Combining isotopic analysis of bulk-skin and individual amino acids to investigate the trophic position and foraging areas of multiple cetacean species in the western South Atlantic

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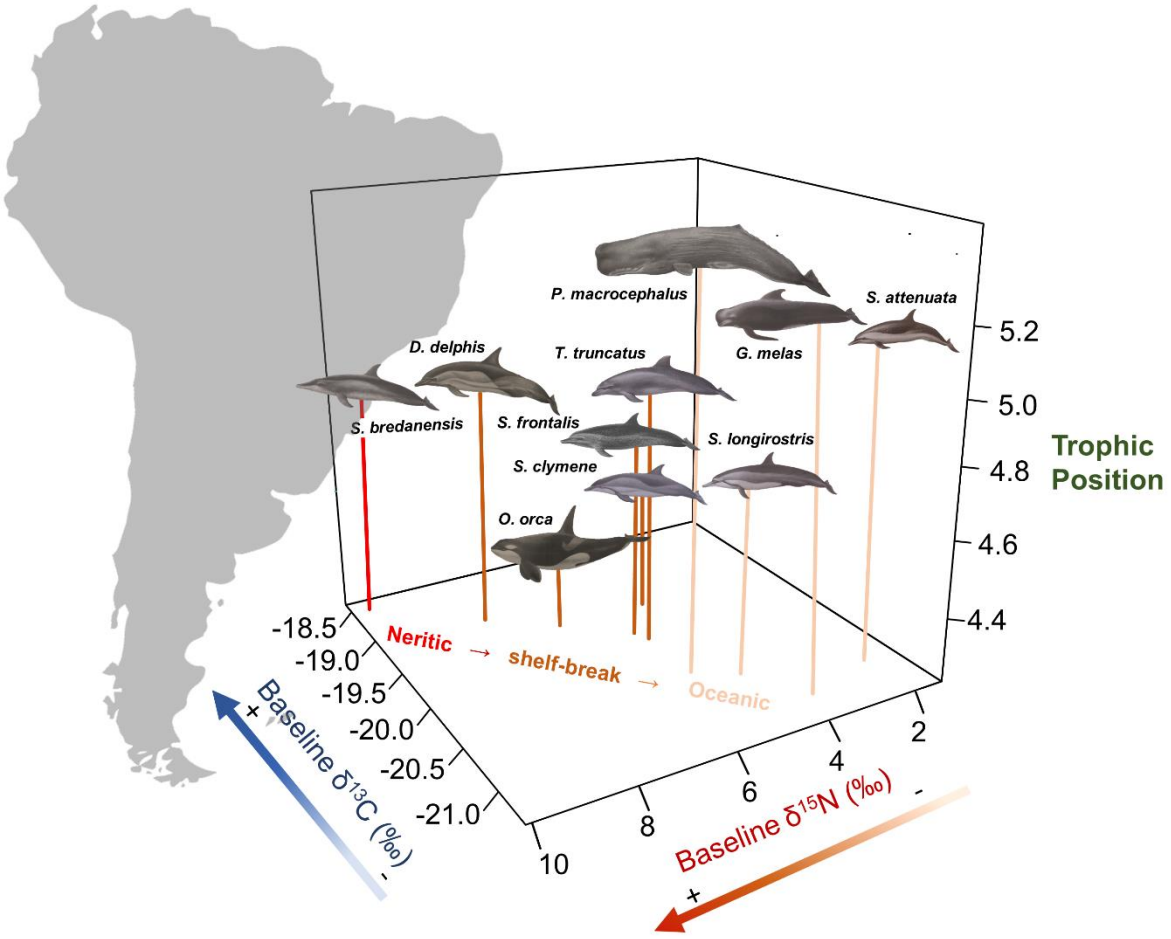
Abstract

We investigated the trophic structure and habitat use of ten cetacean species occurring in the oceanic waters of the western South Atlantic using naturally-occurring stable isotopes. We analyzed $\delta^{15}N$ in individual amino acids (AA) to estimate cetacean trophic position (TP) and to evaluate the spatial differences in baseline $\delta^{15}N$ (source AAs). We adjusted cetacean bulk-skin $\delta^{13}C$ and $\delta^{15}N$ for the effect of trophic level using their estimated TPs, obtaining $\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$, respectively. These values were applied to estimate the overlap in the niche areas of cetacean baseline sources. Our analyses showed spatial segregation between *Steno bredanensis* and the remaining odontocetes, and the high $\delta^{15}N$ in this species reflects its occurrence in neritic waters of the southern region. The highest TPs were observed in *Physeter macrocephalus, Stenella attenuata* and *Globicephala melas*, while the lowest TPs were reported for *S. longirostris*, *S. clymene* and *Orcinus orca*. Overall, source AA-$\delta^{15}N$ showed similar patterns as those of baseline-$\delta^{15}N$ (zooplankton) and were higher in species sampled in the southernmost region of the study area (e.g., *Delphinus delphis)*. Isotopic niche areas estimated using $\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$ suggested high overlap in foraging area between *S. frontalis* and *Tursiops truncatus*, with the latter occupying a higher TP. Our analyses of $\delta^{15}N$ in AAs provide a unique insight into the trophic ecology, forage areas and spatial segregation in resource use among these cetacean populations. Additionally, our work provides AA-$\delta^{15}N$ baseline for future studies on the trophic ecology and habitat use of marine organisms in the western South Atlantic.

Key words: Compound-specific stable isotopes, Odontocetes, Nitrogen, South Atlantic Ocean

Graphical Abstract



Analysis of amino acid $\delta^{15}N$ helped reveal the trophic structure and habitat use of cetaceans from the oceanic waters of the western South Atlantic. Image: Genyffer C. Troina. Cetacean illustration by José Luis Vázquez, adapted from Bastida et al. 2018.

Declarations:

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Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any experiments with human participants. All applicable international, national and institutional guidelines for sampling of animals have been followed and all necessary approvals have been obtained. Sampling was conducted under SIBIO license number 16586-2 (SISBIO- *Sistema de Autorização e Informação em Biodiversidade)* and all procedures performed involving animals were in accordance with the ethical standards of the institution.

Availability of data and material The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

1. Introduction

Identifying marine mammals' preferred habitat and feeding ecology is key to understanding how they shape the structure and are affected by the dynamics of their ecosystems. The main ecological factor affecting cetacean distribution is prey availability (Ballance et al. 2006; Lambert et al. 2014), although their habitat use may also be influenced by interspecific competition for main prey or the presence of potential predators (Wells et al. 1981; Heithaus 2001; Heithaus and Dill 2006). Knowledge of habitat and prey preference allows for the assessment of both actual and potential impacts from anthropogenic interactions, such as fishing activities (bycatch or fish depletion), ship collisions, habitat pollution or degradation, or prey composition changes due to climate change (Rayment et al. 2011; Evans 2018) that are required to inform and implement effective conservation measures (e.g., Bailey and Thompson 2009; Chavez-Rosales et al. 2019).

The offshore waters of the western South Atlantic (WSA) ocean represent an important area for several cetacean species. In this area, killer whale (*Orcinus orca*) occurs along the continental shelf and oceanic waters (Secchi and Vaske Jr. 1998; Di Tullio et al. 2016) and have been reported interacting with longline fisheries of southern Brazil and Uruguay (Secchi and Vaske Jr. 1998; Dalla Rosa and Secchi 2007; Passadore et al. 2015). The sperm whales (*Physeter macrocephalus*) are known to prey upon deep-water squids (Clarke et al. 1980; Santos and Haimovici 2000, 2002), occurring in pelagic waters at approximately 1000 m isobath and beyond (Di Tullio et al. 2016). The long-finned pilot whale (*Globicephalas melas*) prefers cold-temperate waters along the shelf break and slope regions (Di Tullio et al. 2016), where they consume mostly cephalopods (Mansilla et al. 2012; Beasley et al. 2019). The oceanic population of bottlenose dolphins (*Tursiops truncatus*) that occurs along the shelf break and slope waters in the WSA is frequently found in association with other Delphinidae species (Lima et al. 2021). The rough-toothed dolphin (*Steno bredanensis*) in the SWA has been registered mostly in coastal and in the inner continental shelf waters (Ott and Danilewicz 1996; Santos et al. 2017; 2019), but also in offshore areas (Di Tullio et al. 2016; Troina et al. 2020b). The common dolphin (*Delphinus delphis*) has often been sighted in neritic waters off southern Brazil (ECOMEGA, unpublished data) as well as in deeper waters of the outer continental shelf and slope (Tavares et al. 2010; Di Tullio et al. 2016). The Atlantic spotted dolphin (*Stenella frontalis*) is found in nearshore and oceanic waters of warm-tropical regions and in areas associated with upwelling systems (Moreno et al. 2005; Di Tullio et al. 2016). The spinner dolphin (*S. longirostris*), the pantropical spotted dolphin (*S. attenuata*), and the Clymene

dolphin (*S. clymene*) show large overlap in their distributional ranges, which include mainly warm pelagic environments (Moreno et al. 2005; Di Tullio et al. 2016). A large variety of small-to-large meso-to-epipelagic fish and squids comprise the diets of several small delphinids, such as *D. delphis* or those species of the genus *Stenella* (Di Beneditto et al. 2001; Santos and Haimovici 2002; Melo et al. 2010; Lopes et al. 2012).

Stable isotope analysis (SIA) has been widely applied to marine mammals to study migration and forage areas (e.g., Best and Schell 1996; Silva et al. 2019), feeding ecology (e.g., Castro et al. 2016; Borrell et al. 2021) and trophic interactions (e.g., Ryan et al. 2013; Botta et al. 2018). Trophic position can be estimated using bulk-tissue $\delta^{15}N$ given the consistent increase in the heavy isotope (^{15}N) at each trophic level (Minigawa and Wada 1984; Perkins et al. 2014). Such estimates require data on the isotopic values of organisms at the base of the food webs where consumers have been feeding (Post 2002), and usually assume a trophic discrimination factor (TDF) around 2-4‰ (Kelly 2000; McCutchen et al. 2003). Additionally, foraging areas and migratory patterns can be inferred by linking the isotopic values measured in consumers' tissues with local baseline stable isotope patterns (i.e., isotopic landscapes or isoscapes, Hobson et al. 2010; McMahon et al. 2013). The trophic ecology and habitat use of odontocetes that occur in the WSA have been inferred from the analysis of bulk-skin carbon and nitrogen stable isotopes (Troina et al. 2020a). These authors have successfully applied SIA to identify areas used by the different species, which had remarkable latitudinal (north to south) and near-to-offshore gradients in both carbon and nitrogen stable isotope ratios. Additionally, SIA suggested isotopic niche overlaps between *T. truncatus* and *S. frontalis*, or *S. longirostris* and *S. attenuata* (Troina et al. 2020a), species frequently found forming mixed species associations in the SWA (Lima et al. 2021). However, this method was unable to resolve some of the isotopic patterns observed, including the higher $\delta^{15}N$ values in *S. bredanensis* and *D. delphis*, in comparison to those of presumed apex predators (*P. macrocephalus* and *O. orca*).

