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Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modelling methods

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Summary

1. Lipids have more negative $\delta^{13}C$ values relative to other major biochemical compounds in plant and animal tissues. Although variable lipid content in biological tissues alters results and conclusions of $\delta^{13}C$ analyses in aquatic food web and migration studies, no standard correction protocol exists.
2. We compared chemical extraction and mathematical correction methods for freshwater and marine fishes and aquatic invertebrates to better understand impacts of correction approaches on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope data.
3. Fish and aquatic invertebrate tissue $\delta^{13}C$ values increased significantly following extraction for almost all species and tissue types relative to nonextracted samples. In contrast, $\delta^{15}N$ was affected for muscle and whole body samples from only a few freshwater and marine species and had a limited effect for the entire data set.
4. Lipid normalization models, using C : N as a proxy for lipid content, predicted lipid-corrected $\delta^{13}C$ for paired data sets more closely with parameters specific to the tissue type and species to which they were applied.
5. We present species- and tissue-specific models based on bulk C : N as a reliable alternative to chemical extraction corrections. By analysing a subset of samples before and after lipid extraction, models can be applied to the species and tissues of interest that will improve estimates of dietary sources using stable isotopes.

Key-words: chemical tracer, energy transfer, movement, trophic ecology.

Introduction

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes can be used in aquatic ecosystems to decipher movements (Hobson 1999; Rubenstein & Hobson 2004; Cunjak *et al*. 2005) and food habits (Peterson & Fry 1987) of a wide range of species. Carbon stable isotope ratios in consumers are reflected mainly by the photosynthetic pathway used by primary producers. Carbon is a conservative tracer that can be used to trace energy sources in food webs, because isotope values increase only slightly between diet and consumer (0–1‰) during trophic transfers (Fry & Sherr 1984). In contrast, $\delta^{15}N$ values tend to increase with each trophic transfer (DeNiro & Epstein 1981), mainly through preferential excretion of ^{14}N by consumers (Steele & Daniel 1978). Nitrogen isotopes can be used to determine trophic position, increasing about 3·4‰ at each trophic level (Minagawa & Wada 1984; Vander Zanden & Rasmussen 2001; Post 2002). Carbon and nitrogen stable isotopes can also be used to trace movement (Best & Schell 1996; Hansson *et al*. 1997; Fry *et al*. 2003; Cook, Bunn & Hughes 2007) when animal migrations traverse habitats with distinct isotope values ('isotope provinces' *sensu* Hobson 1999).

An issue in stable isotope analysis (SIA) is that lipids have more negative $\delta^{13}C$ values relative to other biochemical compounds due to kinetic isotope effects that occur during the conversion of pyruvate to acetyl coenzyme A in lipid synthesis (DeNiro & Epstein 1977). Variability in tissue lipid content can alter bulk tissue $\delta^{13}C$ values (Focken & Becker 1998), and could be falsely interpreted as dietary or habitat shifts. Researchers can correct for variability in $\delta^{13}C$ values a priori, by extracting lipids from samples, or a posteriori,

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through mathematical corrections. Lipid extractions were performed prior to $\delta^{13}C$ analysis in only 10% of published studies that we reviewed mainly because they used low-lipid tissues such as bird feathers and fish muscle; even fewer have applied lipid correction or normalization techniques (Kline 1999; Matthews & Mazumder 2005).

In stable isotope studies of food webs, reducing interindividual $\delta^{13}C$ variation in improving dietary source proportion estimates is a desirable objective (Post *et al*. 2007). Several different normalization, correction and chemical extraction methods have been applied in stable isotope studies of food webs (Kelly 2000), but a standard protocol for lipid correction does not exist. Most corrections use elemental carbon to nitrogen ratios (C : N) of bulk tissue as a proxy for lipid content in estimating $\delta^{13}C$ of lipid-free tissue (McConnaughey & McRoy 1979; Fry 2002; Kiljunen *et al*. 2006; Sweeting, Polunin & Jennings 2006; Post *et al*. 2007). As lipids are composed mainly of carbon and most lipid classes contain no nitrogen, increases in C : N ratios closely track increases in lipid content (Schmidt *et al*. 2003; Bodin, Le Loc'h & Hily 2007; Post *et al*. 2007). In addition, correct values for protein–lipid $\delta^{13}C$ discrimination and C : N of lipid-free tissue are uncertain, and approaches for a given taxa (e.g. zooplankton, Smyntek *et al*. 2007) may not be appropriate for other unrelated taxa or tissues (Kiljunen *et al*. 2006; Post *et al*. 2007).

Lipids are a major component of energy flow in food webs, and valuable ecological information is lost when they are extracted (Arts, Ackman & Holub 2001). When lipid extractions are performed for $\delta^{13}C$ correction prior to SIA, most researchers analyse the single lipid extracted sample for both $\delta^{13}C$ and $\delta^{15}N$. Common extraction methods alter $\delta^{15}N$ to varying degrees in fish tissue (Sotiropoulos, Tonn & Wassenaar 2004; Murry *et al*. 2006; Sweeting *et al*. 2006), indicating that duplicate samples (one extracted, one nonextracted) are needed for optimal estimation of $\delta^{13}C$ and $\delta^{15}N$.

