

Origin and foraging ecology of male loggerhead sea turtles from southern Brazil revealed by genetic and stable isotope analysis

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Abstract

The southwestern Atlantic Ocean (SWA) represents an important foraging ground for loggerhead sea turtles (*Caretta caretta*). Most studies at the region have focused on adult females and juveniles, and little is known about males. Here, we present the first insights about origin and foraging ecology of male loggerheads from the SWA, by integrating genetic and stable isotope analysis (SIA). Skin samples were obtained from 26 males stranded along the southern coast of Brazil (from 31°21'S–51°05'W to 33°44' S–53°22' W), from February 2014 to March 2017. Samples of potential food sources (benthic and pelagic organisms) were also collected for SIA. A fragment of the mitochondrial DNA control region was sequenced and a Bayesian Mixed Stock

Analysis was performed to estimate natal origins of male loggerheads. Bayesian Stable Isotope Mixing Models were fitted to assess the relative contribution of different food sources assimilated by males. Most males exhibit endemic haplotypes from Brazilian rookeries, followed by a low frequency of a haplotype from the North Atlantic and the Mediterranean Sea, as well as olive ridley (*Lepidochelys olivacea*) haplotype, showing hybridization. SIA showed life stage-related differences in feeding and habitat use by male loggerheads, with benthic invertebrates dominating the diet of adults, while pelagic prey items dominated the diet of juveniles. Our findings demonstrate the importance of southern Brazil neritic and oceanic habitats for male loggerheads and highlight the value of this area for the maintenance of SWA reproductive management units, which are the main contributors to these feeding aggregations.

Introduction

The loggerhead sea turtle *Caretta caretta* has a worldwide tropical and subtropical distribution, but global numbers have been reduced over the last decades due to extensive fishing-related mortality (Wallace et al. 2013). Sea turtles are particularly sensitive to population reductions, especially to mortality of large juveniles and adults, because they are slow-growing and late-breeding animals (Petitet et al. 2012; Avens et al. 2015). Although the abundance of several populations of loggerhead sea turtles is currently stable or increasing, this species is classified as *Vulnerable* in the IUCN Red List and is considered entirely conservation-dependent (Rees et al. 2016; Casale and Tucker 2017).

Similar to others sea turtle species, loggerhead turtles exhibit complex life histories that encompass migrations between nesting and feeding grounds, ontogenetic shifts in diet and habitat use, and natal homing (Bolten 2003; Jensen et al. 2013). A long-standing paradigm predicates that early juveniles spend their first years in the oceanic habitats (~ 12 years for southwestern Atlantic populations; Petitet et al. 2012), feeding opportunistically on pelagic prey items, followed by a period where loggerheads supposedly recruit to neritic habitats where they remain until adulthood, feeding preferentially upon benthic invertebrates (Bjorndal 1997; Bolten 2003; Barros 2010). After reaching sexual maturity at about 30 years of age (Petitet et al. 2012), adult females and males display asynchronous seasonal migrations between foraging and nesting grounds, the latter located at their natal areas (FitzSimmons et al. 1997; Plotkin

2003). However, studies indicate that this generalized life history model is not a rule and that interindividual variation in foraging and migratory behaviour is displayed by juveniles and adult loggerheads among ocean basins (Hatase et al. 2002b; Casale et al. 2007; Mansfield et al. 2009; McClellan et al. 2010; Vander-Zanden et al. 2010; Zbinden et al. 2011). Factors that cause these variations remain poorly understood but could be related to sea surface temperature (Mansfield et al. 2009; Monteiro 2017), resource availability (Pajuelo et al. 2016) and phenotypic plasticity (Hawkes et al. 2006; Watanabe et al. 2011).

Knowledge of ecological features (e.g. habitat and resource use, migratory routes, and connectivity among nesting and foraging grounds) are essential for the development and application of appropriate conservation measures for sea turtle populations (Rees et al. 2016). Stable isotope analysis (SIA) has been extensively applied in ecological research of sea turtles, providing valuable information about diet resources (Dodge et al. 2011), habitat use (Reich et al. 2010; Petitet and Bugoni 2017), and ontogenetic shifts (Arthur et al. 2008; Snover et al. 2010). Stable isotope measurements of nitrogen ($\delta^{15}\text{N}$) can be used to assess the trophic level of a consumer (DeNiro and Epstein 1981; Minagawa and Wada 1984), whereas stable isotope values of carbon ($\delta^{13}\text{C}$) are often used to discriminate habitat use (e.g. neritic *versus* oceanic) and food sources (Fry 2006). Stable isotope values in consumers reflect those of prey items within a timescale that depends on the tissue's turnover rate, which is a species-specific trait (Peterson and Fry 1987; Fry 2006). Therefore, tissue selection is an important aspect for dietary reconstruction (Perkins et al. 2013). In loggerheads, skin provides dietary isotopic signatures over a time frame of ~45 days (Reich et al. 2008) without being modified with the specimen's decomposition (Payo-Payo et al. 2013).

In recent years, studies using SIA and satellite telemetry have revealed a wide variety of movements, habitat use and foraging strategies of loggerhead adult males and females, as well as juveniles, over ocean basins (Hatase et al. 2002b; Hawkes et al. 2006; McClellan et al. 2010; Pajuelo et al. 2012; Pajuelo et al. 2016). These studies have reported an extensive foraging dichotomy in loggerhead populations, which in some cases were related to turtle sizes. In the case of females, a similar pattern was observed in the western Pacific and eastern Atlantic Ocean, where larger adult individuals preferentially use neritic habitats, while smaller ones occurred in oceanic habitats (Hatase et al. 2002b; Hawkes et al. 2006). For adult male loggerheads from the

North Pacific, differences in feeding habitat appeared to also be influenced by body size. While larger males remained in coastal waters (Saito et al. 2015), smaller males inhabited pelagic habitats (Hatase et al. 2002a). In the Mediterranean Sea, however, although polymorphisms in habitat use were observed in adult male loggerheads, the variability in feeding behaviours was not associated with size (Schofield et al. 2010). Moreover, previous studies showed that adult males can display residency behaviour around breeding areas and fidelity to foraging grounds in northwestern and southeastern Atlantic waters (Arendt et al. 2012; Varo-Cruz et al. 2013) and in the Mediterranean Sea (Schofield et al. 2010; Casale et al. 2013), and that some degree of individual specialization can be shown according to resource availability (Pajuelo et al. 2016). Despite the increasing knowledge in different ocean basins, no information is available about the spatial ecology and feeding behaviour of male loggerhead sea turtles in the southwestern Atlantic Ocean (SWA).

