How to account for the lipid effect on carbon stable-isotope ratio ($\delta^{13}C$): sample treatment effects and model bias

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This study investigated the impact of lipid extraction, $CaCO_3$ removal and of both treatments combined on fish tissue $\delta^{13}C$, $\delta^{15}N$ and C:N ratio. Furthermore, the suitability of empirical $\delta^{13}C$ lipid normalization and correction models was examined. $\delta^{15}N$ was affected by lipid extraction (increase of up to 1·65‰) and by the combination of both treatments, while acidification alone showed no effect. The observed shift in $\delta^{15}N$ represents a significant bias in trophic level estimates, *i.e.* lipid-extracted samples are not suitable for $\delta^{15}N$ analysis. C:N and $\delta^{13}C$ were significantly affected by lipid extraction, proportional to initial tissue lipid content. For both variables, rates of change with lipid content (ΔC:N and $\Delta\delta^{13}C$) were species specific. All tested lipid normalization and correction models produced biased estimates of fish tissue $\delta^{13}C$, probably due to a non-representative database and incorrect assumptions and generalizations the models were based on. Improved models need *a priori* more extensive and detailed studies of the relationships between lipid content, C:N and $\delta^{13}C$, as well as of the underlying biochemical processes.
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Key words: carbon; C:N ratio; lipid extraction; mathematical lipid normalization; nitrogen; stable isotopes.

INTRODUCTION

Analysis of the stable carbon (^{13}C:^{12}C) and nitrogen (^{15}N:^{14}N) isotope composition of body tissue is a basic tool in studies on trophic structure and interactions involving fishes in aquatic ecosystems (Gu *et al.*, 1996; Jennings *et al.*, 2002; Campbell *et al.*, 2003). The method is based on isotopic fractionation caused by enzymatic discrimination, which leads to an enrichment of the heavier isotope in animal tissues up the food chain. The ratio of ^{15}N:^{14}N ($\delta^{15}N$) usually

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serves as an indicator of an individual's trophic position within a particular food web, with an increase in $\delta^{15}N$ of $c.$ 3·3‰ with each assimilation step (Minagawa & Wada, 1984; Post, 2002; McCutchan *et al.*, 2003). $^{13}C:^{12}C$ ($\delta^{13}C$) increases by <1‰ per trophic transfer (Rau *et al.*, 1983; Fry & Sherr, 1984; McCutchan *et al.*, 2003), making $\delta^{13}C$ a useful tracer of primary carbon sources (France, 1995; Hobson *et al.*, 2003). $\delta^{13}C$, however, is negatively correlated to tissue lipid content, as lipids are depleted in ^{13}C compared to tissue protein and carbohydrate fractions (Parker, 1964; DeNiro & Epstein, 1978). This ^{13}C depletion in lipids is attributed to kinetic-isotope effects occurring during lipid biosynthesis, mainly during acetyl-CoA formation by pyruvate dehydrogenase (DeNiro & Epstein, 1977; Monson & Hayes, 1982). As lipid content in fishes varies depending on species, tissue, season or lifestyle, lipids are often extracted by polar organic solvents following Folch *et al.* (1957) or Bligh & Dyer (1959) prior to stable-isotope analysis in order to avoid any bias in $\delta^{13}C$ measurement induced by differing tissue fat contents (Gu *et al.*, 1997; Carseldine & Tibbetts, 2005; Watanabe *et al.*, 2006).

Total lipid extraction can affect $\delta^{15}N$ too, but the amount of changes reported varies (Pinnegar & Polunin, 1999; Sotiropoulos *et al.*, 2004; Sweeting *et al.*, 2006). Furthermore, it is yet unknown whether lipid extraction and other tissue treatments may interact in their effect on stable-isotope ratios. Of particular concern is sample acidification, which is commonly applied to remove inorganic carbonate (Wada *et al.*, 1987; Kaehler *et al.*, 2000) and itself is known to affect $\delta^{13}C$ as well as $\delta^{15}N$ (Bunn *et al.*, 1995; Jacob *et al.*, 2005). Though the exact mechanisms involved in the effect of lipid extraction and acidification on tissue $\delta^{15}N$ are still not clarified, both treatments are suspected of leaching organic nitrogen compounds, such as proteins or amino acids (Goering *et al.*, 1990, Sotiropoulos *et al.*, 2004; Sweeting *et al.*, 2006). The treatments may thus potentiate one another in their effect on $\delta^{15}N$ when applied in combination.

Mathematical lipid correction of $\delta^{13}C$ might provide an easy and cost-saving alternative to chemical lipid extraction. Empirically derived lipid normalization models attempt to correct $\delta^{13}C$ measurements of lipid-containing samples according to C:N ratio by making use of the empirical relationships between (1) tissue lipid content and C:N ratio and (2) C:N ratio and lipid-induced differences in $\delta^{13}C$. The most frequently used (Del Giorgio & France, 1996; Kline *et al.*, 1998) lipid-normalization model was developed by McConnaughey & McRoy (1979). More recently, Kiljunen *et al.* (2006) and Post *et al.* (2007) independently presented two new models for fishes (Kiljunen *et al.*, 2006) and for aquatic animals (Post *et al.*, 2007), respectively. The general suitability of most lipid-normalization models, however, remains questionable (Kiljunen *et al.*, 2006; Bodin *et al.*, 2007). Alternatively, $\delta^{13}C$ correction by mass balance has been proposed as an appropriate approach (Fry *et al.*, 2003; Sweeting *et al.*, 2006).

