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ORIGINAL PAPER

Lipid effects on isotopic values in bottlenose dolphins (*Tursiops truncatus*) and their prey with implications for diet assessment

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Abstract Lipid content may affect $\delta^{13}C$ and $\delta^{15}N$ values, thus potentially leading to misinterpretation of isotopic compositions. Solutions to this problem may comprise a priori chemical extraction of lipids or a posteriori arithmetic corrections based on the use of the C:N ratio as a surrogate for lipid content (the so-called lipid normalization equations, LNE). We explored the suitability of LNE vs. chemical extractions to account for the effects of lipids in bulk samples of bottlenose dolphins and their prey, as well as their implications in mass-balance mixing model outputs. Chemical extraction of lipids only affects fish $\delta^{13}C$ values, with greater isotopic differences between bulk and delipidated tissues than analytical errors. Based on a modeled isotopic scenario, we further demonstrated that the most accurate dietary reconstructions were those obtained when using both species-specific (<0.14% deviation from modeled diet) and general LNE for dietary endpoints (<6.5%). In contrast, deviations of up to 60% from the modeled diet were observed when considering the isotopic composition of the bulk diet, especially when the whole fish was used as a dietary endpoint. To reduce time, work load and economic costs, we recommend the use of species-specific LNE to normalize the isotopic composition of diet prior to dietary quantifications when feasible or a general LNE when dealing with generalist predators consuming a high number of prey species.

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Introduction

Stable isotope analyses of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) are widely used as useful tools to investigate food web structure and functioning (Crawford et al. 2008; Layman et al. 2012; Perkins et al. 2014). The principle underlying stable isotope approaches is that the ratio between the heavier and the lighter isotope (e.g., $^{13}C/^{12}C$, commonly expressed in the standard δ-notation, i.e., $\delta^{13}C$) is transmitted in a predictable manner throughout food webs. $\delta^{13}C$ values typically reflect the photosynthetic pathway used by primary producers and is generally used as a suitable indicator of primary sources. In contrast, $\delta^{15}N$ values increase with each trophic level, and are thus used as indicators of trophic positions and trophic interactions (Crawford et al. 2008; Inger and Bearhop 2008; Layman et al. 2012).

An important issue in stable isotope analysis and its interpretation is the potential effect of lipids on the isotopic values obtained. Animal lipids have lower $\delta^{13}C$ values relative to other biochemical compounds as a consequence of kinetic isotope effects occurring during the conversion of pyruvate to acetyl coenzyme-A during lipid

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synthesis (DeNiro and Epstein 1977). For this reason, the amount of lipid content in a specific tissue directly influences its $\delta^{13}C$ values, thus potentially leading to a misinterpretation of the isotopic results (Murry et al. 2006; Logan et al. 2008). To solve this lipid effect, researchers can correct their stable isotope ratios a priori by extracting lipids from samples using different chemical solvents (Kiszka et al. 2011; Ryan et al. 2013; De Stephanis et al. 2015). However, this technique may result in the loss of some non-lipid compounds (e.g., some cellular constituents) or the inadvertent removal of amino acids, which may also alter $\delta^{15}N$ values of delipidated samples in an unpredictable manner (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Murry et al. 2006; Logan et al. 2008; Ehrich et al. 2011). Since ecological investigations commonly require double isotopic analyses for the same tissue, separate determinations of lipid-free $\delta^{13}C$ and bulk $\delta^{15}N$ are desirable, thus increasing time, work load and economic costs. This is disadvantageous when most ecological studies require extensive replication in space and time and when time and funding are limited. Furthermore, the amount of sample that can be obtained from one individual is usually limited, making repeated measurements impossible.

A common alternative to avoid the chemical extraction of lipids is to correct for lipid-based variability in $\delta^{13}C$ values a posteriori, by applying arithmetic corrections (i.e., lipid normalization equations) based on the use of the C:N ratio as a surrogate for lipid content (e.g., Murry et al. 2006; Sweeting et al. 2006; Logan et al. 2008; Ehrich et al. 2011). C:N ratios are derived routinely from mass percentage of carbon and nitrogen during isotope ratio determinations, so these values are generally available for most isotopic studies. General and species-specific lipid normalization models have been published to avoid chemical extraction in a wide range of taxa inhabiting different environments (e.g., McConnaughey and McRoy 1979; Fry et al. 2003; Post et al. 2007; Ehrich et al. 2011). However, thus far, no general model has been found to perform accurately across different tissues and species (Logan and Lutcavage 2008; Ehrich et al. 2011).

