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RESEARCH ARTICLE

Stable isotopes of captive cetaceans (killer whales and bottlenose dolphins)

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SUMMARY

There is currently a great deal of interest in using stable isotope methods to investigate diet, trophic level and migration in wild cetaceans. In order to correctly interpret the results stemming from these methods, it is crucial to understand how diet isotopic values are reflected in consumer tissues. In this study, we investigated patterns of isotopic discrimination between diet and blood constituents of two species of cetaceans (killer whale, *Orcinus orca*, and bottlenose dolphin, *Tursiops truncatus*) fed controlled diets over 308 and 312 days, respectively. Diet discrimination factors (Δ; mean ± s.d.) for plasma were estimated to $\Delta^{13}C=2.3\pm0.6$‰ and $\Delta^{15}N=1.8\pm0.3$‰, respectively, for both species and to $\Delta^{13}C=2.7\pm0.3$‰ and $\Delta^{15}N=0.5\pm0.1$‰ for red blood cells. Delipidation did not have a significant effect on carbon and nitrogen isotopic values of blood constituents, confirming that cetacean blood does not serve as a reservoir of lipids. In contrast, carbon isotopic values were higher in delipidated samples of blubber, liver and muscle from killer whales. The potential for conflict between fisheries and cetaceans has heightened the need for trophic information about these taxa. These results provide the first published stable isotope incorporation data for cetaceans, which are essential if conclusions are to be drawn on issues concerning trophic structures, carbon sources and diet reconstruction.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/4/538/DC1

Key words: captivity, discrimination factor, nitrogen enrichment, *Orcinus orca*, turnover, *Tursiops truncatus*.

INTRODUCTION

Stable isotopes have a broad array of applications in ecology (Kelly, 2000; Crawford et al., 2008), including the investigation of diet composition, the trophic level at which the consumer is feeding, and even habitat use and migratory patterns (e.g. Hobson, 1999; Rubenstein and Hobson, 2004). Nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) stable isotopes are used to denote a species' position within the food web and to trace the origin of trophic resources exploited by the species, respectively. Stable isotope analysis is especially advantageous when investigating the feeding ecology and habitat use of marine mammals, where it is often impossible to directly observe feeding or migratory behaviour (Alves-Stanley and Worthy, 2009; Newsome et al., 2010a), and provides information on assimilated foods (not just ingested foods) as well as time-integrated information (Dalerum and Angerbjörn, 2005). Moreover, the complex spatial, temporal and behavioural variation in trophic systems makes linking foraging behaviour to prey populations difficult (Kelly, 1996). Augmenting traditional dietary information with stable isotope data can improve our understanding of the trophic ecology of marine mammals in many instances. Over the last 10 years, the number of ecological studies using stable isotopes to investigate the diet of marine mammals has grown (reviewed in Newsome et al., 2010a). The methodology has been used to address a broad range of topics, including the foraging ecology and migration patterns of wild populations [e.g. killer whales (Herman et al., 2005; Newsome et al., 2009); dolphins (Knoff et al., 2008; Barros et al., 2010)], exposure to organochlorides and heavy metals [e.g. killer whales (Krahn et al., 2008); dolphins (Borrell et al., 2006)], and historic ecology and paleoecology [e.g. killer whales (Thewissen et al., 1996); dolphins (Walker et al., 1999)].

However, the difficulty in using stable isotope analysis to evaluate resource incorporation is that consumer metabolic processes may discriminate between different isotopes: the isotopic ratio of the consumer may not correspond exactly to the isotopic ratio of the food resource (this difference between the stable isotope composition of an animal's tissue and its diet is the discrimination factor). Moreover, it is imperative to determine the turnover rate (the time it takes for the isotope to be assimilated into the consumer's tissue) of the sampled tissue to assess whether the isotope signature of the tissue represents the most recent diet or the long-term diet. Also, approximately 10 years ago, reviews of the use of stable isotopes in animal ecology predicted explosive growth in this field and called for laboratory experiments to determine among other discrimination factors and turnover rate (Gannes et al., 1997). Their exhortation was heeded and now the field is supported by a stronger collection of experimental studies in all taxa (Caut et al., 2009; Martínez del Rio et al., 2009). However, such studies have been conducted primarily on invertebrates, small laboratory mammals and birds that are amenable to such experiments and it is generally unknown to what extent the results of these studies can be extrapolated to larger wild mammals.

Although the use of stable isotope ratios of carbon and nitrogen in studies of the trophic ecology of marine mammals continues to grow (especially for cetaceans; supplementary material Fig. S1, Table S1), we found only six control studies on marine mammals [pinnipeds (Hobson et al., 1996; Kurle, 2002; Lesage et al., 2002; Zhao et al., 2006) and sirenians (Ames et al., 1996; Alves-Stanley and Worthy, 2009)], but none on cetaceans. Therefore, there is an urgent need to

estimate isotopic discrimination and turnover for these taxa, because studies investigating the feeding ecology of cetaceans, especially those that are declining or are susceptible to the activities of commercial fisheries (DeMaster et al., 2001; Lewison et al., 2004; Ward et al., 2009), will probably continue to increase in the coming years (supplementary material Fig. S1, Table S1). In the present study, we measured diet-tissue discrimination factors ($\Delta^{13}C$, $\Delta^{15}N$) and turnover in whole blood, red blood cells and blood plasma of two species of cetaceans kept in captivity and fed a constant diet for 308 days (killer whales) and 312 days (dolphins) or subjected to a diet shift after 175 days. We estimated the effect of delipidation on blood constituents and different tissues (blubber, liver and muscle) of one killer whale and their discrimination factors relative to diet.

