/ 3.4\right] + 1 \qquad \text{(eq.2b)}$$

where 1 represents trophic level 1 of POM, trophic levels were calculated for each sample. The isoscape model was subdivided into seasons: Jan–Feb, Mar–Apr, May–Oct and Nov–Dec, so we assigned our samples accordingly.

To find the relationship between gladius and muscle δ^{15}N values, a linear model was fitted to a subset of the data (n = 124), in which gladius and muscle tissue were measured for the same individual.

2.4. Statistical analysis

2.4.1. Generalized additive mixed effect models

All calculations, statistics and plots were made using R.4.03 (R Core Team, 2020). Generalized additive mixed effect models (GAMMs) were used in this study firstly because GAMs and GAMMs permit the inclusion of non-linear relationships (Hastie and Tibshirani, 1986), such as cyclicity over the year, and secondly to account for the spatial structure of the data (Zuur et al., 2009). To account for spatial patterns, samples were divided into five sampling areas. This 5-factor variable was incorporated as a random effect in the GAMMs. Maturity stages were pooled into 1–3 as immature/maturing and 4–5 as mature. We fitted Gaussian models (with an identity link function), which appeared to be reasonable for the SI data. Models with all possible combinations of explanatory variables such as DML (continuous), day (continuous; representing day of the year ranging from 1 to 365), maturity (two level factor) and sex (two level factor) and including all biologically relevant two-way interactions between effects of all pairs of explanatory variables were compared based on their Akaike Information Criterion (AIC). Four separate GAMMs (eq. 5a to 5d) with a Gaussian distribution and an identity link were chosen to investigate the relationship between stable isotope values (C and N) for each tissue type (muscle and gladius), day of the year on which the squid were sampled and DML of the squid.

$$gamm\left(\delta^{15}N_{muscle}\ \sim\ s(DML)+s(Day, bs=\mathrm{cc}), random=list(Area=\ \sim 1)\right) \tag{eq.3a}$$

$$gamm\left(\delta^{15}N_{gladius}\ \sim\ ti(DML, Day, bs=(\mathrm{cs},\mathrm{cc}))+s(DML)+s(Day, bs=\mathrm{cc}), random=list(Area=\ \sim 1)\right) \tag{eq.3b}$$

$$gamm\left(\delta^{13}C_{muscle}\ \sim\ ti(DML, Day, bs=(\mathrm{cs},\mathrm{cc}))+s(DML)+s(Day, bs=\mathrm{cc}), random=list(Area=\ \sim 1)\right) \tag{eq.3c}$$

$$gamm\left(\delta^{13}C_{gladius}\ \sim\ ti(DML, Day, bs=(\mathrm{cs},\mathrm{cc}))+s(DML)+s(Day, bs=\mathrm{cc}), random=list(Area=\ \sim 1)\right) \tag{eq.3d}$$

where *s()* describes a smoothing term for a continuous variable; *ti()* describes an interaction between two continuous variables in form of a tensor; *bs* = represents the regression cubic splines in which ‘cc’ stand for cyclic cubic splines, whereas ‘cs’ are cubic splines which represent knots spread evenly through the covariate values; *random* = represents the random effect of the model, in this case ~ Area (the list attribute is used for coding).

The smoother day of the year was chosen, as it represents the seasonal occurrence of the two spawning cohorts. For each combination of C and N of muscle and gladius tissue (4 combinations), the model with the lowest AIC was chosen, removing non-significant co-variates and interactions. The significance of the interaction between the two continuous explanatory variables was quantified using a tensor rather than a ‘three-dimensional smoother’. We used a tensor, because it permits separate evaluation of the significance of the interaction and because of the cyclicity of the factor day of the year (day 1 follows day 365) and tensors can be used with cyclic cubic splines, i.e. penalised cubic regression splines whose ends meet up. We used the *ti()* function rather than the *te()* function from the R package ‘mgcv’, as *ti()* is appropriate when the main effects are to be included separately from the tensor product interaction (Wood, 2017; Zuur et al., 2009). If the interaction was determined as significant, for visualisation purposes, we reran the models, using the *te()* function. The *te()* function incorporates the main effects, also then deleting the separate terms for the two main effects, allowing us to visualise the combined effect of DML and day of year – this is technically still the same model.

Table 2

Overall model performance (R-sq.) and smoothers of the fixed effects of the GAMMs for δ^{15}N and δ^{13}C for each tissue type muscle and gladius; edf = expected degrees of freedom, F value and p-value.

Isotope	Tissue	Model	R-sq. (adj)	smoother	edf	F	p-value
δ^{15}N	Muscle	Eq. 3a	0.08	s (DML)	4.2	6.44	<0.001
				s (Day)	2.1	1.16	0.005
	Gladius	Eq. 3b	0.30	ti (DML, Day)	3.1	1.47	<0.001
				s (DML)	4.5	5.00	0.003
				s (Day)	2.4	3.39	<0.001
δ^{13}C	Muscle	Eq. 3c	0.51	ti (DML, Day)	5.4	4.17	<0.001
				s (DML)	1.0	6.50	0.011
				s (Day)	6.3	31.12	<0.001
	Gladius	Eq. 3d	0.78	ti (DML, Day)	5.4	3.48	<0.001
				s (DML)	5.1	4.14	0.002
				s (Day)	4.7	20.02	<0.001

Another simpler GAM was used to analyse the effect of seasonality on trophic levels assigned with isoscapes of both tissue types, using day of the year as continuous variable, ‘Tissue’ as a 2 level factor and a cyclic cubic spline:

$$gam(TL\ \sim\ s(Day, by=Tissue, bs=\mathrm{cc})) \tag{eq.4}$$

All GAM(M)s were performed using the R package ‘mgcv’ (Wood, 2017). Plots were made using the R package ‘ggplot2’ (Wickham, 2016).

2.4.2. Trophic niche

Data were split into two spawning cohort groups, for individuals belonging to the ASC and SSC and into three size-classes (small ≤8 cm; medium = 8–19 cm; large = ≥20 cm) to make inter-seasonal comparisons as in Büring et al. (2022). These groups (spawning cohorts and size groups) were used to estimate trophic levels and trophic niches, following a Bayesian multivariate model approach, performed using the R package ‘SIBER’ v.2.1.6 (Jackson et al., 2011) and ‘nicheROVER’ (Lysy et al., 2021). Number of Monte Carlo draws (iterations) was set to 1000 and alpha to 95%. Trophic niche in this context is referring to the SI niche when analysing δ^{13}C and δ^{15}N values together (Bearhop et al., 2004).

