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Sensitivity of stable isotope mixing models to variation in isotopic ratios: evaluating consequences of lipid extraction

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Summary

1. Stable isotopes of carbon and nitrogen are increasingly used in studies of animal diet reconstruction via mixing models. However, isotope ratios of both consumer and source tissues can be altered by various amounts of lipids, potentially leading to biased estimates of diet composition when they are not taken into account.

2. We investigated the consequences of lipid correction on the estimation of diet composition with mixing models. Using empirical data from three northern terrestrial trophic systems, we illustrated the direct effects of lipid extraction (LE) on the $\delta^{13}C$ and $\delta^{15}N$ of source and consumer tissues and its ultimate effects on the reconstruction of the consumer's diet.

3. In parallel, we developed a simulation tool in R, called FATSIM, to assess sensitivity of mixing models to variation in isotopic ratios of samples from source or consumer tissues. This tool can be used to assess the effect of shifts in isotopic ratios caused by LE, or other sources of variation, in any trophic system and thus aid in decision making regarding lipid removal.

4. Using FATSIM, we showed that the potential effects of LE on estimates of diet composition cannot be predicted without simulations, even in relatively simple systems. The sensitivity of a mixing model isotopic shift depends on the complexity of the system (number of sources) and on the relative positions of sources and consumers within the isotopic mixing space.

5. Our study confirms that the presence of lipids in tissues can bias the interpretation of diet reconstruction results. In a given trophic system, testing the sensitivity of a mixing model to LE can help decide whether lipid removal is required in order to avoid this bias.

Key-words: carbon and nitrogen, $\delta^{13}C$ and $\delta^{15}N$, FATSIM, IsoSource, isotopic analysis, lipid correction, normalization, Stable Isotope Analysis in R, trophic relationships

Introduction

During the past decades, the use of stable isotope analysis in ecology has increased exponentially (Kelly 2000; Wolf, Carleton, & Martínez del Rio 2009). In particular, stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) have been used to tackle various questions related to trophic ecology, covering a wide array of scales, from the diet of individual organisms (Angerbjörn *et al.* 1994) or populations (Inger *et al.* 2006) to community-wide trophic interactions (Forero *et al.* 2004). Stable isotope analysis (hereafter SIA) of carbon and nitrogen is based on the principle that variations in isotope ratios in the tissues of consumers reflect variations in the ratios of their food sources in a predictable manner (DeNiro & Epstein 1978, 1981). It has been proposed that isotope ratios can, for instance, be used to estimate trophic level (Post 2002) or trophic niche width (Bearhop *et al.* 2004) of consumers. However, the large potential application of SIA to studies in ecology is restricted by biases and pitfalls that include different techniques of sample preservation or treatment (Kelly, Dempson, & Power 2006; Søreide *et al.* 2006; Mateo *et al.* 2008), gaps in our knowledge of the mechanisms and metabolic pathways underlying tissue synthesis and turnover (Wolf, Carleton, & Martínez del Rio 2009), and a more general lack

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of experimental data such as species- and tissue-specific values of discrimination factors (Gannes, O'Brien, & Martínez del Rio 1997; Martínez del Rio *et al.* 2009). As any tool, SIA require some fine tuning in order to take advantage of its full potential for testing ecological hypotheses.

Recently, several SIA-based studies addressed the issue of the effects of lipids on isotope ratios of tissues (Bodin, Le Loc'h, & Hily 2007; Post *et al.* 2007; Logan *et al.* 2008; Mintenbeck *et al.* 2008) and some suggested that it could also influence interpretation of reconstructed diets, for example, by altering the estimates of diet of consumers feeding on lipid-rich vs. lipid-poor sources (Ricca *et al.* 2007). Due to particular biochemical pathways during their synthesis, lipids are relatively depleted in the heavier stable isotope of carbon (^{13}C) compared with other proteinaceous tissues (DeNiro & Epstein 1977). Consequently, it has also been suggested that lipids should be removed from bulk tissue samples prior to any attempt of diet reconstruction based on SIA (Ricca *et al.* 2007); doing so could prevent estimation bias of the contribution of different sources to the consumer's diet (Logan *et al.* 2008). This suggestion is based on the assumption that lipids and proteins from sources are routed differently (Martínez del Rio *et al.* 2009), for example, that carbon in proteinaceous tissues of consumers is not derived from lipids but solely from dietary proteins. In fact, the extent of such routing seems to depend on several factors, including the specific metabolism of the consumer species (Voigt *et al.* 2008) and also their diet's protein content (Podlesak & McWilliams 2006). It is therefore difficult to anticipate the degree of routing for any given species or individual, but a cautious approach is to consider lipid removal or correction only when lipid routing constitutes a realistic assumption.

In trophic or dietary studies where lipids are removed, extraction is usually applied to both consumers and sources (Hobson & Clark 1992). However, lipid extraction (LE) can also cause an alteration in the nitrogen isotope ratio (Murry *et al.* 2006; Sweeting, Polunin, & Jennings 2006), probably due to leaching of nitrogenous compounds (Mintenbeck *et al.* 2008), although theoretically this should not happen when lipid removal is conducted properly. As a conservative approach, samples can be duplicated to perform SIA both with and without LE, enabling use of carbon isotope ratios from lipid-free tissue samples and nitrogen isotope ratios from bulk tissue samples (Forero *et al.* 2004); such an approach is time consuming and expensive. In addition, there is currently no consensus regarding the need to extract lipids prior to SIA (Post *et al.* 2007; Bennett & Hobson 2009). In fact, the amplitude of the shifts in isotope ratios after LE probably depends on both the species and tissue considered (Bodin, Le Loc'h, & Hily 2007). It was thus concluded that the choice to remove lipids was case specific and should be based on factors such as biological questions of interest, C:N ratios of tissues and degree of overlap among tissue isotope ratios (Post *et al.* 2007; Logan *et al.* 2008).