MATERIALS AND METHODS

General

Five killer whales *Orcinus orca* (Linnaeus 1758) [three females (FRE, SHA and WIK) and two males (INO and VAL)] and three bottlenose dolphins *Tursiops truncatus* (Montagu 1821) [two females (ECU and JO) and one male (ECL)] were studied in captivity at Marineland, Antibes, France. All individuals were kept on constant diets for at least 6 months before the beginning of the experiment, when they were assigned to different diet situations (Table 1): (1) three captive dolphins (ECL, ECU and JO) switched on day 175 from diet A to diet B, (2) two captive killer whales (VAL and WIK) switched on day 175 from diet C to diet D, (3) one captive killer whales (INO) ate a constant diet D throughout the experiment and (4) two captive killer whales (FRE and SHA) ate a constant diet E throughout the experiment.

It was very difficult to change the diet of these captive cetaceans, in particular because they participated in shows every day. When the diet was not appropriate, they did not work and/or they could become aggressive with the staff. Moreover, it was essential for the experiment that they should begin the new diet immediately, with no period of adaptation (very difficult for killer whales, in particular). This explains the small difference in diet composition (and isotopic values) between the different treatments. However, dietary proportions were strictly respected on all days and the quantity provided was calculated based on the energy requirements of each individual, taking into consideration its sex, sexual maturity, age and activity expenditure during the shows. Finally, as the basis of such isotopic studies lies in the precise control of diet, each individual had daily a specific bucket and the fish were given individually during and after the show.

Blood samples were obtained from accessible flipper vein using butterfly blood-collection kits and were directly separated into red blood cells (RBC) and plasma components by centrifuge. At the end of experiment we sampled whole blood for all individuals. The killer whale INO was not sufficiently trained in the sampling of blood collection. Therefore, he was only sampled from day 140. Because the killer whale SHA unfortunately died of bacterial infection during the experiment, we sampled liver, muscle, kidney, skin and blubber. Consistency of dietary isotopic composition was investigated by measuring the isotopic signature of whole fish and fish muscle of each species randomly taken from the stock throughout the experiment (two fish per month for herring, capelin and whiting, one fish per month for sprat). All samples were kept at –20°C until analysis.

Sample preparation and isotopic analysis

Fish muscle, whole fish and cetacean tissues (plasma, RBC, whole blood, liver, muscle, kidney, skin and blubber) were freeze-dried and ground to a fine powder. For fish muscle, whole fish, the different SHA tissues (liver, muscle, kidney, skin and blubber) and the last two blood sampling analyses of each different diet for each individual (plasma and RBC: days 154, 175, 291 and 312 for dolphins and days 154, 175, 259 and 308 for killer whales; whole blood: days 291 and 312 for dolphins and days 259 and 308 for killer whales) we compared isotopic values before and after lipid extraction [using the Folch method (Folch et al., 1957)]. Isotopic analyses were performed using a mass spectrometer (Optima, Micromass, Manchester, UK) coupled to a CNS elemental analyser (Carlo Erba, Milan, Italy). Ratios are presented as δ values (‰), expressed relative to the Vienna Peedee Belemnite (VPDB) standard and to atmospheric N_2 for carbon and nitrogen, respectively. Reference materials were IAEA-CH-6 (–10.4‰) and IAEA-N1 (+0.4‰) for $\delta^{13}C$ and $\delta^{15}N$, respectively. One hundred replicate assays of internal laboratory standards indicate measurement maximum errors (s.d.) of ±0.2 and ±0.15‰ for stable carbon and nitrogen isotope measurements, respectively.

Statistical analysis

Isotopic incorporation data were fitted using a Marquardt non-linear fitting routine (NLIN, SAS, Cary, NC, USA) using the following equation:

$$y = a + be^{ct}, \quad (1)$$

where y is δX (^{13}C or ^{15}N), a is the value approached asymptotically [$\delta X_{(\infty)}$], b is the total change in value after the diet switch ($\delta X_{(\infty)}$–$\delta X_{(t)}$), c is the turnover rate and t is the time (days) since the switch. Turnover rate was also expressed in terms of half-life ($t_{1/2}$), the time it takes for the isotopic composition of the tissue to reach a midpoint between the initial and final values:

$$t_{1/2} = (\ln 0.5) / c. \quad (2)$$

When individuals were fed the two constant diets during the two periods of the shift experiment, we made two fitting equations, one for each period (i.e. for killer whales VAL and WIK and bottlenose dolphins ECL, ECU and JO).