The SWA is an important habitat for loggerheads, with several significant nesting and feeding grounds (Marcovaldi and Marcovaldi 1999; Vélez-Rubio et al. 2013; Carman et al. 2016; Monteiro et al. 2016), but also harboring extensive fishery activities that overlap with sea turtle distributions (Sales et al. 2008; Wallace et al. 2013; Monteiro et al. 2016). This high overlap increases the probability of turtle bycatch and is the main source of the thousands of dead loggerhead strandings in southern Brazil over the last twenty years (Monteiro et al. 2016). However, the effects of this mortality on the demographic and genetic structure of loggerhead populations remain unclear. The Brazilian rookeries host one of the largest numbers of nests in the world (ca. 7000 to 8000 nests/year; Marcovaldi et al. 2018), with nesting grounds ranging over a wide latitudinal area from Sergipe (northeastern) to Rio de Janeiro (southeastern) (Fig. 1; Marcovaldi and Chaloupka 2007). Satellite tracking data of adult female loggerheads from the Bahia nesting population revealed high fidelity to foraging grounds in the Brazilian coast during breeding and post-breeding periods. The movements between nesting and foraging grounds occurred along the continental shelf, where the northern coast of Brazil stood out as the most important foraging ground for reproductive females (Marcovaldi et al. 2010). A phylogeographic study using mitochondrial DNA (mtDNA) have recognized globally, 18 demographically independent management units (MUs – *sensu* Moritz 1994) for loggerhead sea turtles (Shamblin et al. 2014). MUs are defined as rookeries with significant genetic distinction, and can be defined by

differences in mtDNA haplotype frequencies (Moritz 1994). Brazilian rookeries hold endemic haplotypes, which provide a unique profile (Reis et al. 2010b), and were recognized as three MUs within the SWA: (1) the northeastern coast (Sergipe and Bahia), (2) Espírito Santo, and (3) Rio de Janeiro (Shamblin et al. 2014). The genetic differentiation of nesting populations worldwide enables the estimation of the origin of turtles sampled in foraging grounds, which are composed of individuals from multiple rookeries (i.e. mixed stocks), and offer great insights about the migratory behaviour and connectivity of sea turtle populations (Rees et al. 2017; Tolve et al. 2018; but see Prosdocimi et al. 2015). Southern Brazil is an important foraging ground for adults and juveniles of loggerheads in both neritic and oceanic habitats (Barros 2010; Monteiro et al. 2016). Oceanic feeding aggregations at the region are composed mainly by juvenile loggerheads from Brazilian rookeries, and in lower proportion by juveniles from North Atlantic, Mediterranean, and Pacific nesting grounds (Reis et al. 2010b; Shamblin et al. 2014). In the current study, we integrated SIA and genetic analysis of mtDNA to infer natal origins, movements and feeding ecology of male loggerhead sea turtles from southern Brazil foraging grounds and to provide first insights about the life history of these animals in the SWA.

Methods

Sample collection

Tissues samples were collected from male loggerhead sea turtles stranded dead on the coast of Rio Grande do Sul (RS), southern Brazil, between Lagoa do Peixe (31°21'S, 51°05'W) and Arroio Chuí (33°44' S, 53°22' W) (Fig. 1). Sampling took place mainly during austral summer of 2014 through 2017, but a few specimens were sampled in late spring, early autumn and early winter (Table S1). For each specimen, curved carapace length (CCL) was measured with a flexible metric tape (± 0.1 cm), from the midline of the nuchal notch to the posterior end of the posterior marginal scute (Bolten 1999). Sex was determined by the examination of gonads during necropsy (Wyneken 2001) and/or by tail length (for adults > 90 cm CCL; Wibbels 1999; Casale et al. 2005). The stranded specimens were also classified according to the decomposition state of carcasses, as follows: 1 = freshly dead (eyes present); 2 = initial decomposition (without eyes); 3 = moderate decomposition (swollen body, fluids apparent in the orifices, loss of carapace scutes and/or head scales); 4 = intermediate decomposition (shriveled body,

carapace peeling, conspicuous bone plates); and 5 = advanced decomposition (carcass mummified). For genetic analysis, fragments of skin were collected and stored in absolute ethanol or DMSO solution. Skin samples obtained for SIA were stored in plastic bags and frozen at -20°C until laboratory procedures. Additionally, samples of potential food items of loggerhead sea turtles from neritic and oceanic environments, as observed by Bugoni et al. (2003) and Barros (2010), were also collected. These items included the following taxonomic groups: the gastropod *Buccinanops monoliferum*, the squid *Dorytheuthis plei*, the hermit crabs *Dardanus insignis* and *Loxopagurus loxochelis*, the spider crab *Libinia spinosa*, salps (Class Thaliacea, Order Salpida, Family Salpidae), and the fishes whitemouth croaker *Micropogonias furnieri*, banded croaker *Paralonchurus brasiliensis* and cutlassfish *Trichiurus lepturus*. Anemones (Class Anthozoa, Order Actinaria) associated with hermit shells and the pelagic jellyfish *Lychnorhiza lucerna* (Class Scyphozoa, Order Rhizostomeae) were also collected. Cnidarians have no calcareous or chitinous structure (with exception of some hydrozoans such as *Verella vellella*; Francis 1985) and are rapidly digested and difficult to detect in the gastrointestinal tract when consumed by sea turtles (Van Nierop and Hartog 1984). Although these taxa have not been observed in stomach contents of loggerhead sea turtles evaluated in southern Brazil (Bugoni et al. 2003; Barros 2010), they were reported as prey in the North Atlantic, Mediterranean Sea, and Australian coasts (Jones and Seminoff 2013). Crustacean, anemone, cephalopod and fish specimens were obtained from commercial fisheries that operate at the continental shelf adjacent to the study area. Salps were collected at the shelf-break and slope off southern Brazil, between the 550 and 3000 m depth isobaths on oceanographic cruises carried out onboard the R/V *Atlântico Sul* of the Universidade Federal do Rio Grande (FURG). The remaining prey species were collected during beach surveys conducted at the study area.

Genetic analysis

For genetic analysis, DNA was extracted from tissues with a PureLink™ Genomic DNA Kit. A fragment of 818 base-pairs (bp) of the mtDNA D-loop region was amplified through Polymerase Chain Reactions (PCR) with the primer pair LCM15382 (5- GCT TAA CCC TAA AGC ATT GG -3') and H950 (5- GTC TCG GAT TTA GGG GTT TG -3') (Abreu-Grobois et al. 2006). PCR reactions contained 20 to 50 ng of genomic DNA, 5U of Platinum Taq Polymerase or Recombinant Taq Polymerase

(Invitrogen 10966-030 and 11615-010, respectively), 0.2 μM of each primer, 0.4 mM of dNTPs, 1x PCR Buffer and 0.1 mM MgCl_2 . Negative controls were included to detect possible contaminations during the amplification process. PCRs were performed with an Applied Biosystems Veriti 96-well Thermocycler under the following conditions: denaturation of 5' at 94°C; 36 cycles of 30'' at 94°C, 30'' at 50°C, 1' at 72°C; and a final extension of 10' at 72°C. The obtained PCR products were purified with a PureLink™ Quick Gel Extraction and Purification Combo Kit. Purified PCR products were sequenced in forward and reverse direction using a ABI PRISM 3730XL Analyzer.