Based on tissue samples of two Antarctic fish species, the aims of the present study were to investigate (1) the effect of lipid extraction, sample acidification and a combination of both treatments on muscle tissue $\delta^{15}N$, C:N ratio and $\delta^{13}C$, (2) the effect of initial lipid content and C:N ratio on the magnitude of treatment-related changes and (3) whether the $\delta^{13}C$ lipid normalization and correction models of McConnaughey & McRoy (1979), Kiljunen *et al.*

(2006), Post *et al.* (2007) and Sweeting *et al.* (2006) are suitable for the two fish species investigated here.

MATERIALS AND METHODS

ANIMALS

This study is based on two notothenioid species that were sampled in the north-eastern Weddell Sea (Antarctica) during December and January 2003–2004. In notothenioids, buoyancy is adjusted by lipid storages owing to the lack of a swimbladder (Eastman & DeVries, 1982). Tissue lipid content is, therefore, closely associated with the species' lifestyle.

Trematomus pennellii Regan is strictly demersal, living directly on the sea floor. Muscle lipid content in *T. pennellii* is uniform and comparatively low (Eastman & DeVries, 1982; Phleger *et al.*, 1999). The pelagic *Pleuragramma antarcticum* Boulenger has subcutaneous and intramuscular lipid sacs that maintain neutral buoyancy (DeVries & Eastman, 1978). On average, muscular lipid deposits of this species account for *c.* 46% of dry mass (M_D) (Reinhardt & Van Vleet, 1985, 1986; Friedrich & Hagen, 1994), but the subcutaneous lipid sacs are not evenly distributed throughout the muscle (DeVries & Eastman, 1978). Lipid storages in notothenioids mainly consist of triacylglycerols, and to a minor extent of wax esters (Phleger *et al.*, 1999; Hagen *et al.*, 2000).

A piece of lateral white muscle tissue with overlying skin was sampled from 10 adult individuals each of both species. Samples were stored deep-frozen at $-30°$ C until further preparation.

SAMPLE TREATMENT AND STABLE-ISOTOPE ANALYSIS

Frozen tissues were lyophilized for 24 h and subsequently ground to fine powder. Each sample was divided into four sub-samples (coded NN, LN, NA and LA). In two sub-samples (LN and LA) the lipid compounds were removed following Bligh & Dyer (1959), DeNiro & Epstein (1978) and Pinnegar & Polunin (1999): A volume of 2 ml methanol:chloroform:water solution (10:5:4) was added per 100 mg samples. The mixture was agitated, exposed to ultrasound for 30 s and agitated again. Then the mixture was spun down in a centrifuge with 1800 g and the supernatant was discarded. The whole procedure was repeated three more times, but using pure methanol instead of methanol:chloroform:water in the final run. The residual pellet was dried at $60°$ C and subsequently ground. Tissue lipid content was determined gravimetrically by weighting the sample before and after lipid extraction (precision 0·0001 g) and expressed in % M_D. One sample of *P. antarcticum* tissue was discarded due to a failure during lipid extraction (*i.e.* final $n = 9$).

One of the lipid-containing and one of the lipid-extracted sub-samples (NA and LA) were additionally acidified by adding a few drops of 1 N hydrochloric acid (HCl) as recommended by Jacob *et al.* (2005). Finally, all samples were re-dried in an oven at $60°$ C and ground to fine powder prior to analysis.

Stable-isotope analysis and concentration measurements of nitrogen and carbon (mg per sample) were performed simultaneously with a THERMO/Finnigan MAT Delta plus isotope-ratio mass spectrometer (Thermo Finnigan MAT GmbH, Bremen, Germany), coupled to a THERMO NA 2500 elemental analyser (CE Instruments, Milan, Italy). Stable-isotope ratios are given in the conventional delta notation ($\delta^{13}C, \delta^{15}N$) relative to atmospheric nitrogen (Mariotti, 1983) and PDB (PeeDee Belemnite standard) in ‰. The S.D for repeated measurements of laboratory standard material (peptone) was better than 0·15‰ for nitrogen and carbon, respectively. The C:N ratio was calculated by mass. The S.D. of concentration measurements of replicates of laboratory standard were <3% of the concentration analysed.

DATA ANALYSIS

The general relationship between lipid content [% M_D], C:N and the effect of species and interaction between species and covariate on this relationship in untreated sample tissues was investigated by full factorial ANCOVA.

Full factorial ANOVA followed by Tukey's HSD *post hoc* comparison on differences between means were applied to test for the effect of treatment and species on $\delta^{15}N$, C:N ratio and $\delta^{13}C$. The effect of treatment on variation among samples was investigated by CV [(%) CV = $100y\bar{x}^{-1}$, where y = S.D.]. In order to determine whether complete lipid extraction from sample tissue was achieved by the method of Bligh & Dyer (1959), initial lipid content (% M_D) and sample C:N after lipid extraction ($CN^{-1}_{LN,LA}$) was tested again for significant correlation.

The relationships between sample initial lipid content [% M_D], changes (Δ) in $\delta^{15}N$, C:N ratio and $\delta^{13}C$ induced by lipid extraction ($\Delta\delta^{15}N$, ΔC:N and $\Delta\delta^{13}C$) and the effect of acidification and species, and interaction between species and covariate on these relationships were investigated by full factorial ANCOVA. The relationship between initial C:N ratio (C:N_{NN}) and $\Delta\delta^{13}C$ was investigated by means of ANCOVA, as well.