Other approaches, such as mathematical correction (also known as normalization), can be used to correct isotope ratios depending on the amount of lipids in a tissue, which can be estimated from its C:N ratio (McConnaughey & McRoy 1979; Kiljunen *et al.* 2006). Like chemical extraction, normalization can be tissue- and species-specific and might not give appropriate results in all situations (Logan *et al.* 2008; Mateo *et al.* 2008; Oppel *et al.* 2010). In addition, the range of normalization's application is still limited as it was tested primarily on marine species, with models showing a poorer performance in terrestrial animals (Post *et al.* 2007). It therefore seems crucial to find alternative ways to deal with the presence of lipids in SIA samples.

Although several studies have quantified the direct effects of LE on tissue isotope ratios (Schlechtriem, Focken, & Becker 2003; Sotiropoulos, Tonn, & Wassenaar 2004; Ingram *et al.* 2007; Kojadinovic *et al.* 2008), the question of its ultimate effects on the interpretation of diet composition has not been addressed directly, except by Kiljunen *et al.* (2006). Indeed, some isotopic shifts, although statistically significant, might be of little importance for a specific ecological question if the effect on the estimated variable is negligible (Ricca *et al.* 2007). In diet reconstruction studies, the variable of interest is the relative proportion of different sources in a consumer's diet. It is therefore critical to investigate the potential influence of LE on this variable. Analysing sensitivity of estimates of diet composition to the potential effects of LE could provide guidelines towards the decision to remove lipids, or correct for their presence. Such a framework could then translate into better interpretation of interactions between different elements of a given trophic system.

The main objective of our study was to explore potential effects of LE on estimates of diet composition. To achieve this objective we used a three-tiered approach using both empirical data and simulations. *First*, we examined isotope ratios of sources (prey) and consumers (predators) with varying lipid contents, using data from three case studies based on northern terrestrial food webs located in Canada, Russia and Norway. We sampled tissues of consumers and their potential prey in each food web and analysed each sample before and after LE to obtain isotope ratios of carbon and nitrogen on bulk and lipid-extracted tissues. The consumers we chose are the main predator species from each of the case studies (the arctic fox, *Vulpes lagopus*, Linnaeus 1758 or the least weasel, *Mustela nivalis,* Linnaeus 1766). *Second*, we used isotope mixing models to reconstruct two diets for each of these consumer species: one based on isotopic ratios of bulk tissues and one based on isotopic ratios of lipid-extracted tissues. *Third*, we tested the sensitivity of mixing models to various shifts in carbon and nitrogen isotope ratios of the sources or consumer samples. To this end we developed a tool to simulate, before any extraction of lipids, the potential effects of lipid removal on estimates of diet composition in a given trophic system. We illustrated our approach by applying this simulation function to data from one case study.

Materials and methods

CASE STUDIES

We define a trophic system as a system composed of consumer species (predators) and their potential food sources (prey). We summarize

below the main components of the three trophic systems that were used to exemplify our approach. Sample sizes are summarized in Table 1. To facilitate interpretation of the case studies, we used data from one individual predator per trophic system and further discuss the potential implications of this choice. However, our approach could also be implemented using the average isotopic ratios of a group of individuals. Details on the methods used to collect, prepare and analyse samples, including LE, are presented in Appendix S1 (Supporting Information).

Case study 1 (Canada)

The first trophic system consisted of one consumer (an arctic fox) and five sources [ringed seal (*Pusa hispida*, Schreber 1775), greater snow goose (*Chen caerulescens atlanticus*, Linnaeus 1758), greater snow goose eggs, collared lemming (*Dicrostonyx torquatus*, Pallas 1778) and brown lemming (*Lemmus sibiricus*, Kerr 1792)].

Case study 2 (Russia)

The second trophic system consisted of one consumer (an arctic fox) and five sources [reindeer (*Rangifer tarandus*, Linnaeus 1758), Charadriiform eggs, willow ptarmigan (*Lagopus lagopus*, Linnaeus 1758) eggs, variable hare (*Lepus timidus*, Linnaeus 1758) and root vole (*Microtus oeconomus*, Pallas 1776)]. The Charadriiform eggs, considered as a single source, included four species: Temminck's stint (*Calidris temminckii*, Leisler 1812), ringed plover (*Charadrius hiaticula*, Linnaeus 1758), parasitic jaeger (*Stercorarius parasiticus*, Linnaeus 1758) and snipe (*Gallinago* sp.).

Case study 3 (Norway)

The third trophic system consisted of one consumer (a least weasel) and three sources [Norwegian lemming (*Lemmus lemmus*, Linnaeus 1758), grey-sided vole (*Myodes rufocanus*, Sundevall 1846) and root vole].

MIXING MODELS AND DIET RECONSTRUCTION

Two Bayesian mixing models have been recently proposed that allow estimation of the proportions of each source in an isotopic mixture: MixSIR (Moore & Semmens 2008) and the R package siar (Stable Isotope Analysis in R; Parnell *et al.* 2008, 2010). When working with n isotopes in systems containing more than $n + 1$ sources, Bayesian models offer several advantages over non-Bayesian approaches, such as IsoSource (Phillips & Gregg 2003), namely the possibility to (a) interpret the resulting posterior distributions as probability densities, (b) incorporate specific error terms for the discrimination factors and, (c) in siar (but not MixSIR) take into account carbon and nitrogen concentration in samples (but see Phillips & Koch 2002).

The posterior probability distribution resulting from Bayesian estimation allows calculation of credible intervals around measures of central tendency such as mean, median or mode. When no prior information is used, these credible intervals are considered to be similar to the confidence intervals used in frequentist statistics (McCarthy 2007). Here, we used siar, due to the advantages mentioned earlier, and also because it is based on the open-source statistical application R in which new functions are easy to implement. As IsoSource is still commonly used (Zeug & Winemiller 2008; Anderson *et al.* 2009; but see Dennard, McMeans, & Fisk 2009), we also performed our analyses with this mixing model to allow comparisons. All values used as mixing model parameters are detailed in Appendix S1.