Ecological inferences based on bulk-tissue isotope data of highly mobile marine predators require information on the distinct isotopic baselines between the areas they may occur (Graham et al. 2010). Isoscapes have been described for baseline organisms in the oceanic waters of the WSA, showing increasing latitudinal (N-S) and longitudinal (offshore-to-nearshore) gradients in $\delta^{15}N$ values (Troina et al. 2020b). Nevertheless, temporal mismatches can occur between the isotope values of consumers' tissues and baseline organisms, due to difference in time required to achieve isotopic equilibrium with the environment. Turnover time may be longer in some consumers than the seasonal isotopic fluctuations at the base of the local

food webs (McCutchan and Lewis 2001; O'Reilly et al. 2002). Baseline isotopic information is often obtained from short lived organisms (e.g., copepods' ~ 20 d, Irvine and Waya 1999) whose isotopic values result from high turnover rates that capture short term variations in underlying biogeochemical processes (e.g., Troina et al. 2020b). Conversely, predators' isotopic measurements are usually carried out using tissues with considerably longer turnover times (e.g., > 2 months in skin; Dalerum and Angerbjörn 2005; Giménez et al. 2016). Applying adequate (species- and tissue-specific) diet-to-consumer TDF and carefully matching consumers tissue and baseline organisms considering their respective turnover rates are essential to represent approximately equivalent temporal scales and to allow for comparisons of relative trophic positions between species.

Compound-specific stable isotope analysis of individual amino acids (CSIA-AA) reveals the isotopic differences among the amino acids (AAs) that build up the proteins in tissues. Within a consumer tissue, source AAs (e.g., phenylalanine, Phe; lysine, Lys; Popp et al. 2007) have $\delta^{15}N$ values that are comparable to those in baseline organisms (e.g., phytoplankton, zooplankton) due to minimal fractionations (e.g., 0.4‰ in Phe; Chikaraishi et al. 2009), preserving baseline $\delta^{15}N$ values within consumers with higher trophic positions (Lorrain et al. 2009; Dale et al. 2011; McMahon et al. 2019). Therefore, baseline $\delta^{15}N$ values can be resolved with the analysis of source AAs, following the assumption that $\delta^{15}N$ in these AAs reflect baseline isotopic data from where the consumers had been feeding. Conversely, trophic AAs (e.g., glutamic acid, Glu; Popp et al. 2007; Chikaraishi et al. 2009) undergo larger isotopic fractionations (e.g., 8.0‰ in Glu; Chikaraishi et al. 2009) due to transamination and deamination during metabolism (McMahon and McCarthy 2016; O'Connell 2017). The differences in $\delta^{15}N$ between the weighted averages of the trophic and source AAs ($\delta^{15}N_{Tr}$ - $\delta^{15}N_{Sr}$) allow for the estimation of a consumer's trophic position by applying a TDF between these AA groupings, which are usually estimated from controlled feeding experiments (e.g., Chikaraishi et al. 2009; Germain et al. 2013; Bradley et al. 2014; McMahon et al. 2015a,b). Consequently, CSIA-AA has the advantage of providing, based on a single sample, information on both the baseline isotopic values from where the consumer has been feeding and its trophic position (McClelland and Montoya 2002; Popp et al. 2007; Lorrain et al. 2009). Furthermore, some AAs are considered "metabolic" (e.g., threonine, Thr; serine, Ser), whose fractionations vary depending on dietary protein (Fuller and Petzke 2017), the consumer's physiological state, and nutritional condition (Lübcker et al. 2020). In this context, CSIA-AA of $\delta^{15}N$ has helped to investigate the foraging and nursery areas of marine organisms (Lorrain et al. 2009; Dale et al.

2011), food web structure and trophic interactions (Chikaraishi et al. 2014; Choy et al. 2015; McMahon et al. 2016), and anthropogenic related temporal changes in the feeding habits and in ocean biogeochemistry (Pomerleau et al. 2017; McMahon et al. 2019).

In this study, we apply CSIA-AA to estimate the trophic position of free-ranging odontocetes from the WSA and combine their source AA $\delta^{15}N$ with bulk $\delta^{15}N$ values in zooplankton to assess the spatiotemporal gradients in baseline $\delta^{15}N$. Specifically, we address whether 1) intra- and inter-specific variations in cetacean bulk-skin isotopic values result from spatial gradients in $\delta^{15}N$ at the base of the food web or from differences in the trophic position within and among species; and 2) the $\delta^{15}N$ values of odontocetes source AAs reflect habitat use by being associated with the latitudinal patterns in baseline $\delta^{15}N$. We expect to see higher $\delta^{15}N$ values in the source AAs of cetaceans that have a more inshore distribution (e.g., *S. bredanensis* and *D. delphis*) in comparison to the oceanic species (e.g., *P. macrocephalus* and *G. melas*); and that the smaller delphinids (e.g., *D. delphis*; *Stenella* spp.) have relatively lower TPs than *P. macrocephalus, G. melas* and *O. orca*, which will be evidenced by baseline-corrected TP estimates. This is the first time that $\delta^{15}N$ values are analysed in individual AAs of free-ranging odontocetes to assess the trophic ecology and interspecific patterns in habitat use among the different species that occur in the oceanic waters of the WSA.

2. Materials and methods

2.1. Study area

The oceanic waters along the Brazilian outer continental shelf (~150 m isobath) and slope (~2.000 m isobath, Fig. 1) have latitudinal and longitudinal gradients in their oceanographic conditions that affect baseline nitrogen stable isotopes (Troina et al. 2020b). Therefore, the region has been divided into two areas: southeast (SE, 24°S–28°S) and south (S, 28°S–34°S, Fig. 1). The SE area is mainly influenced by tropical waters transported southwards by the Brazil current and by upwellings of the South Atlantic Central Water along the shelf-break (Acha et al. 2004; Brandini et al. 2018). The S area is seasonally influenced by different water masses: the tropical waters of the Brazil current dominate the region in warmer months, whereas the subtropical shelf water (STSW) dominates in colder periods. The STSW is formed by the encounter and mixing of tropical waters, continental waters from Rio de la Plata and Patos-Mirim Lagoon system, and the Subantarctic shelf water transported by the northward flowing

Malvinas current that reaches up to 32°S (Acha et al. 2004; Möller Jr. et al. 2008; Piola et al. 2008).

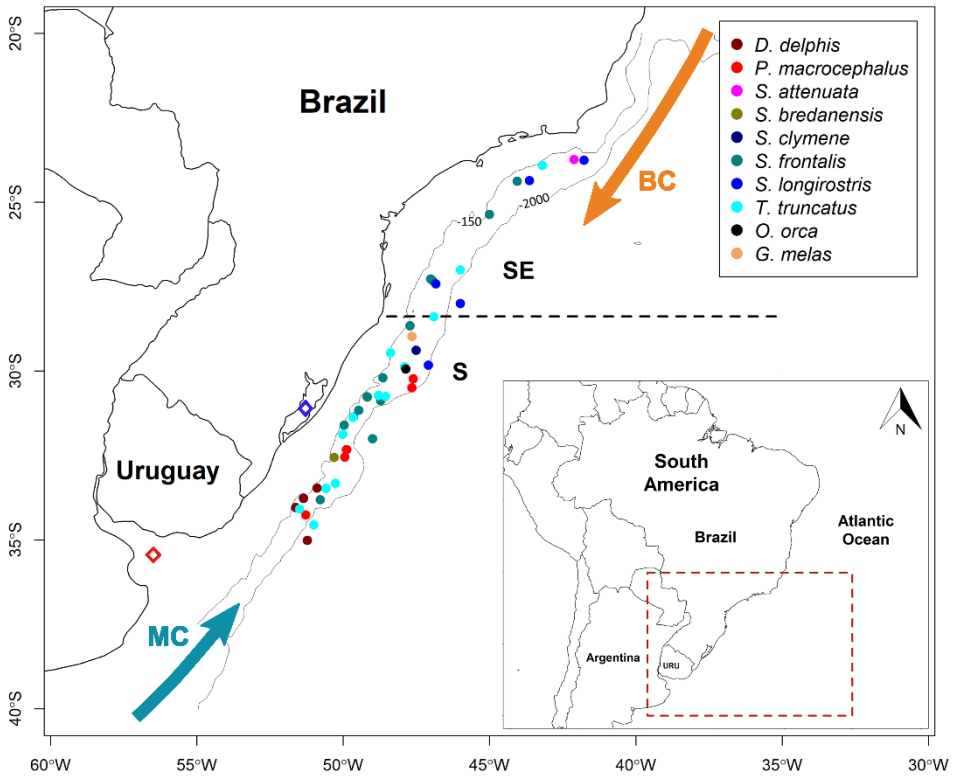

Figure 1: Study area along the outer continental shelf (~150 m isobath) and offshore (up to ~2000 m isobath) waters in the western South Atlantic Ocean. Cetaceans' sampling locations are indicated with the solid circles and coloured by species. Dotted line separates the southeast (SE) and the southern (S) regions. Southward and northward arrows represent the Brazil Current (BC) and Malvinas Current (MC), respectively. Red and blue open diamond indicate Río de la Plata and Patos Lagoon, respectively, which are the main sources of continental waters influencing the S region.

