

## FORUM

# Biases in the analysis of stable isotope discrimination in food webs

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## Summary

1. Recently, Caut, Angulo & Courchamp (2009, *Journal of Applied Ecology*) published a review on diet-tissue isotopic shifts in animals. They concluded that diet-tissue shifts are influenced by the isotopic composition of the diet for both <sup>13</sup>C and <sup>15</sup>N in a wide range of animal taxa.
2. We suggest that the conclusion of Caut, Angulo & Courchamp is in error, and provide a discussion of sources of error in the assessment of diet-tissue discrimination.
3. Errors in the derivation of diet-tissue shifts include imprecise definitions, mathematical artefacts and invalid statistical analysis. It is likely that the work also suffers from experimental bias. The mathematical artefacts and statistical invalidity result from using the same variable (diet isotopic composition) in the independent and dependent variable for regression analysis and failure to correct for the resulting bias. Experimental bias can result from the incomplete turnover of body pools after diet switches or during natural fluctuations in diet isotope composition. Unfortunately, the main sources of error work in the same direction, strengthening the biased relationship between the diet-tissue shift and diet isotope composition. Therefore, the analysis of Caut *et al.* (2009) does not provide proof of a relationship between diet-tissue shift and diet isotope composition.
4. *Synthesis and application.* Future work on diet-tissue discrimination factors should (i) follow the mathematical rules resulting from how isotope data are presented, (ii) be based on appropriate statistics analysis that avoids or corrects for spurious self-correlations, and (iii) consider possible complications associated with the presence of slowly turning-over stores and non-equilibrating 'dead' body pools.

**Key-words:** C-13, carbon, diet-tissue shift, feed, fish, invertebrates, mammals, N-15, nitrogen

## Introduction

Caut, Angulo & Courchamp have recently reviewed carbon and nitrogen isotope discrimination factors in mammals, birds, fish and invertebrates (Caut, Angulo & Courchamp 2009). This extended an earlier study on an omnivorous consumer (Caut, Angulo & Courchamp 2008). These authors concluded that discrimination factors are influenced by the isotopic composition of the diet in all taxonomic groups, with statistically significant effects in most comparisons. If true, this finding is important for all studies of diet-tissue shifts, including nutritional ecology, archaeology and forensics. However, other

recent compilations of discrimination (or diet-tissue shifts) have not identified such effects (e.g. McCutchan *et al.* 2003; Sponheimer *et al.* 2003a; Hwang, Millar & Longstaffe 2007; Männel, Auerswald & Schnyder 2007). Also the conclusion of Caut, Angulo & Courchamp conflicts with the rate law of chemical reactions that states that the rate constant of a reaction is independent of the concentration (IUPAC 1997; Wright 2004). This applies to kinetic isotope effects, which are given by the ratio of the reaction constants for the heavier and the lighter isotopes (Baertschi 1952). In this comment, we draw attention to general sources of bias in isotopic food chain experiments and we suggest that the effect reported by Caut *et al.* (2009) is flawed by invalid mathematical/statistical procedures and possibly experimental bias.

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## Definitions

First, we note that the term ‘discrimination factor’ (or ‘fractionation factor’), as used by Caut, Angulo & Courchamp and recently also by others (e.g. Pearson *et al.* 2003; Robbins, Felicetti & Sponheimer 2005; Carleton *et al.* 2008; Fisk *et al.* 2009; Wolf, Carleton & Del Rio 2009) was originally defined in a different way in the bio- and geosciences (e.g. Craig 1954; Farquhar, Ehleringer & Hubick 1989; Cerling & Harris 1999; Mook 2001). This is leading to ambiguous use of the term, a source of scientific error (Ioannidis 2005). Originally and still in most cases the discrimination factor, abbreviated  $\alpha$  (cf. Baertschi 1952, 1953; Craig 1954; Farquhar *et al.* 1989; Mook 2001), was defined as the ratio of the reaction constants  $k$  of the heavier ( $^{heavy}k$ ) and the lighter isotope ( $^{light}k$ ).