SIMULATING THE EFFECTS OF LIPID EXTRACTION ON DIET ESTIMATES

We developed a simulation tool, called fatsim, to explore the effects of variation in isotopic ratios from source or consumer tissues on estimates of diet reconstruction (R-script and reference manual available at http://www.arctic-predators.uit.no/ISOTOPESWORKSHOP.html). fatsim is a function in R (R Development Core Team, 2009) that uses the siar package to generate successive estimates of diet composition by incrementally varying the $\delta^{13}C$ of sources or consumers. It is also possible to apply a constant (i.e. not incremental) shift in $\delta^{15}N$ to sources or consumers. The main output generated by fatsim is a summary table presenting, for each combination of shifts in isotope ratios, the maximum difference in mean proportion in the diet, for any source, between the unmodified (bulk) and the modified (e.g. lipid extracted) system (95% credibility intervals around the mean proportions for all sources are also provided). Every line in the output table corresponds to a particular combination of shifts in isotope ratios (e.g. in a three-source system one of the multiple combinations could be: $+1{\cdot}0‰$ $\delta^{13}C$ for source 1, $+3{\cdot}5‰$ $\delta^{13}C$ for source 2, $+0{\cdot}5‰$ $\delta^{13}C$ for source 3 and $+2{\cdot}0‰$ $\delta^{13}C$ for the consumer). This table can be read and manipulated as a spreadsheet in order to help quickly identifying the combinations that create important variations in diet outputs. Finally, a more detailed examination of the output distributions can be achieved by using plots of the results (i.e. the siar proportions of sources in diet) generated by fatsim for any combination in the output table.

Although any combination of shifts in isotope ratios can be implemented in fatsim, users should test configurations of shifts that are realistic in their study system. For instance, it would be inappropriate to apply a shift in $\delta^{13}C$ to purely keratinous tissues that are mainly comprised of proteins; the shift for such a tissue in fatsim should be set to zero. For most animal tissues (e.g. egg, blood, muscle and bone collagen) C:N ratios of bulk tissues can be derived from published results (Kelly 2000; Logan *et al.* 2008). However, this information is normally provided, along with $\delta^{13}C$ and $\delta^{15}N$ values, by laboratories performing SIA and the actual C:N ratios should always be checked before using mixing models. Without necessarily giving precise estimates of shifts in isotope ratios, C:N ratios can be used in normalization equations to assess the amplitude of the shifts in $\delta^{13}C$ and $\delta^{15}N$ (Post *et al.* 2007; Logan *et al.* 2008). Indeed, knowing the range of the potential isotopic shifts induced by LE is sufficient to use fatsim simulations and consequently make appropriate decisions regarding the need to extract lipids from samples.

In this paper we illustrate results obtained from fatsim using case study 3 (Norway) because this system is simple (i.e. three sources and one consumer) and demonstrative (i.e. effects of LE are apparent on both sources and consumer isotope ratios, due to high lipid content). To obtain realistic values of maximum shifts in isotope ratios, we used the results from SIA on bulk vs. lipid-extracted samples (Table 1). We then used these values in fatsim to simulate changes in isotope ratios of the sources or consumer whose isotope ratios were most influenced by LE, i.e. least weasel (up to $+3{\cdot}0‰$ $\delta^{13}C$) and the two vole species (up to $+2{\cdot}0‰$ $\delta^{13}C$). Given that all shifts in $\delta^{15}N$ were smaller than our overall measurement precision ($\pm 0{\cdot}2‰$; see Appendix S1) for case study 3, it was not necessary to include shifts of $\delta^{15}N$ in this simulation.

Table 1. Mean $\delta^{13}C$ and $\delta^{15}N$ (‰ ± SD) estimates from bulk tissue, mean shifts in C:N ratios, and mean shifts in $\delta^{13}C$ and $\delta^{15}N$ (‰ ± SD) following lipid extraction in samples of consumers (underlined) and their food sources from three trophic systems

