FORUM

Trophic experiments to estimate isotope discrimination factors

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Summary

1. In Caut, Angulo & Courchamp (2008a) rats were fed with experimental diets of distinct isotopic values (δ^{13}C and δ^{15}N) in order to infer the discrimination factors. We showed negative relationships between discrimination factors and diet isotopic values. In Caut, Angulo & Courchamp (2009), our aim was to generalize these relationships to other taxonomic groups with a view to providing ecologists with a general and flexible method to obtain discrimination factors for diet reconstruction studies when difficult to obtain otherwise.

2. Perga & Grey (2010) claims that there is an artefact of experimental design (due to protein contents) and partly reanalyzes our data, taking into account the type of diet (mixed or not; carnivorous, omnivorous or herbivorous). However, (i) the role of protein content through isotopic routing is not clear; (ii) a reanalysis of our data shows that the relationships between discrimination factors and diet isotopic values remain significant even when following Perga's suggestions.

3. Auerswald *et al.* (2010) criticized the imprecise definitions, mathematical artefacts and invalid statistical analysis of Caut, Angulo & Courchamp (2009). However, (i) their arguments are not new; they challenge the use of stable isotope in ecological trophic studies in general, and lack both alternative suggestions and ecological perspective; (ii) none of their statistical and mathematical objections has a significant effect on the results.

4. *Synthesis and applications.* Neither reply reflects the current context of debate. The complementarity between experimental studies and their application in field studies is indispensable for the use of stable isotopes in ecology. Protocols should be very well assessed in order to represent field conditions with accuracy. Further analyses are necessary to remove some of the flaws in the use of stable isotopes (carrying out an in-depth analysis of isotopic routing, turnover, and dynamic during trophic experiments to ensure accurate attainment of equilibrium), but none of the issues raised by Perga & Grey (2010) and Auerswald *et al.* (2010) alter our main conclusions.

Key-words: diet dependent discrimination factor, diet reconstruction, dietary protein source, diet-tissue shift, fractionation factor, isotopic routing, mixing model

Introduction

Compared to other sciences, ecology is in its infancy and there is a need for more and better ecological studies focusing on quantitative trophic interactions in multispecies settings. In this context, new methodological and theoretical approaches are necessary to move from laboratory species systems to more realistic multitrophic interactions. Stable isotope analysis is one such technique. As a method in a current state of ongoing development, stable isotopes analyses for trophic ecology are still far from flawless.

In this regard, we welcome the endeavour of Perga & Grey (2010, hereafter '*Perga*'), and Auerswald *et al.* (2010, hereafter '*Auerswald*'), in providing criticisms of two of our previous studies (Caut, Angulo & Courchamp 2008a, 2009, hereafter '*Caut*'). Space contraints prevent us from dealing fully with these criticism but we respond below to the main points in each and further details are available from: http://digital.

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Reply to *Perga*

Perga suggest that a bias exists in the method we proposed (to obtain discrimination factors from diet isotopic values) due to isotopic routing. Although we agree that isotopic routing could pose some problems in estimating discrimination factors, the vast majority of the criticisms of *Perga* focus too heavily on our data specifically. They do not present a comprehensive description of the current controversies or a rigorous management of the data from the selected articles cited to support their arguments.

ISOTOPIC ROUTING

Isotopic routing is the result of the differential allocation of dietary components into different tissues (Schwarcz 1991): carbohydrates and lipids should be essentially metabolized, while amino-acids are likely to be routed preferentially to muscle and liver and, to a lesser extent, to hair (Ambrose & Norr 1993). *Perga* suggest that a bias in discrimination factors of our rat experiment is caused by the constituents they call 'protein sources' (fish meal, alfalfa, and casein) against 'non-protein sources' (corn flour).

We concur that isotopic routing may reduce the precision of our estimates, as already outlined in a number of previous studies (Schwarcz 1991; Gannes, O'Brien & Martinez Del Rio 1997; Podlesak & McWilliams 2006). However, we show below that isotope routing does not bias our conclusions.

Perga's consideration of a poor or high protein source is based on a trade-off between C/N ratios and $\delta^{15}N$ values of each constituent. Fish meal and casein with C/N ratios around four are clear protein sources, but the cases of corn flour and alfalfa are not as simple as they depict. With the highest C/N ratios (31·15 and 14·5, respectively), corn flour is considered a non-protein source with a $\delta^{15}N$ value between that of casein and fish meal (6·4), while alfalfa is considered a protein source despite possessing the lowest $\delta^{15}N$ value (−0·7). Moreover, corn flour consists of 9% protein, which cannot be disregarded compared to the 15·5% protein in alfalfa meal. In this context, *Perga* failed to consider the different percentage of protein in each protein source (e.g. 66·2% of proteins in fish meal, 95% in casein), which could have a significant effect. For example, Ambrose & Norr (1993) stated that the diet to bone collagen difference is a function of the $\delta^{13}C$ of dietary protein, but is modulated by the proportion of protein in the total diet.

