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Accounting for the effects of lipids in stable isotope ($\delta^{13}C$ and $\delta^{15}N$ values) analysis of skin and blubber of balaenopterid whales

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RATIONALE: Stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) of darted skin and blubber biopsies can shed light on habitat use and diet of cetaceans, which are otherwise difficult to study. Non-dietary factors affect isotopic variability, chiefly the depletion of ^{13}C due to the presence of ^{12}C-rich lipids. The efficacy of *post hoc* lipid-correction models (normalization) must be tested.
METHODS: For tissues with high natural lipid content (e.g., whale skin and blubber), chemical lipid extraction or normalization is necessary. C:N ratios, $\delta^{13}C$ values and $\delta^{15}N$ values were determined for duplicate control and lipid-extracted skin and blubber of fin (*Balaenoptera physalus*), humpback (*Megaptera novaeangliae*) and minke whales (*B. acutorostrata*) by continuous-flow elemental analysis isotope ratio mass spectrometry (CF-EA-IRMS). Six different normalization models were tested to correct $\delta^{13}C$ values for the presence of lipids.
RESULTS: Following lipid extraction, significant increases in $\delta^{13}C$ values were observed for both tissues in the three species. Significant increases were also found for $\delta^{15}N$ values in minke whale skin and fin whale blubber. In fin whale skin, the $\delta^{15}N$ values decreased, with no change observed in humpback whale skin. Non-linear models generally out-performed linear models and the suitability of models varied by species and tissue, indicating the need for high model specificity, even among these closely related taxa.
CONCLUSIONS: Given the poor predictive power of the models to estimate lipid-free $\delta^{13}C$ values, and the unpredictable changes in $\delta^{15}N$ values due to lipid-extraction, we recommend against arithmetical normalization in accounting for lipid effects on $\delta^{13}C$ values for balaenopterid skin or blubber samples. Rather, we recommend that duplicate analysis of lipid-extracted ($\delta^{13}C$ values) and non-treated tissues ($\delta^{15}N$ values) be used.

The stable isotope ratios of carbon and nitrogen in consumer tissues reflect those of the diet in a predictable manner and can thus be used to infer dietary information at the time and location of tissue synthesis.[1,2] Nitrogen isotopes are relatively strongly fractionated during nitrogen metabolism, and thus increase principally as a function of mean trophic level. The stable isotopes of carbon do not exhibit the same degree of trophic enrichment and tissue carbon isotopes are more indicative of the isotopic composition of primary production fuelling a food web.[3] Baseline $\delta^{13}C$ values vary geographically or between habitats, allowing variation in consumer $\delta^{13}C$ values to be associated with differences in habitat use.[4–6] Stable isotope ratios are thus intrinsic markers from which quantitative information on trophic status, seasonal distribution and foraging area can be derived.

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Most stable isotope investigations of diet and movement in animal systems explicitly target proteinaceous tissues, as the isotopic composition of a consumer's protein is tightly linked to the protein component of diet.[7] A key consideration before carrying out stable isotope analysis on a tissue that may contain multiple molecular components (e.g., muscle, skin or blood) is the lipid-content of the tissue being analyzed. Lipids are enriched in ^{12}C relative to bulk proteins in a given tissue resulting in a decrease in bulk tissue $^{13}C/^{12}C$ and hence $\delta^{13}C$ values.[1] Lipid content may be highly variable between ecological samples (both between and within species) and the potential influence of lipid content on bulk tissue $\delta^{13}C$ values must be considered. There are two common approaches used to account for the effect of lipid on $\delta^{13}C$ values; an *a priori* approach using chemical extraction of lipids from tissue samples, and an *a posteriori* approach using mathematical correction (normalization). The latter is based on the carbon:nitrogen elemental ratio (C:N ratio), as tissues enriched in lipids have a greater relative proportion of C than tissues with low lipid concentrations. Both methods have complications and accounting for lipids has

been given considerable attention in stable isotope ecology.[8–15] Chemical lipid extraction definitively removes the influence of lipids on bulk tissue $\delta^{13}C$ values, but may lead to unpredictable changes in tissue $\delta^{15}N$ values due *inter alia* to the inadvertent removal of amino acids.[9,14,16,17] This is problematic given that both isotopes are typically recorded simultaneously from the same sample, in order to reduce the cost of analysis. Furthermore, studies of large marine taxa, e.g., whales, often rely on the use of remotely darted biopsies (untethered sampling darts fired from a moving boat, at an unrestrained target animal). Using this technique, only a small amount of tissue is available, sometimes preventing duplication of samples for analysis.

Retrospective, arithmetic correction of measured $\delta^{13}C$ values for lipid content is based on tissue C:N ratios. As lipids do not contain nitrogen, the presence of lipids in bulk tissue will increase tissue C:N ratios and decrease $\delta^{13}C$ values proportionally.[18] Correction of bulk tissue $\delta^{13}C$ values should then be possible through regression. A full regression model should include parameters such as the C:N value of lipid-free tissue and the protein-lipid $\delta^{13}C$ discrimination value. These values are often unknown for the tissues and species in question, and are likely to vary; thus, the fundamental assumptions of the models can be difficult to test.[12] Furthermore, one fundamental assumption of most lipid normalization models is that both lipid and protein are derived from the same isotopic source, in order that the lipid-protein discrimination value D is constant. However, this assumption is often violated given the differential turnover rates of those tissue components.[19]

Normalization models for specific tissues or whole-body homogenates have been published for terrestrial mammals,[9] fish,[10] invertebrates[12] and cetaceans.[11] However, the authors caution against using these models for tissues with high lipid content where the relationship between C:N ratios and bulk tissue $\delta^{13}C$ values becomes non-linear. The use of mathematical correction over lipid extraction is favourable, given the risks posed by exposure to some solvents used in the extraction process (e.g., chloroform is carcinogenic). Another consideration, on which there is no unanimous consensus in the literature,[8,20] is the effect of lipid extraction on $\delta^{15}N$ values. There are several commonly used lipid-extraction techniques using various solvents with a range of polarities. These techniques have the potential to solubilize amino acids to differing degrees, and the lipid-extraction technique employed may thus have an effect on $\delta^{15}N$ values.[21,22]

