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ORIGINAL PAPER

Stable isotope analysis in two sympatric populations of bottlenose dolphins *Tursiops truncatus*: evidence of resource partitioning?

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Abstract Skin and muscle from 43 bottlenose dolphins (38 juveniles/adults, 5 calves) stranded in NW Spain were analysed to determine whether stable isotope ratios (δ^{13}C and δ^{15}N) could be used to assess dietary variation, habitat segregation and population substructure. Results were compared with published stomach contents data. Stable isotope ratios from 17 known prey species were also determined. Isotope ratios of the main prey (blue whiting, hake) varied significantly in relation to fish body size. Dolphin calves showed significant heavy isotope enrichments compared to adult females. Excluding calves, δ^{15}N decreased with increasing dolphin body size, probably related to an ontogenetic shift in diet towards species at lower trophic levels, e.g. on blue whiting as suggested by stomach content results. Bottlenose dolphins were divided into two putative populations (North, South) based on previous genetic studies, and values of δ^{13}C and δ^{15}N differed significantly between these two groups, confirming the existence of population structuring.

Introduction

Bottlenose dolphins (*Tursiops truncatus*, Montagu 1821) have a cosmopolitan distribution and are found in most warm temperate to tropical seas including coastal and offshore waters (Wells and Scott 2002). Throughout their range, coastal populations often occur in discrete locations either seasonally or year-round and several small resident coastal populations of bottlenose dolphins have been described in European waters including those in the Moray Firth, Scotland (Wilson et al. 1997), Cardigan Bay, Wales (Evans 1980), the Shannon Estuary, Ireland (Ingram and Rogan 2002) and the Sado Estuary, Portugal (dos Santos and Lacerda 1987).

A dedicated survey of cetacean abundance in shelf Atlantic waters of the Iberian Peninsula in 2005 gave an abundance estimate of 3,935 (CV 0.38) bottlenose dolphins (SCANS-II 2008) while another dedicated survey in 2007 in Galician offshore waters estimated a population of 876 (CV 0.82) individuals (CODA 2009). Previously, based on opportunistic sightings from fishing boats in 1998–1999, López et al. (2004) estimated bottlenose dolphin abundance in Galician coastal and shelf waters to be 664 (95% confidence limits 251–1,226). This latter estimate is best viewed as a minimum figure since it was not possible to estimate the proportion of animals present but not detected by observers, and 100% detection rate was therefore assumed. *Tursiops truncatus* is the most frequently seen cetacean species in coastal waters of Galicia, inhabiting the series of rías (coastal inlets) along the southern coastline

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(Aguilar 1997; López et al. 2002, 2004; Pierce et al. 2010). Although bottlenose dolphins are more commonly found in the coastal waters of southern Galicia, they are also frequent in the northern region, mainly in the summer months from June to September (López 2003) and are also present throughout the Galician shelf (VVAA 2007).

Residency patterns have been observed in bottlenose dolphins from southern Galicia although long-distance movements (>500 km) have been also registered (López 2003; VVAA 2007; López et al. 2009), which suggest the coexistence of highly mobile individuals with other animals that are perhaps more dependant on the habitat provided by the rías. Recent genetic studies seem to support this, and Fernández et al. (in press) found evidence of population substructure within Galician bottlenose dolphins based on mitochondrial and nuclear DNA. Thus, dolphins from northern and southern Galicia were identified as two differentiated genetic units. However, the nature of this coexistence remains unclear as potential migrants between putative genetic populations were also identified (Fernández 2010; Fernández et al. in press). In addition, the two bottlenose dolphin populations appear to be sympatric at least in some areas (e.g. the Galician shelf) even when a certain degree of resource partitioning or habitat segregation is expected to occur in order to reduce intra-specific competition among genetically differentiated units. Previous studies in the research area based on stomach contents showed that Galician bottlenose dolphins have a varied diet, including at least 23 families of fish and 12 species of cephalopods, although it was strongly dominated by blue whiting, *Micromesistius poutassou*, and hake, *Merluccius merluccius*, which, respectively, represented 73 and 8% by number and the 48 and 30% by weight of the total prey items (Santos et al. 2007). However, the possibility of population differences in diet preferences was not assessed in previous analyses.

The bottlenose dolphin, *Tursiops truncatus*, is recorded in the EU Habitats Directive as a Species of Special Interest (Directive 92/43/CEE), the protection of which requires the designation of Special Areas of Conservation (SACs) by the EU Member States. Knowledge of population structure and habitat use is, therefore, required to be able to design and apply effective conservation measures.

Based on the fact that predator stable isotope compositions reflect those of their prey (de Niro and Epstein 1978, 1981), ratios of the natural abundance of stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) have been used extensively in studies of cetacean ecology to assess dietary variation in space and time (e.g., Abend and Smith 1995; Mendes et al. 2007; de Stephanis et al. 2008; Knoff et al. 2008). Turnover rates in different body tissues may vary depending on the metabolic activity of the tissue analysed (e.g. turnover rates for skin are faster than those for teeth; Abend and Smith 1995), and this in theory allows the exploration of the dietary history of individual animals over different time windows (Abend and Smith 1995), in contrast to stomach contents that normally comprise only the prey consumed shortly before the stomachs were collected (Pierce and Boyle 1991). Stable isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) are expressed as parts per thousand (‰) deviations from international standards, using the delta (δ) notation (McKinney et al. 1950).

In addition to information on diet, $\delta^{13}C$ and $\delta^{15}N$ values can act as chemical tracers that reflect characteristics of the ecosystem where an animal lives. As a consequence, stable isotopes have wide applications, including studies of migration patterns (e.g. Mendes et al. 2007) or population substructure (e.g. Borrell et al. 2006; Barros et al. 2009).

Food webs show an enrichment of both $\delta^{13}C$ and $\delta^{15}N$ with trophic level although $\delta^{13}C$ enrichment is generally small, around 1‰ (de Niro and Epstein, 1978). Therefore, $\delta^{13}C$ is more informative regarding the base of the food chain (Mendes et al. 2007), and carbon stable isotope ratios can reveal information, such as inshore verses offshore feeding preferences (Hobson et al. 1994). On the other hand, $\delta^{15}N$ enrichment per trophic level in marine food webs is normally considerably higher, with a mean trophic enrichment factor of around 3‰ (de Niro and Epstein, 1981). Nitrogen isotope ratios are thus more useful as an indicator of trophic position. We should note that enrichment factors also vary depending on the tissue analysed (Hobson et al. 1996; García-Tiscar, 2009) as, for example, metabolically active tissues show less enrichment in $\delta^{13}C$ over diet than inactive or keratinous tissues (Hobson et al. 1996). This represents a potential confounding factor when using isotope ratios in different tissues to indicate feeding over different time periods. However, enrichment factors are considered to be relatively conservative for the same tissue among marine mammal species (Lesage et al. 2001).