When considering the protein percentages of only what *Perga* define as protein sources (fish meal, casesin, and alfalfa), the artefact on which their main criticism is based disappears (Fig. 1a,b). We have used the same correction for the nitrogen, which we think is a better approximation than considering only the C/N ratios. Two of the four relationships shown by *Perga* are non-significant (Fig. 1c,d). The two remaining relationships lose much of the variability highlighted by *Perga* (Fig. 1d).
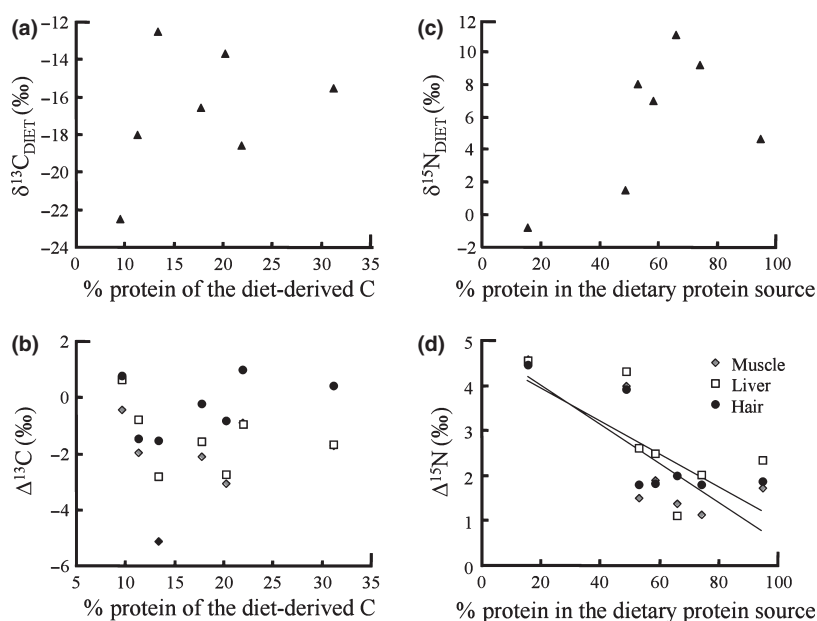

Fig. 1. Changes in *Perga* results when considering the percentage of protein in each protein source in relation to: (a) the diet $\delta^{13}C$ values ($R^2 = 0.12$, $t = 0.82$, $P = 0.447$), (b) the $\Delta^{13}C$ of three rat tissues ($R^2 = 0.01$, $t = 0.22$, $P = 0.84$; $R^2 = 0.13$, $t = -0.85$, $P = 0.43$; $R^2 = 0.15$, $t = 0.94$, $P = 0.39$, for muscle, liver and hair respectively), (c) the diet $\delta^{15}N$ values ($R^2 = 0.33$, $t = 1.58$, $P = 0.175$) and (d) the $\Delta^{15}N$ of three rat tissues ($R^2 = 0.59$, $t = -2.70$, $P = 0.042$; $R^2 = 0.52$, $t = -2.34$, $P = 0.066$; $R^2 = 0.61$, $t = -2.78$, $P = 0.039$, for muscle, liver, and hair respectively). Only significant relationships have a trend line. Thus, six of the eight 'coincidental relationships' *Perga* described are non-significant. $N = 7$ for all samples.

In summary, although we agree that discrimination factors could be biassed by the isotopic values of the proteins that constitute the mixed diets, *Perga* failed to demonstrate that it is the case with our data because they did not account for the proportion of protein in the total diet.

RANGES OF DISCRIMINATION FACTOR VALUES

Another major criticism of our method by *Perga* is that the discrimination factors obtained in our rat experiment and then used to test for relationships between diet $\delta^{13}C$ values, and $\Delta^{13}C$ span a '*considerable range compared to those reported in previous literature reviews*'. Besides more technical arguments (see online SI), we point out that our data remain within the range of previous reviews and that the large range was purposely expected in our experiment.