The effects of lipid extraction on $\delta^{13}C$ values in skin and blubber of humpback whales have been studied.[23] However, the effects of lipid extraction on nitrogen isotope values in these tissues were not investigated. Significant decreases in $\delta^{15}N$ values due to lipid extraction have been reported in the skin of Balaenopteridae (although only for fin, humpback and minke whales when pooled together), while significant increases were found in other cetacean taxa.[11] This suggests that changes in tissue $\delta^{15}N$ values caused by lipid extraction may be species-specific, even among closely related taxa. The study supported the need for species-specific models for lipid normalization of $\delta^{13}C$ values[14] in cetacean skin, and for a greater understanding on the effects of lipid extraction on other isotope ratio values such as $\delta^{15}N$.[11]

EXPERIMENTAL

Sampling, sample preparation and stable isotope analysis

Tissue samples were taken from live fin (*Balaenoptera physalus*) and humpback (*Megaptera novaeangliae*) whales with a *Barnett Panzer V* re-curve crossbow (150 lb draw-strength) using modified bolts and sterilised steel 40 mm biopsy tips (designed by Ceta-Dart, Dr F. Larsen, Copenhagen, Denmark) between winter 2008 and 2011 at two study sites in Ireland (fin and humpback whales) and Boa Vista, Cape Verde (humpback whales only), under permit from the respective national authorities. Skin and blubber were also sampled using a scalpel from dead fin, humpback and minke whales (*B. acutorostrata*) found stranded around the coast of Ireland. Only those carcases exhibiting a code 2 or above on a standardized decomposition scale[24] were considered, to circumvent the possible effects of decay on the integrity of the samples. All samples were initially stored at –20 °C, then transferred to a –80 °C freezer. While still frozen, samples were duplicated (halved longitudinally) and skin and blubber were separated for analysis. As blubber is stratified into biochemically distinct layers in the species concerned,[25–27] only the outer blubber layer was analyzed and this stratum was identified by eye for those samples from stranded specimens.[28] Samples were freeze-dried and, for duplicates, lipids were extracted by Soxhlet reflux using *n*-hexane and acetone (1:1) for 12 h.[29] Both lipid-extracted and whole samples were ground to a fine powder, ~0.50 mg weighed into tin capsules and analyzed by continuous-flow elemental analysis isotope ratio mass spectrometry (CF-EA-IRMS) in three runs at two different laboratories (University of Southampton and the Chrono Centre, Queen's University, Belfast). At the University of Southampton, a EA 3000 elemental analyzer (EuroVector, Milan Italy) combined with a 20-20 isotope ratio mass spectrometer (Europa Scientific, Crewe, UK) was used. At Queen's University Belfast, a Delta V Advantage EA-IRMS system (Thermo Scientific, Bremen, Germany) was used.

Isotope ratios are presented in delta notation as parts per thousand differences from international standards according to the following equation:

$$\delta^{y}X = \left[\left(R_{\text{sample}}/R_{\text{standard}}\right) - 1\right] \times 1000$$

where R denotes the heavier:lighter isotope ratio and Y is the atomic mass of the stable isotope X ($\delta^{13}C$ or $\delta^{15}N$). Internal lab standards, L-alanine and L-glutamic acid (Acros Organics, Geel, Belgium), for the Southampton and Belfast laboratory, respectively, which were calibrated with International Atomic Energy Agency (IAEA, Vienna, Austria) standards, were used: Vienna Pee Dee Belemnite (for carbon), atmospheric N_2 (for nitrogen). Internal standards of known carbon and nitrogen composition (nicotinamide and L-glutamic acid) were routinely analyzed between samples in order to determine instrument precision. Based on the standard deviation of these internal standards, the lowest analytical precision of all three runs was 0.2 ‰ for nitrogen, and 0.1 ‰ for carbon.

Data analysis

Changes in $\delta^{13}C$ values after lipid extraction (i.e. $\delta^{13}C_{\text{lipid-free}} - \delta^{13}C_{\text{bulk}}$) were compared with lipid-free $\delta^{13}C$ values estimated using six published linear and non-linear normalization

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models, originally produced to estimate lipid-free $\delta^{13}C$ values in a variety of taxa. The efficacy of models for normalizing $\delta^{13}C$ values was investigated by comparing Akaike information criterion (AIC) values and mean square error (MSE) of the model fits. Furthermore, the percentage of the predicted $\delta^{13}C$ values that fell within 0.5 ‰ (> twice the mean instrument error of the $\delta^{13}C$ values in most ecological studies) of the lipid-extracted $\delta^{13}C$ value was estimated.[9] The following previously published lipid-normalization models were considered:

Equation (1)[10] is a non-linear equation based on McConnaughey and McRoy[18] fitted for whole body marine vertebrates and invertebrates with three variables: lipid content (L), C:N ratio and an isotopic discrimination factor between pure lipid and protein of the sample, D (6.4 ‰ for cetacean skin).[11] I is a constant, assigned a starting value of –0.02.[18]

$$\delta^{13}C' = \delta^{13}C + D \times \left\{ I + \frac{3.90}{1 + 287/L} \right\} \quad (1)$$

where

$$L = \frac{93}{1 + [0.246 \times (C:N) - 0.775]^{-1}}$$

Equation (2)[11] is a generalized linear model which estimates $\delta^{13}C_{lipid\text{-}free}$ values as a function of $\delta^{13}C_{bulk}$ values, irrespective of the C:N ratio and hence lipid content. This model is deemed to be appropriate for lipid-normalizing skin in Balaenopteridae;[11] however, it was not tested for individual species or for blubber.