Case study (trophic system)	Species	Tissue	*N*	Mean isotopic ratios of bulk tissue (‰ ± SD)		Mean C:N ratio (± SD)		Shift in $\delta^{13}C$ (‰)		Shift in $\delta^{15}N$ (‰)	
				$\delta^{13}C$	$\delta^{15}N$	Bulk tissue	Lipid extracted	Mean (± SD)	Range	Mean (± SD)	Range
1 (Canada)	**Arctic fox**	Blood	1	−22·6	9·3	3·5	3·4	−0·1	NA	0·1	NA
	Ringed seal	Muscle	7	−19·1 ± 0·7	17·2 ± 1·5	3·6 ± 0·4	3·3 ± 0·1	0·5 ± 0·8	[−0·2, 2·0]	0·1 ± 0·3	[−0·5, 0·4]
	Snow goose	Muscle	19	−22·4 ± 4·0	7·1 ± 1·3	3·5 ± 0·1	3·2 ± 0·1	−0·1 ± 0·4	[−0·9, 0·6]	0·1 ± 0·2	[−0·5, 0·4]
	Snow goose	Egg	6	−27·2 ± 1·2	6·3 ± 0·4	7·6 ± 1·8	3·6 ± 0·1	2 ± 1·1	[0·7, 3·1]	0·4 ± 0·2	[0·1, 0·7]
	Collared lemming	Muscle	5	−26·0 ± 0·5	0·7 ± 1·0	3·4 ± 0·2	3·2 ± 0·1	0·3 ± 0·7	[−0·9, 0·9]	0·1 ± 0·1	[0, 0·2]
	Brown lemming	Muscle	30	−26·3 ± 0·4	4·0 ± 1·4	3·4 ± 0·2	3·3 ± 0·2	0 ± 0·6	[−1·1, 0·8]	0 ± 0·3	[−0·9, 0·6]
2 (Russia)	**Arctic fox**	Muscle	1	−25·7	8·7	3·3	3	0·1	NA	0·1	NA
	Reindeer	Muscle	4	−23·8 ± 0·5	3·4 ± 0·1	3·4 ± 0·1	3·0 ± 0	0·3 ± 0·1	[0·1, 0·4]	0·1 ± 0·2	[−0·1, 0·3]
	Charadriiform	Egg	4	−26·8 ± 0·5	5·7 ± 0·6	7·9 ± 2·9	3·6 ± 0·1	2·5 ± 0·9	[1·3, 3·5]	0·6 ± 0·2	[−0·3, 0·8]
	Willow ptarmigan	Egg	2	−28·4 ± 1·8	2·9 ± 1·1	7·5 ± 3·9	3·5 ± 0	3·5 ± 0·9	[2·8, 4·1]	0·2 ± 0·8	[0·4, 0·9]
	Hare	Muscle	4	−26·0 ± 0·2	3·8 ± 1·3	3·4 ± 0·1	3·0 ± 0·1	−0·2 ± 0·4	[−0·6, 0·2]	0·2 ± 0·3	[−0·2, 0·5]
	Root vole	Muscle	3	−27·2 ± 0·3	4·7 ± 0·3	3·4 ± 0·3	3·1 ± 0·1	0·2 ± 0·1	[0·1, 0·2]	0 ± 0·1	[−0·1, 0·1]
3 (Norway)	**Least weasel**	Muscle	1	−27·9	6·4	6·2	3·4	2·7	NA	−0·2	NA
	Norwegian lemming	Muscle	5	−29·0 ± 1·3	1·4 ± 1·0	4·3 ± 0·6	4·1 ± 0·7	−0·1 ± 0·5	[−0·8, 0·4]	0·1 ± 0·2	[−0·1, 0·3]
	Grey-sided vole	Muscle	4	−27·2 ± 1·1	0·3 ± 1·7	4·2 ± 0·8	3·4 ± 0·1	0·8 ± 0·9	[−0·1, 1·8]	0 ± 0·2	[−0·2, 0·1]
	Root vole	Muscle	6	−28·4 ± 0·9	2·9 ± 1·2	4·3 ± 0·7	3·4 ± 0·2	0·9 ± 0·7	[0, 1·6]	−0·1 ± 0·1	[−0·3, 0]

Results

EFFECT OF LIPID EXTRACTION ON δ^{13}C AND δ^{15}N

The mean effect of LE on carbon isotope ratios for the samples of the three case studies (Table 1) shows that when C:N < 4, LE had a limited influence (mean shift ≤0·5‰ δ^{13}C). There was a significant correlation between shift in δ^{13}C and C:N ratio of bulk tissue (all species and tissues combined: Pearson's $r = 0{\cdot}83$, $P < 0{\cdot}001$, d.f. = 100). Higher lipid content in bulk tissue (C:N > 4) led to larger shifts (+0·8‰ to +3·5‰ δ^{13}C on average), except for Norwegian lemming (−0·1‰). Despite a significant correlation between shift in δ^{15}N and C:N ratio of bulk tissue (all species and tissues combined: Pearson's $r = 0{\cdot}45$, $P < 0{\cdot}001$, d.f. = 100), the effect on nitrogen isotope ratios was generally within our overall measurement error (±0·2‰) except for eggs (average shift ≤ +0·6‰ δ^{15}N).

Lipid extraction affected the configuration of the two-dimensional (δ^{13}C, δ^{15}N) trophic systems (Fig. 1), thereby transforming the geometry of the mixing space (*sensu* Phillips *et al.* 2003). For case study 1 (Canada), the mixing polygon changed little following LE, while its shape and size changed clearly for case studies 2 (Russia) and 3 (Norway). The case study 3 system was the only one in which LE modified the δ^{13}C of the consumer (least weasel; + 2·7‰ δ^{13}C after LE; Table 1). It is noteworthy that in this case the consumer was not included within the mixing polygon before LE (Fig. 1c). However, it was still possible to estimate a diet in this case, due to the large standard deviations of prey isotopic ratios.

Lipid extraction did not systematically reduce the variability of isotope ratios in any species or tissue (Fig. 1): after LE, standard deviations remained, on average, similar for δ^{13}C (mean difference in SD −0·1‰) and for δ^{15}N (mean difference in SD +0·1‰) but changed more for some species or tissues (Table 1). For instance, standard deviation of δ^{13}C decreased for willow ptarmigan eggs (−0·9‰ δ^{13}C) but increased for Charadriiform eggs (+0·7‰ δ^{13}C). However, our sample sizes were too small to address this question statistically.

EFFECT OF LIPID EXTRACTION ON ESTIMATES OF DIET COMPOSITION

Arctic fox tissues (blood and muscle) were lean, whereas the least weasel tissue (muscle) had higher C:N ratio and thus lipid content (Table 1); this represented two contrasting situations regarding the effect of lipid removal, i.e. when only source, or both consumer and source isotope ratios shifted following LE. Lipid extraction affected estimates of relative contribution of certain sources to the consumer diet in case studies 2 (arctic fox) and 3 (least weasel), but to a lesser extent in case study 1 (arctic fox; Fig. 2). Thereafter, we present all values of source proportions in the diet as the mean of SIAR posterior distributions calculated for each of the three illustrative trophic systems. Although results are presented in terms of mean, we strongly recommend close examination of the complete output distributions, including 95% credibility intervals (A. Parnell, personal communication). For comparison purposes, we provide results from the IsoSource mixing models in Appendix S1.

