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METHODS

Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses

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Abstract Within an organism, lipids are depleted in ^{13}C relative to proteins and carbohydrates (more negative $\delta^{13}C$), and variation in lipid content among organisms or among tissue types has the potential to introduce considerable bias into stable isotope analyses that use $\delta^{13}C$. Despite the potential for introduced error, there is no consensus on the need to account for lipids in stable isotope analyses. Here we address two questions: (1) If and when is it important to account for the effects of variation in lipid content on $\delta^{13}C$? (2) If it is important, which method(s) are reliable and robust for dealing with lipid variation? We evaluated the reliability of direct chemical extraction, which physically removes lipids from samples, and mathematical normalization, which uses the carbon-to-nitrogen (C:N) ratio of a sample to normalize $\delta^{13}C$ after analysis by measuring the lipid content, the C:N ratio, and the effect of lipid content on $\delta^{13}C$ ($\Delta\delta^{13}C$) of plants and animals with a wide range of lipid contents. For animals, we found strong relationships between C:N and lipid content, between lipid content and $\Delta\delta^{13}C$, and between C:N and $\Delta\delta^{13}C$. For plants, C:N was not a good predictor of lipid content or $\Delta\delta^{13}C$, but we found a strong relationship between carbon content and lipid content, lipid content and $\Delta\delta^{13}C$, and between and carbon content and $\Delta\delta^{13}C$. Our results indicate that lipid extraction or normalization is most important when lipid content is variable among consumers of interest or between consumers and end members, and when differences in $\delta^{13}C$ between end members is <10–12‰. The vast majority of studies using natural variation in $\delta^{13}C$ fall within these criteria. Both direct lipid extraction and mathematical normalization reduce biases in $\delta^{13}C$, but mathematical normalization simplifies sample preparation and better preserves the integrity of samples for $\delta^{15}N$ analysis.

Keywords Stable isotopes · Lipid extraction · Lipid concentration · Mathematical normalization · C:N · Plants · Animals

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Introduction

Stable isotopes have emerged as important tools for addressing questions of plant and animal physiology (Peterson and Fry 1987; Gannes et al. 1997; Dawson et al. 2002), paleoecology (Schwarcz and Schoeninger

1991; Keeling et al. 1995; Finney et al. 2002), material cycling (Bilby et al. 1996; Kitchell et al. 1999), animal migration (Hobson 1999), diet composition (Vandermerwe and Vogel 1978; Phillips 2001), niche shifts (Post 2003) and trophic structure (Ponsard and Arditi 2000; Post et al. 2000; Finlay et al. 2002; Post 2002; Layman et al. 2005, 2006). All of these applications take advantage of natural variations in stable isotopes ratios (e.g., $\delta^{13}C$, which is the ratio of ^{13}C to ^{12}C relative to a reference standard) that result from the chemical or biological processes that cause isotopic discrimination (of fractionation), where discrimination is a change in the ratio of heavy to light isotopes in a compound after uptake, processing or transformation. Carbon is particularly valuable for estimating diet sources, identifying animal movement patterns, and establishing baselines to estimate trophic position (Peterson and Fry 1987; Hobson 1999; Post 2002) because it expresses little trophic fractionation (DeNiro and Epstein 1978; Peterson and Fry 1987; Post 2002; McCutchan et al. 2003). While trophic fractionation has received considerable recent attention (DeNiro and Epstein 1978; Peterson and Fry 1987; Post 2002; McCutchan et al. 2003), the synthesis and accumulation of lipids, which are depleted in ^{13}C and typically have $\delta^{13}C$ values that are more negative than those for proteins and carbohydrates within an individual organism (DeNiro and Epstein 1977; McConnaughey and McRoy 1979), have the potential to influence stable isotope analyses using $\delta^{13}C$, but have not been well integrated into current analyses.

The potential for lipids to influence $\delta^{13}C$ analyses emerges from two sources of variation. First, fractionation of $\delta^{13}C$ during lipid synthesis (DeNiro and Epstein 1977) results in differences (D) in $\delta^{13}C$ between lipids and other tissues, such as protein and carbohydrates, of around 6–8‰ (DeNiro and Epstein 1977; McConnaughey and McRoy 1979). Second, there exists considerable heterogeneity in lipid content among aquatic and terrestrial organisms. For example, the lipid content of muscle tissue of fish found in north temperate lakes can vary from 3 to >34% (Post 2000), and between 1 and 55% for animals in the Bering Sea (McConnaughey and McRoy 1979). This variation in lipid content derives from differences in foraging dynamics and life history constraints and is of considerable interest to ecologists and evolutionary biologists (Schultz and Conover 1997; Gasser et al. 2000; Post and Parkinson 2001; Arrington et al. 2006). Similar heterogeneity exists among tissue types within a single organism (McConnaughey and McRoy 1979; Hobson and Clark 1992; Sweeting et al. 2006). Considerable bias in analyses based on $\delta^{13}C$ could be introduced by the combination of both relatively large differences in $\delta^{13}C$ between lipids and other tissue types and by the considerable heterogeneity in lipid content among samples. For example, assuming a D of 6‰ and all other things being equal (same diet, same body size, etc.), a fish with 36% lipids (e.g., lake trout) would have a $\delta^{13}C$ that is 2‰ more negative than a fish with 3% lipids (e.g., northern pike).

