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ORIGINAL ARTICLE

Feeding ecology of harbour porpoises: stable isotope analysis of carbon and nitrogen in muscle and bone

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Abstract

Harbour porpoises are the most common small cetaceans in the North Sea and Dutch coastal waters. To study their trophic level and feeding location, stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were analysed in muscle and bone samples collected from 157 porpoises stranded along the Dutch coast (2006–2008). In addition, samples from 30 prey species were analysed. Prey samples showed high $\delta^{15}N$ values in species of higher trophic level. In addition, geographic differences in isotopic composition were found, with higher $\delta^{15}N$ and $\delta^{13}C$ values in prey from more southern, coastal and estuarine areas. Based on muscle $\delta^{15}N$ values, we found neonatal enrichment and that larger porpoises, in particular males, seem to feed on lower trophic level species, compared to smaller individuals. Also bone $\delta^{15}N$ values show that larger animals had fed on lower trophic levels in distant times. Porpoises from the Eastern Scheldt reveal distinct $\delta^{13}C$ values in muscle, but not in bone. This shows that these animals had foraged in the Eastern Scheldt for a longer time period but were not born there. Seasonal variation in bone $\delta^{15}N$ and $\delta^{13}C$ values revealed two distinct groups of porpoises along the Dutch coast, a winter group (mainly males) that migrated from neighbouring regions and a Dutch subpopulation in summer. These results furthered our insight about shifts in trophic level and feeding location of harbour porpoises from the southern North Sea over time.

Key words: Phocoena phocoena, *$\delta^{13}C$, $\delta^{15}N$, trophic ecology, The Netherlands*

Introduction

The harbour porpoise (*Phocoena phocoena* Linnaeus, 1758) is widely distributed throughout the temperate and cold waters of the Northern hemisphere (Gaskin 1984; Hammond et al. 2002) and is the most common small cetacean in the North Sea and Dutch coastal waters. Population estimates for the North Sea at large are approximately 350,000 individuals in 1994 and 2005 (Hammond 2006; SCANS-II 2008). Their abundance and distribution in the southern North Sea has changed significantly over the past decades (Camphuysen 2004; Thomsen et al. 2006). A southern shift in distribution has been documented (SCANS-II 2008), which is also reflected in Dutch coastal waters with a peak in sightings and strandings in 2006 (Camphuysen et al. 2008; Reijnders et al. 2009; Camphuysen 2011). Changes in porpoise abundance and distribution are hypothesized to result from changes in prey availability (Camphuysen 2004; MacLeod et al. 2007).

As direct observations of feeding marine mammals are extremely rare, commonly used methods to study the feeding ecology in marine mammals are the analysis of stomach contents, fatty acids and stable isotopes (e.g. Hyslop 1980; Hobson 1999; Iverson et al. 2004). Whereas stomach contents provide information on recently ingested prey (Pierce & Boyle 1991), fatty acids in organisms reflect the assimilated diet over weeks to months (Budge et al. 2006) and stable isotopes over periods varying from

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hours to years, depending on the tissue analysed (Tieszen et al. 1983; Dalerum & Angerbjörn 2005; Phillips & Eldridge 2006).

Isotopic ratios of nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$) and carbon ($^{13}C/^{12}C$, expressed as $\delta^{13}C$) have been used to analyse diet composition, trophic level and origin in terrestrial and marine species (Michener & Kaufman 2007; Newsome et al. 2010). Predators are generally enriched in ^{15}N compared to their prey (approximately 3–4‰ higher; DeNiro & Epstein 1981; Caut et al. 2009), and $\delta^{15}N$ values can therefore be used as indicators of relative trophic level (Post 2002). In general, $\delta^{13}C$ values are more similar between predator and prey (approximately 0.1–1‰ higher in predator; DeNiro & Epstein 1978; Caut et al. 2009), but geographic differences in $\delta^{13}C$ can be used to indicate feeding location (e.g. offshore versus inshore; Hobson 1999; Barnes et al. 2009). Isotopic discrimination of carbon and nitrogen, however, has been shown to vary among tissues, diet and taxa (Caut et al. 2008; Bond & Diamond 2011). Diet–tissue fractionation rates are relatively well studied for muscle (Hobson & Clark 1992a, b; Hobson et al. 1996), but less so for bone. There are also no isotopic discrimination rates published specifically for porpoises.

Tissues integrate isotopic composition of diet at different rates depending on their own turnover rate. Muscle tissue reflects assimilated diet of weeks or months prior to sampling (Kurle & Worthy 2002). Bone tissue, in contrast, displays a more long-term integration, reflecting assimilated diet of 8–12 months in young animals, with an increasingly larger time period in older animals (Sealy et al. 1995; Richards et al. 2002; O'Regan et al. 2008). This offers the opportunity to examine shifts in diet or feeding location within the same individuals over time.

The purpose of the present study is to use isotope analysis on porpoises and their prey (1) to gain insight into the trophic level and feeding location (e.g. coastal versus offshore) of harbour porpoises from the southern North Sea during a period of high porpoise abundance and stranding frequency (2006–2008), (2) to assess how individual characteristics (e.g. sex, age and location) can explain variability in isotopic composition among individuals, and (3) to define possible shifts in trophic level or feeding location by comparing the isotopic composition between muscle and bone. To that end we have analysed 157 harbour porpoises and 30 prey species and have assessed sex- and age-related, seasonal and geographic effects on the isotopic composition ($\delta^{15}N$ and $\delta^{13}C$) of porpoise muscle and bone, and the difference between these tissues.

Material and methods

Sample collection

This study was conducted on 157 harbour porpoises stranded on the Dutch coast between 2006 and 2008 (Figure 1). Stranding date and location were reported for each animal and during post-mortem examinations, general morphometric data were collected, e.g. sex (male, female, unknown) and length (cm). For each animal, age was determined based on total body length: neonates < 90 cm, juveniles 90–130 cm and adults > 130 cm; unless teeth or reproductive organs indicated differently (Table I). Muscle samples were taken from the ventral mid region, while for bone tissue, the fifth rib was collected. Sixty-three porpoises were sampled concurrently for both muscle and bone, allowing the comparison between the two tissue types. Depending on the state of decomposition and/or sampling protocol, some animals were only sampled for muscle ($n = 39$) or only for bone ($n = 55$), resulting in a total of 102 muscle samples and 118 bone samples which were available for the separate analyses of these tissues. Muscle was sampled mostly from very fresh and fresh animals, and only some samples were considered putrefied or very putrefied. Bone was sampled regardless of decomposition state, ranging from very fresh to very putrefied.

