Reprinted from:
Physiological and Ecological Adaptations to Feeding in Vertebrates
J. Matthias Starck and Tobias Wang (Editors)
Science Publishers 2005, Enfield, New Hampshire
ISBN 1-57808-246-3

6

Mass-Balance Models for Animal Isotopic Ecology

Carlos Martínez del Rio[1] and **Blair O. Wolf**[2]
[1] University Wyoming, Department of Zoology Physiology,
Laramie, WY, USA,
[2] University New Mexico, Department of Biology,
Albuquerque, NM, USA

SYNOPSIS

Analysis of natural stable isotope ratios has created a methodological upheaval in animal ecology. Because the distribution of stable isotopes in organisms follows reliable patterns, their analyses have become established useful methods for animal ecologists. However, because animal ecologists have adopted a phenomenological approach to the use of stable isotopes, the mechanisms that create isotope variation patterns remain unexplored. The mass-balance models that can provide a mechanistic, and hence predictive foundation for animal isotopic ecology are presented here. We review and elaborate the current mixing models used to reconstruct animal diets and develop new mathematical models to explain one of the most widely used patterns in animal isotopic ecology: enrichment in ^{15}N observed across trophic levels. Construction of element and isotope budgets is central to testing the mass-balance models described herein. Because the concept of a budget is central to all animal physiological ecology, development of a mechanistic and predictive framework for isotopic animal ecology falls naturally on physiological ecologists. We argue that progress in isotopic animal ecology hinges on laboratory experiments that explore mechanism, documentation of pattern in the field, and theoretical integration of mechanism and pattern.

INTRODUCTION

Democritus was right: living organisms are collections of interacting atoms. We now believe that atoms are made of electrons clouding around a

nucleus made up of protons and neutrons. The numbers of charged particles (electrons and protons) within the atom are equal, so the whole atom is electrically neutral. The neutrons stop the nucleus from tearing itself apart. They work as a glue that bonds with the protons and provides cohesion within the nucleus. Elements with the same number of protons but a different number of neutrons are called isotopes and vary in mass. Most of these isotopes are stable (do not undergo radioactive decay) and can be distinguished by their mass. Many physicochemical processes are sensitive to differences in the dissociation energies of molecules, which often depend on the mass of the elements of which these molecules are made [Ball (2002) provides a particularly good introduction to atoms, elements, and isotopes].

The enzymatic pathways that organisms use to manufacture and transform organic molecules for example, can be isotopically discriminating. In general, it is easier to form, or break, bonds that contain lighter isotopes. The result is that molecules that contain the lighter isotope are preferentially incorporated into the products of incomplete reactions. As a result, the unreacted residues become enriched in the heavier isotope (Hoeffs, 1997). These isotopic effects are useful. The isotopic composition of many materials, including the tissues of organisms, often contains a label of the process that created it. Ecologists and physiologists can use these labels or isotopic signatures to detect the imprint of processes at a variety of scales. Plant physiologists, atmospheric scientists, and geochemists have relied on the measurement of natural stable isotope signatures for decades (Lajtha and Michener, 1994). Animal physiologists and ecologists, on the other hand, have been tardy in joining the isotopic research enterprise. Only one chapter in a recent review on the use of stable isotopes to integrate biological, ecological, and geochemical processes deals with animals (Griffith, 1998). Interestingly, the animals that this chapter deals with are extinct (Cerling et al., 1998 in Griffith, 1998). Indeed, paleontologists and archaeologists have been unusual among zoologists in their reliance on stable isotopes as tools in the reconstruction of the diets and habits of extinct animals and ancient humans (Koch et al., 1994 and references therein). Although zoologists have been latecomers, we have recently been active. The number of publications in animal ecology and physiological ecology that use stable isotopes has doubled every 3 years over the last 10 years (Fig. 6.1). This is a phenomenal rate of increase for the incorporation of any scientific methodology. As the following, almost certainly incomplete list attests, a large variety of phenomena in animal ecology can be informed by an isotopic approach. Stable isotopes have been used to reconstruct animal diets (Hobson et al., 1994), determine patterns of resource allocation to reproduction (O'Brien et al., 2002), track animal migration (Hobson, 1999), assess the flux of materials from the sea into terrestrial food webs (Ben David et al., 1998), assign trophic levels (Post, 2002), and to determine the structure of food webs (France, 1995).

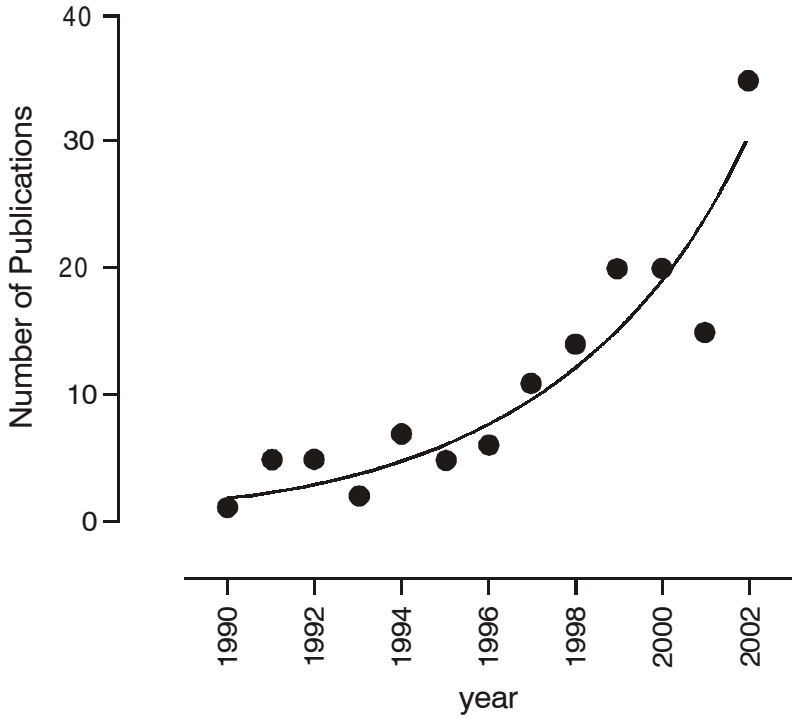

Fig. 6.1. Number of publications on animal ecology and physiological ecology that rely on stable isotopes has increased exponentially ($r^2 = 0.81$) in the last 10 years with a proportional rate of increase of 23%. Number of publications in this data set obtained by searching Biological Abstracts using "stable isotopes" and at least one of the following terms as key words: "animal", "diet", "food web", and "migration".

In geochemistry, plant physiology, physiological ecology, progress in the use of stable isotopes relies on vigorous interaction between theory, laboratory research, and field study [the chapters in Griffiths (1998) volume are superb examples]. With few exceptions (some of which are reviewed below), animal ecologists have adopted a different pathway. The vast majority of our field isotopic studies are phenomenological and a well-developed theoretical edifice does not inform our laboratory experiments. The objective of this chapter is to outline what we believe are some of the elements of a mechanistic theoretical framework for the isotopic ecology of animals. Like work in plant physiology and geochemistry, we too rely on mass-balance models to disentangle the relative importance of the factors that determine animal tissue stable isotopic composition. The two broad themes considered are (1) what is the timescale of incorporation of an isotopic signal into an animal's tissues and (2) why does the isotopic composition of animal tissues often differ from that of the resources they use. We review and elaborate on the current mixing models used to reconstruct animal diets and develop new models to explain one of the most widely used patterns in animal isotopic

ecology: enrichment in ^{15}N observed across trophic levels. Although we focus on the isotopes of carbon and nitrogen for clarity, mass-balance models described here can be easily applied to other elements.

Geochemists developed an arcane, but precise isotopic jargon. Before dealing with mass-balance models, we must introduce the terminology that isotopic ecology shares with the atmospheric and geological sciences.

STABLE ISOTOPES: TERMINOLOGY PRIMER

The isotopic composition of a sample is measured as the ratio of one isotope to another: In most cases the abundance of one isotope (generally the lightest) exceeds that of the other by a large margin. For example, ^{13}C makes up only 1.1% of the total carbon on earth and ^{15}N constitutes only 0.37% of the nitrogen (Richardson and McSween, 1989). Consequently this ratio can be a very small number. To make measurements of the relative abundance of two isotopes graspable, geochemists express the isotopic composition of most materials as the normalized ratio of the sample to a standard in parts per thousand (per mil, ‰):

$$\delta X = [\frac{R_{sample} - R_{standard}}{R_{standard}}]x1000 \quad (1)$$

where X is an element, and R_{sample} and $R_{standard}$ are the ratios of the heavy to the light isotopes for the sample and standard, respectively. In some cases, it is useful to transform from fractions or percentages to ratios and δ values with the transformation

$$R_{sample} = \frac{f_H}{1 - f_H} \quad (2)$$

where f_H is the fraction of the heavy isotope. For values of $f_H < 0.1$, R_{sample} can be approximated very closely by f_H (R_{sample} ≈ f_H).

Although some of the standards chosen by geochemists seem capricious to biologists, at this point we have no say in the matter. A marine belemnite for the Pee Dee Formation (VPDB) and ocean water (standard mean ocean water = SMOW) are used as standards for nitrogen and carbon respectively. Thus, isotope ratios are commonly expressed as ‰ SMOW or ‰ VPDB. The words "depleted" and "enriched" refer to the heavy, and often less abundant isotope of a pair: Depleted means a more negative δ value whereas enriched means a more positive δ value.

Fractionation

As mentioned in the introduction, the natural variation in the relative abundance of stable isotopes in any substance is the consequence of tiny mass differences that cause the isotopes to behave differently in both physical

processes and chemical reactions. In general, the lighter isotope (^{12}C, or ^{14}N) tends to form weaker bonds and to react faster than the heavier isotope (^{13}C, or ^{15}N). As a consequence, the abundance of stable isotopes of an element will vary among the reactants and products of a chemical reaction. The change in isotopic abundance between chemical species (i.e. reactants or products) resulting from physical and chemical processes is called fractionation. Fractionation ($\alpha_{A\text{-}B}$) between the chemical species A and B is described in terms of the ratio in delta (δ) values between the species:

$$\alpha_{A\text{-}B} = \frac{R_A}{R_B} = \frac{(1000 + \delta_A)}{(1000 + \delta_B)} \quad (3)$$

Values of α are usually very close to 1, so the difference between two delta values is often reported and denoted by the discrimination factor $\alpha_{A\text{-}B}$ ($\alpha_{A\text{-}B} = \delta_A - \delta_B$).

Two types of fractionation have relevance for biologists. Equilibrium fractionation occurs among chemical molecules linked by equilibria as a result of bond strength differences between the isotopic species. For example, carbonate in bone is probably derived from blood bicarbonate. Carbon and oxygen isotopes are rapidly exchanged among blood bicarbonate, dissolved blood carbon dioxide, and body water by the following equilibria:

$$CO_2(aq) + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$

The isotope equilibrium of bone carbonate is controlled by the composition of dissolved CO_2, which is produced by respiration, and fractionation associated with equilibrium exchanges of carbon. Suppose that one is attempting to estimate the isotopic composition of the diet of an extinct mammal from the carbon in the apatite of its teeth. At mammalian body temperatures, the fractionation (ε) from CO_2 to HCO_3 is about 8‰ (Mook, 1986). Assuming that ε between dissolved bicarbonate and carbonate in apatite is 1‰ or 2‰ (the ε value for calcium carbonate), then apatite carbonate should have a $\delta^{13}C$ value approximately 9‰ to 10‰ greater than that of respired CO_2 which presumably reflects that of diet.