Discrimination factors between a food resource (W) and a consumer (Y) are described in terms of the difference in delta (δ) values using the Δ notation, where $\Delta=\delta Y-\delta W$. For individuals that were fed the shift diet, we did not calculate Δ when the fitting equation did not significantly converge.

We performed factorial ANOVAs to test the effect of delipidation on isotopic and C/N ratios of different cetacean tissues (plasma, RBC and whole blood) and fish tissues (muscle and whole body). The statistics were performed using STATISTICA 6.0 (StatSoft Inc. 2001, Tulsa, OK, USA). The level of significance for the statistical analysis was P=0.05.

The small sample size prevented statistical comparison of the isotopic values. However, this is the first isotopic analysis of cetaceans in captivity and it is very difficult to obtain access to more individuals. For example, the five killer whales used in the present study represent 12% of the total world population in captivity (actually 42 individuals, see http://www.orcahome.de/orcastat.htm).

RESULTS

Whole fish and muscle had different isotopic signatures. Whole fish were depleted in ^{13}C and ^{15}N compared with muscle tissue (Table 1A). The difference between whole specimens and muscle was species dependent: whiting and capelin differed in $\delta^{13}C$ and herring and sprat differed in $\delta^{15}N$. Isotopic values of lipid-extracted tissues also showed significantly different $\delta^{13}C$ but not $\delta^{15}N$ values for whole fish and muscle (except for whole whiting, which had

the lowest C/N ratios, ~3; Table 1A). Moreover, we observed a significant difference in C/N ratio after lipid extraction (with values of ~3), confirming its success (Table 1A). Finally, we calculated diet isotopic values using the lipid-extracted $\delta^{13}C$ values and non-lipid-extracted $\delta^{15}N$ values (Table 1B). In the following analysis (especially with regards to the discrimination factor), we used the diet isotopic values of whole fish because they better represent the prey as it is consumed in nature by cetaceans and because, after delipidation, there was no significant difference in the values between whole fish and muscle (Table 1A).

$\delta^{15}N$ and $\delta^{13}C$ in dolphin plasma significantly fitted the exponential model for diet A ($F_{2,37}$=155.30, P<0.001 and

Table 1. Mean $\delta^{13}C$, $\delta^{15}N$ and C/N ratio values (±s.d.) for the (A) constituents (whole fish or muscle, delipidated or not) and (B) composition of cetacean diets (with no delipidated constituents for $\delta^{15}N$) over time (*t*, days)

	N	$\delta^{13}C$ (‰)	*F*	*P*	$\delta^{15}N$ (‰)	*F*	*P*	C/N	*F*	*P*
A. Fish constituents										
Herring (*Clupea harengus*)										
Muscle	23	–20.4±0.2	0.98	0.332	12.8±0.1	25.38	**<0.001**	4.8±0.2	0.57	0.459
Whole fish	23	–20.7±0.2			11.7±0.2			5.1±0.2		
Delipidated muscle	23	–18.7±0.1	54.28	**<0.001**	13.0±0.1	2.93	0.101	3.3±0.1	41.93	**<0.001**
Delipidated whole fish	23	–18.6±0.2	61.26	**<0.001**	11.8±0.2	0.29	0.597	3.1±0.1	58.18	**<0.001**
			0.07	0.795		38.59	**<0.001**		2.62	0.120
Capelin (*Mallotus villosus*)										
Muscle	22	–21.7±0.1	59.27	**<0.001**	11.4±0.1	0.62	0.439	3.8±0.2	28.23	**<0.001**
Whole fish	22	–23.3±0.2			11.6±0.2			5.5±0.3		
Delipidated muscle	22	–20.9±0.2	13.47	**0.001**	11.6±0.2	0.58	0.454	3.2±0.0	9.95	**0.005**
Delipidated whole fish	22	–21.2±0.1	115.59	**<0.001**	11.6±0.2	0.00	0.992	3.3±0.0	59.25	**<0.001**
			1.44	0.243		0.01	0.935		7.27	0.013
Sprat (*Sprattus sprattus*)										
Muscle	6	–20.8±0.3	2.87	0.129	12.5±0.1	8.30	**0.021**	6.2±0.4	8.77	**0.018**
Whole fish	6	–21.9±0.6			11.9±0.2			7.6±0.4		
Delipidated muscle	6	–18.1±0.1	99.16	**<0.001**	12.7±0.1	1.11	0.324	3.3±0.1	64.97	**<0.001**
Delipidated whole fish	6	–18.2±0.1	36.33	**<0.001**	12.1±0.1	1.08	0.330	3.3±0.1	152.69	**<0.001**
			0.03	0.858		11.30	**0.010**		0.09	0.770
Whiting (*Merlangius merlangus*)										
Muscle	19	–20.2±0.1	10.66	**0.004**	11.4±0.1	0.49	0.494	3.1±0.1	20.20	**<0.001**
Whole fish	19	–21.2±0.3			11.3±0.2			3.9±0.2		
Delipidated muscle	19	–20.1±0.2	0.05	0.820	11.5±0.2	0.07	0.793	3.1±0.0	4.49	**0.048**
Delipidated whole fish	19	–20.3±0.2	8.27	**0.010**	11.3±0.2	0.05	0.818	3.2±0.0	14.63	**0.001**
			0.25	0.622		0.38	0.544		8.20	**0.010**