Case study 1

The largest effect of LE in this system was on the proportion of snow goose eggs (increased from 15% to 20%) and muscle (decreased from 25% to 20%) in the arctic fox diet (Fig. 2a). However, this did not represent a major change in overall diet. Contribution of other sources remained similar (within ±2%) following LE. The shift in δ^{13}C of only one source (snow goose eggs) did not influence the contribution of all other sources evenly but instead exclusively affected the contribution of the main source in the diet before LE (snow goose muscle).

Case study 2

The relative proportions of the two main sources in the arctic fox diet decreased from 30% to 20% (Charadriiform eggs) or increased from 24% to 38% (root vole muscle) (Fig. 2b). Removing lipids from lipid-rich sources (Ptarmigan and Charadriiform eggs) globally decreased their estimated contribution to the diet of the arctic fox, thereby increasing that of another, leaner, source (root vole). Hence, after LE, root vole muscle became the main source in the estimated diet, while Charadriiform eggs shifted from first to third rank, in terms of diet contribution. By comparison, changes in relative proportions for the three other sources (reindeer, ptarmigan eggs and hare) were at least two times smaller (≤5%). The change observed in the configuration of the mixing space after LE (Fig. 1b) could have helped to anticipate which source proportions would be affected by LE (e.g. that root vole muscle would become the main source due to its geometric proximity to the consumer). However, it was difficult to estimate the amplitude of the changes in all proportions, especially for sources situated geometrically distant from the consumer in the two-dimensional mixing space.

Case study 3

This trophic system was the only one in which the δ^{13}C of the consumer (least weasel) was strongly affected by LE (Table 1, Fig. 1c). Following LE, the relative proportion of grey-sided vole muscle increased from 15% to 31%, while that of Norwegian lemming muscle decreased from 50% to 31%. The relative contribution of root vole muscle to diet changed little (+3%) after LE. Overall, we observed a change in diet composition following LE, with root vole becoming the major food source (instead of Norwegian lemming) and grey-sided vole contributing twice as much as calculated without LE.

SIMULATING EFFECTS OF LIPID EXTRACTION ON DIET ESTIMATES

We illustrate the use of FATSIM by applying it to case study 3 (Norway), where LE affected both sources and consumer

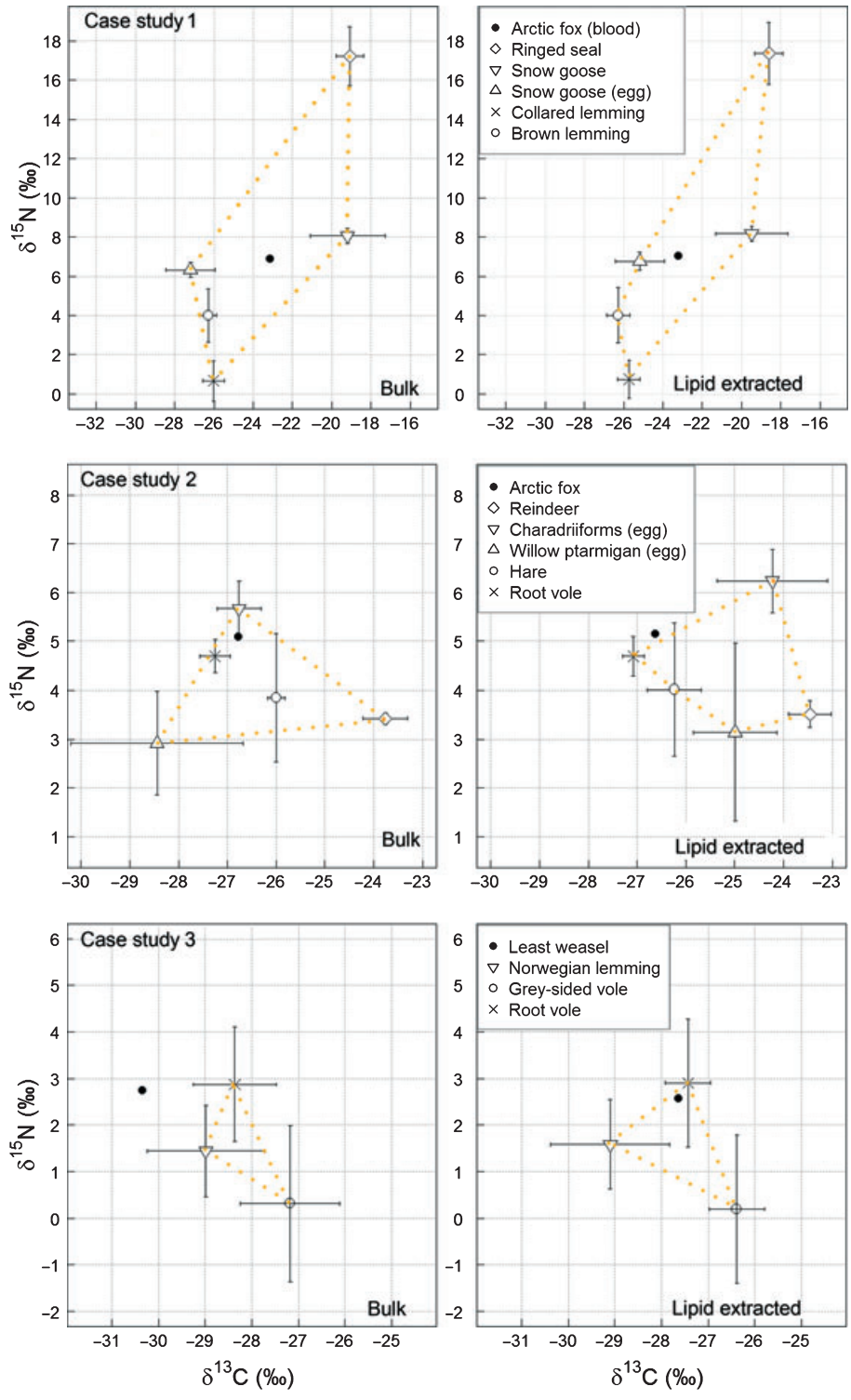

Fig. 1. Scatterplots of the $\delta^{13}C$ and $\delta^{15}N$ (±SD) values for consumers (closed symbols) and prey (open symbols) for bulk tissues (left column) and lipid-extracted tissues (right column) in three trophic systems. To facilitate interpretation, dotted lines show the convex hull around potential sources, that we defined as mixing polygons (descriptive purpose only). Animal tissue used for SIA is muscle, unless otherwise specified in the legend. Consumers are represented as single individuals and discrimination factors were subtracted from their isotopic ratios (see the Materials and methods section for details).