Kinetic fractionation effects occur because of differences in the rate of transport or rate of reaction of isotope species. For reactions catalyzed by enzymes, the magnitude of fractionation can be used to approximate the relative affinity of an enzyme for a compound with one isotope or another. An example of α kinetic fractionation is the reaction catalyzed by the enzyme glutamic oxaloacetic transaminase. This enzyme catalyzes the symmetrical reaction that transfers an amino group from glutamic acid to oxalacetic acid to yield α-ketoglutaric acid and aspartic acid (Macko et al., 1986). Glutamic oxaloacetic transaminase transfers $^{14}NH_2$ from glutamic acid to aspartic acid 1.0083 times faster than $^{15}NH_2$. In the reverse reaction $^{14}NH_2$ is incorporated

into α-ketoglutarate 1.0017 times faster than ^{15}NH. Transaminases catabolize nitrogen transfers for 12 other amino acids. Their kinetic discrimination against ^{15}N may explain the observed ^{15}N enrichment between diet and nonessential amino acids, as well as the progressive enrichment in ^{15}N across trophic levels (Gaebler et al., 1966; Post 2002; and this chapter).

Terminology : Caveat

Many processes can lead to differences in composition between an organism's tissues and its diet. For example, the enzymatic fractionation resulting from the action of transaminases described above, produces tissue proteins that most likely tend to be enriched in ^{15}N relative to diet. Some of the differences between the isotopic composition of diet and a consumer's tissues are the result of fractionating processes. Others are the result of stoichiometric effects and what has been called isotopic routing (see subsequent sections). Because several processes can lead to differences in the isotopic composition of diet and animal tissues, it is inappropriate to call these differences "fractionation". Cerling and Harris (1999) proposed the term discrimination factor ($\Delta_{diet} = \delta_{tissues} - \delta_{diet}$) for the difference between the isotopic composition of diet and that of consumer tissues. Using the term "fractionation" to describe differences between the isotopic composition of a resource and the tissues of a consumer is inappropriate for two reasons: (1) it confuses pattern with process (fractionation is only one of the processes that produce discrimination) and (2) it is inconsistent with usage in other fields.

MIXING MODELS: GUIDE FOR THE PERPLEXED

Stable isotopes are widely used to reconstruct animal diets. The basic idea has been summarized in the phrase "animals are what they eat". The isotopic composition of an animal tissue reflects the contribution of dietary components with different isotopic compositions (DeNiro and Epstein, 1978, 1981). Two types of approaches have been used to reconstruct animal diets from isotopic data: Euclidean distance methods and mixing mass-balance models (reviewed by Phillips, 2001). Phillips (2001) demonstrated that Euclidean distance methods do not estimate diet proportions correctly. Thus, we do not deal with these models here. Rather, we review in some detail mass-balance mixing models and their assumptions. Our description of mixing models relies heavily on the papers by Phillips (2001) and Phillips and Koch (2001).

Linear Mixing Models

The simplest of the mass-balance mixing models assumes that the isotopic composition of their tissues equals the weighed average of the isotopic composition of the diet constituents. For two diet constituents:

$$\delta X_{tissues} = p\,\delta X_A + (1-p)\,\delta X_B \quad (4)$$

where p equals the fraction of diet A and δX_A and δX_B are the isotopic composition of diet components A and B.

Provided that the isotopic composition of two elements is used, eqn (4) can be extended to estimate the fraction (p_i) of the diet comprised by three types of items (Phillips, 2001, Ben David and Schell, 2001). For carbon (C) and nitrogen (N) this requires solving the following system of linear equations in which A, B, and C are three different food types, and $p_A + p_B + p_C$ are the contributions of each food type to the animal diet:

$$\begin{aligned} \delta^{13}C_{tissues} &= p_A\delta^{13}C_A + p_B\delta^{13}C_B + p_C\delta^{13}C_C \\ \delta^{15}N_{tissues} &= p_A\delta^{15}N_A + p_B\delta^{15}N_B + p_C\delta^{15}N_C \\ 1 &= p_A + p_B + p_C \end{aligned} \quad (5)$$

In general, one can use n-1 isotopes to discriminate the contribution of n food sources. Because eqns (4) and (5) depend linearly on p, the mixing relations they depict can be labeled "linear mixing models".

Although eqns (4) and (5) look reasonable, they contain a variety of unrealistic assumptions. First, they assume that food types are stoichiometrically identical, i.e. that food type A and B contain exactly the same relative carbon and nitrogen contents. Second, they assume that all dietary items are assimilated with equal efficiency. Lastly, eqns (4) and (5) assume that isotopes are completely homogenized in the consumer's body prior to tissue synthesis. Phillips and Koch (2001) refined mixing models to incorporate differences in food stoichiometry and assimilation efficiency. We deal with the homogeneity assumption in a later section.

Concentration-dependent Mixing Models

Phillips and Koch's (2001) concentration-dependent mixing models assume that the contribution of a given dietary item to an animal's carbon (or nitrogen) pool depends on how much carbon (or nitrogen) that item contains. The difference in the results of using linear mixing models and concentration-dependent mixing models is best illustrated with one isotope and two diet types. Let us call B the total assimilated biomass, p the fraction of total assimilation contributed by diet 1, (1-p) the fraction of total assimilation contributed by diet 2, and $[C_1]$ and $[C_2]$ the concentrations of element X in diets 1 and 2, respectively. The relative contribution of diet 1 to the pool of element X in the consumers tissues will be

$$p_1 = \frac{Bp[C_1]}{B(p[C_1]+(1-p)[C_2])} = \frac{p[C_1]}{p[C_1]+(1-p)[C_2]} \quad (6)$$

and the isotopic composition of the pool of element X will be

$$\delta X = \left(\frac{1}{p[C_1]+(1-p)[C_2]}\right)(\delta X_1 p[C_1] + \delta X_2 (1-p)[C_2]) \quad (7)$$

Figure. 6.2 illustrates the potentially large errors that can be committed by assuming a linear mixing model when the two diets differ significantly in elemental composition. The concentration-dependent mixing model can be easily modified for more than one isotope and more than one diet. Again, n-1 isotopes can be use to differentiate among n diets. For more than one isotope, pi is the fraction of total assimilated biomass (B) contributed by item i and P_{xi} represents the fraction of assimilated element X

$$p_{xi} = \frac{Bp_i x_i}{B\sum_{j=1}^{n} p_j x_j} = \frac{p_i x_i}{\sum_{j=1}^{n} p_j x_j}, \quad (8)$$

For three food sources, and two elements for example, carbon and nitrogen with concentrations $[C_i]$ and $[N_i]$ (i = 1, 2, and 3) and isotopic compositions $^{13}C_i$ and $^{15}N_i$, we have that:

$$\begin{aligned} \delta^{13}C_{tissues} &= \frac{1}{(p_1[C_1] + p_2[C_2] + p_3[C_3])}(p_1[C_1]\delta^{13}C_1 + p_2[C_2]\delta^{13}C_2 + p_3[C_3]\delta^{13}C_3) \\ \delta^{15}N_{tissues} &= \frac{1}{(p_1[N_1] + p_2[N_2] + p_3[N_3])}(p_1[N_1]\delta^{15}N_1 + p_2[N_2]\delta^{15}N_2 + p_3[N_3]\delta^{15}N_3) \\ 1 &= p_1 + p_2 + p_3 \end{aligned} \quad (9)$$

Of course, eqn (9) reduces to eqn (5) if all the diet components have the same elemental composition (i.e. $[C_1] = [C_2] = [C_3]$, and $[N_1] = [N_2] = [N_3]$). The error caused by neglecting concentration dependence increases as the differences in elemental composition among dietary components increase. With a bit of algebra, eqn (9) can be written in matrix form as a system of 3 linear equations in 3 unknowns:

$$\mathbf{AP = B} \quad (10)$$

where

$$A = \begin{bmatrix} (\delta^{13}C_1 - \delta^{13}C_{tissue})[C_1] & (\delta^{13}C_2 - \delta^{13}C_{tissue})[C_2] & (\delta^{13}C_3 - \delta^{13}C_{tissue})[C_3] \\ (\delta^{15}N_1 - d^{15}N_{tissue})[N_1] & (\delta^{15}N_2 - d^{15}N_{tissue})[N_2] & (\delta^{15}N_3 - d^{15}N_{tissue})[N_3] \\ 1 & 1 & 1 \end{bmatrix}$$

and

$$P = \begin{bmatrix} p_1 \\ p_2 \\ p_3 \end{bmatrix}, \text{ and } B = \begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix}$$

Phillips and Koch (2001) provide an algorithm to solve for vector **P** in eqn (10).

The concentration-dependent mixing model proposed by Phillips and Koch (2001) assumes that all elements in a diet are assimilated with the same efficiency, which is not necessarily the case. Fortunately, element-dependent

variation in assimilation efficiency can be incorporated into the model. Let us call B' the total biomass ingested and e_{xi} the efficiency with which element X is assimilated in diet i. Then eqn (8) must be modified as

$$p_{x_i} = \frac{B' e_{X_i} p_i x_i}{B' \sum_{j=1}^{n} e_{X_j} p_j x_j} = \frac{e_{X_i} p_i x_i}{\sum_{j=1}^{n} e_{X_j} p_j x_j}, \quad (11)$$

Equation (9) has to be modified accordingly and the value of e_{xi} must be estimated experimentally. Although physiological ecologists estimate the assimilation efficiency for food types and even specific nutrients routinely, there are few accounts of the efficiency with which different elements are assimilated. We emphasize that the term e_x in eqn (11) represents "true" assimilation efficiency rather than the apparent assimilation efficiency so often reported. True assimilation efficiency is the fraction of the ingested element absorbed (i.e. e_x = amount of element x not assimilated/amount of element x ingested), whereas apparent assimilation efficiency includes endogenous fecal losses (apparent assimilation efficiency = [amount of element x not assimilated + endogenous fecal losses]/amount of element x ingested). Readers can find a lucid explanation of the difference between true and apparent assimilation in Karasov (1990).

Incorporating food stoichiometry in mixing models requires more empirical work. It requires analyzing (or at least estimating) the food's elemental composition and may require determining the efficiency with which different elements in each diet are assimilated. Field researchers may understandably complain that concentration-dependent models require more additional data and assumptions than simple linear mixing models (Robbins et al. 2002). However, simple models that make seriously wrong assumptions can yield seriously erroneous results. The "collect-combust-and infer" approach that has characterized animal isotopic ecology so far has been fruitful. Although it will probably remain the approach of choice for some problems that can be solved by qualitative approaches, it has serious limitations. The simple linear mixing models that dominate the literature are misleading if the elemental composition of diet components differs substantially (Fig. 6.2). Considering the potential effect of food's elemental composition and differential assimilation on isotopic incorporation adds realism to mixing models. In some cases, however, even the detail provided by concentration-dependent models may not suffice and additional assumptions may need to be incorporated.