3. Results

For $N_{Gladius}$, C_{Muscle} and $C_{Gladius}$ (eqs. (3b)–(3d)) an interaction between DML and day of the year was found to improve model fit (AIC; Table S1 supplementary material). Day of the year and DML were the most important co-variates for both muscle and gladius carbon and

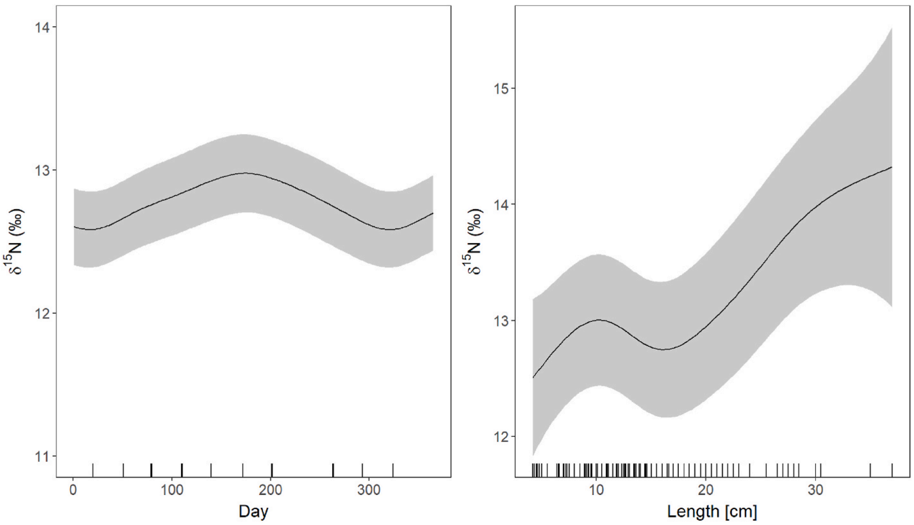

Fig. 3. Estimated smoothing curves from the GAMM for δ^{15}N values of muscle tissue vs. DML [cm] and day of the year; grey shaded area representing 95% confidence interval.

nitrogen signatures (4 separate models; eqs. (3a) to (3d)), whereas sex and maturity did not improve the model fit. δ^{15}N values of muscle tissue were significantly affected by DML and day of the year (GAMM, Table 2), but an interaction between these co-variates did not seem to improve model fit, with an AIC of 667 vs an AIC of 664 in (Table S1, supplementary material), therefore the simplest model specification eq. (3a) was chosen.

3.1. δ^{15}N values

3.1.1. Muscle tissue

δ^{15}N values increased towards austral winter months (Jun–Aug) and were the lowest during austral summer months (Nov–Feb) and increased with increasing DML (Fig. 3). The effects of day of the year and DML were both significant ($p < 0.001$, GAMM smoother, Table 2), even though deviance explained by the model was only 8%. P-values of GAMs and GAMMs are only approximate and should be interpreted with care (Zuur et al., 2009).

3.1.2. Gladius tissue

Like muscle tissue, δ^{15}N values in gladius tissue were significantly affected by DML and day of the year, explaining 30% of the deviance (Table 2). In gladius tissue, a pronounced positive trend of increasing δ^{15}N values with DML could be observed (Fig. 4B) and the interaction of DML with day of the year was found to be significant (Table 2, Fig. 4C).

We reran the model with the *te()* smoother to account for the significant interaction.

$$gamm\left(\delta^{15}N_{gladius} \sim te(DML, Day, bs = (\mathrm{cs}, \mathrm{cc})), random = list(Area = \sim 1)\right) \tag{eq.5a}$$

According to the model (eq. (5a)), small ASC individuals in February had higher δ^{15}N values compared to small SSC individuals in September (Fig. 4D). Large individuals of the ASC (found in July–August) had higher δ^{15}N values later on in the year compared to large SSC individuals (found in December–January).

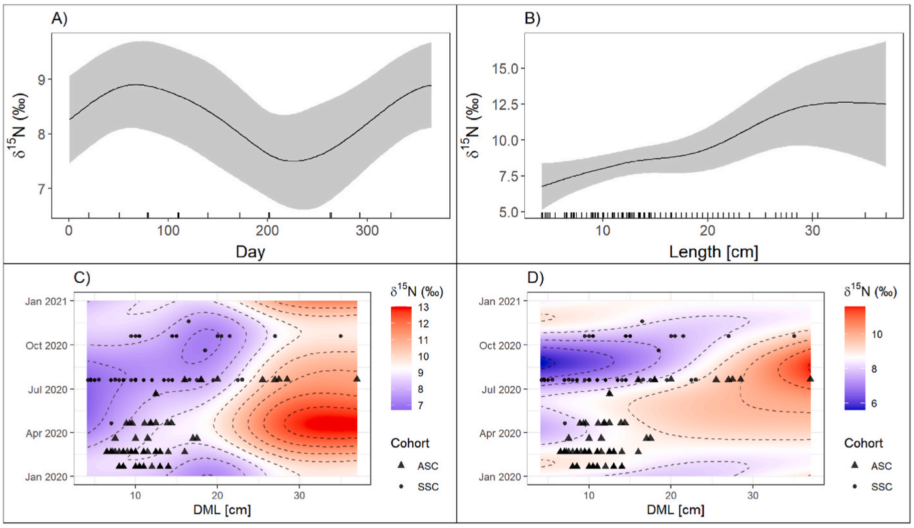

Fig. 4. Estimated smoothing curves from the GAMM for δ^{15}N values of gladius tissue vs. A) DML [cm] and B) day of the year; C): ti-tensor (interaction excluding main effects) of day of the year and DML [cm]; D): te-tensor (interaction including main effects) of day of the year and DML [cm]; triangles = ASC, circles = SSC.