MATHEMATICAL LIPID NORMALIZATION AND CORRECTION OF $\delta^{13}C$

The normalization model of McConnaughey & McRoy (1979) is based on empirical relationships described by two equations. The proportional lipid content L of a sample is calculated from sample C:N ratio:

$$L = 93\{1 + [0.246(R_{CN}) - 0.775^{-1}]\}^{-1} \tag{1}$$

Lipid normalized $\delta^{13}C'$ is calculated from L and measured $\delta^{13}C$ of the sample:

$$\delta^{13}C' = \delta^{13}C + D(I + 3.90[1 + 287L^{-1}]^{-1}) \tag{2}$$

where D is the isotopic difference between lipids and protein (D is assumed to be 6‰; McConnaughey & McRoy, 1979), and I is a constant ($I = -0.207$).

Kiljunen *et al.* (2006) proposed a modification of the McConnaughey & McRoy (1979) model for normalizing $\delta^{13}C$ of fish muscle tissue, with re-estimated variables $D = 7.018$‰ and $I = 0.048$ (see equation 2). Both, the model of McConnaughey & McRoy (1979) and the modified model of Kiljunen *et al.* (2006) assume non-linear relationships between tissue lipid content and C:N ratio, and between lipid-induced differences in $\delta^{13}C$ ($\Delta\delta^{13}C$) and C:N.

Post *et al.* (2007), in contrast, found the relation between $\Delta\delta^{13}C$ and C:N ratio to be linear and developed a simplified normalization model for aquatic organisms:

$$\delta^{13}C' = \delta^{13}C - 3.32 + 0.99R_{CN} \tag{3}$$

Another method to correct $\delta^{13}C$ for different lipid contents is the mass balance approach. The mass balance correction of $\delta^{13}C$ was introduced by Fry *et al.* (2003) and recently adjusted by Sweeting *et al.* (2006) to sea bass *Dicentrarchus labrax* (L.) muscle tissue. This approach assumes that the analysed sample tissues are exclusively composed of proteins and lipids, and requires the knowledge of C:N of pure protein (Sweeting *et al.*, 2006):

$$\delta^{13}C'_{protein} = \{(\delta^{13}CR_{CN}) + [7(R_{CN} - R_{CN\,protein})]\}R_{CN} \tag{4}$$

Following Sweeting *et al.* (2006) a lipid ^{13}C depletion (D in equation 2) of 7‰ was assumed. C:$N_{protein}$ is supposed to be equal to C:N of lipid-free sample tissue

(C:N$_{LN}$). To account for species-specific differences in C:N$_{protein}$, mean C:N$_{LN}$ of the respective species was used in equation (4).

The suitability of these four models was tested by comparing effects of lipid extraction (δ^{13}C$_{LN}$) and model correction (δ^{13}C') by pair-wise ANCOVA of δ^{13}C v. source (normalization and correction or measurement) and covariate log$_{10}$(C:N$_{NN}$) (untreated sample).

RESULTS

Total lipid content in muscle samples of *T. pennellii* was on average 28·06% M_D (Table I). Mean lipid content in *P. antarcticum* was higher (32·39% M_D) and more variable, ranging from 25 to 46% M_D. Both C:N ratio and δ^{13}C in untreated tissue samples showed a similar pattern. C:N ranged from 3·37 to 3·76 in *T. pennellii* and from 3·63 to 7·14 in *P. antarcticum*. δ^{13}C ranged from −25·84 to −24·50‰ in *T. pennellii* and from −28·97 to −25·47‰ in *P. antarcticum*. δ^{15}N values were similar in both species and varied from 8·64 to 9·90‰ (Table I).

RELATIONSHIP BETWEEN LIPID CONTENT AND C:N RATIO

Tissue lipid content and C:N ratio were positively correlated. ANCOVA identified significant ($P < 0.05$) effects of species and of lipid content on C:N ratio in untreated tissue samples, with both factors interacting significantly such that the slope of C:N with lipid content differed among species (Fig. 1).

IMPACT OF SAMPLE TREATMENT

δ^{15}N, C:N ratio and δ^{13}C were significantly affected by both sample treatment and species, but only in C:N treatment and species interacted significantly (Table II). In all three variables, NN and NA samples were not significantly different, *i.e.* there was no isolated effect of acidification detectable.

TABLE I. Tissue lipid content and composition of untreated samples (NN) of *Trematomus pennellii* ($n = 10$) and *Pleuragramma antarcticum* ($n = 9$). Means, minimum and maximum values of lipid content, C:N ratio, and of δ^{13}C and δ^{15}N

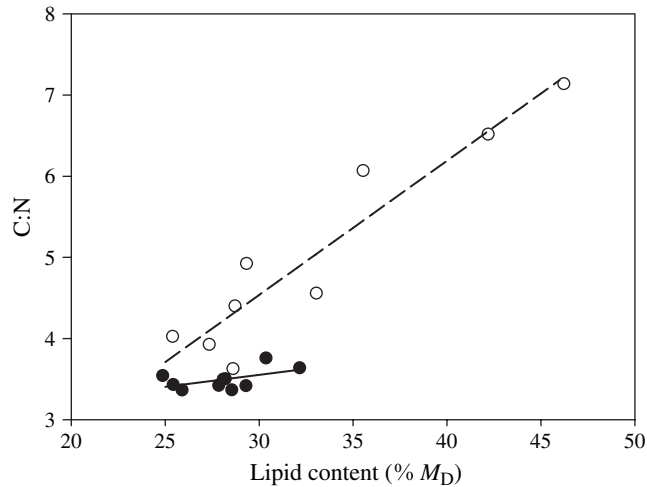| Species	Lipid (% M_D)	C:N	δ^{13}C (‰)	δ^{15}N (‰)
T. pennellii				
Mean	28·06	3·50	−25·26	9·05
Minimum	24·86	3·37	−25·84	8·79
Maximum	32·15	3·76	−24·50	9·54
P. antarcticum				
Mean	32·93	5·02	−27·23	9·47
Minimum	25·39	3·63	−28·97	8·64
Maximum	46·22	7·14	−25·47	9·90

M_D, dry mass.