We conducted an analysis of $\delta^{13}C$ and $\delta^{15}N$ for a broad range of freshwater and marine fishes and invertebrates, without treatment and following chloroform–methanol lipid extraction in order to explore extraction effects on SIA results. Models using tissue C : N and bulk $\delta^{13}C$ were tested as an alternative approach to correct for lipid $\delta^{13}C$ alteration in fish and invertebrate tissues. Our objective was to provide practical solutions for researchers using SIA who are dealing with the potential confounding influence of lipids in aquatic food web and migration studies.

Methods

SAMPLE COLLECTION AND PROCESSING

Samples of 431 marine and freshwater fishes and invertebrates representing 18 families were analysed for $\delta^{13}C$ and $\delta^{15}N$ with and without chloroform–methanol lipid extraction treatment (Table 1). All samples were stored frozen for a maximum of seven months before analysis. Freezing has not altered $\delta^{13}C$ or $\delta^{15}N$ values for fish (Sweeting, Polunin & Jennings 2004) or marine invertebrate tissues (Bosley & Wainright 1999), but has affected freshwater zooplankton (Feuchtmayr & Grey 2003). Our comparisons of $\delta^{13}C$ and $\delta^{15}N$ between lipid extracted and bulk tissue samples were based on aliquots of dried, homogenized samples subjected to identical frozen storage conditions.

Liver, muscle and gonad samples were removed from selected fish species (Table 1), thawed, lightly rinsed with deionized water, transferred to glass scintillation vials and dried at 60 °C for at least 48 h. The remaining fishes and all invertebrates were dried whole, with pooled samples of two to 20 individuals used for freshwater invertebrates and whole individuals used for marine invertebrates. Larger whole Atlantic herring *Clupea harengus* (Linnaeus), Atlantic mackerel *Scomber scombrus* (Linnaeus), silver hake *Merluccius bilinearis* (Mitchill) and shortfin squid *Illex illecebrosus* (Leseur) were lightly rinsed with deionized water, then finely minced and dried in aluminium weigh boats at 60 °C for at least 48 h until they reached a constant weight over 3 h. Dried samples were then homogenized with either a Wig-L-Bug® ball and capsule amalgamator (Crescent Industries, Auburn, ME, USA) and stainless steel grinding vials or a mortar and pestle, depending on tissue volume.

Two aliquots were removed from each homogenized sample; one aliquot was immediately prepared for SIA (see below), while the second underwent lipid extraction using a modification of the Bligh & Dyer (1959) method. To extract lipids, dried powdered samples were placed in glass centrifuge tubes and immersed in a 2 : 1 ratio of chloroform : methanol with a solvent volume about three to five times greater than sample volume. Samples were then mixed for 30 s, left undisturbed for greater than 30 min, then centrifuged for 10 min at 1318 ***g***. The supernatant containing solvent and lipids was then discarded. This process was repeated at least three times or more until the supernatant was completely clear and colourless following centrifugation. Samples were dried at 60 °C for 24 h to remove remaining solvent. Euphausiid samples also underwent acid washing after lipid extraction and drying to remove exoskeletal carbonates. Acid washing consisted of addition of 1 N HCl until bubbling ceased (Jacob *et al*. 2005), and the samples were redried at 60 °C for 24 h.

To determine the relationship between bulk tissue C : N and percentage lipid, a quantitative modification of the Bligh & Dyer (1959) method was performed on four fish species (bony bream *Nematalosa erebi* (Günther), golden perch *Macquaria ambigua* (Richardson), silver tandan *Porochilus argenteus* (Zeitz) and spangled perch *Leiopotherapon unicolor* (Günther)). Briefly, *c.* 0·2 g of dry sample was weighed into a centrifuge tube, to which chloroform, methanol and distilled water were added at a ratio of 2 : 2 : 1·8. The mixture was shaken then centrifuged at *c.* 265 ***g***. for 10 min. The bottom solvent layer was withdrawn and passed through a micropipette column packed with sodium sulphate into an aluminium weigh boat. The fraction remaining in the centrifuge tube was subjected to a second extraction with a 9 : 1 ratio of chloroform : methanol, centrifuged, the bottom layer withdrawn and passed through sodium sulphate as before. The fraction in the weigh boat (the lipid fraction) was evaporated in a drying oven and weighed to determine percentage lipid using the formula percentage lipid = (lipid weight/dry weight) × 100.

