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ORIGINAL PAPER

Effects of lipid extraction and the utility of lipid normalization models on $\delta^{13}C$ and $\delta^{15}N$ values in Arctic marine mammal tissues

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Abstract Animals store lipids, which are ^{13}C-depleted, in their tissues that often must be extracted to correctly interpret $\delta^{13}C$ data. However, chemical lipid extraction (CLE) can alter $\delta^{15}N$ values and lipid normalization (LN) models are not consistent across fauna. We determined whether lipids should be extracted by assessing effects of CLE and validating LN models for liver and muscle from seven and eight marine mammal species, respectively, and skin from one species. In liver, CLE significantly increased $\delta^{13}C$ and $\delta^{15}N$ values for all species, whereas only a significant increase in $\delta^{13}C$ occurred in skin. For muscle, $\delta^{13}C$ and $\delta^{15}N$ values were generally greater after CLE, but this was not consistent across species. Extracted lipids were depleted by approximately 7 and 5 ‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively, in both muscle and liver compared with protein in all species. The reliability of LN models varied between tissues and species; thus, their use is largely dependent on the precision of stable isotope values needed to address the objectives of a study. A decision framework to decide whether CLE or LN models is required for ecological interpretation of stable isotopes based on species, tissue and study objectives is presented.

Keywords Carbon · Lipids · Marine mammals · Nitrogen · Stable isotopes

Introduction

Stable isotope analysis has become a well-established tool in ecological studies to assess trophic interactions and energy flow through ecosystems (Peterson and Fry 1987), trace animal movements and habitat use (Rubenstein and Hobson 2004) and provide time-integrated information of assimilated foods for diet reconstruction (DeNiro and Epstein 1978, 1981) via stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios. However, the synthesis and storage of lipids, which are depleted in ^{13}C relative to protein and carbohydrates (DeNiro and Epstein 1977), in animal tissues vary considerably due to tissue type, life-stage, season, foraging behavior (Fagan et al. 2011) and reproductive status (Bowen et al. 1987). Thus, lipids normally need to be removed to reduce inter-individual variation in lipid content and differences between tissue types in an individual to provide comparable $\delta^{13}C$ values and avoid erroneous interpretations of ecological relationships (Kiljunen et al. 2006; Post et al. 2007).

The issue of lipids influencing $\delta^{13}C$ values has long been recognized, and lipids are removed from tissues by either chemical lipid extraction (CLE) or mathematical normalization (DeNiro and Epstein 1977; Logan et al. 2008). CLE is a time- and labor-intensive process,

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especially for studies dealing with large sample sizes (Kelly 2000). One of the most common methods for CLE is the Bligh and Dyer's (1959) method, which uses a chloroform/methanol solvent to reduce lipids. The use of polar organic solvents removes simple lipids but may also extract more complex lipophilic amino acids and polar lipids that are bound to membrane proteins (Sweeting et al. 2006). Therefore, CLE has been found to alter $\delta^{15}N$ values across a number of taxa, usually resulting in an ^{15}N-enrichment of $\delta^{15}N$ (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Murry et al. 2006; Sweeting et al. 2006; Lesage et al. 2010; Hussey et al. 2012b; Elliott et al. 2014), although a number of studies have reported depletion in ^{15}N or no effect on $\delta^{15}N$ values following lipid extraction (Bodin et al. 2007; Ingram et al. 2007; Ricca et al. 2007; Barrow et al. 2008; Horstmann-Dehn et al. 2012).

The effects of CLE on $\delta^{13}C$ and $\delta^{15}N$ values differ considerably between species and tissues with varying lipid content where changes to $\delta^{13}C$ values range from −0.2 to 5.0 ‰ (Ehrich et al. 2011; Hussey et al. 2012a, b) and −0.2 to 2.9 ‰ for $\delta^{15}N$ (Sotiropoulos et al. 2004; Logan et al. 2008). To mitigate the potential change in $\delta^{15}N$ caused by CLE, a few studies have suggested analyzing two aliquots of a sample, one lipid-extracted (LE) to determine $\delta^{13}C$ and one non-lipid-extracted (BULK) for $\delta^{15}N$ (Sotiropoulos et al. 2004; Murry et al. 2006; Sweeting et al. 2006), but this greatly increases time involved and doubles cost of analyses. Several generalized (McConnaughey and McRoy 1979; Fry et al. 2003; Post et al. 2007) and species-specific (Lesage et al. 2010; Ehrich et al. 2011) lipid normalization (LN) models have been established to estimate lipid-free $\delta^{13}C$ values in aquatic organisms without the need for CLE. But, these models have produced inconsistent results as no single model has consistently performed best across tissues, species and species-groups, suggesting species- and tissue-specific effects (Logan et al. 2008; Lesage et al. 2010; Ehrich et al. 2011; Ryan et al. 2012). In addition, the effects of CLE on $\delta^{13}C$ and $\delta^{15}N$ values and the applicability of generalized and species-specific LN models have not been formally tested for most biota, especially in the Arctic.

Arctic species, especially marine mammals, experience high variation in lipid storage and percent blubber content relative to season, which is a fundamental specialization to their environment due to their highly variable food supply throughout the year (Lee 1974; Ryg et al. 1990; Falk-Petersen et al. 2000). In Arctic marine mammals, seasonal changes in body mass mainly due to fluctuations in percent blubber content are common (Ryg et al. 1990) and in turn affect the routing of lipids to proteinaceous tissues (Martínez del Rio et al. 2009). Therefore, the lipid physiology of Arctic marine mammals offers a unique opportunity to determine the effects of lipid extraction and normalization on $\delta^{13}C$ and $\delta^{15}N$ values. We quantified the $\delta^{13}C$, %C, $\delta^{15}N$, %N and C:N values of lipids extracted from liver and muscle to provide insight on the mechanisms that may drive stable isotope differences between tissues. This is the first study to analyze in combination with the $\delta^{13}C$, %C, $\delta^{15}N$, %N and C:N values of lipids extracted from multiple tissues of any animal. The objectives of this study were as follows: (1) to determine the effects of CLE on the $\delta^{13}C$, $\delta^{15}N$ and C:N values of liver, skin and muscle for eight Arctic marine mammal species, (2) to evaluate six LN models that utilize non-lipid-extracted (BULK) C:N and $\delta^{13}C$ values to estimate lipid-free $\delta^{13}C$ for tissues with varying lipid content by species and (3) develop a decision framework to determine whether CLE should be undertaken prior to SIA dependent on species and stable isotopes of interest.

Materials and methods

Paired liver and muscle samples from bearded seals (*Erignathus barbatus*), harbor seals (*Phoca vitulina*), harp seals (*Pagophilus groenlandicus*), ringed seals (*Pusa hispida*), walrus (*Odobenus rosmarus*) and beluga (*Delphinapterus leucas*), as well as paired skin, liver and muscle samples from narwhal (*Monodon monoceros*) and muscle samples from bowhead whales (*Balaena mysticetus*) were collected opportunistically throughout the year by Inuit hunters from across the Canadian Arctic as part of their subsistence harvests from 1996–2010 (Table 1). This study provides novel analysis into the biochemistry of walrus tissues, which has not been well studied. For some individuals, a sample of only one tissue type was provided (Table 1). All tissue samples were placed in plastic bags (Whirl-Pak™) immediately after sampling and then stored frozen at −20 °C, which is the preferred preservation method for higher-order fauna (Hobson et al. 1997a, b; Bosley and Wainright 1999; Sweeting et al. 2004).