$$\alpha = \text{light } k / \text{heavy } k \quad \text{eqn 1}$$

Equation 1 is equivalent to the ratio  $R_S/R_P$ , where  $R$  denotes the abundance ratio of heavy to light isotopes and the indices S and P indicate the substrate (diet) and the product (animal tissue or body) of a reaction chain. Following the definition of  $\delta$  ( $\delta = (R_{\text{sample}}/R_{\text{standard}}) - 1$ , where  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the ratios of the heavy to the light isotopes in the sample and a standard; McKinney *et al.* 1950) eqn 1 is also equivalent to the ratio  $(1 + \delta_S)/(1 + \delta_P)$ , where  $\delta_S$  denotes the isotopic composition of the substrate and  $\delta_P$  that of the product.

This definition of  $\alpha$  is important as the rate law for chemical reactions implies that the reaction constants and hence their ratio  $\alpha$  is independent of the isotopic composition of the reactant (i.e. the substrate of the reaction which is the diet in trophic relations) (IUPAC 1997; Wright 2004). The discrimination factor in its strict definition can hence not be influenced by the isotopic composition of the diet. Synonymous terms for  $\alpha$  are ‘isotope effect’, ‘fractionation factor’ or ‘enrichment factor’ (Park & Epstein 1960; O’Leary 1981; Farquhar *et al.* 1989; Cerling & Harris 1999). As a ratio  $\alpha$  can be used in multiplication operations with the isotopic composition of the diet (or more generally: the source) and is hence called factor and differs from discrimination.

Discrimination,  $\Delta$  (*sensu* Blackmer & Bremner 1977; Farquhar *et al.* 1989) equals the difference between  $\alpha$  and unity:

$$\Delta = \alpha - 1 \quad \text{eqn 2}$$

Equation 2 is equivalent to

$$\Delta = \frac{\delta_S - \delta_P}{1 + \delta_P} \quad \text{eqn 3}$$

A synonymous term for  $\Delta$  is ‘enrichment’ (Craig 1954; Cerling & Harris 1999).

The ‘discrimination factor’ as named by Caut, Angulo & Courchamp (here designated  $\Delta'$ ) is the numerical difference between product and substrate isotope composition:

$$\Delta' = \delta_P - \delta_S \quad \text{eqn 4}$$

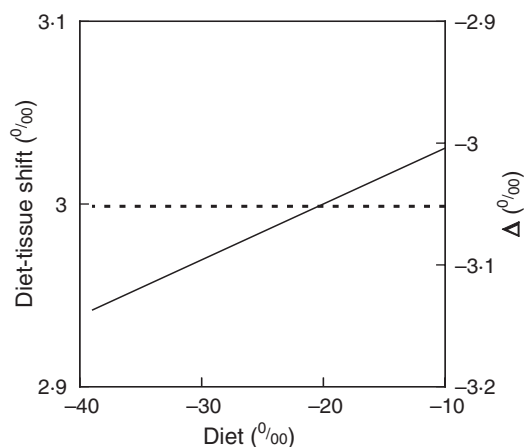
So, the ‘discrimination factor’ reported by Caut, Angulo & Courchamp should be termed ‘diet-tissue shift’ (or similar) (e.g. Ambrose 2000; Darr & Hewitt 2008; Auerwald *et al.* 2009). This distinction is particularly important for diet-tissue discrimination studies with a wide range of diet isotopic composition, because discrimination (eqn 3) is independent of the isotopic composition of the diet, whereas the diet-tissue shift (eqn 4) is not (Fig. 1). Although the magnitude of the effect is small, it does affect the relationship between the diet-tissue shift and diet isotope composition presented by Caut, Angulo & Courchamp, since it reduces the (negative) slope.

