Influence of lipids on stable isotope ratios in mammal hair: highlighting the importance of validation

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Abstract. Carbon (\(\delta^{13}C\)) and nitrogen (\(\delta^{15}N\)) stable isotope ratios are increasingly used in ecological studies to evaluate diet composition and trophic relationships. However, lipids may influence stable isotope ratios due to the depletion of \(^{13}C\) in adipose tissues relative to proteins and carbohydrates. \(\delta^{13}C\) values can be corrected by lipid extraction or normalization models. The aims of our study were to evaluate the effects of lipid extraction on stable isotope ratios in a terrestrial mammal, the caribou (Rangifer tarandus caribou), and to propose relevant lipid normalization models that are method- and tissue-specific for \(\delta^{13}C\) values. We also evaluated whether four \(\delta^{13}C\) lipid normalization and correction models proposed in the literature were applicable to our study species. Stable isotope ratios were obtained for hair, plasma, and red blood cell samples of 44 caribou in the Gaspé National Park (Québec, Canada). The effects of lipid extraction on stable isotope ratios were tested using a paired \(t\)-test. A simple linear model was used to correct for the effects of lipid extraction and to assess its performance compared to that of published equations. Lipid content significantly influenced \(\delta^{13}C\) values in caribou hair. The four lipid normalization equations commonly used in the literature did not accurately predict \(\delta^{13}C_{\text{lipid-free}}\) values of caribou hair. Based on our results, we recommend controlling systematically for lipids in terrestrial systems and analyzing \(\delta^{13}C\) (lipid-free) and \(\delta^{15}N\) (bulk) from two separate aliquots to reach a greater precision. We also recommend controlling for lipids in hair tissue. If not possible, we recommend using a lipid normalization model that is tissue-, method-, and species-specific or applying a model that has been previously validated for the tissue and species of interest.

Key words: \(\delta^{15}N\); \(\delta^{13}C\); lipid correction; lipid extraction; mathematical normalization; Rangifer tarandus caribou; stable isotopes; terrestrial ecosystems.

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INTRODUCTION

Understanding the foraging ecology of a species, a population or an individual is a fundamental question in ecology, conservation, and management. Foraging is a key behavior as it influences the growth, reproduction, and survival of an individual and may thus influence its fitness (Brown 1992, Abramsky et al. 2002). It is thus important to improve constantly the knowledge regarding the diet of wild species. Because direct observation of feeding behaviors in wild species is difficult and often impossible (Litvaitis 2000), other techniques were developed to acquire information about the diet of a species. Researchers often have to use indirect methods to assess diet, such as the analysis of prey parts from scats and stomach contents (McInnis et al. 1983, Hewitt and Robbins 1996, Deb 1997). More recently, biogeochemical markers like stable
isotopes have become a key tool to study the feeding ecology of wild species (Peterson and Fry 1987, Kelly 2000).

Carbon (δ13C) and nitrogen (δ15N) stable isotope ratios are increasingly used in ecological studies to provide information on sources of primary productivity (Ramsay and Hobson 1991, France 1995) and energy flow (Peterson and Fry 1987) as well as habitat use and migratory patterns (Rubenstein and Hobson 2004). Stable isotope ratios are useful to provide insights into trophic relationships (Hobson and Welch 1992, Post 2002) and diet composition (Ben-David et al. 1997, Phillips et al. 2005). Isotope analyses are based on the principle that stable isotope ratios in the tissues of consumers reflect the ratios of their diet (DeNiro and Epstein 1978, 1981, Hobson et al. 1996). On the other hand, the relatively high (+3–4‰) and predictable enrichment in 15N from one trophic level to the next makes nitrogen isotopes useful indicators of trophic position (DeNiro and Epstein 1981, Minagawa and Wada 1984, Peterson and Fry 1987). The lower trophic enrichment for 13C (typically −1‰) limits the usefulness of this element as an index of trophic position but makes it particularly suitable for delineating carbon sources (DeNiro and Epstein 1978, Peterson and Fry 1987).