FIG. 1. Relationship between lipid content (% dry mass, M_D) and C:N ratio in untreated tissue samples of *Trematomus pennellii* (●, ——) and *Pleuragramma antarcticum* (○, - - -). $y = 1.119 + 0.097x + 0.517z + (x - 30.366) 0.068z$, where z (species) = 1 for *P. antarcticum* and −1 for *T. pennellii*) ($n = 19$, $r^2 = 0.94$, $P < 0.001$).

Following lipid extraction (LN and LA treatments) sample $\delta^{15}N$ increased, but the effect of the two treatments, LN and LA, differed significantly ($P < 0.05$). After LN treatment, $\delta^{15}N$ increased by on average 1·37‰ (*T. pennellii*) and 1·65‰ (*P. antarcticum*), but by on average 0·91‰ (*T. pennellii*) and 1·23‰ (*P. antarcticum*) after LA treatment. C:N ratio and $\delta^{13}C$ did not differ between LN and LA treatment. C:N decreased after lipid extraction by *c.* 0·19 in *T. pennellii* and by *c.* 1·07 in *P. antarcticum*. $\delta^{13}C$ increased by *c.* 1·22‰ in *T. pennellii* and by *c.* 1·85‰ in *P. antarcticum*.

TABLE II. ANOVA of sample treatment effects (NN, no treatment; NA, acidification; LN, lipid removal; LA, lipid removal + acidification) on C:N ratio, $\delta^{13}C$ and $\delta^{15}N$ in *Trematomus pennellii* ($n = 10$) and *Pleuragramma antarcticum* ($n = 9$). Full factorial ANOVA identified significant ($P < 0.05$) effects of treatment and species in all variables, and of treatment × species interaction in C:N. Mean ± S.D. are given for each variable. CV values (%) are given in parentheses. Treatment levels with different upper case letter are significantly ($P < 0.05$) different

Treatment	Species	C:N		$\delta^{13}C$		$\delta^{15}N$	
				Variables			
NN	*T. pennellii*	3·50 ± 0·13 (3·59)	AB	−25·26 ± 0·39 (1·55)	A	9·05 ± 0·25 (2·72)	A
	P. antarcticum	5·02 ± 1·25 (24·96)		−27·23 ± 1·24 (4·56)		9·47 ± 0·48 (5·03)	
LN	*T. pennellii*	3·28 ± 0·05 (1·51)	C	−23·94 ± 0·40 (1·65)	B	10·42 ± 0·39 (3·78)	B
	P. antarcticum	4·00 ± 0·29 (7·34)		−25·13 ± 1·05 (4·18)		11·12 ± 0·65 (5·82)	
NA	*T. pennellii*	3·63 ± 0·10 (2·62)	A	−25·02 ± 0·40 (1·60)	A	8·81 ± 0·31 (3·48)	A
	P. antarcticum	5·29 ± 1·21 (22·93)		−26·65 ± 1·29 (4·85)		9·80 ± 0·58 (5·90)	
LA	*T. pennellii*	3·47 ± 0·08 (2·32)	BC	−23·90 ± 0·39 (1·61)	B	9·96 ± 0·31 (3·12)	C
	P. antarcticum	4·17 ± 0·27 (6·53)		−25·05 ± 0·75 (2·98)		10·70 ± 0·65 (6·04)	

Variation in δ^{15}N slightly increased following all treatments from 2·72% (NN) to up to 3·78% (LN) in *T. pennellii*, and from 5·03% (NN) to up to 6·04% in *P. antarcticum* (Table II). In C:N sample treatment decreased variation in both species. In particular lipid extraction (LN and LA) caused changes in CV, with the largest shift from 24·96% (NN) to 7·34% (LN) in *P. antarcticum*. Changes in CV following acidification (NA) were minor. Variation in δ^{13}C was not affected by any treatment in *T. pennellii* (CV range: 1·55–1·65%). In *P. antarcticum*, a decrease in variation was caused by the combined treatment (LA), while CV was unaffected by lipid extraction and acidification alone (LN and NA).

The positive relationship between initial lipid content (x) and C:N ratio (C:N$_{LN, LA}$; y) was still present after lipid extraction in tissue samples of *P. antarcticum*, albeit with a distinctly lower slope than in lipid-containing NN samples (ANCOVA, interaction term $P < 0.001$; $y = 3.111 + 0.029x$, $n = 18$, $r^2 = 0.507$, $P < 0.001$; see Fig. 1 for comparison).