In animals in general (e.g., Sotiropoulos et al. 2004; Mateo et al. 2008) and marine mammals in particular (e.g., Lesage et al. 2010; Ryan et al. 2012), lipid extractions through chemical procedures are known to affect $\delta^{13}C$ and $\delta^{15}N$ values in a species-specific fashion. Accordingly, different lipid normalization equations have been published for different species of cetaceans as an alternative to chemical extraction, but these have shown contrasting accuracies/performances (e.g., Lesage et al. 2010; Ryan et al. 2012; Wilson et al. 2014; Yurkowski et al. 2014). While some authors argue against arithmetical normalization due to the poor predictive power of the models (Ryan et al. 2012), others recommend the use of equations obtained from tissue and species-specific models (Lesage et al. 2010), cautioning that their use is largely dependent on the precision of the stable isotope values needed to address the research question (Lesage et al. 2010; Yurkowski et al. 2014).

Here, we explore the suitability of lipid normalization equations vs. the more conventional chemical extractions used to account for the effect of lipids in bulk samples of bottlenose dolphins and their prey. Furthermore, we simulated the effect of different lipid normalization procedures on the dietary estimates obtained through mass-balance isotope mixing models to reconstruct the modeled diet of a potential predator within different isotopic scenarios. Given the results obtained, we provide some recommendations on the most desirable treatments to achieve the most accurate dietary estimates.

Materials and methods

Sample collection and preparation

Samples of bottlenose dolphin skin, fish muscle and whole fish were obtained during a controlled feeding experiment carried out at Loro Parque facilities in Tenerife (Canary Islands, Spain), where six individuals of bottlenose dolphin were maintained with controlled diets of sprat (*Sprattus sprattus*), herring (*Clupea harengus*) and capelin (*Mallotus villosus*) for 350 days. The experiment was designed to calculate diet-to-tissue discriminant factor and turnover rates (Giménez et al. 2016), but here the data obtained through the entire experiment are used to explore the effects of lipids on stable isotope signature of bottlenose dolphins and its prey and to construct lipid normalization equations. Dolphin skin samples were obtained with a scalpel from the dorsal fin of each individual every 14 days along the entire experiment. Muscle subsamples of fish prey were obtained from each individual and processed separately from the remaining fish. Both bottlenose dolphin skin and fish samples were kept frozen at −20 °C in plastic vials until further processing. At the laboratory, fish and dolphin skin samples were oven dried at 60 °C for 48 h and powdered with a mortar and pestle. Two aliquots were extracted from each powdered sample. One aliquot was immediately processed for dual isotopic determinations (hereafter $\delta^{13}C_{bulk}$ and $\delta^{15}N_{bulk}$), whereas the other underwent lipid extraction with several rinses of chloroform:methanol (2:1) solution (modified version of Bligh and Dyers 1959 protocol) before dual isotopic determination (hereafter, $\delta^{13}C_{del}$ and $\delta^{15}N_{del}$).

Stable isotopes analyses

Subsamples of powdered materials were weighed to the nearest μg and placed into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ determinations. Isotopic analyses were carried out at the Laboratorio de Isótopos Estables of the Estación Biológica de Doñana (LIE-EBD, Spain; http://www.ebd.csic.es/lie/index.html). All samples were combusted at 1020 °C using a continuous flow isotope ratio mass spectrometry system by means of Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The isotopic compositions are reported in the conventional δ-notation as the per mil (‰) deviation relative to Vienna Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$). Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of ±0.1 and ±0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. C:N ratios are expressed in % of weight. The analytical measurement errors for %C and %N are ±0.1 and ±0.15‰, respectively. The internal standards used were: EBD-23 (cow horn), LIE-BB (whale baleen), and LIE-PA (feathers of razorbill). These laboratory standards were previously calibrated with international standards supplied by the International Atomic Energy Agency (IAEA, Vienna).

Lipid normalization equations

We used C:N ratios for non-lipid-extracted samples (hereafter, $C:N_{bulk}$) as a suitable proxy of sample lipid content to obtain our lipid normalization equations (e.g., Post et al. 2007; Ehrich et al. 2011; Abrantes et al. 2012; Ryan et al. 2012 but see Fagan et al. 2011 and Wilson et al. 2014). Following Post et al. (2007), least-squares linear regressions were built between $C:N_{bulk}$ and the difference between $\delta^{13}C$ values for delipidated ($\delta^{13}C_{del}$) and non-lipid-extracted samples ($\delta^{13}C_{bulk}$). In prey models, the species was introduced as a three-level factor to explore species-specific differences in these relationships, i.e., the role of the species in modulating the lipid effect on $\delta^{13}C$ values.

