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Stable isotope analysis: modelling lipid normalization for muscle and eggs from arctic mammals and birds

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Summary

1. Lipids are more depleted in ^{13}C than proteins. Variable lipid contents in tissues affect therefore the measurements of stable carbon isotope ratios. Model based (also called mathematical) normalization has been suggested to correct $\delta^{13}C$ values using the ratio of carbon to nitrogen (C/N) as a proxy for lipid content. This approach has not been thoroughly validated for terrestrial animals and it is not clear to what extent it is generally applicable or species/tissue specific.

2. Ratios of stable carbon isotopes ($\delta^{13}C$) were obtained for muscle samples of 22 mainly terrestrial arctic mammal and bird species and for egg samples of 32 bird species from nine sites in the circumpolar Arctic. We used linear and nonlinear equations to model the difference in $\delta^{13}C$ between samples from which lipids had been extracted chemically and bulk tissue samples. Models were compared on the basis of a model selection criterion (AIC) and of prediction error as estimated by cross-validation.

3. For muscle samples, a linear and a nonlinear equation performed equally well. The observed values were also well predicted by a previously published general equation for aquatic organisms. For egg samples, a nonlinear equation fitted separately to waterfowl and non waterfowl bird species fitted the data best. Prediction errors were, however, larger than for muscle samples.

4. The generality of the inferred normalization equations was assessed by applying them to a second data set from a similar ecosystem, but produced in the frame of another study. The predicted lean $\delta^{13}C$ values were within 0·5‰ of the observed values for 73% of the muscle samples, but only for 27% of the egg samples.

5. Based on our results, we recommend model based normalization of $\delta^{13}C$ values as an economic way to deal with varying lipid contents in muscle samples of mammals and birds. For egg samples, on the contrary, model based predictions had large errors. Therefore, we recommend chemical lipid extraction in order to estimate lipid-free $\delta^{13}C$ values for egg content.

Key-words: $\delta^{13}C$, $\delta^{15}N$, C/N ratio, cross-validation, lipid content, lipid correction, lipid extraction, mathematical normalization, model selection, trophic relationships

Introduction

Stable isotope analysis (SIA) is a useful tool for a wide spectrum of ecological applications including palaeoecology, migration studies and different aspects of trophic ecology (e.g. Kelly 2000; Rubenstein & Hobson 2004; West *et al.* 2006; Inger & Bearhop 2008). Studies of trophic relations applying SIA most commonly use the stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) relative to reference standards (expressed as $\delta^{13}C$ and $\delta^{15}N$; Kelly 2000). The diet of individuals or populations can be inferred based on the principle that 'you are what you eat', i.e. that the isotopic ratios in the tissues of consumers reflect the mixture of the isotopic ratios present in the different food items consumed (DeNiro & Epstein 1978, 1981).

In a recent review Wolf, Carleton & Martinez del Rio (2009) emphasized the need for more laboratory experiments to improve and refine the use of stable isotopes in ecology. A technical concern in SIA is that lipids are more depleted in ^{13}C

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relative to proteins and other tissues due to the different biochemical pathways involved in their respective synthesis (DeNiro & Epstein 1977). The $\delta^{13}C$ measured for a certain sample does thus not only reflect the diet of an individual, but is also related to the lipid content of the tissue sample: Fatty tissues have lower $\delta^{13}C$ values than lean tissues. This is first of all a concern for tissues with varying lipid content such as muscle, egg content or whole body samples. Two different approaches have been suggested to increase the accuracy of $\delta^{13}C$ measurements. Lipids can either be removed chemically from the samples prior to SIA (Logan & Lutcavage 2008; Bodin *et al.* 2009), or the isotope ratios can be corrected for lipid content based on a normalization model (Post *et al.* 2007; Logan *et al.* 2008). Whilst appearing to be a logical solution, chemical lipid extraction can affect the measurements of $\delta^{15}N$, because the solvents used can wash out nitrogen containing compounds (Sotiropoulos, Tonn & Wassenaar 2004; Kojadinovic *et al.* 2008; Mintenbeck *et al.* 2008). Some authors thus cautioned that $\delta^{13}C$ and $\delta^{15}N$ should be measured from separate sub samples of lipid free and bulk tissue samples, respectively (Sweeting, Polunin & Jennings 2006; Oppel *et al.* 2010). However, this procedure leads to a considerable increase in work load and price. Therefore, model based lipid normalization seems appealing.

To use a model for correcting $\delta^{13}C$ values, a relationship between the increase in $\delta^{13}C$ resulting from lipid extraction and the lipid content of the sample is needed (McConnaughey & McRoy 1979). This relationship can subsequently be used to normalize the $\delta^{13}C$ values of other samples for lipid content. Most normalization equations use the elemental ratio of C to N (C/N) as a proxy for lipid content in bulk tissue. C/N is easily derived from the percentage element weight commonly reported when measuring isotope ratios with a mass spectrometer. It is proportional to lipid content because lipids are composed mainly of C and most lipid types contain no N (Bodin, Le Loc'h & Hily 2007; Post *et al.* 2007). Constant, linear and nonlinear equations relating the difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$) to C/N have been used for lipid normalization (Kiljunen *et al.* 2006; Post *et al.* 2007; Ricca *et al.* 2007; Kojadinovic *et al.* 2008; Logan *et al.* 2008; Oppel *et al.* 2010). The relation of $\Delta\delta^{13}C$ to C/N is close to linear for C/N values up to about seven (Post *et al.* 2007; Mintenbeck *et al.* 2008) and flattens out for higher values (Ricca *et al.* 2007; Logan *et al.* 2008). Asymptotic, nonlinear equations are thus more appropriate for samples with high lipid content and different models have been proposed (Kiljunen *et al.* 2006; Sweeting, Polunin & Jennings 2006; Logan *et al.* 2008).

The performance of model based normalization has been assessed most thoroughly for aquatic and marine organisms. McConnaughey & McRoy (1979) estimated a normalization equation from a variety of marine animals and proposed it as generally applicable, at least to marine organisms. Post *et al.* (2007) presented a linear relationship between C/N and $\Delta\delta^{13}C$ with a good fit for aquatic animals, but much less so for terrestrial animals, where the sample size was low (only one value for each of 13 species). They concluded that a general normalization equation can be applied to aquatic organisms, but should be better validated for terrestrial animals. Several more detailed studies have shown, however, that the relation of $\Delta\delta^{13}C$ to C/N can vary among groups of organisms (Kiljunen *et al.* 2006; Kojadinovic *et al.* 2008), among tissues (Logan *et al.* 2008) and even between rather closely related species (Mintenbeck *et al.* 2008), thus strongly questioning the general applicability of lipid normalization. For eggs, lipid correction with a constant has been suggested (Ricca *et al.* 2007). More recently, this has been shown to be inadequate, as the relation of $\Delta\delta^{13}C$ to C/N varied strongly between sedentary and migratory birds (Oppel *et al.* 2010).

The effect of different preservation methods on $\delta^{13}C$ and $\delta^{15}N$ must also be considered when undertaking SIA (Sweeting, Polunin & Jennings 2004; Kelly, Dempson & Power 2006). Previous studies investigating lipid normalization used fresh, frozen or dried samples. However, sample preservation in 70% ethanol is often a practical alternative (Barrow, Bjorndal & Reich 2008). Post *et al.* (2007) did not recommend lipid normalization for samples conserved in ethanol, a caution which would considerably limit the applicability of this method.

The aim of this study is to assess whether model based normalization is a useful approach to correct $\delta^{13}C$ values for samples of varying lipid content originating from mostly terrestrial arctic mammals and birds. In addition, we identified a threshold for C/N below which samples can be considered lean and no lipid correction is necessary, assessed the effect of chemical lipid extraction on $\delta^{15}N$ in muscle and eggs, and discussed whether lipid normalization can be used for samples stored in ethanol. A first data set was used to fit lipid normalization equations for muscle and egg content, and to test whether the relation of $\Delta\delta^{13}C$ to C/N differs among groups of species. We then compared our equations with previously published models. Finally, we use a second data set from a similar ecosystem but produced in another laboratory, to test the generality of our findings. We conclude with a set of recommendations on how to deal with lipids in studies of trophic ecology using SIA.