Isotopic Routing

Mass-balance mixing models make a crucial assumption which is almost certainly wrong in many animals. They assume that the isotopes of the elements contained in all dietary sources are completely homogenized ("mixed")

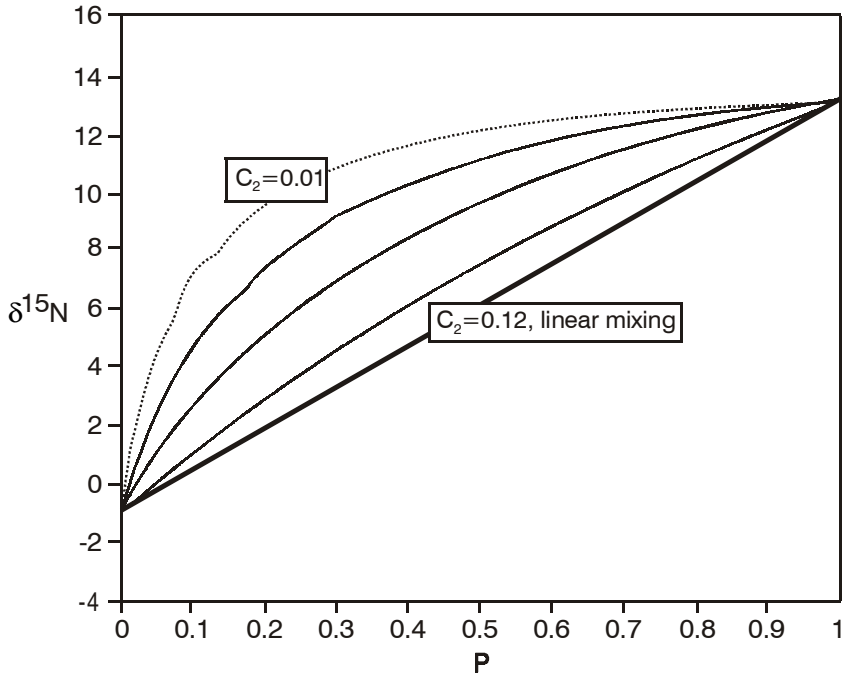

Fig. 6.2. Isotopic composition of a homogeneous mixture of two materials depends on two factors: 1) the fraction of each material in the mixture and 2) the elemental composition of the two materials. In the example depicted by the family of curves, p equals the fraction of material 1 in the mixture and 1-p is the fraction of material 2. The isotopic composition of materials 1 and 2 is $\delta^{15}N_1$=13.2 and $\delta^{15}N_2$ = -0.9 respectively. To construct the curves we maintained C_1 constant (C_1 = 0.12) and varied C_2 from 0.01 (punctate curve) to 0.12 (thick line). The values for C_2 in the remaining curves, from top to bottom are 0.02, 0.04, and 0.08. A linear mixing model [see eqn (4)] assumes that $C_1 = C_2$ and hence always predicts a straight line. Mixing models that account for the elemental composition of the mixture yield curves rather than straight lines if $C_1 \neq C_2$. The isotopic and elemental compositions of this artificial example correspond to the values of salmon (material 1) and plants (material 2) ingested by brown bears (*Ursus arctos*, after Phillips and Koch, 2001).

in the animal body before tissues are synthesized. The animals that best fit this assumption are foregut fermenters in which nutrients are homogenized to the common denominator of volatile fatty acids and bacterial protein before being absorbed. However, even in ruminants many nutrients escape the fermentative chamber and are absorbed intact in the lower gut (Van Soest, 1994). Once absorbed, nutrients enter a variety of metabolic pathways and the elements in them can undergo varying degrees of mixing (Fig. 6.3). The mixing assumption is problematic whenever diet components differ in macronutrient content. Synthesis of one macronutrient from another can be difficult (e.g. glucose and glucogenic amino acids cannot be synthesized from fatty acids) and is always energetically expensive (Fig. 6.3). Thus,

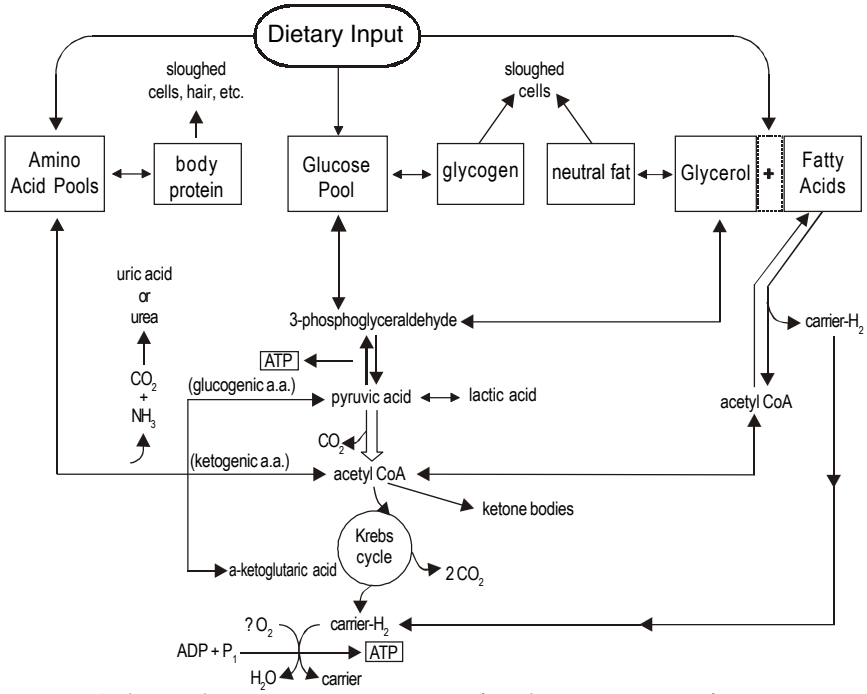

Fig. 6.3. Carbon and nitrogen in organisms are found as components of macronutrients. This scheme outlines the potential interconversions among the primary macronutrients in an animal body. Although there is potential for considerable mixing of elements among the different macronutrient pools, mixing may be energetically expensive. Recall that in general catabolism generates ATP. Synthesis, however, requires both ATP and reducing equivalent (such as NADHP). For example, lipids can be synthesized from both carbohydrates and proteins but lipid synthesis entails a high cost (the synthesis of a single palmitate molecule from 8 Acetyl-CoA requires 7 ATPs and 14 NADPH). In a similar fashion, although dispensable amino acids can be synthesized from the carbon skeletons resulting from both carbohydrate and lipid metabolism, this process is ATP dependent. Furthermore, the addition of amino acids to a peptide chain requires ATP (Mathews et al. 2000). A corollary of this observation is that organisms should route dietary macronutrients.

animals should route macronutrients and the isotopes in them from diet into the same macronutrient types in their tissues. This phenomenon has been called isotopic or nutrient routing.

Paleontologists have recognized the problems posed by isotopic routing for quite some time. For example, anthropologists and paleontologists have traditionally used bone collagen, largely composed of protein, to analyze isotopic composition for dietary reconstruction. Collagen has two problems: (1) it contains 33% glycine, which is a relatively $^{13}C^{-}$enriched amino acid, so collagen tends to be $^{13}C^{-}$enriched relative to other tissues; and (2) collagen is largely composed of protein and the composition of body protein in omnivores often reflects the isotopic composition of dietary protein (Ambrose and

Norr, 1993). Recognition of the principle that in omnivores the isotopic composition of tissue protein often reflects that of dietary protein, and not that of bulk diet, has led researchers to analysis of the carbonates in bone apatite (Tieszen and Fagre, 1993). These are synthesized from circulating bicarbonate derived from CO_2 and hence probably reflect the components of the diet that are catabolized for energy (Ambrose and Norr, 1993). Omnivorous animals feeding on diets with low protein content should allocate dietary protein for tissue maintenance and repair, rather than catabolize it for energy. Consequently apatite carbonates may underestimate the contribution of dietary protein (see below).

Macronutrient Concentration-dependent Mixing Models

The notion that protein should be routed to protein can be formalized in mixing models that depend on the content of macronutrients (protein, carbohydrate, and lipid) in each diet component. For element X (carbon or nitrogen) in protein:

$$\delta X_{\text{tissue protein}} = \left(\frac{1}{p[P_1] + (1-p)[P_2]}\right)(\delta X_{Pl} p[P_1] + \delta X_{P2}(1-p)[P_2]) \quad (12)$$

where $[P_1]$ and $[P_2]$ are the protein contents of diet components 1 and 2 respectively and δX_{P1} and δX_{P2}, are the isotopic compositions of the protein in these components. Equation (12) assumes that the concentration of element X in the protein of components 1 and 2 is the same, which is a reasonable assumption. It also assumes that protein in both components is assimilated with equal efficiency. Differences in the elemental concentration and in assimilation efficiency between the protein contained in diet components can be easily incorporated into eqn (12) [see eqns (8) and (10)]. Figure 6.4 illustrates the difference between the results of a concentration-dependent model and one that incorporates differences in protein content between diet components. The differences between the results of the two models increase as the disparity in protein composition between diets increases. Note the large errors a concentration-dependent model can engender if there is routing. Suppose that $[P_2] = 0.07$ and $\delta^{13}C = -24$ for the example depicted in Fig. 6.4. If there is protein routing this value represents a p of 0.25. The concentration-dependent model would estimate p as 0.75!

A protein concentration-dependent mixing model for n-1 isotopes and n diets can be constructed as:

$$X_{\text{tissue protein}} = \frac{\sum_{i=1}^{n} p_i e_{Pi} \delta X_{Pi} [P_i]}{\sum_{i=1}^{n} p_i e_{Pi} [P_i]} \quad (13)$$