Influence of the lipid normalization procedure on diet assessments

We simulated the effect of different lipid normalization procedures on the dietary estimates obtained through mass-balance isotope mixing models. To this end, we generated four different isotopic scenarios based on the modeled isotopic composition ($\delta^{13}C$ and $\delta^{15}N$ values) of four different dietary endpoints (I–IV; Table 1 and Fig S1) and a potential predator feeding on these food resources in the following proportion: I: 30%; II: 20%;III: 40%; IV: 10%. As the most reliable diet-to-tissue discrimination factors (DTDF), we considered those obtained from double measurements in bulk ($\delta^{15}N_{bulk}$) and lipid-free ($\delta^{13}C_{del}$) prey samples from the experimental trial ($\Delta\delta^{13}C_{del} = 1.01 \pm 0.37$ and $\Delta\delta^{15}N_{bulk} = 1.57 \pm 0.52$) in Giménez et al. (2016). The isotopic composition ($\delta^{13}C_{pred}$ and $\delta^{15}N_{pred}$) of modeled predators could be, therefore, described as follows:

$$\begin{aligned}\delta^{13}C_{pred} = {} & 0.3 \cdot (\delta^{13}C_{del,\,I} + \Delta\delta^{13}C_{del}) \\ & + 0.2 \cdot (\delta^{13}C_{del,\,II} + \Delta\delta^{13}C_{del}) \\ & + 0.4 \cdot (\delta^{13}C_{del,\,III} + \Delta\delta^{13}C_{del}) \\ & + 0.1 \cdot (\delta^{13}C_{del,\,IV} + \Delta\delta^{13}C_{del})\end{aligned}$$

$$\begin{aligned}\delta^{15}N_{pred} = {} & 0.3 \cdot (\delta^{15}N_{bulk,\,I} + \Delta\delta^{15}N_{bulk}) \\ & + 0.2 \cdot (\delta^{15}N_{bulk,\,II} + \Delta\delta^{15}N_{bulk}) \\ & + 0.4 \cdot (\delta^{15}N_{bulk,\,III} + \Delta\delta^{15}N_{bulk}) \\ & + 0.1 \cdot (\delta^{15}N_{bulk,\,IV} + \Delta\delta^{15}N_{bulk})\end{aligned}$$

Different scenarios additionally accounted for differences in the isotopic variability of dietary endpoints, as well as in the magnitude of isotopic differences among dietary endpoints.

To simulate the effect of different lipid treatments on dietary reconstructions, all combinations of DTDFs obtained in the captive feeding experiment of Giménez et al. (2016) were used to quantify any deviation from modeled diet in all modeled scenarios (Table 2). Furthermore, new DTDFs were calculated taking into account the species-specific ($\Delta\delta_{norm1}$) and general normalization equations

Table 1 Simulated scenarios varying the mean and standard deviation (sd) of prey items

Scenario	Prey	$\delta^{15}N$		$\delta^{13}C$	
		Mean	sd	Mean	sd
1	I	16	0.5	−20	0.5
	II	14	0.5	−15	0.5
	III	11	0.5	−20.5	0.5
	IV	12	0.5	−15.5	0.5
2	I	16	1	−20	1
	II	14	1	−15	1
	III	11	1	−20.5	1
	IV	12	1	−15.5	1
3	I	18	0.5	−21	0.5
	II	16	0.5	−14	0.5
	III	9	0.5	−21.5	0.5
	IV	10	0.5	−14.5	0.5
4	I	18	1	−21	1
	II	16	1	−14	1
	III	9	1	−21.5	1
	IV	10	1	−14.5	1

Table 2 DTDFs used to simulate the effect of lipid extraction by chemical solvents and normalization equations

DTDF	Fish muscle	Whole fish	References
$\Delta\delta^{13}C_{bulk}$	1.95 ± 0.6	3.59 ± 0.56	Giménez et al. (2016)
$\Delta\delta^{15}N_{bulk}$	1.57 ± 0.52	1.74 ± 0.55	Giménez et al. (2016)
$\Delta\delta^{13}C_{del}$	1.01 ± 0.37	0.93 ± 0.56	Giménez et al. (2016)
$\Delta\delta^{15}N_{del}$	1.01 ± 0.5	1.49 ± 0.42	Giménez et al. (2016)
$\Delta\delta^{13}C_{norm1}$	1.01 ± 0.47	0.88 ± 0.44	This study
$\Delta\delta^{13}C_{norm2}$	1.18 ± 0.47	1.16 ± 0.44	This study

$\Delta\delta_{norm1}$: species-specific equation, $\Delta\delta_{norm2}$: general normalization equations

($\Delta\delta_{norm2}$) calculated in the present paper to test the effect of arithmetic corrections on the dietary estimates (Table 2).