One limitation of stable isotope analysis, however, is that lipids are more depleted in 13C relative to protein and carbohydrate fractions (DeNiro and Epstein 1977, McConnaughey and McRoy 1979). The depleted δ13C ratios result from the isotopic fractionation that occurs during the conversion of pyruvate to acetyl coenzyme A during lipid synthesis (DeNiro and Epstein 1977). The δ13C values are also influenced by the heterogeneity in lipid content among organisms (Post et al. 2007) and tissue types (McConnaughey and McRoy 1979, Sweeting et al. 2006) and by the chemical extraction methods (Dobush et al. 1985, Logan and Lutcavage 2008, Elliott and Elliott 2016). Lipid extraction methods using polar solvents tend to increase the δ13C values and decrease CN ratios compared to non-polar solvents (Logan and Lutcavage 2008). Thus, not accounting for lipids may bias future interpretation of trophic relationships or diet composition (Lesage et al. 2010, Tarroux et al. 2010). A consensus about the necessity to extract lipids or account for their effects is recognized in the literature (Kelly 2000, Post et al. 2007). Two different approaches have been suggested to do so: controlling variability in δ13C values a priori through lipid extraction or, when the former is impossible, accounting for lipids a posteriori using a previously published normalization model (McConnaughey and McRoy 1979, Fry 2002, Post et al. 2007, Lesage et al. 2010).

During the past decade, lipid extraction was the approach advocated to obtain uniform samples in order to ease comparison (Post et al. 2007). It was generally assumed that the chemical solvents used to remove lipids would not alter the δ15N values of tissue (Sotiropoulos et al. 2004). However, recent studies conducted on a variety of marine and freshwater vertebrates have documented an enrichment in δ15N values caused by the leaching of isotopically light nitrogenous cell components in association with extraction solvents (Sotiropoulos et al. 2004, Mintenbeck et al. 2008, Lesage et al. 2010). In contrast, other studies focusing on marine and terrestrial animals have documented negative or neutral effects of chemical solvents on δ15N values (Bodin et al. 2007, Ricca et al. 2007, Ehrich et al. 2011). The effects of lipid extraction on δ15N values differ considerably between species, tissues, and methods, indicating the need to evaluate solvent effects on various tissues and in multiple species (Sotiropoulos et al. 2004). Some authors have recommended conducting lipid extraction on separate aliquots of samples to determine δ13C and δ15N values because of the variability often observed following lipid extraction (Sweeting et al. 2006, Lesage et al. 2010). However, this procedure is a time- and labor-intensive process and may be very expensive for large sample sizes (Kelly 2000, Kiljunen et al. 2006). Lipid extraction is therefore the approach recommended when a maximum of precision is needed for calculations of diet composition involving mixing models and niche isotopic overlap (Lesage et al. 2010, Tarroux et al. 2010).

Another common strategy used to solve the problem of the lipid effect refers to lipid normalization equations employed a posteriori; several of these equations have been established to estimate lipid-free δ13C values in various aquatic organisms (McConnaughey and McRoy 1979, Fry 2002, Post et al. 2007). These models use the
elemental ratio of C to N (CN ratio) as a proxy for lipid content and the isotopic difference between proteins and lipids (DeNiro and Epstein 1977, McConnaughey and McRoy 1979). However, the relationship between the CN ratio and the δ13C value can vary among tissues (Logan et al. 2008) as well as between closely related species (Mintenbeck et al. 2008), thus questioning the application of these lipid normalization models. Lipid normalization models are not always applicable to all taxa and tissues, so alternative models have been proposed (Kiljunen et al. 2006, Post et al. 2007, Lesage et al. 2010, Tarroux et al. 2006, Post et al. 2007). Kiljunen et al. (2006) re-estimated the isotopic differences between proteins and lipids and improved the precision and applicability of the lipid normalization corrections in various aquatic and marine organisms. Errors introduced by lipids or normalization equations may bias the interpretation of trophic relationships and dietary composition (Post et al. 2007, Lesage et al. 2010). Considering this, Kiljunen et al. (2006) recommended to validate models before applying them to a particular species and tissue, or to use tissue-specific and species-specific models. These specific models have generally fit to the data better and give better predicted values of lipid-free δ13C than generalized models (Logan et al. 2008, Lesage et al. 2010).