$$\delta^{13}C' = \beta 0 + \beta 1 \times \delta^{13}C \quad (2)$$

Equation (3)[8] is a non-linear model with two parameters, developed for whole organisms and muscle for a range of terrestrial and aquatic animal species. This relationship has only been found to be appropriate for tissues with high lipid content (>15%) and was therefore considered suitable for testing with whale skin and blubber. Terms a and b are parameters that are estimated from the data.

$$\delta^{13}C' - \delta^{13}C = a + b \times (C:N) \quad (3)$$

Equation (4)[12] is a generalized log-linear model allowing for the non-linear relationship of the single explanatory variable; bulk C:N.

$$\delta^{13}C' - \delta^{13}C = \beta 0 + \beta 1 \times \ln(C:N) \quad (4)$$

Equation (5)[12] is a derivation of Eqn. (1) in McConnaughey and McRoy,[18] where the protein-lipid discrimination D is replaced by a. This three-parameter non-linear model has previously been tested for whole-body homogenates and individual tissues for a range of aquatic vertebrates and invertebrates. Parameters b and c are estimated from the data.

$$\delta^{13}C' - \delta^{13}C = \frac{a \times (C:N) + b}{C:N + c} \quad (5)$$

Equation (6)[30] is a non-linear equation designed for tissues of freshwater fishes where p and f denote the protein-lipid discrimination and the $C:N_{lipid\text{-}free}$ values, respectively.

$$\delta^{13}C' - \delta^{13}C = p - \left(\frac{p \times f}{C:N} \right) \quad (6)$$

Both linear and non-linear models were fitted by least squares, where normally distributed error terms ($\varepsilon \sim N(0,\sigma^2)$) were assumed. Model selection was carried out based on the lowest AIC and MSE of the estimates. Visual overview of model performance,[8,10,12,18,30] and those specific to skin in Balaenopteridae,[11] was carried out by comparing the predicted values with those derived from lipid-extracted sample. All statistical analyses were performed in R version 2.12.1.[31]

RESULTS

Sampling

Biopsy samples obtained from 22 fin and 32 humpback whales were analyzed. All biopsies comprised a complete epidermis profile and portion of the outer blubber stratum to a depth of 15–25 mm. Samples from six minke whales which stranded on the Irish and British coasts between 1999 and 2010 were also included in the analysis. In total, skin and blubber samples from 60 specimens were analyzed in duplicate, control and lipid-extracted (hereafter referred to as bulk and lipid-free, respectively).

Changes in isotope ratios following lipid extraction

The $\delta^{13}C$ and $\delta^{15}N$ values stratified by both tissue and species were found to be normally distributed before and after lipid extraction. With the exception of the minke whale data, Levene's test for variance indicated that sample variances for $\delta^{13}C$ and $\delta^{15}N$ values did not significantly differ due to lipid extraction within species or tissues. Two-tailed t-tests and Wilcoxon's signed rank tests were thus used to test for significant differences in stable isotope ratios due to lipid extraction. Significant increases in $\delta^{13}C$ values were found for both tissues in all species (Table 1, Figs. 1 and 2). The change in $\delta^{13}C$ values due to lipid extraction was found to be higher for those tissue samples with higher C:N ratios, but this relationship was not observed for changes in $\delta^{15}N$ values (Fig. 1). Increases in $\delta^{15}N$ values (mean ± SD) were significant for fin whale (1.1 ± 1.5 ‰) but not for humpback whale blubber. Significant increases in $\delta^{15}N$ values, following lipid extraction, were detected in skin for minke whales only (1.6 ± 0.1 ‰). While fin whale skin showed a decrease in $\delta^{15}N$ values after lipid extraction; this was less than the analytical precision. A small sample size for minke whale blubber samples precluded their inclusion in the above statistical comparisons.

C:N values for lipid-extracted skin and blubber

Following lipid extraction, the mean C:N ratio was not consistent between species and tissues (Table 2). These C:N values were higher for skin (fin, 3.67 ± 0.35; humpback, 3.30 ± 0.17; minke, 3.24 ± 0.22) than for blubber (fin, 3.15 ± 0.25; humpback, 2.87 ± 0.06; minke, 3.06 ± 0.10). Fin whale C:N values were higher than those for either humpback or minke whale for both tissues. Our C:N values estimated directly from lipid-extracted skin were similar to the pooled species mean of 3.2 presented in Lesage *et al.*[11] (Table 2). By extrapolation, it was possible to

Table 1. Mean (± SD) $\delta^{13}C$ and $\delta^{15}N$ values of skin and blubber of bulk and lipid-extracted samples by species

		Skin						Blubber					
Isotope Ratio	Species	*n*	Bulk	SD	Lipid-extracted	SD	*P*	*n*	Bulk	SD	Lipid-extracted	SD	*P*
$\delta^{13}C$	Fin	22	–20.2	1	–18.2	0.5	<0.01	22	–22.1	1.4	–15.4	0.8	<0.01
	Humpback	32	–21.3	1.4	–19.6	1.2	<0.01	32	–23.2	2.3	–17.3	1.4	<0.01
	Minke	6	–21.8	2.1	–17.9	1.8	<0.05^{V}	3	–25.0	1.1	–16.9	2.0	–
$\delta^{15}N$	Fin	22	12.0	1.2	11.9	1.0	0.43	22	11.6	1.6	12.8	1.1	<0.01
	Humpback	32	12.8	1.1	12.9	1.2	0.10	32	13.5	1.2	13.7	1.5	0.39
	Minke	6	13.0	0.6	13.8	0.7	<0.05^{V}	3	12.7	0.8	14.9	1.7	–