Bayesian mixing models (SIAR package; Parnell et al. 2010) were run with all of the different DTDFs and the outputs were compared with the modeled diet (i.e., I: 30%; II: 20%; III: 40%; IV: 10%). The influence of the different lipid normalization procedures in derived dietary estimates was calculated as the sum of the absolute differences with respect to the modeled diet. As an estimate of the variability ascribed to the dietary estimates obtained, we summed derived variances for each dietary endpoint within each treatment and scenario. All statistical analyses were conducted using the open-source statistical programming language R v.3.1.1 (http://cran.r-project.org).

Results

Changes in isotope ratio values following lipid extraction

Significant increases in $\delta^{13}C$ values following lipid extraction were found in dolphin skin ($t = -12.99$, $df = 122$, p value <0.001, Fig. 1a; Table 3) and in the whole prey and muscle tissues analyzed (all p values <0.001, Fig. 1c, e). Increases in $\delta^{15}N$ values were also significant for dolphin skin ($t = -8.56$, $df = 122$, p value <0.001; Fig. 1b; Table 3) and in prey samples (all p values <0.001, Fig. 1d, f, see also Giménez et al. (2016)). The highest increase after lipid extraction occurred in $\delta^{13}C$ values of whole fish (Table 3).

Lipid normalization models

Regression analysis for bottlenose dolphin skin showed a significant linear relationship between $C{:}N_{bulk}$ and the observed difference between $\delta^{13}C_{bulk}$ and $\delta^{13}C_{del}$ ($\Delta\delta^{13}C_{bulk\text{-}del}$; $R^2 = 0.16$, $F_{1,121} = 23.86$, p value <0.001, Fig. 2a) following the equation:

$$\Delta\delta^{13}C_{bulk\text{-}del} = -4.17 + 1.28\,C{:}N_{bulk} \quad (1)$$

The best model for fish muscle included the species as a significant factor and explained 67% of the isotopic variance ($F_{3,68} = 46.04$, $R^2 = 0.67$, p value <0.001, Fig. 2e), whereas the general model that combined all species explained 60% of observed variance ($F_{1,70} = 103.5$, $R^2 = 0.60$, p value <0.001, Fig. 2c). Derived normalization equations for fish muscle were as follows:

$$\text{Herring}: \Delta\delta^{13}C_{bulk\text{-}del} = -3.58 + 1.12\,C{:}N_{bulk} \quad (2)$$

$$\text{Capelin}: \Delta\delta^{13}C_{bulk\text{-}del} = -3.02 + 1.12\,C{:}N_{bulk} \quad (3)$$

$$\text{Sprat}: \Delta\delta^{13}C_{bulk\text{-}del} = -3.18 + 1.12\,C{:}N_{bulk} \quad (4)$$

$$\text{All species combined}: \Delta\delta^{13}C_{bulk\text{-}del} = -3.14 + 1.10\,C{:}N_{bulk} \quad (5)$$

Similarly, the best model for the whole fish also included the species factor and explained 75% of observed isotopic variance ($F_{3,34} = 34.62$, $R^2 = 0.75$, p value <0.001, Fig. 2b), whereas the general model only explained 41% of the variance ($F_{1,36} = 24.79$, $R^2 = 0.41$, p value <0.001, Fig. 2d). The normalization equations for whole fish were as follows:

$$\text{Herring}: \Delta\delta^{13}C_{bulk\text{-}del} = -1.02 + 0.65\,C{:}N_{bulk} \quad (6)$$

$$\text{Capelin}: \Delta\delta^{13}C_{bulk\text{-}del} = -0.24 + 0.65\,C{:}N_{bulk} \quad (7)$$

$$\text{Sprat}: \Delta\delta^{13}C_{bulk\text{-}del} = -0.47 + 0.65\,C{:}N_{bulk} \quad (8)$$

$$\text{All species combined}: \Delta\delta^{13}C_{bulk\text{-}del} = -0.40 + 0.62\,C{:}N_{bulk} \quad (9)$$

Lipid-correction effects on dietary estimates

Bayesian mixing model (SIAR) results showed that the largest deviations from the modeled diet were observed in those simulations where DTDFs were obtained without lipid extraction (i.e., $\Delta\delta^{13}C_{bulk}$ and $\Delta\delta^{15}N_{bulk}$), and were more pronounced when using whole fish than fish muscle samples (Fig. 3, Fig S2). The stable isotope scenario also played an important role, with larger effects on dietary estimates in those scenarios where dietary endpoints were isotopically closer (scenarios 1 and 2, Fig S1). The most accurate dietary estimates (less than 0.14% deviation from the modeled diet) were obtained when using the DTDF derived from normalizing the isotopic composition of prey through the species-specific lipid normalization equation. Increasing deviations from the modeled diet were observed when using whole fish or normalizing the data with the general equation but never exceeded 10% deviation from the real diet (Fig. 3).