Perga removed the 'extreme' points from our experiments for the regressions between diet $\delta^{13}C$ values and $\Delta^{13}C$ and pointed to a diminished R^2. Despite this, the regressions remain significant ($F_{1,73} = 36.05$ $P < 0.001$) and explains 32% of variability despite the relevance of other factors (e.g. tissues, species). We believe our arguments, the retained significance and high explained variability when concentrating only on part of the data, are sufficient to rebut this criticism.

DISTINCTION BETWEEN MIXED AND MONOSPECIFIC DIETS

Perga claim (but did not test) that the same bias related to isotopic routing might affect ~50% of the published data included in our analyses because the data came from studies that also used mixed diets. We repeated our analyses for mono-specific diet only, considering as mono-specific all diets with only one constituent (excluding vitamins and minerals as constituents). Regression trends remained significant for mammals, for birds (only for $\Delta^{15}N$) and for invertebrates. For fishes, data was not sufficient to perform statistics ($n < 10$). When analyzing mixed diets only, trends were similarly significant for mammals, fishes and invertebrates, but not for birds (Fig. 2). These results confirm the validity of our method. We agree that regression lines between mono-specific and mixed diets in each taxa and for each isotopic value differ. This is caused, first, because the values to obtain the regression are different and, secondly, sometimes sample size is very different.

NATURAL DIETS IN LABORATORY STUDIES

A point is raised concerning the application of experimental results from laboratory studies to those in wild situations. Although we concur that this point merits consideration, we think that the issue is more about the selection of a correct diet item in relation to the actual consumer diet in the wild than the whereabouts of the experimental setting (laboratory or field conditions). A mono-specific diet constituted by one properly selected resource should result in a valid discrimination factor for application in wild studies (but see the possible effect caused by stress below). Looking carefully at the data set from mono-specific diets, we observed that the majority of diet items correspond to wild natural diet items (e.g. salmon, apples, insects or plants).

CARNIVOROUS VS. HERBIVOROUS DIETS

Perga criticized our relationship between $\delta^{15}N$ diet and $\Delta^{15}N$ referring to the effect of the type of diet: carnivorous, omnivorous and herbivorous. They showed significant differences in $\Delta^{15}N$ and $\delta^{15}N$ of diet between these categories, explained by an indirect consequence of N availability, with lower $\Delta^{15}N$ and higher $\delta^{15}N$ values for carnivorous than herbivorous diets. In fact, this is not a problem, as *Perga* claim, because two contrasting hypotheses are debated in the literature concerning this trend. The 'quantity hypothesis' states that $\Delta^{15}N$ will increase as %N increases and C/N ratio decreases in diets, and therefore that $\Delta^{15}N$ increases with the trophic level of the animal (Pearson *et al.* 2003). The counter-argument states that $\Delta^{15}N$ decreases as dietary protein quality increases, and therefore that $\Delta^{15}N$ decreases with the trophic level of the animal (e.g. Roth & Hobson 2000). This is called the 'quality hypothesis'. In distinct reviews Vander Zanden & Rasmussen (2001) found the former pattern, Robbins, Felicetti & Sponheimer (2005) found the latter, while Post (2002) and Vanderklift & Ponsard (2003) found no differences between carnivores and herbivores. Our data supports the quality hypothesis; *Perga* seem to champion the alternative hypothesis, but fail to acknowledge the ongoing debate.

Similarly, *Perga* presents only one aspect of the controversy on nutritional stress in trophic experiments to explain our variations in $\Delta^{15}N$ of mammals (see online SI). Nutritional stress could be problematic but in most of experiments animals are fed according to their needs.

Reply to Auerswald

Caut reviewed carbon and nitrogen isotopic discrimination factors published in the literature and analyzed their relationships with the diet isotopic factors, based on previous studies showing this relationship in rats (Caut, Angulo & Courchamp 2008a) and in bear (*Ursus americanus,* Hilderbrand *et al.* 1996; *Ursus arctos horribilis,* Felicetti *et al.* 2003). *Auerswald* tries to invalidate our relationship arguing mathematical, statistical and experimental bias, which we refute below.

TERMS OR DEFINITIONS?

With the term 'discrimination factor', we mean the difference between isotopic values of a consumer tissue and the diet (δtissue – δdiet). This is the definition that has been used since the earliest studies on trophic ecology.