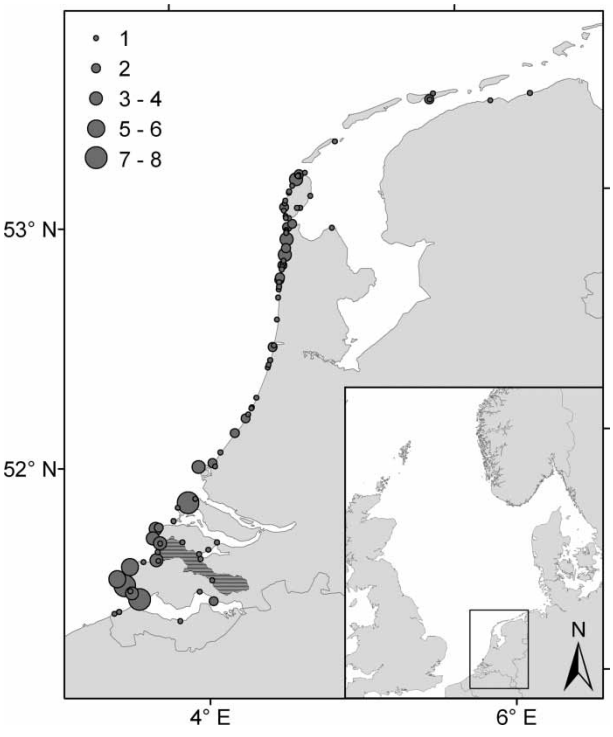

Figure 1. Porpoise (*Phocoena phocoena*) stranding locations and numbers along the Dutch coast analysed in this study (2003–2008). The dark grey area indicates the Eastern Scheldt.

Table I. Porpoise (*Phocoena phocoena*) length and weight measurements, grouped by age-class (adult/juvenile/neonate) and sex (M/F/U) with number of samples (*n*), mean, standard deviation (SD) and range (Min/Max).

			Length (cm)				Weight (kg)			
Age-class	Sex	*n*	Mean	SD	Min	Max	Mean	SD	Min	Max
Neonate	Female	6	76.0	4.3	71	80	7.2	1.2	6	9
	Male	6	81.7	4.2	75	86	8.3	0.8	7	9
Juvenile	Female	45	112.2	10.9	92	133	19.7	6.2	9	41
	Male	63	109.1	12.0	87	141	18.6	5.5	8	37
	Unknown	3	105.7	15.5	88	117	–	–	–	–
Adult	Female	20	152.1	7.3	140	165	41.8	6.7	31	58
	Male	14	141.1	6.7	131	153	38.4	7.3	28	49

Thirty species of fish and squid were collected during ongoing surveys in the North Sea between 2002 and 2008 by the Centre for Fishery Research CVO (http://www.cvo.wur.nl/). Species, length, fishing locality (latitude/longitude) and date were available for each sample ($n = 624$). Fishing localities were grouped into 11 areas (Table II). White muscle tissue samples were collected, then prepared and analysed for stable isotope analysis in the same way as porpoise samples, except that no lipid extraction was performed. Porpoise and prey samples were stored frozen at -20°C until analysis.

Sample preparation

Lipids are depleted in ^{13}C relative to proteins (DeNiro & Epstein 1978; Lidén et al. 1995). Variation in δ^{13}C among animals thus primarily reflects fat content of tissues due to differences in nutritional status, masking possible underlying differences in prey preferences. Lipids were therefore extracted from samples prior to analysis, both in muscle and bone tissue. Bone samples were also acidified to remove non-dietary carbonates and to extract collagen. Muscle samples were freeze-dried for ca. 20 h and homogenized with a pestle and mortar. Ribs were cleaned with a scalpel and bone marrow was removed. Bone fragments were sonicated in Milli-Q purified water and dried overnight at room temperature. Bone samples were then homogenized with an automatic grinder (Retsch MM301), and demineralized in a weak acid solution (2% HCl) for 20 min or until no more gas bubbles were produced (Ambrose 1990). They were then rinsed with Milli-Q purified water to neutralize and dried at 35°C overnight (Moore et al. 1989). Lipids were extracted from muscle and bone powder in a 2:1 chloroform–methanol solution (Folch et al. 1957). Because pre-treatment may sometimes alter δ^{15}N (Lidén et al. 1995), samples were analysed in twofold: one time before pre-treatment to measure δ^{15}N and a second time after lipid extraction and acidification to measure δ^{13}C values.

Stable isotope analysis

Muscle and bone samples (1.5 mg for muscle and 2 mg for bone tissue) were weighed into tin cups. Stable isotope measurements were performed by isotope-ratio mass spectrometry using a mass spectrometer (V.G. Optima Isoprime, UK) coupled to an N-C-S elemental analyser (Carlo Erba) for automated analyses at the Laboratory for Oceanology, Liège University, in Belgium. Stable isotope abundances are expressed in conventional delta (δ) notation in parts per thousand (‰), and are

Table II. Range of latitude and longitude of the fishing localities of prey samples ($n = 624$).

		Latitude (°)		Longitude (°)		δ^{15}N (‰)		δ^{13}C (‰)	
Area	*n*	Min	Max	Min	Max	Mean	SD	Mean	SD
Central North Sea	32	53.91	56.24	1.41	6.16	13.08	1.78	−20.02	1.31
Dutch Coastal Zone	404	35.50	53.91	4.20	6.91	16.14	1.56	−18.26	1.94
Eemsdelta	18	53.29	53.49	7.16	7.47	18.76	1.13	−17.96	0.80
English Channel	28	50.79	50.79	0.86	0.86	15.14	1.09	−18.45	0.75
Firth of Forth	20	56.32	56.71	−1.44	−0.24	14.09	0.54	−18.31	0.48
German Coastal Zone	30	54.00	54.53	8.11	8.32	16.68	1.14	−18.53	1.23
Northern North Sea	22	57.59	58.12	0.55	1.90	12.17	1.10	−20.08	0.88
North-western Moray Firth	18	57.89	58.21	−3.24	−2.70	11.60	1.36	−20.13	0.96
South-eastern UK Coast	8	51.48	53.11	1.73	1.89	15.63	1.25	−18.57	1.09
Southern Bight	27	52.21	53.21	2.12	3.61	15.76	1.19	−18.01	1.10
Outer Delta	17	51.79	51.79	3.52	3.52	18.84	1.24	−18.46	1.82

expressed relative to the international standards Vienna-PeeDee Belimnite limestone (V-PDB) for ^{13}C measurements and atmospheric nitrogen for ^{15}N measurements according to the following equation: $\delta X = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$ where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ isotopic ratio of the sample and X is ^{13}C or ^{15}N. Reference materials used were: IAEA-N1 ($\delta^{15}N = 0.4 \pm 0.2‰$) (mean $\pm$ SD) and IAEA-C6 ($\delta^{13}C = -10.4 \pm 0.2‰$) (IAEA, Vienna, Austria). Internal standards (glycine) were inserted into all runs at regular intervals to assess drift over time. Standard deviation on repeated measurements on glycine and replicated samples was $\pm 0.1‰$ for carbon and $\pm 0.3‰$ for nitrogen, respectively.