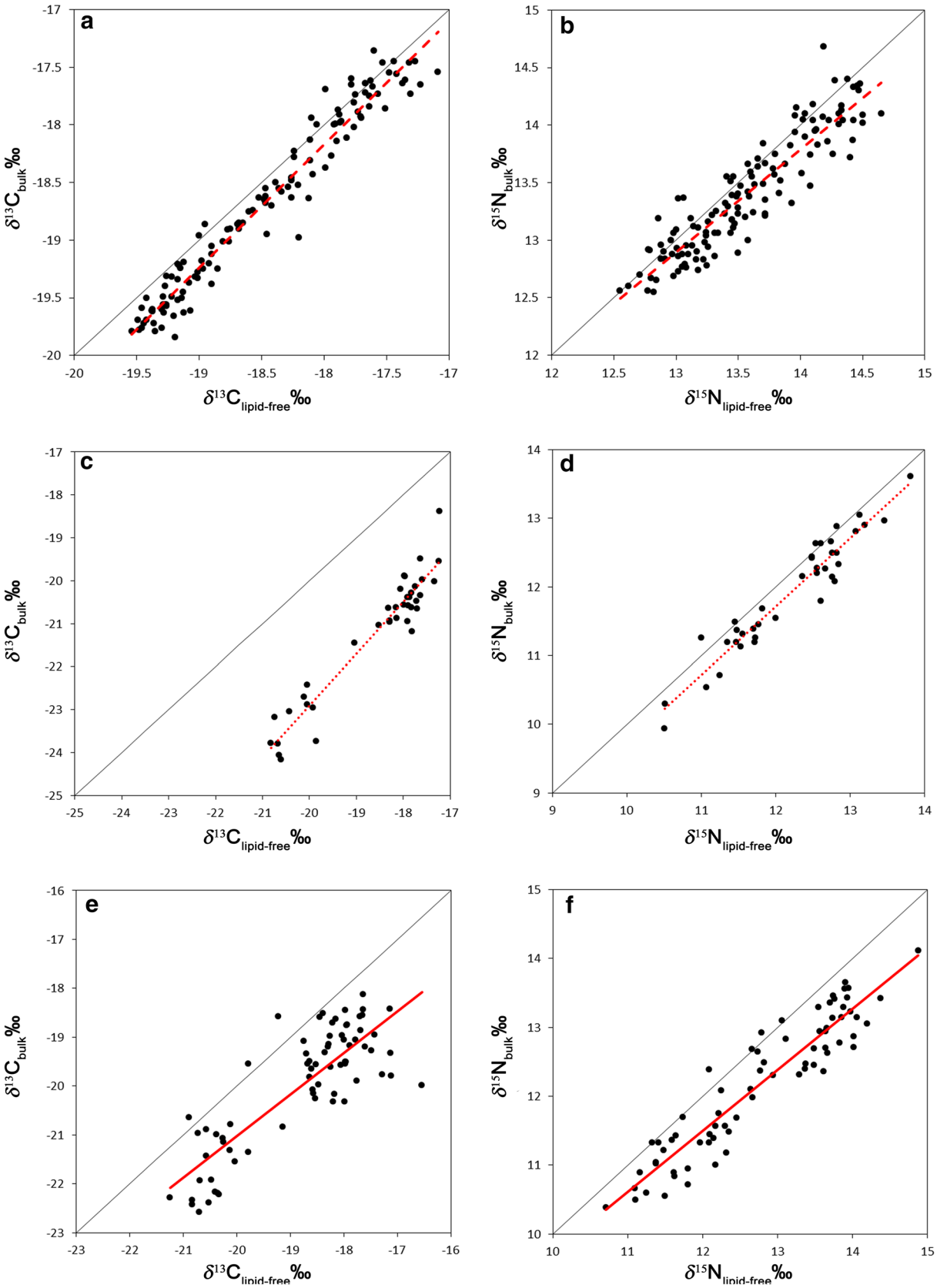

Fig. 1 Relationship between $\delta^{13}C_{bulk}$ and $\delta^{13}C_{lipid\text{-}free}$ samples and between $\delta^{15}N_{bulk}$ and $\delta^{13}N_{lipid\text{-}free}$ samples in bottlenose dolphin skin (**a**, **b**), whole fish (**c**, **d**) and fish muscle (**e**, **f**)

Table 3 Change in isotopic values of bottlenose dolphins, sprat, herring and capelin after delipidation