2.2. Sample collection and preparation

Samples were collected during ten research cruises in the scope of the *Projeto Talude* (Continental Slope Project) between 2009 and 2015 during austral spring (n = 5) and autumn (n = 5). Cetacean skin biopsies were obtained from bow-riding animals from the bow of the RV *Atlântico Sul* (Federal University of Rio Grande, FURG) or from a small boat deployed from the ship. Biopsies were collected using a 120-lb draw weight crossbow with modified darts specifically designed for sampling, with different size tips depending on the target species. Zooplankton data used here have been published in previous work (Troina et al. 2020b) and were collected at pre-determined oceanographic stations as a composite of the whole vertical profile of each station. All samples (zooplankton and cetacean) were immediately stored at -20ºC without any chemical treatment until processing. Zooplankton groups (amphipods,

copepods, euphausiids and chaetognaths) were analyzed separately for stable isotopes, with each taxon subsample including multiple specimens. Zooplankton and cetacean skin samples were rinsed with distilled water, dried in an oven at 60°C for 48 h, and ground to a fine powder using a mortar and pestle. More details about zooplankton sampling and treatment are presented in Troina et al. (2020b). The analyses of bulk-tissue stable isotopes have been described in previous publications for the zooplankton data (Troina et al. 2020b) and for the cetacean samples (Troina et al. 2020a).

2.3. Compound-specific stable isotope analysis

About 2-5 mg of 69 cetacean skin samples were processed for analysis of $\delta^{15}N$ in amino acids. This method is a modified version of the method presented in Chikaraishi et al (2007) and is described in detail by Riekenberg et al. (2020). In short, samples were first acid hydrolyzed, then derivatized into N-pivaloyl/isopropyl derivatives and analyzed in duplicate using a Trace 1310 gas chromatograph coupled to a DeltaV Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen) via a GC Isolink II, at the NIOZ Royal Netherlands Institute for Sea Research (Texel, The Netherlands). This method allowed the measurement of 13 individual amino acids: Phe, Lys, methionine (Met), tyrosine (Tyr), threonine (Thr), serine (Ser), glycine (Gly), alanine (Ala), aspartic acid (Asp), Glu, isoleucine (Ile), leucine (Leu) and Val. Underivatized amino acid $\delta^{15}N$ values used for normalization were determined via EA-irMS and were calibrated against IAEA-N-1 and IAEA-N-2 using the secondary reference materials acetanilide #1 and urea #2 with a precision of ±0.1‰. Both samples and standard $\delta^{15}N$ values were adjusted against an internal reference peak (norleucine), normalized to account for derivatization, and then adjusted for linearity using a 'scaling mix' of 5 amino acids known to have a large range (-2.4‰ to 61.5‰) composed of amino acid reference materials including Gly (USGS65) and Val (USGS74) provided by Arndt Schimmelmann (Indiana University; Schimmelmann et al. 2016). The precision for sample and standard measurements was <0.5‰ with an average sample precision (SD) of 0.24‰ for individual AAs, ranging between 0.2‰ for Leu and 0.3‰ for Thr.

2.4. Cetacean trophic position

Trophic positions estimated using bulk-tissue $\delta^{15}N$ derive from the equation adapted from Germain et al. (2013), which uses a dual trophic discrimination factor (TDF) to account for a TDF between cetacean and their prey ($TDF_{cetacean\text{-}prey}$) of 1.6‰ (Giménez et al. 2016) or 2.4‰

(Caut et al. 2011), and a constant TDF of 3.4‰ (Post 2002) for the remaining trophic connections:

$$TP_{bulk} = \left[\frac{(\delta^{15}N_{\text{bulk-skin}} - TDF_{\text{cetacean-prey}}) - \delta^{15}N_{\text{copepods}}}{3.4}\right] + TP_{\text{copepods}} + 1 \quad [1]$$

where $TP_{copepods}$ is the trophic position of copepods (= 2), $\delta^{15}N_{bulk\text{-}skin}$ and $\delta^{15}N_{copepods}$ are the $\delta^{15}N$ of cetacean bulk-skin and bulk-copepods, respectively. The last + 1 is to account for the cetacean-prey trophic step. Since there are spatial differences in baseline nitrogen stable isotopes throughout the study area (Troina et al. 2020b), estimates were carried out separately for cetaceans sampled in each region using $\delta^{15}N$ values of copepods sampled in each respective location (S or SE). Additionally, since there is also a shelf-break-to-offshore difference in baseline $\delta^{15}N$, we estimated cetacean TP twice, either with $\delta^{15}N$ from copepods sampled along the shelf-break (4.3±1.8‰ in the SE; 6.1±2.3‰ in the S region) or offshore region (1.3±2.1‰ in SE; 3.9±1.6‰ in S, data from Troina et al. 2020b).

Cetacean trophic positions were also estimated using $\delta^{15}N$ in trophic and source AAs. We assessed TP from AA-$\delta^{15}N$ values using several approaches that resulted in similar intra- and inter-specific trends (Appendix 1 and 2). However, we present the results of TP estimates based on multiple AAs, as they usually yield more precise TP estimates (e.g., Nielsen et al. 2015; Ruiz-Cooley et al. 2021). We used the weighted mean $\delta^{15}N$ values of the trophic AAs Ala, Asp, Glu, Ile, Leu and Val ($\delta^{15}N_{Tr\text{-}AA}$) and the source AAs Phe and Lys ($\delta^{15}N_{Sr\text{-}AA}$). We adopted a TDF of 3.1 ± 0.4‰ (Ruiz-Cooley et al. 2021) and a β of 3.4± 0.9‰ determined for multiple AAs by Nielsen et al. (2015).

$$TP_{Tr-Sr} = \left(\frac{\delta^{15}N_{\text{Tr-AA}} - \delta^{15}N_{\text{Sr-AA}} - \beta}{TDF}\right) + 1 \quad [2]$$

Uncertainty in TP estimates was calculated using the propagation of error (e.g., Bradley et al. 2015; Ohkouchi et al. 2017) (see appendix 1 for more details).

As there is a small increase in Phe-$\delta^{15}N$ at each trophic level (0.4‰; Chikaraishi et al. 2009), we used the estimated TP to correct Phe-$\delta^{15}N$ using equation 3:

$$\text{Phe} = \delta^{15}N_{\text{Phe}} - (0.4 \times (TP - 1)) \quad [3]$$

Cetacean bulk-skin $\delta^{15}N$ and $\delta^{13}C$ were also corrected for TP to obtain the baseline values (TP = 2, to be comparable to zooplankton) of their feeding areas, by re-arranging equation 1. We applied a cetacean-prey TDF of 1.6‰ and 1.01‰, for nitrogen and carbon, respectively

(Giménez et al. 2016), and assumed a TDF of 3.4‰ ($\delta^{15}N$) and 1.0‰ ($\delta^{13}C$) (Post 2002) for the remaining trophic connections:

$$\delta^{15}N_{Adjusted} = (\delta^{15}N_{bulk} - 1.6) - (3.4 \times (TP - 2 - 1)) \quad [4]$$

$$\delta^{13}C_{Adjusted} = (\delta^{13}C_{bulk} - 1.01) - (1.0 \times (TP - 2 - 1)) \quad [5]$$

2.5. Statistical analysis

All statistical analyses were carried out using the R version 3.5.3 (R Core Team 2019). Because the data were not normally distributed (Shapiro test, $p < 0.05$), we applied the Spearman rank correlation test to assess the correlation between $\delta^{15}N$ in Phe and bulk-skin; between bulk-skin and the difference in $\delta^{15}N$ between $\delta^{15}N_{Tr\text{-}AA}$ and $\delta^{15}N_{Sr\text{-}AA}$ (Tr-Sr); and between Phe and Tr-Sr. These analyses allowed us to verify whether bulk-skin $\delta^{15}N$ was affected by variation in baseline $\delta^{15}N$ or in trophic position (TP), and if cetacean TP varied spatially following baseline $\delta^{15}N$ changes, respectively. Welch one-way ANOVA was applied to evaluate the spatiotemporal differences in $\delta^{15}N$ (SE and S region in autumn and spring) and to compare estimated TP by different equations. The Welch's test can be applied to compare populations with unequal variances and has good performance when data is not normally distributed (Ruxton 2006; Rasch et al. 2011). When the null hypothesis was rejected, post-hoc analysis was carried out with the Games-Howell Test, a nonparametric test to perform post-hoc analysis that does not assume normality, homoscedasticity or equal sample sizes (Ruxton and Beauchamp 2008; Shingala and Rajyaguru 2015).

To assess the spatial patterns in $\delta^{15}N$ values, we applied a generalized linear mixed-effects model (GLMM) that estimates the probability of $\delta^{15}N$ in baseline (bulk zooplankton, Phe and $\delta^{15}N_{Adjusted}$) to correspond to region S or SE. GLMMs can incorporate random effects and are not limited to normally distributed data, allowing the user to determine the exponential family and link functions, which makes them ideal to apply in ecological studies (Bolker et al. 2009). The model was fit with a binomial distribution family (logit link function) using the glmer function in R package *lme4* (Bates et al. 2015). The variable *group* (Phe, $\delta^{15}N_{Adjusted}$ and pooled zooplankton, including amphipods, copepods and euphausiids) was used as a random effect. The probability that an individual sample (from any random group) comes from region SE was modelled as a function of its $\delta^{15}N$ value. To test model accuracy, the data set was randomly divided for model training (70% of data) and testing (30% of data). The training data

set was used to construct the model and the testing set was applied to predict the model accuracy.

Because sample size greatly differed among species (Table 1), with samples of only one individual for some species (e.g., *S. attenuata*, *S. clymene*, *G. melas*; Table 1), it was not possible to statistically test for inter-specific differences in estimated TPs. Therefore, we applied a hierarchical cluster analysis (Euclidean distance, complete-linkage) to assess the level of similarity between cetacean species based on Phe-$\delta^{15}N$ and estimated TPs. Lastly, we used $\delta^{15}N_{Adjusted}$ and $\delta^{13}C_{Adjusted}$ values (cetacean bulk-skin adjusted for TP) to calculate the standard ellipse areas for each species using the Stable Isotope Bayesian Ellipses in R version 2.1.4 (SIBER; Jackson et al. 2011).