## Possible sources of bias

In our opinion, the analysis of Caut, Angulo & Courchamp is affected by two sources of bias: one is a mathematical/statistical artefact, the other is an experimental bias. As we show, both sources of bias (unfortunately) work in the same direction, mutually amplifying the spurious effect of diet isotope composition on the diet-tissue shift.

## Mathematical artefact

Any measured value of diet isotope composition is an estimate, which is termed ‘random variable’ in statistics. Any random variable is composed of its true value ( $\mu$ ) and a random error ( $\epsilon$ ), which can be treated by statistical procedures. The same applies to parameters derived from random variables like the mean. In addition, bias may occur, which cannot be statistically treated by averaging and other procedures. Assuming no bias for the moment, the measured value of diet isotope composition thus is  $\delta_S = \mu_S + \epsilon_S$ , and  $\delta_P = \mu_P + \epsilon_P$  for the tissue isotope composition.

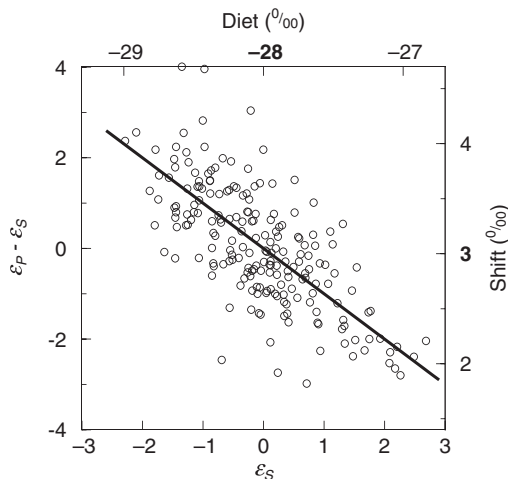


**Fig. 1.** Diet-tissue shift (broken line, left axis) and diet-tissue discrimination  $\Delta$  (solid line, right axis) as related to the isotopic composition of the diet. The calculations were made for the range of carbon isotope composition as given by Caut *et al.* (2009) and in assuming a true diet-tissue shift of 3‰ for a diet of  $-20$ ‰ and no errors associated with the measurements.

There are many sources of error, including the errors of sampling and measurement, which contribute to  $\varepsilon_S$  and  $\varepsilon_P$ . When regressing the difference between measured values of diet and tissue isotope composition (i.e. a ‘measured’ diet-tissue shift:  $\delta_P - \delta_S$ ) against the measured value of diet isotope composition ( $\delta_S$ ), as carried out by Caut, Angulo & Courchamp, then the error term of the independent variable ( $\varepsilon_S$ ) appears in the independent and the dependent variable, causing a erroneous (artificial) relationship between the two variables (Fig. 2), which is termed spurious correlation in statistics (Pearson 1897; Kenney 1982; Kanaroglou 1996).

Therefore, regression analysis and related statistical procedures require that the independent and the dependent variable are independent measurements (Fox 1997; Sachs & Hedderich 2006). This is not the case when the dependent variable includes the independent. So, the regression of diet-tissue shift against diet isotope composition is invalid and invalidates any further statistical evaluation. In support of their approach, Caut, Angulo & Courchamp cited the Hills (1978) paper, but this does not treat this particular statistical issue, and it does not contain arguments in support of their approach.

The reason for this statistical rule is illustrated in Fig. 2 by using a biologically meaningful example: it shows the artefact, which results when the error of the independent variable is included in the dependent variable in a regression analyses. The analysis is based on one pair of samples, a diet and an animal tissue, for which the true isotopic values are known:  $\mu_S -28\text{‰}$  and  $\mu_P -25\text{‰}$  (thus, the diet-tissue shift is  $3\text{‰}$ ). The total error (measurement, sampling and other) was assumed to yield a standard deviation of  $0.4\text{‰}$  for both diet and tissue data. From this we drew 200 random pairs of ‘measured’ diet and tissue isotope values, and plotted the ‘measured’ diet-tissue shift