Species	Tissue	Change in isotopic values after delipidation	
		δ^{13}C (‰)	δ^{15}N (‰)
Bottlenose dolphin	Skin	0.2 ± 0.03	0.17 ± 0.04
Sprat	Muscle	1.37 ± 0.23	0.64 ± 0.13
Herring	Muscle	0.74 ± 0.41	0.33 ± 0.19
Capelin	Muscle	1.16 ± 0.28	0.67 ± 0.13
Sprat	Whole fish	2.60 ± 0.18	0.28 ± 0.13
Herring	Whole fish	2.20 ± 0.35	0.17 ± 0.17
Capelin	Whole fish	2.99 ± 0.33	0.39 ± 0.11

Isotopic variability ascribed to different dietary endpoints apparently played a marginal role in determining the mean relative contribution of different prey types to the diet of consumers (Fig. 3), but resulted in increasing variability in dietary estimates. With regard to the latter point, the greatest variability in dietary estimates was observed in those scenarios where dietary endpoints were isotopically closer, whereas the effect of considered DTDFs was marginal (Fig. 4). This pattern was relatively constant throughout all the simulations, except when no delipidation was conducted.

Discussion

Changes in isotope ratios (δ^{13}C, δ^{15}N) and C:N values following lipid extraction

The observed relationships between C:N ratios in dolphin skin samples and the corresponding $\Delta\delta^{13}C_{bulk\text{-}del}$ values indicated a potential effect of the lipid content on the isotopic composition of this tissue. However, observed changes in the isotopic composition of dolphin skin were smaller than the analytical laboratory measurement error for δ^{15}N values and very close to those for δ^{13}C values, a likely result of the low lipid content in our skin samples. Indeed, observed C:N ratios (ranging from 3.3 to 3.6‰) fell well within the general range for lipid-free samples of aquatic organisms in general (Post et al. 2007), and of marine mammals in particular (Yurkowski et al. 2014). We, therefore, concur with recent studies suggesting that lipid extraction is not required for marine mammal skin samples with C:N < 3.6 (Wilson et al. 2014; Yurkowski et al. 2014).

In contrast, observed changes in δ^{13}C values for fish samples were above the analytical laboratory measurement error, thus indicating the need to account for variations in δ^{13}C values due to the amount of lipids in the sample (see Post et al. 2007). Further, the lipid effect on δ^{13}C values was greater for whole fish than for fish muscle, likely due to the higher amount of fat content typical of other fish tissues, different from the relatively lipid-free muscle tissue (Sotiropoulos et al. 2004). Remarkably, δ^{15}N values also increased after lipid extraction, as occurred in other studies with fish samples (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Sweeting et al. 2006; Murry et al. 2006; Logan et al. 2008), thus underscoring potential misinterpretations of δ^{15}N values when extracting lipids prior to isotopic determinations.

Lipid normalization models

Species-specific lipid normalization equations showed a better goodness of fit than the general equation, which combined all prey species. Thus, tissue- and species-specific lipid normalization equations are desirable (Lesage et al. 2010). Extrapolation of our arithmetical correction beyond the measured range of C:N ratios must be avoided because previous models have predicted a nonlinear relationship between C:N and $\Delta\delta^{13}$C at high C:N ratios (e.g., McConnaughey and McRoy 1979). On the other hand, the derived model for bottlenose dolphin skin only explained 16% of the isotopic variation. This low explanatory power would have introduced a great amount of error if the equation had been used. Due to the low effects after lipid extraction and the low explanatory power of the normalization equation, we recommend using bulk values if the C:N ratio of dolphin samples is in the range of our results.

Lipid-correction effects on isotopic mixing models

The largest deviations of estimated diet from modeled diet were found when using $\Delta\delta^{13}C_{bulk}$ and $\Delta\delta^{15}N_{bulk}$ values for whole fish samples, likely as a result of the greater lipid content of whole fish with respect to the muscle samples. In contrast, the use of species-specific lipid normalization equations for fish muscle resulted in the smallest deviations from the modeled diet.

Sotiropoulos et al. (2004) found marginal and ecologically insignificant values of δ^{13}C depletion in bulk fish muscle compared with whole fish samples, thus providing justification for the lack of lipid extraction in stable isotope studies that used fish muscle. In contrast, our simulations indicated that the lack of lipid extraction in fish muscle results in up to 35% of accumulated error in diet estimates. If duplicate measurements were not feasible, we therefore encourage a priori evaluation of arithmetical corrections taking into account the study objectives and research question, as an alternative method to δ^{13}C determination from delipidated samples. The errors derived from treatment