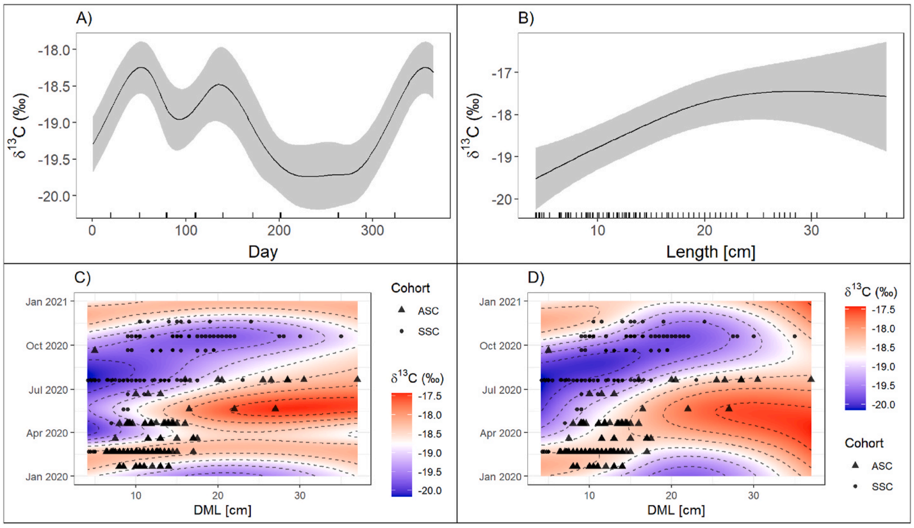

Fig. 5. Estimated smoothing curves from the GAMM for δ^{13}C values of muscle tissue vs. A) DML [cm] and B) day of the year; C): ti-tensor (interaction excluding main effects) of day of the year and DML [cm]
D): te-tensor (interaction including main effects) of day of the year and DML [cm]; triangles = ASC, circles = SSC.

3.2. δ^{13}C values

3.2.1. Muscle tissue

Similar to the model testing N of muscle tissue, the GAMM revealed that δ^{13}C values in muscle tissue were influenced by DML and day of the year (Table 2), explaining 52% of the deviance. δ^{13}C values were the lowest in Sep–Nov (austral spring), highest in Feb–Mar (austral autumn), and steadily increased with increasing DML. The interaction between DML and day of the year was very pronounced ($p < 0.001$, GAMM, Table 2, Fig. 5C), therefore we reran the model (eq. (5b)).

$$gamm\left(\delta^{13}C_{muscle} \sim te(DML, Day, bs = (\text{cs}, \text{cc})), random = list(Area = \sim 1)\right) \tag{eq.5b}$$

Small individuals (~10 cm) of the ASC in February had higher δ^{13}C values (~-18‰) compared to small SSC individuals around September (~-20‰; Fig. 5D).

Large individuals (~20–30 cm DML) of both cohorts were found to have higher δ^{13}C values in the latter part of the year (Fig. 5D). The highest δ^{13}C values of approximately −17‰ δ^{13}C could be found in May in individuals ~30 cm DML.

3.2.2. Gladius tissue

The δ^{13}C values of gladius tissue were significantly influenced by DML and day of the year ($p < 0.001$, GAMM, Table 2). The trends observed in the model were similar to δ^{13}C values of muscle tissue. In contrast to muscle tissue, the δ^{13}C values in the gladius did not follow a

Table 3
Mean δ^{13}C and δ^{15}N values (‰) with standard deviation and number of samples of Euphausiacea, *Munida gregaria* and salps.

	δ^{15}N	δ^{13}C	TL (based on Isoscapes)	N
Euphausia lucens	8.04 ± 1.22	−20.61 ± 0.39	1.81 ± 0.43	3
Munida gregaria	8.80 ± 1.28	−20.24 ± 0.73	2.62 ± 0.61	9
Salps	8.79	−21.88	1.91	1
Total	8.61 ± 1.2	−20.47 ± 0.77	2.38 ± 0.65	13

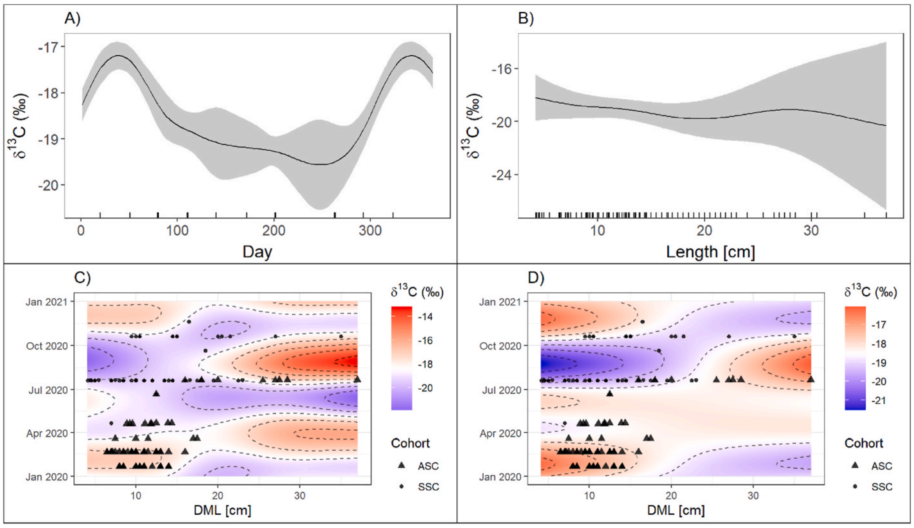

Fig. 6. Estimated smoothing curves from the GAMM for δ^{13}C values of gladius tissue vs. A) DML [cm] and B) day of the year; C): ti-tensor (interaction excluding main effects) of day of the year and DML [cm]
D): te-tensor (interaction including main effects) of day of the year and DML [cm]; triangles = ASC, circles = SSC.

certain trend with increasing DML (Fig. 6B), but a strong interaction between DML and day of the year ($p < 0.001$, Table 2) was found (Fig. 6C). Therefore, the model was reran with the *te()* function (eq. (5c)).

$$gamm\left(\delta^{13}C_{gladius} \sim te(DML, Day, bs = (\mathrm{cs}, \mathrm{cc})), random = list(Area = \sim 1)\right) \quad \text{(eq.5c)}$$

Individuals belonging to the ASC, found mainly during February to May, had higher δ^{13}C values (~-18 to −17‰) compared to SSC individuals found between June and December (~-20 to −18‰, Fig. 6D).