		$\delta^{13}C$			$\delta^{15}N$		
	t (d)	Whole fish	Muscle	Delipidated whole fish	Delipidated muscle	Whole fish	Muscle
B. Diet composition							
Dolphin (ECL, ECU, JO*)							
A: 30% herring + 70% capelin	0–175	–22.5	–21.3	–20.4	–20.2	11.6	11.8
B: 30% herring + 70% sprat	175–312	–21.5	–20.6	–18.3	–18.3	11.8	12.6
Killer whale (WIK, VAL)							
C: 60% herring + 40% capelin	0–175	–21.7	–20.9	–19.6	–19.6	11.7	12.2
D: 70% herring + 30% whiting	175–308	–20.9	–20.3	–19.1	–19.1	11.6	12.4
Killer whale (INO)							
D: 70% herring + 30% whiting	0–308	–20.9	–20.3	–19.1	–19.1	11.6	12.4
Killer whale (FRE, SHA)							
E: 82% herring + 18% whiting	0–308	–20.8	–20.4	–18.9	–19.0	11.6	12.5

Significant values (P<0.05) are in bold. For $\delta^{13}C$ and $\delta^{15}N$, values were determined using ANOVAs between fish muscle and whole fish (for muscle), fish muscle and lipid-extracted muscle (for whole fish), whole fish and lipid-extracted muscle (for delipidated muscle) and lipid-extracted fish muscle and lipied-extracted whole fish (for delipidated whole fish).

Diets before experiments consisted of 50% herring + 25% capelin + 25% sprat for the dolphins, 60% herring + 20% capelin + 20% whiting for killer whales WIK and VAL, 70% herring + 30% whiting for INO and 80% herring + 20% whiting for FRE and SHA.

*JO is the dolphin star of the movie “The big blue” (1988, director Luc Besson).

Table 2. Exponential equations (with R^2) and half-life time ($t_{1/2}$) in days for converging models for bottlenose dolphins ECL, ECU and JO and killer whales VAL and WIK

Tissue		Carbon			Nitrogen		
Animal ID	Diet	Equation	R^2	$t_{1/2}$	Equation	R^2	$t_{1/2}$
Plasma							
ECL, ECU and JO	A	y=–19.63+1.82e–0.0152x	0.85	45.6	y=14.02+1.14e–0.0351x	0.90	19.7
	B	y=–17.90–1.67e–0.0554x	0.90	12.5	y=14.73–0.75e–0.0344x	0.79	20.1
VAL and WIK	C	y=–19.42+1.25e–0.0115x	0.73	60.3	y=13.83+0.27e–0.0166x	0.75	41.8
	D	y=–18.14–1.43e–0.0515x	0.70	13.5	–	–	–
RBC							
ECL, ECU and JO	A	–	–	–	y=12.26+0.98e–0.0055x	0.78	126.7
	B	y=–17.60–1.73e–0.0252x	0.86	27.5	–	–	–

We only calculated and present the half-life and equation when the exponential and NLIN models significantly converged.

$F_{2,37}$=97.52, P<0.001, respectively) and diet B ($F_{2,25}$=43.62, P<0.001 and $F_{2,25}$=110.26, P<0.001, respectively; Table 2, Fig. 1). Nitrogen and carbon half-lives ranged from 19.7 to 20.1 and from 12.5 to 45.6 days, respectively (Table 2). For RBC, the exponential model was significant for δ^{15}N in diet A ($F_{2,38}$=65.04, P<0.001) and δ^{13}C in diet B ($F_{2,25}$=70.94, P<0.001), with half-lives of 126.7 and 27.5 days, respectively.

δ^{15}N and δ^{13}C of killer whale plasma significantly fitted the exponential model for diet C ($F_{2,23}$=31.05, P<0.001; $F_{2,23}$=28.23, P<0.001, respectively), but only δ^{13}C for diet D ($F_{2,16}$=16.63, P<0.001; Table 2, Fig. 1). Indeed, plasma δ^{15}N was fitted and converged for diet D, but was not significant ($F_{2,16}$=2.52, P=0.116), even if the final isotopic values were similar to those for INO (considered at equilibrium in diet D; Fig. 1). The mean nitrogen half-