Frozen liver, muscle and skin samples were freeze-dried for 48 h and then homogenized by hand using a mortar and pestle. Lipids were extracted using 2 ml of 2:1 chloroform/methanol solvent similar to the Bligh and Dyer's (1959) method and established in McMeans et al. (2009). Then, 400–600 µg of LE and BULK skin, liver and muscle were weighed into tin capsules for stable isotope analysis. To determine the stable isotope values in extracted lipid and percent lipid content, a subset of five liver and muscle samples from each species with sufficient sample volume (0.10–0.25 g), which included ringed, bearded and harbor seals, walrus, narwhal and beluga, were extracted with 3 ml of 2:1 chloroform/methanol solution following McMeans et al. (2009). The supernatant was filtered through a No. 1 Whatman filter paper and drained into a

Table 1 Mean ± SE $\delta^{13}C$, $\delta^{15}N$ and C:N values of lipid-extracted (LE) and non-lipid-extracted (BULK) tissue samples from Arctic marine mammal tissues

Species	Genus species	Species code	Tissue	*N*	BULK $\delta^{13}C$ (‰)	LE $\delta^{13}C$ (‰)	BULK $\delta^{15}N$ (‰)	LE $\delta^{15}N$ (‰)	BULK C:N	LE C:N
Pinnipeds										
Bearded seal	*Erignathus barbatus*	Bea	L	18	−21.4 ± 0.3	−19.5 ± 0.3	16.1 ± 0.2	16.3 ± 0.2	4.9 ± 0.1	3.5 ± 0.1
			M	22	−18.9 ± 0.3	−19.1 ± 0.2	15.5 ± 0.2	15.9 ± 0.2	3.4 ± 0.1	3.4 ± 0.1
Harbor seal	*Phoca vitulina*	Hbr	L	11	−19.8 ± 0.3	−18.7 ± 0.4	16.8 ± 0.7	17.1 ± 0.7	4.2 ± 0.1	3.6 ± 0.1
			M	11	−19.1 ± 0.1	−19.1 ± 0.1	16.4 ± 0.2	16.8 ± 0.4	3.4 ± 0.1	3.4 ± 0.1
Harp seal	*Pagophilus groenlandicus*	Har	L	7	−19.5 ± 0.1	−17.8 ± 0.1	15.5 ± 0.2	15.7 ± 0.1	4.5 ± 0.1	3.5 ± 0.1
			M	7	−18.2 ± 0.1	−17.9 ± 0.1	14.9 ± 0.2	15.1 ± 0.4	3.6 ± 0.1	3.3 ± 0.1
Ringed seal	*Pusa hispida*	Rin	L	44	−19.9 ± 0.1	−18.9 ± 0.1	15.5 ± 0.1	15.6 ± 0.1	4.1 ± 0.1	3.5 ± 0.1
			M	58	−18.8 ± 0.1	−18.9 ± 0.1	15.1 ± 0.1	15.3 ± 0.1	3.5 ± 0.1	3.4 ± 0.1
Walrus	*Odobenus rosmarus*	Wal	L	20	−20.8 ± 0.1	−19.6 ± 0.2	12.7 ± 0.1	13.0 ± 0.1	5.0 ± 0.1	4.0 ± 0.1
			M	19	−19.4 ± 0.2	−19.2 ± 0.2	12.3 ± 0.1	12.7 ± 0.1	3.5 ± 0.1	3.3 ± 0.1
Cetaceans										
Beluga	*Delphinapterus leuca*	Bel	L	29	−19.7 ± 0.1	−18.7 ± 0.1	17.0 ± 0.1	17.1 ± 0.1	4.2 ± 0.1	3.6 ± 0.1
			M	31	−18.7 ± 0.1	−18.4 ± 0.1	16.4 ± 0.1	16.7 ± 0.1	3.5 ± 0.1	3.4 ± 0.1
Bowhead	*Balaena mystticetus*	Bow	M	7	−19.8 ± 0.4	−19.4 ± 0.3	12.0 ± 0.3	12.4 ± 0.3	4.2 ± 0.2	3.7 ± 0.1
Narwhal	*Monodon monoceros*	Nar	L	5	−19.5 ± 0.3	−18.8 ± 0.3	16.8 ± 0.1	17.0 ± 0.1	4.0 ± 0.1	3.5 ± 0.1
			M	39	−17.7 ± 0.1	−17.7 ± 0.1	14.7 ± 0.1	15.0 ± 0.1	3.5 ± 0.1	3.4 ± 0.1
			S	39	−18.2 ± 0.1	−17.3 ± 0.1	15.0 ± 0.1	15.1 ± 0.1	4.1 ± 0.1	3.6 ± 0.1

Samples were collected from across the Canadian Arctic in the following locations: Arviat, Chesterfield Inlet, Igloolik, Iqaluit, Grise Fiord, Pangnirtung, Repulse Bay, Resolute and Sanikiluaq, Nunavut and Holman, Northwest Territories

L liver, *M* muscle, *S* skin

pre-weighed aluminum dish, where the supernatant was allowed to evaporate at room temperature in a fume hood for 24 h. Percent lipid was determined gravimetrically. Because lipids have high carbon but low N, 900–3,500 μg of lipid was weighed into tin capsules for an accurate $\delta^{15}N$ value and an additional 800–1,000 μg of lipid was weighed into another tin capsule to avoid excessive carbon and obtain reliable $\delta^{13}C$ values. The $\delta^{15}N$ and $\delta^{13}C$ values were measured by a Thermo Finnigan Delta^{Plus} mass spectrometer (Thermo Finnigan, San Jose, CA, USA) coupled with an elemental analyzer (Costech, Valencia, CA, USA) at the Chemical Tracers Laboratory, Great Lakes Institute for Environmental Research, University of Windsor, Canada. Stable isotope ratios are expressed in parts per thousand (‰) in delta (δ) notation using the following equation:

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

where X is ^{13}C or ^{15}N and R equals $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The standard materials for ^{13}C and ^{15}N are Pee Dee Belemnite and atmospheric N, respectively. The analytical precision based on the standard deviation of replicated analyses of two standards (bovine muscle (NIST 8414) and an internal laboratory standard (tilapia muscle) $n = 58$ for each) was <0.1 ‰ for $\delta^{15}N$ and $\delta^{13}C$. NIST standards (sucrose (NIST 8542) and ammonia sulfate (NIST 8547); $n = 3$ for each) that were analyzed during the study generated values that were within <0.1 ‰ of certified values for $\delta^{15}N$ and $\delta^{13}C$. In addition, triplicates were run for every 13th sample, the standard deviation of replicates for $\delta^{15}N$ and $\delta^{13}C$ was within precision values (<0.1 ‰), and the %C and %N were <0.5 and <0.2 %, respectively.

Stable isotope values for BULK and LE liver, muscle and skin from all species and species-group were normal based on normal quantile–quantile plots and showed no heteroscedasticity based on Levene's tests (all $P > 0.05$); thus, data were not transformed. To quantify the effects of CLE on $\delta^{13}C$ and $\delta^{15}N$ values, differences between BULK and LE muscle, liver and skin samples for each species were assessed using paired t-tests. Linear regression analyses were used to: (1) examine the relationship between $\delta^{13}C_{DIFF}$ (LE–BULK $\delta^{13}C$) and BULK C:N in liver and muscle among all species, (2) determine the relationship between BULK and LE $\delta^{15}N$ values for liver and muscle among all species and (3) investigate the relationships of $\delta^{15}N_{DIFF}$ (LE–BULK $\delta^{15}N$) and lipid content relative to BULK C:N, as well as N (μg) in lipid extracts relative to tissue lipid content among all species.