3.3. Zooplankton δ^{15}N and δ^{13}C values

Euphausiacea, salps and larval *M. gregaria* were assumed to rank between primary and secondary consumers (Castro et al., 2021; Gibbons et al., 1991; Perissinotto and Pakhomov, 1998). Values of δ^{15}N of *M. gregaria* ranged from 7.64 to 11.45‰ (Table 3).

The mean δ^{15}N of the 8 pooled samples of *M. gregaria* was 8.8‰. When adding values from Euphausiacea ($n = 3$) and the value from the pooled salp individuals ($n = 1$), the average δ^{15}N was 8.61. This value as the first method was used as the N baseline value to calculate the trophic level of *D. gahi* using eq. (2a). However, the trophic levels of zooplankton based on isoscapes suggested, that the *M. gregaria* was above trophic level 2 and the average zooplankton baseline slightly above 2. Therefore, we continued our analysis with trophic levels calculated from the isoscape model, which was based on particulate organic matter.

3.4. Trophic level based on isoscapes

The linear equation ($R^2 = 0.458$) for the relationship between δ^{15}N muscle tissue and δ^{15}N gladius tissue was found to be:

$$N_{muscle} = 9.70 + 0.363 * \delta^{15}N_{gladius} \quad \text{(eq.6)}$$

with eq. (6) corrected δ^{15}N values were calculated for gladius tissue.

Using the δ^{15}N baseline values from zooplankton (Fig. 7) and isoscapes, trophic level for each *D. gahi* individual, each cohort and each size class were calculated. The mean δ^{15}N for muscle tissue of adult (mature) *D. gahi* individuals was 12.74 (±1.09) ‰, for ASC 12.83 (±1.03) ‰ and for SSC 12.68 (±1.14) ‰. The mean trophic level of adult squid based on zooplankton and muscle tissue was 3.23 (±0.34), for ASC 3.26 (±0.32) and for SSC 3.21 (±0.36), whereas based on isoscapes it was 3.47 (±0.46) overall, for the ASC 3.52 (±0.51) and 3.44 (±0.42) for the SSC. Trophic level increased with size but was similar between cohorts for each size class. Detailed information about δ^{15}N and trophic level for each cohort and size class per tissue type can be found in Table 4.

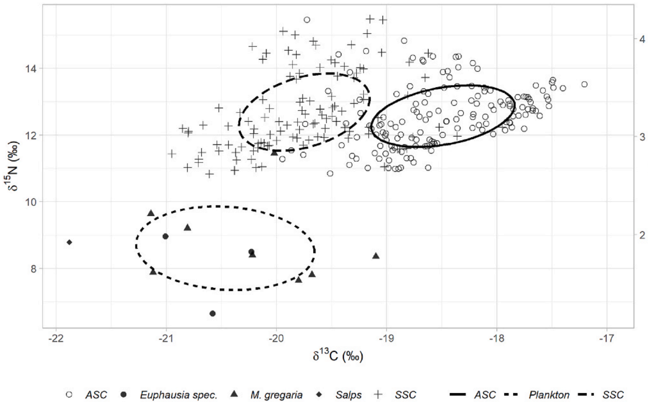

Fig. 7. Stable isotope niche of *D. gahi's* spawning cohorts (muscle tissue) with zoo-planktonic baseline (Euphausiacea, *M. gregaria* and salps).

The trophic levels of gladius tissue based on corrected δ^{15}N values and based on muscle tissue δ^{15}N values were calculated from the isoscape baselines (with eq. (2b)) and analysed (GAM eq. (4), Fig. 8). A clear trend could be seen in both tissues with trophic levels higher in austral winter months (Apr–Oct), although the trend was clearer to observe in muscle tissue.

3.5. Trophic niche based on SIA

Differences in stable isotope values between muscle and gladius tissue were observed. Gladius δ^{15}N values were on average 34.1% lower (8.43 ± 1.72‰) than muscle tissue (12.8 ± 0.88‰) and δ^{13}C values were 2.6% higher in the gladius (−18.60 ± 1.20‰) compared to muscle tissue (−19.1 ± 0.90‰; Fig. 8).

Muscle tissue values of δ^{13}C and δ^{15}N for each size-class and cohort clustered separately, mainly due to their different δ^{13}C values (Standard Ellipse Area, Fig. 9). δ^{13}C values clearly separated both cohorts, where the ASC had values above −19‰, whereas the SSC individuals mostly had values below −19‰. Both cohorts ranged between 11 and 14‰ for δ^{15}N. ASC values clustered around −17.5 and −19.5‰ for δ^{13}C but SSC values clustered around −19.5 and −20.5‰ for δ^{13}C and therefore had a slightly narrower range than the ASC.

Trophic niche of small and medium-sized squid based on muscle tissue overlapped for each spawning cohort (Fig. 9). Highest mean niche overlap in muscle tissue (Fig. 10, Table S2 supplementary material) had the group SSC medium with SSC small (95.77%) and with SSC large (92.58%). ASC small had a high overlap with SSC small (90.5%) and with ASC medium (88.52%). Least overlap had ASC large with SSC small (10.29%) and with SSC medium (14.21%), followed by ASC medium (16.32%) and ASC small (18.46%).

Like muscle tissue, the main difference in gladius tissue between cohorts was seen in the δ^{13}C values, whereas δ^{15}N values were similar in both cohorts. Gladius SSC values clustered between −20 and −18.5‰ for δ^{13}C and between 6 and 11‰ for δ^{15}N. ASC gladius values clustered between −18.5 and ~-16.3‰ for δ^{13}C. The δ^{15}N cluster of the ASC gladius values were narrower compared to the SSC with values between 8 and 11‰. Trophic niche as assessed by gladius tissue looked very similar to that based on muscle tissue, but with large SSC individuals having a wider niche in δ^{13}C and large ASC individuals having a narrower δ^{13}C niche (Fig. 9). Large SSC individuals had a large spread in δ^{15}N in both muscle and gladius tissues, from about 6 to 11‰.

Regarding the niche overlap in gladius tissue (Fig. 11, Table S2), the group ASC medium had the highest overlaps with ASC large (90.61%) and with ASC small (90.33%), followed by SSC medium overlapping with SSC small (77.61%) and ASC medium (75.03%). Least mean niche overlap was found again in ASC large with SSC mall (2.73%) and SSC medium (9.94%), followed by SSC small overlapping with ASC large (12.35%) and ASC small overlapping with SSC large (13.25%).