P value pertains to paired *t*-tests for the effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values of skin and blubber, but to Wilcoxon's signed rank test in the case of minke whale data, denoted by ^{V}

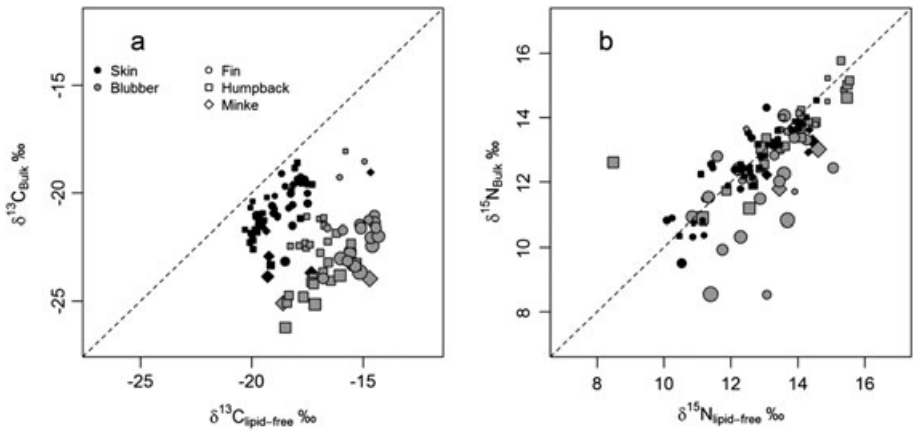

Figure 1. Lipid extraction leads to enrichment of $\delta^{13}C$ values (a) but not $\delta^{15}N$ values (b) for skin. Points are plotted in proportion to log(C:N) values to illustrate that as the lipid-free $\delta^{13}C$ values fall below parity (dashed line); they are more enriched in ^{13}C relative to the untreated (bulk) samples for all species and tissues. No such patterns emerged in $\delta^{15}N$ values.

estimate the theoretical C:N value for which the change in $\delta^{13}C$ values was zero, hereafter referred to as $C{:}N_{lipid\text{-}free}$, for some models only (Table 3). The $C{:}N_{lipid\text{-}free}$ values (upper, lower 95% confidence intervals) derived from Eqn. (6) were within the mean ± one SD of the empirical $C{:}N_{lipid\text{-}free}$ values for fin, humpback and minke whale skin (3.2 (2.6, 3.7), 3.3 (2.9, 3.5) and 2.5 (2.1, 3.1)) and for fin and humpback whale blubber (2.6 (2.6, 3.0) and 2.8 (2.6, 3.0)), respectively. The $C{:}N_{lipid\text{-}free}$ values derived from Eqn. (4) were comparable with empirical values for humpback whale skin only (3.1 (1.7, 5.2)) (Tables 2 and 3).

Lipid normalization models for $\delta^{13}C$ values

Most model predictions underestimated the change in $\delta^{13}C$ values due to lipid extraction compared with the observed values for skin. The models tended to overestimate the change in $\delta^{13}C$ values at higher C:N values, i.e., for blubber. As indicated by both AIC and MSE values, non-linear models performed better for most species and tissues. In general, the levels of error around the model estimates were high. Most models (Eqns. (3), (4), (5) and (6)) consistently underestimated the change in $\delta^{13}C$ values in skin, while some models (Eqns. (3), (4) and (5)) overestimated that change in blubber (Fig. 3). These differentials were species-specific. For example, in fin whales, overestimations ranged from means (± SD) of <0.1‰ (±0.5) for Eqn. (1) to 0.7 ‰ (±0.5) in Eqn. (4) for skin, and ranged from means of <0.1 ‰ (±0.7) for Eqn. (1) to 1.2 ‰ (±1.1) for Eqn. (3) for blubber. The non-linear model by Kiljunen *et al.*[10] (where $\delta^{13}C' - \delta^{13}C$ values are predicted by regressing C:N with the parameters *D* and *I*), gave a robust fit for both skin and blubber (Table 3). Equations (1), (5) and (6) provided the closest and most consistent predictions to parity with observed shifts in $\delta^{13}C$ values following lipid extraction. Equation (6) gave the highest percentage of predicted values fitted within 0.5 ‰ of the lipid-extracted values: 94% for humpback skin and 90% for humpback blubber. Despite being the only model previously applied to balaenopterid skin,[11] Eqn. (2) did not perform better than other models for skin (Table 3 and Fig. 4). There was a significant difference found between the slopes for all species for Eqn. (2) ($F_{6,105} = 151.6$, $p < 0.01$), indicating the need for high model specificity among closely related taxa when using this model (cf. Lesage *et al.*[11]).