In the present paper, isotopic ratios of carbon and nitrogen were measured in skin and muscle from bottlenose dolphins stranded or by-caught in Galicia. Possible causes of variation in the stable isotope composition of the dolphins were assessed. As skin and muscle have different turnover rates, temporal differences between isotopic values of dolphin tissues were explored to assess dietary variation over time. The isotope ratios of known prey species in the research area were also analysed, and relative positions of predator and prey in the trophic web were investigated. Isotopic mixing models were applied to estimate the proportional contribution of each prey source to bottlenose dolphin diet. To provide additional insight into isotopic variability in tissues of bottlenose dolphin in Galicia, we also examined potential sources of variation in the stable isotope composition of their main prey species, namely blue whiting and hake (Santos et al. 2007). Finally, published data on bottlenose dolphin stomach contents in the research area (Santos et al. 2007) were re-analysed, taking into account the division of the sample into two putative populations, and compared to stable isotope results.

We expected to find ontogenetic variation in stable isotope ratios consistent with previous stomach content analyses (Santos et al. 2007), and a certain degree of population substructure and habitat segregation as indicated by genetic studies (Fernández 2010; Fernández et al. in press).

Materials and methods

Sample collection

Necropsies of stranded and by-caught dolphins were performed between March 1998 and April 2007 by the Galician stranding Network, CEMMA, supported by the Natural Environment Department of the Galician Government, which provides all-year-round coverage of the Galician coastline. Animals were measured and sexed, and cause of death was determined when possible. Skin and muscle samples (approximately 3 by 3 cm) were collected from those stranded animals in a sufficiently good state of preservation, following the protocol of the European Cetacean Society (Kuiken 1996). Diagnosis of fishery by-catch mortality in stranded animals also followed Kuiken (1996), being based on signs, such as net and/or ropes attached to carcasses, net marks on the body, missing flukes or lobes from the tail. Gonads and teeth were removed to obtain data on maturity and age. However, since length data were available for a greater number of animals, length is used as a proxy for age and maturity in the present study.

All the fish and cephalopods included in this paper belonged to species known to be eaten by bottlenose dolphins in Galicia (Santos et al. 2007) and were caught near the Galician coast. The majority were acquired in Vigo's fish market although several individuals of hake (*Merluccius merluccius*) and pouting (*Trisopterus luscus*) and all the silvery pout (*Gadiculus argenteus*) analysed were provided by the market sampling programme of the Instituto Español de Oceanografía (IEO). In addition, 21 hake, 19 blue whiting (*Micromesistius poutassou*), 9 Atlantic horse mackerel (*Trachurus trachurus*) and 3 blue jack mackerel (*Trachurus picturatus*) were collected during the PELACUS acoustic survey (IEO) carried out in March and April 2007. Exact fishing haul positions are available only for those fish collected during the oceanographic survey.

All prey and dolphin tissues were preserved frozen at −20°C prior to analysis.

Lipid extraction of the samples

Lipid extraction was carried out following a protocol based on Morin and Lesage (2003). A small amount of skin and muscle (a few grams) was collected from each individual sample with a scalpel and left to dry for 24 h at 70°C. Only one tissue (muscle) was analysed for fish and cephalopod species following the same process, as applied to dolphin samples, although fish species with high fat content (e.g. sardine, *Sardina pilchardus)* were left to dry for longer periods (up to 4 days). Dried tissues were ground, and 0.2 g from each sample was taken for further processing. Lipid extraction was achieved with several rinses in a 2:1 chloroform: methanol mixture. Samples were then left to dry for at least 12 h at room temperature, washed 3 times in de-ionized water, desiccated, ground and freeze dried.

Stable isotope analyses

Between 0.6 and 0.9 mg of each sample was placed in a tin capsule. Carbon and nitrogen isotope analyses were then performed simultaneously using continuous-flow isotope ratio mass spectrometry (NERC LSMSF, East Kilbride, Scotland). Results are expressed as parts per thousand (‰) deviations from international standards, in delta (δ) notation (McKinney et al. 1950). Replicate measurements of internal laboratory standards (gelatin and alanine) indicate precisions of 0.2 and 0.1‰ for δ^{15}N and δ^{13}C, respectively.

Assignment of individuals to populations

Previous genetic analysis highlighted that bottlenose dolphins from northern and southern Galicia represent different genetic populations (Fernández 2010; Fernández et al. in press). Punta Queixal (Queixal Point) adjacent to Monte (Mount) Louro, 5 km north of the town of Muros, was identified as the geographic boundary between the four southern rías and the northern Galician coast, and therefore, animals stranded in the area delimited by the border with Portugal and "Punta Queixal" (see Fig. 1) were assigned to one putative population (southern Galicia, SGAL, $n = 29$), and dolphins stranded in the area extending from "Punta Queixal" to the border with Asturias were assigned to the second population (northern Galicia, NGAL, $n = 13$). For one individual, stranding location was unknown, and classification as belonging to SGAL or NGAL was therefore not possible.

Data analysis

A quadratic discriminant analysis was carried out with the software Minitab 15 for the small sample ($n = 7$) of dolphins from which both isotopic and genetic data were available.

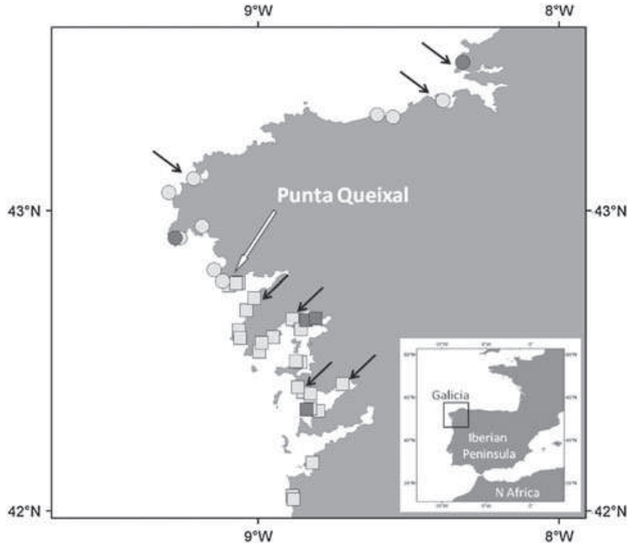

Fig. 1 Map of the study area (Galicia, NW Spain) showing the stranding locations of the bottlenose dolphins on which stable isotope analyses were performed. *Squares* dolphins from southern Galicia, SGAL. *Circles* dolphins from northern Galicia, NGAL. *Dark grey squares* and *circles* indicate the stranding locations from the five calves analysed. The symbol ↓ indicates the stranding locations of the seven dolphins from which both stable isotope and genetic data were available

Dolphins were genetically assigned to two groups (populations), and animals were then reclassified into two sets based on their isotopic values. The efficiency of the discrimination parameters (δ^{15}N and δ^{13}C) was assessed.