STABLE ISOTOPE SAMPLE PREPARATION

Aliquots (0·2–1·2 mg) of lipid-extracted and bulk tissue samples were weighed to the nearest 0·001 mg and packed into tin capsules in preparation for SIA. Samples were flash combusted at 1100 °C and resultant gases delivered via continuous-flow for analysis of $\delta^{13}C$, $\delta^{15}N$, percentage carbon and percentage nitrogen using either

Table 1. Freshwater (FW) and marine (M) fish and invertebrate species and tissue types (L = liver, M = muscle, G = gonad, W = whole body) analysed for $\delta^{13}C$ and $\delta^{15}N$ for bulk tissue and samples lipid extracted using chloroform–methanol. *P*-values indicate significant increases ($^+$) or decreases ($^-$) in $\delta^{13}C$ or $\delta^{15}N$ following lipid extraction with an overall α of 0·05. Additional data for European sea bass *Dicentrarchus labrax* (Linnaeus) liver and muscle (Sweeting *et al*. 2006) were also used for model fitting

Taxa	*n*	Habitat	Tissue	Bulk C : N range	$\delta^{13}C$ *P*-value	$\delta^{15}N$ *P*-value
Fishes						
American eel *Anguilla rostrata* (Lesueur)	50	FW	M	3·3–9·8	< 0·001$^+$	< 0·001$^+$
Atlantic salmon *Salmo salar* (Linnaeus)	6	FW	M	3·0–3·2	0·109	0·075
Brook trout *Salvelinus fontinalis* (Mitchill)	12	FW	M	3·1–3·6	< 0·001$^+$	0·792
Slimy sculpin *Cottus cognatus* (Richardson)	8	FW	M	3·2–3·2	< 0·001$^+$	0·339
Atlantic salmon *Salmo salar* (Linnaeus)	6	FW	L	4·0–4·3	< 0·001$^+$	0·409
Brook trout *Salvelinus fontinalis* (Mitchill)	6	FW	L	3·9–7·1	0·006$^+$	0·410
Slimy sculpin *Cottus cognatus* (Richardson)	8	FW	L	4·0–8·9	< 0·001$^+$	0·942
Bony bream *Nematalosa erebi* (Günther)	36	FW	W	2·9–8·0	< 0·001$^+$	< 0·001$^+$
Golden perch *Macquaria ambigua* (Richardson)	36	FW	W	3·2–6·4	< 0·001$^+$	0·520
Silver tandan *Porochilus argenteus* (Zeitz)	36	FW	W	3·3–12·1	< 0·001$^+$	< 0·001$^+$
Spangled perch *Leiopotherapon unicolor* (Günther)	29	FW	W	2·7–9·3	< 0·001$^+$	< 0·001$^+$
Slimy sculpin *Cottus cognatus* (Richardson)	8	FW	G	2·5–4·3	< 0·001$^+$	0·107
Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus)	82	M	M	3·1–6·5	< 0·001$^+$	< 0·001$^+$
Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus)	44	M	L	3·9–12·0	< 0·001$^+$	0·048
Atlantic herring *Clupea harengus* (Hildebrand)	11	M	W	6·3–12·5	< 0·001$^+$	0·664
Atlantic mackerel *Scomber scombrus* (Linnaeus)	3	M	W	3·8–9·2	0·083	0·172
Silver hake *Merluccius bilinearis* (Mitchill)	12	M	W	3·5–4·1	< 0·001$^+$	< 0·001$^-$
Invertebrates						
Alderflies (Megaloptera)	7	FW	W	3·7–5·7	0·011$^+$	0·807
Caddisflies (Hydropsychidae)	7	FW	W	4·5–5·7	< 0·001$^+$	0·643
Dragonflies (Gomphidae)	5	FW	W	4·0–4·4	0·002$^+$	0·651
Mayflies (Heptageniidae)	26	FW	W	5·2–10·7	< 0·001$^+$	0·514
Stoneflies (Perlidae)	15	FW	W	4·3–5·3	< 0·001$^+$	0·002$^+$
Water pennies (Psephenidae)	12	FW	W	4·3–7·5	< 0·001$^+$	0·523
Water striders *Aquarius remigis* (Say)	12	FW	W	4·1–6·0	< 0·001$^+$	0·051
Water strider nymphs *Aquarius remigis* (Say)	6	FW	W	4·0–5·7	< 0·001$^+$	0·501
Krill (Euphausiidae)	10	M	W	3·7–4·3	< 0·001$^+$	0·001$^+$
Shortfin squid *Illex illecebrosus* (Leseur)	6	M	W	4·0–4·5	< 0·001$^+$	< 0·001$^-$

a DELTA^{plus} or DELTA^{plus} Advantage isotope ratio mass spectrometer at the University of New Brunswick (UNB) and Northern Arizona University (NAU), respectively. C : N ratios were determined from percentage element weight. Measurements of commercially available reference materials across all runs were both accurate and precise with mean ± SD of –33·6 ± 0·16‰ for $\delta^{13}C$ and –3·1 ± 0·18‰ for $\delta^{15}N$ for acetanilide (n = 214) at UNB and mean ± SD of –25·9 ± 0·04‰ for $\delta^{13}C$ and 2·0 ± 0·14‰ for $\delta^{15}N$ for NIST 1547 (peach leaves, n = 149) at NAU. Replicate analyses of samples produced SD of 0·16‰ for $\delta^{13}C$ and 0·16‰ for $\delta^{15}N$ (n = 108) at UNB and SD for $\delta^{13}C$ of 0·05‰ and 0·07‰ for $\delta^{15}N$ at NAU (n = 61). Samples were also routinely analysed at both labs to ensure data were comparable (e.g. smallmouth bass muscle: UNB $\delta^{13}C$ = –23·2 ± 0·11‰, $\delta^{15}N$ = 12·5 ± 0·18‰, n = 19; NAU $\delta^{13}C$ = –23·3 ± 0·02‰, $\delta^{15}N$ = 12·4 ± 0·11‰, n = 3). All C and N isotope data are reported in δ notation according to the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$ where X is ^{13}C or ^{15}N and R is the ratio ^{13}C : ^{12}C or ^{15}N : ^{14}N (Peterson & Fry 1987). Standard materials are Vienna Pee Dee belemnite (VPDB) for carbon and atmospheric N2 (AIR) for nitrogen. All $\delta^{13}C$ and $\delta^{15}N$ values were normalized on the VPDB and AIR scales with IAEA CH6 (–10·4‰), CH7 (–31·8‰), N1 (0·4‰) and N2 (20·3‰).