Material and methods

SAMPLE COLLECTION

The samples were collected in the frame of two projects addressing trophic interactions in terrestrial arctic tundra ecosystems. Both projects are part of the International Polar Year initiative Arctic Wildlife Observatories Linking Vulnerable EcoSystems (Arctic-WOLVES). In both projects, a large number of samples were analyzed for stable isotope ratios of C and N. A subset of 229 samples is the subject of this study. The majority of the subset was chosen at random, but for muscle tissue the random samples were, however, supplemented by samples chosen because of their high C/N ratios, indicating high lipid content.

A first data set (A) was used to fit the normalization equations and consisted of muscle samples from several mammal and bird species, as well as of egg samples from several bird species (Table 1). Samples were collected in eight localities in the Eurasian Arctic, from Svalbard (Norway, 78°N, 17°E) to Wrangel Island (Russia, 71°N, 180°E). To test the generality of the obtained equations, we applied them to a

Table 1. Average, minimum and maximum values of the carbon : nitrogen ratio (C/N), the difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$), and the difference in $\delta^{15}N$ between lipid extracted and bulk tissue samples ($\Delta\delta^{15}N$) for each tissue and species

Species	*n*	C/N (range)	$\Delta\delta^{13}C$ (range)	$\Delta\delta^{15}N$ (range)	loc
Data set A, muscle					
Dicrostonyx torquatus	2	4·78 (4·40; 5·16)	1·65 (1·63; 1·67)	−0·07 (−0·23; 0·10)	Y
Dicrostonyx vinogradovi	2	3·91 (3·70; 4·12)	0·38 (0·36; 0·39)	−0·25 (−0·26; −0·24)	W
*Lemmus lemmus**	5	5·01 (4·08; 6·77)	0·01 (−0·76; 0·54)	0·49 (−0·09; 2·16)	V
*Lemmus sibiricus**	2	5·31 (4·63: 5·99)	1·14 (0·58; 1·69)	−0·03 (−0·06; 0·00)	W
Microtus oeconomus	9	4·04 (3·26; 5·08)	0·66 (0·04; 1·55)	0·01 (−0·21; 0·14)	N, V
Myodes rufocanus	4	4·22 (3·35; 4·98)	0·79 (−0·10; 1·76)	−0·12 (−0·26; −0·02)	V
Sorex minutus	1	3·24	−0·24	0·01	N
Lepus timidus	4	3·43 (3·33; 3·52)	−0·16 (−0·54; 0·21)	−0·11 (−0·61; 0·20)	N
Rangifer tarandus	23	4·12 (3·23; 8·14)	0·81 (0·11; 2·51)	−0·03 (−0·52; 0·26)	N, S
Mustela nivalis	1	6·24	2·67	−0·18	V
Vulpes lagopus	9	4·21 (3·24; 5·94)	1·40 (−0·12; 3·30)	−0·02 (−0·31; 0·12)	N, S, W
Odobenus rosmarus	2	3·24 (3·18; 3·29)	0·06 (0·03; 0·08)	−0·05 (−0·18; 0·08)	W
Anthus cervinus	1	3·18	0·00	0·20	Y
Anthus pratensis	1	3·58	0·06	0·02	Y
Carduelis flammea	1	3·55	−0·23	−0·03	Y
Plectrophenax nivalis	1	3·94	1·09	0·03	W
Anas acuta	1	4·42	0·70	0·02	N
Clangula hyemalis	1	4·09	0·78	0·14	Y
Somateria mollissima	3	5·41 (3·76; 7·71)	1·19 (0·56; 1·90)	−0·21 (−0·48; 0·20)	W
Anser caerulescens	2	4·43 (3·43; 5·42)	0·38 (0·28; 0·48)	−0·12 (−0·12; −0·11)	W
Pluvialis squatarola	1	4·16	1·18	−0·06	W
Lagopus muta	2	5·08 (4·65; 5·51)	0·9 (−0·06; 1·86)	−0·20 (−0·25; −0·14)	S
Bubo scandiaca	1	4·33	1·58	0·70	W
Total	79				
Data set A, egg content					
Uria lomvia	1	3·96	0·00	−0·10	S
Anas crecca	1	12·69	3·58	1·35	Y
Anas penelope	1	10·41	3·59	0·64	Y
Clangula hyemalis	4	10·27 (8·56; 12·61)	3·56 (3·32; 3·94)	0·86 (0·53; 1·58)	D, Y
Somateria mollissima	3	7·29 (5·95; 8·45)	1·30 (0·93; 1·84)	0·24 (0·01; 0·45)	W
Somateria spectabilis	3	10·38 (8·87; 11·96)	2·88 (2·57; 3·13)	0·65 (−0·76; 1·49)	L, T
Anser albifrons	4	14·09 (9·34; 20·22)	2·89 (1·12; 3·78)	1·01 (0·62; 1·57)	T
Anser brachyrhynchus	3	8·45 (3·34; 11·71)	2·58 (0·28; 3·83)	0·68 (0·31; 1·03)	S
Branta bernicla	4	9·36 (5·04; 13·71)	3·41 (1·33; 5·07)	0·71 (−0·02; 1·32)	T
Branta leucopsis	2	10·53 (10·50; 10·55)	4·26 (3·66; 4·85)	1·15 (1·14; 1·15)	S
Anser caeruluscens	5	9·584 (8·08; 10·66)	2·73 (2·23; 3·08)	0·19 (−0·20; 0·42)	W
Larus heuglini	4	9·31 (6·78; 16·54)	3·97 (3·20; 5·76)	0·54 (0·13; 1·46)	T
Larus hyperboreus	1	18·43	4·53	1·25	T
Stercorarius longicaudus	1	9·5	3·88	0·93	Y
Stercorarius parasiticus	2	9·22 (7·01; 11·42)	3·60 (3·47; 3·73)	0·17 (−0·05; 0·39)	N, Y
Calidris alpina	2	9·49 (8·19; 10·79)	4·20 (4·08; 4·32)	0·58 (0·54; 0·61)	T
Calidris minuta	1	7·7	4·03	0·25	T
Calidris temminckii	2	6·94 (4·60; 9·27)	2·59 (1·29; 3·88)	0·28 (0·13; 0·43)	N, L
Charadrius hiaticula	2	8·27 (7·71; 8·82)	3·25 (2·63; 3·87)	0·64 (0·59; 0·68)	N, T
Phalaropus fulicarius	1	11·81	5·02	0·59	T
Pluvialis apricaria	1	7·72	3·33	0·35	Y
Pluvialis fulva	2	10·87 (10·28; 11·46)	4·52 (4·32; 4·72)	0·77 (0·54; 0·99)	T
Pluvialis squatarola	3	10·42 (5·73; 12·97)	3·42 (1·51; 4·44)	0·93 (0·59; 1·15)	T
Tringa glareola	1	10·14	5·03	0·90	Y
Gallinago sp.	1	6·85	2·74	0·88	N
Lagopus lagopus	3	7·21 (4·72; 10·24)	3·31 (2·81; 4·1)	0·12 (−0·32; 0·75)	N, Y
Lagopus muta	3	8·75 (7·87–9·23)	4·40 (4·07; 4·57)	0·35 (0·06; 0·67)	T
Anthus cervinus	1	9·30	4·00	1·00	Y
Anthus sp.	1	6·15	1·98 (1·98; 1·98)	−0·15 (−0·15; −0·15)	N
Calcarius lapponicus	1	7·42	2·85 (2·85; 2·85)	−0·04 (−0·04; −0·04)	N
Emberiza sp.	1	5·84	1·72 (1·72; 1·72)	0·63 (0·63; 0·63)	N
Plectrophenax nivalis	3	10·35 (9·03–12·7)	4·06 (3·81; 4·19)	0·57 (0·28; 0·88)	T
Total	68				

Table 1. (Continued)