EFFECTS OF INITIAL LIPID CONTENT AND C:N ON CHANGES Δ

Due to the effect of combined lipid extraction and acidification on δ^{15}N, the relationship between initial lipid content and $\Delta\delta^{15}$N in *T. pennellii* and *P. antarcticum* was *a priori* investigated separately for non-acidified and acidified samples. In acidified samples $\Delta\delta^{15}$N was independent of lipid content and species. In non-acidified samples $\Delta\delta^{15}$N was significantly affected by initial lipid content (ANCOVA, $P < 0.05$), but the relation was independent of species. This relationship, however, disappears ($P > 0.05$) if the sample with highest lipid content is excluded [see Fig. 2(a)].

ΔC:N was significantly affected by species and by lipid content (ANCOVA, $P < 0.001$), with both factors interacting significantly ($P < 0.001$), *i.e.* the relationship between ΔC:N and lipid content differed in slope among species [Fig. 2(b)].

$\Delta\delta^{13}$C and tissue lipid content were significantly related, too ($P < 0.001$). The relationship was species dependent ($P < 0.05$) but variables did not interact, thus, the species effect consisted of differences in intercepts only [Fig. 2(c)]. $\Delta\delta^{13}$C was directly proportional to C:N$_{NN}$ of the tissue sample ($P < 0.001$) but this relationship was independent of species [Fig. 2(d)]. The relationships between $\Delta\delta^{13}$C, lipid content [Fig. 2(c)] and C:N$_{NN}$ [Fig. 2(d)] were additionally affected by acidification with HCl, resulting in a shift of intercepts but not in different slopes (no significant interaction).

MATHEMATICAL LIPID NORMALIZATION AND CORRECTION OF δ^{13}C

None of the tested δ^{13}C lipid normalization and correction models was able to predict the δ^{13}C$_{LN}$ measured in lipid-extracted tissue samples appropriately. All four models show the same bias: they do not catch the full leverage effect of lipid content on δ^{13}C (ANCOVA, interaction term $P < 0.05$; Fig. 3).
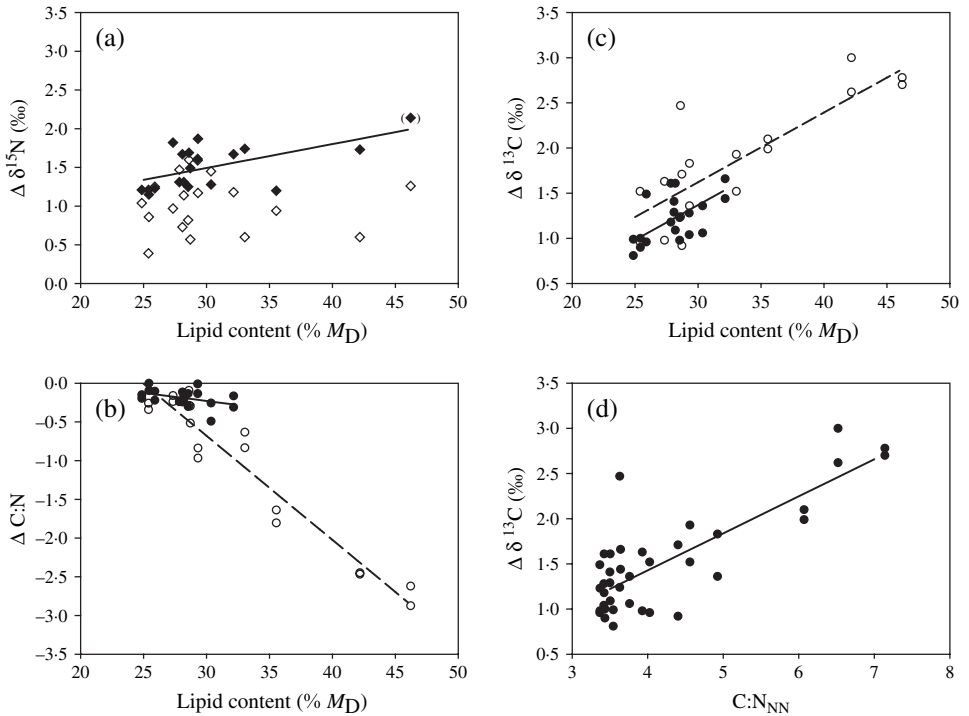
FIG. 2. Effects of lipid extraction on $\delta^{15}N$, C:N ratio and $\delta^{13}C$. Plot shows the difference Δ in variable (treated minus untreated sample) v. initial lipid content (% dry mass, M_D) and C:N ratio (C:N_{NN}). Only significant terms of the full factorial model are shown. Significant effects of HCl treatment are included in regression equations but not shown in the plot. (a) $\delta^{15}N$ non-acidified samples ($\blacklozenge$, ——) and lipid content: $y = 0.568 + 0.031x$ [$r^2 = 0.36$, $n = 19$, $P < 0.01$, but no significant relationship if the sample with highest lipid content is excluded (value in parentheses)]. In acidified samples ($\Diamond$) no significant relationship between lipid content and $\Delta\delta^{15}N$ was detected. (b) C:N and lipid content in *Trematomus pennellii* ($\bullet$, ——) and *Pleuragramma antarcticum* ($\bigcirc$, - - -): $y = 1.879 - 0.078x - 0.246z + (x - 30.366)(-0.057x)$ ($r^2 = 0.94$, $n = 38$, $P < 0.001$). (c) $\delta^{13}C$ and lipid content in *T. pennellii* ($\bullet$, ——) and *P. antarcticum* ($\bigcirc$, - - -): $y = -0.819 + 0.077x + 0.127z + 0.172w$ ($r^2 = 0.83$, $n = 38$, $P < 0.001$). (d) $\delta^{13}C$ and C:N ratio: $y = -0.212 + 0.410x + 0.172w$ ($r^2 = 0.74$, $n = 38$, $P < 0.001$). Where z (species) = 1 for *P. antarcticum* and -1 for *T. pennellii* and w (HCl) = 1 for non-acidified samples and -1 for acidified samples.