Sequences were manually edited and aligned using the program BioEdit ver 7.0.9 (Hall 1999) and classified according to previously described haplotypes recorded in the Archie Carr Center for Sea Turtle Research database (<http://accstr.ufl.edu/>) and GenBank (<http://ncbi.nlm.nih.gov>). Throughout this paper we use standardized haplotype nomenclature established by both databases. The original haplotype denomination is based on short mtDNA fragments (380bp) and were recorded with subsequent numbers. Haplotypes based on the larger fragment (~800 bp) remained with their original short sequence designations, but receive numeral suffixes to indicate polymorphisms observed within the expanded sequences (Shamblin et al. 2014). Haplotype (h) and nucleotide (π) diversities (Nei 1987) were calculated using the program DNAsp 5.10 (Rozas et al. 2003). There is little information on standard diversity indices based on long sequences of loggerheads sea turtles, making it difficult to compare with previous studies carried out in loggerhead foraging grounds in the Atlantic. Thus, here we calculated haplotype and nucleotide diversities for short and long sequences. A 'many-to-one' Bayesian Mixed Stock Analysis (MSA) was performed in order to estimate the natal origins of male loggerhead sea turtles (Pella and Masuda 2001). MSA was carried out using R software version 3.4.2 (R Core Team 2017) through the package *mixstock*, which estimates the contribution from source populations to one or more mixed stocks (Bolker et al. 2007). As a baseline, we used the haplotype frequencies matrix of loggerhead rookeries provided by Shamblin et al. (2014). A Markov Chain Monte Carlo (MCMC) was applied to obtain the posterior distributions of stocks contributions, integrating the data likelihood with an uninformative prior, through four chains of 20,000 iterations with an initial discard of the first 10,000, resulting in posterior distributions with 95% credibility intervals (CrI 95%). The Gelman-Rubin reduction factor was used to verify convergence of chains by

comparing variances between them. Values below 1.2 for all parameters indicate that convergence was achieved and the corresponding estimates are reliable (Bolker et al. 2007).

MSA is a powerful tool to understand the migration patterns of sea turtles, but the frequency of some haplotypes in source populations can affect the model fit, providing results with large uncertainty. The occurrence of common haplotypes that are shared among rookeries but are rare in mixed stocks results in contribution estimates that are biologically unreliable (e.g. haplotype CC-A2 and variants, reported for the northwestern Atlantic, Mediterranean and South Africa loggerhead rookeries; Jensen et al. 2013; Shamblin et al. 2014). Previous studies suggested the exclusion of nesting grounds that increase error and generate unlikely stock contributions (Engstrom et al. 2002; Rees et al. 2017). To perform MSA, hybrid individuals should also be excluded because they represent orphaned haplotypes due to the lack of baseline data of hybrid haplotypes in Atlantic rookeries (Bolker et al. 2007).

Stable isotope analysis

Male loggerhead skin and prey tissues were rinsed with distilled water, oven dried to a constant mass at 60°C for 48 to 72 h, and powdered using a mortar and pestle. About 0.7 mg of each sample was weighed and placed in individual 4 x 6 mm tin cups. The prey tissues processed for SIA were: muscle from crustaceans and fishes, the longitudinal muscle of the column of anemones, the bell of jellyfishes, the mantle of gastropods and cephalopods, and the whole body of salps.

Lipid content of a tissue is a potential confounding factor in SIA, particularly in studies using mixing models (Kiljunen et al. 2006). In general, tissues that exhibit C:N ratios higher than 3.5 have lipid content with the potential to alter $\delta^{13}\text{C}$ values (Post et al. 2007). In such cases, lipid extraction or mathematical normalization is required (Post et al. 2007; Logan et al. 2008; Petitet and Bugoni 2017). According to previous studies carried out with green turtles *Chelonia mydas*, C:N ratios of sea turtles' skin are below the threshold of 3.5 and lipid content of this tissue does not appear to alter $\delta^{13}\text{C}$ values (Vander Zanden et al. 2012; Bergamo et al. 2016). For this reason, most skin samples were analyzed without lipid extraction. However, six adult males presented C:N ratios > 3.5, indicating high lipid content that could alter $\delta^{13}\text{C}$ values. Therefore, these skin

samples were lipid-extracted using a Soxhlet apparatus with a 2:1 solvent mixture of chloroform and methanol for 6 h (Medeiros et al. 2015), and re-analyzed. Regarding prey samples, only anemones showed evidence of high lipid content. In this case, we normalized carbon isotopic values according to D’Ambra et al. (2014).

Samples of male loggerhead turtles, jellyfishes, spider crabs and gastropods were analyzed using a Costech 4010 elemental analyzer coupled to a Thermo Scientific Delta V isotope ratio mass spectrometer at the University of New Mexico Center for Stable Isotopes (UNM–CSI). For the remaining samples, SIA was performed at the Stable Isotope Core Laboratory, Washington State University (SICL–WSU), with an elemental analyzer Costech 4010 connected to a Delta PlusXP isotope ratio mass spectrometer Thermofinnigan. Stable isotope values are expressed in δ -notation as parts per thousand (‰) differences from the international standard material, Vienna Pee Dee Belemnite limestone and atmospheric nitrogen (Air) for carbon and nitrogen, respectively, according to the following equation (as in Bond and Hobson 2012):

$$\delta X = (R_{\text{sample}}/R_{\text{standard}}) - 1 \quad (1)$$

where X is the ^{13}C or ^{15}N value, and R is the corresponding ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ (Peterson and Fry 1987). Both laboratories use internal standards of known carbon and nitrogen composition in order to estimate instrument precision. The analytical precision (standard deviation – SD) of the internal laboratory standards used by UNM–CSI was measured at $< 0.2\text{‰}$ for $\delta^{15}\text{N}$ and $< 0.04\text{‰}$ for $\delta^{13}\text{C}$; for SICL–WSU the SD was $< 0.1\text{‰}$ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

Samples analyzed in different laboratories can be compared directly only after a calibration procedure. Isotopic values of feathers of the yellow-nosed albatross *Thalassarche chlororhynchos* revealed significant differences in $\delta^{13}\text{C}$ values between paired-samples analyzed both UNM–CSI and SICL–WSU laboratories (Leal 2018). Therefore, $\delta^{13}\text{C}$ values of our samples analyzed in SICL–WSU were corrected using following equation:

$$\delta^{13}\text{C}_{\text{corrected}} = (-1.59) + 0.92 (\delta\text{WSU}) \quad (2)$$

where δ WSU represents the $\delta^{13}\text{C}$ values obtained at the Stable Isotope Core Laboratory, Washington State University (Leal 2018).