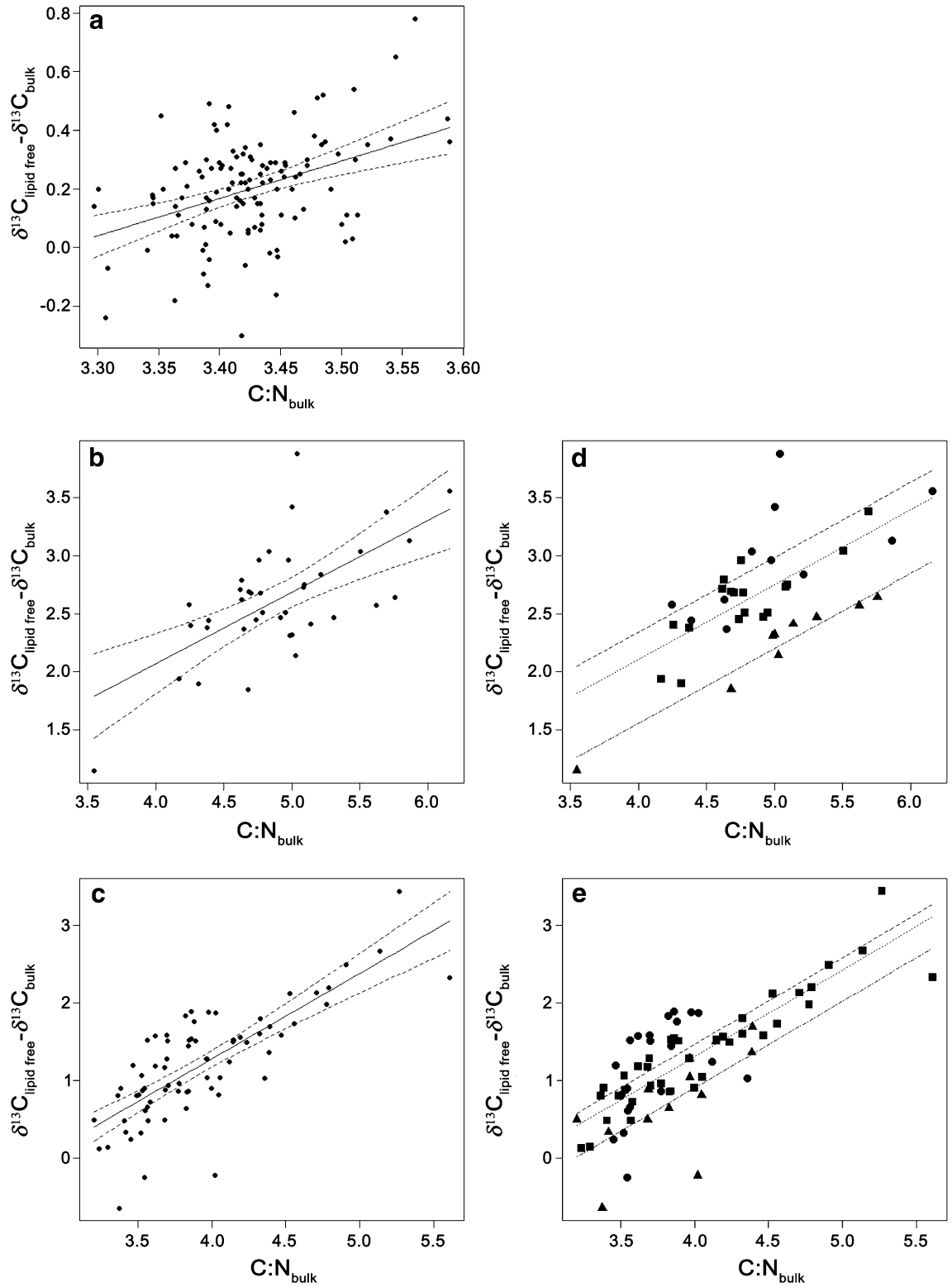

Fig. 2 Model fitting the bottlenose skin (**a**), whole fish (**b**) and fish muscle (**c**). Species-specific models are presented for whole fish (**d**) and fish muscle (**e**). *Square* sprat, *circle* capelin, *triangle* herring