In terrestrial animals, however, only a few studies have focused on the effect of lipids on δ13C and δ15N values (but see Post et al. 2007, Tarroux et al. 2010, Ehrich et al. 2011). Post et al. (2007) proposed a linear normalization equation using the CN ratio but recommended validating it for terrestrial animals because their sample size was small. Before applying a model developed for another species, tissue, or a different lipid extraction protocol, it is necessary to take into account the potential biases associated with such an arithmetical correction of δ13C values for lipids (Lesage et al. 2010, Tarroux et al. 2010). Furthermore, it is commonly assumed in the literature that no lipid extraction or normalization is necessary when using low-lipid tissues (CN ratio < 4 for terrestrial animals and <3.5 for marine animals; Post et al. 2007). However, some studies observed a significant increase in δ13C after lipid extraction despite a CN ratio below 3.5, and therefore do not support this assumption (Lesage et al. 2010, Yurkowski et al. 2015).

Our study aimed to (1) evaluate the effects of lipid extraction on δ13C, δ15N, and CN ratio values in plasma, red blood cells, and hair of a terrestrial mammal, the woodland caribou, (2) propose tissue-, species-, and method-specific lipid normalization models for δ13C values, (3) evaluate whether four of the most commonly used δ13C lipid normalization and correction models throughout literature (McConnaughey and McRoy 1979, Fry 2002, Post et al. 2007, Ehrich et al. 2011) are suitable to correct δ13C estimates for our studied species, and (4) determine whether those four models are efficient to deal with varying lipid contents in different types of mammalian tissues.

**Materials and Methods**

**Study area**

We focused our study on the range of the Atlantic–Gaspésie caribou population, a small and relict herd using bare habitats found at high elevations (~700 m) in the Gaspésie National Park and the surrounding Matane, Dunière, and Chic-Chocs Wildlife Reserves (Ouellet et al. 1996, Fig. 1). Three groups of summits are used by caribou, namely (from west to east) the Logan ridge (Mount Logan: 1128 m), Albert plateaus (Mount Albert: 1154 m), and McGerrigle area (Mount Jacques-Cartier: 1268 m). The altitudinal gradient determines three distinct ecological zones characterized by differences in vegetation type. The montane area (100–900 m) is mainly characterized by the balsam fir (Abies balsamea)–white birch (Betula papyrifera) bioclimatic domain, while the subalpine forest (900–1050 m) is a transition zone where tree height decreases with increasing elevation, and the alpine tundra (>1050 m) is characterized by lichens, mosses, graminoids, and ericaceous shrubs. The population was designated as Endangered in 2000 under the Canadian Species at Risk Act (SARA) and is considered as an irreplaceable component of Canada’s biodiversity (COSEWIC 2011). The estimated population size ranged between 69 and 82 caribou in the 2017 aerial survey (Morin 2017).

**Data collection and sample preparation**

We collected hair and blood samples from 44 caribou captured in 2013 and 2014 across the Gaspésie caribou range. Animals were captured
using a net gun fired from a helicopter; our capture and manipulation protocol was authorized by the Animal Welfare Committee [Université du Québec à Rimouski (hereafter UQAR) certificate #CPA-52-13-112; Ministère des Forêts, de la Faune et des Parcs (hereafter MFFP) certificate #CPA FAUNE 13-08]. We collected caribou blood with a sodium heparin tube. On the day of capture, we centrifuged whole blood kept on ice after capture to obtain plasma and red blood cells that were frozen at −20°C until they were processed. We dried and stored hair samples in paper bags at ambient temperature until they were processed.

We washed hair samples using a solution of 2:1 chloroform–methanol in an ultrasonic bath to remove all possible surface contamination and external lipids (Hobson et al. 2000). Samples were then rinsed in distilled water and oven-dried at 50°C for 24 h (Hobson et al. 2000). Hair and blood samples were freeze-dried for 48 h and ground to a fine powder (Bosley and Wainright 1999, Jardine et al. 2003). We used a Cryomill with cooling option (liquid nitrogen at −196°C) for hair samples. We divided samples into two parts: One part of the subsamples (bulk) received no further treatment prior to isotope analyses, and the second part (lipid-free) was lipid-extracted.

**Lipid extraction and stable isotopic analyses**

We conducted lipid extraction using 7 mg of powdered hair (to remove internal lipids, Dunnett 2005, Dunnett and Lees 2003) or 1 mL of blood material and a solvent consisting of a mixture of chloroform and methanol (2:1 v/v; Folch et al. 1957). We shook the mixture and stored it overnight at 4°C. We centrifuged the mixture at 11,200 g for 10 min and discarded the supernatant (Folch et al. 1957). We repeated the whole procedure two times. After three extractions, samples were dried by evaporation overnight,
rinse with distilled water, oven-dried overnight at 50°C, and powdered again.