Species	*n*	C/N (range)	$\Delta\delta^{13}C$ (range)	$\Delta\delta^{15}N$ (range)	loc
Data set B, muscle					
Dicrostonyx groenlandicus	6	3·48 (3·20; 3·80)	0·25 (−0·90; 0·90)	0·13 (0·00; 0·40)	
Lemmus trimucronatus	30	3·43 (3·00; 4·30)	−0·01 (−1·1; 0·80)	0·01 (−0·90; 0·60)	
Pusa hispida	7	3·59 (3·20; 4·30)	0·44 (−0·20; 2·00)	0·11 (−0·50; 0·40)	
Anser caerulescens	19	3·49 (3·30; 3·80)	−0·06 (−0·09; 0·70)	0·12 (−0·50; 0·40)	
Calcarius lapponicus	9	3·70 (3·20; 4·90)	0·48 (0·10; 1·10)	0·09 (−0·20; 0·40)	
Total	71				
Data set B, egg content					
Anser caerulescens	11	9·45 (5·60; 14·70)	1·87 (−0·10; 3·10)	0·50 (0·00; 0·80)	

Data set A is composed of samples from eight sites in arctic Eurasia and data set B comprises samples from Bylot Island (Nunavut, Canada; 73°N 80°W). For data set A, loc indicates the location where the samples were collected: S – Svalbard (Norway; 78°N, 15·5°E), V – Varanger Peninsula (Norway; 70·4°N, 30°E), N – Nenetskiy AO (Russia; 68·3°N, 53·2°E), D – Dolgiy Island (Russia; 69·25°N, 59·1°E), Y – southern Yamal (Russia; 62·2°N, 69·15°E), T – western Taymyr (Russia; 74·15°N, 86·8°E), L – Lena Delta (Russia; 72·7°N, 127°E), W – Wrangel Island (Russia; 71°N, 180°E).
*The *Lemmus sibiricus* samples and three of the *L. lemmus* samples had C/N values above 4 after lipid extraction and were thus excluded from the analysis. An additional *L. lemmus* sample was an outlier with C/N = 6·77 and $\Delta\delta^{13}C$ = 0·54.

second data set from a similar ecosystem, but processed for another project, consisting of samples from Bylot Island (Canada, 73°N, 80°W). This data set (B) comprised muscle samples from three mammal and two bird species, as well as snow goose egg samples (Table 1). All samples were from freshly dead animals and were stored in 70% ethanol.

SAMPLE PREPARATION AND STABLE ISOTOPE ANALYSIS

Muscle samples were frozen at −80 °C, dried for at least 48 h at 60 °C and ground to a fine powder using a Mixer Mill (MM301; Retsch GmbH & Co. Haan, Germany; data set A) or a mortar and pestle (data set B). Whole egg samples were homogenized before being dried for at least 48 h at 60 °C. Grinding fatty muscle samples and whole egg resulted in a more or less solid paste. After grinding, samples were subdivided into two aliquots. One aliquot was directly weighed for SIA and the second was subjected to lipid extraction using a modification of the Bligh & Dyer (1959) method. For data set A lipid extraction was performed as follows: 1 ml of a 2:1 chloroform–methanol mixture was added to the powdered samples (solvent to powder ratio ≥ 20). The samples were then vigorously shaken several times and, after 15 min, centrifuged for 10 min. The supernatant was discarded and the procedure repeated at least once or until the supernatant was clear and colourless after centrifuging. Finally, remains of solvent were evaporated for 24 h. Lipid extraction for data set B was conducted at the Stable Isotopes in Nature Laboratory (SINLAB), New Brunswick (Canada), following a similar procedure. All samples were weighed to the nearest 0·01 mg, packed into tin capsules and sent to SINLAB, where they were combusted in a Carlo Erba NC2500 Elemental Analyzer before delivery to a Finnigan Mat Delta Plus mass spectrometer (Thermo Finnigan, Bremen, Germany). Isotope ratios were expressed as ratios in permil (‰) referenced against Peedee belemnite carbonate for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$ (Kelly 2000). C/N ratios were determined from percentage element weight measured by the mass spectrometer. The measurement error was assessed by repeating lipid extraction and SIA of bulk and lipid extracted aliquots for six samples, and precision was found to be good: After lipid extraction, C/N ratios differed on average by 0·05 (max = 0·12), $\delta^{13}C$ by 0·24‰ (max = 0·50‰) and $\delta^{15}N$ by 0·24‰ (max = 0·39‰). The values measured for bulk tissue samples differed more, because the lipid content differed between aliquots of egg content. Consequently the measurement error was also larger for $\Delta\delta^{13}C$ (average error 0·55‰, max = 1·45‰). The measurement variance of the C/N values was much smaller than the range of C/N values before extraction (the maximal error for the lipid extracted samples was 28 times smaller than the range for muscle and 140 times smaller for eggs). It was thus unlikely to bias the estimation of the normalization equations (Fuller 1987).

DATA ANALYSIS

To investigate the potential of lipid normalization, we fitted different equations previously used to assess the relation of $\Delta\delta^{13}C$ to C/N. We used both a constant and a linear model, and in addition we fitted the nonlinear models used by Logan *et al.* 2008. The first model (equation 1a in Logan *et al.* 2008) was modified from McConnaughey & McRoy (1979) by aggregating the parameter for protein-lipid discrimination with the parameters relating $\Delta\delta^{13}C$ to lipid content and lipid content to C/N into three parameters to be estimated from the data (a, b and c)

$$\Delta\delta^{13}C = \frac{a \times (C/N) + b}{(C/N) + c}. \quad \text{eqn 1}$$

The second model (equation 2 in Logan *et al.* 2008) was based on Fry (2002):

$$\Delta\delta^{13}C = p - \frac{p \times f}{C/N} \quad \text{eqn 2}$$

where p represents protein–lipid discrimination and f the C/N value of lipid free tissue. The third model was a log-linear model proposed Logan *et al.* (2008, equation 3) with an intercept β_0 and a slope β_1

$$\Delta\delta^{13}C = \beta_0 + \beta_1 \ln(C/N) \quad \text{eqn 3}$$

All analyses were carried out in R version 2·9·2 (R Development Core Team 2009). Models were fitted by least squares, assuming a constant variance, using the functions lm, nls and nlsList (package nlme in R, Pinheiro & Bates 2000). For muscle samples, we compared a general model to models distinguishing between birds and mammals. For egg samples, we investigated whether subdividing the samples into six or

two groups of species [seabirds, waterfowl, Laridae (gulls and skuas), waders, ptarmigans and passerines; or waterfowl and non waterfowl] would give better predictions. Models were first compared using Akaike's Information Criterion corrected for small sample sizes (AIC_c), and the model with the lowest AIC_c was considered the most appropriate (Burnham & Anderson 2002). Models with a difference in AIC_c to the best model (ΔAIC_c) of less than 2 also have good support. We further compared the predictive error of the different models using *K*-fold cross-validation. The data set was split randomly into *K* sets, of which *K*-1 were used to fit the model and the prediction error was calculated when predicting the last set (Hastie, Tibishrani & Friedman 2001). This procedure was repeated 1000 times. We considered three prediction criteria: mean squared error (MSE), mean absolute error (MAE) and the proportion of predicted values within 0·5‰ of the observed $\Delta\delta^{13}C$ values. This threshold corresponds to twice the average measurement error and smaller differences in $\delta^{13}C$ are likely not to be biologically significant in most ecological applications. As low values of *K* can give biased estimates and large values of *K* may result in high variance of the estimates, we used $K = 5$ and $K = 10$ as recommended by Hastie, Tibishrani & Friedman (2001). In addition, we used the leave-one-out approach, corresponding to cross-validation with *K* equal to the sample size. The $\Delta\delta^{13}C$ values predicted with our equations were also compared to values predicted using published equations (Post *et al.* 2007; Logan *et al.* 2008). Finally the inferred normalization equations were applied to data set B and the predicted $\Delta\delta^{13}C$ values were compared to observed values using the same criteria as above.