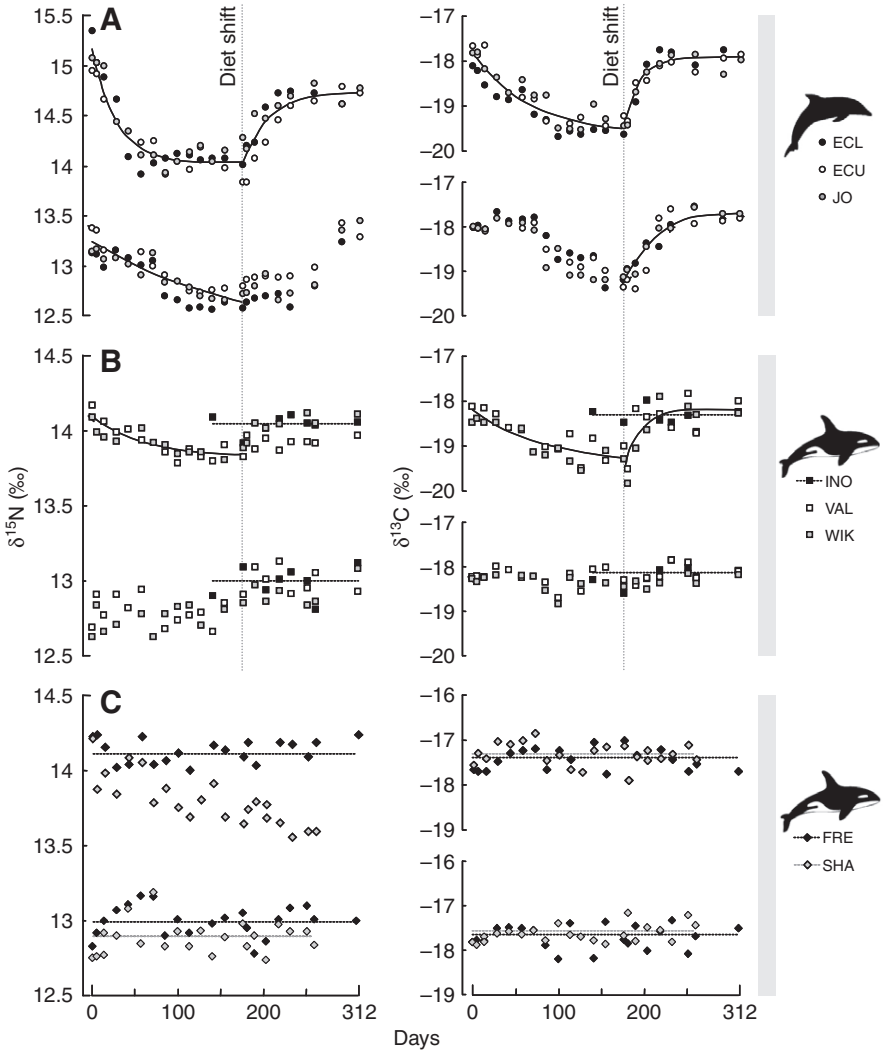

Fig. 1. Individual nitrogen and carbon isotopic values of plasma (top) and red blood cells (bottom) for: (a) three captive dolphins (ECL, ECU and JO) switched on day 175 from diet A to diet B; (b) two captive killer whales (VAL and WIK) switched on day 175 from diet C to diet D, and one captive killer whale (INO) that ate a constant diet D throughout the experiment; and (c) two captive killer whales (FRE and SHA) that ate a constant diet E throughout the experiment. For dolphin (ECL, ECU and JO) and killer whale groups (VAL and WIK), exponential fits are only shown when significant. For individuals that ate a constant diet, we present the mean isotopic values (dotted lines) (except for nitrogen plasma values for SHA).

Table 3. Carbon and nitrogen discrimination factors (Δ, ‰) in different tissues (calculated with whole fish, delipidated for $\delta^{13}C$ and not delipidated for $\delta^{15}N$) of the bottlenose dolphins ECL, ECU and JO and the killer whales VAL, WIK, INO, FRE and SHA

		Carbon			Nitrogen		
Animal ID	Diet	ΔPlasma	ΔRBC	ΔWhole blood	ΔPlasma	ΔRBC	ΔWhole blood
ECL-ECU-JO	A	0.8	–	–	2.4	0.7	–
	B	0.4	0.7	0.1	2.9	–	1.7
	Mean	0.6			2.6	–	
VAL-WIK	C	0.3	–	–	2.0	–	1.4
	D	0.5	–	0.8	–	–	–
INO	D	0.8	1.0	1.1	2.5	1.4	1.5
FRE	E	1.5	1.3	1.5	2.5	1.4	1.6
SHA	E	1.6	1.3	0.8	–	1.4	–
	Mean	1.0	1.2	1.1	2.3	1.4	1.5

For individuals with a diet shift, we only calculated discrimination factors when the exponential and NLIN models significantly converged (indicated with a '–', see Table 2). For INO, FRE and SHA, we estimated Δ using the means of isotopic values during the experiment.

life was 41.8 days whereas the carbon half-life was very variable, ranging from 13.5 to 60.3 days (Table 2), probably due to the low carbon isotopic diet shift (Table 1).