We agree with *Auerswald* that there is no consensus in the term that researchers use for the δtissue – δdiet. This is due to both the rapid evolution of a relatively recent method and its use in very different fields of science. While the 66 studies covered by our review showed a wide variability in the terms used
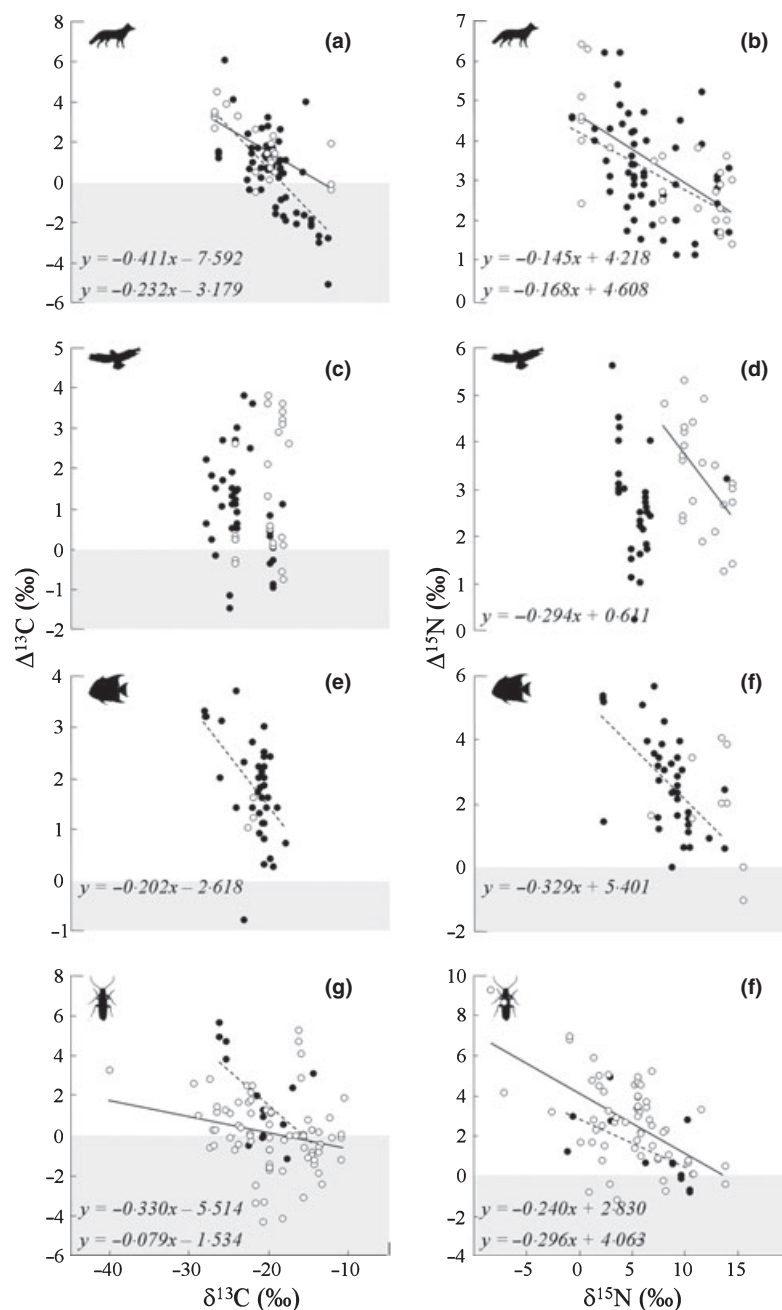
Fig. 2. Relationships between $\Delta^{13}C$ and $\delta^{13}C$ and between $\Delta^{15}N$ and $\delta^{15}N$ of mono-specific and mixed diets for mammals (🐺), birds (🐦), fishes (🐟), and invertebrates (🪲). The complete data set of Caut, Angulo & Courchamp (2009) was used, separating data when individuals were fed with mono-specific diets (white dots) or mixed diets (black dots). Regression lines are only shown when significant, in continuous line for mono-specific diets and in dotted line for mixed diets (for mammals: $R^2 = 0.53$, $F_{1,25} = 28.55$, $P < 0.001$ and $R^2 = 0.51$, $F_{1,24} = 25.61$, $P < 0.001$ for $\Delta^{13}C$ and $\Delta^{15}N$ respectively; for $\Delta^{15}N$ of birds: $R^2 = 0.29$, $F_{1,21} = 8.60$, $P = 0.008$; and for invertebrates: $R^2 = 0.05$, $F_{1,74} = 4.42$, $P = 0.039$ and $R^2 = 0.31$, $F_{1,63} = 29.21$, $P < 0.001$ for $\Delta^{13}C$ and $\Delta^{15}N$ respectively).

for the definition of δtissue – δdiet, all authors used the same definition. Thus, *Auerswald*'s suggestion to distinguish between this definition (eqn 4) and the definition of discrimination coming from a reaction chain (eqn 3) to avoid confusion has no sense in our review. *Auerswald* state that the '*magnitude of this effect is small*': 0·15‰ for a 30‰ of range in diet in their Fig. 1, similar to the error of precision of a mass spectrometer. Indeed, this effect is the result of the denominator in eqn 3, which could be overlooked due to the precision of trophic isotopic works (Goericke, Montoya & Fry 1994).