STATISTICAL MODELS AND ANALYSES

Differences in mean changes in C and N isotopes ($\delta^{13}C' - \delta^{13}C$ and $\delta^{15}N' - \delta^{15}N$) between lipid extracted ($\delta^{13}C'$ and $\delta^{15}N'$) and bulk tissue $\delta^{13}C$ and $\delta^{15}N$ samples (Table 1) were tested. Differences were determined using paired t-tests (α = 0·05) and a subsequent Holm test to reduce the probability of committing type I errors as a result of multiple comparisons.

To compare lipid corrected $\delta^{13}C$ estimates among models, log-likelihood values (assuming normally distributed errors) were calculated for models described by McConnaughey & McRoy (1979) and Fry (2002). The Akaike Information Criterion (AIC_c) value was also calculated for each model. AIC_c values are determined according to the equation

$$AIC_c = -2 * \text{log-likelihood} + 2k + \frac{2k(k+1)}{n-k-1}$$

where k equals the number of parameters and lower AIC_c values reflect improved model fits. AIC_c values are also presented as AIC differences (Δ_i) according to the equation

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

where AIC_{ci} corresponds to the AIC_c value for model i and min AIC_c is the model with the lowest AIC_c value among tested models (Burnham & Anderson 1998). AIC_c differences were calculated between individual models. Models with Δ_i of about 0–2 have substantial support as best model fits, Δ_i of 4–7 indicates considerably less support, and $\Delta_i > 10$ provides essentially no support for a given

model (Burnham & Anderson 1998). All tested models used bulk tissue C : N as a predictor of $\delta^{13}C' - \delta^{13}C$.

The first model form that we tested is based on the McConnaughey & McRoy (1979) model (eqn 1),

$$\delta^{13}C' - \delta^{13}C = D\left(\theta + \frac{3{\cdot}90}{1 + 287/L}\right) \quad \text{eqn 1}$$

$$\text{where } L = \frac{93}{1 + (0{\cdot}246 * C:N - 0{\cdot}775)^{-1}}$$

and L and D represent sample lipid content and protein–lipid discrimination, respectively. The McConnaughey & McRoy (1979) model (eqn 1) was fit with D and θ based on our data set. A new generalized model based on eqn 1 was developed that maintained the nonlinear relationship of the difference in $\delta^{13}C$ between bulk tissue and lipid extracted tissue, but aggregated assumed values into three parameters,

$$\delta^{13}C' - \delta^{13}C = \frac{a * C:N + b}{C:N + c} \quad \text{eqn 1a}$$

The y-asymptote, or D in eqn 1, corresponds to a in eqn 1a. The model estimate $C:N_{lipid\text{-}free}$ is represented by $-b/a$ (x-intercept), whereas b/c (y-intercept) is the $\delta^{13}C$ difference corresponding to a C : N value of zero.

The second mathematical lipid correction approach that we tested is based on the Fry (2002) equation (eqn 2),

$$\delta^{13}C' - \delta^{13}C = P - \frac{P * F}{C:N} \quad \text{eqn 2}$$

where P and F represent protein–lipid $\delta^{13}C$ discrimination and $C:N_{lipid\text{-}free}$, respectively. The Fry (2002) equation (eqn 2) was fit with P and F based on our data set.

A new model of the difference in $\delta^{13}C$ between bulk and lipid-extracted tissue and log-transformed C : N,

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

was also explored. The model estimate of $C:N_{lipid\text{-}free}$ is represented by $e^{(-\beta_0/\beta_1)}$.

Normally distributed error terms, $\varepsilon \sim N(0, \sigma^2)$, were assumed and models were fit based on eqns 1, 1a, 2, and 3 to data from different tissue types and species with sample sizes greater than or equal to 10 (Table 1). For fish species, three nested models of a given type were fitted. The simplest model assumed all model parameters were the same across species and tissue types, the intermediate model assumed that the parameters were tissue-specific and the full model assumed that parameters were both tissue- and species-specific. For invertebrate species, two models of a given type were fitted where the simpler model again assumed that all model parameters were the same across species and the full model assumed parameters were species-specific. Likelihood ratio tests were performed for each model type and species group (fishes or invertebrates) to determine the most parsimonious models. Parameters were estimated for all models using least-squares procedures available in R (R Development Core Team 2006).