Statistical analysis

Generalized Additive Models (GAMs) were fitted to test for age-related and temporal trends in isotope values and to examine whether variation in isotope values is associated with sex or stranding location. A total of 18 sets of models were fitted using six possible response variables (i.e. bone $\delta^{13}C$, muscle $\delta^{13}C$, bone $\delta^{15}N$, muscle $\delta^{15}N$, $\delta^{13}C_{bone}$–$\delta^{13}C_{muscle}$ expressed as $\Delta^{13}C_{bone-muscle}$ and $\delta^{15}N_{bone}$–$\delta^{15}N_{muscle}$ expressed as $\Delta^{15}N_{bone-muscle}$) and using data from all individuals, excluding outliers. Outliers were identified using the Chauvenet's criterion (Chauvenet 1863). Samples of unknown sex or without length measurements were also removed from the analysis. The explanatory variables included are a smooth function of Length (in cm, acting as proxy for age), Month, Sex (female, male) and whether the individual was found in the Eastern Scheldt (an inshore tidal bay) or along the Dutch coast. The smooth function (with a maximum number of 4 degrees of freedom) enables the estimation of a non-linear relation between the response and the explanatory variable. For Month, a cyclic smoother was used which ensures that the model estimates at the beginning and end are identical. To arrive at the best model, forward model selection based on Akaike's Information Criterion (AIC) was used. The model with the lowest AIC was used, but only if the change in AIC from one to the next was larger than 2 (Burnham & Anderson 2002). Data are presented as mean $\pm$ SD unless stated otherwise. Statistical analysis was carried out in the computing environment R (R 2.92; R Development Core Team 2009).

Results

Porpoises

This study included a total of 157 porpoises stranded in three consecutive years, 36, 50 and 71 animals in 2006, 2007 and 2008, respectively. There were slightly more male ($n = 83$) than female porpoises ($n = 71$), and of three animals the sex was not determined. Most animals were juveniles ($n = 111$), compared to 34 adults and 12 neonates. Length and weight measurements per age-class and sex are given in Table I. In summary, length ranged from 71 to 165 cm (116 cm $\pm$ 22) and weight ranged from 6 to 58 kg (23 kg $\pm$ 11). Porpoises were collected along the entire Dutch coast, including 16 animals that have stranded inside the Eastern Scheldt (Figure 1). Samples were available for each month but two distinct stranding periods can be recognized, comparable with the seasonal pattern of all recorded strandings along the Dutch coast (Figure 2). The first stranding period includes animals stranded in winter and spring (December until May) with a distinct peak of strandings in March. The second

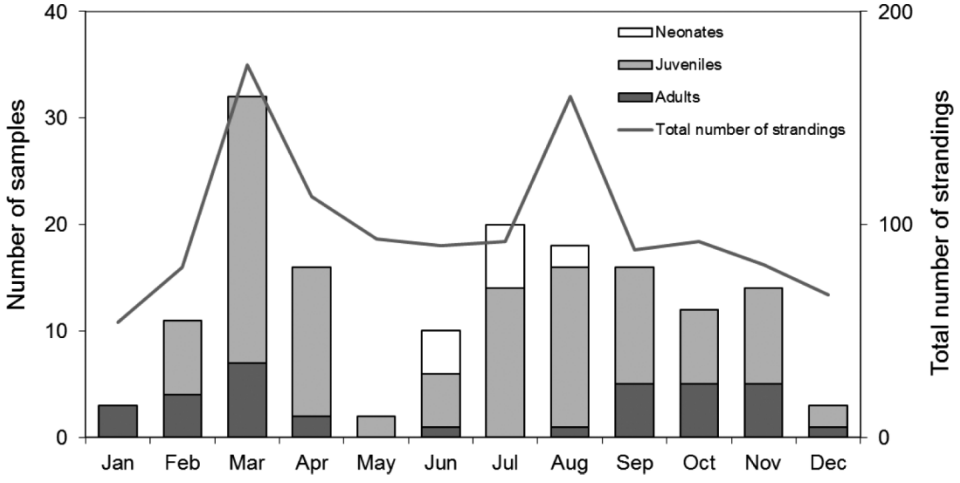

Figure 2. Porpoise (*Phocoena phocoena*) strandings per calendar month ($n = 157$) analysed in this study (2006–2008), separated by age-class. The solid line shows the total number of strandings on the Dutch coast from 2006 to 2008 (www.walvisstrandingen.nl).

Table III. Porpoise (*Phocoena phocoena*) isotopic composition ($\delta^{15}N$ and $\delta^{13}C$) of muscle samples, grouped by age-class and sex with number of samples (*n*), C : N ratio (C : N), mean, standard deviation (SD) and range (Min/Max).

Muscle Age-class	Sex	*n*	C : N	$\delta^{15}N$ (‰)				$\delta^{13}C$ (‰)			
				Mean	SD	Min	Max	Mean	SD	Min	Max
Neonate	Female	3	3.50	17.28	1.76	15.46	18.98	−17.99	0.88	−19.00	−17.46
	Male	3	3.51	18.23	0.91	17.27	19.07	−17.76	0.08	−17.82	−17.68
Juvenile	Female	33	3.34	16.66	1.24	14.18	18.73	−18.19	0.51	−19.31	−17.26
	Male	41	3.38	16.41	1.26	13.92	18.56	−18.29	0.49	−19.69	−17.21
Adult	Female	11	3.38	16.17	1.35	13.68	17.65	−18.11	0.61	−19.09	−16.82
	Male	11	3.29	15.15	1.34	13.45	17.52	−18.32	0.57	−19.21	−17.02

period includes animals stranded in summer and autumn (June until November). In this latter period, samples used in our study were more evenly distributed compared to the total number of recorded strandings along the Dutch coast. Neonates were only found in summer (June until August).

Based on criteria in Kuiken (1996), 21 porpoises were diagnosed as bycatch (13.3%) and another 30 porpoises that were diagnosed as possible or probable bycatch (19.1%), animals that were mostly also suffering from infectious disease and lung oedema. The remaining 104 animals mostly died of emaciation, infectious diseases and lung oedema (66.2%), while two fairly emaciated porpoises were life strandings (1.3%). Most porpoises showed signs of emaciation (75.1%): 19 animals slight, 26 moderate and 73 severe emaciation.