Ontogenetic variability was explored for blue whiting and hake isotopic composition (δ^{15}N, δ^{13}C) using additive models. For each isotope, fish length was considered in the model as an explanatory variable. For hake, origin of the samples (Vigo's fish market vs. oceanographic survey) was also included as an explanatory variable.

Mann–Whitney U tests were carried out to determine the significance of differences in isotopic compositions between calves (<180 cm) and juvenile/adult (>199 cm) bottlenose dolphins. As suckling dolphins are placed one trophic level above their mothers, mean δ^{13}C and δ^{15}N for adult females (>277 cm, López 2003) were subtracted from those of the calves (<180 cm) to estimate isotope enrichment factors for each of the two populations considered.

Factors affecting δ^{13}C and δ^{15}N values in skin and muscle of juvenile/adult dolphins (>199 cm) were analysed using GLMs (Generalized Linear Models) and GAMs (Generalized Additive Models). Since the two response variables were continuous and appeared to have an approximately normal distribution, Gaussian models were fitted to the data. The explanatory variables considered were dolphin length, dolphin sex, year, quarter (season), assigned population (i.e. north or south) and cause of death (by-catch or other). Effects of adding interaction terms were also considered.

Univariate analyses were also carried out to determine potential differences between skin and muscle isotopic compositions for the same individuals, which could be indicative of a change of diet. For each dolphin, δ^{13}C and δ^{15}N values from skin and muscle were compared using the non-parametric Wilcoxon signed-rank test for dependent samples. In addition, Euclidean distances between the isotopic compositions of skin (s) and muscle (m) were calculated for each animal following the equation:

$$\mathrm{Distance}(s,m) = \sqrt{(\delta^{13}Cs - \delta^{13}Cm)^2 + (\delta^{15}Ns - \delta^{15}Nm)^2}$$

Gaussian linear and additive models were run to quantify the potential influence of the explanatory variables (dolphin length, dolphin sex, year, quarter, population and cause of death) on Euclidean distance values.

Binomial GAMs were fitted to prey presence–absence data from previous published stomach contents analysis (see Santos et al. 2007). Presence of blue whiting and hake in the stomachs was modelled in relation to the same explanatory variables considered to model δ^{13}C and δ^{15}N in dolphin skin and muscle. This analysis thus differs from the published analysis by considering possible population (area) differences.

Gaussian linear models were fitted to quantify relationships of δ^{13}C and δ^{15}N values in skin and muscle with the proportion of blue whiting and hake in the stomach contents (number of fish of the relevant prey species/total number of prey).

All models were optimized using a forwards selection procedure, finally accepting the one with the lowest AIC (Akaike Information Criterion) provided that no outliers or large "hat" values (indicating influential data points) were detected and no serious patterns remained in the residuals. During the process of model selection, when the models showed very similar AIC values (differences in AIC <2), an F test was performed to compare and select between nested models.

All models and statistical tests mentioned above were performed using the software R.2.7.

Isotopic mixing models were applied with the software SIAR (Parnell et al. 2010) to estimate the proportional contribution of sources (dietary items) within the mixture (consumer tissue). The four main prey categories contributing to the total diet by weight were considered as sources: blue whiting, hake, conger eel, *Conger conger*, and mullet, *Mugil* sp. (Santos et al. 2007). The enrichment factors considered were derived from data obtained from the present paper (see the section entitled *Suckling dolphins* under *Results*), being 3 ± 0.3‰ for δ^{15}N and 1.5 ± 0.5‰ for δ^{13}C. The number of interactions used was 400,000.

Separate analyses were run for each tissue (skin and muscle) and population (SGAL and NGAL).

Results

Composition of the sample of bottlenose dolphins and prey

Sampled dolphins comprised 20 females and 23 males stranded in Galicia between 1998 and 2007. Length was determined on every occasion and ranged from 130 to 331 cm, although for three individuals, total body length had to be estimated due to the absence of some body parts (e.g. fluke). Eight animals were classified as definitely by-caught. For one adult female, muscle samples were not available. Stranding locations are shown in Fig. 1.

Sample sizes of prey species ranged from $n = 2$ for gilthead seabream, *Sparus aurata*, and sardine to $n = 42$ for hake.

Analysis of stable isotopic composition and variability in prey species

Isotopic compositions of fish and cephalopods are summarized in Table 1. Values of δ^{15}N ranged from 11.1‰ for one of the gilthead seabreams to 14.3 ± 0.8‰ for pouting. Pouting also showed the highest δ^{13}C value (−15.4 ± 0.5‰), whereas blue jack mackerel had the lowest δ^{13}C (−18.0 ± 0.4‰) (Table 1). Comparing isotopic values of dolphins and prey species, juvenile and adult bottlenose dolphins from southern Galicia were placed among the top positions of the trophic chain, although the average δ^{15}N value is around 0.4‰ lower than that for pouting (Fig. 2). On the other hand, dolphins stranded in northern Galicia showed stable isotope values lower than those of several fish and cephalopod species, including hake.

Values of δ^{15}N in hake muscle increased linearly with fish body length ($P < 0.001$). However, the origin of the samples also significantly influenced δ^{15}N composition ($P < 0.001$), and hake obtained in the scientific survey displayed lower trophic levels. The model explained the 58.1% of the deviance. Hake length did not affect δ^{13}C values, but hake caught during the survey showed significantly lower δ^{13}C ($P < 0.001$).