isotopic ratios (Table 1). Results of simulations are summarized in Table 2 and show the change in the estimates of diet composition following shifts in $\delta^{13}C$ (i.e. the absolute change in the relative proportion for any of the sources as estimated before vs. after the isotopic shifts). In other words, a change of 25% indicates that the proportion of at least one of the

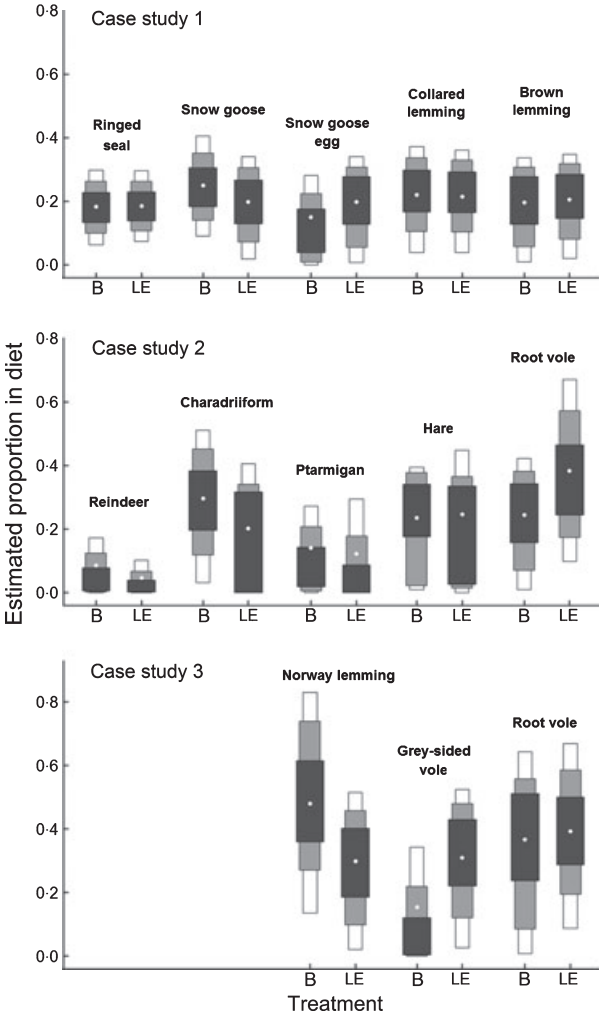

Fig. 2. Diet reconstruction outputs from SIAR mixing models: for each source, we show median (white dot) 50%, 75% and 95% credibility intervals (respectively dark grey, light grey and white boxes) of the posterior probability distributions of proportions in diet, when using bulk tissue (B) and lipid-extracted (LE) samples.

sources in the diet has, for example, increased from 5% to 30% or decreased from 50% to 25%. In order to interpret these changes in more detail, FATSIM allows users to plot, for each line: (i) the consumer and sources in the mixing space before and after the isotopic shift (such as those in Fig. 1) and (ii) the SIAR estimates of diet composition before and after the isotopic shift (such as those in Fig. 2).

An overview of the results indicates that changes ≥10% in diet composition should be expected for most combinations tested using case study 3. Moreover, the relative contribution of at least one source can change by up to 21% (Table 2). Considering the large potential effect of small variations in isotopic ratios on diet composition and the range of potential isotopic shifts induced by LE in samples (C:N ratio >4·0; Table 1), we would conclude that lipids need to be removed for both consumer and sources in that specific trophic system.

Discussion

EFFECT OF LIPID EXTRACTION ON δ^{13}C AND δ^{15}N

Our results show that LE induces shifts in isotope ratios of tissues when their C:N ratio is >4·0, a threshold value corresponding to lipid content ≤10% in terrestrial animals (Post *et al.* 2007). This is in line with results from previous studies which tested direct effects of LE on isotope ratios (Logan *et al.* 2008). For C:N ratios <4·0, the shift in isotopic ratios was generally smaller than, or close to, our overall measurement error (±0·2‰). When the C:N ratio ≈ 4·0 (e.g. lemming and vole, Table 1), the shift in δ^{13}C was not consistent among samples, making predictions of a shift in δ^{13}C difficult when using normalization equations. The fact that LE also induced a change in δ^{15}N of whole egg (up to +0·9‰) is probably due to some leaching of nitrogenous compound (Sotiropoulos, Tonn, & Wassenaar 2004). However, the cause of this nitrogen leaching remains unclear and the amplitude of the isotopic shift was always three to four times smaller for nitrogen than for carbon.

EFFECT OF LIPID EXTRACTION ON ESTIMATES OF DIET COMPOSITION

In each of the three trophic systems, estimates of consumer diet composition reacted differently to LE. Although in case studies 1 and 2 the systems shared the same predator and had the same number of sources, it was difficult to transpose the consequences of LE between systems for diet reconstructions due to differences in the general configuration of the mixing space. In case study 3 the strong effect of LE on the consumer's isotopic ratio led to different reconstructed diets. This had an influence on the biological interpretation of this consumer's diet and not removing lipids, in this case, would have led to incorrect conclusions.