The difference in $\delta^{15}N$ between lipid extracted and bulk tissue samples ($\Delta\delta^{15}N$) was summarized by species and tissue type. We used a paired *t*-test to estimate a confidence interval for the difference and a linear model to assess whether there was a relation between $\Delta\delta^{15}N$ and the C/N of the bulk tissue samples.

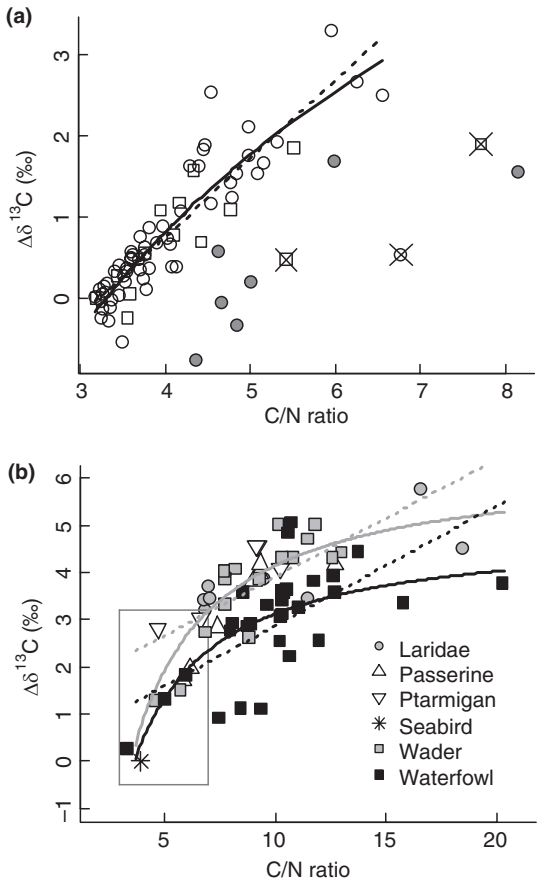

Fig. 1. The difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$) plotted against the ratio of carbon : nitrogen (C/N) for the samples used to fit the normalization equations (data set A). (a) Muscle samples. Open circles represent mammal samples and open squares bird samples. Grey circles represent samples which were excluded because their C/N value after lipid extraction was above 4, and the crosses indicate three additional outliers. The line shows the nonlinear normalization equation (eqn 3) as fitted to the data, and the dotted line shows the linear equation. (b) Egg samples. The symbols represent eggs of different taxonomic groups of birds as indicated in the legend. The lines show nonlinear normalization equations (eqn 2) as fitted to the data for two groups of birds and the dotted lines show linear equations. Black lines indicate equations for waterfowl and seabirds, and grey lines for other species. The square shows the range of the muscle samples used for model fitting.

Results

LIPID NORMALIZATION FOR MUSCLE

In data set A, the C/N ratios of muscle samples ranged from 3·18 to 8·14 (mean = 4·24) for bulk tissue samples and from 2·89 to 4·77 (mean = 3·35) for lipid extracted samples. The distribution of C/N ratios after lipid extraction indicated that samples with values above four were outliers (supplementary Fig. S1). High C/N values after extraction may be due either to incomplete removal of lipids or to a higher than average C/N ratio of lean muscle. $\Delta\delta^{13}C$ was on average 0·76‰ (range = −0·76 to 3·3‰). Fig. 1a shows the relation of $\Delta\delta^{13}C$ to C/N (values for each species are in Table 1). We excluded the seven samples with a $C/N > 4$ after lipid extraction, as most of these samples appeared as outliers also on Fig. 1a. These were five lemmings of the genus *Lemmus* Link, one reindeer (*Rangifer tarandus* L.) and one rock ptarmigan (*Lagopus muta* Montin). Three additional outliers (*Lemmus lemmus* L., *Anser caerulescens* L. and *Somateria mollissima* L.) were removed before fitting the normalization models (Fig. 1a).

Based on ΔAIC_c, mean squared predictive error and mean absolute predictive error as estimated from cross-validation, the nonlinear equation proposed by Logan *et al.* (2008, eqn 3) was the most appropriate for the muscle data. The results from cross-validation using different number of groups ($K = 5$, $K = 10$ and leave-one-out) were similar (Table S1). Although the simplest linear model had considerably less support ($\Delta AIC_c = 5·26$; Table S1), the associated normalization relation was very similar to that shown by eqn 3, and the deviation from linearity in the C/N range covered by our data was not large (Fig. 1a). When comparing model performance by the percentage of $\Delta\delta^{13}C$ values predicted within 0·5‰ of the observed value by cross-validation, the linear equation performed slightly better than eqn 3 (Table 2). All tested models were able to predict between 84% and 87% of the $\Delta\delta^{13}C$ values at this level of precision. Models distinguishing between mammals and birds had lower support from ΔAIC_c (Table S1). The distribution of the residuals of the linear model and of eqn 3 revealed a clear outlier, which was removed for parameter

Table 2. Normalization equations and parameter estimates with 95% confidence intervals (95% CI)

Model	Equation	Parameters (95% CI)	MSE	pred (%)
Muscle				
eqn 3	$\Delta\delta^{13}C = \beta_0 + \beta_1 \times \ln(C/N)$	β_0 = −4·996 (−5·586; −4·407) β_1 = 4·186 (3·760; 4·611)	0·116	86
Linear	$\Delta\delta^{13}C = a + b \times (C/N)$	a = −3·113 (−3·474; −2·653) b = 0·968 (0·851; 1·052)	0·127	87
Logan *et al.* (2008) equation 3 (fish muscle)	$\Delta\delta^{13}C = \beta_0 + \beta_1 \times \ln(C/N)$	β_0 = −4·763 (−5·041; −4·485) β_1 = 4·401 (4·207; 4·595)	0·371	49
Post *et al.* (2007) linear (aquatic organisms)	$\Delta\delta^{13}C = a + b \times (C/N)$	a = −3·32# b = 0·99	0·132	85
Egg content				
eqn 2	$\Delta\delta^{13}C = p - \frac{p \times f}{C/N}$	Waterfowl: p = 4·938 (4·322; 5·553) f = 3·665 (3·056; 4·274) Non waterfowl: p = 6·394 (5·604; 7·184) f = 3·505 (2·923; 4·087)	0·534	59
eqn 1	$\Delta\delta^{13}C = \frac{a \times (C/N) + b}{(C/N) + c}$	Waterfowl: a = 6·060 (3·146; 8·974) b = −21·106 (−30·955; −11·256) c = 2·777 (−4·687; 10·240) Non waterfowl: a = 6·400 (4·238; 8·562) b = −22·403 (−29·190; −15·615) c = 0·017 (−5·537; 5·571)	0·561	56
eqn 3	$\Delta\delta^{13}C = \beta_0 + \beta_1 \ln(C/N)$	Waterfowl: β_0 = −2·901 (−4·539; −1·264) β_1 = 2·574 (1·853; 3·295) Non waterfowl: β_0 = −1·936 (−3·621; −0·251) β_1 = 2·604 (1·833; 3·375)	0·552	51
Linear	$\Delta\delta^{13}C = a + b \times (C/N) + \text{group}$	a = 1·391 (0·753; 2·028) b = 0·253 (0·189; 0·316) waterfowl = −1·040 (−1·435; −0·646)	0·704	49

In addition to the equations fitted to our data, parameters of two published equations are given for comparison. The prediction error of the models is given as mean squared error (MSE) and the proportion of values predicted within 0·5‰ of the observed values (pred) as estimated from leave-one-out cross-validation for the equations fitted in this study. For the published models, MSE and pred are reported for the comparison of predicted and observed values.
MSE, mean squared error.
#Post *et al.* (2007) did not report the precision of their estimates.

estimation, but included in model comparison statistics. The model selection results were however similar when removing this sample. The 95% confidence interval (CI) of the parameters estimated for eqn 3 included the values of parameters estimated by Logan *et al.* (2008) for fish muscle (Table 2). For the linear model, the 95% CI of the parameters estimated from our data encompass the values inferred by Post *et al.* 2007 for aquatic organisms. However, the slope of the equation from the linear model (Table 2) is steeper than that of 0·83 estimated by Kojadinovic *et al.* (2008) for marine birds. Fitting a linear equation to only our bird sample data resulted in a slope of 0·85 (95% CI = 0·48–1·22) which is close to Kojadinovic *et al.*'s (2008) estimate. The equation proposed by Post *et al.* (2007) for aquatic organisms predicted $\Delta\delta^{13}C$ values which were very close to our observed values, whereas Logan *et al.*'s (2008) model for fish muscle overestimated $\Delta\delta^{13}C$ for most samples (Fig. 2).