A paired *t*-test was also conducted to verify significant differences between lipid-extracted and non-extracted skin samples, separately for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. A general linear model (GLM) was fitted to verify significant differences in stable isotope values among size classes (i.e. adults and juveniles) and genetic composition (i.e. haplotype classification) of male loggerheads. Individual turtles were considered juveniles if CCL was smaller than the minimum maturation size (88.2 cm CCL) estimated for male loggerheads from the North Atlantic (Avens et al. 2015). The GLM was fitted with a Gamma distribution, where $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were the response variable, and size classes and haplotypes were the categorical explanatory variables with two and five levels, respectively. The model fit was verified through residual diagnostics (e.g. quantile-quantile plots and Cook's distance). The variables that significantly affected $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were used to separate male loggerheads in different groups. The significance level for both paired *t*-test and GLM was $\alpha = 0.05$.

Stable Isotope Mixing Models (SIMM) integrate variability in resource and consumer isotope values, providing a framework for understanding trophic ecology (Parnell et al. 2013). For each group defined through the GLM, we estimated the relative contribution of potential prey to the diet of male loggerheads by fitting a Bayesian mixing model with the package *simmr* (Parnell 2016) developed for software R. Before running SIMM, we assessed three trophic discrimination factors (TDFs) provided by previous studies, as well the feasibility of the prey database using simulated mixing polygons with a Bayesian statistical framework through the packages *sp* and *splancks* (Smith et al. 2013). The mixing polygon simulation is visualized with a mixing region (i.e. convex hull), which is calculated by testing a grid of values for point-in-polygon bounded by the proposed food sources, providing a quantitative basis for model rejection, consumer exclusion (those outside the 95% mixing region) and the evaluation of the most appropriate TDFs (Smith et al. 2013). One set of TDFs is derived from a study carried out with captive early juvenile loggerhead sea turtles (Reich et al. 2008), which estimated the mean values (\pm SD) of residence time and TDF of several tissues. For skin, the estimated residence time was 46.1 ± 8.9 days for $\delta^{13}\text{C}$ and 44.9 ± 3.1 days for $\delta^{15}\text{N}$. The TDF was $1.11 \pm 0.17\text{‰}$ for $\delta^{13}\text{C}$ and $1.60 \pm 0.07\text{‰}$ for $\delta^{15}\text{N}$. The other two TDFs applied in mixing polygons simulations consisted of values estimated for skin

of captive large juveniles and adults of green turtles from Cayman Turtle Farm in Grand Cayman, British West Indies (Vander Zanden et al. 2012). The estimated values for juveniles were $1.87 \pm 0.56\text{‰}$ for $\delta^{13}\text{C}$ and $4.77 \pm 0.40\text{‰}$ for $\delta^{15}\text{N}$, and for adults $1.62 \pm 0.61\text{‰}$ for $\delta^{13}\text{C}$ and $4.04 \pm 0.04\text{‰}$ for $\delta^{15}\text{N}$. After running SIMM, a diagnostic matrix plot was produced, providing the correlation among sources. This is a useful tool to identify when the model is fitting well, indicated by low correlations between sources, or when the model is unable to differentiate food items (Parnell 2016). Based on that, some prey species can be grouped according to the similarity in their isotope signatures, taking into account their habitat and resource use preferences (Phillips et al. 2005). Due to the similarity in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of crustaceans *L. spinosa* and *D. insignis*, and the fishes *M. furnieri* and *P. brasiliensis*, provided by the diagnostic plots of SIMM, we lumped these prey items in two groups, hereafter called as crabs and croakers, respectively. All statistical analyses involving isotopic values were carried out using R software version 3.4.2 (R Core Team 2017).

Results

A total of 26 male loggerhead sea turtles were sampled, of which 19 were adults and 7 were juveniles (Table S1). The CCL of all specimens ranged from 24 to 114 cm (mean = 88.8 ± 21.9 cm). One adult male could not be measured because the carapace was damaged by the carcass decomposition process. However, it was possible to infer that it was an adult male due to the large body size and long tail.

Genetic analysis

A total of five haplotypes were identified: CC-A2.1 (n = 1), CC-A4.1 (n = 7), CC-A4.2 (n = 15), CC-A4.3 (n = 1) and one haplotype typical of olive ridley (*Lepidochelys olivacea*) sea turtles (n = 2), haplotype F (hereafter referred to as Cc × Lo haplotype). Haplotype F was identified through the short sequence (380pb), since genetic characterization with long sequences has not been conducted for olive ridley rookeries in the Atlantic Ocean. Haplotype and nucleotide diversities are summarized in Table 1.

A preliminary MSA including all previously described rookeries provided unrealistic contribution estimates due to the presence of one male containing CC-A2.1 in our samples (data not shown). Therefore, a second MSA was performed removing the male with CC-A2.1 haplotype and the north Atlantic and Mediterranean nesting areas, resulting in a matrix only with Brazilian lineages. This second MSA showed that males in southern Brazil originated in a slightly greater proportion from Espírito Santo state, followed by Rio de Janeiro, Bahia and Sergipe states (Table 2).

Stable isotope analysis

Lipid-extracted and non-extracted skin samples from males differed significantly in $\delta^{13}\text{C}$ values ($t = -5.64$, $P < 0.01$), but not for $\delta^{15}\text{N}$ values ($t = 0.64$, $P = 0.54$). After lipid extraction procedures, all skin samples had C:N ratios < 3.5 (Table S1). All males showed $\delta^{13}\text{C}$ values ranging from -18.01 to -12.99‰ ($-14.73 \pm 1.30\text{‰}$) and $\delta^{15}\text{N}$ values ranging from 6.80 to 18.59‰ ($14.77 \pm 2.99\text{‰}$). In adult males, $\delta^{13}\text{C}$ values ranged from -16.44 to -12.99‰ ($-14.71 \pm 0.90\text{‰}$) and $\delta^{15}\text{N}$ values ranged from 10.69 to 18.59‰ ($15.96 \pm 1.69\text{‰}$). In juveniles, $\delta^{13}\text{C}$ values ranged from -18.01 to -14.54‰ ($-16.19 \pm 1.13\text{‰}$) and $\delta^{15}\text{N}$ values ranged from 6.80 to 14.26‰ ($11.16 \pm 3.02\text{‰}$). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were significantly lower in juveniles than adult males. The model also indicated a significant difference of $\delta^{15}\text{N}$ values of male hybrids compared to male with loggerhead haplotypes only (Table S2). Based on GLM results, we performed the mixing polygon simulations and the SIMM for adults and juveniles, separately. For adults, the mixing polygon simulation revealed that the most suitable TDFs for mixing models were the estimated values for adults of green sea turtles (Fig. 2b). The convex hull output also indicated that one adult individual was outside the 95% mixing region; therefore, this consumer was excluded from SIMM (Fig. 2b). For juveniles, the mixing polygon simulation revealed that the TDFs estimated for juvenile loggerheads was the most appropriate to use in our SIMM, and that one hybrid individual occurred outside the outermost contour of convex hull, thus being excluded from further analysis (Fig. 2c). Mixing polygon models using the other two sets of TDF values showed more individuals falling outside the convex hulls (Fig. 2a and d). Therefore, the two subsequent SIMMs were run with the sets of TDF values with the better fit for adults and juveniles, excluding one individual each (Table S1).