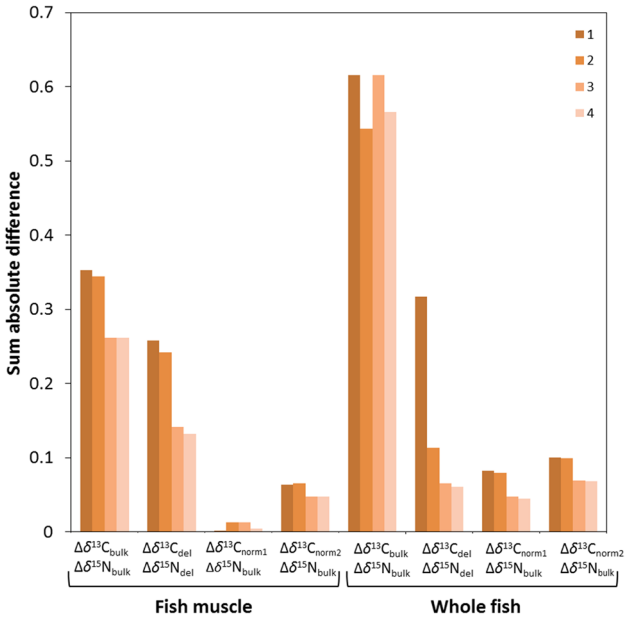

Fig. 3 Sum of the absolute difference of the mean contribution between the correct treatment ($\delta^{13}C_{del}$ $\delta^{13}N_{bulk}$) and the different treatments and normalization for each scenario (*1–4*). Norm1 = DTDF derived from values normalized with the species-specific equation, Norm2 = DTDF derived from values normalized with the general equation

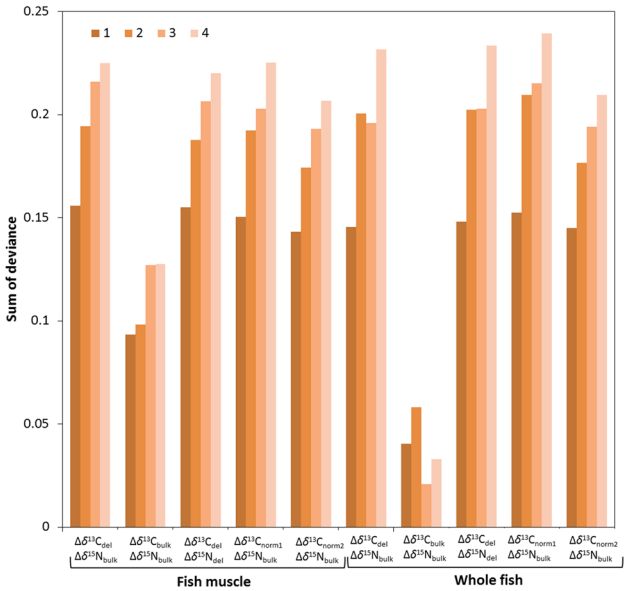

Fig. 4 Sum of the deviance of each treatment or normalization for each scenario (*1–4*). Norm1 = DTDF derived from values normalized with the species-specific equation, Norm2 = DTDF derived from values normalized with the general equation

or the normalization will have an ecological significance depending on the specific question that is addressed in a given study (Ricca et al. 2007; Tarroux et al. 2010).

As seen in other cetacean species (Lesage et al. 2010) and other aquatic organisms (Post et al. 2007), the effect associated with sample treatment and lipid normalization on the mean relative contribution of different prey types to the diet of consumers increases as the isotopic distance among prey sources decreases. However, the standard deviation of dietary sources does not seem to have a great influence in the estimates (Fig. 3).

In addition, the effect of sample treatment or the arithmetical normalization showed a marginal effect on the variability of derived dietary estimates. The factor contributing the most to this variation was likely related to the isotopic differences among dietary endpoints and the standard deviation of different prey sources. In particular, observed variability in diet estimates decreased when dietary endpoints were isotopically closer or showed smaller isotopic variability (Fig. 4).

In conclusion, in order to reduce time, work load and economic costs, we recommend the use of species-specific LNE to normalize the isotopic composition of diet before dietary quantifications through mixing models. This will be only feasible when dealing with specialists or non-wide generalists, for which the number of potential food resources might be relatively small. In generalist consumers, with a large number of potential food resources, the use of general LNE might be more practical.

We analyzed the effect of lipids on stable isotope values of dolphins at two different scales: the individual scale (i.e., how lipid extraction affects isotope ratios) and the system scale (i.e., how lipid extraction induces changes in diet assessments). At the system scale, we tested four different scenarios varying the relative position of sources and consumers within the isotopic space (i.e., the mean value of sources and their standard deviation). Nevertheless, the sensitivity of a mixing model output also depends on the complexity of the system (i.e., number of sources; Tarroux et al. 2010). Accordingly, further studies in more complex systems are strongly encouraged to provide additional insights into the actual effects of lipid normalization procedures on stable isotope-based dietary studies.

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Compliance with ethical standards

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

Ethical approval All animal procedures performed were in accordance with the ethical standards of EBD-CSIC through the evaluation of its ethical committee. The project was approved and funded by the Spanish Ministry of Economy and Competitiveness [CGL2011-25543, EcoCet Project].

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