The potential influence of lipids on $\delta^{13}C$ has caused some researchers to use either direct chemical lipid extraction or a mathematical normalization technique to standardize lipid content (McConnaughey and McRoy 1979; Hobson and Clark 1992; Kling et al. 1992; Alexander et al. 1996; Post et al. 2000; Lesage et al. 2001; Fry et al. 2003; Sweeting et al. 2006). Chemical extraction methods typically use a methanol–chloroform solution to physically remove lipids from samples (Folch et al. 1957; Bligh and Dyer 1959; Pinnegar and Polunin 1999), reducing lipid concentrations to a uniformly low level. These techniques are advantageous because they remove the majority of lipids, creating uniform samples for comparison; however, they may cause fractionation in $\delta^{15}N$ (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004) and they are time-consuming. The mathematical normalization technique proposed by McConnaughey and McRoy (1979) uses the C:N ratio and an estimate of D to produce a post hoc ''lipid-normalized'' $\delta^{13}C$ for each sample. The adjustment proposed by McConnaughey and McRoy (1979) derives from the relationship between C:N and percent lipid, and a relationship between percent lipid and a correction factor for $\delta^{13}C$ based on the fractionation of $\delta^{13}C$ during lipid synthesis. This technique is attractive because it could account for variation in lipid concentration using the C:N ratio of a sample, which is measured during most analyses for $\delta^{13}C$ and $\delta^{15}N$.

To date, there is no consensus on whether or not researchers need to address lipids in analyses using $\delta^{13}C$. Some analyses have found an effect of lipids on $\delta^{13}C$, while others have not (McConnaughey and McRoy 1979; Hobson and Clark 1992; Kling et al. 1992; Pinnegar and Polunin 1999; Chaloner et al. 2002), and many studies using stable isotopes have ignored lipid effects altogether. Neither has there been any rigorous evaluation of the effectiveness and reliability of the two methods for accounting for variation in lipid content. In particular, while a number of authors have used versions of the mathematical normalization technique (McConnaughey and McRoy 1979; Alexander et al. 1996; Lesage et al. 2001; Fry et al. 2003; Schmidt et al. 2003; Sweeting et al. 2006), it remains poorly developed and virtually untested.

Here we address the questions of: (1) if and when is it important to remove or account for variation in lipid

content and the potential effect of lipids on $\delta^{13}C$; and (2) if it is important, which method(s) are reliable and robust for dealing with lipids? To address these questions we perform direct lipid extraction on plants and animals with wide variations in lipid composition and that are drawn from a variety of terrestrial and aquatic ecosystems. We analyze the relationships between C:N ratio, lipid concentration, and the difference in $\delta^{13}C$ before and after lipid extraction for plants and animals, and use these data to evaluate lipid-normalized $\delta^{13}C$ using mathematical techniques. Finally, we discuss the advantages and disadvantages of chemical and mathematical methods for lipid normalization and make recommendations for dealing with lipids in stable isotope studies.

Methods

Samples and analyses

For our analysis, we selected animals and plants that covered a wide range of lipid concentrations. A full list of samples can be found in Appendix A, but briefly they included 16 aquatic animals, 13 terrestrial animals and 17 plant species drawn from tropical and temperate ecosystems. Samples were either muscle tissue or whole organisms (which is dominated by muscle tissue in most cases). We chose these tissues types because they are the most common samples used for stable isotope analyses, and the two types of samples most likely to contain high and variable lipid contents. All samples were either frozen before processing, or dried and processed immediately after collection. To account for potential differences among organisms and ecosystem types, we present separate analyses for animals found in aquatic ecosystems, animals found in terrestrial ecosystems, and plants (aquatic and terrestrial, vascular and nonvascular combined). For each sample, we measured lipid content as percent of dry mass (% lipid), the $\delta^{13}C$ and $\delta^{15}N$ of samples before and after direct lipid extraction, and the carbon-to-nitrogen (C:N) ratio by mass of each sample before lipid extraction. The effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ were calculated as $\Delta\delta^{13}C = \delta^{13}C_{extracted} - \delta^{13}C_{untreated}$ and $\Delta\delta^{15}N = \delta^{15}N_{extracted} - \delta^{15}N_{untreated}$, respectively. The C:N ratio, % lipid, and $\Delta\delta^{13}C$ were used to evaluate the mathematical normalization technique by testing relationships between (a) the C:N ratio and % lipid, (b) % lipid and $\Delta\delta^{13}C$, and (c) the C:N ratio and $\Delta\delta^{13}C$. Percent carbon was also used to evaluate lipid normalization for plants.

Lipid extraction and quantification

Lipids were extracted and quantified using methanol and chloroform following Folch et al. (1957), as revised by Post and Parkinson (2001) and Arrington et al. (2006). Briefly, a 0.5 ± 0.0001 g portion of powered tissue was loaded into a 30 ml test tube, to which 8 ml chloroform and 8 ml of methanol were added (resulting in a 50:50 methanol–chloroform solution). The mixture was heated in a 61 °C water bath until it boiled, cooled to room temperature, and increased in volume to 25 ml through the addition of chloroform. The entire volume was filtered through a No. 1 Whatman filter paper into a 125 ml separatory funnel, to which 10 ml of 0.9% saline solution was added. The separatory funnel and its contents were shaken vigorously, the mixture was allowed to separate, and the bottom methanol–chloroform layer was drained into a pre-weighed aluminum dish. These contents were evaporated on a hot plate at 70 °C. The weighing dish was cooled to room temperature and weighed to the nearest 0.0001 g. The lipid remaining in the aluminum dish represented the mass of lipid per 0.5 g of dry tissue.