No relationship was found between δ^{15}N and blue whiting length, although the models showed a non-linear increase in δ^{13}C with fish length. The best model explained the 49.6% of the deviance, and the smoother term ($df = 2.29$, $P = 0.013$) showed an increase in δ^{13}C up to around 200 mm fish length, when the relationship starts to be less clear (Fig. 3). The

Table 1 Species, number of samples, length (mm) and muscle δ^{15}N and δ^{13}C (‰) of prey species of fish and cephalopods from the research area

Taxa	Common name	Code	n	Length range, mm	δ^{15}N	δ^{13}C
Fish						
Gadidae						
Micromesistius poutassou	Blue whiting	BW	21	171–275	11.7 ± 0.3	−17.7 ± 0.4
Trisopterus luscus	Pouting	PO	11	160–285	14.3 ± 0.8	−15.4 ± 0.5
Trisopterus minutus	Poor cod	PC	4	196–210	12.8 ± 0.3	−16.1 ± 0.4
Gadiculus argenteus	Silvery pout	SP	10	92–125	11.5 ± 0.2	−16.8 ± 0.2
Sparidae						
Diplodus sargus	White seabream	WS	5	210–340	13.3 ± 0.3	−16.2 ± 0.2
Sparus aurata	Gilthead seabream	GS	2	307/308	11.1/14.3	−16.5/−14.7
Pagellus sp.	Seabream	SE	3	270–290	13.9 ± 0.3	−16.3 ± 0.2
Other						
Merluccius merluccius	Hake	HA	42	205–685	13.6 ± 0.4	−16.7 ± 0.5
Trachurus trachurus	Atlantic horse mackerel	HM	18	127–353	13.0 ± 0.4	−16.9 ± 0.5
Trachurus picturatus	Blue jack mackerel	JM	8	180–230	12.6 ± 0.2	−18.0 ± 0.4
Chelon labrosus	Thicklip grey mullet	GM	6	445–470	11.7 ± 1.0	−17.4 ± 1.2
Conger conger	Conger eel	CE	7	680–1645	13.5 ± 1.1	−15.6 ± 0.3
Atherina presbyter	Sand smelt	SS	3	100–120	13.6 ± 0.3	−15.8 ± 0.2
Sardina pilchardus	Sardine	SA	2	103/172	12.0/11.5	−16.4/−17.8
Cephalopods						
Todaropsis eblanae	Lesser flying squid	FS	7	100–166	13.9 ± 0.3	−17.1 ± 0.4
Sepia officinalis	Cuttlefish	CU	5	47–240	13.0 ± 0.6	−15.5 ± 0.5
Eledone cirrhosa	Curled octopus	CO	5	65–80	11.7 ± 0.4	−16.7 ± 0.3

For fish species, total length was recorded whereas mantle length was measured for cephalopods

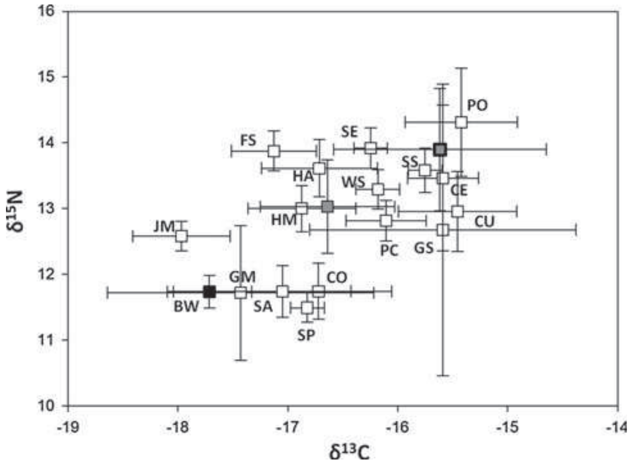

Fig. 2 Muscle δ^{15}N and δ^{13}C of fish, cephalopods and juvenile/adult (>199 cm) bottlenose dolphins from the study area. *Black square*, blue whiting. *Grey square bold border*, dolphins from southern Galicia, SGAL. *Grey square plain border*, dolphins from northern Galicia, NGAL. *White squares*, other prey species. Species codes correspond to those in Table 1

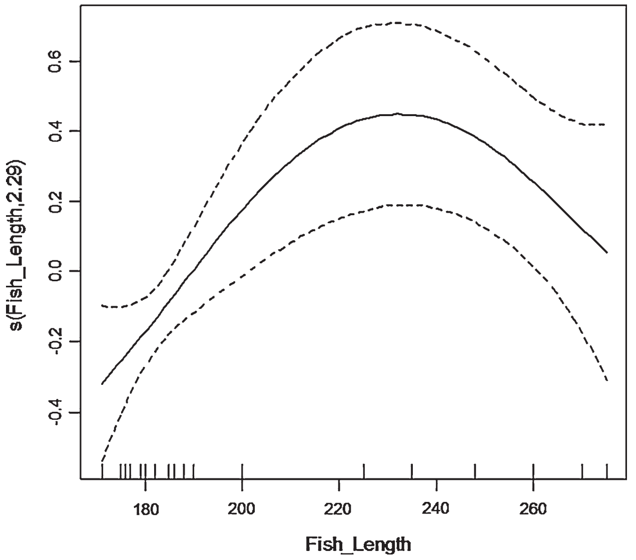

Fig. 3 Smoother for the effect of fish length (mm) on muscle δ^{13}C (‰) for blue whiting. The *Y*-axis represents the trend (positive or negative) in muscle δ^{15}N in relation to fish body length. Tick marks in the *X*-axis represent sampled fish. Dotted lines are the approximate 95% confidence limits

influence of sample origin on blue whiting isotopic composition was not explored as all but two individuals were obtained during the oceanographic survey.

Suckling dolphins

Isotopic compositions of dolphins are summarized in Table 2. Five calves, with body lengths between 130 and 179 cm, showed δ^{15}N and δ^{13}C values significantly higher than the rest of the animals both for muscle (Mann–Whitney U; $P < 0.001$ for δ^{15}N and $P = 0.018$ for δ^{13}C) and skin (Mann–Whitney U; $P = 0.001$ for δ^{15}N and $P = 0.006$ for δ^{13}C) (Fig. 4). These isotopic values are consistent with suckling animals, and therefore, these five calves were not included in further statistical analyses.

Skin of suckling dolphins from the SGAL population displayed an enrichment of 2.7‰ δ^{15}N and 1.9‰ δ^{13}C compared to adult females from the same population while the enrichment factor calculated for NGAL dolphins was 1.8‰ δ^{15}N and 1.4‰ δ^{13}C. Muscle enrichment factors were estimated as 3.3‰ δ^{15}N and 2.0‰ δ^{13}C for the SGAL population (Table 3).

Quadratic discriminant analysis

For seven dolphins, both genetic and stable isotope data were available (see Fig. 1). Four of the animals (stranded in southern Galicia) were genetically classified as SGAL, and three animals (stranded in northern Galicia) were identified as NGAL. A quadratic discriminant analysis was applied based on the δ^{15}N and δ^{13}C values of the seven dolphins both for muscle and skin. Although small sample size makes it difficult to ascribe much importance to the results, all dolphins were correctly assigned to their genetic group.