The nonlinear normalization equation (eqn 3) fitted to the muscle data had a value of zero for C/N = 3·30 and the linear equation crossed zero at C/N = 3·21. This indicates that lean muscle samples have a C/N value around 3·25. Taking into account some noise in the data, we thus suggest that samples with a C/N value below 3·5 can be considered as lean and do not need to be corrected for lipid content. In agreement with the suggested threshold, the mean $\Delta\delta^{13}C$ for samples with C/N between 3·0 and 3·5 was 0·02‰ (range = −0·54 to 0·42‰), whereas the mean $\Delta\delta^{13}C$ for samples with C/N between 3·5 and 4 was 0·47‰ (range = −0·23 to 1·09‰).

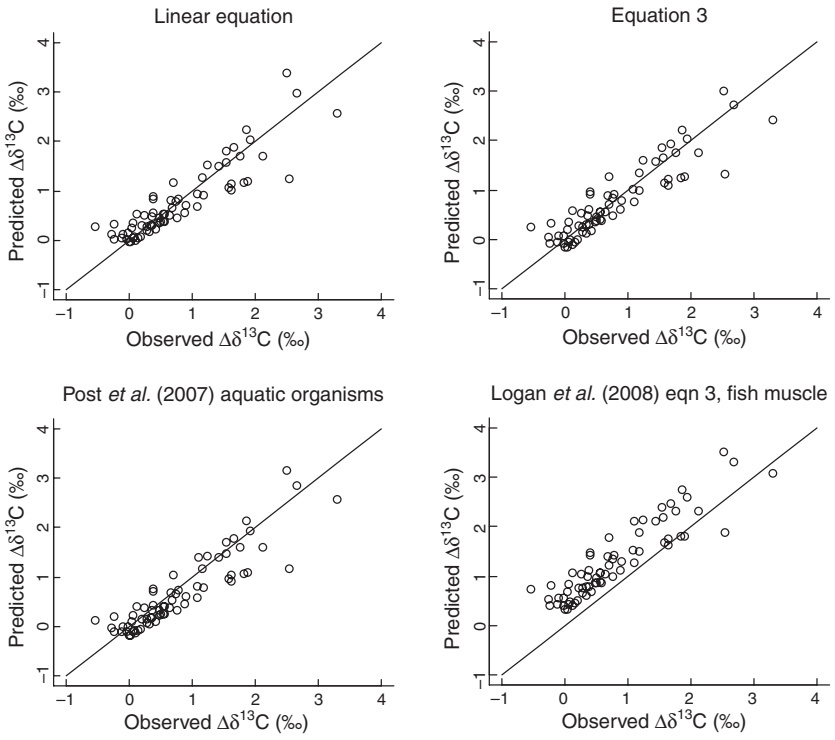

Fig. 2. Difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$) predicted by four normalization equations for muscle plotted against the observed values (data set A). The parameters of the linear equation and of equation 3 were estimated from data set A, and predicted values were obtained by leave-one-out cross-validation. The two other normalization equations were taken from the literature.

LIPID NORMALIZATION FOR EGGS

The C/N ratio of bulk egg content ranged from 3·34 to 20·22 (mean = 9·52), indicating a much higher lipid content than in muscle (Table 1). After lipid extraction, C/N was on average 3·52 (range = 3·19–4·02). In agreement with higher lipid content, $\Delta\delta^{13}C$ values were also on average higher for egg content than for muscle (mean = 3·32‰, range = 0–5·76‰). Fig. 1b shows the relation of $\Delta\delta^{13}C$ to C/N (values for each species are presented in Table 1).

The species were first subdivided into six groups according to taxonomy (Fig. 1b). Exploratory analyses of the residuals of linear and nonlinear models fitted to $\Delta\delta^{13}C$ against C/N showed that waterfowl and the only seabird (*Uria lomvia* L.) had negative mean residuals, whereas mean residuals were positive for the other groups (Fig. S2). We therefore divided the data into one group containing waterfowl and *U. lomvia* and a second group containing the other species (Table 1). Preliminary model comparison based on ΔAIC_c confirmed that categorization using two groups was more appropriate than the initial five groups. *Uria lomvia* was excluded from this analysis as there was only one sample of this species. Considering the total data set, the model specified by eqn 2 distinguishing between waterfowl/seabirds and other species had clearly the highest support from ΔAIC_c (Table S2) as well as from cross-validation (Table 2). Parameter estimates for a linear model and the three nonlinear models corresponding to eqns 1 to 3 are reported in Table 2 and plots of predicted against observed $\Delta\delta^{13}C$ values are shown in Fig. 3.

As the data were very scattered and only a few egg samples had low C/N values, the threshold below which egg samples can be considered lean could not be estimated precisely (Fig. 1b). The f parameter estimated in eqn 2, which corresponds to the C/N value of lipid free tissue, was 3·67 (95% CI = 3·06–4·27) for waterfowl and 3·51 (95% CI = 2·92–4·09) for other birds, indicating that a threshold of 4 may be adequate.

TEST WITH THE DATA FROM BYLOT ISLAND

Among the muscle samples of data set B, 22 had C/N values above 3·5, the threshold determined for applying a lipid correction. The normalization equations estimated for data set A fitted the data well, except for the negative $\Delta\delta^{13}C$ values observed for some samples (Fig. 4a). Applying eqn 3, $\Delta\delta^{13}C$ could be predicted within 0·5‰ of the observed values for 15 of 22 samples (68%). The linear equation performed equally well and predicted $\Delta\delta^{13}C$ values within 0·5‰ of the observed values for 16 of 22 samples (73%; Fig. S3). For egg content, on the contrary, the normalization equations fitted to data set A overestimated $\Delta\delta^{13}C$ consistently (Fig. 4b). On average the $\Delta\delta^{13}C$ values measured for the snow goose eggs from data set B were considerably lower than the values measured for the eggs of data set A with comparable C/N values. Consequently the $\Delta\delta^{13}C$ values predicted on the basis of eqn 2 were not well correlated with the observed values (Fig. S3). The difference between the predicted and the observed values ranged from −0·34‰ to 3·26‰ and only three out of 11 (27%) predicted values were within 0·5‰ of the observed values.

EFFECTS OF LIPID EXTRACTION ON $\delta^{15}N$

For muscle samples of data set A, lipid extraction did not have a strong influence on values of $\delta^{15}N$. $\Delta\delta^{15}N$ was on average

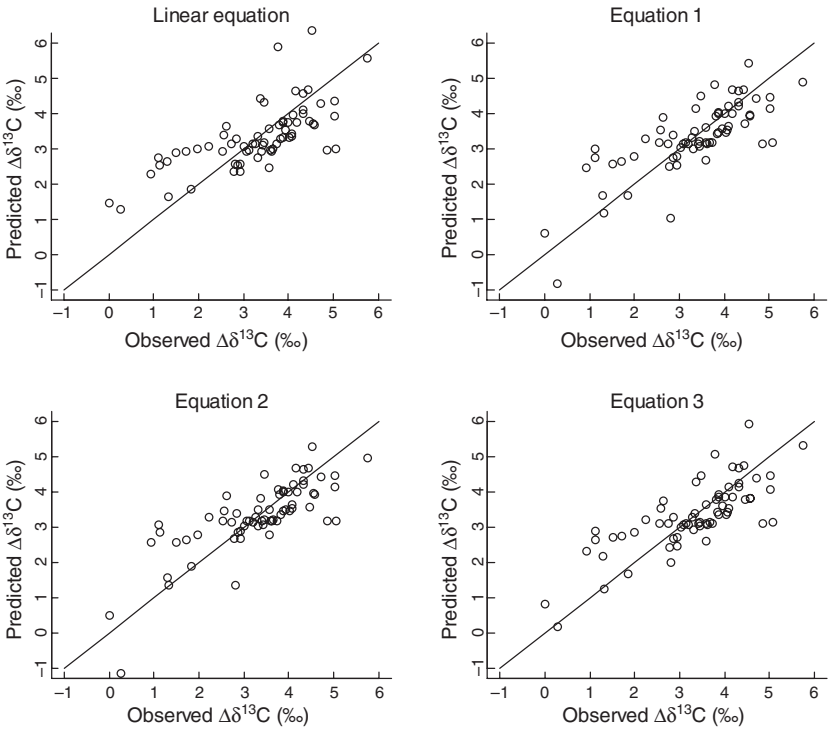

Fig. 3. Difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$) predicted by four normalization equations for eggs plotted against the observed values (data set A). The parameters of all four equations were estimated from data set A, and predicted values were obtained by leave-one-out cross-validation.