Stable isotope analyses

Samples were dried for >48 h at 40–50 °C and ground into a fine powder. Lipids were extracted and quantified from a portion of the sample while another portion was retained without lipid extraction. Stable isotope analyses were performed using continuous flow on either a Europa Geo 20/20 isotope ratio mass spectrometer at the Cornell Laboratory for Stable Isotope Analysis (CLSIA), or on a ThermoFinnigan DeltaPlus at the Yale Earth System Center for Stable Isotopic Studies (ESCSIS). All stable isotope values are reported in the δ notation, where $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1]1000$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The global standard for $\delta^{13}C$ is PeeDee belemnite and for $\delta^{15}N$ is atmospheric nitrogen. The CLSIA working standard for animals was CBT ($\delta^{13}C = -25.1$, $\delta^{15}N = 17.4$; 12.2% N, 54.9% C) and the standard deviations of replicate samples of CBT analyzed at CLSIA were 0.05‰ for $\delta^{13}C$ and 0.18‰ for $\delta^{15}N$. The ESCSIS working standard for animals was OBT ($\delta^{13}C = -28.3$, $\delta^{15}N = 15.2$; 13.1% N, 49.9% C) and for plants it was YGC ($\delta^{13}C = -26.1$, $\delta^{15}N = 0.41$; 1.9% N, 37.1% C). The standard deviation of replicate samples of OBT analyzed at ESCSIS were 0.14‰ for $\delta^{13}C$ and 0.22‰ for $\delta^{15}N$, and for YGC they were 0.06‰ for $\delta^{13}C$ and 0.15‰ for $\delta^{15}N$.

Results

There was a significant positive relationship between $\Delta\delta^{13}C$ and % lipid for plants and animals in both aquatic and terrestrial ecosystems (Fig. 1; Table 1). Percent lipid explained nearly 90% of the variation in $\Delta\delta^{13}C$ for aquatic animals and 80% of the variation for plants (Table 1). Although a positive relationship, % lipid explained only 40% of the variation in $\Delta\delta^{13}C$ for animals from terrestrial ecosystems (Table 1) and there appeared to be little relationship between % lipid and $\Delta\delta^{13}C$ at lipid levels below around 15% (Fig. 1). There were significant differences in the slope of the relationship between % lipid and $\Delta\delta^{13}C$ among aquatic animals, terrestrial animals, and plants (ANCOVA, interaction term, $F_{(2,55)} = 2.92$, $P = 0.004$), with aquatic and terrestrial animals having steeper slopes than plants. There was no significant difference in slope between aquatic and terrestrial animals (ANCOVA, interaction term, $F_{(1,38)} = 0.22$, $P = 0.51$), but there was a significant difference in the intercept between terrestrial and aquatic animals ($F_{(1,39)} = 5.71$, $P = 0.021$) with a more negative intercept for the relationship between % lipid and $\Delta\delta^{13}C$ for terrestrial animals (Table 1).

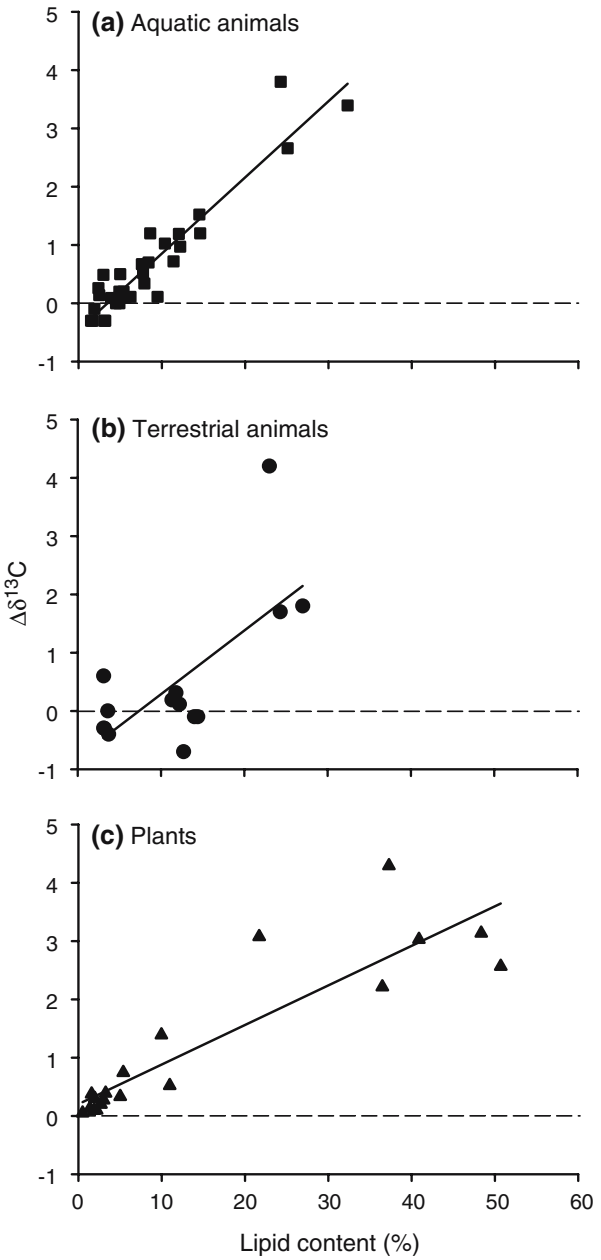

Fig. 1a–c Relationship between lipid content and the $\Delta\delta^{13}C$ for **a** aquatic animals (16 species; *filled squares*), **b** terrestrial animals (13 species; *filled circles*), and **c** plants (17 species; *filled triangles*). See Methods and the Appendix for more information. See Table 1 for equations

There were significant positive relationships between the C:N ratio and % lipid for both aquatic and terrestrial animals, but no relationship between C:N and % lipid for plants (Fig. 2; Table 1). The C:N ratio explained 94 and 91% of the variation in % lipid for aquatic and terrestrial animals, respectively (Table 1). The slope of the relationship between the C:N ratio and % lipid was significantly different for aquatic and terrestrial animals (ANCOVA, interaction term, $F_{(1,32)} = 12.3$, $P = 0.001$).