The standard ellipse areas (representing the isotopic niche) and the niche overlap estimated with Bayesian procedures clearly differed for squids of different size groups, with the group ASC large having least niche overlap with other groups in muscle and gladius tissue. Group SSC small had the largest niche overlaps in muscle tissue, whereas group ASC medium showed the largest niche overlaps in gladius tissue (Figs. 9–11).

4. Discussion

This study represents the first attempt to investigate the trophic niches of two seasonal cohorts of *Doryteuthis gahi* using stable isotope analysis, with samples of muscle to indicate recent diet (weeks) and samples of gladius to represent diet over a longer time-scale (months). Inclusion of the Area as a mixed effect factor improved the models, however we did not investigate further the spatial differences in this study and left them for further studies.

As expected, δ^{15}N and the related trophic level increased with increasing body size. This was shown in many other squid species such

Table 4
Mean δ^{13}C and δ^{15}N values (‰) and trophic level based on a) zooplankton and b) isoscapes per cohort, tissue (for gladius based on the enrichment factor E) and size-class of *D. gahi* with standard deviation (SD). N = number of individuals.

Cohort	Type	Size-class	Mean δ^{15}N ± SD	Mean δ^{13}C ± SD	Mean TL (Zooplankton)± SD	Mean TL (Isoscapes) ± SD	Mean DML ± SD	n
ASC	Muscle	small	12.61 ± 1.03	−18.66 ± 0.4	3.12 ± 0.3	3.43 ± 0.27	6.95 ± 0.75	12
		medium	12.46 ± 0.86	−18.51 ± 0.6	3.08 ± 0.25	3.44 ± 0.50	11.49 ± 2.35	133
		large	13.92 ± 0.43	−18.07 ± 1.21	3.51 ± 0.13	3.77 ± 0.43	26.6 ± 5.10	10
	Gladius	small	8.47 ± 0.89	−17.60 ± 0.64	3.17 ± 0.1	3.41 ± 0.36	7.03 ± 0.42	7
		medium	8.86 ± 1.23	−17.69 ± 1.00	3.21 ± 0.13	3.26 ± 0.55	11.57 ± 2.55	58
		large	10.09 ± 0.61	−17.87 ± 0.36	3.34 ± 0.06	3.27 ± 0.06	27.58 ± 5.51	6
SSC	Muscle	small	12.4 ± 1.13	−19.6 ± 0.82	3.06 ± 0.33	3.51 ± 0.27	5.67 ± 1.13	22
		medium	12.69 ± 1.09	−19.81 ± 0.51	3.14 ± 0.32	3.48 ± 0.41	13.25 ± 3.18	81
		large	13.25 ± 1.51	−19.61 ± 0.63	3.31 ± 0.45	3.40 ± 0.33	24.77 ± 4.77	11
	Gladius	small	7.29 ± 0.86	−19.37 ± 0.85	3.04 ± 0.09	3.44 ± 0.39	5.85 ± 1.26	11
		medium	7.64 ± 1.45	−19.29 ± 0.65	3.08 ± 0.15	3.33 ± 0.42	12.46 ± 3.14	35
		large	8.37 ± 2.18	−19.12 ± 1.27	3.15 ± 0.23	3.36 ± 0.38	24.21 ± 5.28	7

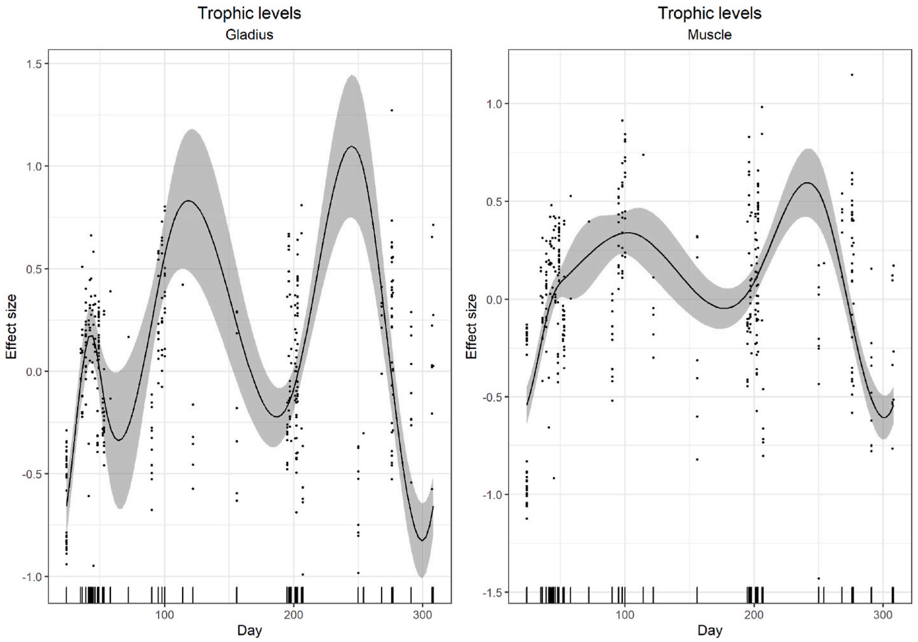

Fig. 8. Effect size of trophic levels of *D. gahi* over the year 2020 separate for gladius and muscle tissue
Smoothers for both tissue types were significant ($p < 0.001$, edf = 4.96 and edf = 4.87 resp.), as was the intercept (estimate = 3.43, $p < 0.001$) and 40.4% of the overall deviance was explained by the model.

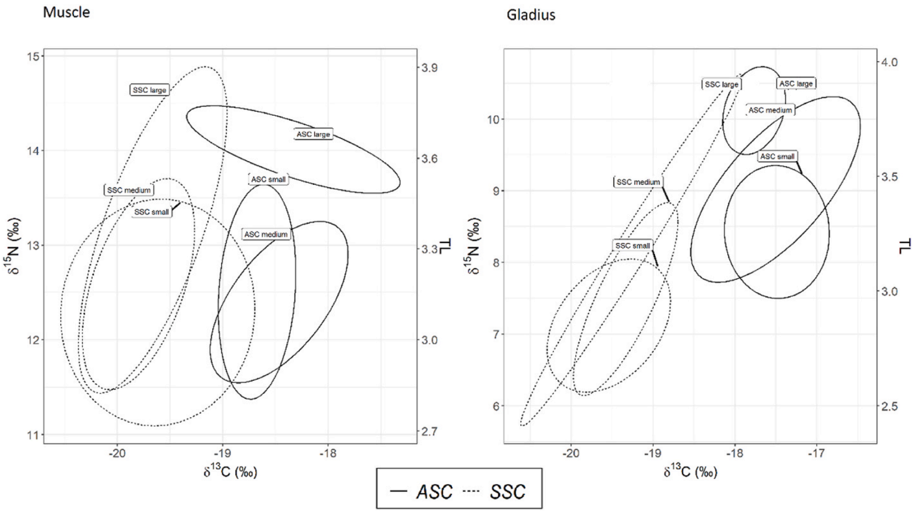

Fig. 9. Standard ellipse of δ^{13}C and δ^{15}N (‰) values per size class of autumn spawning cohort (ASC, solid line) and spring spawning cohort (SSC, dashed line); left: muscle tissue, right: gladius tissue.