Three outliers were identified in the response data, one in the bone $\delta^{15}N$ values (17.7‰), one in the $\Delta^{13}C_{bone-muscle}$ values (3.3‰) and one in the $\Delta^{15}N_{bone-muscle}$ values (−0.1‰). In- or excluding these outliers did not lead to different models being selected in the forward model selection procedure. The final six models presented here were based on all animals, excluding outliers.

Isotopic composition in muscle

Muscle $\delta^{15}N$ ranged from 13.4 to 19.1‰ (16.4 ± 1.4‰), with $\delta^{13}C$ ranging from −19.7 to −16.8‰ (−18.2 ± 0.5‰) (Table III). GAMs revealed that length and sex explained a significant part of the variation of $\delta^{15}N$ ($R^2 = 0.243$, deviance explained = 26.1%). The $\delta^{15}N$ value of an individual of 80 cm was ca. 2.6‰ lower compared to an individual of 160 cm, and females had on average ca. 0.5‰ higher $\delta^{15}N$ values than males (Figure 3A). GAMs revealed that the area of stranding (Eastern Scheldt versus Dutch coast) explained a significant part of the variation of $\delta^{13}C$ ($R^2 = 0.0733$, deviance explained = 8.25%). Animals stranded along the Eastern Scheldt had on average 0.5‰ higher $\delta^{13}C$ values compared to animals stranded along the Dutch coast (Figure 3B).

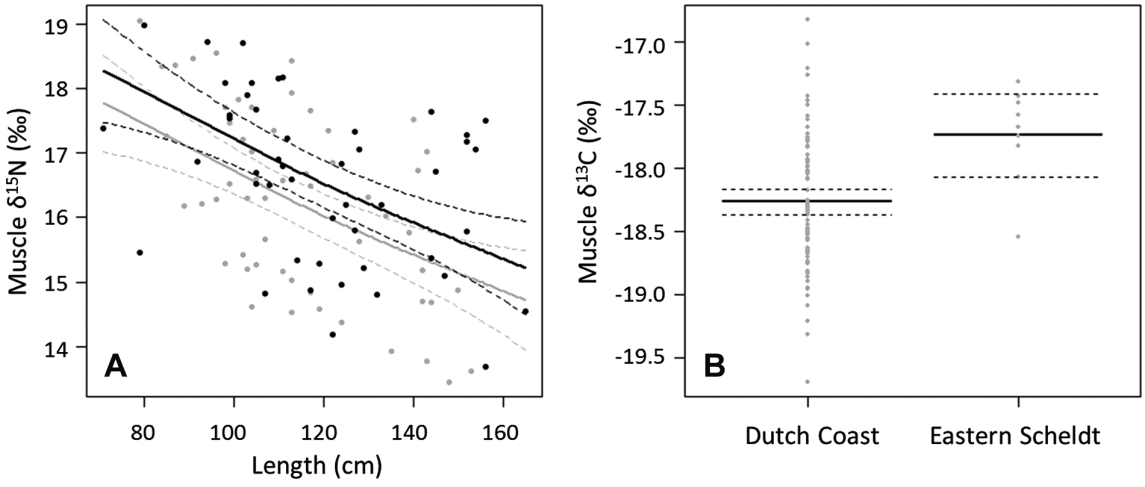

Figure 3. Porpoise (*Phocoena phocoena*) isotopic composition of muscle; effect of length and sex (black = females, grey = males) on $\delta^{15}N$ (A) and the effect of stranding location (Dutch coast versus Eastern Scheldt) on $\delta^{13}C$ (B). Solid line presents the mean model estimate and the dotted lines the 95% confidence intervals.

Table IV. Porpoise (*Phocoena phocoena*) isotopic composition ($\delta^{15}N$ and $\delta^{13}C$) of bone samples, grouped by age-class and sex with number of samples (*n*), C : N ratio (C : N), mean, standard deviation (SD) and range (Min/Max).

Bone Age-class	Sex	*n*	C : N	$\delta^{15}N$ (‰)				$\delta^{13}C$ (‰)			
				Mean	SD	Min	Max	Mean	SD	Min	Max
Neonate	Female	5	3.35	19.10	1.89	15.93	20.69	−15.03	0.89	−16.59	−14.46
	Male	7	3.33	17.93	1.32	16.17	19.77	−15.21	0.62	−16.14	−14.56
Juvenile	Unknown	3	3.68	14.75	1.21	13.77	16.11	−16.46	0.21	−16.68	−16.27
	Female	37	3.48	16.24	1.65	12.97	19.21	−15.58	0.68	−17.31	−14.01
	Male	39	3.44	16.08	1.37	12.31	18.67	−15.47	0.66	−17.05	−14.29
Adult	Female	18	3.56	15.64	1.55	11.33	17.49	−14.85	0.63	−15.77	−13.81
	Male	9	3.47	15.87	1.17	13.69	17.72	−14.77	0.48	−15.68	−14.29

Isotopic composition in bone

Porpoise bone $\delta^{15}N$ ranged from 11.3 to 20.7‰ (16.3‰ ± 1.7), with $\delta^{13}C$ ranging from −17.3 to −13.8‰ (−15.4‰ ± 0.7) (Table IV). GAMs revealed that length and month explained a significant part of the variation of both $\delta^{15}N$ and $\delta^{13}C$ ($R^2 = 0.331$, deviance explained = 35.1% and $R^2 = 0.19$, deviance explained = 22.1%, respectively). The $\delta^{15}N$ value of an individual of 80 cm was on average 3.0‰ higher compared to an individual of 160 cm (Figure 4A). Highest $\delta^{15}N$ values were found in August (Figure 4C). Individuals of 105 cm had the lowest $\delta^{13}C$ values, 0.5‰ lower than an individual of 80 cm and 0.7‰ lower than an individual of 160 cm (Figure 4B). Lowest values for $\delta^{13}C$ were found in April and highest $\delta^{13}C$ values in September (Figure 4D).