The best model fit consisted of nine food sources (values of potential food items are summarized in Table S3). Results from SIMM demonstrated greater contribution of the hermit *L. loxochelis* in the diet of adult male loggerheads (CrI 95% = $40.4 \pm 18.4\%$, ranging from 3.9 to 70.8%; Fig. 3a), followed by the gastropod *B. monoliferum* ($12.4 \pm 10.2\%$, ranging from 1.1% to 39.5%). For the remaining food sources, the estimated contributions were homogeneous and close to zero. For juveniles, SIMM showed a greater contribution of salps ($23.9 \pm 11.3\%$, ranging from 3.1 to 45.8%; Fig. 3b), followed by the hermit *L. loxochelis* ($13.9 \pm 12.8\%$, ranging from 1.2 to 48.9%).

Discussion

The SWA represents an important foraging ground for loggerhead sea turtles at different life stages. However, most studies have focused on adult females and juveniles, and little is known about males. To the best of our knowledge, this study provides the first insights about the origins, movements and foraging ecology of male loggerhead sea turtles in the SWA, and highlights the usefulness of genetic and stable isotopes analysis as complementary methods to address life history features of sea turtles. Our study also describes for the first time hybridization in sea turtle males.

Origin and migrations

Genetic results showed that most males (88.5%) that occur in foraging grounds in southern Brazil exhibit haplotypes endemic from Brazilian rookeries (CC-A4.1, CC-A4.2 and CC-A4.3; Shamblin et al. 2014), accompanied by a low frequency of the haplotypes CC-A2.1 (3.8%) and $Cc \times Lo$ (7.7%). All adults sampled in our study were from Brazilian lineages. Haplotype CC-A2.1 (Genbank EU179445; Shamblin et al. 2012) is the most geographically widespread loggerhead sea turtle haplotype and is present in almost all of the western/eastern Atlantic and Mediterranean nesting grounds, except for those in Brazil (Shamblin et al. 2014). Haplotypes CC-A4.1, CC-A4.2 and CC-A4.3 (Genbank KF840723-25; Shamblin et al. 2014) are variants of the haplotype CC-A4 (Genbank AJ001077; Bolten et al. 1998), which is the ancestral haplotype exclusive to the Brazilian loggerhead populations (Reis et al. 2010b). Haplotype F, observed in hybrid males, has been recorded in olive ridley sea turtle rookeries from Brazil, Suriname and Guinea Bissau (Genbank AF051773; Bowen et al. 1998).

Analysis of the long mtDNA fragment greatly increased diversity estimates when compared to short haplotypes due to the higher proportion of polymorphic sites (Table 1). Based on the short mtDNA sequence, haplotype and nucleotide diversities found in males were lower than the ones found for unsexed turtles at oceanic feeding aggregations in southern Brazil ($h = 0.714 \pm 0.031$ and $\pi = 0.017 \pm 0.001$; Reis et al. 2010b) and foraging grounds in Uruguayan waters ($h = 0.431 \pm 0.087$ and $\pi = 0.014 \pm 0.007$; Caraccio et al. 2008), but higher than unsexed loggerheads from neritic aggregations of the Buenos Aires province, Argentina ($h = 0.032 \pm 0.031$ and $\pi = 0.000089 \pm 0.0003$; Prosdocimi et al. 2015). Previous studies that assessed the genetic composition of loggerhead sea turtles in foraging grounds in the SWA, without sexual differentiation, observed that coastal waters of Uruguay and Argentina are composed exclusively by adults and large juveniles from Brazilian rookeries (Caraccio et al. 2008; Prosdocimi et al. 2015). In oceanic waters off Uruguay and Brazil, however, although Brazilian haplotypes are still the most frequent, foraging grounds are composed by smaller juveniles with higher genetic variability than those from neritic areas, in which haplotypes from the North Atlantic, Mediterranean Sea, and Pacific Ocean were reported (Caraccio et al 2008; Reis et al. 2010b).

Globally, hybridization events have been reported occasionally through genetic analysis (Karl et al. 1995; Seminoff et al. 2003; James et al. 2004). However, a high incidence of hybridization among Cheloniidae sea turtle species has been observed in Brazil (Lara-Ruiz et al. 2006; Reis et al. 2010a). The causes of this extensive hybridization are still unclear, but are likely a consequence of anthropogenic pressures that caused historic population declines and uneven sex ratios within populations and among species (Vilaça et al. 2012). The observed olive ridley haplotype is common at several rookeries in the Atlantic. However, to date, Cc × Lo hybrids have only been reported in the Sergipe rookery, possibly facilitated by the spatial and temporal overlap of the nesting seasons of both species (Reis et al. 2010a; Vilaça et al. 2012). Based on this, and considering the high frequency of endemic Brazilian haplotypes observed among males, we propose that hybrid males come from the Sergipe rookery.

MSA estimates indicate that male loggerheads from southern Brazil originated in a slightly greater proportion from nesting grounds in Espírito Santo, followed by Rio de Janeiro, Bahia and Sergipe rookeries (Fig. 1, Table 2). These results corroborate a sex ratio study that estimated that loggerhead sea turtle nests in Espírito Santo and Rio

de Janeiro produce less female hatchlings than those in Sergipe and Bahia (Marcovaldi et al. 2016). While the northeastern nesting grounds produce a mean of 94% females (ranging from 83% to 99% in Sergipe, and from 79% to 98% in Bahia), at the southeastern region female offsprings correspond to a mean of 53% of nests (ranging from 33% to 81% in Espírito Santo and 18% to 81% in Rio de Janeiro). Sea turtle species have temperature-dependent sex determination, where the pivotal incubation temperature that leads to offspring with a 50:50 proportion of each sex is $\sim 29^{\circ}\text{C}$ (Wibbels 2003). The proportion of female offspring decreases from north to south due to lower sand temperature in higher latitude beaches (Marcovaldi et al. 2016). Although MSA provided valuable insights on the origin of male loggerhead sea turtles, results must be interpreted with caution due to the large confidence intervals of contribution estimates. Brazilian loggerhead sea turtle rookeries are genetically distinct from others around the world (Reis et al. 2010b; Shamblin et al. 2014), and three distinct management units in Brazil have been suggested based on long mtDNA sequences (Shamblin et al. 2014). Although the analysis of long mtDNA fragments have refined our understanding of the genetic structure among nesting grounds, further analysis of Brazilian rookeries including larger sample sizes and other genetic markers it is necessary to better determine the boundaries of management units (Shamblin et al. 2014), as well as improve baseline data and overcome limitations of MSA (Engstrom et al. 2002; Jensen et al. 2013).