Six general and species-specific linear and nonlinear LN models that use BULK C:N and $\delta^{13}C$ parameters were evaluated to determine the best model fit among and between Arctic marine mammal species using data from tissues of all species.

Table 2 Mean ± SE $\delta^{13}C$, %C, $\delta^{15}N$, %N and C:N values of lipids extracted from Arctic marine mammal liver and muscle

Species code	Tissue	*N*	Lipid			$\delta^{13}C$			$\delta^{15}N$	
			Content (%)	$\delta^{13}C$ (‰)	C (%)	*D* (‰)	$\delta^{15}N$ (‰)	N (%)	*D* (‰)	C:N
Pinnipeds										
Bea	L	5	24.5 ± 4.0	−27.4 ± 0.7	74.3 ± 1.8	7.6 ± 0.6	11.1 ± 1.1	1.9 ± 0.3	4.9 ± 0.5	39.5 ± 6.6
Har	L	5	14.9 ± 1.1	−26.0 ± 1.2	69.9 ± 2.3	7.3 ± 0.6	11.2 ± 1.8	2.4 ± 0.4	6.0 ± 0.6	29.4 ± 3.8
	M	5	7.9 ± 0.9	−26.4 ± 0.9	67.1 ± 2.3	7.5 ± 0.5	10.6 ± 1.6	3.5 ± 0.5	5.3 ± 0.4	19.9 ± 3.8
Rin	L	5	20.1 ± 10.2	−25.6 ± 0.7	67.4 ± 1.9	6.9 ± 0.8	10.3 ± 2.0	3.0 ± 0.6	5.2 ± 2.0	24.0 ± 6.4
	M	3	7.9 ± 1.61	−26.1 ± 0.6	66.5 ± 4.1	7.0 ± 0.6	9.0 ± 0.7	4.4 ± 1.2	6.2 ± 0.3	16.6 ± 6.8
Wal	L	5	19.4 ± 2.4	−27.6 ± 0.2	71.5 ± 3.1	7.7 ± 0.5	7.8 ± 1.4	2.1 ± 0.6	5.1 ± 1.6	36.7 ± 8.8
	M	5	17.1 ± 1.0	−27.4 ± 0.4	67.1 ± 2.6	7.8 ± 0.4	5.8 ± 0.7	3.9 ± 0.6	6.5 ± 0.6	17.8 ± 3.8
Cetaceans										
Bel	L	3	13.9 ± 3.6	−26.7 ± 0.3	68.1 ± 7.4	7.9 ± 0.2	12.4 ± 1.6	2.3 ± 0.6	4.3 ± 1.4	30.2 ± 4.0
	M	5	9.3 ± 1.1	−26.2 ± 1.0	63.0 ± 2.5	7.7 ± 1.1	11.4 ± 0.7	4.5 ± 0.8	4.9 ± 0.7	14.3 ± 2.7
Nar	L	3	11.7 ± 0.8	−26.8 ± 0.1	67.1 ± 4.0	7.4 ± 0.9	11.7 ± 0.6	3.2 ± 1.8	5.3 ± 1.0	30.0 ± 4.4
	M	4	9.5 ± 0.6	−25.0 ± 1.1	60.8 ± 3.3	6.6 ± 0.9	11.9 ± 0.7	5.0 ± 1.0	4.1 ± 0.6	12.6 ± 3.4

D represents the isotopic difference in $\delta^{13}C$ and $\delta^{15}N$ between LE tissue and lipid

L liver, *M* muscle

1. A generalized nonlinear model developed by McConnaughey and McRoy (1979) estimated lipid-free $\delta^{13}C$ ($\delta^{13}C'$) for several marine invertebrates and vertebrates using the following two equations :

$$L = \frac{93}{1 + ((0.246 \times \text{C:N}) - 0.775)^{-1}}$$
$$\delta^{13}C' = \delta^{13}C + D \times \left(I + \frac{3.90}{1 + 287/L}\right) \quad (1)$$

where *L* represents proportional lipid content, C/N is the ratio of C and N in the BULK sample, *D* signifies the isotopic difference between protein and lipid and *I* is a constant (−0.207) (McConnaughey 1978). The *D* was estimated for liver and muscle of each species, although a *D* of 6.0 ‰ was used when it could not be quantified due to low sample volume (refer to Table 2 for *D* values). A *D* of 6.4 ‰ was used for cetacean skin (Lesage et al. 2010).

2. Post et al. (2007) developed a new approach by using 16 aquatic species from several ecosystems to develop a linear model to estimate $\delta^{13}C'$ by only using BULK C:N:

$$\delta^{13}C' = \delta^{13}C - 3.32 + 0.99 \times \text{C:N} \quad (2)$$

3. Fry et al. (2003) developed a mass-balance approach to calculate $\delta^{13}C'$ using BULK C:N, C:N of pure protein (lipid-extracted sample; $\text{C:N}_{\text{protein}}$) and *D*:

$$\delta^{13}C' = \frac{(\delta^{13}C \times \text{BULK C:N}) + [D(\text{BULK C:N} - \text{C:N}_{\text{protein}})]}{\text{BULK C:N}} \quad (3)$$

4. Lesage et al. (2010) proposed and developed a species-specific linear model for whale skin using $\delta^{13}C$ of BULK tissue in relation to $\delta^{13}C_{\text{DIFF}}$.

$$\delta^{13}C' - \delta^{13}C = \beta 0 + \beta 1(\text{Bulk } \delta 13C) \quad (4)$$

5. Ehrich et al. (2011) developed a species-specific linear model for bird and mammal muscle using the C:N of BULK tissue in relation to $\delta^{13}C_{\text{DIFF}}$.

$$\delta^{13}C' - \delta^{13}C = \beta 0 + \beta 1(\text{Bulk C:N}) \quad (5)$$

6. Logan et al. (2008; Eq. 3) developed a linear model for aquatic vertebrate and invertebrates using log-transformed BULK C:N in relation to $\delta^{13}C_{\text{DIFF}}$.

$$\delta^{13}C' - \delta^{13}C = \beta 0 + \beta 1 \ln(\text{C:N}) \quad (6)$$

We compared the validity of each LN model in relation to our data by species using Akaike information criterion corrected for small sample sizes using residual sums of squares from regression analysis between observed $\delta^{13}C_{\text{DIFF}}$ and predicted $\delta^{13}C_{\text{DIFF}}$ (AIC_c; Burnham and Anderson 2002). AIC_c values were calculated using the following equation:

$$AIC_c = n^* \text{LN}(\text{RSS}/n) + \frac{2k(k+1)}{n-k-1}$$

where RSS is residual sums of squares, *n* represents sample size and *k* is the number of parameters in the model, and in this case is three which includes the intercept, slope and predicted $\delta^{13}C_{\text{DIFF}}$. Model fit was assessed using r^2, mean square error (MSE) and AIC_c values where higher r^2 values and smaller MSE and AIC_c values equate to better model