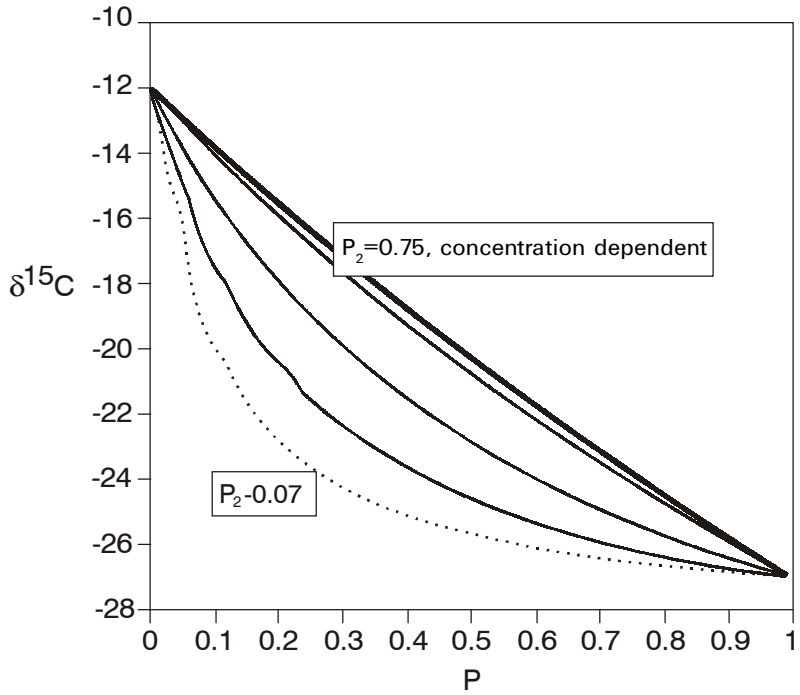

Fig. 6.4. Mixing models that assume isotopic routing of dietary protein into tissue protein yield results different from those of concentration-dependent models. In this example, we applied a protein routing model [eqn (11)] to two diets with contrasting isotopic composition ($\delta^{13}C_1$ = -27 and $\delta^{13}C_2$ = -12). Diet 1 had a fraction of assimilated biomass equal to p and a protein content ($[P_1]$ on a dry mass basis) of 0.75. The protein content of diet 2 ($[P_2]$) varied from 0.07 (dashed line) to 0.75 (thick line). The values for P_2 in the remaining curves, from top to bottom, are 0.14, 0.28, and 0.54. The concentration-dependent model [from eqn (7)] assumes that $[C_1]$ = 57.7 and $[C_2]$ = 46.8 (i.e. the values of lean deer meat and corn respectively. The thick line represents both the concentration-dependent model and a protein routing model that assumes identical protein contents in diet components 1 and 2. Because the carbon contents of the two diet components are similar, the concentration-dependent model yields a linear mixing relationship.

where p_i is the fraction of total biomass ingested comprised by component i, e_{Pi} the efficiency with which the protein contained in diet component i is assimilated, [Pi] its protein content, and δX_{Pi} the isotopic composition for element X of the protein in component i. For N and C, the matrix form of this linear system is

$$\mathbf{A_P P = B}, \tag{14}$$

where

$$A_P = \begin{bmatrix} (\delta^{13}C_1 - \delta^{13}C_{tissue})e_{P1}[P_1] & (\delta^{13}C_2 - \delta^{13}C_{tissue})e_{P2}[P_2] & (\delta^{13}C_3 - \delta^{13}C_{tissue})e_{P3}[P_3] \\ (\delta^{15}N_1 - \delta^{15}N_{tissue})e_{P1}[P_1] & (\delta^{15}N_2 - \delta^{15}N_{tissue})e_{P2}[P_2] & (\delta^{15}N_3 - \delta^{15}N_{tissue})e_{P3}[P_3] \\ 1 & 1 & 1 \end{bmatrix}$$

and

$$P = \begin{bmatrix} p_1 \\ p_2 \\ p_3 \end{bmatrix}, \text{ and } B = \begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix}$$

The mixing model described by eqn (12) assumes that protein is always routed into protein. This assumption is likely to be correct if, when eaten alone, all diet components satisfy the animal's protein requirements. However, many interesting situations involve one dietary component that does not provide sufficient protein but which is abundant and provides energy in the form of carbohydrates and lipids. Good examples of these situations are fruit- and nectar-eating birds that satisfy most of their protein requirements with insects (Martínez del Rio, 1994) and carnivorous mammals that ingest fruit and plants in addition to meat (Pritchard and Robbins, 1990). When one of the diets is protein deficient, at low ingestion levels of the protein-rich alternative there is probably significant routing of carbon from carbohydrates and, to a lesser extent from lipids, to protein. A mixing model that incorporates this added level of realism requires many assumptions and cannot be solved analytically for p. It is useful, however, to ascertain how wrong the results of our mixing models can be.

We explored the potential effect of isotopic routing among macronutrients with an artificial situation. We assumed a large animal (65 kg) that consumes two diet components with contrasting isotopic and macronutrient compositions (Fig. 6.5). The protein content of these two diets is such that only one of the diet components (component 1) has sufficient protein to satisfy the animal's minimal protein requirements (PR_{min} g day^{-1}) if it ingests enough of it to satisfy its energy needs. The other diet component (component 2) is protein deficient. We assumed this animal to ingest enough mass of each diet component to maintain neutral energy balance. However, we also assumed that the animal protein intake was insufficient to satisfy its minimum protein requirements when it ate only component 2. Assuming the total protein consumption as equal to or higher than PR_{min}, we calculated the animal tissue protein $\delta^{13}C$ using eqn (11). However, were protein ingestion less than PR_{min}, we assumed that the carbon needed to synthesize the protein deficit was derived from amination of carbon skeletons derived from carbohydrates in component 2 (the carbohydrate-rich, but protein-deficient component):

$$\delta^{13}C_{\text{tissue protein}} = \begin{cases} \left(\frac{B(p[P_1]+(1-p)[P_2])}{PR_{min}}\right)\left(\frac{1}{p[P_1]+(1-p)[P_2]}\right)\left(\delta^{13}C_{P_1}p[P]_1 + \delta^{13}C_{P_2}(1-p)[P_2]\right) + \\ \left(\frac{(PR_{min} - B(p[P_1]+(1-p)[P_2]))}{PR_{min}}\right)\delta^{13}C_2 \quad \text{if } PR_{min} > B(p[P_1]+(1-p)[P_2]) \\ \text{and} \\ \frac{1}{p[P_1]+(1-p)[P_2]}\left(\delta^{13}C_{P_1}p[P_1] + \delta^{13}C_{P_2}(1-p)[P_2]\right) \quad \text{if } PR_{min} < B(p[P_1]+(1-p)[P_2]) \end{cases} \tag{15}$$

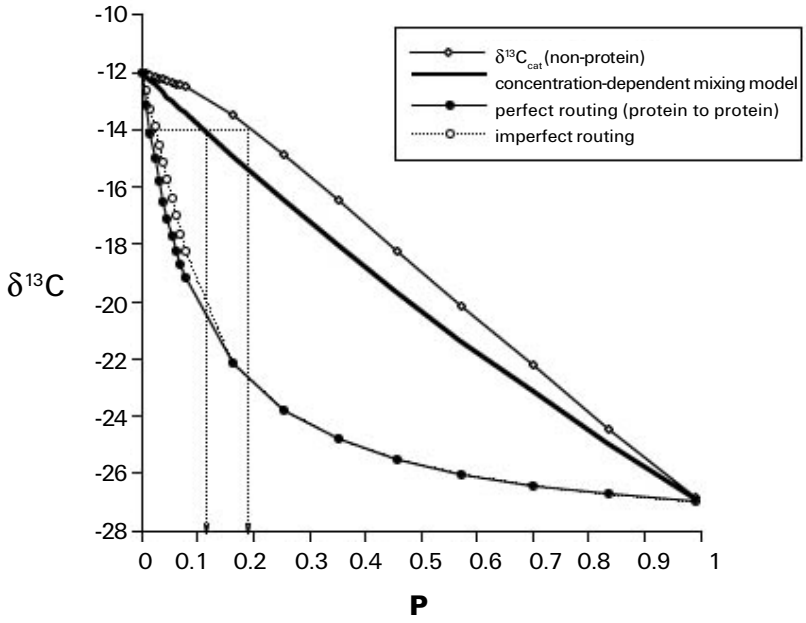

Fig. 6.5. A more realistic model of isotopic routing for two diet components differing in protein content. The protein content and isotopic composition of diet components 1 and 2 are identical to those in Fig. 6.4. The energy content of diet component 1 was 25.1 kJ g^{-1} and that of diet component 2.17 kJ g^{-1} and we assumed that the animal ingested a mixture of diet components that satisfied its daily maintenance energy requirements (11,241 kJ day^{-1}). We also assumed that the minimal protein requirements (PR_{min}) equaled 77 grams day^{-1} (these data correspond to a 65 kg human exercising moderately; Reeds and Becket, 1996). Finally, we assumed that diet component 1 contained 25% lipid and no carbohydrates and component 2 contained 89% carbohydrate and 4% lipid. The thick curve assumes concentration-dependent mixing. Note that assuming that some carbohydrate is routed to protein (curve with open circles, "imperfect routing") at low protein intakes does not yield results very different from those of a mixing curve that assumes that only protein is routed into protein. The curve labeled with diamonds assumes that the isotopic composition of the nutrients catabolized for energy reflects a mixture of the components that remain after protein has been allocated to satisfy minimal protein requirements. If animals route protein to protein and allocate carbohydrates and lipids to energy production, a concentration-dependent mixing model underestimates the contribution of the protein-rich component. Using a concentration dependent-model a value of $\delta^{13}C$ = -14‰ in exhaled breath (or bone apatite) corresponds with a p ~ 0.12. However, if there is protein routing p = 0.19 (dotted lines).

As Fig. 6.5 shows, making this more realistic assumption yields results very similar to those obtained by assuming a simple protein-to-protein routing model (the maximal difference between the two models is 1.4‰). Equation (15) assumes that only carbohydrate carbon from diet component 2 is incorporated into protein. An alternative is to assume that the carbon used to compensate the protein deficit is derived from aminating carbon skeletons from a mix of all macronutrients. This assumption yields almost identical results as eqn (15).

The critical assumption of eqn (15) is that carbon skeletons of carbohydrates can be aminated. Why would an animal with limited protein transfer an amino group from protein into carbohydrate. The reason lies in the inefficiency of metabolism. Even animals in negative nitrogen balance continue to catabolize protein. O'Brien et al. (2000, 2002) have shown that hawkmoths feeding on sucrose-rich but nitrogen-free nectar, use nitrogen stored as larvae to synthesize dispensable amino acids that are incorporated into egg proteins. These authors found the carbon isotopic composition of nectar sugars in dispensable ("nonessential") amino acids, but not in indispensable ("essential"). We speculate that when nitrogen is limited, the ammonia resulting from inevitable protein turnover will be incorporated into carbon skeletons derived from carbohydrates to synthesize dispensable (nonessential) amino acids. Of course, essential amino acids are always derived exclusively from dietary protein. This hypothesis can be tested by analyzing the composition of specific amino acids (see O'Brien, 2002).

Equations (12) and (15) can be used to predict the carbon isotopic composition of tissue protein. Assuming that protein is routed to protein, the isotopic composition of the mixture of macronutrients catabolized for energy ($\delta^{13}C_{cat}$) should reflect the isotopic composition of carbon contributed by the macronutrients that remain after allocation to protein. Estimating $\delta^{13}C_{cat}$ is a simple exercise in accounting but because the resultant equation is long and awkward, it is not presented here. The value of $\delta^{13}C_{cat}$ reflects the increased importance of the protein-deficient diet as a source of energy. If there is protein routing and a concentration-dependent mixing model is used to estimate diet composition from the $\delta^{13}C$ of nonprotein tissue (e.g. breath CO_2 and bone apatite; Hatch et al., 2002), the fraction of the protein-rich component (p) will be underestimated (Fig. 6.5). Several authors have pointed out that the carbon isotope ratio of breath CO_2 is a reliable indicator of the carbon isotope composition of bulk diet (Hatch et al., 2002 and references therein). The results depicted in Fig. 6.5 cast doubt on this assertion. Indeed, if diet components differ in protein content, breath CO_2 will have a carbon isotope composition closer to bulk diet than the $\delta^{13}C$ of body protein. Yet $\delta^{13}C_{cat}$ will have a value biased toward that of the protein-deficient component of the diet. This bias should increase with (a) the protein requirements of the animal and (b) the disparity in protein content between dietary components.