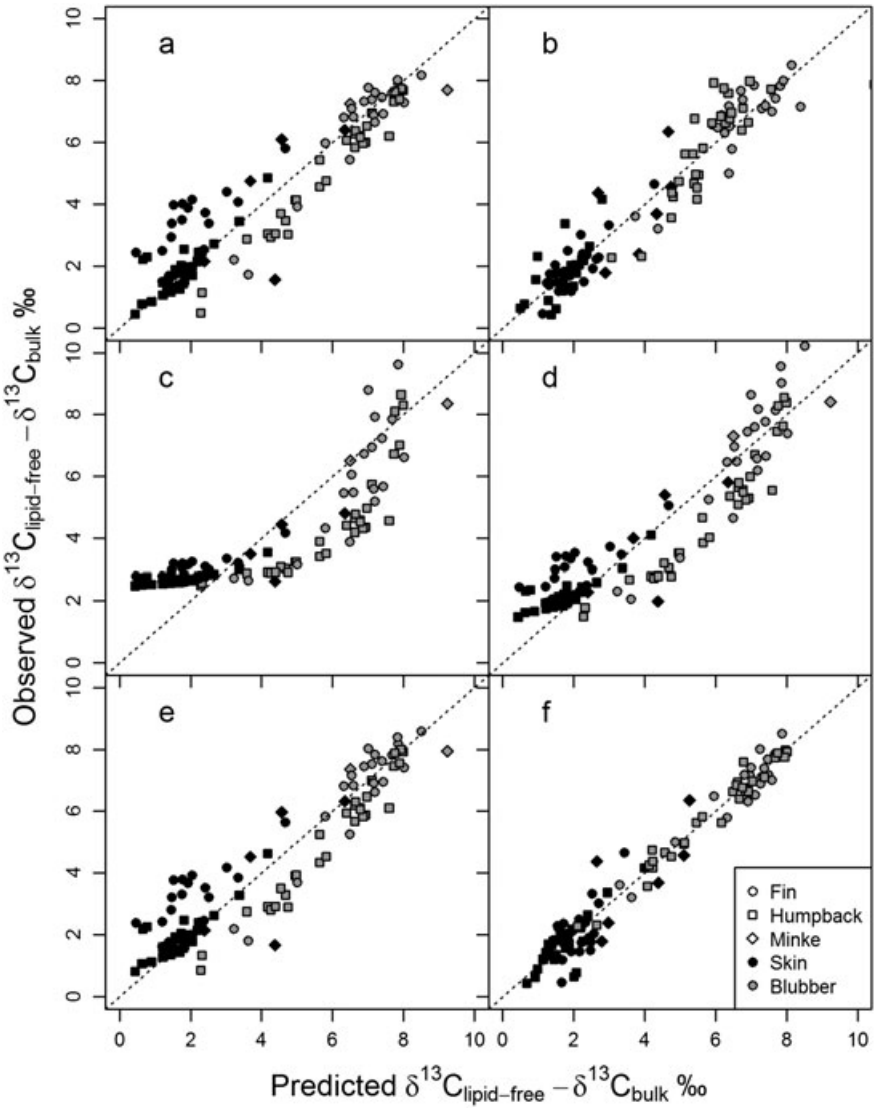

Figure 2. Comparison between observed (lipid-extracted) and predicted (normalized) changes in $\delta^{13}C$ values. Predicted values were obtained by modeling our data using previously published lipid normalization models: (a) Eqn. (1),[10] (b) Eqn. (2),[11] (c) Eqn. (3),[8] (d) Eqn. (4) (corresponding to Eqn. (3) in[12]), (e) Eqn. (5) (corresponding to Eqn. (1)a in[12]), (f) Eqn. (6).[30]

Table 2. Mean (±SD) C:N ratios following lipid extraction for samples used in subsequent analysis

			Bulk C:N			Lipid-extracted C:N		
Tissue	Species	n	Mean	SD	Range	Mean	SD	Range
Skin	Fin	22	5.57	1.31	4.19 - 10.02	3.67	0.35	3.29 - 4.19
	Humpback	32	4.49	0.72	3.67 - 7.68	3.30	0.17	2.99 - 3.90
	Minke	6	7.35	3.60	4.23 - 12.37	3.24	0.22	2.89 - 3.53
Blubber	Fin	22	18.61	9.80	4.32 - 42.55	3.15	0.25	2.77 -3.50
	Humpback	32	10.98	6.54	3.69 - 26.75	2.87	0.06	2.70 - 2.98
	Minke	3	22.22	4.86	18.78 - 25.66	3.06	0.10	2.99 - 3.13

Table 3. Selected linear and non-linear models with the best fits including parameters (±95% confidence intervals) and modeled C:N ratio for lipid-free tissue where computable