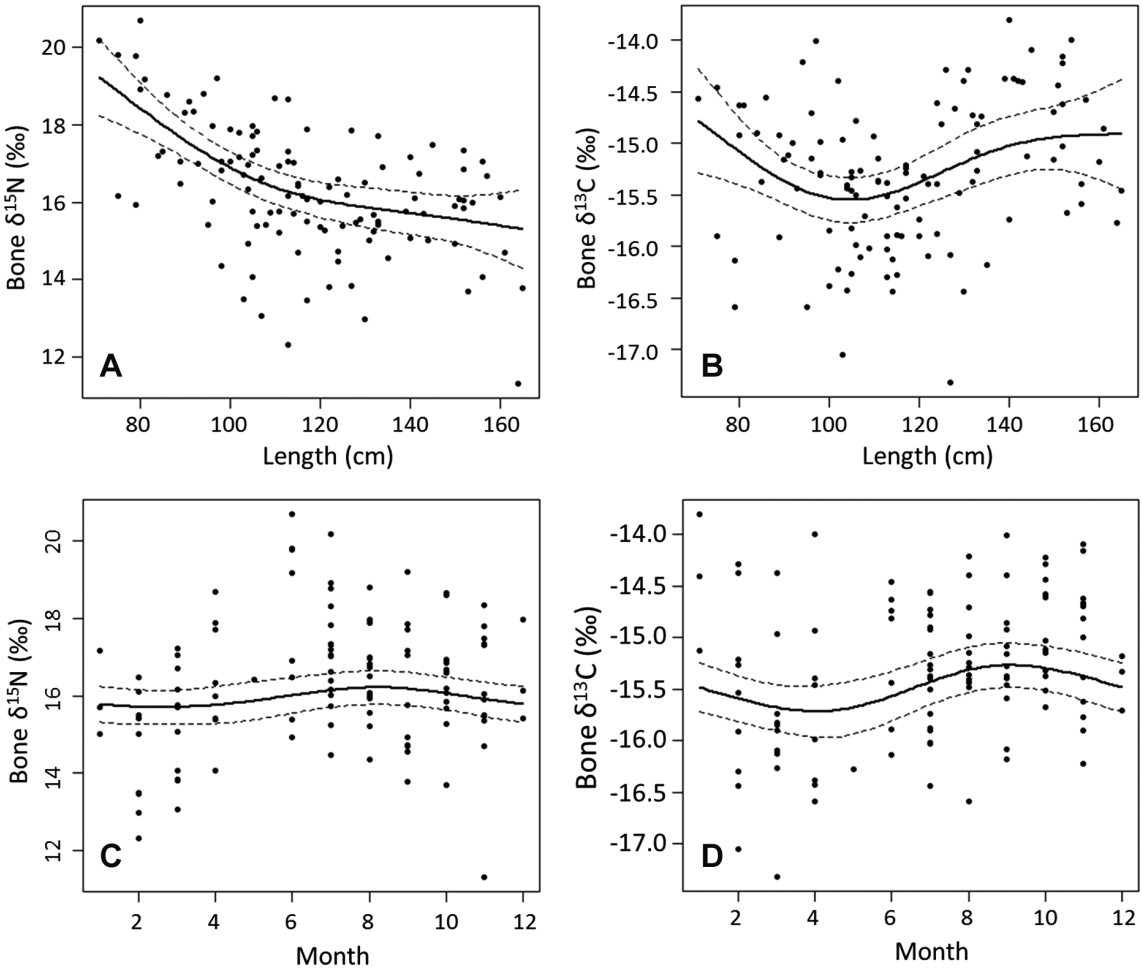

Figure 4. Porpoise (*Phocoena phocoena*) isotopic composition of bone; effect of length on $\delta^{15}N$ (A) and $\delta^{13}C$ (B), effect of month on $\delta^{15}N$ (C) and $\delta^{13}C$ (D). Solid line presents the mean model estimate and the dotted lines the 95% confidence intervals.

Muscle versus bone

Porpoises showed slightly but not significantly lower values ($-0.3‰$, *t*-test, df = 45, $p = 0.062$) in bone $\delta^{15}N$ ($16.1‰ \pm 1.5$) relative to muscle $\delta^{15}N$ ($16.4‰ \pm 1.3$). In contrast, bone $\delta^{13}C$ ($-15.2‰ \pm 0.7$) was significantly higher ($+3.1‰$, *t*-test, df = 45, $p < 0.001$) relative to muscle $\delta^{13}C$ ($-18.3‰ \pm 0.4$). GAMs revealed that length explained a significant part of the variation of $\Delta^{15}N_{bone-muscle}$ and $\Delta^{13}C_{bone-muscle}$ ($R^2 = 0.139$, deviance explained = 17.9% and $R^2 = 0.214$, deviance explained = 22.7%, respectively). Lowest $\Delta^{15}N_{bone-muscle}$ values were found in animals of 105 cm with higher $\Delta^{15}N_{bone-muscle}$ values in both smaller and larger animals (Figure 5A). The $\Delta^{13}C_{bone-muscle}$ of an individual of 80 cm was $1.2‰$ lower compared to an individual of 160 cm (Figure 5B).

Prey samples

This study included a total of 624 prey samples of 30 fish and squid species, collected throughout the North Sea. About two-thirds of all prey samples were collected from the Dutch Coastal Zone (64.7%, $n = 404$), while other locations each accounted for between 1.3% and 5.1% of the samples (Table II). The $\delta^{13}C$ and $\delta^{15}N$ values of each prey species are presented in Table V. Mean $\delta^{15}N$ values of potential prey species ranged from 11.6 to $18.8‰$ ($15.8 \pm 2.4‰$), with mean $\delta^{13}C$ values ranging from -20.1 to -18.0 ($-18.5 \pm 0.8‰$).

Prey samples showed large geographic differences in $\delta^{15}N$ and $\delta^{13}C$ values (Table II). There were three clusters of locations with similar, partly overlapping isotopic composition (Figure 6). The main cluster included locations from the southern North Sea, including the Dutch, German and southeastern UK coasts and the Southern Bight and English Channel. One cluster included the two delta areas, the Outer and Eems Delta, both characterized by relatively high $\delta^{15}N$ values. Another cluster included the northern locations Northwest Moray Firth and the Central North Sea, characterized by comparatively low $\delta^{15}N$ and $\delta^{13}C$ values. The Firth of Forth had somewhat lower $\delta^{15}N$ values compared to the main cluster of locations, comparable to the northern locations but similar $\delta^{13}C$ values as the main cluster. This shows that estuarine areas are characterized by relatively high $\delta^{15}N$ values while coastal areas are characterized by relatively high $\delta^{13}C$ values. Northern and central areas from the North Sea are characterized by relatively low $\delta^{15}N$ and $\delta^{13}C$ values, while the remaining areas fall into one large cluster.

Excluding samples from the northern and central North Sea and the delta areas, top predators such as smelt (*Osmerus eperlanus*), seabass (*Dicentrarchus labrax*), whiting (*Merlangius merlangus*) and cod (*Gadus morhua*) showed the highest $\delta^{15}N$ values, characteristic for their high trophic level, feeding on other fish species. Flounder (*Platichthys flesus*) and long rough dab (*Hippoglossoides platessoides*) also showed relatively high $\delta^{15}N$ values. Although their main food source are small invertebrates, mature specimens have been shown to feed on small fish species (Knijn et al. 1993). In contrast, mackerel (*Scomber scombrus*) showed the lowest $\delta^{15}N$ values, characteristic for the low trophic level of a filter feeder. Common squid (*Alloteuthis subulata*) and dab (*Limanda limanda*) also showed relatively low $\delta^{15}N$ values, as small individuals feed mainly on small crustaceans. Despite feeding on plankton, herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) did not show distinctly low $\delta^{15}N$ values (Knijn et al.