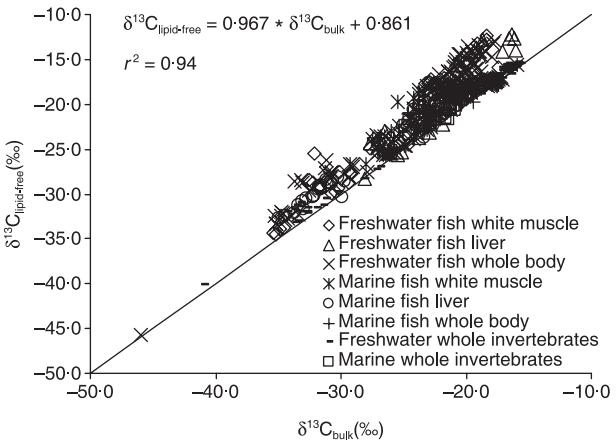

Fig. 1. Bulk tissue and lipid-extracted $\delta^{13}C$ for a range of freshwater and marine fishes and invertebrates (Table 1) with 1 : 1 reference line.

Results

CHLOROFORM–METHANOL LIPID EXTRACTION COMPARISONS

We observed significant increases in $\delta^{13}C$ following lipid extraction for all invertebrate species and all fish tissues, except whole Atlantic mackerel (n = 3) and Atlantic salmon smolt white muscle (n = 6). The best fit for all data was $\delta^{13}C_{lipid\text{-}free} = 0{\cdot}967 \times \delta^{13}C_{bulk} + 0{\cdot}861$ with 95% confidence intervals of 0·001 and 0·024 for slope and intercept, respectively (Fig. 1; Table 1). For all tissues, mean increases ranged from 1·1 ± 0·11 for marine fish white muscle to 3·0 ± 0·22 for marine fish liver (Table 2). Lipid extracted fish C : N ranged from 3·2 to 6·4 for liver (n = 64), 3·0–3·4 for white muscle (n = 158), and 2·7–4·4 for whole body samples (n = 163). Bulk tissue C : N was a good predictor of the observed change in $\delta^{13}C$ due to extraction in fish tissues (Fig. 2) and was also an

Table 2. Summary of mean change in $\delta^{13}C$ and $\delta^{15}N$ (± SE) following lipid extraction in chloroform–methanol for different taxa and tissues

Taxa	n	Tissue	$\delta^{13}C$	$\delta^{15}N$
Marine fishes	82	Muscle	1·1 ± 0·11	0·7 ± 0·06
Marine fishes	44	Liver	3·0 ± 0·22	–0·2 ± 0·09
Marine fishes	26	Whole	2·8 ± 0·33	–0·2 ± 0·08
Freshwater fishes	76	Muscle	2·0 ± 0·19	0·3 ± 0·04
Freshwater fishes	20	Liver	1·7 ± 0·12	0·0 ± 0·06
Freshwater fishes	137	Whole	1·5 ± 0·09	0·3 ± 0·04
Marine invertebrates	16	Whole	1·3 ± 0·11	–0·1 ± 0·12
Freshwater invertebrates	94	Whole	1·3 ± 0·07	0·0 ± 0·05

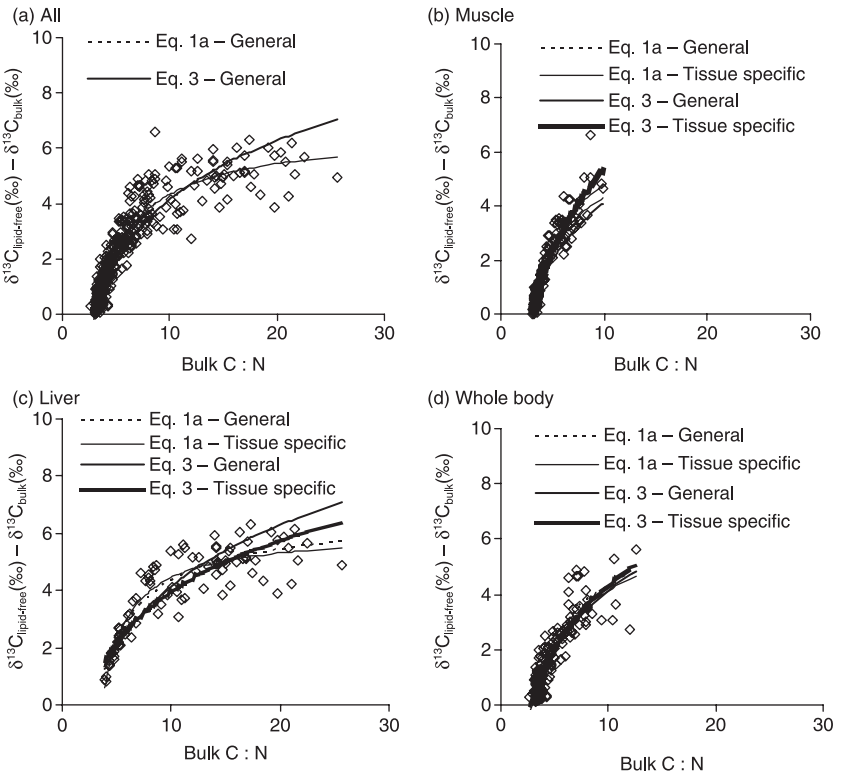

Fig. 2. Model fits to differences in fish tissue bulk $\delta^{13}C$ and lipid-free $\delta^{13}C$ following lipid extractions with 2 : 1 chloroform : methanol. Models were fit (a) to a general data set of fish muscle, liver and whole body paired $\delta^{13}C$ samples, and (b–d) to tissue-specific data sets. Equations defined in Methods.