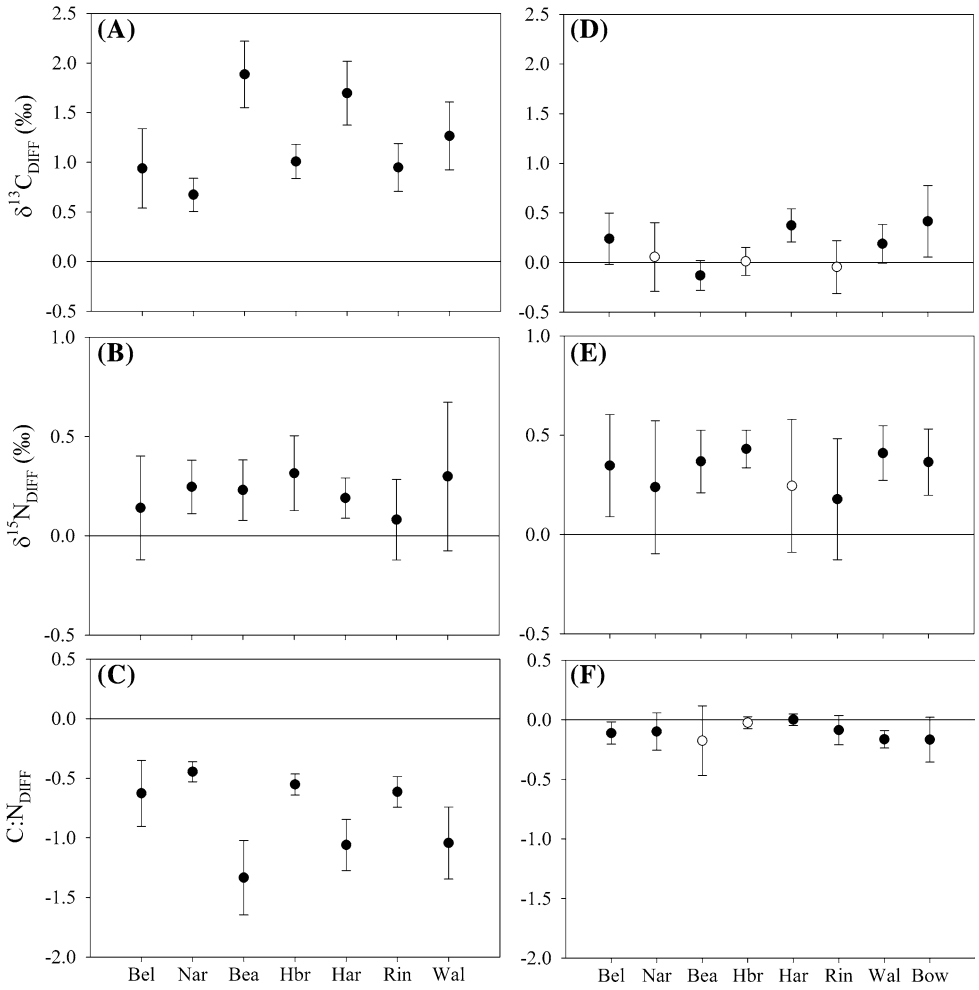

Fig. 1 Differences in $\delta^{13}C$ (**a**, **d**), $\delta^{15}N$ (**b**, **e**) and C:N ratio (**c**, **f**) between lipid-extracted (LE) and non-lipid-extracted (BULK) liver (**a**–**c**) and muscle (**d**–**f**) samples from Arctic marine mammals. *Circles* represent mean values (±SD) with significance (*black circles*) and nonsignificance (*open circles*) in paired *t* tests between LE and BULK values (see Supplementary material). See Table 1 for species codes and sample size

fit and accuracy. In addition to determining the model with the best fit, we calculated AIC_c weights that measure the weight in support of the model given the data and the difference between the lowest AIC_c and the other models (Δ_i) using the equation:

$$\Delta_i = AIC_{ci} - \min AIC_c$$

where AIC_{ci} is the AIC_c value for model *i* and min AIC_c is the minimum AIC_c value between all models (Burnham and Anderson 2002). Models with a Δ_i value ≤ 2 have the most support, whereas Δ_i between 4 and 7 have considerably less support and $\Delta_i > 10$ have no support (Burnham and Anderson 2002). The proportion of predicted $\delta^{13}C_{DIFF}$ values that were within 0.1 ‰ (i.e., analytical precision) of the observed $\delta^{13}C_{DIFF}$ values ($P_{0.1}$) was calculated to determine LN model accuracy. Statistical analyses were performed using Systat 11.0 (Systat Software Inc., Chicago, Illinois) with $\alpha = 0.05$.

Results

Liver and skin

Differences in $\delta^{13}C$, $\delta^{15}N$ and C:N values between CLE and BULK were species and tissue specific (Fig. 1a–f). Mean LE $\delta^{13}C$ and $\delta^{15}N$ values were significantly greater compared with BULK $\delta^{13}C$ and $\delta^{15}N$ values ($\delta^{13}C_{DIFF}$ and $\delta^{15}N_{DIFF}$), ranging from 0.7 (narwhal) to 1.9 ‰ (bearded seal; $t_{4-43} = 8.98–26.03$; all $P < 0.001$) for C and 0.1 (ringed seal and narwhal) to 0.3 ‰ (harbor seal and walrus; $t_{4-43} = 2.67–6.14$; all $P < 0.02$) for N in liver (Fig. 1a, b; Table 1 and Online Resource 1). In addition, the C:N value significantly decreased after CLE in liver among all species ($t_{4-43} = 9.64–31.80$; all $P < 0.001$) by an average of 0.5 (narwhal) to 1.4 (bearded seal). The $\delta^{13}C_{DIFF}$, $\delta^{15}N_{DIFF}$ and $C:N_{DIFF}$ values (difference between LE and BULK tissue) of liver were not consistent within species with the highest

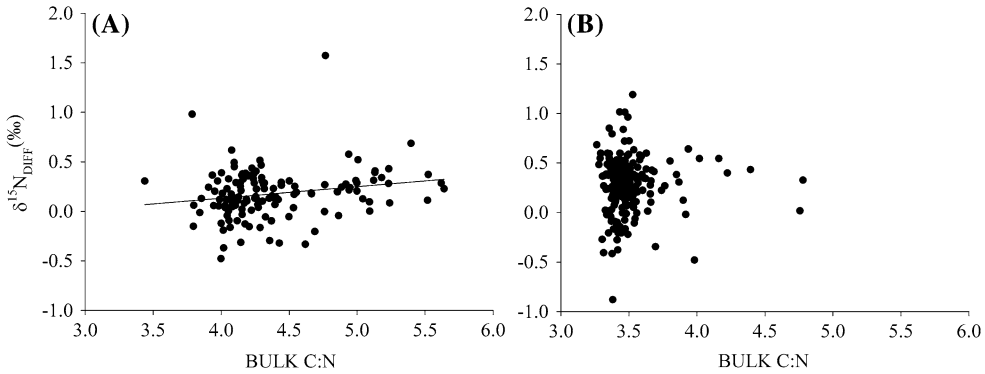

Fig. 2 Relationship between the difference in $\delta^{15}N$ of lipid-extracted and BULK liver (**a**) and muscle (**b**) samples and the C:N value of non-lipid-extracted (BULK) from Arctic marine mammals. Linear regression for **a** was significant ($\delta^{15}N_{DIFF} = 0.12x - 0.33$, $r^2 = 0.04$, $F_{1,132} = 5.54$, $P < 0.05$) for liver, but no significant relationship was found for muscle

amount of variation in $\delta^{13}C_{DIFF}$ and $\delta^{15}N_{DIFF}$ occurring in beluga and walrus, respectively (Fig. 1a, b). Among individuals within a species, the maximum $\delta^{15}N_{DIFF}$ values occurred in walrus (1.6 ‰) and beluga (1.0 ‰), but generally was <0.5 ‰ for most species. As well, the largest amount of variation in $C:N_{DIFF}$ values occurred in beluga (1.5), walrus and bearded seals (1.9; Fig. 1c). In narwhal skin, there was a significant increase in $\delta^{13}C$ by 0.9 ‰ ($t_{38} = 15.25$; $P < 0.001$) and difference in C:N ($t_{38} = 10.84$; $P < 0.001$) between LE and BULK samples, whereas $\delta^{15}N$ remained unchanged ($t_{38} = 1.64$; $P > 0.05$).