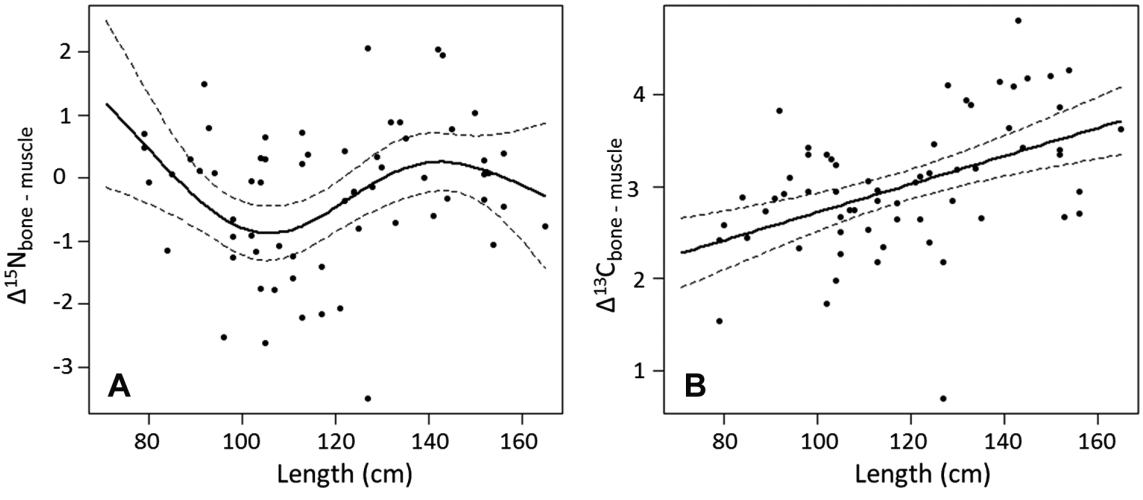

Figure 5. Porpoise (*Phocoena phocoena*) isotopic composition, effect of length on $\Delta\delta^{15}N_{bone-muscle}$ (A) and $\Delta\delta^{13}C_{bone-muscle}$ (B). Solid line presents the mean model estimate and the dotted lines the 95% confidence intervals.

Table V. Isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of prey species collected from the North Sea between 2002 and 2008.

Species		*n*	C : N	$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
				Mean	SD	Mean	SD
Alloteuthis subulata	Common squid	19	3.6	−18.52	0.52	13.85	0.74
Ammodytes tobianus	Lesser sandeel	10	3.2	−18.76	0.80	15.08	1.02
Arnoglossus laterna	Scaldfish	20	3.3	−18.28	0.83	15.98	1.03
Buglossidium luteum	Solenette	20	3.3	−17.93	0.77	16.51	0.59
Callionymus lyra	Dragonet	15	3.1	−19.26	1.32	15.75	0.61
Ciliata mustela	5bearded-rockling	20	3.2	−17.14	0.56	16.96	0.63
Clupea harengus	Herring	20	3.2	−18.59	0.37	15.82	1.18
Dicentrarchus labrax	Seabass	21	3.3	−15.13	1.22	18.18	1.14
Gadus morhua	Cod	33	3.1	−18.75	1.50	15.68	2.54
Gobius niger	Black goby	13	3.3	−18.34	0.60	19.46	0.48
Hippoglossoides platessoides	Long rough dab	19	3.2	−18.70	1.00	14.40	3.06
Hyperoplus lanceolatus	Greater sandeel	20	3.2	−18.07	0.95	15.19	0.63
Limanda limanda	Dab	31	3.3	−18.47	0.65	14.21	1.74
Loligo forbesi	Veined squid	20	3.5	−20.54	0.95	12.46	0.45
Merlangius merlangus	Whiting	30	3.1	−18.19	0.86	16.38	1.60
Osmerus eperlanus	Smelt	20	3.4	−18.50	0.97	18.48	0.28
Platichthys flesus	Flounder	30	3.2	−19.98	3.44	17.20	0.67
Pleuronectes platessa	Plaice	27	3.1	−18.06	0.80	15.53	0.68
Pomatoschistus microps	Common goby	20	3.5	−15.66	0.72	16.73	0.48
Pomatoschistus minutus	Sand goby	20	3.3	−16.61	1.00	16.96	0.41
Scomber scombrus	Mackerel	20	6.4	−22.42	0.63	13.45	1.50
Scophthalamus maximus	Turbot	20	3.6	−17.63	0.74	16.71	0.38
Solea solea	Sole	15	3.2	−17.72	1.12	16.34	1.56
Sprattus sprattus	Sprat	20	4.4	−20.57	0.89	14.96	0.71
Syngnathus acus	Greater pipefish	5	3.3	−18.33	0.83	15.21	0.81
Syngnathus rostellatus	Nilsson's pipefish	21	4.3	−19.05	1.21	15.97	0.51
Trachurus trachurus	Scad	20	3.7	−18.91	1.30	16.18	0.73
Trisopterus esmarkii	Norway pout	20	3.3	−19.68	0.83	11.56	1.34
Trisopterus luscus	Bib	20	3.2	−17.65	0.49	18.02	1.96
Trisopterus minutus	Poor cod	35	3.1	−18.54	0.63	14.58	0.77

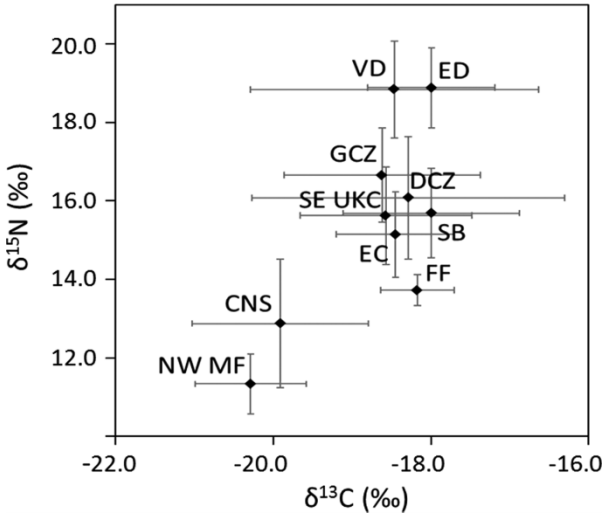

Figure 6. Isotopic composition $\delta^{15}N$ and $\delta^{13}C$ of prey species grouped by fishing localities. VD, Voordelta; ED, Eemsdelta; GCZ, German Coastal Zone; DCZ, Dutch Coastal Zone; SB, Southern Bight; SE UKC, South-eastern UK Coast; EC, English Channel; FF, Firth of Forth; CNS, Central North Sea; NW MF, North-western Moray Firth.