Analysis of stable isotopic composition and variability in dolphins

Dolphin body length was not significantly different between populations (Mann–Whitney U test; $P = 0.667$). Gaussian GAMs showed a significant relationship between muscle δ^{15}N and both dolphin body length ($df = 3.3$, $P = 0.013$) and population ($P = 0.012$). The final model explained 46.2% of the deviance. The shape of the smoother for length (Fig. 5) indicates that δ^{15}N decreases with increasing dolphin length (up to around 250 cm) although the relationship is less clear for bigger body sizes. δ^{15}N was lower for dolphins classified as belonging to NGAL than for those belonging to SGAL (Fig. 6).

The δ^{15}N of skin decreased linearly with dolphin body length ($P = 0.038$) and was higher in SGAL than in NGAL ($P = 0.002$; Fig. 6). This GLM explained 33.2% of deviance.

GLMs showed that δ^{13}C values in muscle and skin differed significantly between populations ($P = 0.004$ and $P = 0.003$, respectively) and dolphins from SGAL displayed heavier stable isotope ratios for both elements (Fig. 6).

Although fishery by-catches could go undiagnosed (so that our "other causes of death" category potentially includes some by-caught animals), results provide no

Table 2 δ^{15}N and δ^{13}C values (‰) of muscle and skin of bottlenose dolphins stranded in the Galician coast; SGAL, southern Galicia, NGAL, northern Galicia

Group	Length, cm	δ^{15}N		δ^{13}C	
		Muscle	Skin	Muscle	Skin
Calves	130–179	$n = 5$			
		16.8 ± 0.8	16.3 ± 0.8	−14.4 ± 1.2	−14.2 ± 1.1
		$P = 0.187$		$P = 0.438$	
Juvenile–adults, SGAL	200–328	$n = 26$			
		13.9 ± 0.9	14.2 ± 1.0	−15.6 ± 1.0	−15.6 ± 1.0
		$P = 0.013$		$P = 0.861$	
Juvenile–adults, NGAL	208–331	$n = 10$	$n = 11$	$n = 10$	$n = 11$
		13.0 ± 0.7	13.2 ± 0.7	−16.6 ± 0.6	−16.6 ± 0.6
		$P = 0.769$		$P = 0.922$	

Calves included 3 individuals from SGAL and 2 animals form NGAL. Significant differences between tissues (muscle vs. skin) for each category are indicated by bold face

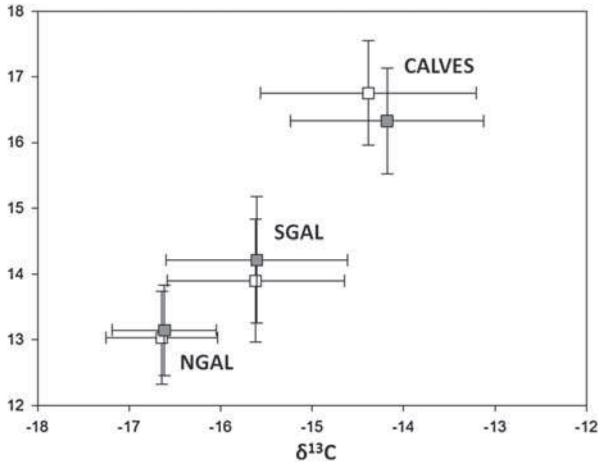

Fig. 4 δ^{15}N and δ^{13}C for calves (<180 cm) and juvenile/adult bottlenose dolphins (>199 cm) from southern Galicia (SGAL) and northern Galicia (NGAL). *Grey squares*, skin compositions. *White squares*, muscle compositions

Table 3 Estimated enrichment factors for δ^{15}N and δ^{13}C in muscle and skin based on the isotopic signatures of calves (<180 cm) and adult females (>277 cm)

			Enrichment factor
Muscle			
SGAL	Calves $n = 3$	Females $n = 5$	
δ^{15}N	17.1 ± 1.0	13.8 ± 1.0	3.3‰
δ^{13}C	−13.8 ± 1.0	−15.8 ± 0.9	2.0‰
NGAL	Calves $n = 2$	Females $n = 0$	
δ^{15}N	16.3 ± 0.1	–	–
δ^{13}C	−15.2 ± 1.1	–	–
Skin			
SGAL	Calves $n = 3$	Females $n = 5$	
δ^{15}N	16.8 ± 0.4	14.1 ± 1.1	2.7‰
δ^{13}C	−13.7 ± 1.1	−15.6 ± 1.2	1.9‰
NGAL	Calves $n = 2$	Females $n = 1$	
δ^{15}N	15.6 ± 0.7	13.8	1.8‰
δ^{13}C	−14.9 ± 0.5	−16.3	1.4‰

evidence that the isotopic profiles of by-caught animals are in any way atypical since no significant differences in either isotope ratio were found in relation to cause of death.

Comparison between skin and muscle isotopic values

For juvenile/adult dolphins, significant differences were found between δ^{15}N values in skin and muscle (Wilcoxon signed-rank test; $P = 0.010$). No differences between the two tissues were found for δ^{13}C (Wilcoxon signed-rank test; $P = 0.587$). When samples were analysed separately for both putative populations, the same results were found for SGAL (Wilcoxon signed-rank test; $P = 0.013$ for δ^{15}N and $P = 0.861$ for δ^{13}C), but no significant differences between the two tissues were found for the smaller sample of dolphins classified as NGAL (Wilcoxon signed-rank test; $P = 0.769$ for δ^{15}N and $P = 0.922$ for δ^{13}C; Table 2).

Euclidean distances between skin and muscle isotopic values were used as a proxy of potential diet/habitat shifts. None of the explanatory factors considered (dolphin length, dolphin sex, year, quarter, population, cause of death) had any significant effect on Euclidean distance values.