Muscle

The effects of CLE on $\delta^{13}C$, $\delta^{15}N$ and C:N values for muscle were much more variable between species than liver. The $\delta^{13}C$ values were significantly higher for LE muscle samples in relation to BULK muscle samples by a mean of 0.2–0.4 ‰ ($t_{6-34} = 3.05$–7.24; $P < 0.001$) in harp seals, walrus, bowhead and beluga (Table 1), whereas there was no significant effect of CLE on $\delta^{13}C$ values for harbor and ringed seals, and narwhal ($t_{10-57} = 0.27$–1.27; $P > 0.05$; Fig. 1d). The $\delta^{13}C$ values of bearded seal muscle became significantly depleted in ^{13}C (−0.2 ‰; $t_{21} = 4.31$; $P < 0.01$) and the $\delta^{15}N$ values were significantly enriched in ^{15}N after CLE in muscle for all species (t_{6-57} range = 2.77–15.14; all $P < 0.01$) by an average of 0.1–0.4 ‰, except for harp seals ($t_6 = 1.94$; $P > 0.05$; Fig. 1d, e). The C:N values significantly decreased after CLE in muscle for harp and ringed seals, walrus, bowhead, beluga and narwhal ($t_{6-57} = 3.90$–6.18; all $P < 0.005$), except for bearded ($t_{21} = 1.27$; $P = 0.22$) and harbor ($t_{10} = 1.04$; $P > 0.05$) seals. The highest amount of variation in $\delta^{13}C_{DIFF}$, $\delta^{15}N_{DIFF}$ and $C:N_{DIFF}$ in muscle occurred in bowhead, beluga, narwhal, harbor seals and ringed seals (Fig. 1d, e). The maximum increases in $\delta^{15}N_{DIFF}$ occurred in beluga (1.2 ‰), narwhal (1.0 ‰) and ringed seals (1.0 ‰), but generally $\delta^{15}N_{DIFF}$ increased <0.5 ‰ for most species. The largest amount of variation in $C:N_{DIFF}$ values occurred in bowhead whales (1.0; Fig. 1f). Among marine mammals, regressions of $\delta^{15}N$ values between BULK and LE samples showed a 1:1 relationship (slope = 0.97 for liver and 0.96 for muscle), indicating that the effect of CLE was uniform across different species and C:N values. Among all species, a significant increase between $\delta^{15}N_{DIFF}$ and BULK C:N only occurred in liver, but with low fit ($r^2 = 0.04$; Fig. 2).

Tissue lipid content

Percent lipid content was higher in liver than muscle for all species (Table 2) where BULK C:N was a better indicator of lipid content in liver than muscle (Fig. 3). Slightly higher mean %C values occurred in lipids extracted from liver (67.1–74.3 %) compared with muscle (60.8–67.1 %), whereas mean %N values were slightly higher in muscle (3.5–5.0 %) than liver (1.9–3.2 %) among all species (Table 2). As a result, mean C:N values were much higher in lipids extracted from liver (24.0–39.5) than muscle (12.6–19.9; Table 2). The $\delta^{13}C$ values of lipids extracted from both muscle and liver were similar among each species (mean difference = 0.2–0.5 ‰, except narwhal = 1.8 ‰; Table 2). Lipid $\delta^{13}C$ values were lower than the $\delta^{13}C$ values of LE muscle (D; 6.6–7.8 ‰) and liver (6.9–7.9 ‰) for each species (Table 2). The $\delta^{15}N$ values of lipids were lower than $\delta^{15}N$ values of BULK tissues. Although lipids extracted from muscle had slightly higher %N values, $\delta^{15}N$ values were lower in comparison with liver for all respective species, except narwhal (Table 2). The isotopic difference of $\delta^{15}N$ between lipid and BULK liver and muscle (D) was generally higher in muscle than liver, but less variation occurred in liver (Table 2). Among marine mammal liver and muscle, no relationship occurred between tissue lipid content (%) and N (μg) in lipid extracts (Fig. 4).

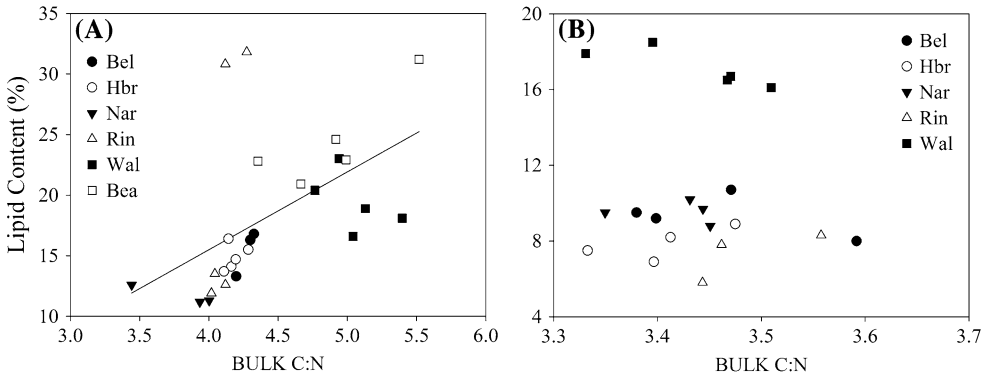

Fig. 3 Relationship between BULK C:N and lipid content (%) for liver (**a**) and muscle (**b**) of six and five Arctic marine mammals, respectively. See Table 1 for species codes. The linear regression for **a** was $y = 6.42x - 10.19$, $r^2 = 0.28$, $P < 0.01$ and **b** was not significant

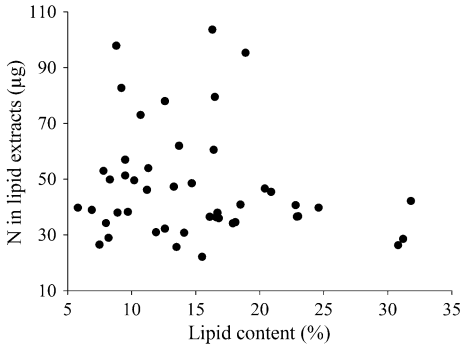

Fig. 4 Relationship between lipid content and N of lipid extracts in Arctic marine mammal muscle and liver. No significant relationship occurred

LN models

Among all individuals, a significant linear increase in $\delta^{13}C_{DIFF}$ in relation to BULK C:N occurred in both liver and muscle (Fig. 5). After estimating the $\delta^{13}C_{DIFF}$ using LN models, best model fit was evaluated using ΔAIC_c, MSE, r^2 and $P_{0.1}$ with results varying considerably between species and tissues. The species-specific linear models (i.e., models 4 and 5) were the most appropriate for liver among species ($P_{0.1}$ range = 45–86 %; r^2 range = 0.21–0.97; Table 3; see Online Resource 2 for parameter estimates). Model 4 fit the best for harbor and harp seals, walrus and narwhal, whereas model 5 was the best fit for bearded seals, ringed seals, and beluga (Table 3). Model 5 fit best for narwhal skin ($P_{0.1} = 32$ %; $r^2 = 0.54$; Table 3). For muscle, model 5 had the lowest ΔAIC_c for some species (harp and ringed seals, and walrus) and generally had the best fit ($P_{0.1}$ range = 47–75; r^2 range = 0.18–0.59). Model fit varied among the other species, as model 4 had the lowest ΔAIC_c for bearded and harp seals, and bowhead whales, and model 3 had the best fit for harbor seals, beluga and narwhal. Model 6 had very poor fit for both liver and muscle of all species (Fig. 6d, h). Overall, the generalized LN models (models 1, 2 and 3, and 6; Fig. 5) were not as accurate in predicting lipid-free $\delta^{13}C$ values in most tissues and species compared with the species-specific linear models (models 4 and 5).