1993; Pierce et al. 2010). This suggests that a coastal distribution is also associated with higher $\delta^{15}N$ values. This is confirmed by other typical coastal species such as gobies (*Pomatoschistus minutus* and *Pomatoschistus microps*) and 5-bearded rockling (*Ciliata mustela*) that show relatively high $\delta^{15}N$ in combination with high $\delta^{13}C$ values, even though they mostly feed on small benthic prey species (Knijn et al. 1993). Flounder showed a large variation in $\delta^{13}C$ values, covering both its winter distribution along the coast and its summer distribution in the brackish waters. Cod and long rough dab showed a large variation in $\delta^{15}N$ values, due to the large size range, covering small individuals feeding mainly on benthic species and large individuals that also feed on small fish (Knijn et al. 1993). The remaining species can all be found throughout the entire North Sea, covering both coastal and deeper waters. They feed mainly on benthic species such as crustaceans, molluscs, polychaetes and sometimes very small fish, resulting in relatively average $\delta^{13}C$ and $\delta^{15}N$ values (Knijn et al. 1993).

Discussion

This study included porpoises stranded in a period of high porpoise abundance and stranding frequency. Samples covered both sexes, all age-classes, and animals stranded along the Dutch coast throughout the year. Individual characteristics (i.e. length, sex, stranding area and month) were accountable for part of the variation in isotopic composition of the animals and gave insight in the trophic level and feeding location.

Age-related effects

Age-related changes in isotopic composition (derived from length; Lockyer 2003) were identified both in muscle and bone tissue. We found that neonatal enrichment for nitrogen values was very distinct from older age-groups. Generally, foetal tissue has the same isotopic composition as the mother's tissue (Richards et al. 2002). After birth, when the young suckles, neonatal enrichment in ^{15}N occurs relative to their mothers' isotopic values as the offspring is theoretically 'feeding' on their mothers tissues (Jenkins et al. 2001; Witt 2001).

In muscle, smaller individuals had higher $\delta^{15}N$ values but similar $\delta^{13}C$ values. Studies based on stomach contents have shown smaller, more benthic, coastal prey in young porpoises (e.g. shrimp, small fish and squid) compared to larger more pelagic, offshore prey in adult porpoises (e.g. gadoids and flatfishes) (Smith & Read 1992; Börjesson et al. 2003; Santos et al. 2004). It is also assumed that young animals start preying on small prey species such as gobies, small flatfishes and shrimp before preying on larger species (Santos & Pierce 2003). Our data confirm that young porpoises stay in coastal waters and feed mainly on small species, i.e. gobies. These prey are small in size, found in high numbers along the Dutch coast and show high $\delta^{15}N$ values. Even though $\delta^{13}C$ values of adult porpoises were similar to those of younger individuals, the lower $\delta^{15}N$ values reflect offshore feeding. We cannot confirm that adult porpoises feed mainly on larger gadoids (e.g. cod and whiting) as the high trophic level of large gadoids is not reflected in porpoise tissues.

A relation between length and $\delta^{13}C$ was less distinct than for nitrogen and found in bone only, presumably caused by differences in trophic enrichment between carbon and nitrogen. We found that muscle showed temporal differences in prey choice faster (Figures 4A and 5A,B), resulting in more individual variation in isotopic composition compared to bone. The gradual decrease in signal acquired during suckling when animals become older, is caused by a faster dilution in muscle compared to bone due to differences in turnover times (Jenkins et al. 2001; Habran et al. 2010). In bone, $\delta^{13}C$ values were high in young individuals and in animals with a length of approximately 135 cm and longer, suggesting that they were feeding more coastally. Higher $\delta^{13}C$ values in young animals can be explained both by neonatal enrichment due to recent suckling as well as differences in the foraging ecology of porpoises of different age-classes based on their diving and hunting experience or feeding location.

Sexual segregation

In muscle, females generally showed slightly higher $\delta^{15}N$ values compared to males, suggesting that they, to some extent, fed on higher trophic level prey. Intersexual differences in nitrogen values were more explicit in adult porpoises and only reflected in muscle but not in bone tissue, indicating sexual segregation at maturity, where females feed at a relatively higher trophic level. This has previously been documented for porpoises (Das et al. 2004a) and for other marine mammal species (Hobson 1999; Lesage et al. 2001). This is confirmed by Smith & Gaskin (1983), who suggest that adult females stay with their young while adult males migrate further offshore, possibly preying on different prey species. Even though previous studies on the diet of porpoises (Aarefjord et al. 1995) suggest that a higher consumption need of lactating females may result in feeding at larger and different prey species, stomach contents analysis on porpoises from Dutch coastal waters has shown that female porpoises had ingested more small gobies compared to males that fed more on larger gadoids (Santos & Pierce 2003). The high $\delta^{15}N$ values of gobies in our study confirm the findings from stomach contents analysis that adult females feed more coastal and on similar prey together with their young.

Seasonal effects

Bone showed slightly higher $\delta^{15}N$ values in porpoises stranded in August, while $\delta^{13}C$ values were lowest in April and highest in September. As bone is considered a long-term integrator and since the effect of length is corrected for by the model, this does not reflect a seasonal effect or merely the occurrence of neonates in summer months, but suggests that animals from each respective period belong to two groups composed of different animals that have used a different habitat during their period of rapid growth. Genetic analyses of porpoises support this hypothesis as they found that porpoises (mainly

males) stranded along the Dutch coast in winter had migrated from neighbouring regions, most probably from British and Danish coastal waters (Andersen et al. 2001). Porpoises stranded along the Dutch coast in summer are considered to be part of a Dutch subpopulation of the southeastern North Sea population (Yurick & Gaskin 1987; Walton 1997).

Porpoises in the Eastern Scheldt

Porpoises stranded within the Eastern Scheldt had distinct (higher) $\delta^{13}C$ values in muscle compared to porpoises stranded along the Dutch coast. The Eastern Scheldt is a tidal bay, created by dams isolating the former estuary from freshwater input of the river Scheldt (Nienhuis & Smaal 1994). Although no baseline isotopic values are available for the Eastern Scheldt, our data confirms that prey from the delta areas differ significantly from the marine system (Clementz & Koch 2001). The distinct isotopic composition of porpoises stranded in the Eastern Scheldt indicates that they have been feeding in the area long enough to integrate this distinct isotopic pattern and that they do not frequently leave the area to forage offshore. It is plausible that movement of marine mammals is limited since the building of the storm-surge barrier. Having entered the Eastern Scheldt, porpoises may stay there for most of the time. As this distinct Eastern Scheldt isotopic signature was not observed in bone tissue, these animals were not born in the Eastern Scheldt but entered the area relative recently.