The message of our admittedly simplistic routing models may be disappointing for ecologists eager to use stable isotopes to find out what their study animals eat. Our models suggest that isotopic ecology and nutritional ecology are inextricably linked. To understand incorporation of the isotopic signal of different diet components into animal tissues it appears that we must know not only the macronutrient content of these components, but also the efficiency with which these macronutrients are assimilated. Using concentration-dependent or macronutrient content-dependent models to reconstruct animal diets using stable isotopes cannot be done with

confidence until we validate the relative performance of these models in the laboratory. The models described in this section outline a research agenda.

Although we believe that isotopic ecology can benefit from the adoption of more realistic mixing models, there will always be a place for the simple mixing models outlined in eqns (4) and (5). These models can be used to accurately describe *the proportion of different diets incorporated into a given tissue*. As emphasized above, these proportions may/may not represent the proportion in which these diets are ingested. Using any of the models described above is "correct" provided that in each case the assumptions of the models are identified and that the limitations of the inferences that can be derived from them are recognized.

Compound Specific Isotopic Analysis: Is It a Way Out?

The ability to measure the isotopic composition in specific biochemical compounds (e.g. fatty acids, cholesterol, and amino acids) suggests an alternative to avoid the problem of nutrient routing in diet reconstruction (Hammer et al., 1998). The isotopic composition of indispensable ("essential") nutrients that the animal cannot synthesize must reflect the composition of the mix of these nutrients in the diet (O'Brien et al., 2002). There is enormous potential for use of the isotopic composition of individual essential nutrients to sort out an animal's dietary components. However, this new level of technological sophistication does not liberate us from mixing models. It is an easy exercise to modify eqn (12) to determine diet components from the isotopic composition of an essential nutrient (just substitute P_i for E_i, the concentration of the essential nutrient E in diet, and δX_{Pi} for δX_{Ei}). The isotopic composition of an essential nutrient in animal tissues is the result of the concentration of this nutrient in each diet component and thus its isotopic composition in the diet in toto.

DYNAMICS OF ISOTOPIC INCORPORATION

Mixing models assume equilibrium. They assume that an animal has ingested the diet components in a fixed combination long enough for the isotopic composition of its tissues to have reached a steady state. Because animals shift diets such is rarely the case. Indeed, stable isotopes have proven useful tools in documenting diet shifts in animals (Wolf and Martínez del Rio, 2000). The isotopic composition of a tissue is the result of the integration of isotopic inputs over some time in the past. Thus, using tissues with different turnovers will give information of the past diet of an animal over different time intervals. The turnover rate of tissue constituents governs the time window of isotopic incorporation (Tieszen et al., 1983).

The dynamics of incorporation of an isotopic "signature" into a tissue depends on the rate at which the materials in the tissue turn over. Consider

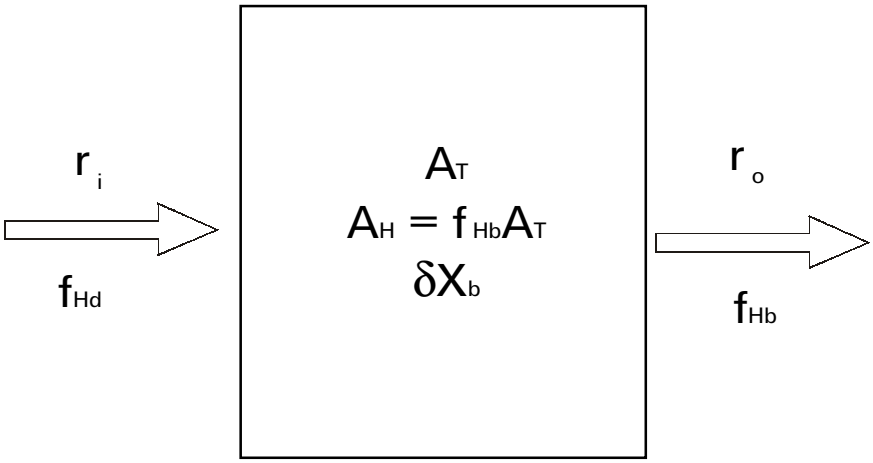

Fig. 6.6. In a pool of element X of size A_T, the rate at which the amount of heavy isotope $(\frac{df_H A_T}{dt})$ changes equals the difference between the rate at which this isotope enters the pool ($A_T r_i f_{Hd}$) minus the rate at which it exits the pool ($A_T r_o f_{Hb}$). A_T is the size of the pool (in mols), r_o and r_i are the fractional rates of input and output of element X into the pool (with units equal to time^{-1}) respectively, f_{Hb} the fraction of heavy isotope in the pool, and f_{Hd} the fraction of heavy isotope in diet.

a tissue that contains A_T mols of the element in question. We can envision A_T as the pool of element X in a tissue. Then consider the amount ($A_H = f_{Hb}A_T$) of the heavy isotope (^{13}C or ^{15}N) in this pool. Let us call r_i and r_o (with units equal to time^{-1}) the fractional rates at which the element enters and leaves the pool respectively, and f_{Hd} and f_{Hb} the fractions of the heavy isotope in the incoming materials (d for diet) and in the pool (b for body) respectively (Fig. 6.6). Then

$$\frac{dA_H}{dt} = \frac{d(A_T f_{Hb})}{dt} = f_{Hb}\frac{dA_T}{dt} + A_T\frac{df_{Hb}}{dt} \qquad (16)$$

and

$$\frac{dA_H}{dt} = A_T(r_i f_{Hd}(t) - r_0 f_{Hb}). \qquad (17)$$

Combining eqns (16) and (17), and because , $(\frac{1}{A_T})\frac{dA_T}{dt} = (r_i - r_0)$ we have.

$$\frac{df_{Hb}}{dt} = (r_i f_{Hd}(t) - r_o f_{Hb}) - f_{Hb}(\frac{1}{A_T})\frac{dA_T}{dt} = r_i(f_{Hd}(t) - f_{Hb}). \qquad (18)$$

Assuming that at time 0, $f_{Hb} = f_{Hb}(0)$, and that $f_{Hd}(t)$ is constant ($f_{Hd}(t) = f_{Hd}$), we may then integrate eqn (18) to yield:

$$f_{Hb}(t) = f_{Hd} - (f_{Hd} - f_{Hb}(0))e^{-r_i t}. \quad (19)$$

Several studies have investigated the change in tissue isotopic composition after an animal has been subjected to two diets that differ markedly in isotopic composition (O'Brien et al., 2000 and references therein). These authors used coexponential functions of the form

$$\delta X_{tissue}(t) = \delta X_{diet} - (\delta X_{diet} - \delta X_{tissue}(0))e^{-rt} \quad (20)$$

to describe their data. Because the heavy isotope is usually rare (i.e. $f_H << 0.1$), $f_H \approx R_{sample}$ and. $f_H \approx R_{standard}(1000\ \delta X + 1)$. Substituting this expression for f_H into eqn (19) leads to eqn (20). At steady state (i.e. $r_i = r_0 = r$), the exponent r in eqns (19) and (20) represents the fractional turnover rate of element X in a given tissue. Figure 6.7 shows incorporation of the isotopic signal from diet into tissues with contrasting fractional turnover rates.

Some tissues, such as liver and plasma proteins, have high turnover rates, and their isotopic composition reflects integration of recent inputs. In Japanese quail for example, carbon in liver has a half-life of approximately 3 days

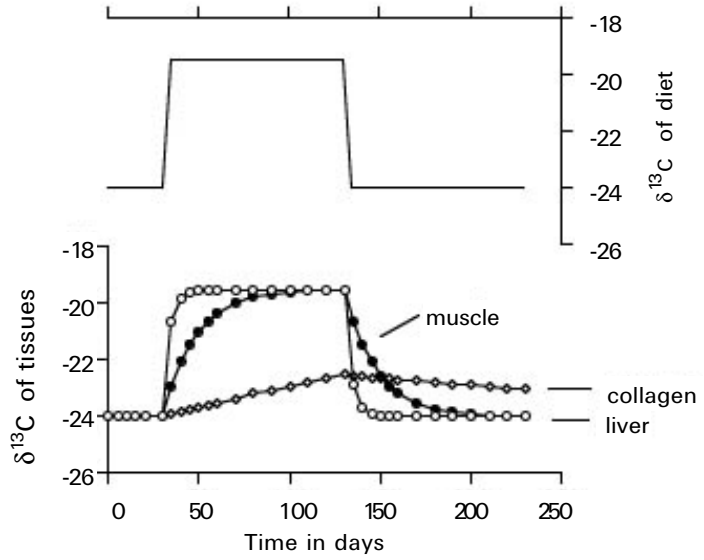

Fig. 6.7. Equations (19) and (20) can be used to investigate the carbon isotopic turnover of different tissues. The plot above was modified from data presented in Hobson and Clark (1992). In the example, an animal raised on a diet with $\delta^{13}C$ = -24‰ is shifted to a diet with $\delta^{13}C$ = -19.5. Liver has a high turnover (r = 0.27 d^{-1}) and its isotopic composition tracks diet changes. In contrast, tissue with low turnover such as collagen (r = 0.004 d^{-1}) reflect past diets rather than actual diet, even 100 days after a diet change. For simplicity, we assumed no isotopic discrimination between diet and tissues–which is not the case.

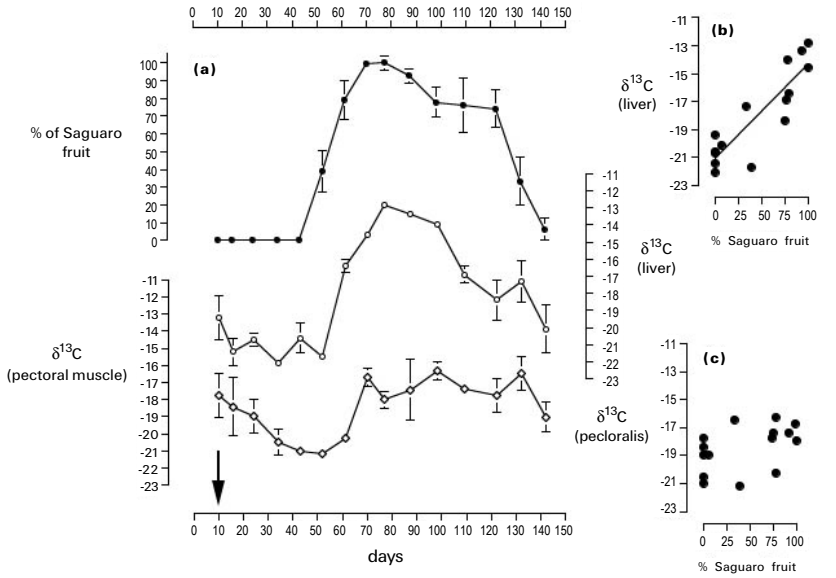

Fig. 6.8. Tissues with different carbon turnover rates show different patterns of isotopic incorporation in response to changes in diet. Wolf and Martínez del Rio (2000) measured the percentage by dry mass of saguaro fruit (a CAM plant with a $\delta^{13}C = 13.1$) in the crop of white-winged Doves (*Zenaida asiatica*). They also measured the carbon isotope composition of tissue with high and low carbon turnover (liver and pectoral muscle respectively). Note that $\delta^{13}C_{liver}$ "tracks" the percentage of saguaro in dove crops, whereas $\delta^{13}C_{pectoralis}$ fails to reflect changes in the isotopic composition of diet. Indeed, $\delta^{13}C_{liver}$ and percent saguaro in diet corelate positively (b), whereas $\delta^{13}C_{pectoralis}$ and percent saguaro in diet do not (c). Each point in this Figure is the mean from measurements of 6 to 10 individuals and bars are standard errors.