Discussion

Effect of CLE on $\delta^{13}C$ and $\delta^{15}N$

The need for CLE depends on the study question being addressed, but the importance of standardizing $\delta^{13}C$ values is vital when investigating individual- and population-level dietary and habitat differences (Hobson et al. 2002; Newsome et al. 2009). The effect on $\delta^{13}C$ and $\delta^{15}N$ values after CLE varied considerably between tissues and species demonstrating the importance of a more quantitative understanding of tissue and species effects when using stable isotopes in ecological studies. As expected, CLE significantly increased $\delta^{13}C$ values in higher-lipid marine mammal tissues, but not always for lower-lipid tissues (i.e., muscle). Lipid content may explain much of the variability in elemental composition such as $\delta^{13}C$ between Arctic marine mammal species, which has been reported in fishes and related to life-stage, sex, breeding status, foraging ecology, season and geographic location (Hendrixson et al. 2007; Fagan et al. 2011). Due to the opportunistic sampling, lack of biometrical information for most samples and the paucity of samples by season and species, we are unable to investigate intra-annual and intra-species variation in lipid content for Arctic marine mammals.

CLE also significantly influenced $\delta^{15}N$ values, generally causing an increase although results were species and tissue dependent. This increase in $\delta^{15}N$ was verified by isotope analysis of lipid extracts, which had a small amount of ^{15}N-depleted nitrogen. Lesage et al. (2010) found that CLE had a small but significant effect on $\delta^{15}N$ values for cetacean skin potentially as a result of water washing (Jacob et al. 2005), whereas Horstmann-Dehn et al. (2012) and

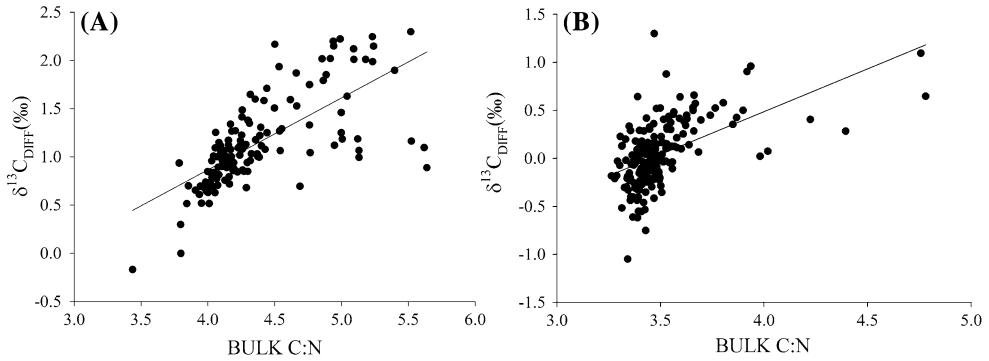

Fig. 5 Linear regressions for $\delta^{13}C_{DIFF}$ (difference between BULK and LE $\delta^{13}C$ values) relative to BULK C:N among all species for liver (**a**) and **b** muscle. Significant relationships occurred for both **a** ($\delta^{13}C_{DIFF} = 0.75x - 2.12$, $r^2 = 0.48$, $F_{1,132} = 123.9$, $P < 0.001$) and **b** ($\delta^{13}C_{DIFF} = 0.89x - 3.08$, $r^2 = 0.29$, $F_{1,191} = 76.7$, $P < 0.001$)

Ehrich et al. (2011) observed no significant changes in $\delta^{15}N$ for cetacean skin and muscle, and bird and terrestrial mammal muscle, respectively. Both studies, as well as ours, used a chloroform/methanol solvent to extract lipids, but the effects on mammal tissues varied considerably between studies, likely due to differences in tissue lipid content, variation in specific extraction techniques or differences in analytical precision (<0.1 ‰ for $\delta^{13}C$ and $\delta^{15}N$—this study; 0.2 ‰ for $\delta^{13}C$ and $\delta^{15}N$—Horstmann-Dehn et al. 2012; Ehrich et al. 2011).

The mean enrichment of $\delta^{15}N$ values in liver (0.2 ‰) and muscle (0.3 ‰) of all species after CLE was greater than analytical variability. Within species, a high amount of variation in $\delta^{15}N$ values (up to 1.6 ‰ difference) occurred after CLE, similar to other studies (Murry et al. 2006; Ryan et al. 2012). This can cause trophic misinterpretations at a population or species level in terms of isotopic niche analyses (Newsome et al. 2007), particularly when using community-wide metrics (i.e., convex hull; Layman et al. 2007). In addition, increases in $\delta^{15}N$ by CLE could alter trophic level estimates via a scaled framework (Hussey et al. 2014) in higher-order taxa, as well as diet reconstructions by mixing models (Tarroux et al. 2010), which are also sensitive to diet–tissue discrimination factors (DTDF = $\delta^{15}N_{pred} - \delta^{15}N_{prey}$; Bond and Diamond 2011). A DTDF of less than 2.5 has been reported for birds (Hobson and Clark 1992; Caut et al. 2009), large animals (Hobson et al. 1996; Hussey et al. 2010, 2012a; Caut et al. 2011; Varela et al. 2011) and animals that consume high-$\delta^{15}N$ prey (Caut et al. 2009; Hussey et al. 2014).

Lipid content

BULK C:N was not a reasonable indicator of lipid content in marine mammal liver and muscle, which is similar to results from Fagan et al. (2011) for lake whitefish (*Coregonus clupeaformis)* but contrasts with previous studies on other fishes, crustaceans and combined aquatic and terrestrial species (Bodin et al. 2007; Post et al. 2007; Logan et al. 2008). Biological factors, such as a species' feeding ecology, nutritional status, age and reproductive status, play a role in lipid content and elemental composition variability which may lead to these discrepancies between studies (Fagan et al. 2011). As well, the effect of lipid content on $\delta^{13}C$ values at low C:N values ($\leq$3.5) varies among species and tissues, and tissues should not necessarily be considered lean (i.e., lipid extraction not required) in samples where C:N $\leq$ 3.5. In general, we found that tissues with C:N < 3.5 do not require CLE, consistent with Post et al. (2007). However, a significant increase in $\delta^{13}C$ occurred after CLE in beluga and walrus muscle despite C:N $\leq$ 3.5, suggesting that caution should be used when applying the "C:N 3.5 rule." Lesage et al. (2010) observed a similar result, finding that lipids can have a relatively large effect on $\delta^{13}C$ in cetacean skin with low C:N. The range of $\delta^{13}C$ *D* (isotopic difference between lipids and LE tissue) was similar for liver and muscle among all species, but slightly higher than that for tissues of pelagic seabirds (4.2–6.8 ‰; Thompson et al. 2000), fish (5.5–7.3 ‰; Focken and Becker 1998; Gaye-Siesseggar et al. 2003; Schlechtriem et al. 2003) and cetacean skin (6.4 ‰; Lesage et al. 2010). This suggests that lipids in marine mammal liver and muscle may be more depleted in ^{13}C, but requires further investigation via compound-specific stable isotope analysis.