We weighed a subsample of 0.500–0.700 mg (±0.001 mg) of powdered tissue (bulk and lipid-free) into a tin capsule and analyzed it for stable carbon and nitrogen isotope ratios using a COST-ECHECS 4010 Elemental Analyser coupled to a DeltaPlus XP Isotope Ratio Mass Spectrometer (IRMS, Thermo Electron Co, Marine Chemistry and Mass Spectrometry Laboratory, UQAR). By convention, 13C and 15N isotope abundances were expressed in delta notation (‰), as

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

where X is 13C or 15N, and Rsample is the corresponding ratio 13C/12C or 15N/14N; Rstandard represents the ratios for the respective standards, that is, Vienna Pee Dee Belemnite (PDB) and atmospheric nitrogen (AIR). We evaluated the accuracy of our isotopic analyses using commercially certified material (B2151) and the precision of measurement by randomly duplicating a subset of our samples. Replicates using certified materials (n = 7) indicated a systematic error of ± 0.28 and ± 0.25 ‰ for δ13C and δ15N, respectively, whereas the average deviations observed between replicates of hair and blood samples (n = 24) indicated an analytical error of 0.043 ‰ for δ13C and 0.045 ‰ for δ15N.

**Statistical analyses**

Using paired t-tests, we tested for an effect of lipid extraction on the δ13C, δ15N, and CN ratios while controlling for inter-individual variation. We used linear and nonlinear regression analyses to examine the relationship between \( \delta^{13}C_{\text{lipid-free}} - \delta^{13}C_{\text{bulk}} \), CN ratio, \( \delta^{15}N_{\text{bulk}} \), \( \delta^{15}N_{\text{lipid-free}} \), \( \delta^{13}C_{\text{bulk}} \), and \( \delta^{13}C_{\text{lipid-free}} \). We assessed the normality of residuals using normal quantile-quantile plots and assessed heteroskedasticity with a plot of standardized residuals against fitted values (Quinn and Keough 2002).

We proposed simple linear models from our data using \( \delta^{13}C_{\text{bulk}} \) values and CN ratios to correct lipid extraction effects and to assess its performance compared to published equations.

\[ \delta^{13}C_{\text{lipid-free}} = \beta_1 \times \delta^{13}C_{\text{bulk}} + \beta_0 \]  

(1)

\[ \delta^{13}C_{\text{lipid-free}} = \beta_1 \times \delta^{13}C_{\text{bulk}} + \beta_2 \times \text{CN} + \beta_0 \]  

(2)

We used four linear and nonlinear models integrating 13C and CN ratio parameters to investigate the potential application of lipid normalization equations and their literature parameters on caribou tissues. These four models are quite commonly used in the peer-reviewed literature, but their reliability for caribou tissues has not been evaluated. The first model, developed by McConnaughey and McRoy (1979) for various marine vertebrates and invertebrates, estimated lipid-free \( \delta^{13}C \) values using the following two equations:

\[ L = \frac{93}{1 + \left[ 0.246 \times \text{CN} - 0.775 \right]} \]  

(3)

\[ \delta^{13}C_{\text{lipid-free}} = \delta^{13}C_{\text{bulk}} + D \times \left( I + \frac{3.90}{1 - 0.207} \right) \]  

(4)

where L is the lipid content, CN is the ratio of C and N in the untreated samples, D is the isotopic difference between pure lipid and pure protein, and I is a constant. The McConnaughey and McRoy model (hereafter referred to as MM model) assumed a D of 6‰ and an I of −0.207 (McConnaughey 1978). Post et al. (2007) developed a simplified model using the CN ratio in the untreated samples for a wide variety of aquatic and terrestrial animals (see Eq. 6 in Post et al. 2007):

\[ \delta^{13}C_{\text{lipid-free}} = -3.44 + 1.00 \times \text{CN} + \delta^{13}C_{\text{bulk}} \]  

(5)

Fry (2002) developed a mass-balance approach and requires information on the CN ratio of untreated samples, pure protein (CNprotein), and D:

\[ \delta^{13}C_{\text{lipid-free}} = \delta^{13}C_{\text{bulk}} + D - \frac{D \times \text{CNprotein}}{\text{CN}} \]  

(6)

where CNprotein is determined from the lipid-extracted samples when no lipids remained in the sample and is assumed to be 3.7 (Fry 2002), and D is assumed to be 6‰ (McConnaughey 1978). Ehrich et al. (2011) developed a species-specific linear model for bird and mammal muscles using CN in the untreated sample:

\[ \delta^{13}C_{\text{lipid-free}} = -3.113 + 0.968 \times \text{CN} + \delta^{13}C_{\text{bulk}} \]  

(7)

We also estimated the parameters D and I by fitting the original equations of the MM model to our observed data, the parameter D from the Fry equation, and the coefficients...
\( \beta_0 \) and \( \beta_1 \) from the linear model of Post using the least-squares procedures in R (see Appendix S1: Table S1).