Feeding ecology and habitat use

In SIA, an important methodological issue is that lipids have low $\delta^{13}\text{C}$ values compared to other molecules, which is a potential confounding factor for stable isotope values, leading to erroneous ecological interpretations (Post et al. 2007). Skin samples of six adult males showed high lipid content capable to alter $\delta^{13}\text{C}$ values, and a defatting approach was applied. Although previous studies performed with juvenile green sea turtles indicated that skin does not have significant high lipid content (Bergamo et al. 2016), the increase in $\delta^{13}\text{C}$ values observed in our samples after lipid extraction suggests that this methodological step could be necessary to accurately determine $\delta^{13}\text{C}$ values in skin of loggerhead sea turtles. The high lipid content observed in the skin of adult males could be associated with the preferential ingestion of benthic organisms, since loggerhead prey in the neritic habitat had higher energetic values than in oceanic

habitat (Barros 2010). As no significant changes in $\delta^{15}\text{N}$ values after lipid extraction with chloroform-methanol were detected, we again suggest that the extraction of lipids could be carried out from the beginning of sample processing, as a way to optimize the cost of analyses and reduce processing time in the laboratory (Medeiros et al. 2015).

Over the past decades, a foraging dichotomy between oceanic and neritic habitats has been reported for loggerhead sea turtles (Hatase et al. 2002b; Hawkes et al. 2006; McClellan et al. 2010). Our results indicated size-related differences in feeding and habitat use of male loggerheads in southern Brazil. Mixing model outputs demonstrated consistent foraging behaviour among adult males, which were shown to occur mainly in coastal waters and consume benthic organisms, preferentially the hermit crab *L. loxochelis* followed by the gastropod *B. monoliferum*. On the other hand, juvenile males showed higher variability in habitat and resource use as evidenced by the large contribution of both oceanic (i.e. salps) and neritic (the hermit crab *L. loxochelis*) to their diet. Similarly, adult male loggerheads from the Mediterranean that were satellite-tracked showed a smaller and more neritic home range (Casale et al. 2013) than unsexed juveniles tracked in the same region (Casale et al. 2012). Also in the Mediterranean Sea, a long-term sea turtle tagging study showed a polymodal pattern of movement and habitat use among juvenile and adult loggerheads, and observed that loggerheads found in the oceanic environment are larger than turtles inhabiting neritic areas (Casale et al. 2007). In the Northwest Atlantic, adult male loggerheads of two feeding grounds showed distinct foraging strategies: while some males exhibit long-term consistency in habitat use and individual specialization, others display less site fidelity and a more variable feeding behaviour (Pajuelo et al. 2016). These patterns of temporal consistency were similar to that reported in adult female loggerheads sampled in Florida rookery, USA (Vander Zanden et al. 2010). In Brazil, satellite tracking of adult female loggerheads from the nesting population of Bahia revealed high fidelity of females to neritic foraging grounds in the northeastern (internesting) and northern (postnesting) coasts of Brazil (Marcovaldi et al. 2010). Distinct foraging strategies in adult male loggerheads were also observed in the northern Pacific (Hatase et al. 2002a; Saito et al. 2015) and in Boa Vista, West Africa (Varo-Cruz et al. 2013), and in the same way were reported to be similar to what was observed for females (Hatase et al. 2002b; Hawkes et al. 2006). The variation in foraging ecology of male loggerheads was associated with seasonal changes in sea surface temperature and with the diversity of

available resources in feeding grounds, which directly affects foraging site fidelity and the degree of individual specialization in a population (Saito et al. 2015; Pajuelo et al. 2016).

The variability in the isotopic values observed in juveniles of our study could be a result of the ontogenetic habitat shift, from the oceanic to the neritic environment. In southern Brazil, it was estimated that recruitment occurs when loggerheads reach in mean 65 cm CCL (ranging from 55.7 to 77.9 cm, Monteiro 2017). Our results showed that the lowest isotopic values of males were observed in the individuals with CCL < 65 cm, indicating that skin still reflect oceanic isotopic signatures. Furthermore, previous studies reported that recruitment to neritic environments does not represent a concomitant abrupt change in habitat and diet, and that juveniles remain to feed upon oceanic-pelagic organisms for an unknown period (Barros 2010; McClellan et al. 2010). Our results also corroborate previous studies that analyzed gut contents of juvenile and adult loggerheads in southern Brazil. Although more than 45 prey have been identified, it was reported that early juveniles using the oceanic environment consumed predominantly salps and pyrosomes, whereas large juveniles and adults foraging in neritic zones mainly prey upon hermit crabs and gastropods (Bugoni et al. 2003; Barros 2010). On the other hand, a high degree of individual specialization in resource use and a long-term fidelity to foraging grounds were observed in large juvenile of loggerheads from southern Brazil through the association of skeletochronology and SIA, and confirmed by satellite telemetry data (Monteiro 2017). Both oceanic and neritic loggerheads showed similar temporal consistency in habitat use, but the degree of individual specialization was higher in neritic turtles than in oceanic turtles. Differently from what we observed in males, this foraging polymorphism was not associated with the size of individuals, but with the variation of sea surface temperature and currents in the region (Monteiro 2017). During spring and summer, with the predominance of the tropical waters of the Brazil Current, loggerheads exhibit a greater habitat fidelity to neritic foraging grounds. However, in late autumn and winter, with the intrusion of cold waters carried by the Malvinas/Falkland Current, some loggerheads migrate to oceanic habitat while others remain close to the shore (Monteiro 2017). Although the variation in isotopic values could also be reflecting the use of different foraging areas along the coast, based on the described habitat use patterns it is unlikely to be the origin of the isotopic variation found. Nevertheless, the limited sample size of our study precludes a

precise interpretation on the feeding behaviour of juvenile male loggerheads, and we recommend more extensive and targeted studies to address the variability in stable isotope values and identify possible ontogenetic shifts and polymodal foraging patterns.

In general, foraging aggregations of sea turtles are mixed stocks composed of individuals from several nesting populations (Jensen et al. 2013), and differences in diet and habitat use could be associated with the genetic variability related to such varied sources. Since male loggerheads foraging in southern Brazil have similar origins this relationship was not observed. However, there were significant differences in $\delta^{15}\text{N}$ values between male loggerheads and hybrids. Our sample size does not allow us to affirm that it is due to genetic variation. The male hybrids sampled in our study had the smallest CCL among males, and the lower $\delta^{15}\text{N}$ values could be a result of the initial life phase, in which young juveniles feed opportunistically on small pelagic organisms (Jones and Seminoff 2013). Recent studies showed the occurrence of immature hawksbill (*Eretmochelys imbricata*) and loggerhead sea turtle hybrids along the Brazilian coast, and suggested that hybrids could be adopting ecological traits typical of loggerheads, such as feeding in southern Brazil aggregations (Proietti et al. 2014). Our results could be an indication that the same foraging and developmental ground of juvenile loggerheads is also being occupied by the offspring of loggerhead \times olive ridley hybrids, but further investigations, with larger sample size and integrating genetics to SIA and telemetry, are required to address this hypothesis.