There was a significant positive relationship between the C:N ratio and $\Delta\delta^{13}C$ for both aquatic and terrestrial animals, but not for plants (Fig. 2; Table 1). For aquatic animals, C:N explained 90% of the variation in $\Delta\delta^{13}C$ (Table 1), but for terrestrial animals, C:N explained only 28% of the variation in $\Delta\delta^{13}C$ (Table 1). The slope of the relationship between the C:N ratio and $\Delta\delta^{13}C$ was similar for both aquatic and terrestrial animals (ANCOVA, interaction term, $F_{(1,32)} < 0.01$, $P = 0.97$).

For plants, there was an overall significant relationship between % carbon and % lipid, and between % carbon and $\Delta\delta^{13}C$ (Fig. 3; Table 1). These significant relationships were, however, driven by samples with % lipid >40% (Fig. 3; Table 1). There was little relationship between % carbon and % lipid, or between % carbon and $\Delta\delta^{13}C$ among samples with % carbon <40% (Fig. 3; all marine algae or seagrass species). It appears % carbon is the best predictor of $\Delta\delta^{13}C$ for samples with % carbon >40%.

There was a small but significant effect of lipid extraction on the $\delta^{15}N$ of animal (paired t test; $n = 37$, $t = 9.0$, $P < 0.001$) but not plant samples ($n = 19$, $t = 1.84$, $P = 0.09$). The mean paired difference in $\delta^{15}N$ of animal samples before and after lipid extraction was 0.25 (SD = 0.18). For animals, $\Delta\delta^{15}N$ was correlated

Table 1 Linear regression equations and diagnostic statistics for aquatic and terrestrial animals and plants

	Sample size (n)	Significance (P values)	Variance explained (R^2)
Aquatic animals			
$\Delta\delta^{13}C = -0.47 + 0.13 \times$ % lipid (Eq. 1)	28	<0.001	0.898
% lipid = –20.54 + 7.24 × C:N (Eq. 2)	22	<0.001	0.941
$\Delta\delta^{13}C = -3.32 + 0.99 \times$ C:N (Eq. 3)	22	<0.001	0.907
Terrestrial animals			
$\Delta\delta^{13}C = -0.81 + 0.11 \times$ % lipid (Eq. 4)	14	0.016	0.396
% lipid = –30.57 + 10.74 × C:N (Eq. 5)	14	<0.001	0.907
$\Delta\delta^{13}C = -3.44 + 1.00 \times$ C:N (Eq. 6)	14	0.069	0.250
Plants			
$\Delta\delta^{13}C = 0.20 + 0.07 \times$ % lipid (Eq. 7)	19	<0.001	0.801
% lipid = 15.23 + –0.001 × C:N (Eq. 8)	19	0.968	0.000
$\Delta\delta^{13}C = 1.25 + -0.00 \times$ C:N (Eq. 9)	19	0.919	0.000
% lipid = –35.10 + 1.03 × % Carbon (Eq. 10)	19	<0.001	0.534
$\Delta\delta^{13}C = -3.02 + 0.09 \times$ % Carbon (Eq. 11)	19	< 0.001	0.664
Plant samples with carbon >40%			
% lipid = –64.16 + 1.56 × % Carbon (Eq. 12)	16	<0.001	0.620
$\Delta\delta^{13}C = -5.83 + 0.14 \times$ % Carbon (Eq. 13)	16	<0.001	0.841

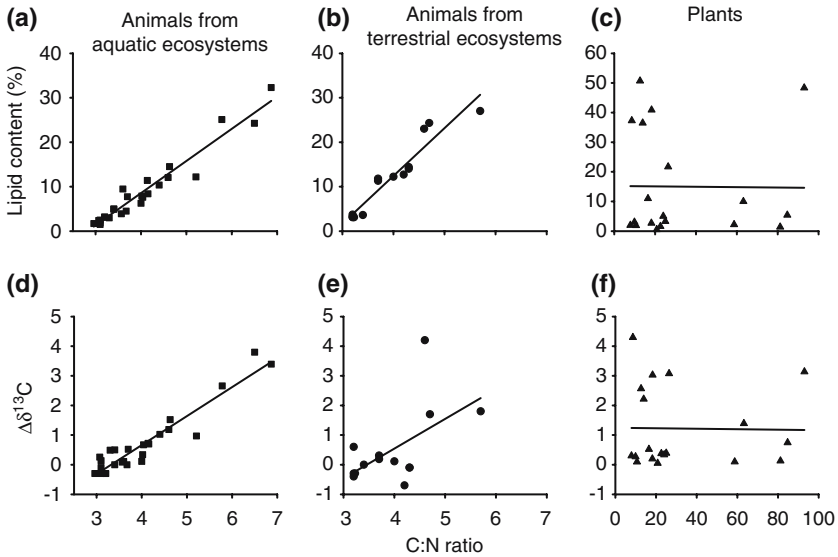

Fig. 2a–f Relationship between C:N ratio and lipid content for **a** aquatic animals *filled squares*, **b** terrestrial animals *filled circles*, and **c** plants *filled triangles*, and between C:N and $\Delta\delta^{13}C$ for **d** aquatic animals *filled squares*, **e** terrestrial animals *filled circles*, and **f** plants *filled triangles*

with % lipid ($n = 34$, $r = 0.52$, $P < 0.01$) and $\Delta\delta^{13}C$ ($n = 34$, $r = 0.64$, $P < 0.01$). For plants, $\Delta\delta^{15}N$ was not correlated with % lipid ($n = 19$, $r = 0.02$, $P = 0.94$) or $\Delta\delta^{13}C$ ($n = 19$, $r = 0.08$, $P = 0.74$).