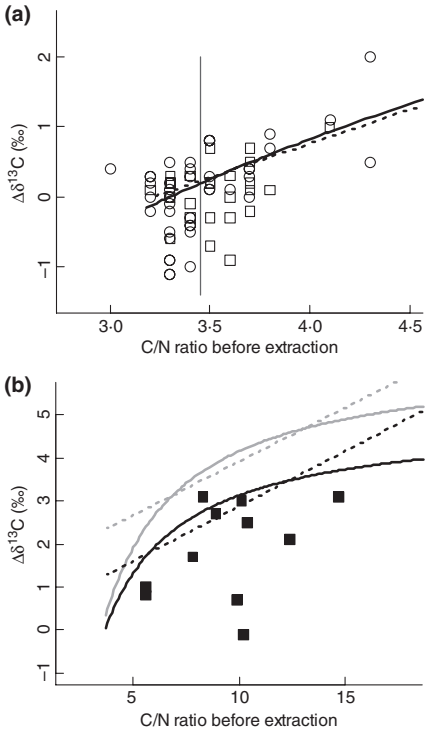

Fig. 4. The difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$) plotted against the ratio of carbon : nitrogen (C/N) for the samples from data set B. (a) Muscle samples. Circles represent mammal samples and squares bird samples. The black line shows the nonlinear normalization equation (eqn 3) as fitted to data set A, and the dotted line shows the linear equation. The vertical grey line indicates the threshold of C/N = 3·5 below which samples can be considered lean. (b) Egg samples. The lines show nonlinear normalization equations (eqn 2) as fitted to data set A and the dotted lines show linear equations. Black lines indicate equations for waterfowl and seabirds, and grey lines for other species.

−0·03‰ (95% CI = −0·08 to 0·06‰), and did in most cases not exceed the measurement error of maximum 0·39‰ (87% of the values). Values for the different species are shown in Table 1. There was no relation between $\Delta\delta^{15}N$ and C/N. For egg content (data set A), the effect of lipid extraction on $\delta^{15}N$ was larger and in general positive (87% of the samples; Table 1). On average, $\Delta\delta^{15}N$ was 0·58‰ (95% CI = 0·46–0·70‰). There was a significant positive relation between C/N and $\Delta\delta^{15}N$ (Fig. S4). An increase of 1 in C/N corresponded to an increase of 0·10‰ in $\Delta\delta^{15}N$ (95% CI of the slope = 0·08–0·13; excluding one outlier value with the most negative $\Delta\delta^{15}N$ after inspection of the residuals).

Similar tendencies were observed in data set B. The effect of lipid extraction on $\Delta\delta^{15}N$ was rather small for muscle (mean = 0·07‰; 95% CI = 0·01–0·13‰), whereas it was larger for egg content (mean = 0·49‰; 95% CI = 0·34–0·65‰; Table 1). There was also a significant relation between C/N and $\Delta\delta^{15}N$ for egg content (slope = 0·055, 95% CI = 0·006–0·104).

Discussion

Our results suggest that model-based lipid normalization is a good method to deal with varying lipid content in muscle samples of mammals and birds. Applying cross-validation to assess prediction error for the estimated normalization equations, we showed that predicted $\Delta\delta^{13}C$ values were within 0·5‰ of the observed values for over 85% of the samples. The MAE of the predicted values was 0·26‰ and thus very close to our estimate of the measurement error of $\delta^{13}C$ after lipid extraction (0·24‰). The model given by Post *et al.* (2007) for aquatic organisms had similar parameter estimates and gave the same level of precision. For most applications of SIA in ecology, this

level of precision is of the same order of magnitude, or better, than the precision obtained considering other sources of incertitude, such as individual variation or the incertitude associated with isotope discrimination factors (Wolf, Carleton & Martinez del Rio 2009). The necessary level of precision and the potential effect of shifts in $\delta^{13}C$ due to lipids depend indeed on the particular biological questions addressed. Considering mixing models, they depend also on the relative positions of the predators and the potential prey in the isotopic space and should thus be assessed for each study specifically (Tarroux *et al.* 2010).

Our analyses did not support different equations for mammal and bird muscle. Kojadinovic *et al.* (2008) proposed, however, a normalization equation for marine birds with a lower slope than the one inferred here. Fitting a linear model to only the bird sample data (data set A), indeed resulted in an equation closer to that of Kojadinovic *et al.* (2008). This indicates that the slope of the equation relating $\Delta\delta^{13}C$ to C/N is likely to be lower for bird muscle than for mammal muscle. Our samples sizes were, however, not sufficient to address this question thoroughly. The high C/N ratios obtained after lipid extraction for five out of seven *Lemmus* samples in data set A may indicate that Siberian and Norwegian lemmings have higher C/N ratios in lean muscle than the other species analysed here, an observation which could be worth further investigations. This seemed, however, not to be the case for the brown lemmings from data set B.

For data set B, predictions from the estimated equations were not as good, but still within a range which makes them useful for many ecological applications. In data set B there were samples with more negative $\Delta\delta^{13}C$ values than in data set A. Negative $\Delta\delta^{13}C$ values are not expected when assuming that $\Delta\delta^{13}C$ results only from the difference in lipid content between lipid extracted and bulk tissue samples. As most samples of data set B had low lipid content, these negative values may be explained by measurement error. Imperfect sample homogenization, as grinding was carried out with a mortar and pestle for data set B, may have enhanced the differences between different aliquots, and thus the measurement error for this data set.

Altogether, our results indicate that lipid normalization is a rather robust method for muscle samples with moderate lipid content and C/N values up to seven. Extending the conclusions of Post *et al.* (2007), lipid normalization appears to be applicable to a wide variety of organisms including terrestrial mammals and birds. Our sample represented in fact a mixture of terrestrial and marine organisms. Many arctic animals, notably predators and shorebirds, use a mixture of terrestrial and marine resources during their life making the distinction between the terrestrial and the marine resource environment not as clear-cut in the Arctic as in some other biomes (Roth 2003; Gauthier *et al.* 2004). Despite the cautions advanced by Post *et al.* (2007) and Sweeting, Polunin & Jennings (2004), lipid normalization seems to be applicable also to samples stored in 70% ethanol. Although we did not directly investigate the effect of storing samples in 70% ethanol by comparison of our isotope measurements with measurements from identical samples stored by other means, our results show that the relation between $\Delta\delta^{13}C$ and C/N, on which normalization is based, is similar for samples stored in 70% ethanol as for samples stored frozen or dried.