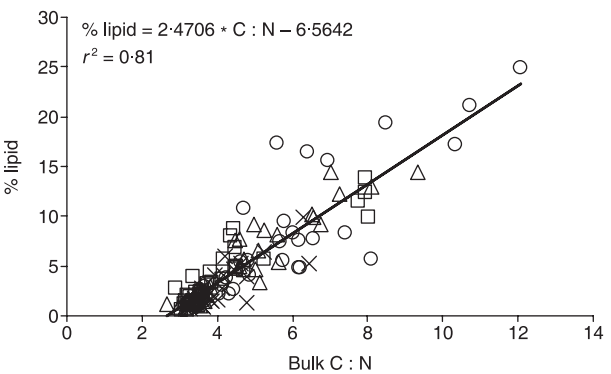

Fig. 3. Percentage lipid estimates for four freshwater Australian fish species (silver tandan ○, bony bream □, spangled perch △ and golden perch ×) in relation to bulk tissue C : N.

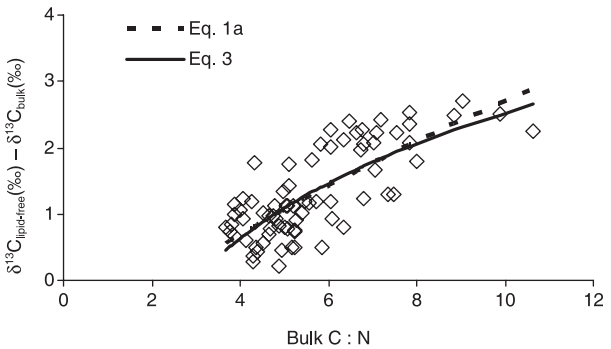

Fig. 4. Model fits to differences in whole body invertebrate bulk $\delta^{13}C$ and lipid-free $\delta^{13}C$ following lipid extractions with 2 : 1 chloroform : methanol. Equations defined in Methods.

indicator of percentage lipid in the Australian freshwater fish samples ($r^2 = 0{\cdot}81$; Fig. 3). The *x*-intercepts for our simplest fish tissue models (liver, muscle and whole body combined) were 3·0 (eqn 1a) and 0·962 (eqn 3; Appendix 1), indicating a C : N of 2·6 (calculated as $e^{0{\cdot}962}$) for eqn 3 for pure protein, or the C : N at which no change in $\delta^{13}C$ occurred due to lipid extraction. A best fit equation for quantitative lipid extractions of whole body Australian fishes produced a C : $N_{lipid\text{-}free}$ of 2·7 (Fig. 3).

Following extractions, $\delta^{15}N$ significantly increased for Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus) and American eel *Anguilla rostrata* (Lesueur) white muscle and whole body bony bream, silver tandan and spangled perch, while $\delta^{15}N$ significantly decreased for whole body silver hake (Table 1). In marine fishes, alteration of $\delta^{15}N$ ranged from –0·2 ± 0·09 for liver to 0·7 ± 0·06 for white muscle (Table 2). Overall, there was limited evidence for a major $\delta^{15}N$ alteration associated with lipid extraction (Tables 1 and 2).

After lipid extraction in whole body invertebrates, C : N ranged from 3·3 to 4·8 ($n = 94$; freshwater) and 3·2–3·5 ($n = 16$; marine). For invertebrates, C : N accounted for less of the variation in $\delta^{13}C$ change owing to extraction (Fig. 4). We observed significant decreases (whole shortfin squid) and increases (euphausiids and stoneflies (Perlidae)) in $\delta^{15}N$, but all remaining $\delta^{15}N$ data sets were not significantly altered (Tables 1 and 2). The best fit relationship for $\delta^{15}N$ for all samples (fishes and invertebrates) was $\delta^{15}N_{lipid\text{-}free} = 1{\cdot}018 \times \delta^{15}N_{bulk} + 0{\cdot}020$ with 95% confidence intervals of 0·020 and 0·161 for slope and intercept, respectively.