$\delta^{13}C'$ estimated from sample C:N by the lipid normalization model of McConnaughey & McRoy (1979) was consistently lower than $\delta^{13}C_{LN}$ and this gap increased with increasing $\delta^{13}C$ values up to a maximum difference of 2.9‰. Normalization following the models of Kiljunen *et al.* (2006) and Post *et al.* (2007) resulted in deviations of up to 1.5 and 2.2‰, respectively. For both models, the ratio of $\delta^{13}C':\delta^{13}C_{LN}$ decreased with $\delta^{13}C_{LN}$, from ratios <1 (overestimation) to values >1 (underestimation) (Fig. 3). Except for two values, the mass balance approach of Sweeting *et al.* (2006) consistently resulted in an underestimation of $\delta^{13}C$, although species-specific means of C:$N_{protein}$ were used (4.0 for *P. antarcticum*, 3.28 for *T. pennellii*, see Table II). Maximum deviation of $\delta^{13}C'$ from $\delta^{13}C_{LN}$ accounted for 3.2‰ (Fig. 3). Replacement of mean C:$N_{protein}$ in equation (4) by minimum C:$N_{protein}$ (lowest measured C:N_{LN}: 3.54
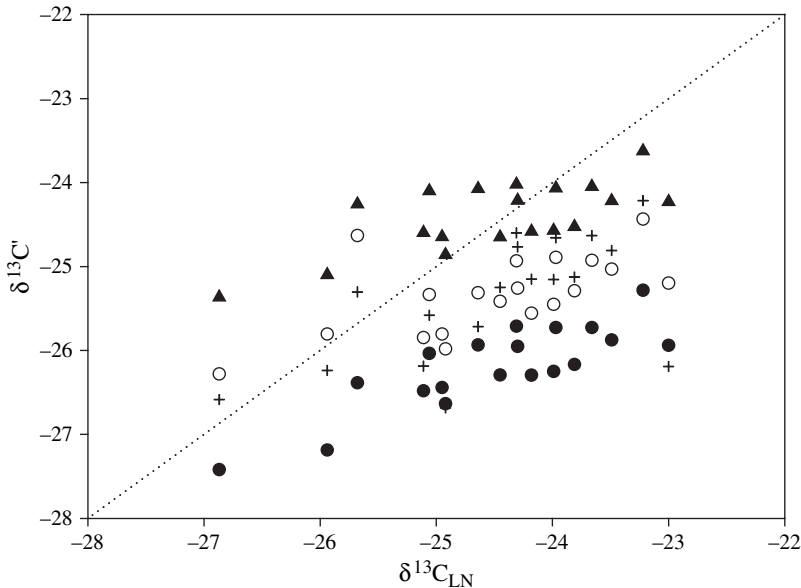
FIG. 3. Relationship between $\delta^{13}C_{LN}$ measured after lipid extraction and lipid normalized and corrected $\delta^{13}C'$ according to models by McConnaughey & McRoy (1979; ●), Kiljunen et al. (2006; ▲), Post et al. (2007; ○) and Sweeting et al. (2006; +). Dotted line indicates $y = x$.

for *P. antarcticum*, 3·19 for *T. pennellii*) lowered maximum deviation to 2·30‰, but the results still differed in slope from the measured relationship (ANCOVA, interaction term $P < 0.05$; data not shown in Fig. 3).

DISCUSSION

The results of this study clearly indicate that sample treatment, in particular chemical lipid extraction, not only affects C:N and $\delta^{13}C$ but also alters $\delta^{15}N$ in fish muscle tissue.

SAMPLE TREATMENT EFFECTS ON $\delta^{15}N$

Tissue $\delta^{15}N$ significantly increased following lipid extraction (LN, +1·37 and +1·65‰), and also, but to a lower extent, when lipid extraction and acidification were combined. Treatment induced increases in $\delta^{15}N$ variation were negligible (increase of 0·40–1·06%) compared to changes reported in other studies (Bunn *et al.*, 1995). Previous studies confirm the extraction-induced effect on $\delta^{15}N$ and reported increases of up to 1·2‰ in crustacean hepatopancreas (Bodin *et al.*, 2007) and up to 2·8‰ in whole juvenile fishes (Sotiropoulos *et al.*, 2004). In fish muscle, the tissue most commonly used for stable-isotope analysis, however, previous studies found maximum $\delta^{15}N$ increases of $<0.8‰$ (Pinnegar & Polunin, 1999; Sotiropoulos *et al.*, 2004; Sweeting *et al.*, 2006), *i.e.* distinctly lower rates of change than observed in this study. The chemistry behind the loss of ^{14}N from the tissue during lipid extraction is poorly

understood, but a leaching of lipid-associated proteins, enriched in ^{14}N, was recently proposed to be the major cause (Sotiropoulos *et al.*, 2004; Sweeting *et al.*, 2006). The positive relationship between initial lipid content and $\Delta\delta^{15}N$ caused by lipid extraction [see Fig. 2(a)] supports this hypothesis, albeit validity of this relationship needs to be verified. The effect of lipid extraction on $\delta^{15}N$, however, is distinctly stronger in the two Antarctic fish species studied here than in other fish species. Compared to other fishes, Antarctic notothenioids contain similar levels of ammonia but distinctly higher levels of trimethylamide-nitrogen (Oehlenschläger, 1991). As trimethylamines are highly soluble in aliphatic alcohols such as the methanol used for lipid extraction, an additional lipid-independent leakage of ^{14}N from trimethylamide compounds is likely.