For egg content, which covered a much larger range of C/N values and included samples with higher lipid content than muscle, it was clear that the relation of $\Delta\delta^{13}C$ to C/N was positive and nonlinear (Fig. 1b). Both the model choice based on AIC_c and cross-validation showed that a nonlinear model was most appropriate for lipid normalization in this case. The difference between the different nonlinear models was, however, rather small. The predictions of $\Delta\delta^{13}C$ for the egg data were clearly not as good as those for the muscle data. Using the best normalization equation (eqn 2), only 59% of the predicted $\Delta\delta^{13}C$ values were within 0·5‰ of the observed, whereas for the muscle data several equations allowed prediction of 85% or more of the data at this level of accuracy. In data set A, there seemed to be a consistent difference between waterfowl/seabird eggs and eggs of other species. The large discrepancy between predicted and observed $\Delta\delta^{13}C$ values obtained for data set B indicates that the estimated equations cannot be generalized. Altogether our results are in accordance with the conclusions of Oppel *et al.* (2010) and show that it is difficult to apply lipid normalization to samples of egg content. The lack of predictability in $\Delta\delta^{13}C$ values is likely to be due to sampling of migratory birds. Nutrients originating from isotopically different environments along migratory routes were probably incorporated into the eggs, confounding different $\delta^{13}C$ values of the nutrients with different lipid content (Oppel *et al.* 2010).

There was no strong effect of chemical lipid extraction on $\delta^{15}N$ values measured in muscle. For egg content, the effect was larger and in general positive, indicating that compounds with lower $\delta^{15}N$ values were preferentially washed out during chloroform-methanol rinsing. As suggested by Sotiropoulos, Tonn & Wassenaar (2004), this can be due to leaching of amino-acids at the same time as structural fats are removed. In addition, $\Delta\delta^{15}N$ of eggs was positively correlated with C/N. Because the fat content of a whole egg mixture depends on the proportion of yolk to albumen in the sample, this positive relation indicates that the ^{14}N-rich compounds were lost from the yolk.

RECOMMENDATIONS

- Model based lipid normalization can be applied to muscle samples of mammals and birds with moderate lipid content and C/N values between about 3·5 and 7. Samples with a C/N ratio below 3·5 can be considered as lean and do not need to be corrected for lipid content.
- For many studies of trophic ecology, a general equation, such as the one estimated here, can be applied. For samples of bird muscle, it is likely that the equation of Kojadinovic *et al.* (2008) is more correct. Further assessment of the range of applicability of these equations would be useful, but needs to be based on samples sizes and ranges in C/N allowing estimation with satisfactory precision.

• Researchers, who prefer to perform chemical lipid extraction for lipid rich muscle samples, in order to obtain maximally precise $\delta^{13}C$ estimates, can also use $\delta^{15}N$ values from lipid-extracted samples since chemical extractions were not shown to alter $\delta^{15}N$ for this tissue.

• We do not in general recommend lipid normalization for samples of egg content. For these samples, the best results are obtained when running samples in duplicate, with and without chemical lipid extraction. If, however, the required accuracy is low, and sample size is large, lipid normalization may be applied, but requires estimating a study specific equation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Distribution of the carbon : nitrogen ratios measured after chemical lipid extraction. The samples with values above four were considered outliers and excluded for the estimation of the normalization equation.

Figure S2. Residuals of four different models fitted to the egg data (data set A) plotted per taxonomic group for birds. (a) linear model, (b) equation 1, (c) equation 2 and (d) equation 3.

Figure S3. Difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples ($\Delta\delta^{13}C$) predicted by the normalization equations plotted against the observed values for data set B. For muscle samples, the linear equation with parameters estimated from data set A was used. For egg content, equation 2 with parameters estimated from data set A was used.

Figure S4. The difference in $\delta^{15}N$ between lipid extracted and bulk tissue samples ($\Delta\delta^{15}N$) plotted against C/N for egg content (data set A). Symbols refer to the different taxonomic groups of birds as detailed in the legend.

Table S1. Comparison of different models fitted to the relation of $\Delta\delta^{13}C$ (the difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples) to C/N (the ratio of carbon : nitrogen), as measured for muscle samples of mainly terrestrial mammals and birds. Models were compared using Akaike's information criterion corrected for small sample sizes (AIC_c), expressed relatively to the AIC_c value for the best model (ΔAIC_c) and cross-validation with five or ten groups (K) as well as the leave-one-out approach (corresponding to cross-validation with K = sample size). The prediction error estimated from cross-validation was summarized as mean squared error (MSE), mean absolute error (MAE) and the proportion of $\Delta\delta^{13}C$ values predicted within 0·5‰ of the observed values (pred). The number of parameters (par) estimated for each model is also given. The best model(s) according to each criterion is highlighted in bold.

Table S2. Comparison of different models fitted to the relation of $\Delta\delta^{13}C$ (the difference in $\delta^{13}C$ between lipid extracted and bulk tissue samples) to C/N (the ratio of carbon : nitrogen), as measured for whole egg samples of 32 bird species nesting in the arctic tundra. Exploratory analyses suggested a subdivision of the species into one group containing waterfowl and *Uria lomvia* and a second group containing the other species (two groups). Models were compared using Akaike's information criterion corrected for small sample sizes (AIC_c), expressed relatively to the AIC_c value for the best model (ΔAIC_c) and cross-validation with five or ten groups (K) as well as the leave-one-out approach (corresponding to cross-validation with K = sample size). The prediction error estimated from cross-validation was summarized as mean squared error (MSE), mean absolute error (MAE) and the proportion of $\Delta\delta^{13}C$ values predicted within 0·5‰ of the observed values (pred). The number of parameters (par) estimated for each model is also given. The best model according to each criterion is highlighted in bold.

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			cross-validation *K*=5			cross-validation *K*=10			leave-one-out		
model	par	ΔAIC_c	MSE	MAE	pred	MSE	MAE	pred	MSE	MAE	pred
$\Delta\delta^{13}C$ ~ C/N	3	5.26	0.127	0.257	**0.864**	0.129	0.260	0.860	0.127	0.259	**0.870**
$\Delta\delta^{13}C$ ~ C/N+class	4	6.88	0.130	0.262	0.853	0.131	0.264	0.849	0.130	0.263	0.841
$\Delta\delta^{13}C$ ~ C/N+class+C/N:class	5	8.62	0.132	0.263	0.851	0.133	0.264	0.856	0.133	0.264	0.855
$\Delta\delta^{13}C$ ~ C/N+C/N:class	4	6.69	0.130	0.262	0.853	0.131	0.264	0.845	0.130	0.263	0.841
eqn1	4	2.00	0.121	0.260	0.846	0.121	0.261	0.836	0.121	0.261	0.841
eqn2	3	2.05	0.119	0.260	0.861	0.119	0.259	0.856	0.119	0.261	0.841
eqn3	3	**0.00**	**0.116**	**0.255**	0.853	**0.116**	**0.256**	0.847	**0.116**	**0.256**	0.855
eqn1 by class	8	9.03	0.339	0.288	0.835	0.129	0.273	0.839	0.130	0.276	0.841
eqn2 by class	6	5.80	0.120	0.266	**0.868**	0.119	0.263	**0.878**	0.120	0.266	**0.870**
eqn3 by class	6	4.66	0.118	0.260	0.853	0.118	0.260	0.847	0.118	0.260	0.855

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			cross-validation K=5			cross-validation K=10			leave-one-out		
model	par	ΔAIC_c	MSE	MAE	pred	MSE	MAE	pred	MSE	MAE	pred
$\Delta\delta^{13}C$ ~ constant	2	70.74	1.417	0.919	0.358	1.410	0.922	0.344	1.428	0.924	0.353
$\Delta\delta^{13}C$ ~ 2groups	3	64.064	1.289	0.885	0.371	1.287	0.883	0.378	1.293	0.884	0.368
$\Delta\delta^{13}C$ ~ C/N	3	42.104	0.953	0.777	0.378	0.919	0.772	0.364	0.965	0.787	0.382
$\Delta\delta^{13}C$ ~ C/N+2groups	4	20.21	0.703	0.659	0.476	0.674	0.647	0.489	0.704	0.661	0.485
$\Delta\delta^{13}C$ ~ C/N+2groups+C/N:2groups	5	22.53	0.777	0.680	0.473	0.739	0.667	0.485	0.779	0.681	0.485
$\Delta\delta^{13}C$ ~ C/N+C/N:2groups	4	23.19	0.742	0.680	0.445	0.711	0.670	0.453	0.741	0.684	0.441
eqn1	4	27.88	0.762	0.691	0.446	0.746	0.692	0.444	0.761	0.699	0.441
eqn2	3	25.62	0.716	0.673	0.453	0.711	0.675	0.448	0.729	0.683	0.441
eqn3	3	30.70	0.781	0.713	0.410	0.765	0.712	0.404	0.795	0.723	0.397
eqn1 for 2 groups	8	4.01	34.843	0.682	0.544	1.453	0.620	0.542	0.561	0.570	0.559
eqn2 for 2 groups	6	**0.00**	**0.533**	**0.544**	**0.572**	**0.529**	**0.544**	**0.572**	**0.534**	**0.540**	**0.588**
eqn3 for 2 groups	6	4.76	0.548	0.577	0.521	0.538	0.572	0.515	0.552	0.576	0.515

Figure S1. Distribution of the carbon to nitrogen ratios measured after chemical lipid extraction. The samples with values above four were considered outliers and excluded for the estimation of the normalization equation.