Recent papers discussing the terminology suggest using the term 'discrimination factor' (Pearson *et al.* 2003; Martínez del Rio *et al.* 2009). *Auerswald* do not provide a useful clarification of this term for ecological studies and proposed a new term

'diet-tissue shift', which could lead to confusion with experimental diet shifts.

MATHEMATICAL AND STATISTICAL BIASES

Auerswald claims that the negative correlation found between isotopic composition of the diet and discriminant factor for different animal groups and elements is spurious because δdiet affects both the dependent and independent terms of the regression. *Auerswald* presents the question as completely solved in 1897 by Pearson (1897), when in fact it has been the focus of debate since then (Prairie & Bird 1989; Smith 1999).

The risk of spurious correlations decreases as the correlation between variables increases and depends on the coefficients of

variation of variables (Kenney 1982). The use of stable isotopes for the analyses of diet is based on the already well demonstrated fact that the isotope composition for $\delta^{13}C$ and $\delta^{15}N$ of an organism is not independent of the isotope composition of its diet (in our data set, we have calculated that these correlations varied between 0·80 and 0·95). Consequently, the basic premise of *Auerswald*'s simulation is false: the isotopic composition of the organism (δ_p) is not independent of the isotopic composition of the diet (δ_s).

Discrimination factor is not a mathematical construct but reflects real changes in isotopic concentration as elements flow from one organism to another. However, we don't aim to solve here the long-standing question on how to regress parts on a whole (see previous references). If we are interested in the relationship between liver and body mass, is it better to regress the mass of the liver against the mass of the body minus the mass of the liver? Or should we regress liver mass against body mass, although the mass of the liver will affect both variables? In the case of isotopic change from the diet to the tissue we chose the former approach, but *Auerswald* prefer the latter. But does this really affect our conclusions?

To answer this question, we calculated the relationship between isotopic composition in the diet and the tissues using Ordinary Least Squares (OLS) regression and Major Axis (MA) regression. With this approach slopes equal to 1 indicate that isotopic discrimination is not affected by diet, while slopes significantly lower than 1 would confirm the effect of diet on isotopic discrimination factor. For these analyses, MA regression is more appropriate when dependent and independent variables are measured in the same units and with similar errors (Sokal & Rohlf 1995), as OLS may bias estimates of slopes in such cases. All correlations were significant at $P < 0.05$. Of all the correlations considered by *Caut*, only one changed qualitatively, while no change occurred for the other nine (see Table 1, note that if we use OLS as suggested by *Auerswald*, all the 10 regressions support *Caut*'s initial conclusions).

The second argument against the validity of *Caut* is more serious. Isotopes are measured with error, something intrinsic to most variables used in ecology, from individual counts, morphological measurements, behavioural observations or organism's condition estimation. *Auerswald* suggest that the significant relationships between diet isotope composition and diet shift reported in *Caut* are an artefact because measurement error in diet will affect both the dependent and independent term of the regression. As they suggest, any variable is composed of its real value and a random error (random errors have a mean of 0). Consequently, as we are using mean values for each estimate of each study, measurement error does not bias estimates but increases the standard error of each study means (Taylor 1999). Variance of measurements from the same experiment will also reflect physiological differences between diet items and not only measurement error. In fact we are confident that the dataset contains a lot of true variation (mean carbon isotopic signature ranges from −40·20‰ up to −10·48‰ and from −8·50‰ to 17·80‰ for nitrogen) that will also be affected by a small amount of measurement error in isotope composi-

tion (classically 0·2–0·3‰). As for their first criticism, the conclusions of the analyses questioned by *Auerswald* do not depend on ignoring error in one of the axes, as demonstrated by using MA regression, see for example Lytle (2001).

EXPERIMENTAL BIAS: DISCRIMINATION FACTORS AND EQUILIBRIUM

Auerswald base all their criticisms of experimental bias on the assumption that the discrimination factors used in our review (and in most of the studies estimating discrimination factors) were not at equilibrium. Indeed, we are not sure that all the cited studies have reached equilibrium because most of them do not show isotopic values' dynamics, but we reduced this possible source of error by excluding '*any estimates of experiments when the authors explicitly stated that isotopic equilibrium was not attained.*' *Auerswald* also criticize the role of the memory effect on discrimination factors. However, once it is assumed that the discrimination values are calculated at equilibrium, this excludes any memory effect. Memory effect, or state immediately after the diet shift, has a role during the process of equilibration (altering parameters of the dynamics, e.g. half-lives) but not on the value of discrimination factor, that is, at equilibrium.