Comparison between isotopic value profiles and stomach contents

For 20 animals, both stomach contents (Santos et al. 2007) and isotopic results were available. There were significant negative linear relationships between δ^{15}N and δ^{13}C values

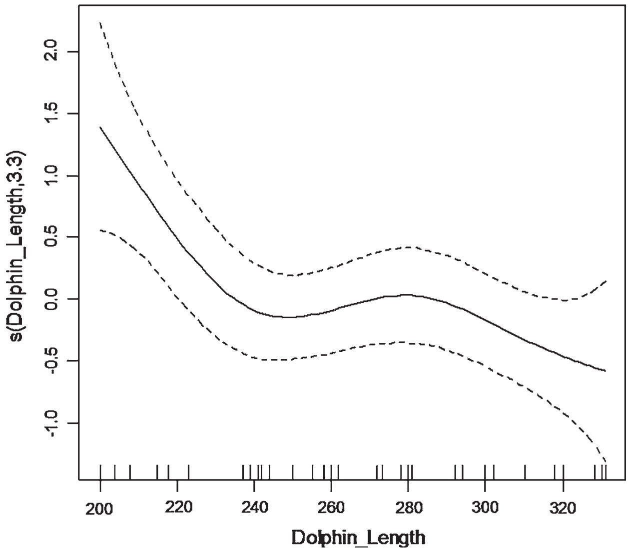

Fig. 5 Smoother for the effect of dolphin length (cm) on muscle $\delta^{15}N$ (‰). The *Y*-axis represents the trend (positive or negative) in muscle $\delta^{15}N$ in relation to dolphin body length. Tick marks in the *X*-axis represent sampled dolphins. Dotted lines are the approximate 95% confidence limits

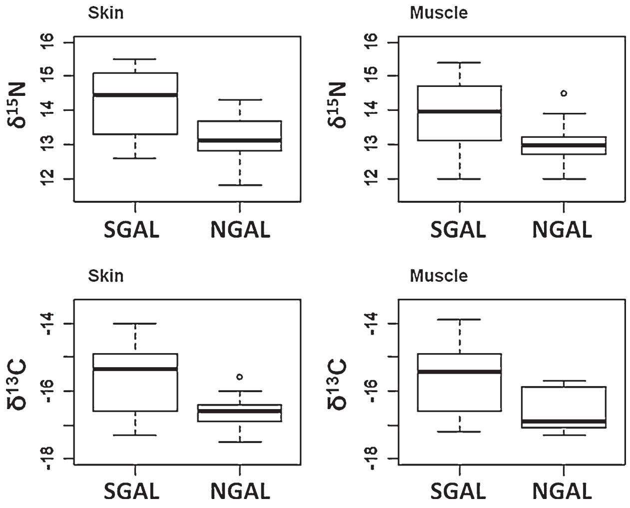

Fig. 6 *Box plots* of isotopic values verses dolphin population (SGAL, NGAL). Only juvenile/adult dolphins (>199 cm) were considered. The midpoint of each *box* represents the median, and the 25% quartiles define the hinges. Differences between hinges show the spread of the data. Whiskers represent maximum and minimum values. An observation beyond 1.5 times the spread is considered an outlier (e.g. observation in top-right and lower-left *box plot*)

and the proportion of blue whiting in the stomach (Table 4). Proportion of hake in the diet did not influence the isotopic compositions of dolphins.

We modelled the presence of blue whiting and hake in the diet using binomial GAMs. For blue whiting, effects of both dolphin body length and population were found to be significant, and the best model explained 29.9% of the deviance. The smoother term for dolphin length ($df = 2.9$, $P = 0.020$) shows a positive relationship between dolphin length and presence of blue whiting up to around 280 cm when numbers of blue whiting start to decrease slightly (Fig. 7). Presence of blue whiting in the stomach was higher for dolphins classified as NGAL ($P = 0.005$). The final model for hake explained the 20.7% of the deviance, and although the effect of dolphin body length was not statistically significant ($P = 0.119$), its inclusion improved the model's AIC. Population had a significant effect on the presence of hake in the diet ($P = 0.001$) with a higher prevalence of this prey species in dolphins from NGAL.

Table 4 Results of GLMs for data on bottlenose dolphin $\delta^{15}N$ and $\delta^{13}C$ (‰) and proportion of blue whiting in the stomach contents (Rel.Blw)

GLM	*P*	DE %
$\delta^{15}N$.muscle ~ Rel.Blw	<0.001	53.47
$\delta^{13}C$.muscle ~ Rel.Blw	<0.001	78.01
$\delta^{15}N$.skin ~ Rel.Blw	<0.001	54.89
$\delta^{13}C$.skin ~ Rel.Blw	<0.001	63.11

The table gives values of probability (*P*) for each model and associated deviance explained (DE, %)

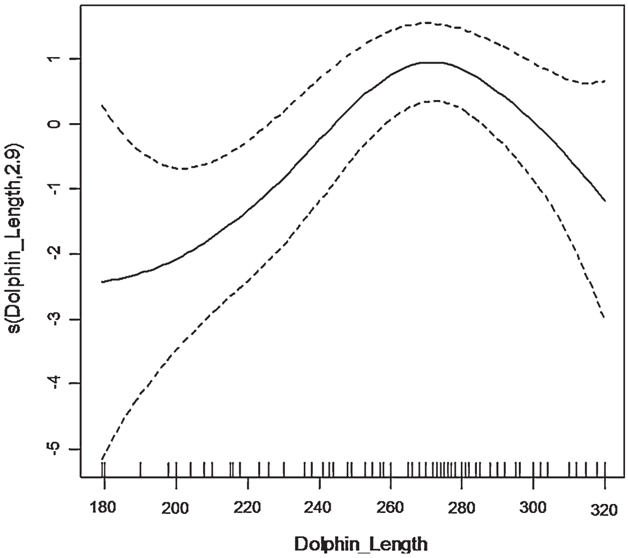

Fig. 7 Smoother for the effect of dolphin length (cm) on the presence of blue whiting in the stomach contents. The *Y*-axis represents the trend (positive or negative) in the presence of blue whiting in relation to dolphin body length. Tick marks in the *X*-axis represent sampled dolphins. Dotted lines are the approximate 95% confidence limits

Results from isotopic mixing models also suggest a higher contribution of blue whiting and hake to the diet of bottlenose dolphins form NGAL based on both muscle and skin isotopic values, although confidence intervals were wide (Table 5). Grey mullet is found to be the most important prey species in southern dolphins and second most important in northern individuals. However, as can be

Table 5 Isotopic mixing models results based on δ^{15}N and δ^{13}C (‰) values

Tissue	Population	Source	Low 95%	High 95%	Mode	Mean
Muscle	SGAL	Blue whiting	0.006	0.628	0.249	0.326
		Hake	0.000	0.145	0.015	0.049
		Conger eel	0.000	0.174	0.021	0.065
		Grey mullet	0.217	0.894	0.639	0.560
	NGAL	Blue whiting	0.150	0.911	0.424	0.498
		Hake	0.000	0.230	0.017	0.073
		Conger eel	0.000	0.138	0.012	0.046
		Grey mullet	0.007	0.706	0.405	0.384
Skin	SGAL	Blue whiting	0.009	0.623	0.391	0.323
		Hake	0.000	0.146	0.015	0.051
		Conger eel	0.000	0.174	0.019	0.068
		Grey mullet	0.201	0.908	0.506	0.557
	NGAL	Blue whiting	0.206	0.945	0.484	0.556
		Hake	0.000	0.167	0.015	0.060
		Conger eel	0.000	0.111	0.011	0.038
		Grey mullet	0.000	0.668	0.401	0.346

Dietary items (sources): blue whiting, hake, conger eel and thicklip grey mullet. Consumer tissue (mixture): muscle and skin from juvenile and adult (>199 cm) bottlenose dolphins from SGAL (South Galicia) and NGAL (North Galicia).