BEST MODELS FOR PREDICTING $\delta^{13}C' - \delta^{13}C$

Model fits improved significantly with increased model specificity. For fish species, the tissue-specific model fit the data significantly better than the simplest model (with parameters constant across all tissue types) and the species–tissue-specific model fit the data significantly better than the tissue-specific model ($P < 0{\cdot}001$ for all comparisons; see Appendix 1 for full list of parameter estimates). Model estimates of $C : N_{lipid\text{-}free}$ ranged from 1·9 to 3·7 among fish models and tissue types with a mean of $3{\cdot}0 \pm 0{\cdot}09$. Protein–lipid discrimination estimates ranged from 5·5 to 7·4 with a mean of $6{\cdot}5 \pm 0{\cdot}17$ among tissue types for eqns 1, 1a and 2. We could not derive a value for protein–lipid discrimination for eqn 3 as it did not have a *y*-asymptote (Fig. 2). The species-specific model also fit invertebrate data significantly better than the simpler model with parameters constant across species for all models ($P < 0{\cdot}001$ for all comparisons).

Lowest AIC_c values (min AIC_c) were not consistently produced by a single model form. For fish samples, eqn 1a had the lowest AIC_c value among general and tissue-specific fitted models while for species–tissue-specific models, eqns 2 and 3 had similar lowest AIC_c values. For invertebrate samples, eqn 2 had the lowest AIC_c value among general models and eqns 1 and 2 had lowest AIC_c values for species-specific models.

Discussion

LIPID IMPACTS ON $\delta^{13}C$

Lipid extractions caused significant increases in $\delta^{13}C$ for almost all species and tissue types, indicating the need to correct for lipid carbon isotope effects, especially when fine scale $\delta^{13}C$ differences are compared. Species- and tissue-specific models could meet this need by reducing the time and uncertainty associated with lipid extraction procedures, and by improving estimates of dietary proportions derived from stable isotope mixing models (Phillips & Gregg 2001).

While lipid extractions caused statistically significant increases in $\delta^{13}C$ relative to bulk tissue samples, observed differences were not always biologically significant, with some tissues showing limited $\delta^{13}C$ change. Fish white muscle, for example, contains minimal lipids and may not benefit from lipid correction or extraction (Pinnegar & Polunin 1999). In fish muscle samples, bulk C : N values < 3·4 generally produced $\delta^{13}C$ changes < 0·7‰. In contrast, fish liver has high lipid content and should be lipid-corrected (Sweeting *et al*. 2006); our tuna liver samples had C : N > 10 and lipid extraction changed $\delta^{13}C$ > 5‰. As lipid content of whole body fish and invertebrate samples are highly variable, bulk C : N provided a less clear predictor of change in $\delta^{13}C$ from lipid removal. Lipid-free C : N varied by species and certain tissues showed a moderate $\delta^{13}C$ response to extraction despite high $C : N_{bulk}$. For example, mayflies (family Heptageniidae) have C : N > 10, but lipid extraction increased $\delta^{13}C$ by < 3‰.

Given the broad range of ecological studies that use carbon isotopes, the biological significance of $\delta^{13}C$ changes will vary by study and the ecological questions being posed. With estimation of marine vs. terrestrial (Bearhop *et al*. 1999) or C3 vs. C4 or CAM primary producer contributions to diet (Peterson, Howarth & Garritt 1985; Wolf, Martínez del Rio & Babson 2002), $\delta^{13}C$ end members may be sufficiently distinct, and lipid correction will not alter isotope data interpretation. Where end members are less isotopically distinct (Abend & Smith 1997), proper lipid correction may be critical in determining food sources for consumers. Decisions regarding lipid correction will ultimately be decided by the scientific questions being addressed in a given study.

Overall, each tissue data set for marine and freshwater systems had greater than a per mil mean increase in $\delta^{13}C$ following extraction, deviations that would alter interpretations of results in many food web studies. The implication is that an analysis of bulk tissue C : N values and correction of $\delta^{13}C$ through chemical extractions or modelling approaches may be necessary. Alternatively, transitioning from bulk $\delta^{13}C$ and $\delta^{15}N$ analyses to compound-specific analyses (Hammer, Fogel & Hoering 1998; Fantle *et al*. 1999) could remove variability due to isotopic heterogeneity. When data are not lipid corrected, authors should provide additional information on bulk tissue C : N to identify potential lipid impacts on $\delta^{13}C$.

CHEMICAL EXTRACTIONS: EFFECTS ON $\delta^{15}N$

Lipid extractions with chloroform–methanol altered sample $\delta^{15}N$ to varying degrees (e.g. sample $\delta^{15}N$ range relative to nontreated samples of –2·4 to +2·9‰). We observed increases in $\delta^{15}N$ only for some fish white muscle and fish and invertebrate whole body samples. Fish liver $\delta^{15}N$ values were not significantly altered by chloroform–methanol extractions and our total data set had minimal $\delta^{15}N$ alteration. Previous studies for individual species have found significant increases in $\delta^{15}N$ associated with lipid extraction, including tissues such as fish white muscle, whole bodies and liver (Pinnegar & Polunin 1999; Sotiropoulos *et al*. 2004; Murry *et al*. 2006). These conflicting results support caution in subjecting all samples to lipid extraction prior to $\delta^{15}N$ analysis and the need for additional studies to determine the specific mechanism for $\delta^{15}N$ alteration.