Ingram et al. (2007) found a significant negative relationship between $\delta^{15}N_{DIFF}$ and BULK C:N for fish muscle and proposed that the effects of CLE on $\delta^{15}N$ values decrease with higher C:N. Our data do not support this finding, as an isometric relationship between $\delta^{15}N$ with increasing BULK C:N for muscle and a significant positive relationship with low fit occurred in liver. A small amount of nitrogen was observed in lipids extracted via CLE from liver and muscle in all species. The amount of N in the lipid

Table 3 Summary of Akaike's information criterion corrected for small sample sizes and expressed as ΔAICc values to determine the LN model with the best fit for Arctic marine mammal tissues

Model	MSE	r^2	ΔAIC_c	Akaike weight	$Pred_{0.1}$ (%)	$Pred_{0.5}$ (%)
Liver						
Pinnipeds						
Bearded seal						
2	0.04	0.86	2.50	0.19	61	100
5	0.04	0.65	0.00	0.66	61	100
Harbor seal						
3	0.02	0.50	0.67	0.40	64	100
4	0.01	0.46	0.00	0.56	73	100
Harp seal						
4	0.02	0.79	0.00	0.97	86	100
5	0.05	0.44	6.93	0.03	57	100
Ringed seal						
3	0.04	0.19	29.80	0.00	43	100
5	0.02	0.62	0.00	1.00	68	100
Walrus						
4	0.09	0.21	0.00	0.91	50	90
5	0.11	<0.00	4.67	0.09	45	85
Cetaceans						
Beluga						
2	0.05	0.74	7.31	0.03	55	100
5	0.04	0.74	0.00	0.97	61	100
Narwhal						
4	0.01	0.97	0.00	0.59	80	100
5	0.02	0.96	2.03	0.22	80	100
Muscle						
Pinnipeds						
Bearded seal						
4	0.01	0.38	0.00	1.00	80	100
5	0.02	0.02	10.15	0.00	70	100
Harbor seal						
3	0.01	0.79	0.00	1.00	100	100
4	0.01	0.20	12.79	0.00	73	100
Harp seal						
4	0.02	0.33	1.40	0.33	71	100
5	0.01	0.45	0.00	0.67	71	100
Ringed seal						
4	0.06	0.16	6.11	0.04	60	98
5	0.05	0.25	0.00	0.96	62	98
Walrus						
4	0.03	0.54	1.02	0.32	74	100
5	0.03	0.56	0.00	0.54	58	100
Cetaceans						
Beluga						
3	0.03	0.69	0.00	0.97	65	97
4	0.03	0.60	7.06	0.03	48	97
Bowhead						
4	0.02	0.83	0.00	0.93	71	100
5	0.04	0.64	5.21	0.07	57	100
Narwhal						
3	0.06	0.62	0.00	0.62	38	100
5	0.06	0.48	1.18	0.35	44	97
Skin						
Narwhal						
4	0.06	0.48	5.16	0.07	0	100
5	0.05	0.54	0.00	0.93	38	97

Only the top two models are shown for each species and tissue; corresponding models can be found in the methods. Coefficient of determination (r^2), mean square error (MSE), $P_{0.1}$ and $P_{0.5}$ represent the proportion between predicted and observed $\delta^{13}C_{DIFF}$ values within 0.1 and 0.5 ‰, respectively

extracts was not correlated with tissue lipid content, indicating that this lipid-associated N is likely some combination of cell membrane proteins associated with both nonpolar and polar lipids, such as glycolipids, phospholipids and sphingolipids (Sotiropoulos et al. 2004; Bodin et al. 2007), and nitrogenous waste product, such as urea or ammonia, as suggested but not verified by other studies (Bearhop et al. 2000; Fisk et al. 2002; Hussey et al. 2012b). In mammals, urea is synthesized from ammonia in the liver (Balter et al. 2006); thus, if a significant amount of metabolic waste products were removed via CLE, one would expect much more N and higher $\delta^{15}N$ *D* in lipids from liver than muscle in all species, which did not occur. The magnitude of *D* for $\delta^{15}N$ varied slightly between muscle and liver, but N in lipid extracts was relatively low among all species, suggesting that CLE extracts a minor amount of both metabolic waste compounds and cell membrane proteins.

LN models

The use of LN models largely depends on the precision of stable isotope values needed for one's study objectives. Models specific to species and tissue generally fit best, and despite observing a significant linear relationship with modest fit between BULK C:N and $\delta^{13}C_{DIFF}$ for both Arctic marine mammal liver and muscle, linear LN model performance varied considerably between tissues and species. However, the linear LN models using BULK C:N (models 3 and 5) and BULK $\delta^{13}C$ (model 4) were generally the top-performing models when predicting lipid-free $\delta^{13}C$. Overall, the reliability of a common LN model for Arctic