All cetaceans had plasma, RBC and whole blood enriched in ^{15}N and ^{13}C relative to their diet (Table 3). $\Delta^{15}N$ was more similar between diet and species than between tissues. Mean $\Delta^{15}N$ values in dolphins were 2.0‰ for plasma and 0.7‰ for RBC, and in killer whales were 2.3‰ for plasma and 1.4‰ for RBC (Table 3). However, $\Delta^{13}C$ was more similar between species and tissues than between diets. Mean $\Delta^{13}C$ values in dolphins were 0.6‰ for plasma and 0.7‰ for RBC whereas the values in killer whales were 1.0‰ for plasma and 1.2‰ for RBC (Table 3). Logically, mean $\Delta^{15}N$ and $\Delta^{13}C$ values of whole blood were between plasma and RBC values (Table 3). Whales kept on constant diets throughout the experiment showed remarkably consistent isotope values. In the case of SHA, nitrogen values of plasma were very variable, probably due to a nutritional stress and lower consumption of fish during the end of its life. No significant effect of delipidation was found on carbon and nitrogen isotopic values between blood tissues (delipidated: $F_{1,117}$=0.32, P=0.481 and $F_{1,117}$=0.29, P=0.181; delipidated × tissue, $F_{2,117}$=0.55, P=0.581 and $F_{2,117}$=0.70, P=0.521, for carbon and nitrogen, respectively; Fig. 2). However, for tissues other than blood (SHA), an important delipidation effect appears for $\Delta^{13}C$, but not for $\Delta^{15}N$ (Table 4).

DISCUSSION

In the absence of estimates for cetaceans, authors have generally assumed a nitrogen isotopic discrimination factor of ~3‰ and a carbon isotopic discrimination factor of ~1‰, probably based on the information available for other marine taxa (e.g. muscle, liver, skin and blubber) (Das et al., 2000; Bode et al., 2003; Hermann et al., 2005; Lusseau and Wing, 2006). With respect to stable nitrogen enrichment, our results were lower than the general range of 2 to 5‰ that has commonly been reported for endothermic taxa (Vanderklift and Ponsard, 2003; Caut et al., 2009). Specifically, $\Delta^{15}N$ values for RBC and plasma were in the same range as those reported for harbour, grey and harp seals (1.1 to 2.2‰ for RBC, 2.3 to 3.8‰ for plasma) (Hobson et al., 1996; Lesage et al., 2002), but all values were much lower than those reported for northern fur seals (4.1‰ for RBC and 5.2‰ for plasma) (Kurle, 2002). $\Delta^{13}C$ values in RBC and plasma were in the same range as all previously published data for marine mammals (0.9 to 1.9‰ for RBC; 0.4 to 1.3‰ for plasma) (Hobson et al., 1996; Kurle, 2002; Lesage et al., 2002). Furthermore, the difference observed between the $\delta^{15}N$ values obtained from plasma and RBC samples in this study corresponds to what has been found in other species of marine mammals (Kurle, 2002; Lesage et al., 2002). Kurle (Kurle, 2002) attributed this difference to differences in the primary proteins and subsequent amino acids present in the different blood components, because $\delta^{13}C$ and $\delta^{15}N$ values of individual amino

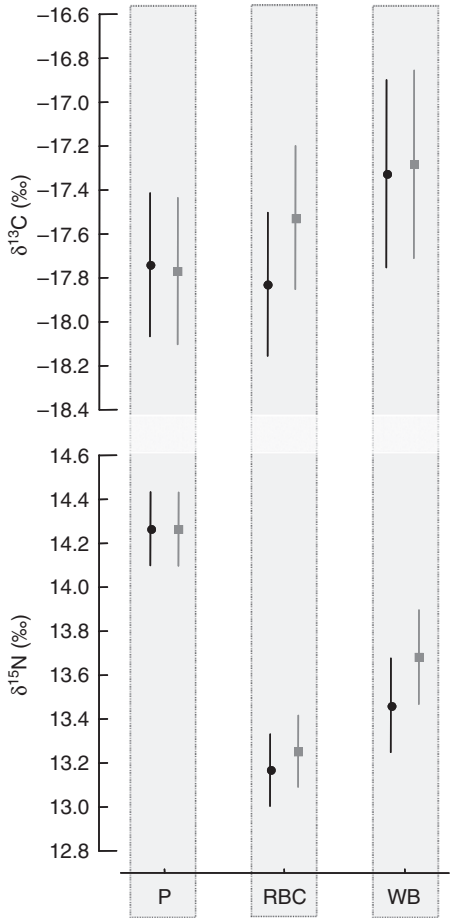

Fig. 2. Carbon and nitrogen isotopic values of blood tissues [plasma (P), N=24; red blood cells (RBC), N=24; and whole blood (WB), N=14], delipidated (grey) or non-delipidated (black), for the last two sampling dates of each diet for each individual (dolphins and killer whales). No significant effect of delipidation was found on carbon and nitrogen isotopic values for any of the blood tissues.