CORRECTION APPROACHES FOR AQUATIC INVERTEBRATES

Our invertebrate data were derived from homogenized whole organisms, and relationships between C : N and change in $\delta^{13}C$ with lipid removal were less pronounced than in fish tissues. While many aquatic invertebrate species store considerable amounts of lipid (Lee, Polhemus & Cheng 1975; Meier *et al*. 2000; Iverson, Frost & Lang 2002; Fisk *et al*. 2003), greater heterogeneity in whole organism invertebrate samples that contain high proportions of chitin, in addition to lipids and protein, may weaken the observed relationship between bulk tissue C : N and $\delta^{13}C$ (Kiljunen *et al*. 2006). In modelling approaches, analysis of soft tissue components may produce a stronger relationship than whole organism analyses. For

whole organism samples, poor model fits that do not demonstrate a clear relationship between C : N and changes in $\delta^{13}C$ (Fig. 4) indicate that lipid removal using chloroform–methanol and acid treatment provide better $\delta^{13}C$ estimates of lipid-free tissue.

CORRECTION APPROACHES FOR FISHES

By using a correction approach rather than extracting lipids prior to analysis, the movement of lipids through food webs can be preserved in ecological studies (Arts *et al*. 2001). Multiple models fit to our data set of fish tissues tracked changes in $\delta^{13}C$ between bulk tissue and lipid extracted samples with increasing bulk tissue C : N. The asymptotic relationship between C : N_{bulk} and $\delta^{13}C$ indicates that a linear fit correction (e.g. Post *et al*. 2007) may not be appropriate over a wide range of C : N_{bulk}.

When dealing with new taxa for which necessary C : N_{bulk}, $\delta^{13}C_{bulk}$ and $\delta^{13}C_{lipid\text{-}free}$ data do not exist, extracting a subset of samples of the species and tissues of interest to develop the necessary correction equations is recommended. These new correction equations can then be applied to the remaining data to account for a large lipid load and biased $\delta^{13}C$. This will reduce sample preparations, eliminate the need to analyse all samples before and after extraction, and decrease $\delta^{13}C$ variability among individuals yielding improved estimates of dietary source proportions in mixing models (Phillips & Gregg 2001). Most analytical laboratories provide percentage C and percentage N data with isotope data, making it easy to evaluate whether lipids bias $\delta^{13}C$ measurements. Values for C : N ratios can be reported based on uncorrected percentage element data or with correction for differences in atomic weight between elements (i.e. $\%C/\%N \times 1{\cdot}6667$). If either method is used consistently, results will not be affected, but applying both methods in a single analysis would reduce accuracy of $\delta^{13}C$ estimates (Sweeting *et al*. 2006). All C : N values used in conjunction with our reported parameter estimates should use uncorrected elemental C : N.

The significant χ^2 differences for all nested models demonstrate that species- and tissue-specific parameters provide the best $\delta^{13}C_{lipid\text{-}free}$ estimates. Model parameters (slope and intercept) relate to protein–lipid $\delta^{13}C$ discrimination and baseline C : N of lipid-free tissue. As model fits improved with specificity, the data used to generate parameters is more important than the specific model selected. Developing equations specific to the tissue and taxa of interest should produce more accurate estimates of diet and habitat.

No single model performed best across nested models but instead model performance was related to parameter specificity. Our modification of the McConnaughey & McRoy (1979) model, eqn 1a, had the lowest AIC_c among general and tissue-specific models, but the highest AIC_c among species- and tissue-specific models. The opposite pattern was observed for our eqn 3. When tissue-specific parameters (e.g. fish liver) are used instead of species- and tissue-specific parameters, our modification of the McConnaughey & McRoy (1979) model (eqn 1a) is most appropriate. The Fry (2002) equation using parameters fit to our data set had essentially indistinguishable AIC_c values ($\Delta_i \sim 2$) from eqn 3 for the species–tissue-specific fit. As eqn 3 failed to accurately reflect protein–lipid discrimination, our Fry (2002) equation fit (eqn 2) is more appropriate for use with species- and tissue-specific parameters (e.g. brook trout muscle).

For fish tissues, data sets could be created through pairwise isotope analysis of a study's bulk tissue and lipid extracted tissue for each species and tissue type presented. Models (based on tissue C : N) could then be fit to each data subset, and fit parameters could be used to predict lipid-free $\delta^{13}C$ for remaining samples. Alternatively, literature parameter estimates from this study or previous published studies (Kiljunen *et al*. 2006; Post *et al*. 2007) could be applied to data sets with similar species and bulk tissue C : N ranges. This approach would be similar to current techniques that estimate trophic position of consumers by using diet–tissue discrimination factors ($\delta^{15}N$ consumer – $\delta^{15}N$ diet) from the literature (Post 2002; McCutchan *et al*. 2003; Vanderklift & Ponsard 2003), or ideally, with species- or taxon-specific estimates in controlled laboratory rearing experiments (Hobson, Gilchrist & Falk 2002). Continued application of this approach in published literature will help to build a library of parameter values across a broader range of taxa and tissue types for use in SIA.

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Supplementary material

The following material is available for this article.

Appendix S1. Parameter estimates for models fit to a dataset of freshwater and marine fish liver, muscle, and whole body and invertebrate whole body samples.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2656.2008.1394.x
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