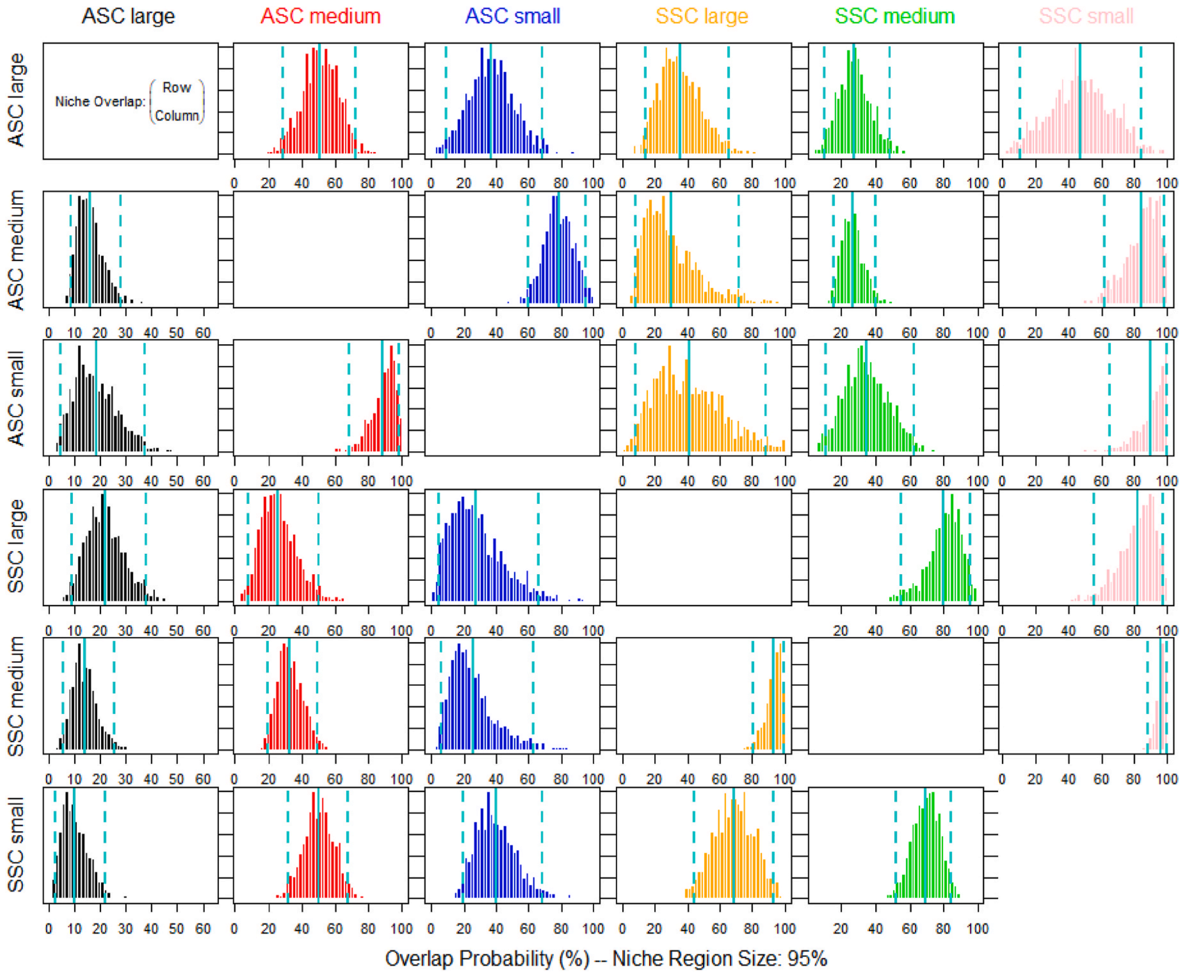

Fig. 10. Niche Overlap Probability for each cohort and sizeclass at a 95% alpha level for muscle tissue.

as the ommastrephid squid *Dosidicus gigas* (Ruiz-Cooley et al., 2006), the gonatid squid *Gonatus fabricii* (Golikov et al., 2018), and in *D. gahi* (Rosas-Luis et al., 2017). Büring et al. (2022) found a size-dependent diet change in *D. gahi*. Small individuals of *D. gahi* fed on Euphausiacea, whereas larger individuals were found to prey upon fish such as Myctophidae or on small squid, including cannibalism (Büring et al., 2022; Rosas-Luis et al., 2014). These variations in diet reflect the development of morphological features related to feeding, e.g. growth of arms and tentacles, or the beak (Boucher-Rodoni et al., 1987). Larger sized individuals are feeding on larger prey, which itself occupies higher trophic levels (Boucher-Rodoni et al., 1987). Larger individuals were sampled in the ASC (+2 cm larger on average), which could be one factor explaining the very distinct niche of large ASC individuals. The model fit was lowest for muscle tissue δ^{15}N values explained by DML of squid.