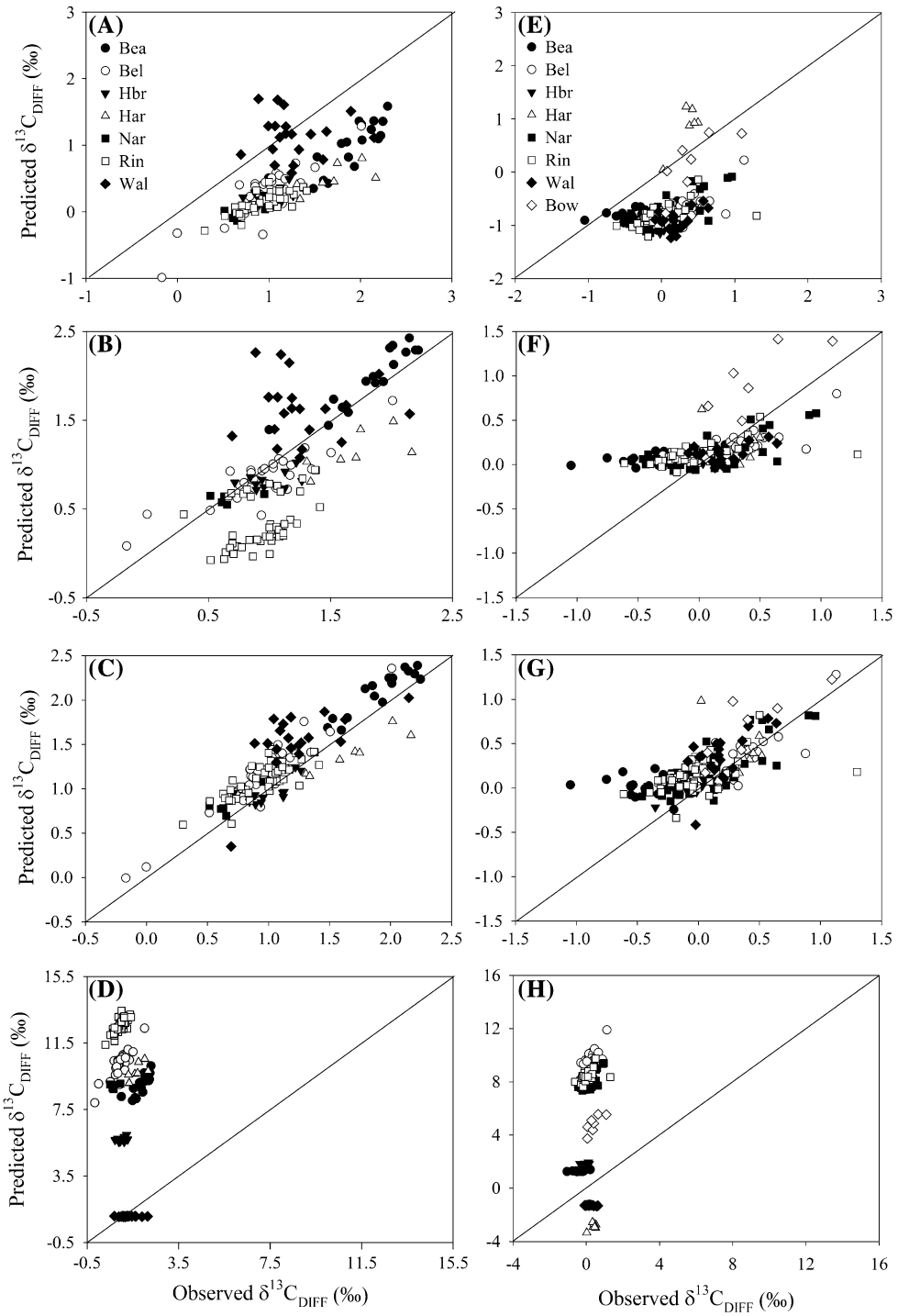

Fig. 6 Predicted difference in $\delta^{13}C$ between lipid-extracted and non-lipid-extracted ($\delta^{13}C_{DIFF}$) Arctic marine mammal liver (**a**–**d**) and muscle (**e**–**h**) by model 1 (**a**, **e**), model 2 (**b**, **f**), model 3 (**c**, **g**) and model 6 (**d**, **h**) in relation to observed values. The *solid line* represents a 1:1 relationship. See Table 1 for species codes. Legend in **a** corresponds to **b**–**d**, whereas legend in **e** corresponds to **f**–**h**

marine mammal muscle and liver did not occur, similar to observations from tissues of other mammalian (Lesage et al. 2010; Ryan et al. 2012) and fish (Logan et al. 2008; Mintenbeck et al. 2008) species.

The proportion of predicted $\delta^{13}C_{DIFF}$ values that were within 0.1 ‰ of the observed $\delta^{13}C_{DIFF}$ values ($P_{0.1}$) for the best fit LN models was generally low for most species and tissues, except in harp seal and narwhal liver, and bearded seal, harbor seal, harp seal, walrus and bowhead muscle where $P_{0.1}$ was ≥70 %. Our results contrast sharply with Ehrich et al. (2011) who concluded that several LN models (models 2, 3 and 5) were able to accurately predict $\delta^{13}C_{DIFF}$ values in Arctic bird and mammal muscle. Caution must be used with their recommendation due to their approach where a predicted $\delta^{13}C_{DIFF}$ value within 0.5 ‰ of the observed $\delta^{13}C_{DIFF}$ value ($P_{0.5}$) was considered

Table 4 Framework to determine whether lipid extraction is required for Arctic marine mammal (individual, population and species-group levels) tissues prior to stable isotope (SI) analysis of $\delta^{13}C$ and $\delta^{15}N$

Stable isotope	Bearded seal	Harbor seal	Harp seal	Ringed seal	Walrus	Beluga	Bowhead	Narwhal
Muscle								
$\delta^{13}C$	R	N	R	N	R	R	R	N
$\delta^{13}C$ and $\delta^{15}N$	A	N	R	N	A	A	A	N
Liver								
$\delta^{13}C$	R	R	R	R	R	R	–	R
$\delta^{13}C$ and $\delta^{15}N$	A	A	A	A	A	A	–	A
Skin								
$\delta^{13}C$	–	–	–	–	–	R†	R*	R
$\delta^{13}C$ and $\delta^{15}N$	–	–	–	–	–	R†	R*	R

R lipid extraction is required, *A* lipid extraction is required but significantly alters $\delta^{15}N$, *N* no significant difference between LE and BULK sample

* From Lesage et al. (2010)

† From Horstmann-Dehn et al. (2012)

– Signifies unstudied

acceptable. When $P_{0.5}$ was applied to our data for the best fit LN model, prediction accuracy increased substantially with the majority of species reaching 100 % predictive efficiency in liver, muscle and skin, but we do not recommend its use when maximal precision is required. A $\delta^{13}C_{DIFF}$ of 0.5 ‰ is over twice the average analytical variability (0.2 ‰) for stable isotope analysis and would have a variance of 1.0 ‰, which is comparable to the trophic discrimination factor in carbon between prey and consumers for numerous taxa (DeNiro and Epstein 1978; Caut et al. 2009). A variance of 1.0 ‰ for $\delta^{13}C$ can lead to considerable bias when estimating trophic discrimination factors and when using Bayesian stable isotope mixing models to estimate dietary proportions. In a two-end member $\delta^{13}C$ mixing model where sources were 2.0 ‰ apart, Lesage et al. (2010) found that a 0.5 ‰ error due to sample treatment in consumer $\delta^{13}C$ resulted in a bias of 30 % in prey contributions. Thus, when determining how well the LN model predicted $\delta^{13}C_{DIFF}$ compared to the observed $\delta^{13}C_{DIFF}$, we recommend using a value within analytical precision.

Decision framework

The question of whether CLE is required to standardize data is dependent on individual research questions. We developed a summary decision framework to guide researchers on the effects of CLE based on our study species and stable isotopes of interest (Table 4). When solely using $\delta^{13}C$ to investigate habitat use, such as two-source primary production models, CLE is always required in liver and skin (i.e., higher-lipid content) for marine mammals. However, the effects of CLE in marine mammal muscle (i.e., lower-lipid content) were species specific and is likely a result of lipid content affecting elemental composition in muscle due to potential underlying differences in age, breeding and nutritional status, and feeding ecologies (Hendrixson et al. 2007; Fagan et al. 2011). Therefore, to mitigate intra- and inter-species variability in lipid content of marine mammal muscle, we recommend using CLE to standardize $\delta^{13}C$ values.

When using $\delta^{13}C$ and $\delta^{15}N$ in combination to address factors, such as estimating dietary proportions of prey items, diet–tissue discrimination factors, niche size, nutritional stress and turnover rate analysis, lipids must be accounted for, but a significant enrichment in $\delta^{15}N$ values after CLE occurred in most species which can lead to misinterpretations of ecological relationships. For liver and muscle of all study species, the best fit linear LN model performed reasonably well with $P_{0.1} \geq 50$ % but we recommend the use of LN models with a $P_{0.1} \geq 70$ %. This recommendation is conditional on the level of precision needed based on one's study objectives as the vast majority of LN models were 100 % accurate within 0.5 ‰, which has been used in other studies (Ehrich et al. 2011; Ryan et al. 2012). In marine mammal skin, LN models did not perform well; however, no significant alterations in $\delta^{15}N$ after CLE across species occurred, and thus, we advocate using CLE on marine mammal skin. Overall, our results highlight that species-specific linear LN models used with their own estimated parameters should be used in most cases.

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