Studies on the impact of acidification on $\delta^{15}N$ (Bunn *et al.*, 1995; Bosley & Wainright, 1999; Pinnegar & Polunin, 1999) are inconclusive so far, regarding both the existence and the direction of such effect. In more recent studies, acidification has been found to decrease $\delta^{15}N$ (Jacob *et al.*, 2005; Kennedy *et al.*, 2005), indicating the loss of isotopically heavier (*i.e.* enriched in ^{15}N compared to the bulk material) nitrogen compounds. In the present study, $\delta^{15}N$ was significantly affected by acidification only in combination with lipid extraction (Table II). The two treatments, however, do interact but do not potentiate one another in their effect on $\delta^{15}N$, as assumed: in the combined treatment (LA) sample $\delta^{15}N$ values were significantly lower than after lipid extraction alone (LN).

The mechanisms underlying this phenomenon are unknown, but lipid extraction, in particular ultrasound exposure, most likely causes disintegration of cellular structures in the sample tissue. Goering *et al.* (1990) suggested that changes in total $\delta^{15}N$ following acidification may be caused by differential leaching of organic nitrogen compounds (*e.g.* nucleic acids or amino acids) with different $\delta^{15}N$ values. Amino acids, for example, are known to vary widely in $\delta^{15}N$ signatures, with ^{15}N obviously accumulates primarily in non-essential amino acids (Gaebler *et al.*, 1966; Ostle *et al.*, 1999; McClelland & Montoya, 2002). Amino acids are, moreover, differentially sensitive to acidification, and particular amino acids as for example the non-essential cysteine, might react with HCl already at comparatively low molar concentrations in the presence of oxygen (Lottspeich & Zorbas, 1998). Analysis of amino acid composition in nototheniid muscle tissue detected high concentrations of taurine, the oxidative degradation product of cysteine (Partmann, 1981). Structural disintegration by lipid extraction is thus supposed to facilitate and enhance HCl induced leaching of particular, isotopically heavy, nitrogen compounds such as non-essential amino acids, which finally results in the effect observed in the combined treatment. Whether acidification affects the relationship between initial lipid content and $\Delta\delta^{15}N$ caused by lipid extraction remains unclear [see Fig. 2(a) and Sweeting *et al.*, 2006].

In stable-isotope based food-web studies, which usually assume a per trophic step increase in $\delta^{15}N$ of *c.* 3·3‰ (Minagawa & Wada, 1984; Post, 2002), a bias of the magnitude found here affects trophic level estimates. Shifts in $\delta^{15}N$ following lipid extraction, moreover, might vary depending on biochemical tissue composition (concerning nitrogen-containing compounds). A clear picture of cause and effect in the relation between changes in $\delta^{15}N$, tissue lipid content

and sample acidification, however, has not yet emerged from the evidence produced so far. Further studies across a wider range of taxonomic groups and tissue types, standardized treatment procedures, as well as a deeper understanding of lipid extraction and of acidification effects on δ^{15}N at the molecular level are required to push this topic forward. The safest method to avoid biased δ^{15}N estimates in multi-isotope studies is to determine δ^{15}N and δ^{13}C in separate, *i.e.* lipid-containing and lipid-extracted, samples.

RELATIONSHIP BETWEEN TISSUE LIPID CONTENT AND C:N RATIO

The results of this study confirm the basic, positive relationship between tissue lipid content and C:N ratio described previously. The relationship observed in the two Antarctic fish species was distinctly linear, at least in the observed range of lipid contents between 24·86 and 46·22% M_D, and C:N ratios between 3·37 and 7·14, respectively. This result is in accordance with recently published observations (Bodin *et al.*, 2007; Post *et al.*, 2007) but contradicts the assumption of non-linearity between lipid content and C:N made by McConnaughey & McRoy (1979). Post *et al.* (2007), moreover, found the relationship between tissue lipid content and C:N to differ in slope between aquatic and terrestrial animals. In the present study, the relationship even turned out to be species specific, probably reflecting lipid-independent, diet-derived differences in C:N ratio between the closely related *P. antarcticum* and *T. pennellii*.

Species-specific differences in the slope of the relationship between lipid content and C:N ratio might obscure linearity when too many data from different taxonomic groups are pooled. More data from different taxonomic groups and with higher taxonomic resolution (*i.e.* species level or genus level), however, are required (1) to identify factors determining this relationship (*e.g.* metabolism, lipid-class composition, lipid-class specific ^{13}C depletion, lifestyle and environment) and (2) to identify species or groups sharing a common relationship between lipid content and C:N.