3. Results

3.1. $\delta^{15}N$ in cetacean bulk skin and in individual amino acids

We measured $\delta^{15}N$ in individual AAs from skin samples of 69 free-ranging odontocetes from 10 different species (Table 1, see appendix 3-6 for $\delta^{15}N$ values in each AA). We compare these new data with those of bulk-skin stable isotopes for the same individuals presented previously in Troina et al. (2020a). *S. bredanensis* had the highest mean $\delta^{15}N$ in bulk-skin and in the majority of the AAs, followed by *D. delphis* (Fig. 2). The highest $\delta^{15}N$ values were measured in Val, followed by Leu, Glu, Ile, Ala and Asp. Ser had intermediate $\delta^{15}N$, comparable to those of bulk-skin (Fig. 2), Gly, Lys and Phe had lower $\delta^{15}N$, while the lowest $\delta^{15}N$ values were observed in Thr. There was significant positive correlation between $\delta^{15}N$ in cetacean bulk-skin and Phe ($r_S = 0.7$, $p < 0.001$, appendix 7), and weak correlation between $\delta^{15}N$ in bulk-skin and Tr-Sr ($r_S = 0.25$, $p < 0.05$). Spearman rank correlation was non-significant between Phe and Tr-Sr ($r_S = -0.2$, $p > 0.05$).

Table 1 Number of samples (N); mean ± standard deviation (SD) values of bulk-skin measured ($\delta^{13}C$ and $\delta^{15}N$) and trophic adjusted($\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$) stable isotopes, $\delta^{15}N$ values in trophic (Tr) and source (Sr) amino acids (weighted average) and trophic-adjusted phenylalanine (Phe), and the estimated trophic position (mean ± SD, and uncertainty in TP estimates - σ^2) for each cetacean species sampled in the southwestern Atlantic Ocean. TP estimates obtained using the multiple-amino acids equation (see methods). For species sampled in both southeast (SE) and southern (S) regions, values are presented as overall mean (SD) and for each region separately.

Species		**Bulk-skin**				**Amino acids**			**TP**
		$\delta^{13}C$	**$\delta^{15}N$**	**$\delta^{13}C_{Adjusted}$**	**$\delta^{15}N_{Adjusted}$**	**Tr**	**Sr**	**Phe**	
	N	**Mean ± SD (‰)**							**Mean ± SD (σ2)**
D. delphis	10	-16 ± 0.7	15.7 ± 1.5	-19 ± 0.8	7.2 ± 2.1	26.5 ± 1.3	10.7 ± 1.8	9.2 ± 2	5 ± 0.3 (0.6)
G. melas	1	-17.8	13	-21.1	3.8	24.1	7.6	5.2	5.2 (0.7)
O. orca	3	-17 ± 0.2	12.8 ± 0.2	-19.6 ± 0.3	6 ± 0.3	24.8 ± 2.6	10.4 ± 2.6	10.4 ± 2.4	4.5 ± 0.1 (0.6)
P. macrocephalus	6	-17.2 ± 0.2	14.4 ± 0.6	-20.5 ± 0.5	5.1 ± 1.8	24.8 ± 0.6	8.2 ± 1.1	6.6 ± 1.9	5.3 ± 0.5 (0.7)
S. attenuata	1	-17.7	11	-20.9	2	23.3	6.9	4.6	5.2 (0.7)
S. bredanensis	5	-15.6 ± 0.2	18 ± 0.6	-18.6 ± 0.2	9.5 ± 0.9	27.9 ± 0.6	12 ± 0.9	11.6 ± 1	5 ± 0.2 (0.6)
S. clymene	1	-16.8	10.9	-19.5	3.6	23.6	8.7	8.5	4.7 (0.6)
S. frontalis	16	-17 ± 0.6	12.8 ± 1.5	-19.9 ± 0.7	4.8 ± 1.8	23.5 ± 1.2	8.2 ± 1.2	6.4 ± 1.3	4.8 ± 0.2 (0.6)
S	10	-16.7 ± 0.5	12.9 ± 1.7	-19.6 ± 0.5	5.1 ± 1.9	23.7 ± 1.2	8.4 ± 1.2	6.6 ± 1.3	4.8 ± 0.2 (0.6)
SE	6	-17.5 ± 0.4	12.4 ± 1	-20.4 ± 0.5	4.2 ± 1.7	23 ± 1.1	7.7 ± 1.1	6 ± 1.3	4.8 ± 0.2 (0.6)
S. longirostris	9	-17.8 ± 0.2	11.1 ± 0.6	-20.4 ± 0.4	4 ± 1.2	21.6 ± 0.8	6.9 ± 0.9	5.6 ± 0.8	4.7 ± 0.3 (0.6)
S	3	-17.9 ± 0.2	11.2 ± 0.8	-20.6 ± 0.2	3.7 ± 0.4	22 ± 0.9	7 ± 0.2	5.3 ± 0.5	4.7 ± 0.4 (0.6)
SE	6	-17.7 ± 0.2	11.1 ± 0.6	-20.3 ± 0.4	4.1 ± 1.5	21.5 ± 0.7	6.8 ± 1.2	5.8 ± 1	4.6 ± 0.3 (0.6)
T. truncatus	17	-16.9 ± 0.4	13 ± 1.1	-19.9 ± 0.5	4.6 ± 1.1	24 ± 1.2	8.2 ± 1	7 ± 1.7	5 ± 0.3 (0.6)
S	14	-16.8 ± 0.3	13 ± 1.2	-19.8 ± 0.5	4.5 ± 1.2	24 ± 1.3	8.2 ± 1.1	7 ± 1.9	5 ± 0.3 (0.6)
SE	3	-17.1 ± 0.4	12.8 ± 0.6	-20 ± 0.2	4.6 ± 1.1	23.7 ± 0.8	8.1 ± 0.3	6.7 ± 0.1	4.9 ± 0.3 (0.6)

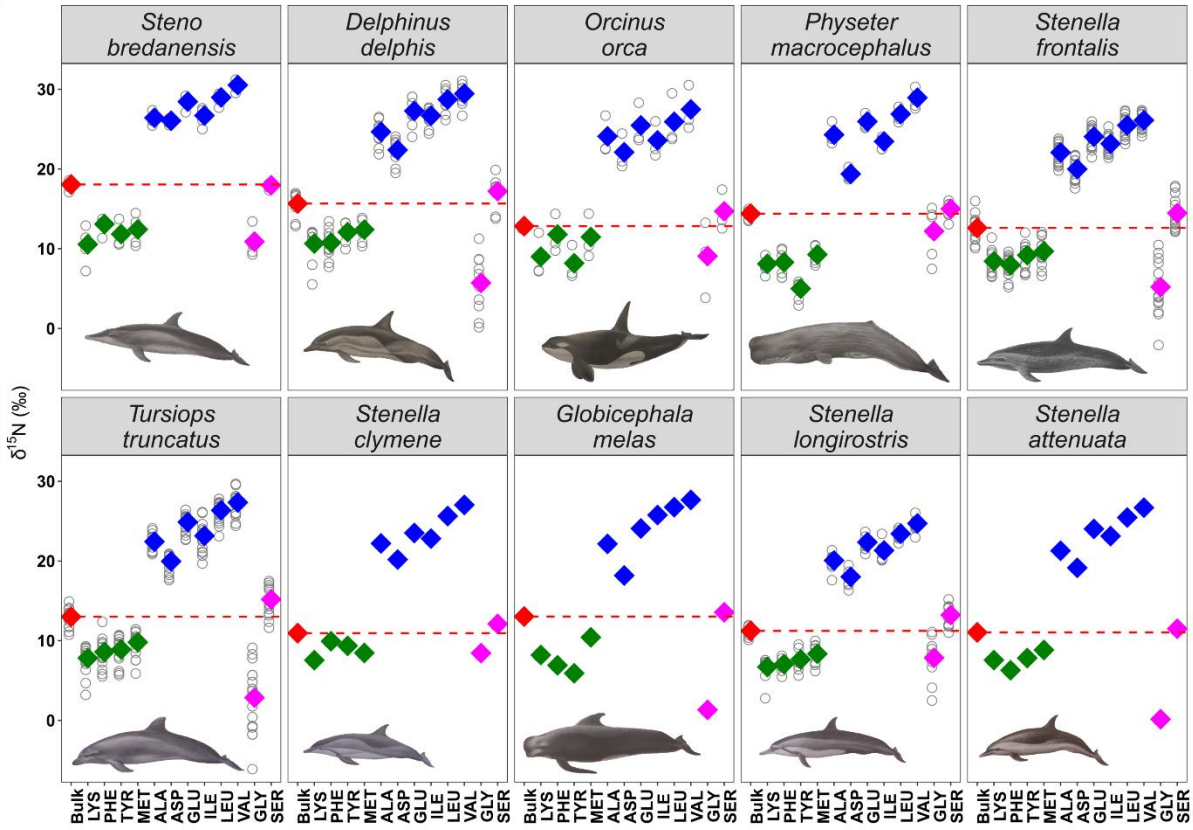

Figure 2 Individual (grey open circles) and mean (solid diamonds) $\delta^{15}N$ measured in amino acids (AA) of skin samples of cetaceans from the western South Atlantic. AAs are sorted by type, with different colours of solid diamonds (mean $\delta^{15}N$) representing AA type: source AA in green (lysine LYS, phenylalanine PHE, tyrosine TYR, methionine MET); trophic AA in blue (alanine ALA, aspartic acid ASP, glutamic acid GLU, isoleucine ILE, leucine LEU, valine VAL); and metabolic AA in pink (glycine GLY, serine SER). Mean $\delta^{15}N$ in bulk-skin from each cetacean species is indicated with the red diamond and the horizontal dashed line. Threonine is not shown in this graph due to the strong differences in $\delta^{15}N$ (negative values) in comparison to the other AAs, but $\delta^{15}N$ for this AA can be seen in the appendix 3 and 6. Cetacean illustration by José Luis Vázquez, adapted from Bastida et al. 2018.