(i.e. r = 0.27; Hobson and Clark, 1992). Thus, 90% of the carbon found in the liver of these animals was incorporated at any moment over approximately 9 days. Other tissues exhibited low turnover and their isotopic composition reflected integration of inputs over a longer time period. In young quail, carbon in collagen had a half-life of 173 days (r = 0.056, Hobson and Clark, 1992). Therefore 90% of the carbon in this tissue was incorporated over 575 days. The choice of tissue for a dietary reconstruction study depends on the question asked. Tissues with high nutrient turnover rates will track isotopic changes in diet closely (Fig. 6.8) whereas tissues with low nutrient turnover rates will integrate an isotopic signature from a large temporal window resulting in a smoother, less steep curve. If the question is to determine how animals track the availability of resources, a tissue with high turnover rate (i.e. plasma proteins and liver) must be used. Conversely, if determination of the importance of various items in the diet of an animal over a long time period is the obyective, then a tissue with low turnover must be used.

POTENTIAL MECHANISMS BEHIND A MAGICAL NUMBER WHY DOES $\delta^{15}N$ INCREASE WITH TROPHIC LEVEL?

The simple model for the dynamics of isotopic incorporation described in the previous section assumes that the isotopic composition of the element leaving the pool equals that of the pool. Modifying this assumption may hold the answer to a perplexing pattern. Many animals are enriched in ^{15}N relative to their diet. In a classic paper DeNiro and Epstein (1981) documented an average 3.4 ‰ enrichment in $\delta^{15}N$ value for whole-body samples over diet. This enrichment in ^{15}N is very useful because it provides ecologists with a tool for estimating the trophic position of an animal. Post (2002) summarized the method for estimating the trophic position from nitrogen isotope measurements in a single equation:

$$\text{trophic position} = \lambda + \frac{(\delta^{15}N_{\text{secondary consumer}} - \delta^{15}N_{\text{base}})}{\Delta_n} \qquad (21)$$

where λ is the trophic position of the organism used to estimate $\delta^{15}N$ base (i.e. $\lambda = 1$ for primary producers), $\delta^{15}N$ is estimated by collecting and combusting the whole consumer, and Δ_n is the enrichment in $\delta^{15}N$. Typically, ecologists assume that n equals 3.4‰.

The 3.4‰ enrichment per trophic level has acquired a magical status (see Eggers and Jones, 2000). Although there is significant evidence suggesting that there is a ^{15}N enrichment with trophic level, the magnitude of this enrichment is quite variable (it ranges from –1‰ to 6‰; Peterson and Fry, 1987; Post, 2002). A variety of factors can determine variation in ^{15}N enrichment but most of them remain unexplored. $\delta^{15}N$ enrichment varies among tissues within an individual (reviewed by Kelly, 1999), among individuals depending on the C:N ratios of diet (Adams and Sterner, 2000), and among species depending on diet type (vertebrate and invertebrate diets, Kelly, 1999). The following sections do not answer why there is a 3.4‰ enrichment in ^{15}N across each trophic level. Instead, we identify the measurements needed for answering this question. We also recognize the potential reasons why the ^{15}N enrichment across trophic levels should vary.

Mass-Balance Model for Body Nitrogen

Why is there a ^{15}N enrichment across trophic levels? It appears that answering this question in a mechanistic fashion is the key to understanding and then interpreting correctly the ^{15}N enrichment associated with increased trophic level. Surprisingly, the physiological mechanisms that determine this enrichment are poorly understood. It is believed that during catabolism, amino acids with amine groups containing ^{15}N are disproportionately retained relative to those with amine groups containing ^{14}N (Macko and Epstep, 1984; Gaebler et al., 1966). The result is that excreted urinary nitrogen tends

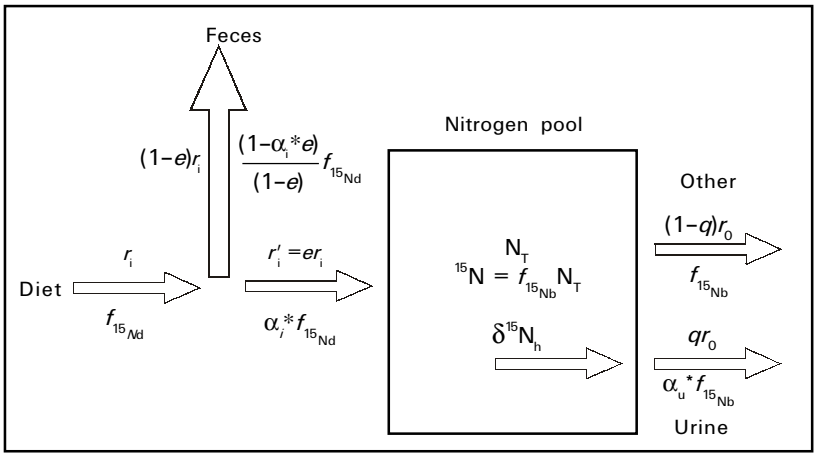

Fig. 6.9. Mass-balance model for whole body nitrogen isotopic composition. The model assumes that the animal ingests food containing a fraction $f_{15\,Nd}$ of ^{15}N at a rate r_i. The animal assimilates a fraction e of this food and the assimilation process has an apparent fractionation equal to α_i^*. The nitrogen in food enters the body nitrogen pool which contains N_T moles of nitrogen. ^{15}N represents a fraction $f_{15\,Nb}$ in this pool. The animal voids nitrogen at a rate equal to r_o. A fraction q of all voided nitrogen exits the animal through a fractionated route (urine). The apparent fractionation of urinary nitrogen equals α_u^*. We assume that fecal nitrogen (with a fraction 1-q of all nitrogen lost) is voided with no fractionation. Equations (22) and (23) summarize this model.

to be isotopically light (^{15}N depleted) relative to an animal's diet or tissues (Steele and Daniel, 1978, Minagawa and Wada, 1984). Figure 6.9 formalizes this observation in a mass-balance model. Substituting the corresponding terms in eqn (18) we obtain a mass-balance model for ^{15}N:

$$\frac{df_{15\,Nb}}{dt} = (r_i'\alpha_i^* f_{15\,Nd} - r_o((1-q)f_{15\,Nb} + q\alpha_u^* f_{15\,Nb}) - f_{15\,Nb}(\frac{1}{N_T})\frac{dN_T}{dt} \qquad (22)$$

which simplifies to

$$\frac{df_{15\,Nb}}{dt} = r_i'(\alpha_i^* f_{15\,Nd} - f_{15\,Nb}) + r_o q f_{15\,Nb}(1-\alpha_u^*) \qquad (23)$$

The terms in eqn (23) are the fractional rates of nitrogen assimilation and excretion (r_i and r_o respectively), the apparent fractionations associated with assimilation and urine production (α_i^* and α_u^* respectively), the fraction of nitrogen excreted through a fractionated route (q), and the fractions of ^{15}N in diet and animal body ($f_{15\,Nd}$ and, $f_{15\,Nb}$ respectively). We call α_i^* and α_u^*, apparent, because these values are quotients of fractions rather than of ratios (i.e. $\alpha_i^* = \frac{f_{15\,N(assimilated)}}{f_{15\,Nd}}$), and because a variety of processes can

lead to differences in the isotope composition between diet and assimilated nitrogen and between body and urinary nitrogen.

Effect of Diet Varying in Protein Content on $^{15}N_{body\text{-}diet}$

Omnivores can ingest diets that vary enormously in nitrogen content. What effect might this variation have on their nitrogen isotope composition? Physiologists recognize two types of excreted nitrogen: endogenous urinary nitrogen (EUN) and metabolic fecal nitrogen (MFN). EUN is composed primarily of nitrogenous waste products (ammonia, uric acid, urea, and creatinine) whereas MFN is composed of nonabsorbed digestive enzymes, intestinal cellular debris, and undigested bacteria and mucus (Robbins, 1993). The parameter q in eqn (23) can be interpreted as:

$$q = \frac{EUN}{EUN + MFN} \tag{24}$$

During protein catabolism the amino group must be removed from the carbon skeletons of amino acids and the resultant nitrogen voided in urine (Fig. 6.3). It is widely believed that the nitrogen in urine is depleted in ^{15}N relative to the pool of nitrogen in the body (i.e. $\alpha_u^* < 1$; Macko et al., 1986 and references therein). Thus the parameter q should be of crucial importance for the interpretation of an animal's nitrogen isotopic composition.

The relationship between q and protein intake is not simple. If the animal is in negative protein/nitrogen balance and catabolizing body tissue, q probably decreases with protein intake. Conversely, if the animal is in neutral protein/nitrogen balance, q should increase with protein intake (Jackson, 1998). Here we consider only the second option. The effect of negative nitrogen balance on an animal's nitrogen isotopic composition is examined in the next section. If the animal is not growing, is in neutral protein balance, and is consuming the same diet over time, then

$r_i' = r_o$ and $\frac{df_{15\,Nd}}{dt} = 0$. Thus,

$$\hat{f}_{15\,Nb} = \frac{\alpha_i^* f_{15\,Nd}}{(1 + q(\alpha_u^* - 1))} = \frac{\alpha_i^* f_{15\,Nd}}{(1 - q + q\alpha_u^*)} \tag{25}$$

Under this assumption, equilibrium nitrogen isotopic composition ($\hat{f}_{15\,Nb}$) increases with q in an accelerating fashion (i.e.). $\frac{d\hat{f}_{15\,Nb}}{dq} > 0$ and $\frac{d^2\hat{f}_{15\,Nb}}{dq^2} > 0$ if $q < 1$

For values of $\alpha_u 1$, the relationship between $\hat{f}_{15\,Nb}$ and q is well approximated by a straight line (Fig. 6.10):

$$\hat{f}_{15\,Nb} \approx \alpha_i^* \hat{f}_{15\,Nd} - a_i^* \hat{f}_{15\,Nd} q(\alpha_\mu^* - 1) \tag{26}$$

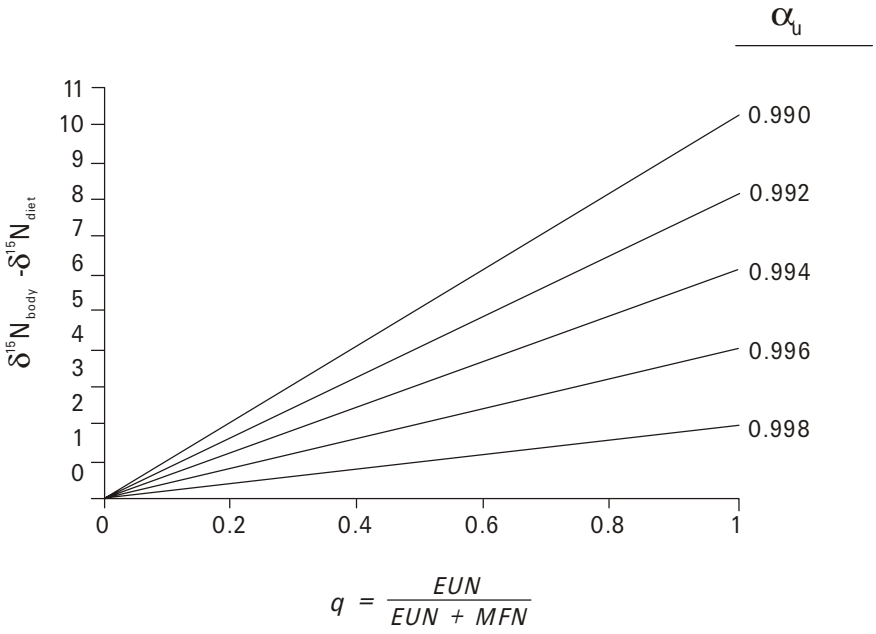

Fig. 6.10. If the animal is at neutral energy balance, the discrimination factor between body and diet ($^{15}N_{body\text{-}diet}$) depends on q, the fraction of total nitrogen lost that is excreted as urine. The curves in this figure were generated using eqn (25). We assumed that $\delta^{15}N_{body}$ = 10 and that there is no apparent fractionation during food assimilation (α_i^*= 0). Each curve represents a different apparent fractionation of urinated nitrogen (α_u).