Model	Tissue	Species	Parameters	95% CI (lwr)	95% CI (upr)	Parameters	95% CI (lwr)	95% CI (upr)	MSE	% predicted	AIC	$C:N_{lipid\text{-}free}$
			D			*I*						
Eqn. (1)	Skin	Fin	5.4	2.7	8.2	−0.01	−0.2	0.4	0.44	54.4	48.1	3.2
Kiljunen *et al.*[10]		Humpback	7.7	5.8	9.7	−0.05	−0.1	0.02	0.18	193.5	39.4	3.6
(Non-linear)		Minke	5.6	−1.6	12.8	0.23	−0.15	n/a	1.56	16.7	23.3	3.9
	Blubber	Fin	7.4	6.2	8.6	0.18	0.05	0.36	0.2	63.6	29.3	3.7
		Humpback	8.4	7.8	9	0.11	0.06	0.16	0.08	90	14.9	3.6
		Minke	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
			β0			β1						
Eqn (2)	Skin	Fin	–13.7	–18.3	–9.1	–0.78	–1	–0.5	0.23	68.2	33.8	n/a
Lesage *et al.*[11]		Humpback	–8.6	–13.2	–4.1	–0.49	–0.7	–0.3	0.34	77.4	58.8	n/a
(Linear)		Minke	–5.5	–25.5	14.5	–0.43	–1.3	0.5	2.36	16.7	25.7	n/a
	Blubber	Fin	–12.3	–17.8	–6.8	–0.86	–1.1	–0.6	0.5	45.5	49.1	n/a
		Humpback	–6.9	–10.7	–3	–0.55	–0.7	–0.4	1.02	40	89.7	n/a
		Minke	66.5	n/a	n/a	2.39	n/a	n/a	n/a	0	n/a	n/a
Eqn. (3)	Skin	Fin	–0.8	–2.2	0.6	0.5	0.3	0.8	0.6	63.6	271.3	2.8
Post *et al.*[8]		Humpback	–2.1	–3.8	–0.5	0.9	0.5	1.2		84.4		3.1
(Non-linear)		Minke	1.2	–0.3	2.7	0.4	0.2	0.5		33.3		1.5
	Blubber	Fin	4.7	4	5.4	0.1	0.1	0.1		36.7		0.6
		Humpback	3.6	3.1	4.1	0.2	0.2	0.2		40		1.1
		Minke	6.8	4.7	8.9	0	0	0.1		33.3		0.2
			β0			β1						
Eqn. (4)	Skin	Fin	–3.1	–5.4	–0.9	3	1.7	4.4	0.4	68.2	45	2.8
Logan *et al.*[12]		Humpback	–5.2	–6.9	–3.5	4.6	3.5	5.8	0.2	75	39.6	3.1
(Linear)		Minke	–1.1	–6.9	4.7	2.6	–0.4	5.6	1.4	33.3	22.4	1.5
	Blubber	Fin	1.2	0	2.3	2	1.6	2.4	0.3	59	38.9	0.6
		Humpback	–0.2	–1.1	0.6	2.7	2.4	3.1	0.3	16.7	53.1	1.1
		Minke	2.7	–96.8	102.2	1.6	–27.7	30.9	2.8	33.3	14.3	0.2
			a			*b*						
Eqn. (5)	Skin	Fin	1.9	n/a	n/a	–13.47	n/a	n/a	0.3	68.2	42.6	6.9
Logan *et al.*[12]		Humpback	9.4	4.7	n/a	–29.6	n/a	–16.1	0.2	93.6	40.9	3.2
(Non-linear)		Minke	3.9	n/a	n/a	–34.7	n/a	n/a	2.3	0	25.9	8.9
	Blubber	Fin	6.8	n/a	n/a	–64	n/a	n/a	1.4	36.4	71.8	9.5
		Humpback	8.9	8.5	9.37	–25.6	–27.8	–22.7	0.1	86.7	16.8	2.9
		Minke	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
			C									
	Skin	Fin	–6.9	n/a	n/a							
		Humpback	2.4	–1.6	n/a							
		Minke	–9.5	n/a	n/a							
	Blubber	Fin	–9.5	n/a	n/a							
		Humpback	–0.2	–1.1	0.922							
		Minke	n/a	n/a	n/a							
			p			*f*						
Eqn. (6)	Skin	Fin	5.1	3.9	6.298	3.2	2.6	3.7	0.3	50	192.9	3.2
Fry[30]		Humpback	7	5.4	8.641	3.3	2.9	3.5		93.8		3.3
(Non-linear)		Minke	6.6	5.5	7.794	2.5	1.8	3.1		0		2.5
	Blubber	Fin	8.4	8	8.787	2.6	2.2	3		68.2		2.6
		Humpback	8.9	8.5	9.389	2.8	2.6	3		90		2.8
		Minke	10.2	8.5	11.971	5.6	1.5	8.5		0		5.6

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Model choices

Of the models considered for lipid normalization of skin, Eqn. (6) was the most appropriate given the overall higher percentage of fitted values (Table 3), and the logical parameters of the model which allows for the derivation of $C:N_{lipid\text{-}free}$. For blubber, non-linear models fitted the data better than linear ones (Fig. 4). Of the models tested, Eqn. (6) was again the most appropriate for normalizing $\delta^{13}C$ values in blubber. This two-parameter model provided the lowest AIC and MSE values, along with the highest percentage of values predicted to within 0.5 ‰ of the observed change in $\delta^{13}C$ values due to lipid extraction. Furthermore, this model includes protein-lipid discrimination values, which allows for greater model specificity. However, no models satisfactorily fitted the data such that predicted lipid-extracted $\delta^{13}C$ values could be used for, e.g., mixing models given the high level of error introduced (Table 3).

Changes in $\delta^{15}N$ values due to lipid extraction

While the changes in $\delta^{15}N$ values before and after lipid extraction were found to be significant only for blubber in fin whales and skin in minke whales (Fig. 1 and Table 1), those changes for skin were mostly increases. This is consistent with a loss of solvent-soluble amino acids that are depleted in ^{15}N. While the observed changes in blubber were greater, they were not as unidirectional, making it difficult to account for these changes.

When species were pooled, 47 % and 66 % of the $\delta^{15}N_{lipid\text{-}free}$ values for skin and blubber, respectively, were greater than the precision of the instrument (0.2 ‰). The relationships between both C:N and $\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk}$ values and the change in $\delta^{15}N$ values were examined by least-squares regression. In all instances explanatory variables were normally distributed and comparison of residual and fitted values indicated constant variance. The non-linear relationship $\log a(\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk})$ best explained the change in $\delta^{15}N$ values due to lipid extraction in skin, where a was found to be significantly different from zero ($t = 13.93$, 58 d.f., $p < 0.01$) and was estimated to be 0.61 (95% CI; 0.52, 0.70) (Fig. 5). The change in $\delta^{13}C$ values was poor at explaining the corresponding change in $\delta^{15}N$ values for blubber (intercept = –0.18 (95% CI; –0.60, 0.69), giving a slope (0.12 (95% CI; 0.00, 0.07) which was not significantly different from zero ($F_{1,51} = 1.20$, $p = 0.28$). The relationships between the change in $\delta^{15}N$ values and both bulk C:N and $C:N_{lipid\text{-}free} - C:N_{bulk}$ (as proxy for lipid content) of bulk skin and blubber samples were examined; however, no significant relationships were found.