Conservation implications

The SWA represents an important foraging and development ground for loggerhead sea turtles at different life stages, and also holds extensive commercial fisheries. The significant overlap between fisheries and sea turtle distribution is currently considered the main cause of the high fishing-related mortality and the decline of several loggerhead turtle populations (Domingo et al. 2006; Wallace et al. 2013; Monteiro et al. 2016). Our findings demonstrated the importance of the foraging ground in southern Brazil for male loggerheads, mainly for Brazilian populations, which are the main stock contributors to this group. In this area, loggerhead sea turtles are highly vulnerable to bycatch in longline fisheries in the oceanic habitat (Sales et al. 2008), whereas in the neritic environment incidental capture occurs mainly in trawl and driftnet fisheries that operate along the continental shelf (Fiedler et al. 2012; Monteiro et al.

2016). The foraging dichotomy observed through SIA warns us that these individuals are exposed to both oceanic and neritic fisheries, putting the structure of populations at risk. Therefore, we strongly recommend that future fisheries management plans take into account the implementation of time-area closures in summer months in coastal areas of southern Brazil where trawling is intense, aiming to reduce the impact of fisheries on large juvenile and adult males, and bycatch mitigation measures in offshore pelagic longlines, protecting small turtles, in order to guarantee population stability and to preclude bottleneck events that could severely impact genetic diversity and survival of these populations.

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Compliance with ethical standards

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

Ethical approval: This article does not contain any studies with human participants. All applicable international, national, and institutional guidelines for the care and use of animals were followed. Sampling was conducted under SIBIO licenses Nos. 15962-5 and 49019-1 to 49019-3 (SISBIO- *Sistema de Autorização e Informação em Biodiversidade*). No live animals were used for experiments or sampling. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Table 1 Standard diversity indices (mean \pm standard deviation) calculated for male loggerhead sea turtles in southern Brazil with short and long sequences. N corresponds to the number of haplotypes, h is haplotype diversity, and π is nucleotide diversity

Sequence size	N	h	π
380pb	2	0.083 \pm 0.075	0.003 \pm 0.003
818pb	4	0.543 \pm 0.085	0.003 \pm 0.002

Table 2 Estimated contributions of Brazilian loggerhead rookeries to the male aggregation at the southern Brazil foraging ground, based on Bayesian Markov Chain Monte Carlo mixed stock analysis. Mean values are shown with standard deviation (SD). The 2.5 and 97.5% values indicate the upper and lower bounds of the 95% credibility interval

Stock	Mean	SD	2.5%	Median	97.5%
Sergipe	0.2399	0.1371	0.1325	0.2386	0.5102
Bahia	0.2476	0.1358	0.1439	0.2494	0.5150
Espírito Santo	0.2619	0.1228	0.1709	0.2555	0.5180
Rio de Janeiro	0.2506	0.1310	0.1508	0.2553	0.5230

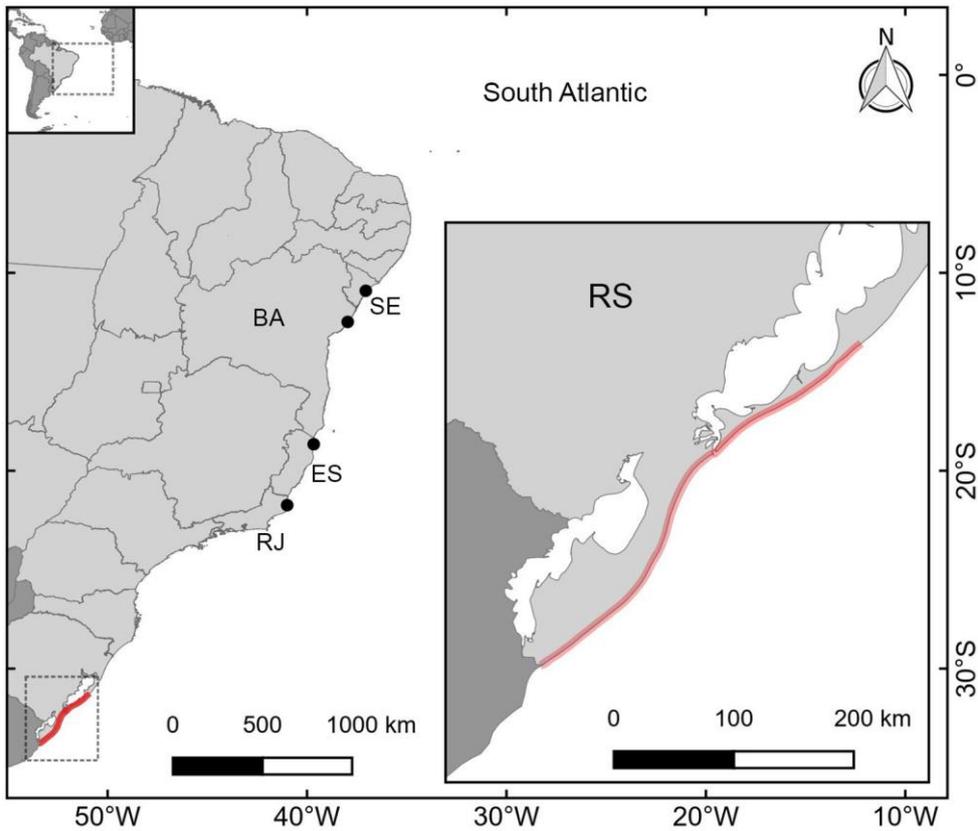


Fig. 1 Map of the Brazilian coast, with black dots indicating the main loggerhead sea turtle nesting sites in Brazil, located in the states of Sergipe (SE), Bahia (BA), Espírito Santo (ES) and Rio de Janeiro (RJ). Zoomed map indicates the study area along the Rio Grande do Sul (RS) state coastline, in southern Brazil, with red line indicating sampling location