The estimated 95% confidence intervals, mode and mean are given for each diet item, tissue and population

seen from Fig. 2, grey mullet and blue whiting have rather similar isotopic compositions, with grey mullet having slightly higher δ^{13}C values, which could have influenced the results when applying mixing models.

Discussion

Carbon and nitrogen isotopic compositions were consistent with values for bottlenose dolphins stranded in other locations along the Iberian Peninsula (Borrell et al. 2006), although the two Galician populations were isotopically separable, highlighting the existence of ecological differences in the bottlenose dolphin community in agreement with previous genetic findings (Fernández 2010; Fernández et al. in press). Using stranding area as a proxy for genetic population might lead to some misclassifications as it may not always reflect the true provenance of samples (i.e. where the animals lived as opposed to where the carcases were found). Although sample size was small ($n = 7$), limiting confidence in the reliability of the result, quadratic discriminant analysis correctly assigned individuals to genetic populations based on stable isotopic values, and our analyses confirmed the existence of two clear groups within Galician bottlenose dolphins.

Animals classified as northern Galician show less variability in both δ^{15}N and δ^{13}C than animals from southern Galicia. However, the smaller sample size for northern animals makes it difficult to interpret this result in terms of dietary specialization. Only dolphins classified as southern Galician were found to have significant differences between skin and muscle δ^{15}N values, but again, this could be related to sample size. Contrasting isotope ratios from skin and muscle can indicate movement between different habitats, or alternatively, changes in trophic level as a result of a shift in prey sources. The relatively high variability in isotopic values of dolphins from southern Galicia could indicate a broader diet as might be expected if they regularly feed within the species-rich ría environment. However, further samples are needed, especially for northern Galicia.

Significantly higher δ^{13}C values were associated with dolphins from southern Galicia when compared to dolphins from the north. These isotopic differences suggest the existence of habitat segregation between both populations as coastal environments (such as the rías) are enriched in δ^{13}C while more pelagic environments are characterized by lower δ^{13}C values (France 1995; Hobson 1999; Lesage et al. 2001; Das et al. 2003). Dolphins from southern Galicia also showed a significantly higher trophic level (δ^{15}N) than dolphins from the north which could be related to resource partitioning, with northern Galician animals being more dependent on shelf prey species at lower trophic levels. The importance of both blue whiting (low trophic level) and hake (high trophic level) as part of the diet was higher in northern Galician dolphins. In addition, isotopic mixing models suggest a higher consumption of thicklip grey mullet and conger eel by southern Galician animals. Nevertheless, blue whiting was identified as the main contributor by weight to the total diet (Santos et al. 2007), and despite small sample size, higher numbers of blue whiting in the diet were related to lower isotopic values. Therefore, higher proportions of blue whiting in the diet would explain the lower δ^{15}N value found in northern Galician dolphins. Bottlenose dolphins in Galician waters would, consequently, rely on habitat segregation and resource partitioning to reduce potential intra-specific competition.

Dolphins with body lengths less than 180 cm showed the expected isotopic values of suckling animals (i.e. higher than in juveniles or adults). Length at birth is estimated to be up to 132 cm (Mead and Potter 1990), and bottlenose dolphins can nurse for 1–2 years before weaning (Wells and Scott 2002). Stable isotope findings shown here indicate a shift from a milk-based diet to a fish-based diet at dolphin lengths between 180 and 200 cm, in agreement with previous reports. The smallest bottlenose dolphin stranded in Galicia with solid food in its stomach was 179 cm in length (López 2003).

Estimations on skin and muscle of harp seals (*Pagophilus groenlandicus*) held on a constant diet of herring gave mean trophic enrichment factors of 2.3‰ δ^{15}N/2.8‰ δ^{13}C and 2.4‰ δ^{15}N/1.3‰ δ^{13}C, respectively (Hobson et al. 1996). Das et al. (2003) reported a mean enrichment of 2.2‰ δ^{15}N between harbour porpoise (*Phocoena phocoena*) pups and adult female muscle. In addition, García-Tiscar (2009) found average enrichment factors of 1.58‰ δ^{15}N and 1.63‰ δ^{13}C between captive killer whale, *Orcinus orca*, skin and their prey. In the present paper, the enrichment factor for δ^{15}N (3.3‰) measured in muscle in southern Galician dolphins was found to be around 1‰ higher than previous estimates on harp seals (Hobson et al. 1996) and harbour porpoises (Das et al. 2003). On the other hand, the enrichment factor for δ^{13}C (1.9‰) measured in skin of bottlenose dolphins from the same population was around 1‰ lower than calculations by Hobson et al. (1996) based on harp seals and more similar to the estimates on captive killer whales by García-Tiscar (2009). Several sources of variation, including the methodology of analysis and dietary differences, have been proven to affect δ^{15}N and δ^{13}C enrichment factors (McCutchan et al. 2003; Vanderklift and Ponsard 2003). In the present study, enrichment factors per trophic level should be interpreted with caution as they were estimated based on rather few samples. Moreover, trophic enrichment between mother and calf is species dependent (Jenkins et al. 2001; Das et al. 2003), and this finding therefore needs further investigation.

Juvenile and adult bottlenose dolphins from southern Galicia are placed among the top predators of the food web. However, dolphins stranded in northern Galicia show δ^{15}N values lower than some typically ichthyophagous fish species such as hake, and differences in stable isotope values between the two populations of dolphins were higher than differences found between several fish and cephalopod species. Our results also placed pouting, and a small pelagic fish, sand smelt *Atherina presbyter,* at the top of the trophic chain. These last two species feed on small fish, fish larvae, crustaceans and cephalopods (Svetovidov 1986; Quignard and Pras 1986) and such a high trophic level were unexpected. The lowest δ^{15}N values were registered in species that feed on crustaceans (silvery pout, *Gadiculus argenteus,* and curled octopus, *Eledone cirrhosa*), on algae and detritus (thicklip grey mullet, *Chelon labrosus*), on plankton (sardine) or on small crustaceans, cephalopods and small fish (blue whiting) (Sorbe, 1980; Boyle 1983; Ben-Tuvia 1986; Cohen et al. 1990; Olaso and Rodriguez-Marin 1995; Bode et al. 2003).

