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Mixing models in analyses of diet using multiple stable isotopes: a response

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Phillips (2001) provides a thorough evaluation of several existing mixing models used in determination of animal diets from stable isotope analysis (Ben-David et al. 1997a, 1997b; Kline et al. 1993; Szepanski et al. 1999; Whitledge and Rabeni 1997). The author not only evaluates and criticizes the existing models but also proposes a mathematically correct model for three endmember situations (Phillips 2001). This linear mixing model, which is based on a standard mathematical solution for three unknowns using three equations, estimates proportions in the diet for cases with three potential food sources (Phillips 2001). The ability to simulate diets (i.e., arrive at isotopic values from predetermined proportions) is a strength of the linear mixing model compared with the models based on Euclidean distances.

Phillips (2001) tested the performance of the linear mixing model and compared the results of this model with the Euclidean-distance-based models using data published by Szepanski et al. (1999). The Euclidean distance models are based on the concept that a shorter distance between a food item and the isotopic ratios of the consumer implies greater contribution of this food to the diet. Based on this concept the relation between distance (Euclidean) and relative contribution is an inverse one. Unfortunately, the models proposed by Kline et al. (1993) and Whitledge and Rabeni (1997) fail to correctly denote this relation mathematically. Therefore, we will restrict our discussion to comparisons between the model described by Ben-David et al. (1997a, 1997b; Eq. 3 in Phillips 2001) and the linear mixing model proposed by Phillips (2001; Eq. 4). Phillips (2001) demonstrated that the proportions of moose, caribou, and salmon in the diet of wolves which are derived using Eq. 3 result in differing isotopic ratios when these proportions are used to re-calculate the isotopic ratios using the linear mixing model. For comparison, Phillips (2001) conducts the same analysis using the linear mixing model and derives different proportions for moose, caribou, and salmon in the diet of wolves. Phillips (2001) then proceeds to demonstrate that the proportions derived from the linear mixing model result in the original wolf isotopic ratios when those are re-calculated by the same formula. Because the linear mixing model is based on mass balance equations (Phillips 2001) this result is to be expected. Nonetheless, to independently evaluate the performance of the two mixing models, both models should be applied to data consisting of isotopic values of food and consumer tissues when the true proportions of the food are known.

We conducted a controlled feeding study on isotopic fractionation and response curves in captive adult mink (*Mustela vison*) fed three different diets (Ben-David 1996). One group of ten animals was fed a pure salmon diet ($\delta^{13}C$=–20.82‰, $\delta^{15}N$=12.27‰), a group of eight was fed beef diet ($\delta^{13}C$=–24.22‰, $\delta^{15}N$=6.19‰), and a third group of seven individuals was fed a mixture of 50% salmon and 50% beef. All foods were finely ground and well mixed to ensure complete consumption of diet. The salmon diet had low fat content (less than 5%) and the isotopic values of the extracted fat portions were similar to those of the lean, proteinaceous tissue. The beef diet, in contrast, contained 50% fat and 50% lean tissue with identical $\delta^{15}N$ value but radically different $\delta^{13}C$ values (fat $\delta^{13}C$=–26.18‰, lean tissue $\delta^{13}C$=–22.28‰). Thus, the mixed-diet group of seven individuals received 25% lean beef tissue, 25% beef fat, and 50% salmon. The actual isotopic signatures of the mixed diet were $\delta^{13}C$=–22.7‰, $\delta^{15}N$=9.59‰. This signature translates to 55% beef when carbon is used in a single-isotope linear mixing model and 44% beef when