Discussion

The strong relationship between lipid content of samples (% lipid) and changes in $\delta^{13}C$ after lipid extraction ($\Delta\delta^{13}C$) highlights the concentration dependence of the lipid bias—the bias introduced by lipids increases as the concentration of lipids increases (Fig. 1). A sample with high lipid concentration that has not been lipid-extracted or normalized would be 3–4‰ more negative than an extracted or normalized sample. In contrast, at low lipid concentrations, lipid extraction has very little impact on the $\delta^{13}C$ of animal and plant samples. This general result appears robust across plants and animals, for organisms from marine, freshwater, and terrestrial ecosystems, and for organisms found in tropical and temperate environments (Appendix A).

The concentration dependence of the lipid effect explains the contradictory views in the literature concerning the necessity of lipid extraction. A number of authors have found differences between lipid-extracted or normalized samples and regularly extract lipids from samples to minimize the potential for

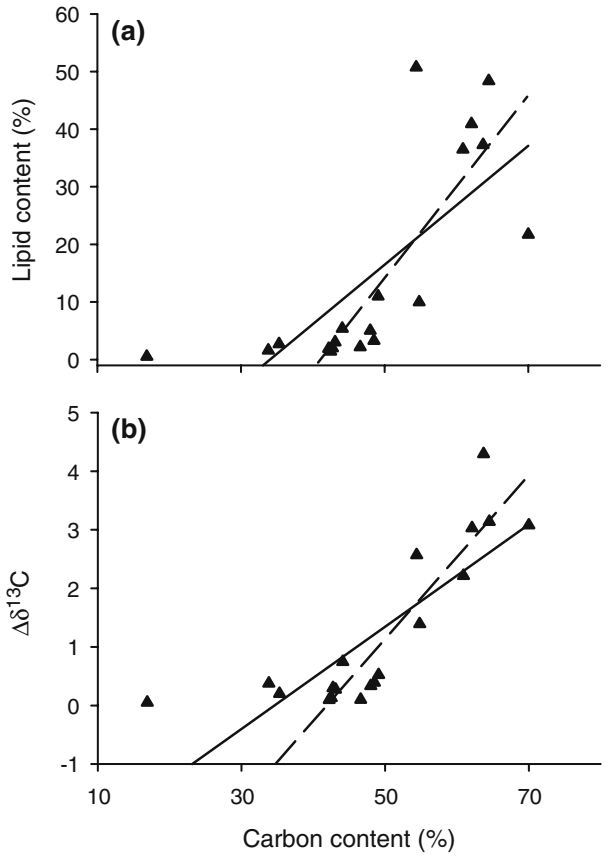

Fig. 3a–b Relationship between **a** carbon content and lipid content, and **b** carbon content and $\Delta\delta^{13}C$ for plants. The least squares regression is shown for all samples (*solid line*) and for samples with carbon content >40% (*dashed line*). See Table 1 for equations

lipid-induced biases (e.g., McConnaughey and McRoy 1979; Hobson and Clark 1992; Kling et al. 1992; Post et al. 2000; Sweeting et al. 2006), while other researchers have found little effect of lipid extraction on $\delta^{13}C$ (Pinnegar and Polunin 1999; Chaloner et al. 2002). It is quite possible that both approaches were appropriate. Our results indicate that it is important to account for lipids when lipid content is high, or when comparing species with variable lipid content. It is not, however, necessary to account for lipids in animal samples when lipid content is consistently low—below around 5% lipid (C:N < 3.5) for aquatic animals and 10% lipid for terrestrial animals (C:N around 4; although our sample size is small). For plants, the intercept for the relationship between % lipid and $\Delta\delta^{13}C$ is positive, suggesting that researchers need to account for lipids in plant samples, even when lipid content is very low. These observations apply to all organisms used in the analysis, including predators, prey, and baseline organisms used to estimate trophic position or diet source.

Sensitivity and bias

The importance of variation in lipid content depends upon both the organism under study and the scientific question of interest. In general, the potential bias depends upon the signal-to-noise ratio, where *noise* is described by the range of variation in lipid content among samples and *signal* is described by the magnitude of difference in $\delta^{13}C$ among end members (diet items, food webs, etc.) or processes of interest (e.g., spatial and temporal trends in $\delta^{13}C$). We provide as an example the bias introduced when estimating diet source using a two end-member mixing model: $\alpha = (\delta^{13}C_{consumer} - \delta^{13}C_{em2})/(\delta^{13}C_{em1} - \delta^{13}C_{em2})$, where α is the proportion of carbon in a consumer ($\delta^{13}C_{consumer}$) derived from end member 1 ($\delta^{13}C_{em1}$). Versions of this and more complicated multiple end-member mixing models have been used to estimate the diets of aquatic and terrestrial animals, to estimate the $\delta^{15}N$ baselines required to estimate trophic position, and to evaluate habitat coupling and material flow (Vandermerwe and Vogel 1978; Peterson et al. 1985; Hobson 1999; Post et al. 2000; Phillips 2001; Post 2002; Vander Zanden and Vadeboncoeur 2002). We calculated the percent error introduced into a two-end-member mixing model as a function of the bias in $\delta^{13}C$ due to variation in lipid concentrations ($\Delta\delta^{13}C$ calculated from percent lipid using Eq. 1 in Table 1) and the absolute value of the difference in $\delta^{13}C$ between the two end members (Fig. 4), assuming that the $\delta^{13}C$ of the end members is not biased by lipids (error = $100 \times \Delta\delta^{13}C/|\delta^{13}C_{em1} - \delta^{13}C_{em2}|$).