**Fig. 2.** Shift between two random numbers ( $\varepsilon_S$  and  $\varepsilon_P$  equivalent to the errors  $\varepsilon$  of the diet and the tissue) depending on random number  $\varepsilon_S$ ; both random numbers were generated for a true mean of 0 and a true standard deviation of 1;  $n = 200$ ; the line has a slope of  $-1$ . The top axis and the right axis exemplify the effect of the error terms  $\varepsilon$  for a true isotopic value of  $-28\text{‰}$  of the diet and a true shift of  $3\text{‰}$  assuming standard deviations for the measurements of diet and tissue of  $0.4\text{‰}$ .

against diet isotope composition (Fig. 2). Subsequently, we performed a regression analysis. This yielded a strong dependence of the diet tissue-shift on diet isotope composition. As is apparent, the artefact resulted solely from including the random error of the independent variable in both the independent and the dependent variable.

Based on statistical reasoning and the example given above, we suspect that the effect of diet isotope composition on the diet-tissue shift, as reported by Caut, Angulo & Courchamp, is mainly a mathematical/statistical artefact. Of course, the data set of Caut, Angulo & Courchamp also contains true variation of diet isotope composition, but this does not fix the error. In our ideal example: if we avoided the artefact by regressing the ‘measured’ values of tissue against the ‘measured’ values of diet (not shown), then the slope of the relationship between the two was one. This meant that estimates of diet and tissue isotope composition varied in parallel, and the error was randomly distributed around the regression. So, there was no effect of diet isotope composition on discrimination, consistent with the initial specifications.

Kenney (1982) and Kanaroglou (1996) have shown formally for the case where the dependent ( $\delta_P - \delta_S$ ) is a difference with the independent ( $\delta_S$ ) that the equation of the correlation coefficient  $r$  becomes:

$$r_{\delta_S, \delta_P - \delta_S} = \frac{s_{\delta_P} / s_{\delta_S} \times r_{\delta_S, \delta_P} - 1}{\sqrt{1 + (s_{\delta_P} / s_{\delta_S})^2 - 2 \times s_{\delta_P} / s_{\delta_S} \times r_{\delta_S, \delta_P}}} \quad \text{eqn 5}$$

where  $s$  denotes the standard deviation of  $\delta_P$  and  $\delta_S$ . For our example, where we assumed no correlation between  $\delta_P$  and  $\delta_S$  and an equal  $s$  for  $\delta_P$  and  $\delta_S$ , eqn 5 predicts  $r_{\delta_S, \delta_P - \delta_S} = 0.707$ , which matches what we found ( $r_{\delta_S, \delta_P - \delta_S} = 0.708$ ). Eqn 5 can then be used to predict and subtract the spurious component ( $r_s$ ) of the correlation coefficient  $r_{\delta_S, \delta_P - \delta_S}$  (Kenney 1982; Kanaroglou 1996):

$$r_s = \frac{-1}{\sqrt{1 + (s_{\delta_P} / s_{\delta_S})^2}} \quad \text{eqn 6}$$

Applying eqn 6 to the data sets of Caut, Angulo & Courchamp turns all previously significant correlations insignificant (Table 1) proving that the effect found by Caut, Angulo & Courchamp is entirely spurious in their data set. The sign of the correlation coefficient even changed from negative to positive after correction, which agrees with the prediction in Fig. 1 that the calculation of a shift instead of discrimination should cause a small positive effect.

Equation 5 implicitly predicts that the range and the strength of the relationship in Fig. 2 depend on the errors of the two measured variables, which may not be the same. If the error for the tissue is lower than that for the diet, then the relation in Fig. 2 becomes tighter (higher  $r^2$ ). If tissue measurements have no error (which could approximately be true where measurements of the same tissue are repeated many times) then the  $r^2$  equals