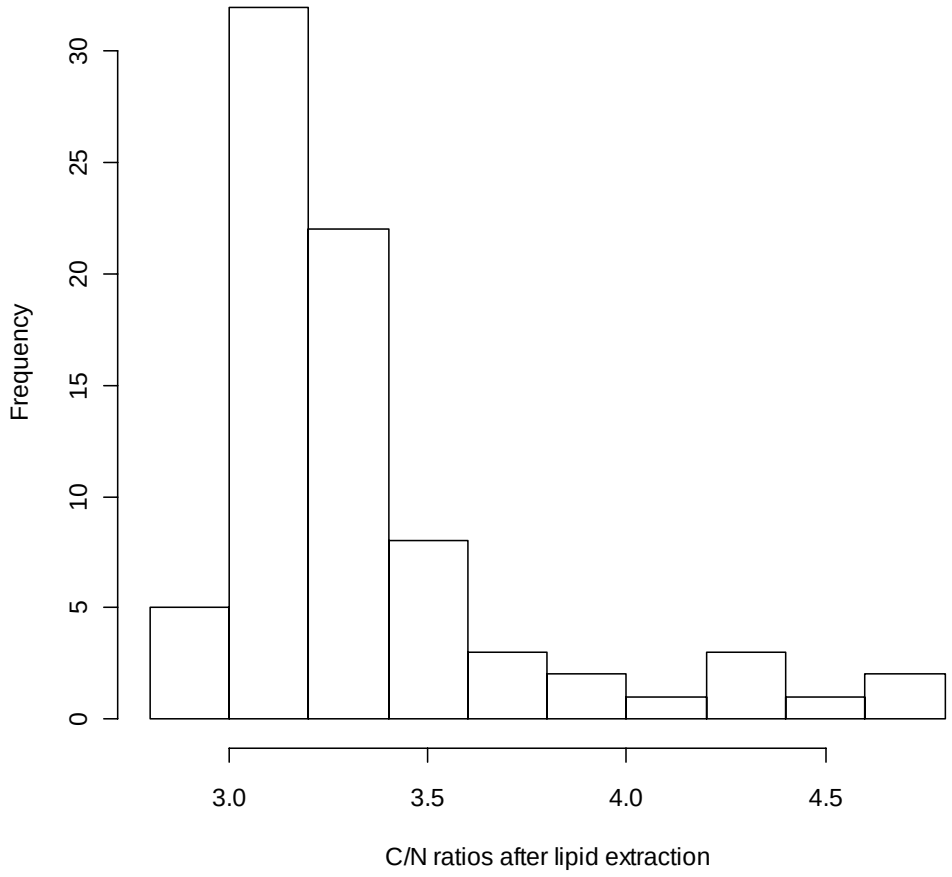
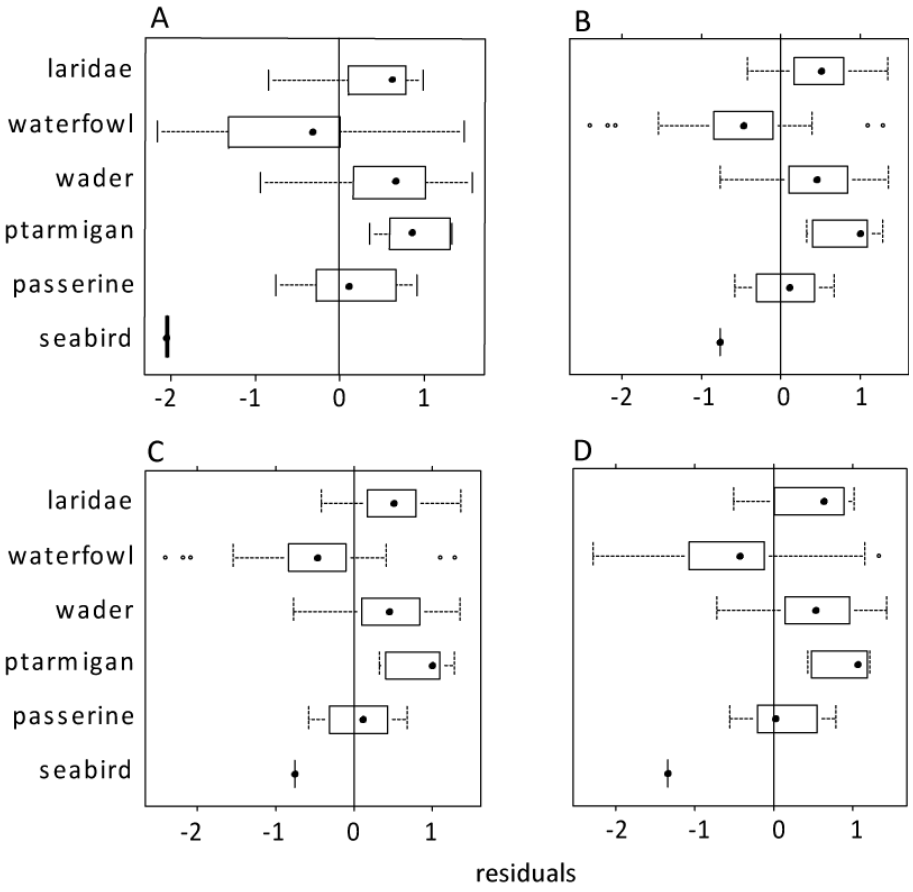


Figure S2. Residuals of four different models fitted to the egg data (dataset A) plotted per taxonomic group for birds. A) linear model, B) equation 1, C) equation 2 and D) equation 3.



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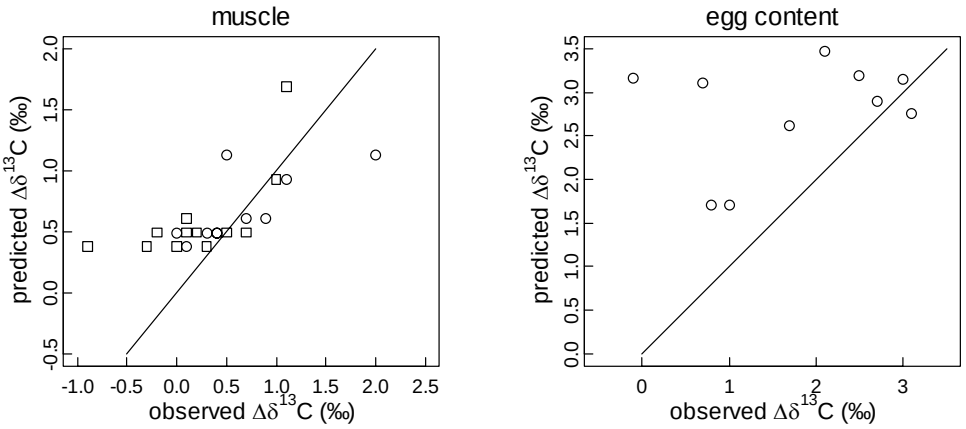

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