DISCUSSION

Effects of lipid concentration on bulk tissue $\delta^{13}C$ values

In accordance with other stable isotope studies on animal tissues, chemical lipid extraction resulted in significant increases in $\delta^{13}C$ values of bulk tissue for both skin and blubber (Fig. 1). Our findings indicate that, despite implementing high model specificity, lipid normalization models introduce high levels of error (range of mean square error for all models considered) in predicted lipid-free $\delta^{13}C$ values for skin (0.10–2.36) and blubber (0.08–2.8). The intended use of stable isotope data will determine whether or not lipid normalization is appropriate. Some attention has been paid to the effect of introducing tissue-treatment-derived error when estimating prey assignment by mixing models.[10,11,32,33] The probability of erroneous diet assignments arising from the increased error introduced by lipid extraction in cetacean tissues will be dependent on the isotopic distinction between the potential prey items. Lesage *et al.*[11] showed that a 100% prediction error can arise when sample treatment errors exceed 0.5 ‰. While the non-linear models proposed by Fry[30] and Kiljunen *et al.*[10] provided the best fit out of the six models considered, poor fit corresponding to high C:N values was prevalent for all models (Figs. 2 and 5). No model provided a satisfactory fit for $\delta^{13}C$ values to within 0.5 ‰ of the observed values (Figs. 2, 4 and 5). Where the ultimate use for stable isotope values of balaenopterid skin and blubber is in prey-assignment models, we recommend lipid extraction rather than normalization of $\delta^{13}C$ values.

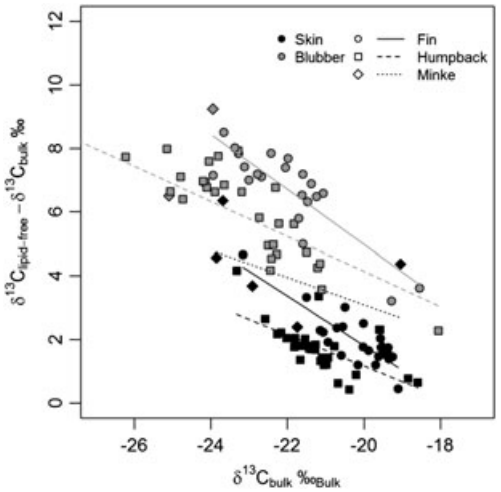

Figure 3. Lipid-normalized $\delta^{13}C$ values as predicted by Eqn. (2):[11] a linear model proposed for lipid normalization of $\delta^{13}C$ values for cetacean skin. The model was also tested for blubber and shows species and tissue-specific effects. All regression coefficients with 95% confidence intervals are presented in Table 3.

C:N ratio values

The C:N ratio provides a useful and low-cost proxy for lipid content and has become the standard explanatory variable for normalizing $\delta^{13}C$ values.[18] However, Fagan *et al.*[34] found no support for the predictive relationship between C:N and percentage lipid in freshwater fishes. The empirical $C:N_{lipid\text{-}free}$ values for fin, humpback and minke whale were 3.7, 3.3 and 3.2, respectively, for skin and 3.2, 2.9 and 3.1 for blubber. These values will provide benchmarks when assessing the effectiveness of the lipid-extraction process in future studies. Two non-linear models (Eqns. (1) and (6)) predicted $C:N_{lipid\text{-}free}$ ratios comparable with those empirical values, confirming that bulk C:N can be a useful explanatory variable for the lipid normalization of balaenopterid skin and blubber (cf. Fagan *et al.*[34]) (Tables 2 and 3; Fig. 4). However, our findings indicate that this relationship breaks down for samples with a high C:N ratio, i.e. >4.5 for skin and >15 for blubber (Fig. 4). The

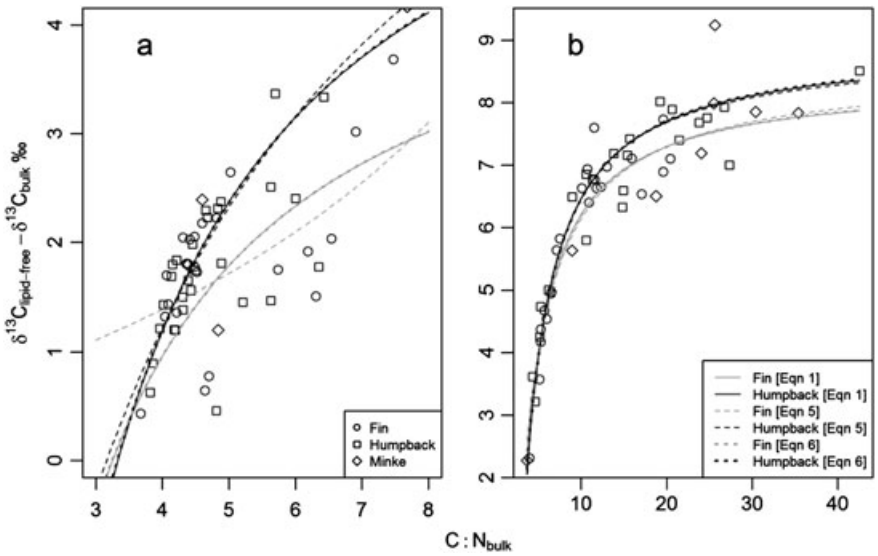

Figure 4. Plots of those three models (Eqns. (1), (5) and (6)) which best described the change in δ^{13}C values with C:N for fin and humpback skin (a) and blubber (b) based on lowest AIC and MSE (Table 3).