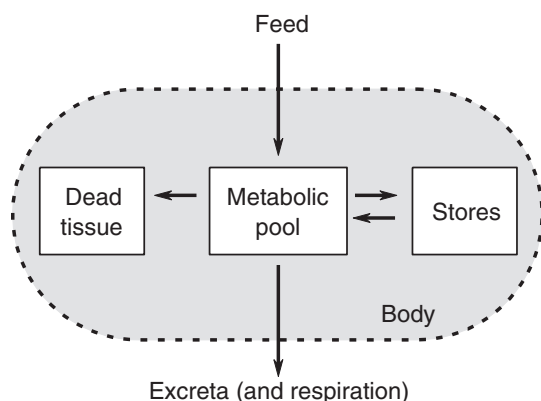
**Table 1.** Correlation coefficients between the diet-tissue shift and the isotopic composition of the diet ( $r_{\delta S, \delta S - \delta P}$ ) as calculated by Caut *et al.* (2009) and correlation coefficients corrected for the spurious component according to Kenney (1982) ( $r_{corr}$ ) for the nitrogen and carbon isotopic composition and four classes of animals

Class		<i>n</i>	$r_{\delta S, \delta S - \delta P}$	$r_{corr}$	Change in significance from $r_{\delta S, \delta S - \delta P}$ to $r_{corr}$
$\delta^{13}C$	Mammals	96	-0.69	-0.01	$P < 0.05 \rightarrow NS$
	Bird	61	0.04	0.25	$NS \rightarrow NS$
	Fish	40	-0.50	0.17	$P < 0.05 \rightarrow NS$
	Invertebrates	90	-0.29	0.20	$P < 0.05 \rightarrow NS$
$\delta^{15}N$	Mammals	79	-0.39	0.20	$P < 0.05 \rightarrow NS$
	Bird	53	0.04	0.38	$NS \rightarrow P < 0.05$
	Fish	37	-0.59	0.24	$P < 0.05 \rightarrow NS$
	Invertebrates	74	-0.59	0.14	$P < 0.05 \rightarrow NS$

one. Conversely, if the error for the tissue is larger than for the diet, then  $r^2$  is smaller than in Fig. 2. In general, we assume that it is quite difficult to maintain a diet with constant isotopic composition for a sufficiently long period of time (see Experimental bias). Thus, achieving an isotopic equilibrium between diet and tissue and subsampling of diet is more demanding than sampling of tissue, generating larger errors for diet.

### Experimental bias

An animal body comprises several compartments, which can be assigned to one of three functional groups, which are relevant to isotopic exchange kinetics: (i) metabolic pools which are supplied by digesta, (ii) stores which exchange carbon or nitrogen compounds with metabolic pools (thus showing turnover), and (iii) inert (dead) tissues which receive carbon or nitrogen from metabolic pools during formation, but do not exchange with them (no turnover) (Fig. 3). While metabolic pools respond rapidly to a change in the isotopic composition of the diet, they do not reach isotopic equilibrium until stores



**Fig. 3.** Conceptual model of an animal as composed of three functional compartments: (i) a metabolic pool, (ii) stores which exchange matter with the metabolic pool, and (iii) inert ('dead') tissue, which unidirectionally receives matter from the metabolic pool during formation.

are fully equilibrated (Ayliffe *et al.* 2004). Stores include liver, muscle, bone collagen and fat stores, which differ in turnover times (Telang, Buck & Wheeler 2002; Ojeda-Avila, Woods & Raguso 2003; Ayliffe *et al.* 2004; Sponheimer *et al.* 2006; Cerling *et al.* 2007; Zazzo *et al.* 2007). 'Dead' tissues include hair, feathers or chitin skeletons, which do not equilibrate with metabolic pools and stores (Montagna & Ellis 1958; O'Connell & Hedges 1999; Roth & Hobson 2000). The presence of 'dead' pools makes it impossible to achieve isotopic equilibrium in experiments with whole-body analysis except if they are renewed by shedding or moulting. The presence of stores also hampers the assessment of true diet-tissue discrimination, as they can have half-lives which are orders of magnitude higher than those of metabolic pools, which complicates their detection (Ayliffe *et al.* 2004). This problem, failure to equilibrate isotopically, causes an experimental bias.