In a prior analysis on aged *D. gahi* individuals, based on statolith growth increment counts averaged from the last 20 years, it was found that for squid measuring 10 cm DML, the last fifth part of the gladius represented approximately the last 41 days (±3 days) of squid life, in 20 cm DML squid ~the last 51 days (±4 days) and in 30 cm DML squid ~the last 53 days (±8 days) (Falkland Islands Government - unpublished data). Therefore, the gladius tissue of the ASC might have preserved the summer peak in δ^{15}N, whereas the ASC muscle tissue reflects lower autumn δ^{15}N values and their feeding at the lower trophic levels. In the SSC, these contrasting effects of feeding on higher trophic level prey (Amphipods, Chaetognaths, fish and squid) on the one hand but experiencing a lower δ^{15}N baseline in winter months and in greater water depths on the other hand, might have balanced each other out. The results were similar in δ^{15}N muscle values of the SSC compared to the ASC but the SSC was lacking the δ^{15}N summer peak evident in the gladius tissue of the ASC, which might be therefore lower compared to the ASC. Results of trophic levels based on the isoscape baseline (Fig. 8) confirmed higher trophic levels in winter months despite lower δ^{15}N values and are in agreement with findings of the diet study by Büring et al. (2022).

Highly productive areas such as the Patagonian Shelf seem to cause an increased uptake of nitrates by phytoplankton, resulting in high δ^{15}N isoscapes. Glew et al. (2021) modelled nitrogen isoscapes of the Southern Ocean and found δ^{15}N to be dependent on the distance to the coast and water depth. The authors isoscape models predicted higher δ^{15}N values during the summer months (Nov–Dec) compared to the winter months (May–Oct) for the Southern Ocean and around continental shelves of the South Atlantic. Considering the results of the diet study from Büring et al. (2022), the SSC were expected to have higher δ^{15}N values (for both muscle and gladius tissue) due to a diet consisting of species of higher trophic level (Chaetognatha, Amphipoda, fish). This was not reflected in this study. Instead, the ASC was found to have similar muscle tissue values, higher gladius δ^{15}N values and occupied a higher trophic level when compared to the SSC. Therefore, not only must the diet composition of each cohort be considered but also the overall trophic baseline each cohort experiences throughout its development.

The most pronounced difference in stable isotope values between the two spawning cohorts of *D. gahi* was δ^{13}C. Carbon stable isotope values are based on primary producers, altering very little within the food chain (Cherel and Hobson, 2007; DeNiro and Epstein, 1978; Tieszen et al., 1983) and are influenced by environmental factors such as

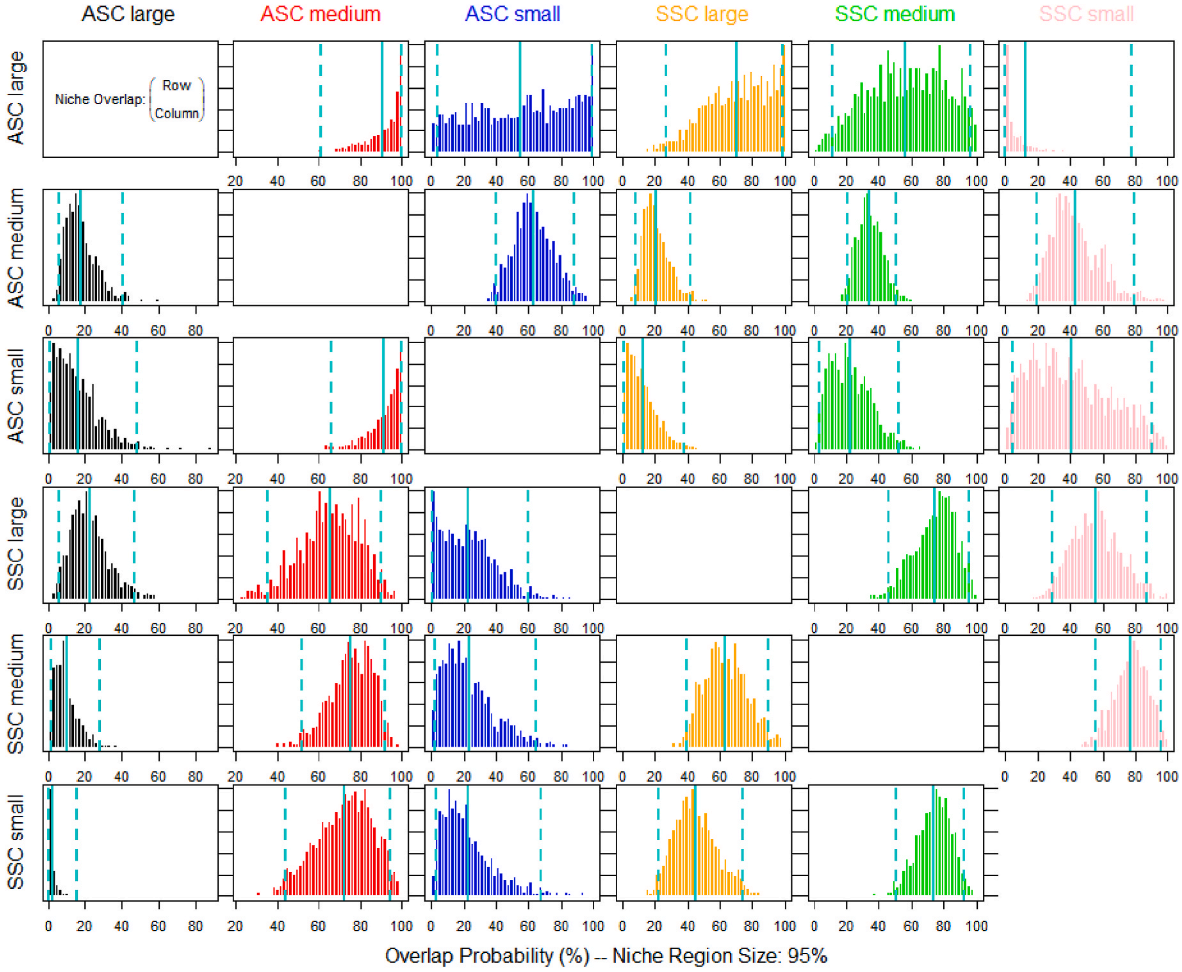

Fig. 11. Niche Overlap Probability for each cohort and sizeclass at a 95% alpha level for gladius tissue.

temperature, light intensity and currents (France, 1995). As our models accounted for potential spatial variability, the differences in δ^{13}C values found between the two seasonal spawning cohorts of *D. gahi* were most likely to reflect seasonal differences in δ^{13}C values of primary producers of the Patagonian Shelf ecosystem. The ASC feed during the austral summer and therefore in warmer and shallower shelf waters compared to the SSC, which have their feeding period during the austral winter and occur at deeper depths (Arkhipkin et al., 2004, 2013). This might explain the lower δ^{13}C values in muscle and gladius tissues of the SSC, as deeper offshore waters are normally more depleted in δ^{13}C than more productive coastal waters (Miller et al., 2008). Furthermore, Quillfeldt et al. (2015) found seasonal variability in δ^{13}C and δ^{15}N around the Falkland Islands, with higher δ^{13}C values in summer than in spring, supporting the findings of this study.