Table 4. Discrimination factors (Δ, ‰, calculated with whole fish, delipidated for $\delta^{13}C$ and not delipidated for $\delta^{15}N$) and C/N ratio of different killer whale (SHA) tissues, before (Δ, C/N) and after delipidation (Δdel, C/Ndel)

			Carbon			Nitrogen		
Tissue (SHA)	C/N	C/Ndel	Δ	Δdel	Δ–Δdel	Δ	Δdel	Δ–Δdel
Muscle	3.40	3.19	0.34	1.26	–0.92	1.21	1.23	–0.02
Liver	4.75	3.46	–1.82	1.27	–3.09	2.92	2.78	0.14
Skin	3.64	2.93	0.09	2.43	–2.34	3.05	3.18	–0.13
Blubber	9.69	3.06	–5.39	2.54	–7.93	3.34	3.15	0.19

acids range widely (Fantle et al., 1999) and the most abundant plasma protein is serum albumin, whereas it is hemoglobin in RBC. However, Kurle (Kurle, 2002) and Lesage et al. (Lesage et al., 2002) also found that the $\delta^{13}C$ values of serum and plasma were significantly lower than those of RBC. Although they attributed these differences in part to the variability in amino acids, they ascribed a greater importance to the differences in the amount of lipid present in each blood component (unfortunately these authors did not consider the delipidated blood component in their comparison, see below).

Indeed, a current practice when analysing $\delta^{13}C$ values of marine mammals is to extract the ^{13}C-depleted lipids because they may greatly influence $\delta^{13}C$ values and bias interpretations in tissues serving as reservoirs of lipids. However, although it is common to extract the ^{13}C-depleted lipids from the muscle and blubber of marine mammals, this has generally not been done for RBC, serum or plasma (e.g. Hilderbrand et al., 1996; Hobson et al., 1996; Kurle, 2002; Lesage et al., 2002). Values between pure lipids and proteins differ by 6‰ in ^{13}C, and the $\delta^{13}C$ value of a tissue containing 15% lipids, such as the serum of harbour seals (Lesage et al., 2002), could bias interpretations. In this study, delipidation had no significant effect on $\delta^{13}C$ and $\delta^{15}N$ values in whole blood, RBC or plasma (Fig. 2). These results stand in opposition to the predictions of Kurle (Kurle, 2002) and Lesage et al. (Lesage et al., 2002) and show that cetacean plasma contains little serum albumin, which is a major carrier of fatty acids in the blood (Lehninger, 1982). In contrast, $\delta^{13}C$ values were higher in the delipidated samples of blubber, liver and muscle (0.92–7.93‰; Table 4). Our results confirm the strong relationship between the C/N ratio and lipid content and the recommendation of Post et al. (Post et al., 2007) for aquatic animals, i.e. to extract lipid or normalize values if C/N>3.5 [see Table 4; C/N ratios were greater than those after delipidation (C/N_{del}) and the difference between the discrimination factor Δ and the discrimination factor after delipidation ($\Delta–\Delta_{del}$) was greater than 0]. For example, for marine mammals, Newsome et al. (Newsome et al., 2010a) has advised using delipidated values only for protein-rich tissues that are known to contain considerable amounts of lipids (e.g. skin, muscle, plasma) and not for whole blood, RBC or metabolically inert tissues (e.g. fur and dentin).

Although the majority of marine mammal studies routinely delipidate consumer tissues, the choice to delipidate the prey tissues as well remains highly arbitrary. Our results show significant differences in the carbon isotopic values of delipidated *versus* undelipidated prey as well as in the discrimination factors. Therefore, it is important to pay attention to this issue when comparing controlled diets with what is found in nature [e.g. diet reconstruction or trophic level (Zhao et al., 2006)]. In our analysis, we considered the impact of different value combinations, delipidated *versus* undelipidated consumer and prey, on our results. Indeed, it is ideal to perform isotopic analyses of delipidated *versus* undelipidated subsamples from individual prey samples when possible (Newsome et al., 2010a). Furthermore, recent work on California sea otters (Newsome et al., 2010b) suggests that delipidated prey samples are inappropriate when keratinaceous tissues are used to examine the diet of consumers feeding upon lipid-rich foods, such as many marine mammals and seabirds.

Another important point to consider when investigating dietary ecology in this system is the choice of prey tissue. A prey's isotopic signature can be defined either as the value for its whole body or its muscle tissue alone (Hobson et al., 1996; Kurle, 2002; Lesage et al., 2002). However, care should be taken in this choice because $\delta^{13}C$ and $\delta^{15}N$ values can differ between individual tissues and the whole body they comprise (see Caut et al., 2009). Indeed, in the different species of fish investigated here, we found consistent differences between the signatures of whole specimens and their corresponding muscle tissue: muscle tissue was enriched in ^{13}C relative to the whole fish. Such differences may result from differential protein turnover in the whole body *versus* in fish skeletal muscle and from differential metabolic routing of dietary elements to various tissues characterized by different macromolecules, including proteins (Cherel et al., 2005). In this context, the use of delipidation could serve to minimize the problem of tissue choice. Indeed, after delipidation, our results showed no significant differences between muscle tissue and whole body in any of the fish species considered. Nonetheless, from a biological point of view, it is better to use data from whole specimens, as dolphins and killer whales swallow their prey whole. Alternatively, for carnivorous species feeding upon flesh only, it would be better to use the isotopic signature of the skeletal muscle. Thus, predator feeding ecology must be taken into account when reconstructing diets (Cherel et al., 2005).