Unfortunately, the mathematical artefact and experimental bias caused by the isotopic memory, work in the same direction, strengthening the bias. The main difference between the two sources of errors is in the value of the slope. Concerning the mathematical artefact: here the slope is always  $-1$  for the diet-tissue shift (or close to 1 for discrimination) provided that there is no real variation in the isotopic composition of the diet (Fig. 2). But, the slope decreases with increasing variation of the diet. That is, the bias gets smaller if diet isotope composition varies much. Theoretically, the slope becomes nil, if the variation of diet isotope composition is infinite.

Concerning the experimental bias: the slope (bias) is equal to the proportion of non-equilibrated body material in non-equilibrium feeding studies. Accordingly, as a special case, the slope is  $-1$  for the diet-tissue shift immediately after a diet switch, when diet and tissue have not started to equilibrate at all.

Clearly, studies with constant chemical and isotopic composition of feed are mainly affected by the mathematical artefact. Conversely, studies with varying feed isotope composition suffer mainly from the memory effect.

As explained above, we cannot be certain about the exact magnitude of experimental bias in the report of Caut, Angulo & Courchamp, as a reinterpretation would require knowledge of the exact kinetics of isotopic composition of metabolic, storage and dead pools of all animals in the review. But these data are usually not reported in the original studies. Yet, in the Caut, Angulo & Courchamp review about 10% of the studies used diet-switches and these are particularly sensitive to isotopic memory effects. Such studies occupy the extremes of the range of diet isotope compositions and therefore have a strong leverage effect on the regression. In addition, about 30% were field studies, which are affected naturally by variation in diet isotope composition.

The problem can only be overcome by studies in which isotopically (and chemically) uniform feed is provided from birth to death, similar to the classical experiments by De Niro & Epstein (1978, 1981). Where maternal material constitutes a significant proportion of body mass, controlled and constant feeding must start with the mothers as in the experiments by Hobson & Clark (1992). If diet-tissue shifts are to be based on metabolic pool samples, then the constancy of diet isotope



composition should be ensured (and documented) by diet sampling over a multiple of the turnover time of relevant body stores. Still, there may be special situations, such as essential amino acids which are derived from only one feed source (e.g. Fantle *et al.* 1999).

The danger of memory effects in the estimation of feed-tissue discrimination can be reduced by avoiding whole body analysis, and focusing on a metabolic pool-like blood serum (Hilderbrand *et al.* 1996; Roth & Hobson 2000; Felicetti *et al.* 2003; Darr & Hewitt 2008) or recent products of metabolic pools, such as new hair growth (Hobson, McLellan & Woods 2000; Cerling *et al.* 2006; Schnyder *et al.* 2006; Zazzo *et al.* 2007, 2008; Mützel *et al.* 2009). A high resolution record of changes in diet isotope composition can be obtained by analysis of the longitudinal variation of isotope composition along a hair (Ayliffe *et al.* 2004). Such analyses have revealed the existence and kinetic properties of body stores and can predict the experimental bias which results from isotopic memory by stores.

## Conclusions

We agree with Caut, Angulo & Courchamp that understanding and estimating discrimination in diet consumer relationships is a very important issue in trophic network studies and diet reconstructions. Avoiding mathematical and experimental artefacts in the estimation of diet-tissue discrimination is essential to identify (and quantify) true effects of diet on discrimination. We argue that the 'Diet-Dependent Discrimination Factor' method (as proposed by Caut, Angulo & Courchamp) results from these artefacts and becomes unnecessary when these errors are avoided. True effects may result from other factors, such as diet protein content and amino acid composition (Sponheimer *et al.* 2003b; Gaye-Siessegger *et al.* 2004), diet selection (Garcia *et al.* 2003), differential digestibility of diet components in mixed rations (Caswell *et al.* 1973; Fantle *et al.* 1999) or routing (Fantle *et al.* 1999; Dalerum & Angerbjörn 2005).

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