Blue whiting and hake constitute the main prey for the bottlenose dolphin in Galicia (Santos et al. 2007). Galician waters are an important nursery ground for blue whiting and hake where, being two of the most abundant fish species, they are targets of directed fisheries and have considerable commercial value.

Trophic level (δ^{15}N) can often vary within a species in relation to animal body size as a consequence of ontogenetic changes in diet. Two opposite trends can be found in nature. First, increasing δ^{15}N values may occur with increasing predator body sizes, for example due to feeding on larger prey. This trend has been found in the squid *Gonatus fabricii* (Hooker et al. 2001) and can partly be caused by certain degree of cannibalism (e.g. Mouat et al. 2001). Second, decreasing δ^{15}N content may be seen with increasing predator body sizes as happens with sardines, in NW Spain, when their main prey changes to species at a lower trophic level (Bode et al. 2003). Different trends were seen in the present study, with increasing trophic level in larger hake, an absence of size-related trophic level variation in blue whiting and a decrease in trophic level in the dolphins.

In the present study, δ^{15}N was seen to increase with hake body length. Young hake was found to prey in crustaceans and small fish, and the importance of fish in the diet rises as hake grows with hake over 12 cm length feeding almost exclusively on active natatory fishes (Guichet 1995; Bozzano et al. 1997). Variation in prey preferences by hake related to hake size has been found in the Bay of Biscay where medium-sized hake shows a preference for clupeids and blue whiting while larger hake feeds preferably on horse mackerel (Guichet 1995). In addition, Guichet (1995) recorded cases of cannibalism on hake nursery grounds where individuals between 15 and 80 cm preyed on smaller hake. Our study agrees with those patterns of ontogenetic variation in diet in that trophic level increased with hake size. Sample origin also influenced δ^{15}N and δ^{13}C patterns in hake muscle, possibly indicating sampling over different habitats.

Juvenile blue whiting (with a length between 7 and 14 cm) are pelagic (Raitt 1968) and at a length of around 16 cm adopt a demersal lifestyle (Bas and Morales 1966). Most of the blue whiting eaten by bottlenose dolphins in Galicia were estimated to be over 16 cm (Santos et al. 2007).

In agreement with a diet based on small crustaceans, cephalopods and small fish (Sorbe 1980; Cohen et al. 1990; Olaso and Rodriguez-Marin 1995), blue whiting was found to be at a lower trophic level compared with other prey taxa present in bottlenose dolphin diet. In the research area, small blue whiting (<20 cm) feed intensively on pelagic crustaceans such as euphausiids whereas bigger fish become more ichthyophagous preying mainly on silvery pout (Olaso and Rodriguez-Marin 1995). Blue whiting shows a certain degree of cannibalism at lengths bigger than 20 cm, and at sizes over 25 cm, the ingestion of silvery pout decreases while cannibalism increases (Olaso and Rodriguez-Marin 1995). Higher trophic levels for larger fish were expected, but in the present study, δ^{15}N did not increase significantly with length. However, our sample set included few individuals bigger than 20 cm, which could account for the lack of an ontogenetic trend. On the other hand, δ^{13}C values increased with fish length up to 20 cm and remained constant thereafter. Sorbe (1980) reported that blue whiting less than 20 cm length were mainly found between 150 and 300 m depth in the Bay of Biscay whereas mature fish inhabited deeper waters. A shift in diet, from pelagic crustaceans to fish, was also recorded at around 20 cm length (Olaso and Rodriguez-Marin 1995). Carbon originating from pelagic phytoplankton generally has lower δ^{13}C values compared with benthic organisms (Quesada 2005), and δ^{13}C values are typically higher in species from coastal or benthic food webs (France 1995; Hobson 1999; Lesage et al. 2001; Das et al. 2003). Higher carbon isotope ratios in bigger blue whiting could reflect movements towards more demersal waters.

The present paper and the study by Santos et al. (2007) show an increase in the importance of blue whiting with dolphin length up to around 280 cm, although median blue whiting length in bottlenose dolphin stomach contents in Galicia was not related to dolphin body length (Santos et al. 2007). Previous analysis also found that larger dolphins took bigger hake while male dolphins also ate bigger (but fewer) hake than females (Santos et al. 2007).

In the present study, δ^{15}N was found to decrease with increasing dolphin size, which indicates a possible change in the targeted prey with bigger dolphins showing preference for species at lower trophic levels. Consequently, changes in trophic level would reflect a preference towards blue whiting as dolphins grow. As suggested by Santos et al. (2007), ontogenetic shifts in bottlenose dolphin diet, with larger dolphins taking more fish of certain species, are likely to reflect increasing experience, improved diving and prey-catching abilities and increased stomach capacity. Therefore, higher percentages of blue whiting in the diet of larger dolphins may be due to an improvement in diving capacity. Most of the blue whiting ingested by bottlenose dolphins in the research area were in the size range that has a demersal life style and may not be available for younger and more inexperienced dolphins. In addition, juvenile dolphins from southern Galicia (inhabiting the rías) could have a diet mainly based on more coastal prey as the suggested offshore foraging trips (Santos et al. 2007) might not be feasible for them.

The present paper confirms the existence of resource partitioning within Galician bottlenose dolphins, consistent with population substructure previously identified from genetic studies. As suggested before, the mechanisms controlling the geographic structuring of bottlenose dolphin populations may be strongly linked to local habitat choice and dependence (Natoli et al. 2005; Segura et al. 2006). Bottlenose dolphins live in fission–fusion societies (Connor et al. 2000) and therefore movements between groups and communities can be expected. Apparent migrants between these two populations (i.e. dolphins which stranded along the part of the coastline inhabited by the other population) have been registered, yet migration rate is not strong enough to eliminate population substructure (Fernández 2010; Fernández et al. in press). Ideally, a larger sample set comprising paired genetic and stable isotope data at the individual level should be obtained to confirm substructure and better resolve the boundaries between these two partially sympatric groups.

In our study area, the coastal southern Galicia population, particularly nursing groups and juvenile dolphins, could face bigger threats than northern Galician dolphins as the rías are more affected by habitat degradation and pollution than open waters. Finally, fisheries interactions could influence the two populations at different levels. The importance of blue whiting and hake is higher for northern Galician dolphins, and therefore, this population could be more affected by ecological interactions with fisheries and by-catch as fishermen and dolphins are exploiting the same resources and areas. However, lower levels of competition with fisheries and by-catch within southern dolphins could have stronger effects in this coastal and semi-isolated population. The different habitat use and characteristics of the two populations should be taken into account in the future when designing and implementing conservation measures such as SACs for the bottlenose dolphin in Galicia.

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