The data needed to test the predictions of eqn (25) are easy to obtain but remarkably scarce. There is very little data on the nitrogen isotopic composition of EUN or MFN (Steele and Daniel, 1978; Minagawa and Wada, 1984) and almost no data on the relationship between q and protein intake (reviewed for humans and rats by Jackson, 1998). One of the few studies that has varied protein intake and measured the isotopic composition of animal tissues shows a pattern consistent with the predictions of eqn (15; Fig. 6.11). It is reasonable to assume that protein intake (and hence q) is larger in carnivores than in herbivores. Hence we conjecture that $^{15}N_{body\text{-}diet}$ should be higher in carnivores than in herbivores. Although in general, carnivores have tissues enriched in ^{15}N over those of herbivores (Kelly, 1999), to our knowledge no study has compared $^{15}N_{body\text{-}diet}$ between herbivores and carnivores in a systematic fashion.

The Effect of Protein Quality on $^{15}N_{body\text{-}diet}$.

A nonintuitive corollary of the dependence of $^{15}N_{body\text{-}diet}$ on q is that $^{15}N_{body\text{-}diet}$ must also depend on the quality of the ingested protein. The term "protein quality" refers to the ability of a dietary protein to provide the needs of an animal. Protein quality is dependent on the match between the amino acid

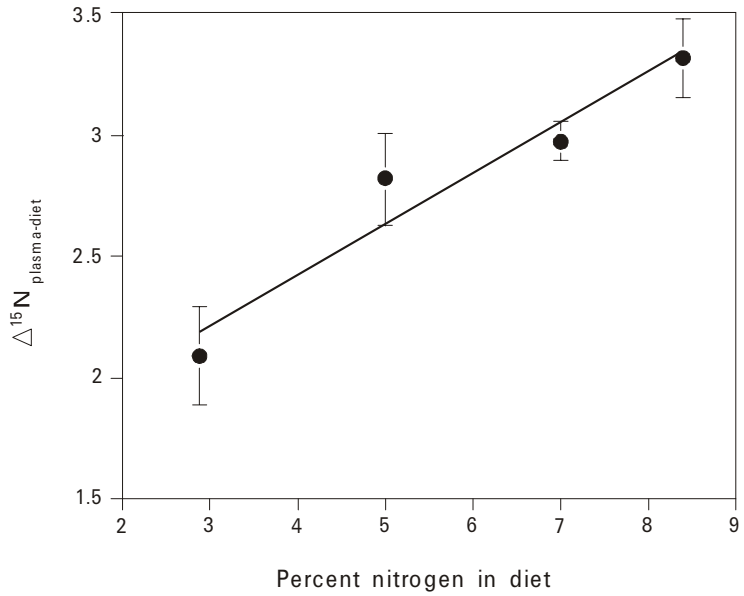

Fig. 6.11. The discrimination factor between diet and plasma proteins ($^{15}N_{plasma\ proteins\text{-}diet}$) in yellow-rumped warblers (*Dendroica coronata*) varied linearly (y = 1.58 + 0.21 X, r^2 = 0.94, regression on the means) with nitrogen intake. To vary nitrogen intake, birds were fed diets containing a homogeneous mixture of bananas (low-protein diet) and mealworms (high-protein diet). Points are means and bars are standard deviations of 6 individulas. Birds were fed on the controlled diets for 21 days (S. F. Pearson et al., unpubl. data).

composition of dietary protein and the amino acid needs of the animal (Robbins, 1993). A deficiency of one, or several essential amino acids will lead to greater protein requirement and ingestion and hence to higher catabolism of the amino acids not needed for protein synthesis (i.e. to an increase in EUN and hence in q). Thus, we predict that $^{15}N_{body\text{-}diet}$ will decrease as protein quality increases.

Do Fasting Animals Get Heavier? Effect of Negative Nitrogen Balance on $\delta^{15}N$

Several authors have hypothesized that animals in negative nitrogen balance become progressively enriched in ^{15}N (reviewed by Ben David et al., 1999). Negative nitrogen balance implies that $r_o > r_i'$. The simplest situation to model is one in which the animal is fasting ($r_i = 0$). Because in fasting animals all nitrogen losses are urinary, and hence q = 1, eqn. (23) reduces to

$$\frac{df_{15\ Nd}}{dt} = r_o f_{15\ Nb}(1-\alpha_u^*) \tag{27}$$

and thus

$$f_{15\ Nb}(t) = f_{15\ Nb}(0)e^{(1-\alpha_u^*)r_o(t)t} \tag{28}$$

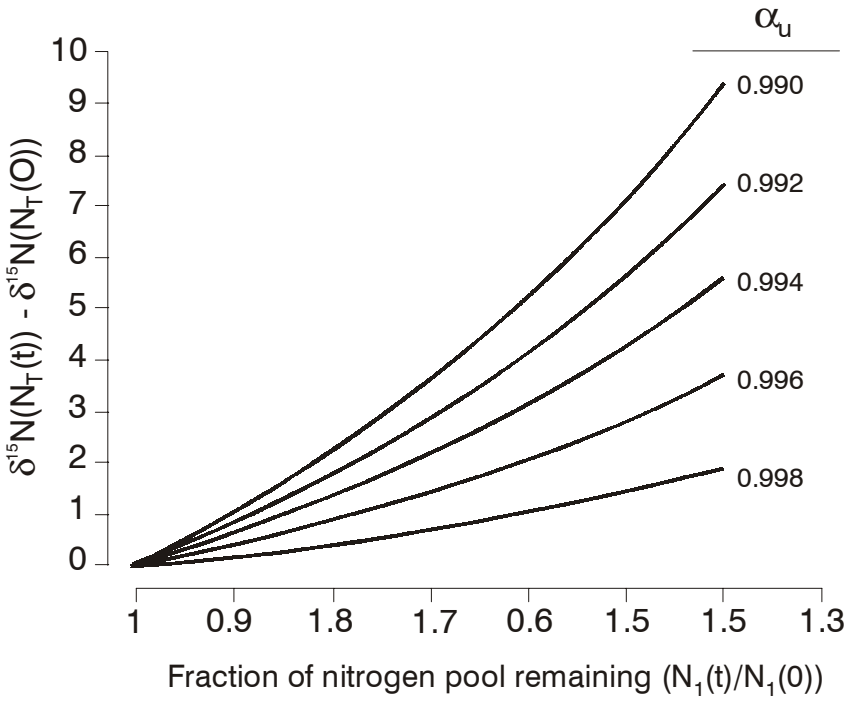

Fig. 6.12. Fasting animals become enriched in ^{15}N relative to their prefast nitrogen isotopic composition when their nitrogen pool decreases as a result of fasting. The magnitude of ^{15}N enrichment decreases as a power function of the fraction of the original nitrogen pool remaining. Curves were constructed using eqn (30) and several values for the apparent fractionation of urinated nitrogen (α_u*).

In fasting animals $\frac{dN_T}{dt} = -r_o(t)N_T(t)$ and hence

$$t = \frac{-\log(\frac{N_T(0)}{N_T(t)})}{r_o(t)} \tag{29}$$

Substituting eqn (29) into eqn (28) and after a bit of algebra, we have

$$f_{15\,Nb}(t) = f_{15\,Nb}(0)(\frac{N_T(t)}{N_T(0)})^{(\alpha_u * - 1)} \tag{30}$$

Equation (30) resembles a Rayleigh distillation (Robinson, 2001). It predicts that $f_{15\,Nb}$ will decrease as a power function of the fraction of the original nitrogen pool remaining (Fig. 6.12). To our knowledge, no study has examined the relationship between nitrogen isotope enrichment and protein loss. Schmidt et al. (1999) measured $\delta^{15}N$ in fasting earthworms (*Lumbricus festivus*)

and found no effect of fast length on nitrogen isotopic composition. However, the worms lost little mass (11%) and their C:N ratios decreased during the 2-month fast, suggesting that protein was spared. This study emphasizes that care must be exercised when using $\delta^{15}N$ in studies that aim to use nitrogen isotopic composition to measure body condition. Hobson et al. (1993) found that Ross' geese (*Chen rossii*) females lost 45% of their pectoral muscle mass and 60% of their liver mass while incubating eggs. In support of our prediction, these losses led to significant ^{15}N enrichment.

The relationship between $\delta^{15}N$ and nitrogen loss has been expected on intuitive grounds for quite some time (Hobson et al., 1993), and has prompted wildlife biologists to speculate that nitrogen isotopic composition can be used as a tool to assess body condition (see Ben David et al., 1999 and references therein). Unfortunately the term "body condition" has a multitude of possible definitions, not all of which are compatible with the use of $\delta^{15}N$ as an index of condition. This multiplicity of meanings is reflected in a plethora of body condition indices (reviewed by Hayes and Shonkwiler, 2001). Body condition indices are numerical estimates believed to reflect health, nutritional status, and/or fat content (Hayes and Shonkwiler, 2001). $\delta^{15}N$ can be used as an index of condition only if loss in body condition is equated with nitrogen loss. As is the case with all condition indices, before $\delta^{15}N$ can be used as a condition index in the field, it must be calibrated in the laboratory (Weatherhead and Brown, 1996).

An animal that is not fasting can be in negative nitrogen balance (i.e. $r_o > r_i'$). Under these conditions, $f_{15\,Nb}$ reaches an asymptotic equilibrium given by

$$\hat{f}_{15\,Nb} = \frac{\alpha_i * f_{15_{Nd}}}{(1 - \frac{r_o}{r_i'} q(1 - \alpha_u *))} \tag{31}$$

(note that eqn (31) reduces to eqn (25) if $r_o = r_i'$). Animals in negative nitrogen balance will always have more positive $\delta^{15}N$ values than animals in neutral and positive nitrogen balance (Fig. 6.13).