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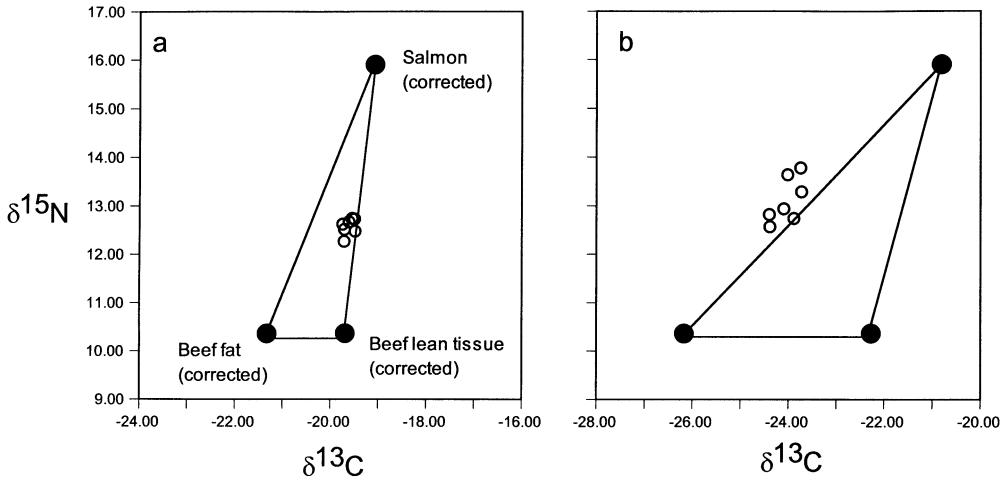

Fig. 1 Stable isotope ratios (*open symbols*) of **a** blood and **b** fat tissues collected from 7 individual captive mink at the end of a 77-day feeding trial. Values for three food items (beef lean tissue, beef fat, and salmon – *filled symbols*) were corrected for trophic fractionation between food and consumer tissues (see Fig. 3). *Lines connecting the three food items* enclose the space in which the linear mixing model will provide valid and mathematically correct results

nitrogen is used and suggests that, indeed, the mixture comprised 50% beef and 50% salmon. Mink were fed these diets for 77 consecutive days and sedated once every week for collection of blood samples (Ben-David 1996). At the end of this period and following the collection of blood, animals were humanely euthanized and tissue samples of liver, muscle, bone, and fat were collected for isotope analysis (Ben-David 1996). During the trial period of 77 days, mink in the mixed diet group gained on average 220 g (±63, SE), which corresponded to 20% of their original body mass.

To independently test the performance of the Euclidean-distance-based model and the linear mixing model, we calculated the expected proportion of each component of the diet from isotopic values of blood samples collected on the last sampling (Fig. 1), at which time equilibration was nearly complete (Fig. 2). Similarly, we obtained results from the fat tissues collected from the same animals on the same day (Fig. 1). Thus, in this study we discuss the performance of the two mixing models in determining proportions of lean beef tissue, beef fat, and salmon in the diet of the seven individual mink fed the mixed diet (Tables 1, 2).

To correct for trophic fractionation between food and consumer blood tissues we used linear regression equations developed by Hilderbrand et al. (1996) from captive feeding trials in bears. We selected these equations to avoid using data from our own captive experiment in an effort to avoid circularity. Our data fit reasonably well with these regression models (Fig. 3) and thus we feel that the resulting fractionation values are appropriate. Nonetheless, we caution against using these equations as an alternative to mixing models. While these equations may be appropriate for studying diets of bears (Hilderbrand et al. 1996) they provide no theoretical bases for the observed relations between isotopic signatures of diet and consumer. For example, fractionation values for bears eating mule deer were closer to those of bears eating apples, than to those of bears eating salmon. Because the biochemical composition of apples (e.g., amino acids, carbohydrates, and fatty acids) is likely less similar to mule deer than salmon, it is unclear what was