The difference in $\delta^{13}C$ between end members ($|\delta^{13}C_{em1} - \delta^{13}C_{em2}|$) depends upon the question being asked. For examples, in lakes, $\delta^{13}C$ is used to address questions about littoral–pelagic coupling and to produce the $\delta^{15}N$ baseline required to estimate trophic position (Post et al. 2000; Post 2002; Vander Zanden and Vadeboncoeur 2002). Pelagic sources (open water) are typically more negative than littoral sources (near shore), and the difference in $\delta^{13}C$ between littoral and pelagic is around 7–8‰ (France 1995; Post 2002). Across the range of lipid contents commonly found in the muscle tissue of fish (5–35%), the error could be greater than 50% (Fig. 4), and even small variations in lipid concentration would introduce an error of 5–10%. For example, a fish with 35% lipid (e.g., lake trout; Appendix A) could have an α of 1 (100% pelagic) before lipid normalization and an α~0.5 after normalization, while a fish with 5% lipids (e.g., lean lake trout, northern pike, Appendix A) would have an α of around 0.5 before and after lipid extraction. A similar application is the use of $\delta^{13}C$ to infer shift in vertebrate diets

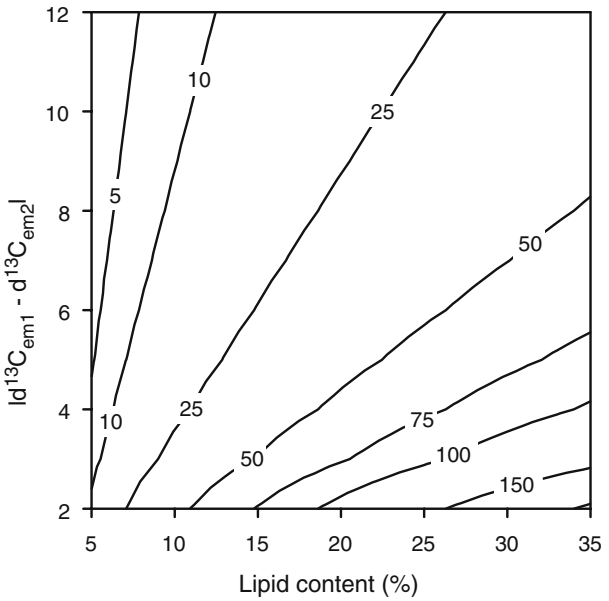

Fig. 4 The percent error introduced into a two-end-member mixing model (isoclines) as a function of variation in lipid concentrations and the absolute value of the difference in $\delta^{13}C$ between the two end members. Percent error was calculated as $100\Delta\delta^{13}C/|\delta^{13}C_{em1} \times \delta^{13}C_{em2}|$, where $\Delta\delta^{13}C$ was calculated from lipid content using Eq. 1 from Table 1

between C3 and C4 plants. C3 plants are more negative than C4 plants and the difference in $\delta^{13}C$ between C3 and C4 plants is large (around 12–14‰). With this large difference in end members, even moderate variations in lipid composition would introduce relatively little error (Fig. 4). In both of these cases, high lipid content biases the $\delta^{13}C$ towards estimate of α that suggest a greater contribution of pelagic carbon or C3 plants. In general, lipid extraction or normalization is most important when lipid content is variable either among consumers of interest, between consumer and end member organisms, or when the difference in $\delta^{13}C$ between end members is <10–12‰. The vast majority of ecological studies using natural variation in $\delta^{13}C$ fall within these criteria.

Chemical lipid extraction

Chemical lipid extraction is an effective and direct method for homogenizing lipid content in samples. A variety of methods are available for chemical lipid extraction, but most of them derive from the methanol–chloroform method outlined in Folch et al. 1957. Because we wanted quantitative estimates of % lipid for our analysis in this paper, we followed closely the Folch et al. (1957) method which produces reliable estimates of lipid content and uniformly low lipid contents. Chemical lipid extraction methods are, however, time-consuming (around 10 min per sample for the Folch method) and can cause small but significant fractionation in $\delta^{15}N$. The average fractionation of $\delta^{15}N$ found here (0.25‰) is similar to that found by previous studies (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Sweeting et al. 2006), and is not much more than the typical analytical error for $\delta^{15}N$ analyses using continuous flow techniques (0.15–0.25‰).

Mathematical normalization

First proposed by McConnaughey and McRoy (1979), mathematical normalization of stable isotopes for lipid concentration offers some obvious advantages over chemical lipid extraction. Mathematical normalization does not require the additional preanalysis step of chemical extraction, and the information required for mathematical normalization—the relative concentrations of carbon and nitrogen in the sample—are typically estimated during analyses of $\delta^{13}C$ and $\delta^{15}N$. With these obvious advantages, why is mathematical normalization not widely adopted? We were initially skeptical of the mathematical normalization techniques (we suspect like many others) because the mathematical predictions made by the equations in McConnaughey and McRoy (1979) did not match our empirical results. We, like Sweeting et al. (2006), found that the McConnaughey and McRoy (1979) equations consistently underestimated the $\Delta\delta^{13}C$ of samples (Fig. 5). Even if the intercept were adjusted (by increasing the value of D in the McConnaughey and