By applying enrichment factors found in this study, it is possible to achieve a better estimate of both the trophic level at which wild cetaceans are feeding and the type of habitat (i.e. nearshore *versus* offshore, benthic *versus* higher in the water column) in which they are foraging. In view of the lack of data for comparison, we recommend that future studies extract (and not) lipids from all blood components to determine how lipid content in the blood influences stable isotope ratios.

The estimation of the isotope turnover rate from different tissues provides an important and minimally invasive tool to interpret temporal variation in foraging ecology [e.g. carbon isotopic values for migration (Rau et al., 1982)] of wild animals. There are very few studies on nitrogen turnover in other species and none in marine mammals. We have shown that plasma provides information about the last 3 months (~time to equilibrium) and RBC provide long-term information (>175 days). Our cetacean nitrogen half-lives (19.9 to 41.8 days for plasma and >175d for RBC) were longer than those of homologous tissues in other terrestrial vertebrate species, such as bears (4 and 28 days, in plasma and RBC, respectively) (Hilderbrand et al., 1996) and birds (<1.8 and <15 days for plasma and whole blood, respectively) (e.g. Bearhop et al., 2002; Hobson and Bairlein, 2003; Pearson et al., 2003; Evans Ogden et al., 2004) but closer to those of a freshwater turtle *Trachemys scripta* (35.6 days in plasma) (Seminoff et al., 2007). This observed contrast in the rate of isotopic incorporation between species is likely to correspond

to differences in tissue growth, catabolic turnover or body mass (see Martínez del Rio et al., 2009). Additionally, the observed differences in the incorporation rates between dolphins and killer whales are likely to be driven by both species contrasting body mass (200 kg for dolphins and ~2000 kg for killer whales). For example, Carleton and Martínez del Rio (Carleton and Martínez del Rio, 2005) have shown that the fractional rate of ^{13}C isotopic incorporation into the RBC in several bird species declined with decreasing body mass to approximately one-quarter of the original rate. Thus, our results for dolphins were probably higher because they have quicker incorporation/turnover rates than killer whales. The combined effect of body size and growth on incorporation rate may accentuate this effect in large animals, such as marine mammals. Field experiments that prove the effects and consequences of these factors on isotopic incorporation are needed in order for ecologists to be able to interpret the results of isotopic patterns (Martínez del Rio et al., 2009).

However, the high degree of variability in the half-lives we measured as well as the non-convergence of some models in killer whales could be explained by the low isotopic shifts between diets, which were very close to measurement errors. Indeed, the difficulty of controlling the diet of these species, given the constraint of their performances, enormously limited potential diets (see 'Materials and methods'). Our results on the half-life of dolphins were more solid and can be applied to killer whales or other cetaceans. The particular case of SHA confirmed the results of time integration in blood; when SHA refused to eat properly (~200 days), the $\delta^{15}N$ values of plasma decreased rapidly whereas $\delta^{15}N$ values in RBC remained constant. The effect of nutritional restriction could depend on species-specific differences in physiological response (Williams et al., 2007) or the level of nutritional stress (Kempster et al., 2007).

The potential for conflict between fisheries and cetaceans has heightened the urgent need for trophic and migration information about the animals within those ecosystems. Nonetheless, proper utilization of this tool requires a fuller understanding of the processes of carbon and nitrogen enrichment. Although controlled-feeding studies of large taxa are often difficult and expensive, they offer our best hope of understanding these processes. Our understanding of these mechanisms has progressed tremendously over the last decade (Gannes et al., 1997), but natural patterns of isotopic variability and the factors influencing discrimination are still not well understood. Our experiment offers the first time-integrated information and baseline values of $\Delta^{15}N$ and $\Delta^{13}C$ for cetaceans and will be very valuable for future studies on endangered species and species in conflict with humans (e.g. food fishes). Using C and N isotopic analysis, we determined, with a non-invasive method, how tissues of cetaceans can provide consistent information on the trophic structures of cetacean species. Although we are aware that blood is still rarely sampled in the wild, we encourage its collection from stranded individuals or fishery bycatch. We hope that new techniques will be developed to sample blood using hollow-tipped syringes or bullets fired by a crossbow, as is currently done for skin and blubber. Indeed, blubber and skin tissues would seem better, but their collection is difficult to achieve on animals in captivity, especially to study the equilibrium value of isotopic tissue, which then requires a dynamic sampling of tissue (i.e. at different times). So many samples from the same individual may cause ethical problems and infection, requires training and may result in public presentation of marked individuals. However, the discrimination factors from the killer whale SHA represent a first opportunity to have estimation of blubber, muscle and skin tissue (e.g. $\delta^{13}C$ and $\delta^{15}N$; Table 4) for cetaceans during a long-term constant diet. Published diet-tissue discrimination values vary widely for carbon and nitrogen (reviewed in Caut et al., 2009), and the effect of selecting one value over another, or of the natural variability of these values, on conclusions concerning trophic structures, carbon sources or diet reconstruction should be investigated further.

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