We compared the validity of each model applied to each tissue (i.e., dried hair, blood) using the residual sums of squares obtained from a linear regression analysis between observed \( \delta^{13} \)C lipid-free values and predicted \( \delta^{13} \)C lipid-free values. We identified the most parsimonious model as the one having the lowest value of Akaike information criterion corrected for small sample size (AICc) among a set of candidate models. In addition, we calculated \( \Delta \text{AICc} \) and \( \text{AICc weights} \ (\omega_i) \), where models with \( \Delta \text{AICc} \leq 2 \) provide a relevant and interesting support to the best model (Burnham and Anderson 2002), and \( \omega_i \) provide the relative preference among a set of candidate models (Vandekerckhove et al. 2014). We assessed the fit of the most parsimonious model using \( \text{R}^2 \).

To evaluate model performance, we determined the precision of the predicted values with the proportion of predicted \( \delta^{13} \)C lipid-free values that were within 0.25% (systematic error) of the observed \( \delta^{13} \)C lipid-free values (\( P_{0.25} \)) and with the mean absolute error (MAE) using repeated k-fold cross-validation (James et al. 2013). To do so, we calculated model coefficients using 90% of the data and withholding the remaining 10% for validation, and repeated this procedure 999 times (1000 iterations) using the library caret (Kuhn 2018). We performed all statistical analyses using R software version 3.4.2 (R Core Team 2017).

**RESULTS**

**Effect of lipid extraction on stable isotope ratios**

Lipid extraction had no effect on \( \delta^{13} \)C values in plasma (paired \( t \)-test, \( t = 0.86, P = 0.40 \)) and red blood cells (paired \( t \)-test, \( t = 1.49, P = 0.15 \)), but it increased \( \delta^{13} \)C values in hair (paired \( t \)-test, \( t = 19.42, P < 0.05 \)) by an average of 0.40\% (Table 1; Fig. 2). Lipid extraction had no effect on \( \delta^{15} \)N values in red blood cells (paired \( t \)-test, \( t = 1.72, P = 0.096 \)) but significantly increased \( \delta^{15} \)N values in hair (paired \( t \)-test, \( t = -3.09, P < 0.05 \)) and plasma (paired \( t \)-test, \( t = -3.94, P < 0.05 \)) by an average of 0.11\% and 0.18\% (Fig. 2). The CN ratios were relatively low, ranging from 3.93 to 4.30 for bulk samples and from 3.65 to 4.13 for lipid-free samples (Table 1). Lipid extraction had a negative effect on CN ratio values in all three tissues (paired \( t \)-test, \( t = 6.28 \) to 29.54, all \( P < 0.05 \)) by an average of 0.06 for red blood cells, 0.28 for hair, and 0.17 for plasma (Fig. 2).

We found a subtle but significant positive linear relationship between \( \delta^{15} \)Cbulk and CN ratios in hair samples only, but CN explained only 15% of the variation in \( \delta^{13} \)Cbulk (\( F_{1,42} = 7.46, r = 0.39, P < 0.05 \); Fig. 3). No linear relationships were observed between \( \Delta \delta^{13} \)C and \( \delta^{13} \)Cbulk in all three tissues (all \( R^2 < 0.1, r < 0.02, P > 0.05 \)). There were no linear or nonlinear relationships between \( \Delta \delta^{13} \)C and CN ratios for all three tissues (all \( R^2 < 0.08, r < 0.30, P > 0.05 \)). There was also no linear relationship between \( \delta^{15} \)Nbulk and CN ratios (all tissues \( R^2 < 0.10, r < 0.40, P > 0.05 \)) and between \( \delta^{15} \)Nbulk and CN ratios (all tissues \( R^2 > 0.08, r < -0.03, P > 0.05 \)). However, a strong significant linear relationship was observed between \( \delta^{15} \)Nbulk and \( \delta^{15} \)Nlipid-free in all three tissues (all \( R^2 > 0.87, r > 0.93, P < 0.001 \); Fig. 2).