3.2. Cetacean trophic position

The Welch ANOVA showed significant differences in TP estimates among the different (bulk vs. AA) equations ($F = 29.8$, $df = 166.3$, $p < 0.001$), with those using bulk-skin $\delta^{15}N$ data (both offshore and shelf-break baseline $\delta^{15}N$) resulting in significantly higher TPs than the equation using AA data ($P < 0.05$). Additionally, because the offshore baseline had a lower $\delta^{15}N$ value ($\delta^{15}N_{copepods}$ in offshore < shelf-break), every cetacean species would have a higher TP ($p < 0.05$) if they were feeding along the offshore area (Fig. 3 and appendix 8). Estimates using bulk-skin $\delta^{15}N$ identified *S. bredanensis* as occupying the highest TP amongst all cetacean species (Fig. 3, appendix 8). Conversely, estimates using AA-$\delta^{15}N$ identified *P. macrocephalus* with the highest TP (TP = 5.3 ± 0.5), followed by *G. melas* (TP = 5.2) and *S. attenuata* (TP = 5.2). Intermediate TPs were observed in *D. delphis*, *T. truncatus*, and *S. bredanensis* (Table 1).

Lower TPs were estimated for *S. frontalis* (TP = 4.8 ± 0.2), *S. clymene* and *S. longirostris* (TP = 4.7), and *O. orca* (TP = 4.5 ± 0.1). Overall cetacean TP did not differ between regions or seasons (F = 1.1, df = 19.6, p > 0.05; Fig. 4).

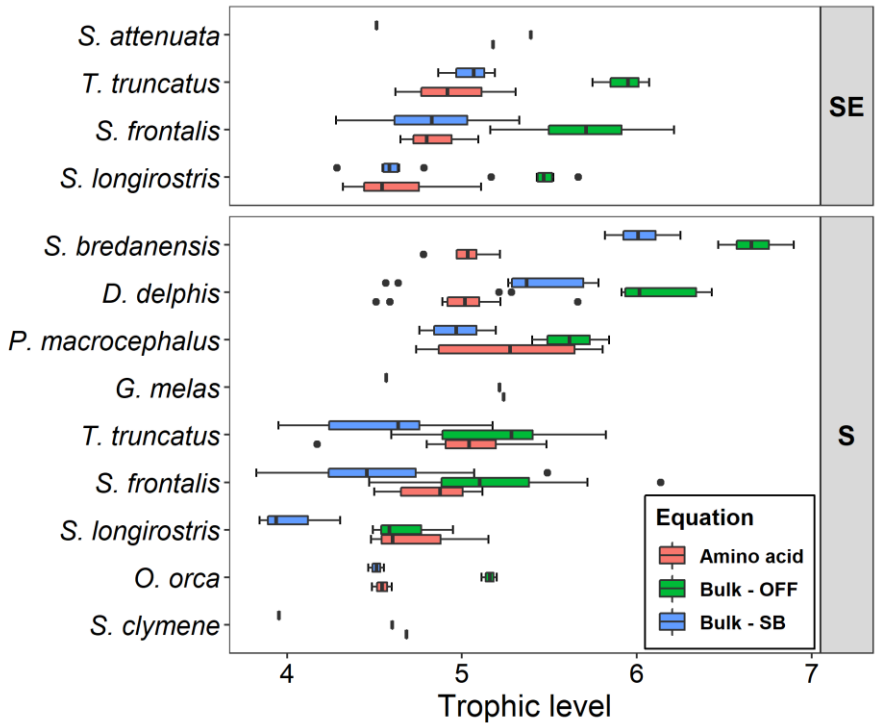

Figure 3 Estimated trophic position (TP) for cetacean species with bulk-tissue (blue and green) and amino acid (red) nitrogen stable isotopes ($\delta^{15}N$). Bulk-tissue equation uses $\delta^{15}N$ in cetacean skin and copepods (baseline, primary consumer) and considers different baseline $\delta^{15}N$ for copepods sampled along the shelf-break (blue boxes) and offshore (green boxes), in the southeast (SE) and southern (S) regions. TP is presented separately for cetacean sampled in each region (SE or S), and used regional baseline $\delta^{15}N$ values for copepods as their $\delta^{15}N$ values differed between the shelf-break (SB) and offshore (OFF). Therefore, TP was estimated using baseline $\delta^{15}N$ from SB = 4.3‰ and OFF = 1.3‰ in the SE; and from SB = 6.1‰ and OFF = 3.9‰ in the S region. The amino acid equation uses $\delta^{15}N$ values of multiple trophic and source amino acids ($TP_{Tr\text{-}Sr}$), a trophic discrimination factor of 3.1‰ and β = 3.4‰.

3.3. Spatiotemporal patterns in $\delta^{15}N$

The seasonal (autumn-spring) and latitudinal (SE-to-S) patterns of $\delta^{15}N$ in bulk zooplankton (amphipods, copepods and euphausiids) and Phe were similar: mean $\delta^{15}N$ value was higher in the south (both seasons) than in the SE, and slightly higher in spring than in autumn (Fig. 4). These spatiotemporal differences were statistically significant for $\delta^{15}N$ values in bulk zooplankton (F = 26.6, df = 73.8, p < 0.001), $\delta^{15}N_{Adjusted}$ (F = 3.3, df = 21.4, p < 0.05), and Phe (F = 6.2, df = 24.8, p < 0.01). Post hoc Games-Howell comparisons showed that latitudinal (SE-to-S) differences were significant (higher $\delta^{15}N$ value in the S region) in zooplankton and Phe in autumn, but not in Phe and $\delta^{15}N_{Adjusted}$ in spring (p > 0.05). Significant

seasonal differences were observed within the S region for zooplankton $\delta^{15}N$ value (higher in spring), while none of the groups showed significant seasonal differences in $\delta^{15}N$ in the SE region. Our logistic regression model confirmed that higher baseline $\delta^{15}N$ values were observed in the S than in the SE region (Fig. 5). The training dataset had 74% predictability accuracy, and the accuracy of the model based on the testing dataset was 75%. The model predicted a decrease of 0.63 (95% CI = 0.52 – 0.78) in the odds of samples being from the S region for each unit decrease in $\delta^{15}N$ (β = -0.45, se = 0.1, $p < 0.001$). Additionally, the small variance of the random intercept (σ^2 = 0.05) indicated that all groups (zooplankton, Phe and $\delta^{15}N_{Adjusted}$) had similar logistic curves (Fig. 5a).

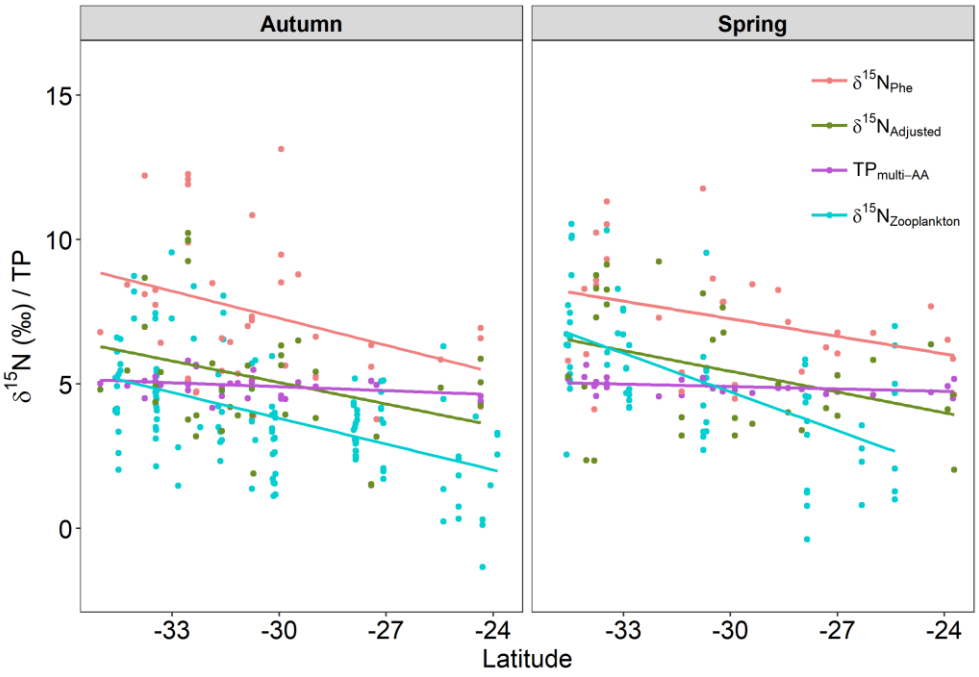

Figure 4 Latitudinal patterns in cetacean trophic position ($TP_{multi\text{-}AA}$), $\delta^{15}N$ values of bulk zooplankton ($\delta^{15}N_{Zooplankton}$) and cetacean trophic-adjusted source amino acid phenylalanine ($\delta^{15}N_{Phe}$) and bulk-skin ($\delta^{15}N_{Adjusted}$), plotted separately for samples obtained in austral autumn and spring.

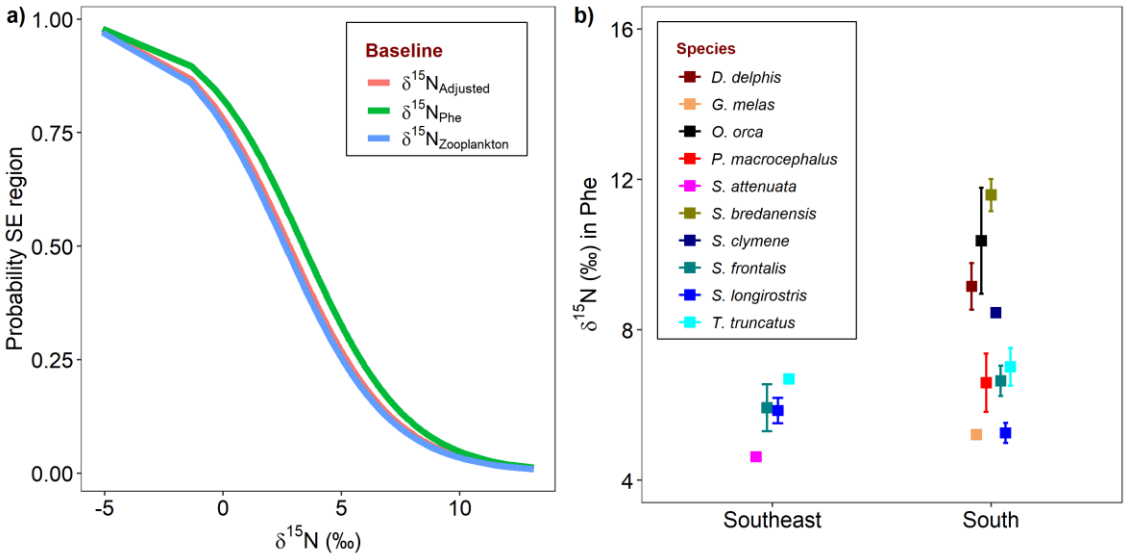

Figure 5 Latitudinal patterns in $\delta^{15}N$ values observed in zooplankton and cetacean bulk-skin and amino acids: a) predicted probabilities from the logistic regression model for region as response to $\delta^{15}N$ with groups (Baseline) as random effects: model predict a decrease in the probability of sample being from the southeast (SE) region as baseline $\delta^{15}N$ values increase. Baseline is represented by pooled zooplankton ($\delta^{15}N_{Zooplankton}$), and cetacean bulk-skin and source amino acid phenylalanine adjusted for trophic position effect ($\delta^{15}N_{Adjusted}$ and $\delta^{15}N_{Phe}$, respectively); and b) shows the species patterns in Phe $\delta^{15}N$ between the two regions.