Shifts in trophic level and feeding location (muscle versus bone)

Muscle $\delta^{15}N$ values were on average only slightly higher compared to bone (0.29‰), while $\delta^{13}C$ values were significantly higher in bone compared to muscle (3.02‰). Differences in isotopic composition between muscle and bone can be due to two factors: tissue-dependent fractionation between diet and tissues, and/or a recent shift in feeding locality before the stranding.

Diet–tissue fractionation rates are well studied for muscle and other tissues (Hobson & Clark 1992a, b; Hobson et al. 1996), but not for bone in marine mammals. In general, $\delta^{15}N$ values are considered very similar among different tissue types. However, $\delta^{13}C$ values are expected to differ between muscle and bone, even in animals on a constant diet. This difference can be estimated around 3‰ as $\Delta^{13}C_{muscle-diet}$ and $\Delta^{13}C_{collagen-diet}$ are approximately 1–2‰ and 4–5‰, respectively (Hedges 2003; Koch 2007). The difference in $\delta^{13}C$ values between muscle and bone found in this study could therefore be explained purely by tissue-dependent fractionation rates.

However, $\Delta^{13}C_{bone-muscle}$ values also showed a relation with length, $\Delta^{13}C_{bone-muscle}$ values were lowest in very young animals and increased with length. This shows that the difference between muscle and bone cannot be generalized and has to be considered age-specific, based on different turnover rates of muscle and bone. Until more information is available on specific turnover rates in tissues of porpoises, it is difficult to unmask a growth or metabolic effect and to expose possible dietary changes later in life.

Individual variation

There was a large amount of unexplained variation by the GAM models, pointing towards a high individual variation in diet. In general, porpoises are considered opportunistic, generalist feeders, relying for their main dietary intake on few species that are easily available in high numbers (Teilmann & Dietz 1998; Christensen & Richardson 2008). Most diet descriptions are based on stomach contents analysis that often fails to account for individual variation. Our study on a population level suggests that porpoises may be considered an opportunistic, generalist feeder but that there is large individual variation. That there may be individual variation in feeding preference has also been suggested by Recchia & Read (1989) and Santos & Pierce (2003).

General assumptions and cautions

Stable isotope analysis is widely used to study the feeding ecology of marine species. Isotopic composition in a predator is subjected to or influenced by three factors: (1) tissue composition and lipid content (e.g. Sotiropoulos et al. 2004; Jacob et al. 2005), (2) tissue turnover rates (Tieszen et al. 1983), and (3) tissue-dependent fractionation (DeNiro & Epstein 1978, 1981). The ability to infer information on trophic level and feeding location of porpoises and possible changes over time depends on the knowledge of the specific influence of these factors on porpoise isotopic composition.

We have extracted lipids prior to analysis to eliminate the influence of differences in nutritional status (varying lipid content of tissues) and to enable the comparison between muscle and bone tissue. Specific turnover rates in porpoise tissues are yet unknown, but can be considered similar to or slightly higher than in other marine mammals, due to their small size (Worthy & Edwards 1990,

Kastelein et al. 1997). Diet–collagen fractionation in marine mammals is still poorly understood. It is therefore difficult to examine whether differences in isotopic composition between muscle and bone is reflecting differences in fractionation, or temporal changes in feeding ecology. The identification of temporal changes in trophic level or feeding location thus remains difficult until specific turnover rates and tissue-dependent fractionation of carbon and nitrogen in porpoises are better understood.

About three-quarters of the porpoises analysed were emaciated, many of them severely. About two-thirds of the animals had died of emaciation, sometimes in combination with infectious diseases and lung oedema. Approximately one-third of the animals were diagnosed as bycatch, either as definite bycatch or possible/probable bycatch. These animals showed no signs of infections or emaciation. With their fast metabolism, porpoises suffer from emaciation relatively fast (Worthy & Edwards 1990; Kastelein et al. 1997), but emaciation is not considered to influence $\delta^{15}N$ and $\delta^{13}C$ values (Das et al. 2004b; Gómez-Campos et al. 2011).

Comparison to other studies

There are two previous studies on the isotopic composition of porpoises in Dutch coastal waters. Das et al. (2003) analysed muscle tissue of 46 porpoises stranded on the French, Belgian and Dutch coasts between 1994 and 2000. The $\delta^{15}N$ values of porpoises from our study were 0.2‰ lower compared to those documented by Das et al. (2003). This shows that porpoises from Dutch and adjacent coastal waters are feeding on similar trophic level prey. The $\delta^{13}C$ values from our study were 1.8‰ lower compared to those documented by Das et al. (2003). This difference is probably due to regional differences in $\delta^{13}C$ baseline values that are reflected in porpoises from France and Belgium, which are included in their study.

Christensen & Richardson (2008) analysed bone tissue of 88 porpoises stranded on the Dutch coast between 1848 and 2002, most animals from 1940 onwards. The $\delta^{15}N$ and $\delta^{13}C$ values of porpoises from our study were approximately 1‰ and 3‰ lower to those reported by Christensen & Richardson (2008) for the period 1978–2002, respectively. This supports that porpoises have gradually been feeding on lower trophic level prey over the last century. They argue that this is due to changes in food web structure with progressively lower trophic level prey available to porpoises. The gradual decrease in $\delta^{13}C$ can only partially be explained by anthropogenic changes in carbon composition in the atmosphere (Cullen et al. 2001), but indicates that the food web structure of the North Sea has also changed over the past century. Similar to our study, both studies (Das et al. 2003; Christensen & Richardson 2008) found sexual segregation in adult porpoises with higher $\delta^{15}N$ in females. In contrast to our study, Das et al. (2003) found that female porpoises had also slightly higher $\delta^{13}C$ values, which could reflect a bias due to different sex ratios of porpoises from the three countries.

Conclusions

We have shown that stable isotope analysis can yield important information on the feeding ecology of harbour porpoises. We have found (1) differences in trophic level and feeding location between animals of different ages, (2) sexual segregation between adult porpoises, and (3) have identified different groups of porpoises that stranded during the summer and winter months. We have also shown that $\delta^{13}C$ values can be used to identify porpoises that have been feeding in the Eastern Scheldt for a longer period of time. We have found no evidence that any of the animals we analysed was born in the Eastern Scheldt, indicating that they have recently entered the Eastern Scheldt. Future stable isotope analysis in bone has the potential to assess whether animals born in the Eastern Scheldt stay there. However, the difference in isotopic composition between muscle and bone cannot be used for determining shifts in porpoise feeding ecology over time until we have better insight into differences in turnover times and isotopic routing of these two tissues.

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