From a geometrical point of view, LE induced an important change (≥10%) in estimated diet in two systems (case studies 2 and 3) that were characterized by the relative proximity of the end-members in the mixing space (Fig. 1), compared with the other system (case study 1). This is in accordance with the idea that greater isotopic distance among end-members should theoretically lead to less influence of a given shift in isotope ratios (Post *et al.* 2007). Other characteristics of a given trophic system, such as complexity, variability in isotope ratios, and degree of consumer specialization on lipid-rich sources, can indicate *a priori* the potential influence that LE (or other sources of isotopic variation) could have on diet estimates. Such characteristics (summarized in Table 3) can be observed when examining the geometric configuration of the system in the mixing space. As a general rule, isotopic variation is likely to have a reduced impact on diet estimates in a complex system with a generalist consumer, compared with a simpler system with a specialist consumer.

Overall, distributions of credible intervals (from SIAR; Fig. 2) and feasible contributions (from IsoSource; Fig. S1) were large, due to the considerable variability of particular prey isotope ratios, such as snow goose muscle. This might have

Table 2. Table adapted from the output generated by FATSIM simulations on the Norwegian data set (case study 3)

Line no.	Combination of shifts in $\delta^{13}C$ (‰) applied to consumer or sources				Maximum absolute difference in mean proportions (%) in diet
	Consumer (least weasel)	Grey-sided vole	Root vole	Norwegian lemming	
0	0	0	0	0	0 (reference level)
1	+1	+1	+1	0	2
2	+1	+2	+1	0	3
3	+2	+2	+2	0	3
4	+1	0	+1	0	7
5	+1	+2	0	0	8
6	+2	+1	+2	0	8
7	+1	+1	0	0	8
8	+2	+2	+1	0	9
9	+1	+1	+2	0	11
10	+1	0	+2	0	11
11	+2	+1	+1	0	12
12	+1	0	0	0	12
13	+1	+2	+2	0	12
14	+2	0	+2	0	13
15	+2	+2	0	0	14
16	+3	+2	+2	0	14
17	+2	0	+1	0	15
18	+2	+1	0	0	16
19	+3	+1	+2	0	17
20	+3	+2	+1	0	18
21	+2	0	0	0	18
22	+3	0	+2	0	19
23	**+3**	**+1**	**+1**	**0**	**20**
24	+3	+2	0	0	21
25	+3	0	+1	0	22

Each line corresponds to a specific combination of shifts in $\delta^{13}C$ for the tissues of consumer and prey. For each combination, the right-hand column indicates the corresponding maximum absolute change in mean proportion for any of the sources in the reconstructed diet. Lines were ranked from the smallest to largest maximum effects of LE on mean proportions in reconstructed diet. Line 23 (bold) corresponds, in terms of shifts in isotopic ratios, to the situation we obtained after extracting lipids from our samples (see the Results section).

contributed to dampening the potential effects of shifts in $\delta^{13}C$ and $\delta^{15}N$ on diet outputs. The exact cause of this variability goes beyond the scope of this study, but high variability in source or consumer isotope ratios can actually be present in a given system, independent of lipid effects, and thus have an influence on diet estimates. Hence, in this context, the costs of LE could outweigh the associated benefits. Using FATSIM, we could have anticipated this for case study 1 and thus have avoided the extra laboratory work of LE.

We used only one individual predator in each study system and this did not allow us to test additional biological hypotheses concerning predator–prey relationships. However, we only wished to illustrate our conceptual approach, so that the sample size of individual predators was not important here. We emphasize that using data from more than one individual predator in any of our case studies would have created a new trophic system, with potentially alternate biological interpretations of differences among systems.

Several experimental studies have demonstrated the existence of statistically significant shifts in isotope ratios following LE, although the amplitude and direction of these shifts vary among studies, species and tissues (Søreide *et al*. 2006; Post *et al*. 2007; Logan *et al*. 2008; Mateo *et al*. 2008). It was suggested that isotopic shifts are likely to become ecologically

Table 3. Summary of the characteristics of a given trophic system and the corresponding relative effect that isotopic shifts can have on diet estimates

	Effect of a given isotopic shift on diet estimates	
	Larger effect	Smaller effect
Complexity of the trophic system (number of sources)	Few	Many
Relative distance among sources in the mixing space	Clumped	Dispersed
Relative distance between shifting sources and consumer in the mixing space	Small	Large
Variability in isotopic ratios relative to distance between sources	Small	Large