Muscle and gladius tissues had distinct stable isotope values. It is known that cephalopod muscle tissue is normally low in lipids but is rich in proteins (Clarke et al., 1994). Apart from proteins, cephalopod hard structures such as beaks or gladius are mainly composed of chitin, which is a polymer of N-acetyl-*b*-D-glucosamine (Arkhipkin et al., 2012; Merzendorfer and Zimoch, 2003). Chitin and purified D-glucosamine of arthropod exoskeletons were found to have lower δ^{15}N ratios compared to muscle tissue; however, similar to muscle tissue, δ^{15}N ratios of gladius tissue increased with increasing trophic level (Schimmelmann and DeNiro, 1986). δ^{13}C values vary little between tissue types (Cherel and Hobson, 2005; Schimmelmann and DeNiro, 1986), due to the different composition of amino acids in hard structures (lacking protein) and muscle tissue (pure protein in the case of cephalopods; Cherel et al., 2019, 2009). *Loligo* sp. were found to have chitin to protein ratios of about 1:2 to 1:1.5 in the gladius (Hunt and Nixon, 1981), which we can assume is similar to our study species *D. gahi*. Cherel and Hobson (2005) found higher δ^{15}N values in muscle tissue compared to δ^{15}N depleted beaks, due to different metabolic pathways for protein and chitin. In this study, gladius tissue was less enriched in ^{15}N and ^{13}C than muscle tissue, but both showed similar trends in seasonality and for δ^{15}N also in size.

To estimate the trophic level of two cohorts in three different size groups, the trophic baseline of the Patagonian shelf ecosystem was calculated based on δ^{15}N values of primary consumers of phytoplankton and on modelled isoscapes by Glew et al. (2021). In this study Euphausiacea, larval and juvenile plankton stages of the lobster krill *M. gregaria* and salps were sampled during the same year, even though not all seasons and areas could be sampled evenly. The use of salps to determine the trophic baseline is contested (Stowasser et al., 2012) as they consume flagellates and cyanobacteria (i.e. *Prochlorococcus*) (Sutherland et al., 2010; von Harbou et al., 2011), which are isotopically depleted compared to large phytoplankton (Del Giorgio and France, 1996; Fawcett et al., 2010; Fry and Wainright, 1991). Selective feeding on flagellates and cyanobacteria could lead to lower isotopic signatures of salps compared to those found for particulate organic matter. Zooplankton species follow a strong seasonal pattern, with highest abundances in spring and summer (Sabatini and Colombo, 2001). This study has the constraints of not covering SI values of zooplankton throughout the whole year of 2020 to access seasonality of primary consumers. Values obtained from autumn and spring were averaged that prevented to describe seasonality.

The trophic level of a species may alter over time or be different in different ecosystems, but rather than using only δ^{15}N values, the trophic level enables us to compare species and studies in a more standardized way (Navarro et al., 2013). On the one hand, using Euphausiacea and Munida specimens sampled within the same year and area as the target species (*D. gahi*) to calculate trophic level seemed very important. But on the other hand, the use of isoscapes, specific for each location and moment of time seemed more reliable due to relative scarcity of our zooplankton samples. According to the isoscapes calculated by Glew et al. (2021), euphausiids and salps had a trophic level of about 2, whereas *M. gregaria* planktonic juveniles had slightly higher levels.

Highest niche overlaps based on muscle tissue isotopes were revealed for the SSC cohort, especially the small individuals with medium and large ones, as well as with ASC small and medium individuals. As the SSC has a shortened egg development time and faster growth rate due to warm temperatures in austral summer months, they can be found in the same water column at the same time (Arkhipkin et al., 2004), hence sharing the same ecotrophic niche. On the other hand, large ASC individuals can be found late in austral winter months (May–July), being the only group in Falkland Island waters with little sharing of their trophic niche with other groups, due to the specific environmental conditions this groups is exposed to. Apart from their sampling size, this would be another factor, explaining the unique econiche of this group.

Classical stomach content analysis may be biased due to factors e.g. rejecting hard parts such as bones, otoliths and beaks, overestimating the importance of soft-tissue items such as crustaceans (Boyle and Rodhouse, 2005; Buckland et al., 2017). Nevertheless, stomach content analysis has advantages when it comes to the identification of prey species compared to SIA, as the latter can only predict possible prey species and are highly dependent on good SI mixing models including all possible prey items (Bearhop et al., 2004; Hyslop, 1980; Jackson et al., 2011; Montecinos et al., 2016). A combination of both methods might achieve the best results when determining the ecology and trophic position of a species (Martínez-Baena et al., 2016; Rosas-Luis et al., 2016). This study with the SIA approach supports the findings of Büring et al. (2022). The spawning cohorts of *D. gahi* might have distinct trophic niches due to the different environmental factors such as depth and temperature regimes (e.g. carbon baselines) the cohorts experience during their lifetime (Arkhipkin et al., 2004).

This study made a further contribution towards understanding the ecology of the two spawning cohorts of *D. gahi*. The knowledge of the ecotrophic niches and trophic levels of two seasonal spawning cohorts of a squid species can provide important information for ecosystem-based management. It will help to better understand the role of *D. gahi* in the ecosystem and its relationships with other species and to predict the impacts of changes in the environment on *D. gahi* and the ecosystem as a whole. Furthermore, it will help developing effective management strategies to conserve *D. gahi* and maintain the ecological balance of the ecosystem.

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CRediT authorship contribution statement

Tobias Büring: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jessica B. Jones:** Writing – review & editing, Supervision. **Graham Pierce:** Writing – review & editing, Validation, Supervision, Project administration. **Francisco Rocha:** Supervision. **Paco Bustamante:** Writing – review & editing, Methodology. **Maud Brault-Favrou:** Resources. **Alexander I. Arkhipkin:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tobias Buring reports financial support was provided by Beauchene Fishing Company Ltd. Tobias Buring reports a relationship with Beauchene Fishing Company Ltd that includes: funding grants.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2023.108300.

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