3.4. Inter-specific trends in trophic position and habitat use

Our hierarchical cluster analysis identified cetacean groups based on the similarity in baseline $\delta^{15}N$ value (adjusted Phe) and trophic position (Fig. 6). *S. bredanensis* and *D. delphis* were grouped together as the species with the highest baseline $\delta^{15}N$ values, indicating a predominantly neritic distribution. These species, along with *O. orca* and *S. clymene* (with relatively lower TPs), were highly dissimilar from the other cetacean species (Fig. 6), that had isotopic baseline indicating predominant distribution towards the outer continental shelf and open oceanic waters. The cluster including the more oceanic species was further split into two groups, according to their relative trophic positions: *P. macrocephalus*, *G. melas* and *S. attenuata* were separated from the remaining cetaceans with relatively lower TPs (Fig. 6). Within this "relatively lower TP" group, the cluster analysis identified isotopic dissimilarity, further splitting *S. longirostris* from the more isotopically similar *S. frontalis* and *T. truncatus* (Fig. 6).

The standard ellipse areas based on $\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$ (cetacean TP-adjusted bulk-skin), representing the isotopic niche of primary consumers in the areas used by each cetacean species, indicated marked inter-specific spatial segregations (Fig. 7). The pairwise percentage overlap between species is shown in appendix 9. The baseline niche area of *S.*

bredanensis did not overlap with those of any other cetacean species, except with *D. delphis* (17% overlap, appendix 9), although the overlap between the corresponding 95% prediction ellipses suggested a small (7%) overlap between *S. bredanensis* and *S. frontalis* (appendix 9). The highest overlap was observed between *T. truncatus* and *S. frontalis* (Fig. 7).

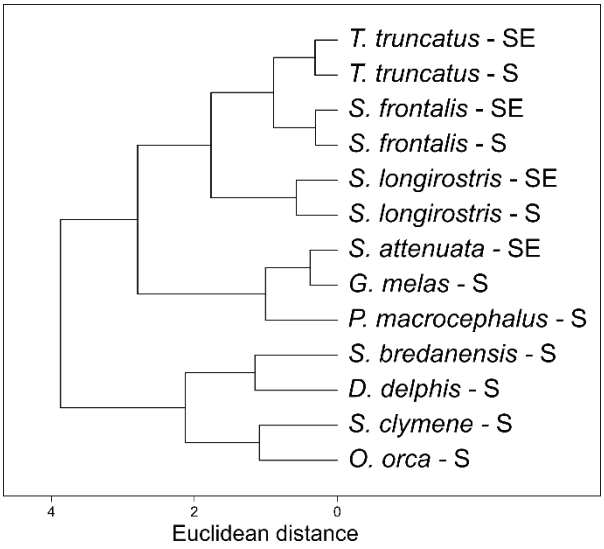

Figure 6 Hierarchical cluster analysis (complete linkage) dendrograms based on estimated trophic position and $\delta^{15}N$ in trophic-adjusted phenylalanine of cetaceans from the oceanic waters of southeast (SE) and southern (S) Brazil.

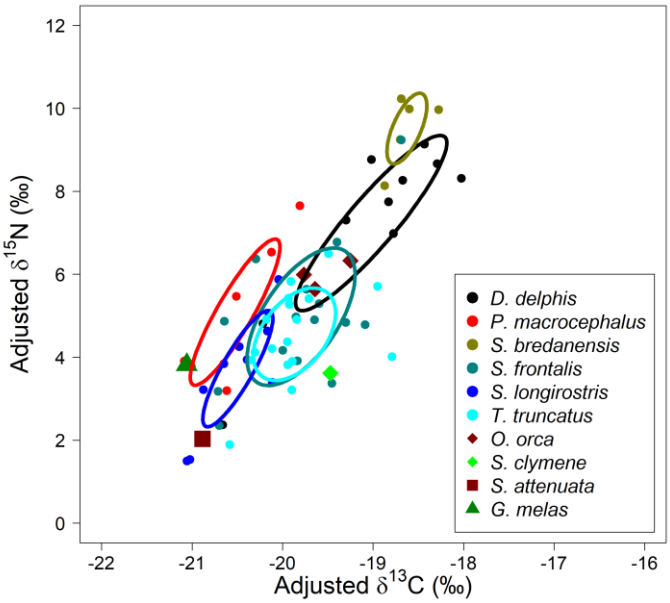

Figure 7 Estimated isotopic niche areas of baseline sources where cetacean species forage, based on trophic adjusted cetacean bulk-skin ($\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$) using SIBER; and $\delta^{13}C$ and $\delta^{15}N$ biplots of baseline sources for cetaceans from which the standard ellipse area could not be estimated due to small sample size ($n < 6$).

4. Discussion

We measured the $\delta^{15}N$ values of individual AAs to investigate the foraging areas and trophic position of odontocetes from the western South Atlantic. AAs generally adhered to source (e.g., Phe, Lys) vs. trophic (Val, Leu, Glu, Ile, Ala and Asp) groupings, which is in line with the expected low or high diet-to-consumer fractionation for source and trophic AAs, respectively (Popp et al. 2007; Chikaraishi et al. 2014; McMahon and McCarthy 2016; O'Connel 2017). The nitrogen isotopic patterns in trophic adjusted Phe measured in cetacean skin were highly associated with baseline (i.e. zooplankton) $\delta^{15}N$, confirming the baseline shift between SE and S regions, and helping to differentiate the cetacean species in terms of their habitat usage. The niche areas of baseline sources, assessed by correcting cetacean bulk-skin $\delta^{13}C$ and $\delta^{15}N$ values for trophic effect, revealed spatial segregation between *S. bredanensis* and the remaining odontocetes, and further confirmed the high overlap in habitat use between *T. truncatus* and *S. frontalis.*

4.1. Spatial patterns in baseline nitrogen stable isotopes

The spatial patterns in the isotopic values of cetacean Phe and bulk-skin were similar to those of bulk zooplankton: $\delta^{15}N$ values were higher in individuals sampled in the S region than in those sampled in the SE (Figs. 4 and 5b), and GLMM estimated increased probabilities of higher $\delta^{15}N$ to be from samples obtained in the S region (Fig. 5a). The spatial variation in $\delta^{15}N$ of Phe was consistent with variation in bulk-skin $\delta^{15}N$ in a much larger data set (Troina et al. 2020a) and with the described nitrogen isoscapes throughout the region (Troina et al. 2020b). This was supported by the significant relationship between bulk-skin and TP-adjusted Phe $\delta^{15}N$ values(Appendix 7), suggesting that cetacean ^{15}N-enrichment in the south is consistent with baseline $\delta^{15}N$ values and not due to higher TPs occupied by the southernmost species. Altogether, our analyses provided further support for the offset in baseline $\delta^{15}N$ values based on location due to different oceanographic conditions throughout the study area that result in the upwelling of nutrient-rich ^{15}N-enriched deep waters towards the south and along the shelf-break (Troina et al. 2020b). The source AA Phe has been successfully applied to study penguins from different oceanic regions, where interspecific gradients in Phe-$\delta^{15}N$ values among individuals sampled along latitudinal gradients represented the differences in the isotopic baseline of their respective foraging areas (Lorrain et al. 2009). Similar results have been described for marine predators in other regions, where AA-$\delta^{15}N$ values observed in predators

sampled along a latitudinal gradient were associated with baseline (zooplankton) $\delta^{15}N$ values (Popp et al. 2007) or with known marine isoscapes (Matthews and Ferguson 2014). Thus, even though cetaceans are highly mobile animals, populations inhabiting the oceanic waters of the western South Atlantic seem to have a high level of fidelity to either southeast (those with low $\delta^{15}N$ values) or southern (high $\delta^{15}N$ values) regions (see section 4.2).

4.2. Cetaceans' trophic ecology and habitat use

The estimated TPs derived from equations based on bulk-tissue isotope values considered local baseline (e.g., $\delta^{15}N$ from copepods sampled in the south to estimate the TP from cetaceans sampled in the same region) and the in-to-offshore gradient in baseline $\delta^{15}N$ values. Equations using the lower $\delta^{15}N$ value from copepods sampled in the offshore waters resulted in higher TPs for all odontocete species (Fig. 3). The inter-specific patterns in relative TPs estimated using equations based on bulk-tissue differed from those using AA data. While bulk-$\delta^{15}N$ equations yielded the highest TPs for *S. bredanensis* and *D. delphis*, AA equations estimated the highest TPs for *P. macrocephalus*, *G. melas* and *S. attenuata*. Trophic position estimates based on bulk-tissue $\delta^{15}N$ rely greatly on the use of correct isotopic values for baseline organisms (e.g., copepods), and the use of inadequate baseline $\delta^{15}N$ will have critical influence on TP estimates. Inadequate isotopic baselines may result from missing baseline data (e.g., from other regions where species occur that were out of the range of our study), or from temporal mismatch between baseline and consumers' stable isotopes, as different isotopic turnover rates may apply for cetacean and zooplankton tissues. Evidently, baseline $\delta^{15}N$ values were resolved with the AA analyses, as source-AA $\delta^{15}N$ values should reflect baseline isotopic patterns of their foraging areas (Chikaraishi et al. 2009; McMahon and McCarthy 2016).