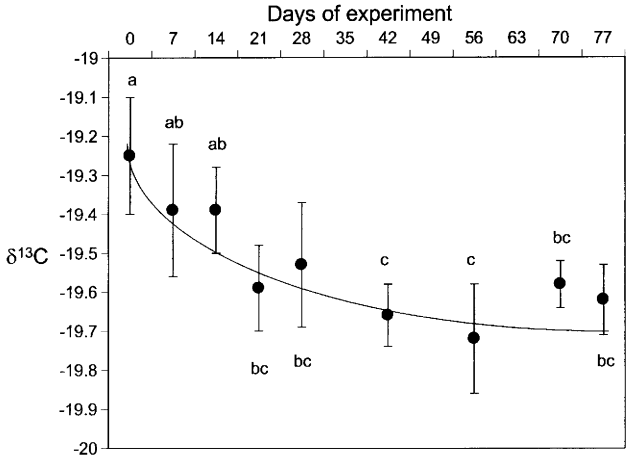

Fig. 2 Response curve of $\delta^{13}C$ in blood cells for captive mink (n=7) fed mixed diet of 25% beef fat, 25% lean beef, and 50% salmon. At the end of the study (77 days) equilibration was nearly complete (non-linear regression, r^2=0.53, P<0.001; Scheffé multiple comparisons with α=0.05; SPSS for Windows)

the cause for the similarity in fractionations in that study. In fact, from these regression equations a novice may conclude that the isotopic ratio of the diet alone determines the fractionation between diet and consumer. Ambrose and Norr (1993), DeNiro and Epstein (1978), and Tieszen and Fagre (1993) provided evidence against such an erroneous conclusion.

Determining carbon fractionation values between diet and fat tissues of consumers presented a challenge because only one other study investigated this relation. DeNiro and Epstein (1978) found no fractionation in carbon between consumer fat tissues and diet in two species of flies (*Calliphora* sp. and *Musca* sp.) fed pork diet but described a 2–3‰ fractionation when horse meat was offered. We chose to make no correction in this study because any fractionation in carbon would have placed the observed values of mink fat tissues well outside the predicted area outlined by the possible foods (Fig. 1). Because we use the same data to compare between the two mixing models we feel this solution is valid.

Table 1 Estimates of the dietary proportions of three foods in the diet of 7 individual captive-mink fed a known diet of 25% beef lean tissue, 25% beef fat, and 50% salmon using an Euclidean distance mixing model (Ben-David et al. 1997a, 1997b) and a linear mixing model (Phillips 2001). Isotope ratios were determined for blood samples collected from each mink at the end of a 77-day dietary trial. Fractionation factors were determined using linear regression equations developed by Hilderbrand et al. (1996) from captive feeding trials in bears

Method	Isotopic ratios of mink blood		Percent in the diet		
	$\delta^{13}C$	$\delta^{15}N$	Beef lean tissue	Beef fat	Salmon
True			25	25	50
Euclidean distance	–19.61	12.47	42	33	25
	–19.72	12.07	47	33	20
	–19.75	12.42	43	33	24
	–19.71	12.32	44	33	23
	–19.55	12.54	42	32	26
	–19.50	12.53	42	32	26
	–19.49	12.28	45	32	23
Linear model	–19.61	12.47	53	9	38
	–19.72	12.07	57	12	30
	–19.75	12.42	47	17	37
	–19.71	12.32	51	14	35
	–19.55	12.54	56	6	39
	–19.50	12.53	59	2	39
	–19.49	12.28	66	0	34

Table 2 Estimates of the dietary proportions of three foods in the diet of 7 individual captive-mink fed a known diet of 25% beef lean tissue, 25% beef fat, and 50% salmon using an Euclidean distance mixing model (Ben-David et al. 1997a, 1997b) and a linear mixing model (Phillips 2001). Isotope ratios were determined for fat samples collected from each mink at the end of a 77-day dietary trial. Fractionation factors for carbon were determined based on results from DeNiro and Epstein (1978)

Method	Isotopic ratios of mink fat		Percent in the diet		
	$\delta^{13}C$	$\delta^{15}N$	Beef lean tissue	Beef fat	Salmon
True			25	25	50
Euclidean distance	–24.1	12.74	39	36	25
	–23.75	13.58	36	31	33
	–23.73	13.09	39	32	29
	–24.02	13.44	36	33	31
	–24.39	12.37	38	41	22
	–24.4	12.62	37	40	23
	–23.89	12.54	41	35	24
Linear model	–24.1	12.74	–6	63	43
	–23.75	13.58	–20	67	53
	–23.73	13.09	–5	63	42
	–24.02	13.44	–24	68	56
	–24.39	12.37	–4	62	42
	–24.4	12.62	–11	64	47
	–23.89	12.54	6	59	34