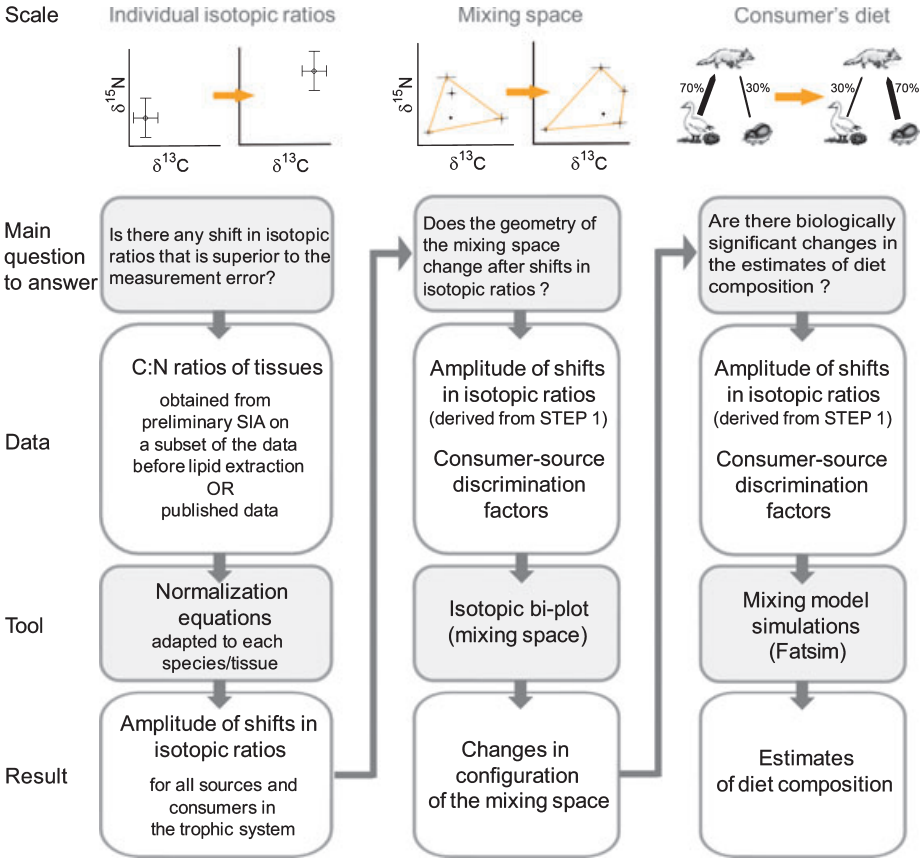

Fig. 3. Summary diagram of the logical steps suggested when addressing issues of lipid extraction (LE) in stable isotope analysis and dietary reconstruction. This can be approached at three different scales, from the individual source (left) to the mixing space (middle), and finally to the trophic system (right). Following these steps should allow a better understanding of the biological significance of the effects of LE at each of these levels.

significant when approaching 5·0‰ δ^{13}C (Ricca *et al.* 2007). Our data show that depending on the system, ecologically significant changes in diet estimates can already occur with shifts of 2·0‰ δ^{13}C. Ecological significance depends on the specific question that is addressed in a given study. While the question of statistical significance remains important, such case-specific variations require caution when translated into ecologically meaningful shifts of δ^{13}C and δ^{15}N (Sotiropoulos, Tonn, & Wassenaar 2004; Ricca *et al.* 2007). Alternatively, absence of statistical significance should not prevent us from verifying and interpreting the ecological implications of small variations in isotope ratios, for instance on the strength of trophic relationships. We acknowledge that potentially important limitations are inherent in the use of isotope ratios at larger ecological scales, such as when basal trophic levels are not taken into account (Hoeinghaus & Zeug 2008). However, our approach suggests that SIA can strengthen ecological understanding of trophic systems when examining proximate and ultimate effects of isotopic variations at several scales, from individual consumers to entire food webs. In diet reconstruction studies, it is more difficult to determine *a priori* what level of shift in isotope ratios is ecologically meaningful. This is especially true when dealing with complex trophic systems, where the mixing space can be composed of many sources. In this case, both trophic dimensions, i.e. δ^{13}C and δ^{15}N, should be taken into account and the effect of a given isotopic shift could be important or negligible depending on the relative positions of the mixture and end-members within the mixing space. With FATSIM, we suggest a method to explore these potential effects on the proportions of each end-member in the mixture.

In SIA, lipid-related issues can be approached at three different scales (Fig. 3): (i) at the scale of the individual source, LE can directly affect isotope ratios, (ii) at the scale of the mixing space, LE can cause changes in the relative geometry of the mixture and end-members, and (iii) at the larger scale of the trophic system, LE can induce changes in the quantification of the strength of consumer–source relationships. Our proposed simulations allow projecting immediate effects of LE on isotope ratios to the larger scale of the trophic system being studied (through interpretation of reconstructed diets). This is equivalent to testing how sensitive a mixing model is to variations in isotope ratios of individual sources or consumers. It is essential to have an idea beforehand of potential amplitude shifts that LE could cause in isotope ratios from different sources of the study system. If FATSIM predicts important changes in diet composition in response to shifts in δ^{13}C and δ^{15}N that are within the range of realistic values, it is critical to

apply LE (or other correction methods if available) to all tissue samples concerned.

SENSITIVITY OF DIET ESTIMATES TO SHIFTS IN ISOTOPE RATIOS

The three case studies presented here as examples were relatively simple trophic systems. Yet, it was not possible, without simulation, to anticipate the amplitude and direction of the changes in diet composition estimates caused by isotopic shifts on as few as two or three sources. Using simulations we demonstrated that these shifts can affect diet estimates to various degrees within a given system. The induced change in diet depends on the amplitude of the isotopic shifts and, equally important, on the combination of sources or consumers to which shifts apply.

FATSIM can help field ecologists evaluating the effects of lipid removal on the interpretation of reconstructed diets in their studied trophic system, and thus facilitate the decision on whether or not to invest time and money in LE of samples. There are multiple sources of variations in isotope ratios (Urton & Hobson 2005) and such uncertainty can influence diet reconstruction at different levels depending on the trophic system considered. FATSIM can be used as a tool to explore effects of other sources of variation in mixing model outputs (e.g. discrimination factors, variability of prey isotopic ratios and routing of dietary macronutrients). Depending on the configuration of the mixing space, a given system might be less sensitive to variability in isotope ratios. Quantifying such effects using simulations would help to interpret resulting diets and better understand the trophic system of interest.

Beyond facilitating the decision to remove lipids or not, FATSIM also has the potential to help increase comprehension of issues related the routing of dietary lipids. FATSIM estimates all possible diets along a gradient from no specific lipid routing (i.e. both lipid and protein fractions of the diet are used to build tissues) to a complete routing (i.e. all lipids are routed to other tissues or to metabolism). Choosing to remove lipids implies that one assumes these are not used by the consumer to build the tissue used for SIA. Therefore, although FATSIM cannot be used to determine the degree of isotopic routing, it can be used to explore the consequences of various degrees of routing on the interpretation of trophic relationships.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Sample collection and preparation.

Figure S1. Output distributions from IsoSource mixing model.

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