The highest TP was observed in *S. bredanensis* amongst all cetacean species, including *P. macrocephalus*, even though equations accounted for the higher baseline $\delta^{15}N$ of copepods sampled in the S region along the shelf-break (where *S. bredanensis* were sampled). This suggested that we were still missing baseline isotopic information and was confirmed by AA-based TPs that yielded relatively lower TP values for this species (5.0 ± 0.2, Table 1). The inconsistency in TP estimates obtained by equations using bulk-skin or AA-$\delta^{15}N$ values (Fig. 3) likely indicates that the baseline $\delta^{15}N$ values used were not representative of *S. bredanensis'* feeding areas. This was supported by the $\delta^{15}N$ values in TP-adjusted Phe, which were higher than in the remaining odontocete species (except for one individual of *O. orca*, Fig. 5b), and by

the estimated niche area of baseline sources based on trophic adjusted bulk-skin $\delta^{13}C$ and $\delta^{15}N$ (Fig. 7). Thus, our results suggest that *S. bredanensis* feeds and occurs in waters with a higher baseline $\delta^{15}N$ value. This would be consistent with foraging in neritic waters along the continental shelf area where lower trophic level organisms have higher $\delta^{15}N$ values (Condini et al. 2015), which was out of the range of this study and therefore not sampled for baseline isotope measurements. Indeed, cetacean species occurring in southern Brazilian coastal regions have considerably higher $\delta^{15}N$ values (Botta et al. 2012; Troina et al. 2016; Secchi et al. 2017), and occurrence in inner shelf waters has been reported for *S. bredanensis* in the western South Atlantic (Ott and Danilewicz 1996; Santos et al. 2017; 2019). Thus, the comparison of TPs derived from these two types of equations (bulk-tissue and AA data) demonstrated that we were missing the isotopic baseline from other regions, resulting in unrealistic bulk-tissue TP estimates (e.g., TP > 6). Therefore, the $\delta^{15}N$ values in Phe and the isotopic niche patterns of baseline sources (Fig. 7) suggest that the high $\delta^{15}N$ values observed in bulk-skin are not due to occupying higher trophic positions, but rather due to foraging in the neritic waters of the southern region.

Similarly, *D. delphis* also had high $\delta^{15}N_{Phe}$ (Fig. 5b) and the second highest bulk-skin $\delta^{15}N$ (Table 1). The estimated TP based on bulk-skin $\delta^{15}N$ value with a shelf break baseline (appendix 8) was comparable to those derived from the $TP_{Tr\text{-}Sr}$ equation (Table 1), whereas TP_{Bulk} equation using the offshore baseline resulted in a considerably higher trophic position for this species (Fig. 3). *D. delphis* was sampled only in the southern region of the study area, where higher $\delta^{15}N$ values have been reported for baseline organisms (Troina et al. 2020b). The surveys for the present work were carried out only in the oceanic waters off the SWA (between the outer continental shelf and slope waters), and *D. delphis* were mainly sighted along the shelf break waters (~150 m isobaths). Furthermore, the species is frequently observed in neritic waters, near the 100 m isobaths (ECOMEGA, unpublished data). Accordingly, our isotopic data indicates that the shelf break isotopic baseline is representative of this species' foraging areas.

Amino acid-based equations estimated the highest TPs for *P. macrocephalus*, *G. melas* and *S. attenuata*. The high TP estimated for *P. macrocephalus* was anticipated as these animals feed upon large squids (presumably high trophic position) at large depths (Clarke et al. 1980; Santos and Haimovici 2000). Bulk-$\delta^{15}N$ data of the short fin squid *Illex argentinus*, an important prey item for sperm whales (Clarke et al. 1980; Santos and Haimovici 2000) ranged between 9 and 13‰ in the same region (Troina 2019), which supports a relatively high trophic position of this prey in these oceanic waters. The high TP estimated for the single *S. attenuata* was

unexpected, as this species has been reported to feed mainly on small epi- and meso-pelagic fish, squids and crustaceans (Robertson and Chivers 1997; Wang et al. 2003; 2012; Perrin 2018). Estimated TP for *G. melas* and *S. attenuata* are derived from a single individual, hence no inference can be made at the population level. Nevertheless, our estimated TP based on bulk-skin were comparable to those of *G. melas* from the Southern Ocean based on a much larger dataset (Fontaine et al. 2015). Additionally, the high TP estimated for *G. melas* using bulk-skin isotope values (Table S5) were consistent with those based on AA data (Table 1), especially when considering an offshore isotopic baseline (both yielding TP = 5.2). *G. melas* feeds primarily on cephalopods (Gannon et al. 1997a, b; Santos and Haimovici 2001; Santos et al. 2014; Beasley et al. 2019), prey with relatively high trophic positions.

The estimated isotopic niche areas of baseline sources indicated little overlap between *P. macrocephalus* and the other odontocete species (Fig. 7). This pattern differed from the high isotopic niche overlap observed among the same species based on bulk-skin stable isotopes (Troina et al. 2020a) and shows how using only bulk-tissue $\delta^{15}N$ values can affect the overall interpretation of trophic structure and habitat use. While high bulk-skin $\delta^{15}N$ values in *P. macrocephalus* were due to the species occupying relatively higher trophic position, as evidenced by the AA-based TP estimates (Fig. 3), high $\delta^{15}N$ in the other odontocetes (e.g., *D. delphis*) was due to occurrence in areas with relatively higher baseline $\delta^{15}N$ values (Fig. 7). Additionally, biplots of bulk-skin $\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$ (Fig. 7) suggest an oceanic distribution for baseline sources used by *P. macrocephalus, G. melas* and *S. attenuata*, which is consistent with these species' distributional patterns (Di Tullio et al. 2016).

The AA data indicated that the lowest TP is occupied by *O. orca.* While the bulk-skin $\delta^{15}N$ value was very similar between *O. orca* and *G. melas*, the AA-$\delta^{15}N$ value suggests that *G. melas* occupies almost 1 TP higher than *O. orca*. The question that remains is whether the higher $\delta^{15}N$ in *O. orca*' source AA Phe than in *G. melas* is due to shifts in baseline $\delta^{15}N$ between the regions where individuals of each species had been feeding, or due to potential trophic level enrichment for this AA (Matthews et al. 2020). When comparing fish- and marine mammal-eating ecotypes of *O. orca*, Matthews and colleagues (2020) found that the latter ecotype had relatively higher $\delta^{15}N_{Phe}$, which resulted in low estimated TPs. The authors suggested that not only trophic but also some source AAs, particularly Phe, may undergo large fractionations that result in ^{15}N-enrichment as trophic level increases. This is consistent with our isotopic data for this species, that had high $\delta^{15}N$ in Phe in comparison with other source AAs (e.g., Lys, Fig. 2). Looking at Lys-$\delta^{15}N$ (Fig. 2), those of *O. orca* were always higher than those of *G. melas*,

strongly suggesting that *O. orca* sampled in the present study occur in areas with relatively higher baseline $\delta^{15}N$. This was also supported by the analysis of bulk-skin $\delta^{13}C_{Adjusted}$ and $\delta^{15}N_{Adjusted}$ values (Fig. 7). Additionally, the multiple AA equation to estimate TP should reduce the effect of large TDF in Phe, if that were the case. As these individuals were feeding on a minke whale calf (*Balaenoptera* spp.) when samples were obtained (Troina et al. 2020a), it is reasonable to assume that they occupy relatively lower TP than those who feed on seals, swordfish *Xiphias gladius* (Dalla Rosa and Secchi 2007; Passadore et al. 2015) or on blue shark *Prionace glauca* (Passadore et al. 2015). Nevertheless, controlled feeding experiments in marine fish have shown that the discrimination in $\delta^{15}N$ values in some AAs between consumer and diet ($\delta^{15}N_{AA\text{-}consumer} - \delta^{15}N_{AA\text{-}diet}$) depends on the quality of dietary protein (McMahon et al. 2015b; McMahon and McCarthy 2016). These authors have demonstrated that large concentrations of high-quality protein (i.e. similar AA composition between prey and predator) seem to yield a lower TDF, while diet with a smaller quantity of low quality protein yields a significantly higher TDF. This is especially relevant for marine mammal-eating *O. orca* ecotypes, whose source-to-trophic AAs TDF could be lower than the values applied here. In this case, the use of incorrect (i.e. higher) TDF values would underestimate TPs, which would justify the relatively low values observed in *O. orca* sampled in this study.

The low TPs estimated for *S. longirostris* based on the AA data confirms that their low $\delta^{15}N$ in bulk-skin reflects both their occurrence in waters with low baseline $\delta^{15}N$ (Troina et al. 2020a) and consumption of lower trophic level prey. Indeed, bulk-muscle $\delta^{15}N$ in epi- and mesopelagic fish, important prey items for this species (Silva-Jr. et al. 2007), are relatively lower than those in *S. longirostris* (~8-10‰ vs. 11.2±0.6‰, respectively; Table 1, Troina 2019). This difference is within the ~1.6‰ (±0.5) range of trophic enrichment factor estimated for $\delta^{15}N$ values in cetacean bulk-skin (Giménez et al. 2016). Furthermore, *S. longirostris* sampled in the SE and S regions were clustered together (Fig. 6) and the lower baseline $\delta^{15}N$ values observed in *S. longirostris* sampled in the S region were consistent with baseline values from the SE region. This indicates that, although some individuals may venture in the oceanic waters of the southern region, they spend most of the time in waters of relatively lower $\delta^{15}N$ values (i.e. in the SE region). This is consistent with bulk-tissue $\delta^{15}N$ values in a much larger dataset, that did not show isotopic differences in $\delta^{15}N$ between *S. longirostris* sampled in these two regions (Troina et al. 2020a). Therefore, our AA data provides further support for the lack of spatial (SE-S) differences in TP and habitat use at the intra-specific level for *S. longirostris*.