Table 1 describes the results from determining the proportions of lean beef, beef fat, and salmon in the diet of mink from stable isotope values obtained from blood samples using the Euclidean distance model (Eq. 3 in Phillips 2001) and the linear mixing model (Eq. 4). In the Euclidean distance model, the estimated proportion of lean beef in the diet varies between 42 and 47%, while in the linear mixing model it varies between 47 and 66%. Both methods overestimate the proportion of lean beef, which was 25%. Similarly, both models underestimated the proportion of salmon in the diet (Euclidean distance model 20–26%, linear mixing model 30–39%). The largest difference is evident in the estimation of proportion of beef fat in the diet. The Euclidean distance model overestimates the percentage of this food in the diet (32–33%) while the linear mixing model underestimates that percentage (0–17%). Visual examination of the data may lead to the conclusion that although the mink ingested the fat portion of their diet, they did not assimilate it. Clearly, the isotopic values of the mink blood and the results of the linear mixing model suggest that this is a correct interpretation. Thus, it seems that the linear mixing model provides a better representation of the assimilation of the different foods by the consumer than the Euclidean distance model. Nonetheless, neither model resulted in an accurate and unequivocal description of the proportions of the different foods ingested by our captive mink.

The conclusion that the mink did not assimilate the ingested fat portion of their diet requires further consideration. Investigation of the results from the fat tissues of the mink indicates that beef fat was assimilated by the mink and deposited in the fat tissues (Fig. 1). This represents a case of specific routing of resources. Other studies demonstrated that different components of the diet (i.e., carbohydrates, lipids, and proteins) are usually routed to different tissues without being first homogenized and repartitioned (Ambrose and Norr 1993; Tieszen and Fagre 1993). Calculations of the proportion of lean beef, beef fat, and salmon in the diet of mink from stable isotope values obtained from fat samples using both models (Eqs. 3, 4 in Phillips 2001) demonstrated that

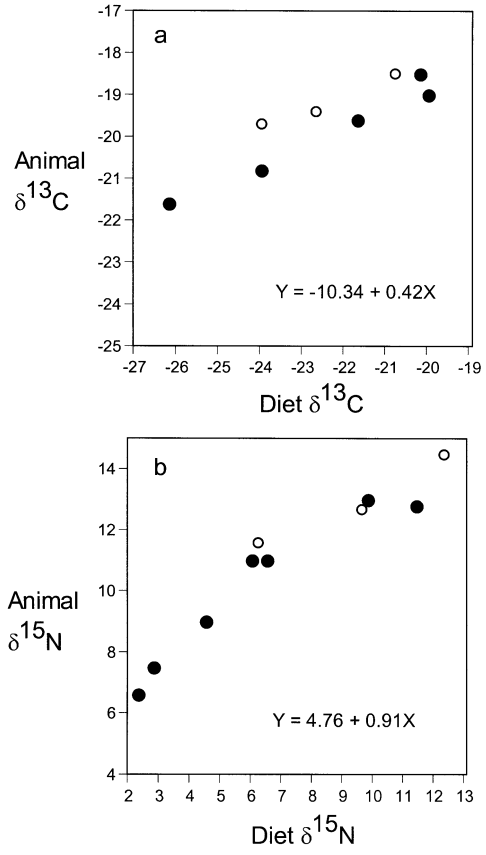

Fig. 3 Relation between **a** carbon and **b** nitrogen isotope ratios of diet and bear blood tissues (*filled symbols*, average values, adapted from Hilderbrand et al. 1996) and those of captive mink fed 100% beef, 100% salmon and a mixed diet of 50% beef and 50% salmon (*open symbols*, average values, adapted from Ben-David 1996). Fractionation values for use in the comparison of the two mixing models were derived solely from the regression equations from the bear data

both methods fail to accurately describe the actual proportions of these items (Table 2), probably because both models assume complete mixing and repartitioning of C and N from all sources.