**Tissue- and caribou-specific linear models**

Our best caribou-specific model to predict \( \delta^{13} \)C lipid-free values in hair included the \( \delta^{13} \)Cbulk parameter alone (Eq. 1) and was as follows:

\[
\delta^{13} \text{C lipid-free} = 0.982(\pm 0.048) \times \delta^{13} \text{Cbulk} - 0.028(\pm 1.15)
\]  

(8)

In this model, \( \delta^{13} \)Cbulk explained 98% of the variation in \( \delta^{13} \)C lipid-free values (\( F_{1,42} = 1718.78, P < 0.05 \)). There was an absolute error of \( \pm 0.11\% \)

| Tissues         | \( n \) | \( \delta^{13} \)Cbulk | \( \delta^{13} \)C lipid-free | \( \delta^{15} \)Nbulk | \( \delta^{15} \)N lipid-free | CN bulk | CN lipid-free |
|-----------------|--------|------------------------|-----------------------------|----------------------|-----------------------------|---------|---------------|
| Red blood cells | 28     | -22.76 ± 0.10          | -22.67 ± 0.13               | 2.11 ± 0.13          | 2.05 ± 0.15                 | 4.05 ± 0.01 | 3.99 ± 0.01   |
| Hair            | 44     | -24.05 ± 0.13          | -23.65 ± 0.13               | 2.99 ± 0.10          | 3.10 ± 0.11                 | 3.93 ± 0.01 | 3.65 ± 0.01   |
| Plasma          | 24     | -21.69 ± 0.09          | -21.62 ± 0.09               | 2.24 ± 0.13          | 2.42 ± 0.13                 | 4.30 ± 0.01 | 4.13 ± 0.02   |

*Note: Bold values indicated significant difference (paired \( t \)-test \( P < 0.05 \)) between bulk and lipid-free samples.*
in the predictions of $\delta^{13}$C values of lipid-extracted samples when compared to untreated samples. This error was estimated from the cross-validation procedure previously described. The addition of the CN ratio parameter in the linear model (Eq. 2) did not improve its fit to the data, this parameter having no significant effect on the relationship ($t = 1.46, P = 0.15$).

**Lipid normalization models**

Because models with $\Delta AIC_c \leq 2$ are generally considered to provide substantial explainable variation in the data (Burnham and Anderson 2002), our analyses suggested that the models proposed by Ehrich et al. (2011) and Post et al. (2007), as well as the MM model (1979) along with the caribou-specific model we developed, were the most parsimonious models to adjust for lipids when determining isotopic values in caribou hair. However, according to the Akaike weights, the probability that the Ehrich et al. (2011) and Post et al. (2007) models are the best models is three times higher ($\omega_i = 0.336$ and $0.309$, respectively) than for the MM (1979; 0.119) and our caribou-specific (0.181) models, although no model really outperformed the others (max $\omega_i = 0.336$). All four models had a strong fit to the data and explained between 97% and 98% of the variability (Table 2). The MAE estimated from the cross-validation procedure was comparable among models (0.11) and indicated that predictions of $\delta^{13}$C$_{\text{lipid-free}}$ values are close to the observed lipid-extracted $\delta^{13}$C values. Nevertheless, when comparing model performance using the percentage of $\delta^{13}$C values predicted within 0.25‰ of the observed value, our tissue- and caribou-specific model was the most accurate. Our model predicted 93% of the $\delta^{13}$C$_{\text{lipid-free}}$ values compared to 43% for the equation proposed by Ehrich et al. (2011), 86% for the one found in Post et al. (2007), and 9% for the MM model (1979). Our tissue-, method-, and caribou-specific model was also the only one for which no significant differences were found between the predicted and the observed lipid-extracted $\delta^{13}$C values (paired $t$-test, $t = -0.27, P = 0.79$; Fig. 4), suggesting our hair- and caribou-specific model is more accurate.