Auerswald also criticize the role of the '*field studies, which are affected naturally by variation in diet isotopic composition*'. It is important to distinguish between real field studies and experimental studies using natural diets (e.g. apples). Only two of the 66 studies we included are really field studies (consumer not in captivity/laboratory), while diet isotopic values were not controlled along the experiment in only seven studies, far from the 30% stated by *Auerswald*. Moreover, the errors associated to those studies should be random, without imposing any direction in our relationships and frequently very close to the margin of error of a mass spectrometer.

Auerswald's point of view is extremely theoretical, focusing on a potential maximal equilibrium: individuals feeding on the same resource from birth to death, even their mothers feeding on the same resource in some cases, to have a 'true' discrimination factor. Fortunately, this is not the case as the isotopic literature is full of studies that have shown and calculated turnovers, equilibrium states in times frames which are both manageable and useful for the purpose of studying the trophic ecology of a species at different times of its life. Note that ecologists are generally interested in knowing what an individual has eaten during a finite time period, rather than the diet of the grandmother of the individual being sampled.

Conclusions and ways forward

We disagree with *Perga* and *Auerswald* that there are artefacts and biases in the analyses of our studies that invalidate our conclusions. We place their various criticisms in the correct current context of discussion, in particular showing that the role of protein content through isotopic routing is not clear. We reject *Auerswald*'s assertion that there is a bias in the analysis of isotopic discrimination factors for trophic studies

Table 1. (a) Schema of the possibilities of regressing parts of a whole: regressing tissue isotopic values (left) or discrimination factors (Δ, right) against diet isotopic values. (b) Estimated slopes of the relationship between diet isotopic values and diet tissue values ($\delta^{13}C$ and $\delta^{15}N$). Slopes lower than one indicate that the isotopic discrimination factor is lower for animals consuming diets with higher isotopic values. Tests are performed with slopes calculated using Ordinary Least Squares (OLS) and Major Axis (MA) regression. For comparison we include the regression coefficient of isotopic discrimination factors and diet isotopic values reported by Caut, Angulo & Courchamp (2009) or calculated by *Auerswald* from Caut, Angulo & Courchamp (2009) data

(a)

(b)

	Class	OLS			MA		Caut, Angulo & Courchamp 2009	
		OLS slope	t	P	MA slope	95% CI Interval	r from OLS	P
$\delta^{13}C$	Mammal	0·59	9·16	<0·0001	0·68	0·57–0·79	−0·69	<0·05
	Bird	1·02	0·33	0·74	1·13	1·00–1·27	0·04	NS
	Fish	0·80	3·58	<0·001	0·86	0·75–0·99	−0·50	<0·05
	Invertebrate	0·89	2·88	0·005	0·96	0·88–1·04	−0·29	<0·05
	All	0·80	8·25	<0·0001	0·89	0·84–0·94	−0·44	<0·05
$\delta15N$	Mammal	0·85	4·65	<0·0001	0·90	0·83–0·97	−0·39	<0·05
	Bird	1·01	0·31	0·76	1·06	0·97–1·16	0·04	NS
	Fish	0·74	4·32	<0·0001	0·82	0·70–0·97	−0·59	<0·05
	Invertebrate	0·70	6·19	<0·0001	0·79	0·69–0·90	−0·59	<0·05
	All	0·84	7·01	<0·0001	0·91	0·86–0·96	−0·40	<0·05

because: the definition of discrimination factor is the same even if there is some confusion in the term used; the claimed statistical bias has no qualitative effect on the conclusions; and the experimental bias due to the lack of equilibrium in controlled studies is not applicable. We completely reanalyzed our data showing that relationships between discrimination factors and diet isotopic values remain significant even after following *Perga* and *Auerswald*'s suggestions.

Despite appreciable progress since Gannes, O'Brien & Martinez Del Rio (1997) called for more laboratory studies of stable isotopes in ecology, our knowledge still relies heavily on estimating discrimination factors using trophic experiments that need to be close to wild conditions while being constrained by animal welfare. This specifically applies to omnivore species for which researchers should not use mono-specific diets, while the requirements of mixed models for diet reconstruction assume that discrimination factors for each potential item come from animals fed with only this item. The ultimate goal of our studies was to provide ecologists with a flexible method to estimate these diet specific discrimination factors, especially for omnivorous species or when data for specialized animals is lacking. Another alternative to these problems is to construct mixed diets (very practical in some cases, e.g. the wide availability of commercial pellets for fish experiments), but this amplifies some of the problems of trophic experiments, such as the aforementioned isotopic routing.