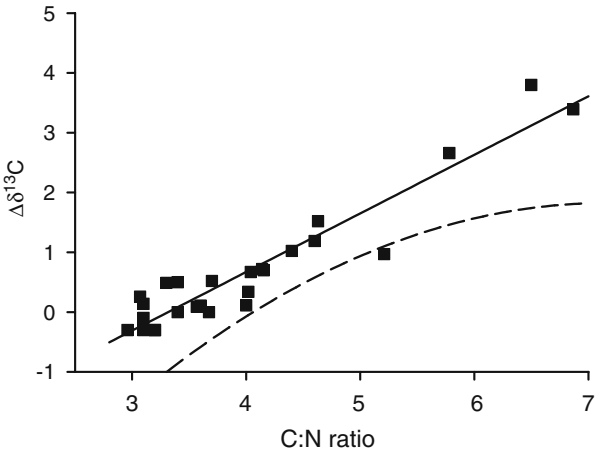

Fig. 5 The relationship between $\Delta\delta^{13}C$ and C:N for aquatic animals *filled squares*. The lipid normalization equations derived from that data (Table 1, Eq. 3; *solid line*) and from equations in McConnaughey and McRoy (1979; *dashed line*) are shown

McRoy equations), the nonlinear form of the McConnaughey and McRoy (1979) model would still not fit our estimates of $\Delta\delta^{13}C$, which for animals was linear over the range of C:N we explored. The lack of fit between model predictions and our empirical results indicates that the McConnaughey and McRoy (1979) model should not be used to mathematically normalize $\delta^{13}C$ for lipid concentration.

Despite the shortcoming of the specific model presented by McConnaughey and McRoy (1979), their observation that C:N predicts % lipid, % lipid predicts $\Delta\delta^{13}C$, and, therefore, that C:N ratios could be used to normalize $\delta^{13}C$ for lipid content, appears correct for animal samples. At least for aquatic organisms, there is a strong relationship between C:N and $\Delta\delta^{13}C$. With that strong relationship, Eq. 3 (Table 1) provides a reliable method for normalizing estimates of $\delta^{13}C$ for lipid concentration where:

$$\delta^{13}C_{\text{normalized}} = \delta^{13}C_{\text{untreated}} + \Delta\delta^{13}C.$$

For aquatic organisms the equation is:

$$\delta^{13}C_{\text{normalized}} = \delta^{13}C_{\text{untreated}} - 3.32 + 0.99 \times \text{C:N}.$$

The resulting $\delta^{13}C_{\text{normalized}}$ provides an estimate of $\delta^{13}C$ that is normalized for the effects of lipid concentration on $\delta^{13}C$ and is comparable to the $\delta^{13}C$ after direct chemical lipid extraction.

For terrestrial organisms, the relationship between C:N and $\Delta\delta^{13}C$ was not strong, with C:N explaining only 33% of the variation in $\Delta^{13}C$ (Table 1). This resulted from a weak relationship between % lipid and $\Delta\delta^{13}C$, despite the strong relationship between C:N and % lipid (Figs. 1, 2; Table 1). Indeed, it appears that there is no relationship between % lipid and $\Delta\delta^{13}C$ at lipid concentrations below 15% because there is no change in the $\delta^{13}C$ with lipid extraction at low lipid concentrations. Our sample size is too small to diagnose the % lipid – $\Delta\delta^{13}C$ relationship for samples with lipid content >15%. We suspect that a larger sample of terrestrial organisms will indicate that the $\Delta\delta^{13}C$ is around 0 for samples with lipid content <15% (C:N < 4), and an increasing function of C:N in samples with lipid content >15%. The weak relationship between % lipid and $\Delta\delta^{13}C$ suggests variation in the discrimination during lipid synthesis and storage for terrestrial animals, which in this case were mostly endotherms (birds and mammals).

For plants, there was no relationship between C:N and % lipid, and therefore no relationship between C:N and $\Delta\delta^{13}C$. C:N is not a good predictor of lipid content in plants because of their plasticity in nitrogen uptake and allocation (Sterner and Elser 2002). Instead, % carbon, particularly for plants with carbon >40%, is a good predictor of % lipid and $\Delta\delta^{13}C$ (Fig. 3). Percent carbon explained nearly 85% of the variation in $\Delta\delta^{13}C$ for plants with % carbon >40% (Table 1).

Caveats

There are few caveats to our analysis that are worth noting. First, all of our analyses were performed on muscle tissue or whole organisms (which is dominated by muscle tissue in most cases). These are the most common samples used for stable isotope analyses, and two of the types of samples most likely to contain high and variable lipid contents. They are not, however, the only tissues used in isotopic studies. For example, liver is used for some questions because of its relatively short turnover time. Preliminary data suggest that the relationships among C:N, lipid content, and $\Delta\delta^{13}C$ are different for liver than for muscle tissue (see also Sweeting et al. 2006). Differences in the relationship between C:N and % lipid likely emerge from tissue-specific differences in the C:N ratios of lipid-free tissue (typically assumed to be pure protein). We see little differences in the C:N ratios of lipid-free tissue when using muscle or whole body samples, but greater variation in lipid-free C:N for specific tissue types. Tissue-specific studies should use our results with caution. Second, our terrestrial animal samples are primarily from endothermic species and further work is needed for terrestrial animals, particularly terrestrial ectotherms which are underrepresented in our dataset.