Similarly as with bulk-skin isotopic data (Troina et al. 2020a), $\delta^{15}N$ values in Phe were slightly higher in *S. frontalis* from the S region than in those from the SE, and higher in spring than in autumn (Appendix 10). These differences were not statistically significant (Appendix 10) likely due to small sample sizes that affected statistical power when comparing intra-specific differences between regions. Nevertheless, AA data provided further support for some level of intra-specific differences in habitat use between *S. frontalis* from the SE and S regions, indicating the existence of discrete populations. Accordingly, spatial (SE vs. S) differences in bulk-skin $\delta^{15}N$ values were not due to distinct trophic positions occupied as individuals from both populations had the same TPs (Table 1), but rather reflect the spatial and seasonal differences in the isotopic baseline between the S and SE regions (Troina et al. 2020b).

Baseline isotopic niche areas indicated resource overlap of *D. delphis* with *S. frontalis* (46%, appendix 9) and with *T. truncatus* (38%). However, the hierarchical cluster based on Phe-$\delta^{15}N$ and estimated TPs did not indicate similarity between *D. delphis* and the latter two species (Fig. 6). This demonstrates that differences in the distributional ranges of these species are affecting their Phe-$\delta^{15}N$ values: while *D. delphis* forages in neritic waters (as discussed above), *S. frontalis* and *T. truncatus* are distributed in further oceanic waters (~250 m and ~500 m isobaths, respectively; Di Tullio et al. 2016). On the other hand, *T. truncatus* and *S. frontalis* showed high isotopic similarity (Fig. 6) and isotopic niche overlap (57%, appendix 9, Fig. 7). Additionally, estimated TPs were similar between *T. truncatus* (both SE and S regions) and *D. delphis* (Fig. 3), while *S. frontalis* had slightly lower TP values. In southern South America, the argentine anchovy (*Engraulis anchoita*) and long-finned squid (*Loligo sanpaulensis*) seem to be important items in the diet of *D. delphis* (Romero et al. 2012), while *S. frontalis* may have a predominantly teuthophagous diet with the long-finned squid *Loligo plei* reported as important prey (Di Beneditto et al. 2001; Lopes et al. 2012). *T. truncatus* is known to have a generalist feeding behaviour, frequently consuming sciaenid, scombrid and mugilid fish (Wells and Scott 2018). Thus, although these species may have similar trophic positions, they most likely consume different prey species or segregate habitat temporarily and/or spatially. This agrees with the patterns of distribution and interspecific interactions reported in the western South Atlantic (Di Tullio et al. 2016; Lima et al. 2021). While *D. delphis* and *S. frontalis* show significant spatial segregation in their areas of occurrence (Di Tullio et al. 2016), *T. truncatus* and *S. frontalis* are often found in mixed-species groups, especially in the SE region (Lima et al. 2021). Accordingly, given the high overlap in the isotopic niches of the baseline sources used by *T. truncatus* and *S. frontalis* (Fig. 7) and the different relative TP (Fig. 3), our analyses

indicate that these two species overlap in the use of areas, while consuming prey at different trophic levels, thereby minimizing interspecific competition.

Finally, our current analyses indicated a relatively low TP (4.7) for the single *S. clymene* sampled. There is still little information about this species' trophic ecology (Jefferson 2018), but mesopelagic fish and squids may be consumed (Fertl et al. 1997; Sakyi et al. 2019). Cephalopod, fish and crustaceans have been reported in the stomach of *S. clymene* from the eastern Atlantic Ocean, with the reef-associated, shoaling fish *Decapterus* sp. being an important prey item (Sakyi et al. 2019). This fish consumes planktonic invertebrates, including copepods and other zooplankton (Berry and Smith-Vaniz 1978). Accordingly, feeding on such low trophic level prey could explain the low TP observed in *S. clymene* in the present study.

It should be noted that the small sample size for some of the species analysed here does not allow us to make inferences at the population level about the trophic ecology and habitat use of cetaceans from the SWA. We compare the data available on amino acid $\delta^{15}N$ values with a much larger data set on bulk-skin $\delta^{15}N$ (Troina et al. 2020a) and baseline organisms throughout the region (Troina et al. 2020b), with the aim to explore the patterns of foraging areas and feeding habits of these oceanic cetaceans. Thus, our work expands the understanding of these species' trophic ecology and habitat use and provides baseline for future research. We highlight the need to continue investigating these oceanic populations aiming at obtaining more robust data on a long term basis to assess changes in their trophic and spatial ecology.

4.3. Limitations and perspective for future research

The high $\delta^{15}N$ values observed in the source AA Phe, particularly in *O. orca* in this and in other study (Matthews et al. 2020), suggest that Phe might not always be the ideal AA to reflect baseline N isotopes, especially for high trophic level consumers. However, biases in estimated TPs should be minimized when adopting the multiple AA approach, that includes $\delta^{15}N$ values of several source AAs (e.g., Nielsen et al. 2015; Ruiz-Cooley et al. 2021). Additionally, a recent controlled feeding study has found that Lys reliably represented baseline nitrogen values in captive sea turtles (Lemons et al. 2020). Given the consistently low $\delta^{15}N$ values observed in Lys in all cetacean species analysed in the present study, as well as their relatively lower $\delta^{15}N$ values in comparison to Phe in *O. orca* (Fig. 2), this AA might be a feasible alternative to represent the isotopic baseline of the regions where these odontocetes have been foraging. Future research should focus on understanding the effect of trophic

discrimination on Lys-$\delta^{15}N$ in higher trophic level organisms. This would allow for trophic corrections, as the ones applied in the current study for Phe-$\delta^{15}N$, for accurate assessment of spatial isoscapes on Lys-$\delta^{15}N$.

Furthermore, trophic position estimates depend on the use of accurate trophic discrimination factors for bulk-tissue ($TDF_{consumer-prey}$) and for predator' source-to-trophic AA $\delta^{15}N$ value ($TDF_{trophic–source}$). Aside from the quality and amount of dietary protein intake that may have substantial effect on TDF (Robbins et al. 2005; McMahon et al. 2015b; McMahon and McCarthy 2016), several other factors may affect $\delta^{15}N$ discrimination between the assimilated food and the consumers' bulk-tissues or individual compounds (e.g., amino acids). Therefore, TDFs differ among taxonomic group (Vanderklift and Ponsard 2003; Germain et al. 2013; McMahon et al. 2015a) and tissue analysed (Hobson and Clark 1992; Caut et al. 2008, 2009), and may be affected by consumers' trophic level and the isotopic values of their prey (Adams and Sterner 2000; Germain et al. 2013; Hussey et al. 2014). Additionally, the form of nitrogen excretion may have an important influence on $TDF_{trophic-source}$, and consumers that excrete urea usually have lower TDF between source and trophic AAs (Germain et al. 2013; McMahon and McCarthy 2016). Controlled feeding experiments involving cetaceans are rare due to logistic and ethical reasons. Therefore, there are only a few instances when prey-to-cetacean bulk-tissue $\delta^{15}N$ TDF have been estimated in such conditions (Caut et al. 2011; Giménez et al. 2016). Only one study has estimated $\delta^{15}N$-TDF between prey and consumer AAs in controlled feeding experiments for marine mammals (harbour seals *Phoca vitulina*, Germain et al. 2013), but no such data are available for cetaceans. Nevertheless, as all species sampled here belong to the same taxon, have the same form of nitrogen excretion (urea), and isotopic measurements were carried out in the same type of tissue (skin), we assume that any of these factors would introduce similar biases in estimated TPs to all odontocetes. Additionally, while the absolute TPs presented here may not be accurate, as they rely on unknowns such as how odontocetes physiology and amino acid metabolism affect AA-$\delta^{15}N$ and TDF values, they do represent relative differences among species as they consider the spatial patterns in baseline $\delta^{15}N$.

5. Conclusion

The present work is the first to analyse $\delta^{15}N$ in individual AAs to study the trophic ecology and habitat use by odontocetes from the western South Atlantic. We observed several

interspecific patterns in the $\delta^{15}N$ of the different AAs, although in the case of *O. orca* conclusions were limited by the lack of knowledge on the effect of baseline, trophic level or metabolic processes (e.g., fasting or nutritional stress, dietary protein quality, metabolic deficiency due to diseases, etc.) on phenylalanine $\delta^{15}N$ discriminations. Continued research can greatly improve the potential for applying AA-$\delta^{15}N$ to help answer a range of ecological questions. Specifically, three research directions are critical for effective use of stable isotopes in AAs to study the trophic structure and habitat use of oceanic cetacean species: 1) define β between source and trophic AAs in oceanic primary producers; 2) define $\Delta^{15}N$ for source AAs (especially Phe and Lys) in marine mammals, to allow for adjustments to obtain baseline isotopic values that remove the trophic effects on these AAs; and 3) estimate TDF values between trophic and source AAs) in high trophic level mammals (see Ruiz-Cooley et al. 2021). Advances in this research topic are especially relevant for studies on species or populations with oceanic distributions, highly migratory species, or to compare past and current populations.

Furthermore, long-term monitoring of cetacean populations is critical to evaluate temporal trends in their trophic ecology and habitat use at the intra- and inter-specific levels. We provide novel data for the oceanic populations of cetaceans from the western South Atlantic, showing the applicability of this method to help distinguish species' foraging areas along with the interspecific differences in trophic position. Additionally, our work provides AA-$\delta^{15}N$ baseline for future studies applying stable isotopes to investigate the trophic ecology and habitat use of marine organisms in the western South Atlantic. The nitrogen isotopic patterns in source AAs were highly associated with baseline $\delta^{15}N$ (zooplankton and cetacean $\delta^{15}N_{Adjusted}$). Therefore, the analysis of $\delta^{15}N$ in source AAs seems to be a good indicator of habitat use by cetaceans along the study area, allowing to differentiate between those individuals that were sampled in each respective region. Additionally, by removing the effect of trophic discrimination on bulk-skin $\delta^{13}C$ and $\delta^{15}N$ values, we have clearly demonstrated the patterns of overlap (e.g., between *T. truncatus* and *S. frontalis*) or segregation (e.g., *S. bredanensis*) in habitat use, that would otherwise have been masked by the trophic effect on these stable isotopes.

Authors' contribution

Genyffer C. Troina: Conceptualization, Methodology, Data curation, Formal analysis and investigation, Writing - original draft preparation, Writing - review and editing, Funding acquisition.

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Eduardo R. Secchi: Conceptualization, Methodology, Writing - review and editing, Funding acquisition.

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