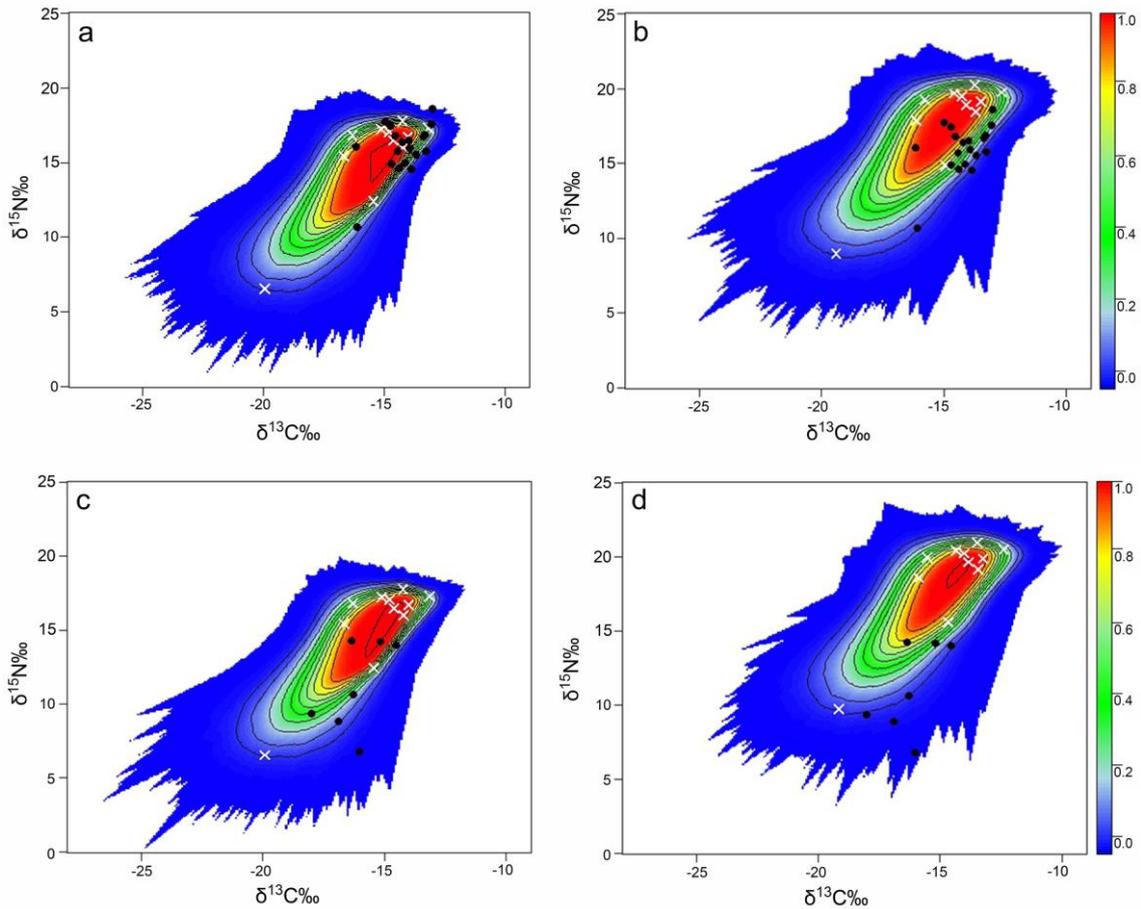


Fig. 2 Simulated mixing models for male loggerhead sea turtles from southern Brazil using three different sets of trophic discrimination values for correcting prey isotopic values. For adults: (a) $1.11 \pm 0.17\text{‰}$ for $\delta^{13}\text{C}$ and $1.60 \pm 0.07\text{‰}$ for $\delta^{15}\text{N}$ (Reich et al. 2008), and (b) $1.62 \pm 0.61\text{‰}$ and $4.04 \pm 0.044\text{‰}$ respectively for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Vander Zanden et al. 2012); for juveniles: (c) $1.11 \pm 0.17\text{‰}$ for $\delta^{13}\text{C}$ and $1.60 \pm 0.07\text{‰}$ for $\delta^{15}\text{N}$ (Reich et al. 2008), and (d) $1.87 \pm 0.56\text{‰}$ for $\delta^{13}\text{C}$ and $4.77 \pm 0.40\text{‰}$ for $\delta^{15}\text{N}$ (Vander Zanden et al. 2012). Position of the consumers (black dots) and the average source signatures (white crosses) are shown. Probability contours (black lines) are at the 5% level (outermost line) and successively at each 10% level

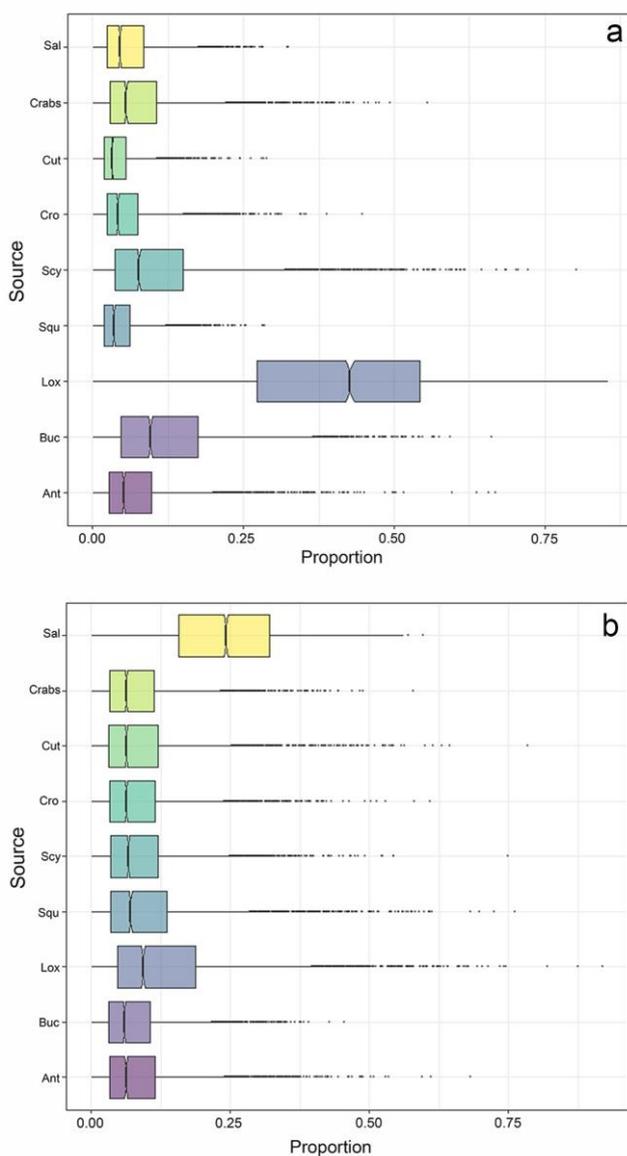


Fig. 3 Results of Bayesian Stable Isotope Mixing Models showing estimated prey contributions (mean, 25% and 75% percentiles) to the diets of (a) adult and (b) juvenile male loggerhead sea turtles. Sal – Salps, Cra – Crabs (i.e. *Libinia spinosa* and *Dardanus insignis*), Cut – Cutlassfish (*Trichiurus lepturus*), Cro – Croakers (i.e. white croaker *Micropogonias furnieri* and banded croaker *Paralichthys brasiliensis*), Scy – Schyphozoa (i.e. jellyfish *Lychnorhiza lucerna*), Squ – Squid (*Dorytheuthis plei*), Lox – *Loxopagurus loxochelis*, Buc – gastropod *Buccinanops monoliferum*, Ant – Anthozoa (i.e. anemones)