In this case, the linear mixing model resulted in negative values (out-of-bound values – Phillips 2001; Fig. 1). This may suggest that an additional food source was not identified or alternatively that the tissue turnover time of fat was longer than the duration of the experiment and our observed values also include residual signatures from previous diets. While no additional foods were available to the mink in our study, it is possible that the observed values were an integrated signature of the experimental and previous diets. Nonetheless, our observation that the captive mink gained on average 20% of their original body mass while feeding on this diet and the fact that all of them were adults make it likely that the weight gain was largely a factor of fat deposition. Thus, it is likely that the sub-sample used in our analysis represented fat deposition from the experimental diet. Our decision to use a trophic fractionation value of zero is supported by our observation that the fractionation between diet and mink fat in the beef-fed group was –0.07 (±0.07 SE). This suggests that the out-of-bound values in our data were not a result of incorrect selection of trophic fractionation value, a failure to identify all potential food sources, or non-equilibrium state of tissues and diet, but rather a result of intrinsic variability in physiological responses of the study animals. That the linear mixing model resulted in negative values under such conditions, which in turn were followed by inflation of the proportions of other foods (Table 2) points to the sensitivity of the model and potentially limits its applications.

Our results demonstrated that mixing models, whether Euclidean-distance-based or linear, provide inaccurate estimates of proportions of foods in the diet and can be misleading in determining proportions of food sources in the diet of wild animals. Our analyses also indicated that the tissue selected for analysis could greatly influence the results of such models. These results emphasize the limitations of both mixing models, which stem from their failure to account for the integration between diet composition, diet selection, and animal nutritional status. Gannes et al. (1997) proposed that patterns of stable isotope ratios observed at the individual level may be a result of an interaction between ecological, physiological, and biochemical processes. Thus, individuals with similar diets may exhibit differing isotopic ratios due to intrinsic variability in assimilation efficiencies, deposition of nutrients (i.e., routing to different tissues) and other internal physiological processes such as nutrient cycling (Ambrose and Norr 1993; Hobson et al. 1993; Schwarcz 1991; Tieszen and Fagre 1993). For example, the nutritional status of the animal (which may in part depend on previous diets, energy and protein balance, and reproductive status) together with the composition of the diet (i.e., carbohydrates, lipids, and protein, as well as amino acid and fatty acid composition) may influence the fate of the different dietary components. While one consumer may assimilate structural lipids and proteins from one source but derive most of its energy from another source, resulting in an underestimate of the latter when the consumer tissues are analyzed (Schwarcz 1991), other individuals with different physiological states may assimilate lipids and proteins from both sources.

Based on our observations, we believe that the primary value of mixing models (Euclidean distance or linear) is in providing a heuristic tool for interpreting dietary results. We encourage investigators to treat both models as methods for obtaining a general index of animal diets rather than as true and correct estimates of proportions in the diet. We also caution that the sensitivity of the linear mixing model to out-of-bound values may reduce its utility. Under such circumstances, researchers may find the Euclidean distance model appropriate for deciphering general patterns in their data.

Phillips (2001) criticizes the application of the Euclidean distance model in situations with more than three end-members (Ben-David et al. 1997b). Indeed, the

occurrence of more than three potential food items creates an under-determined system with multiple potential solutions (Phillips 2001). We fully agree with this criticism. Indeed, the Euclidean distance model creates the misleading impression of a single solution. Nonetheless, situations in which only three potential food sources exist are rare in nature. In such cases, a heuristic investigation of the relative importance of different food types may provide researchers with important insights. We encourage investigators to ascertain the number of endmembers as rigorously as possible and report that their results represent an index rather than a correct estimate of proportions of diet. In addition, we encourage researchers to perform their subsequent statistical analyses on the raw isotopic data rather than on the results of mixing models. We also hope to see additional controlled studies including those that address additional stable isotopes of other elements (Hobson 1999) that will further elucidate the relations between diet composition, metabolic pathways, resulting consumer tissue composition, and associated isotopic data. We hope that such studies will enhance our ability to develop mixing models that will account for both diet and physiological status of animals and will be more accurate and applicable than the current ones.

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