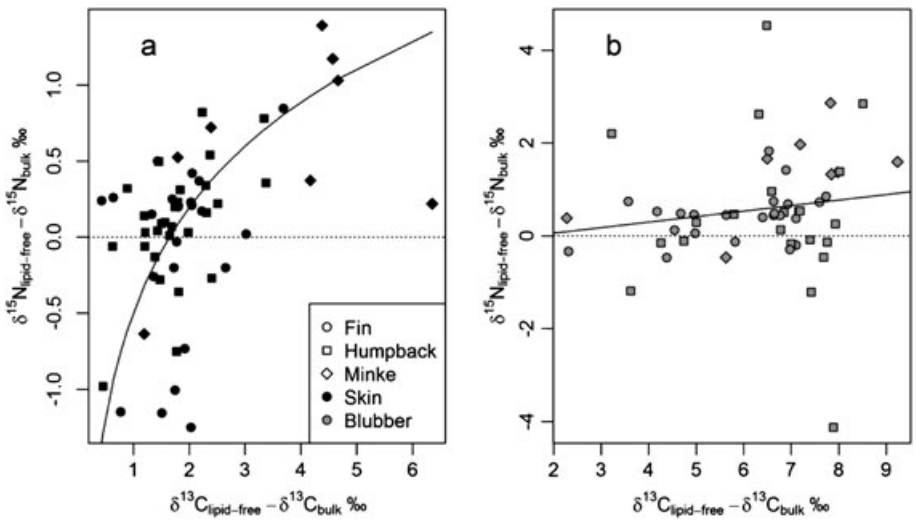

Figure 5. Relationship between the changes in δ^{15}N and δ^{13}C values due to lipid extraction for (a) skin and (b) blubber. Lines were fitted using least squares regression where response variable were normally distributed and residual versus fitted values indicated constant variance. See Results section for model parameters and tests of significance. The broken line denotes no change in δ^{15}N values.

differential tissue- and species-specific C:$N_{lipid\text{-}free}$ values presented here (Fig. 4) indicate that this value should be measured empirically for tissues or species not yet investigated. We have shown that even taxonomically similar species can exhibit differences in (mean ± SD) C:$N_{lipid\text{-}free}$ values of tissues, particularly between skin in fin (3.7 ± 0.4) and minke (3.2 ± 0.2) and between blubber in fin (3.2 ± 0.3) and humpback (2.9 ± 0.1) whales.

Changes in δ^{15}N values following lipid extraction

The observed (mean ± SD) changes in δ^{15}N values following chemical lipid extraction for fin, humpback and minke whales, respectively, were 0.1 ‰ ± 0.7, 0.1 ‰ ± 0.3 and 0.8 ‰ 0.4 for skin and 1.1 ‰ ± 1.5, 0.1 ‰ ± 0.9 and 1.6 ‰ ± 0.1 and were greater than the level of instrumental precision achieved for the δ^{15}N values (0.2 ‰). Sotiropoulos *et al.*[14] proposed that an increase in δ^{15}N values occurs due to the loss of structural lipids that contain nitrogen-rich amino acids. However, those changes observed for skin, in particular, in this study were not unidirectional. The mean shifts in δ^{15}N values observed for skin and blubber were small and were similar to those reported previously for cetacean skin (≤1.6 ‰[11]) and for fish and invertebrates (–0.14 to 1.00 ‰[20]), but were high enough to warrant caution on their use in mixing models.[11]

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Similar to Lesage *et al.*,[11] the present study found changes in δ^{15}N values in skin following lipid extraction. However, we have shown that the magnitude of these changes varies by species within the family Balaenopteridae. The unpredictable changes in δ^{15}N values will have implications when the ultimate goal is to use stable isotope data from lipid-extracted tissues in a diet mixing model, given the increased error introduced into the model.[10,11] The tissue-specific differences in changes to the δ^{15}N values are likely to have arisen from the differential structure and relative abundances of lipid compounds between the tissues.[14,35] Polar structural lipid compounds such as glycolipids, phospholipids and sphingolipids are closely linked with bound amino acids which are depleted in ^{15}N, and will be removed by polar solvents.[35] An incidental co-extraction of structural lipids and their associated amino acids has been widely proposed as the most likely explanation for the increase in δ^{15}N values.[14,15,35] It has been shown, however, that the extraction technique is actually of little consequence.[22] Assuming that C:N is a reliable proxy for the amount of lipid being removed, if increases in δ^{15}N values occurred due to co-extraction of amino acids in structural lipids, the changes in δ^{15}N values and C:N should be correlated. However, the non-significant relationship between these variables does not support the theory of ^{15}N enrichment due to co-extraction of amino acids and lipids in the present study.

Changes in stable nitrogen isotope values have also been found in egg yolk studies.[13,32] The effect was attributed to the migratory nature of the birds in question, whereby the nutrients assimilated into the egg yolk were sourced from multiple isotopically distinct environments.[13,32] Such a scenario is a violation of the fundamental assumption of most lipid normalization models – that lipid and protein are derived from the same source, in order that the lipid-protein discrimination value D is constant. Balaenopterid whales also undertake long-distance migrations,[36,37] and may therefore feed along a broad isotopic cline. If lipids and proteins have differential assimilation rates, lipid normalization of δ^{13}C values in the tissues of balaenopterid whales may be inappropriate.

CONCLUSIONS

Analysis of δ^{13}C and δ^{15}N values is generally undertaken simultaneously for individual tissue samples. However, our findings, which corroborate those of Lesage *et al.*,[11] demonstrate that pre-analysis chemical lipid extraction can have adverse and unpredictable effects on the δ^{15}N values of blubber (cf. Ingram *et al.*[20]). This effect may be related to the lipid content and hence the C:N ratio of the sample. Mathematical lipid normalization of those skin samples with higher C:N values than those analyzed here might also be inappropriate. It is recommended, therefore, that sample aliquots are analyzed separately: with chemical lipid extraction performed prior to measurement of δ^{13}C values, and that δ^{15}N values are determined in tissues with no chemical lipid extraction. We advocate caution against retrospective correction for the effects of lipids on δ^{13}C values in any tissues, before both species- and tissue-specific normalization models have been tested. Furthermore, caution must be taken in comparing lipid-normalization models where the lipid-extraction techniques may differ between studies.[21,22] Thus, duplicate measurement of lipid-extracted and bulk δ^{13}C and δ^{15}N values, respectively, is recommended for the stable isotope analysis of skin and blubber in balaenopterid whales. While analysis of duplicate samples doubles the cost of the analysis, this is required to maximize the accuracy of the results. This study reaffirms the need for more methodological testing, particularly lipid normalization models, before the underlying assumptions of stable isotope analysis in its application to ecological problems can be met.

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