SAMPLE TREATMENT EFFECTS ON C:N RATIO AND δ^{13}C

Lipid extraction resulted in a significant decrease in C:N and a significant increase in δ^{13}C, while the magnitude of these changes (ΔC:N and $\Delta\delta^{13}$C) was proportional to initial lipid content. Variation in C:N was mainly caused by variable lipid content and was distinctly decreased following lipid removal by up to 17·62% in *P. antarcticum*. Similar effects of lipid extraction have been described for muscle tissue from other fish species (Sotiropoulos *et al.*, 2004; Sweeting *et al.*, 2006). Similar to the relationship between lipid content and C:N in untreated sample tissue, the relationship between lipid content and ΔC:N differed in slope among the two species investigated here. The relationship between initial lipid content and $\Delta\delta^{13}$C was species specific, as well, with a higher rate of change in δ^{13}C at a given lipid content in *P. antarcticum*. Tissue C:N ratio, in contrast, seems to be a species-independent indicator of lipid-induced $\Delta\delta^{13}$C (Post *et al.*, 2007), though more data from other taxonomic groups should be analysed to verify this relationship.

C:N and δ^{13}C were unaffected by acidification alone, indicating that the tissues did not contain inorganic carbonates. Combination of lipid extraction and acidification, however, alters the relationship between $\Delta\delta^{13}$C, lipid content and C:N$_{NN}$. Though the slopes of the relationships were unaffected, HCl treatment resulted in a higher rate of change Δ at a given lipid content and C:N$_{NN}$, respectively.

Lipid extraction after Bligh & Dyer (1959) is described as highly efficient in removing lipids from fish muscle tissue (including complex polar lipids bound to membrane compounds; Bligh & Dyer, 1959; Schlechtriem *et al.*, 2003). Nevertheless, the persistence of the correlation between initial lipid content and C:N in lipid-extracted tissue (C:N$_{LN,LA}$) of *P. antarcticum* indicates that extraction was less than total in the lipid-rich tissue samples. Consequently, $\Delta\delta^{13}$C (and ΔC:N) observed in notothenioid muscle tissue will increase even more if the remaining lipids are removed. Such complete extraction of lipids is essential when δ^{13}C is used as a tracer for primary carbon sources, and hence alternative and more efficient extraction methods (Folch *et al.*, 1957; Iverson *et al.*, 2001) should be tested and applied.

POTENTIAL AND LIMITATIONS OF MATHEMATICAL LIPID NORMALIZATION AND CORRECTION MODELS FOR δ^{13}C

A valid model for the lipid correction of δ^{13}C would be a valuable tool, as it would avoid the effort as well as the side effects of chemical lipid extraction on δ^{15}N. Unfortunately, none of the models tested live up to this task. In particular the model of McConnaughey & McRoy (1979) produced strongly biased estimates of δ^{13}C in lipid-free tissue. On the one hand, unclear database and enigmatic model formulation make it difficult to understand the empirical relations underlying this model. On the other hand, the model works with two questionable preconditions: (1) the existence of a common non-linear relationship between tissue lipid content and C:N ratio. This is obviously not the case, at least not in the range of lipid contents present in sampled fish tissue and (2) a constant 6‰ difference in δ^{13}C between lipid and protein. ^{13}C depletion in lipids compared to other biochemical fractions or whole organisms, however, has been found to be highly variable, and, moreover, species dependent (Park & Epstein, 1961; Parker, 1964; Thompson *et al.*, 2000). It is, therefore, not surprising that the model of McConnaughey & McRoy (1979) underestimated δ^{13}C by up to 2·9‰ (Fig. 3). Even though the difference between δ^{13}C$_{LN}$ and δ^{13}C′ appears to decrease towards lower (more negative) δ^{13}C values, it should be considered that these low δ^{13}C$_{LN}$ values refer to lipid-rich samples in which total extraction obviously failed, *i.e.* actual deviation may be even higher.

The model of Kiljunen *et al.* (2006) performed slightly better, but relies on the same questionable basic assumptions as the model of McConnaughey & McRoy (1979). The model of Post *et al.* (2007), in contrast, is exclusively based on the linear relationship between C:N ratio and $\Delta\delta^{13}$C. Basically, this approach appears to be valid, as it produced a linear and species-independent relationship with the data of the two Antarctic fishes, but overall accuracy is low, too (Fig. 3).

Mass balance correction after Sweeting *et al.* (2006) proved to work for sea bass muscle tissue, but also relies on three un-validated assumptions: (1) sample tissue is composed of lipids and protein only. Though carbohydrate content in fish muscle tissue is low, carbohydrates are not completely absent (Oehlenschläger & Rehbein, 1982, Donnelly *et al.*, 1990) and might contribute to C:N and $\delta^{13}C$ values of bulk tissue, (2) a constant difference in $\delta^{13}C$ between lipid and protein (here 7‰), as the models of McConnaughey & McRoy (1979) and Kiljunen *et al.* (2006) and (3) a constant, though species- or taxon-specific, value for C:N$_{protein}$. In LN samples of *T. pennellii*, where complete lipid extraction was obviously successful, a variance in C:N$_{LN}$ of 1·51% was observed. As the mass-balance model is highly sensitive to variations in C:N$_{protein}$ (Sweeting *et al.*, 2006), even small, natural differences between individuals introduce bias into the $\delta^{13}C'$ estimate.

Apparently, all these models lack generality and robustness. Even the models specific for fishes, Kiljunen *et al.* (2006) and Sweeting *et al.* (2006), performed very poorly here. A functional predictive model obviously requires *a priori* a better understanding of the basic relationships and underlying biochemistry. Without appropriate background knowledge, mathematical lipid correction of $\delta^{13}C$ does not provide a reliable alternative to lipid extraction, unless a models' accuracy has been verified specifically for the organisms of interest.

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