Isotopic routing involves serious concerns about the very definition of the discrimination factor, which disregards the diet constitution. In fact, the effect of the proteins of the diet in the estimation of discrimination factors has increasingly been considered. It is not only important in mixed diets when the protein source is different from the carbohydrates source, but also in mono-specific diets because proteins in the diet are generally enriched in 13C by ~4 and 6‰ over carbohydrates and lipids, respectively (Roth & Hobson 2000). Recently, some alternatives have been proposed to reduce this effect, such as calculating a discrimination factor between the tissue of the consumer and the $\delta^{13}C$ values of the protein of the diet rather than of the whole diet (Podlesak & McWilliams 2006) or considering an estimation of the percentage of the diet proteins allocated to the tissue in relation to the carbohydrates and lipids (Arneson & MacAvoy 2005). Although these physiologically based estimations will be crucial for trophic studies, their use by field ecologists (and therefore their ultimate applied usefulness), will surely depend on the feasibility of the associated method both in terms of cost and practicability.

Understanding and estimating discrimination factors remains central to trophic ecology and will certainly continue

to represent a challenge. The use of the same discrimination factor for each potential resource in mixed models has understandably been the norm so far, although it is not correct (Caut, Angulo & Courchamp 2008b). Despite its imperfection, we hope to have demonstrated here that our method remains a significant improvement to obtain more adequate estimates when no detailed data for the studied system exists. We have demonstrated its strength when distinguishing between mono-specific or mixed diets, when removing extreme values or when performing different statistical tests. We welcome constructive criticisms that would contribute to the refinement of our method or the proposal of better alternatives. As a conclusion, we join previous calls for more trophic experiments using natural diets and controlling dynamics in different tissues to achieve equilibrium, to enhance the practical use of isotopic approaches in the study of ecological processes; a call to which our studies aim to make a constructive contribution.

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Trophic experiments to estimate isotope discrimination factors

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This document provides additional information in support of the Forum article by Caut et al. (2010) in the *Journal of Applied Ecology*.

There are many errors and mistakes in Perga & Grey (2010, hereafter *Perga*) and in Auerswald *et al*. (2010, hereafter *Auerswald*) and we will here only report those that affect the arguments of their critique.

PRACTICAL DETAILS IN PERGA & GREY (2010)

They based their rejection of our method largely on literature-based arguments and on reanalysis of some of our data. A closer look at the literature cited shows that it is in part misleadingly selected. To back up their point, *Perga* only cite two reviews on the ranges of discrimination factors, while five more were used in Caut *et al*. (2009) that could have been used (see Table S1 below). As discussed in the main text, Perga describe only one aspect of the controversies to explain the trends of Δ^{15}N, limiting the references to those that support the hypothesis in which they base their argument (e.g. one direction of change of effects of nutritional stress forgetting that the opposite has also be found valid; Hobson *et al*. 1993; Oelbermann & Scheu 2002; Cherel *et al*. 2005; Williams *et al*. 2007; Kempster *et al*. 2007).

Most importantly, some of the data used to demonstrate that our relationships result from artifacts were surprisingly incorrect. This is especially astonishing since the data was made available to one of the authors. For example we have found major inconsistencies between Figure 4a of *Perga* and the data from which it is claimed to come from (*Supporting Information Table S1* of Caut *et al*. 2009). It seems that some of the points have been removed (e.g., Felicetti *et al*. 2003), some have been changed (*e.g*., some Δ^{15}N values from Caut *et al*. 2008), while some other points do not match the values of the original data set.

Together with other imprecisions (*e.g*. the use of 'animals' instead of 'mammals' when they excluded all other animal taxa from their analysis), surely contributed to impede our full appreciation of *Perga* critique.

PRACTICAL DETAILS IN AUERSWALD ET AL. (2010)

Auerswald based their main claims invalidating our method largely on literature-based arguments and simulations of random data. A closer look at the literature cited shows that it is in part misleadingly selected. As discussed in the main text, twice *Auerswald* choose references that did not support their statements: when talking about relationships between discrimination factors and diet isotopic values he states that other 'recent compilations of discrimination factors have not identified such effects' using four references none of which have tested these effects. The same applies to the references used to support their suggestion of using diet-tissue shift instead of discrimination factor, only one the three, a self citation, uses exactly the same term (Definition Section). Similar to *Perga*, *Auerswald* describe only one aspect of the controversies on the spurious correlations, limiting the references to those that support the hypothesis in which they base their argument.