The four lipid normalization equations published in the literature did not predict $\delta^{13}$C$_{\text{lipid-free}}$ values of caribou hair accurately (Fig. 4). We showed that the Ehrich et al. (2011) and Post et al. (2007) equations tended to overestimate $\delta^{13}$C$_{\text{lipid-free}}$, with 95% Confidence Interval (CI) values ranging between 0.017‰ and 0.56‰.
(coefficient of variation (CV) = 46.6%) and −0.19 and 0.36% (CV = 91.1%), respectively. Opposite trends were observed for the MM (1979) and Fry (2002) models: They both underestimated δ13Clipid-free, with values ranging between −0.77 and −0.21% (CV = 29.5%) and −0.34 and 0.24% (CV = 77.8%), respectively.

**DISCUSSION**

**Effect of lipid extraction on stable isotope ratios**

The decision to use lipid extraction prior to stable isotope analysis is a practice that varies across studies, taxa, and tissue types (Hobson 1995, Hilderbrand et al. 1996, Bearhop et al. 2000, Lecomte et al. 2011, Tarroux et al. 2016), and the standardization of lipid extraction in isotopic studies has been recommended as a suitable alternative by Kelly (2000). In several isotopic studies, lipids were not routinely extracted in red blood cells, serum, or plasma (Hobson et al. 1996, Burns et al. 1998, Polischuk et al. 2001, Finstad and Kielland 2011). However, blood serum or plasma may have highly variable lipid contents depending on individual and physiological conditions (Hobson and Stirling 1997, Lesage et al. 2002). The depletion of 13C in adipose tissue relative to proteins and carbohydrates has been reported in the literature (DeNiro and Epstein 1977), so it was recommended to extract lipids from all blood components (Kelly 2000, Lesage et al. 2002). Lesage et al. (2002) observed significant depletions of 13C in phocid seal species, while Tarroux et al. (2016) observed similar depletions in plasma samples of Antarctic fur seals (*Arctocephalus gazella*). However, some studies found no 13C depletion in blood samples of arctic foxes (*Vulpes lagopus*; Tarroux et al. 2012), Antarctic fur seals (Tarroux et al. 2016), or various bird species (Bearhop et al. 2000). The lipid content of whole blood is usually very low, for example, less than 5% of the dry mass of avian (Bearhop et al. 2000) and semi-domestic reindeer blood (*Rangifer tarandus tarandus*; Nieminen and Timisjarvi 1983). Although we did not have lipid percentage values for our caribou blood, we assume that bulk isotope signatures integrated signatures mainly from blood proteins (Bearhop et al. 2000) and that the low lipid content has not affected our δ13Cbulk values. Based on this, lipid extraction of red blood cells and plasma samples does not appear to be a necessary step in the case of caribou.

We showed that lipid extraction influences δ13C values measured in caribou hair. This is an important development in research focusing on isotopic analyses as no other study, to our knowledge, has previously investigated the effects of lipid extraction on δ13Cbulk values in caribou hair.
knowledge, has verified the importance of accounting for the variation in lipid content when measuring δ13C values in mammal hair. Internal lipids are not routinely extracted in mammal hair in the literature (but see Hilderbrand et al. (1996) who have extracted lipids in bear hair). Hair is recognized as a lipid-poor tissue (Tieszen and Boutton 1989, Dunnett and Lees...
2003, Dunnett 2005), and the effect of lipid extraction on $\delta^{13}$C values is recognized to be small compared to lipid-rich tissues (e.g., muscle, liver; Kelly 2000). Although we still have not calculated the proportion of lipid values for our caribou hair per se, foregoing lipid extraction in our hair samples was sufficient to significantly increase $\delta^{13}$C values.

Extracting lipids has significantly increased $\delta^{15}$N values in caribou hair and plasma. However, the differences (0.11 and 0.18‰) were found to be below the commonly used systematic error of 0.25‰ and below the MAE of ±0.18‰ also estimated in our tissues- and caribou-specific linear model correction. We found no strong effect of lipid extraction on $\delta^{15}$N values measured in red blood cells, plasma, and caribou hair tissues, which is supported by other studies conducted on other species. Indeed, Lesage et al. (2010) observed a small effect of lipid extraction on $\delta^{15}$N values in cetacean skin. Ehrich et al. (2011) observed no significant changes in $\delta^{15}$N values in bird and terrestrial mammal muscle but highlighted a positive enrichment in $\delta^{15}$N values in bird eggs. The enrichment in $\delta^{15}$N values is probably due to leaching of isotopically light nitrogenous cell compounds during chloroform-methanol rinsing (Bearhop et al. 2000, Søreide et al. 2006). These different results support the need to further investigate the specific mechanism of $\delta^{15}$N alteration, in addition to testing the effects of lipid extraction protocols on $\delta^{15}$N values for tissues and species of interest before conducting stable isotope analysis.