Finally, extrapolating the relationships presented here beyond the measured range of lipid contents and C:N ratios should be done with caution. Previous models have predicted a nonlinear relationship between C:N and $\Delta\delta^{13}C$ because of the expectation that $\Delta\delta^{13}C$ will converge on D, the discrimination in $\delta^{13}C$ between lipids and lipid-free tissues (typically assumed to be 6–8‰), at high C:N ratios (McConnaughey and McRoy 1979; Alexander et al. 1996). Either that expectation is flawed or, more likely, our samples sit within the region of C:N ratios that is essentially linearly related to $\Delta\delta^{13}C$. Our maximum C:N, 6.9, and lipid content, 32%, were found in lake trout, which can be a very lipid-rich fish. McConnaughey and McRoy (1979), working with lipid-rich animals in the Bering Sea, found that most animals (whole animal or muscle tissue) had C:N ratios of between 3 and 6, with a maximum C:N ratio of 9.

Other applications

Besides lipid normalization of $\delta^{13}C$ for stable isotope analyses, the strong relationship between C:N and lipid content found here should prove useful for studies of energy allocation and lipid storage (e.g., Schultz and Conover 1997; Post and Parkinson 2001). For animals, C:N provides a strong predictor of lipid content. The use of C:N ratios to estimate lipid content for studies of energy allocation and lipid storage will have many of the same inherent advantages as the use of C:N to estimate lipid-normalized values of $\delta^{13}C$. In particular, C:N ratios can be easily measured on an elemental analyzer, used either a standalone unit or as part of a continuous flow isotope ratio mass spectrometer. The equations presented in Table 1 are for lipid content as a percentage of dry mass, so adjustments must be made to calculate lipid content as a percentage of wet weight if that is the measurement of interest (e.g., Post and Parkinson 2001). The use of C:N to predict lipid content is similar to using dry weight to estimate energy density (Hickman and Pitelka 1975; Hartman and Brandt 1995), and the strong relationship between C:N and lipid content found here (Table 1; Fig. 2) suggests that C:N ratios can provide reliable estimates of lipid content in a wide variety of animals.

Recommendations

In light of our findings, we make the following recommendations for dealing with lipids in analyses using $\delta^{13}C$ at natural abundance levels.

1. The first step is to take stock of your samples and question. For animals, if the lipid content or C:N ratio of *all* of the samples is low (% lipid < 5% or C:N < 3.5 for aquatic animals; % lipid <10% or C:N < 4 for terrestrial animals), our data indicate that lipid concentrations are already uniformly low and extraction or normalization will have little influence on the $\delta^{13}C$ values. Lipids need to be accounted for when making comparisons among animals with variable lipid concentrations (or C:N ratios), where end members have variable lipid content, or where end members and animals of interest differ in lipid concentrations. These recommendations apply to all predators and prey, baseline organisms, etc., used to reconstruct diets and trophic position.
2. If you are working with aquatic organisms, which are well represented in this study, the C:N of your sample and Eq. 3 can be used (Table 1) to correct samples for lipid bias. As long as samples have been weighed carefully (we recommend a balance with a resolution of 0.001 mg), and an appropriate reference standard is used (similar composition, well-quantified carbon and nitrogen concentrations), the carbon and nitrogen concentrations provided during $\delta^{13}C$ and $\delta^{15}N$ analysis are sufficient to calculate C:N ratios and perform lipid normalization.
3. When working with a group of organisms or specific tissue types that are not well-represented in this study (e.g., terrestrial ectotherms, liver), chemical extraction should be used to estimate the relationship between $\Delta\delta^{13}C$ and C:N for samples. This is particularly important because variation among taxonomic groups in the C:N ratio of lipid-free tissue (Sweeting et al. 2006) will influence the empirically derived relationship between $\Delta\delta^{13}C$ and C:N. We further recommend that % lipid be estimated in some samples to check the relationships among C:N, lipid content, and $\Delta\delta^{13}C$, although this step is not essential for normalization.
4. Our results suggest that lipid extraction or normalization should generally be performed on plant samples, but that % carbon, rather than C:N, should be used to normalize lipid content. Our results also suggest that the equations for $\Delta\delta^{13}C$ versus % carbon for plants with carbon concentration > 40% should be used (Table 1; Eq. 13)

The above recommendations do not apply to samples that have been chemically preserved in ethanol or formalin, which can have variable and relatively large effects on the $\delta^{13}C$ and $\delta^{15}N$ of tissue samples (Arrington and Winemiller 2002; Sarakinos et al. 2002; Sweeting et al. 2004). We do not generally recommend using chemically preserved samples for stable isotope analyses. However, if they are the only option, we recommend using direct chemical lipid extraction to deal with lipids because it is not clear how chemical preservation affects the relationships between C:N, lipid content, and $\Delta\delta^{13}C$ that make mathematical normalization possible. These recommendations also assume that, when needed, inorganic carbon has already been removed from samples by acidification.

We conclude that, while variation in lipid content among organisms is of considerable interest and importance to ecologists and evolutionary biologists, it is a source of error that must be accounted for when using natural abundance measures of $\delta^{13}C$. It is not the absolute lipid content that matters, but variability in lipid content among samples that introduces bias. Both direct lipid extraction and mathematical normalization are effective techniques for homogenizing lipid content

and eliminating lipid-related biases in $\delta^{13}C$; however, mathematical normalization simplifies sample preparation and can better preserve the integrity of samples for $\delta^{15}N$ analysis. The strong relationship between C:N ratio and lipid content at the center of the mathematical normalization technique may also provide a useful method for estimating lipid contents for studies of energy allocation and lipid storage. Because our results derive from plants and animals drawn from a wide variety of ecosystems (tropical rivers, arctic and temperate lakes and streams, coastal marine, and temperate and tropical terrestrial ecosystems) and they represent many of the taxonomic groups most well-studied by ecologists, we believe mathematical normalization is widely applicable; however, we encourage others to test the relationship among C:N, lipid content, and $\Delta\delta^{13}C$ in other study systems.

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