Some of the analysis and simulations made by *Auerswald* are difficult to understand, and with the actual information we have been unable to replicate. To illustrate the 'spuriouness' of the results presented by Caut *et al*. (2009), *Auerswald* used the formula proposed by Kenney (1982) and Kanaroglou (1996) to correct r when the dependent variable (δp-δs) contains the independent variable (δs). They conclude that none of the significant correlations reported by Caut *et al*. (2009) remains statistically significant, and that 5 out of 8 present slopes of different sign. Probably this is due to applying a formula (eqn 6) that is only valid when x and y are uncorrelated and also applying it in a wrong way (we have been unable to reproduce their results). These formulas assign all variation of the variables to the spurious component and none to the relationship between them (see Kenney 1982). However, as already discussed, all the use of isotopes in food web ecology is based on the fact that δp is related to δs, and this is not a spurious question when questioning the validity of our results.

Table S1. Means and ranges of carbon and nitrogen discrimination factors in previous reviews on stable isotopes. We have included: the number of discrimination factors of each type (n); the topic of each review; the type of data they contain ('L' for data coming from laboratory experiments and 'F' for data coming from the field); and the taxa that each review focuses on (M for mammals, B for birds, F for fishes and I for invertebrates). We have included data coming from Caut, Angulo & Courchamp (2008 and 2009) for comparison. Mean and ranges of nitrogen values reported by Caut, Angulo & Courchamp (2009), considering or removing mixed diets, are within the ranges of previous reported means and ranges, respectively. Means of δ15N coming from the review of Caut, Angulo & Courchamp (2009) differ slightly from those calculated only with mono-specific diets (removing mixed diets), while ranges remained the same. Mean of carbon values reported by Caut, Angulo & Courchamp (2009), considering or removing mixed diets, are always between the ranges of previous reviews, but ranges are higher. Means of δ13C varied little (3‰ for all taxa and 5‰ for mammals) and ranges were reduced (by 1.6‰ for all taxa and 3,3‰ for mammals) when calculated with only mono-specific diets.

Study	$\Delta^{13}C$			$\Delta^{15}N$			Topic	Lab or Field	Taxa
	n	Mean (SD)	[min; max]	n	Mean (SD)	[min; max]			
Minagawa and Wada 1984		-	-	26	3.4 (1.1)	[1.3; 5.3]	Enrichment in food chains	L & F	M,B,F,I
Vander Zanden and Rasmussen 2001	42	0.49 (0.19)	[-2.1; 2.8]	35	2.9 (0.3)	[-0.7; 9.2]	Variation in δ15N and δ13C trophic fractionation	L & F	F,I
Post 2002	-	0.4 (1.3)	[-3; 4]	56	3.4 (0.13)	[0.5; 5.5]	Trophic position estimates	L & F	M,B,F,I
McCutchan et al. 2003	111	0.4 (0.12)	[-2.7; 3.4]	83	2 (0.2)	[-0.8; 5.9]	Variation in trophic shift	L	M,B,F,I
Vanderklift and Ponsard 2003		-	-	134	2.54 (0.11)	[-3.22; 5.89]	Variation in $\Delta^{15}N$	L	M,B,F,I
Robbins et al. 2005		-	-	33	3.21 (0.2)	[1.4; 5.8]	Diet protein quality effect	L	M,B
Sweeting et al. 2007		-	-	56	2.79 (1.46)	[-0.3; 5.6]	Body size & environment effect	L	F
Caut et al. 2008*	21	-1.27 (0.32)	[-5.12; 1.02]	21	2.54 (0.26)	[1.12; 4.59]	Diet isotopic ratio effect	L	M
Caut et al. 2009*	283	0.98 (0.1)	[-5.12; 6,1]	261	2.81 (0.10)	[-3.22; 9.2]	Diet isotopic ratio effect	L& F	M,B,F,I
Only mammals*	76	1.28 (0.18)	[-2.2; 6.1]	69	3.4 (0.14)	[1.4; 6.4]			
Caut et al. 2009**	130	0.66 (0.16)	[-4.4; 5,2]	124	2.87 (0.17)	[-3.22; 9.2]			
Only mammals*	27	1.72 (0.27)	[-0.5; 4.5]	26	3.23 (0.26)	[1.4; 6.4]			

* Without data of unshaved hair

** only data with mono-specific diets

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