Some studies claimed that it is not important to account for lipids in isotopic analyses when the CN ratio is <4.0 (i.e., lipid content around 10%) in terrestrial systems (Post et al. 2007, Ehrich et al. 2011). However, our results suggest that this recommendation is problematic. Indeed, we showed that despite low CN ratios (3.93–4.30), carbon and lipid contents are a potential source of bias for $\delta^{13}$C. Similar results were found for tissues of aquatic vertebrates (e.g., green turtle (Chelonia mydas) muscle, Bergamo et al. 2016; beluga (Delphinapterus leucas) muscle and liver, Choy et al. 2016). Here, we showed that lipid extraction significantly influenced $\delta^{13}$C, $\delta^{15}$N, and CN values in the three tissues for caribou, supporting the necessity to correct for lipid content in tissues from terrestrial mammal species.

**CN ratio as a model parameter**

McConnaughey and McRoy (1979) suggested to use the CN ratio as a proxy of lipid content (in percentage) and observed a greater depletion in $\delta^{13}$C associated with high lipid contents. In contrast, we observed no significant relationship between $\Delta \delta^{13}$C and CN ratio in the three caribou tissues we analyzed and no significant relationship between $\delta^{13}$C and CN ratio in both blood components. However, a weak relationship between $\delta^{13}$C and CN ratio was observed in hair. High CN ratios were not associated with a greater depletion in $\delta^{13}$C in our caribou hair samples. Post et al. (2007) also found a weak link between $\Delta \delta^{13}$C and CN ratio in muscle of terrestrial animals and suggested to estimate empirically the derived relationship between $\Delta \delta^{13}$C and CN when working with a new tissue and species. The strength of this relationship varies among species (Post et al. 2007, Lesage et al. 2010), so using the CN ratio as a proxy of lipid content in all taxa and tissue remains questionable (Kiljunen et al. 2006). Moreover, some authors did not recommend using CN ratios for lipid normalization of $\delta^{13}$C values (Kiljunen et al. (2006) in aquatic invertebrates; Fagan et al. (2011) in fish muscles). Therefore, caution is required when applying lipid normalization models that use CN ratio as a model parameter for calculating $\delta^{13}$C$_{lipid-free}$ values. We recommend using lipid normalization based on $\delta^{13}$C$_{bulk}$ in relation to $\delta^{13}$C$_{lipid-free}$ when dealing with caribou hair, similar to results found by Lesage et al. (2010) for cetacean skin and by Choy et al. (2016) for beluga muscle and liver.

**Lipid normalization models**

Our results suggest that a model specific to the chemical extraction method, the tissue analyzed, and the species of interest usually fits the data better than generalized lipid normalization equations, a recommendation supported by Logan et al. (2008) and Lesage et al. (2010). Our tissue- and caribou-specific models were the most accurate of the five models tested in our study, as no significant difference was found between the predicted and the observed lipid-extracted $\delta^{13}$C values. These tissue- and caribou-specific models also had a better predictive power based on cross-validation procedures. The four lipid normalization equations published in the literature
performed relatively well for caribou hair, with a lower MAE and a strong fit to the data but did not predict $\delta^{13}\text{C}_{\text{lipid-free}}$ values of caribou hair accurately. Lipid normalization appears to be a good method to deal with lipids in stable isotope analyses and to apply to a variety of organisms (Post et al. 2007, Lesage et al. 2010). However, based on our results, we recommend using a tissue-, method-, and species-specific model with fewer assumptions and parameters. If the former is not possible, we recommend validating general equations and parameters published in the literature before using them.

**Conclusions**

Choosing between conducting lipid normalization of $\delta^{13}\text{C}$ values and extracting lipids using common laboratory techniques depends on research objectives, the level of precision required, and the potential consequences of lipid-caused shifts in $\delta^{13}\text{C}$ values on the conclusions of a study. Based on our results, we consider that lipid extraction should be performed when maximum precision is needed to calculate diet composition using mixing models and niche isotopic overlap. As a next step, we recommend testing the effects of lipid extraction on $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and CN ratios for tissues and species of interest before conducting stable isotope analysis. We also support the need for further investigation regarding the effects of lipids on $\delta^{13}\text{C}$ values in mammal hair.

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