Are Growing Animals Lighter? Effect of Growth on $\delta^{15}N$

Equation (31) and Fig. 6.13 suggest that $\hat{f}_{15\,Nb}$ decreases as the ratio of nitrogen assimilation to nitrogen losses (r_i'/r_o) increases. Indeed, $f_{15\,Nb}$ tends asymptotically to $\alpha_i * f_{15\,Nd}$ as this ratio becomes large. These results suggest that growing animals in positive protein balance will have nitrogen isotopic compositions that are more depleted in ^{15}N than those of animals in neutral or negative nitrogen balance. If the growth trajectory of the animal and hence the values of $r_i'(t)$ and $r_o(t)$ are known, then eqn (22) can be integrated and the ontogenetic change in $\delta^{15}N$ predicted. Without this information we are left with a qualitative prediction: $^{15}N_{body\text{-}diet}$ should decrease with growth rate (i.e. with r_i'/r_o).

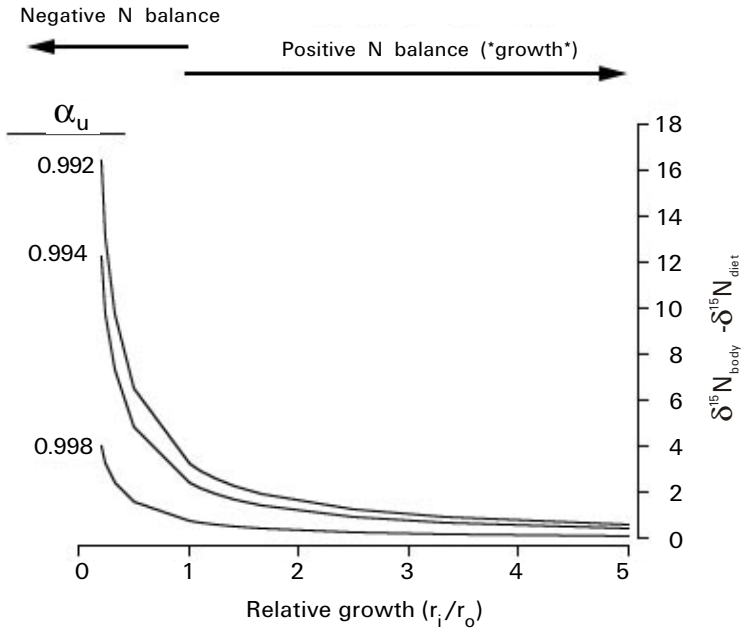

Fig. 6.13. ^{15}N enrichment relative to assimilated nitrogen decreases sharply with relative growth estimated by the ratio of nitrogen assimilation to nitrogen output (r_i'/r_o). A value of $r_i'/r_o = 0$ indicates steady state. Curves were constructed using eqn (31) and several values for the apparent fractionation of urinated nitrogen (α_u).

As far as we know, this prediction has never been examined quantitatively. Several studies have reported variation (and lack of variation) in $\delta^{15}N$ with age. Although the results of these studies are not clear cut, they seem to support the predictions of our model. The first two studies reviewed here appear to contradict the model's prediction. On close assessment, however, the reasons for rejecting the hypothesis become weak. Minagawa and Wada (1984) reported invariant $\delta^{15}N$ with age, and hence presumably with age, in two species of clams. They concluded that there was no growth effect on nitrogen isotopic composition. However, although the clams differed almost fourfold in length, they maintained relatively constant fractional growth rates of the nitrogen pool (Fig. 3 in Minagawa and Wada, 1984). Our model predicts that age per se should have no effect on $^{15}N_{body\text{-}diet}$. We should find ontogenetic changes in $^{15}N_{body\text{-}diet}$ only if age and the growth rate of the nitrogen pool are related. Ambrose (2000) reported no age-specific effects on $^{15}N_{body\text{-}diet}$ in rats but he measured the isotopic composition as a function of age only in rats older than 100 days. At this age, however, rats are not growing at high rates and hence the effect of growth on $^{15}N_{body\text{-}diet}$ should be small.

Several studies have compared $^{15}N_{body\text{-}diet}$ between mammalian young feeding on milk and their mothers (reviewed by Jenkins et al., 2001). Because

young should be growing whereas mothers should have relatively constant masses, our model predicts higher $^{15}N_{body\text{-}diet}$ in mothers than in suckling young. In concordance with this prediction, Jenkins et al. (2001) found that although mothers were significantly enriched in ^{15}N relative to their diets (average $^{15}N_{body\text{-}diet}$ = 4.1 ‰), fast growing nursing offspring were only very slightly enriched in ^{15}N relative to milk (average $^{15}N_{body\text{-}diet}$ = 1.9 ‰). Given that the quality of milk protein for suckling young is likely to be very high (see above: *Effect of Protein Quality on* $^{15}N_{body\text{-}diet}$) this result may not be unexpected and the result of both growth and efficient use of dietary protein. In a widely cited study, Hobson et al. (1993) fed Japanese quail (*Coturnix japonica*) on diets of identical nutrient and isotopic composition but at different rations. One ration allowed the quail to grow while the other sufficed to allow the birds to maintain mass but not to increase it. As predicted by the model, growing birds were depleted in ^{15}N relative to the birds that maintained body mass.

Testing and Refining Nitrogen Isotopic Composition Mass-balance Models

Mathematical models play a variety of roles. They are bookkeeping constructs. They allow us to summarize what we know and to identify what we do not know. At best, they allow making crisp predictions and hence provide a research road map. All models make assumptions, many of which may be unrealistic. Contrasting a model's prediction with data allows distinguishing essential from inconsequential assumptions. Throughout this report we have attempted to identify the assumptions of our models and the experimental measurements needed to test them. Here we reiterate one assumption implicit in our models that may be critical but whose importance is not easy to assess without data. Our models are one-compartment models. We have assumed that assimilated nitrogen enters and then exits a single, well-mixed pool. In principle, testing our models requires measuring the nitrogen isotopic composition of whole body nitrogen.

The single pool assumption is incorrect. As mentioned above, different tissues show characteristic turnovers of carbon and nitrogen, strongly indicating the existence of several element pools. Furthermore, the physiological details of nitrogen flux in animals hint at the existence of distinct, albeit interacting pools. Assimilated amino acids enter the liver where a fraction is deaminated for energy and a fraction passes through to be distributed to replace catabolized amino acids in tissues (Young and Marcini, 1990). Furthermore, different tissues have contrasting protein turnover rates (Johnson et al., 2001), and even different proteins can have widely diverging half-lives (Dice, 1987). The picture becomes even more complicated if we recognize that many animals exhibit significant nitrogen recycling. Urea synthesized by the liver is used by bacteria in the gastrointestinal tract to manufacture protein that can then be assimilated by the host (Lapierre and

Lobley, 2001). The bacterial populations in the gastrointestinal tract and the rest of the body certainly represent two distinct but closely interrelated nitrogen pools.

Do we need to incorporate the existence of many nitrogen pools and the complex, and to a large extent still unclear, fine details of protein metabolism in our model? To answer this question we advocate adopting an approach that Lewontin (2000) branded methodological reductionism. We begin by testing the models that invoke the simplest assumptions and determine their performance by contrasting their predictions with the results of well-designed experiments. Discrepancies between data and predictions should then guide our decision to revise our assumptions and assess the need to include more mechanistic detail in a new generation of models. In essence, we advocate applying the cyclical nature of the hypothetico-deductive method to animal isotopic ecology.

Although we advocate the testing of single pool models, for now we also emphasize that many applications of isotopic ecology must recognize the existence of several nitrogen pools. An appealing feature of the use of stable isotopes to answer ecological questions is that the methodology of ten does not require destructive sampling. We can inform ecological questions by sampling renewable tissues (e.g. blood cells, plasma proteins, muscle samples, hair, and feathers). Using different tissues may yield quan titatively different answers, and interpreting these differences may require recognizing the physiological differences among tissues. Suppose for example, that we are interested in using ^{15}N enrichment as an indicator of protein loss. Protein catabolism during fasting does not occur at the same rate in all tissues (Cherel et al., 1988; Schwilch et al., 2002). In addition, the fraction of amino acids exported intact relative to those deaminated in situ by aminotransferases and dehydratases varies among tissues (Raju et al., 1993). The interaction between these two factors probably dictates the de gree of ^{15}N enrichment in a given tissue as a function of fasting time. We need better data on the behavior of different tissues in response to the variables we have identified as potential influences on ^{15}N composition (protein content in diet, protein loss, and growth). This observation does not invalidate the usefulness of single pool models. It emphasizes the need to use these models to guide research at the same time that we examine their assumptions. Results of experiments needed to test the predictions of one-compartment models can reveal the existence of several pools (Johnson et al 1999) and if need be multicompartment models readily constructed (Faddy and Jones, 1988).

CONCLUDING REMARKS: ECOLOGICAL PATTERN, SEARCH FOR MECHANISTIC EXPLANATIONS, AND ROLE OF PHYSIOLOGICAL ECOLOGISTS

Testing the mass-balance models described in this chapter requires conducting experiments that entail taking fairly simple physiological measurements. In essence, we need to construct detailed budgets of elements and isotopes for animals under a variety of conditions. Later we shall need to unravel the details of the biochemical processes that lead to differences in the isotopic composition of what animals eat and what they defecate and excrete. The concept of a budget is central to all animal physiological ecology (Kooijman, 2000) and thus the creation of a mechanistic and predictive framework for isotopic animal ecology falls naturally on the shoulders of physiological ecologists. We can use the familiar experimental paradigms of physiological ecology to provide the mechanisms that explain important ecological patterns at remarkably broad temporal and spatial scales.

Analysis of natural stable isotope ratios has created a methodological revolution in animal ecology. Stable isotope analyses have become firmly established as useful methods for animal ecologists and one of the central tools for the study of feeding ecology. So far animal ecologists have adopted a phenomenological approach and relied on the search for pattern in the distribution of stable isotope ratios. Because these patterns appear to be robust, the approach has been very useful. Without mechanisms to explain them, however, we cannot ascertain the generality of the patterns discovered nor the boundaries of their usefulness. As Levin (1992) argued, without a mechanism, prediction in ecology is hazardous. The models presented in this chapter attempt to start the quest for a mechanistic and hence predictive foundation for animal isotopic ecology.

Acknowledgments

We thank Denise Dearing and Scott McWilliams for their thorough and constructive comments. Charlie Robbins pointed out the potential connection between $\Delta^{15}N_{body\text{-}diet}$ and the biological value of dietary protein. He also helped us clarify the meaning of wrong and right (as applied to models). We are especially thankful to Max Bastiani for illustrating a previous draft.

REFERENCES

Adams T. S. and Sterner R. W. 2000. The effect of dietary nitrogen content on trophic level ^{15}N enrichment. *Limnol. Oceanogr.* **45**: 601–607.

Ambrose S. H. 2000. Controlled diet and climate experiments on nitrogen isotope ratios of rats. In: *Biogeochemical approaches to paleodietary analysis* S. H. Ambrose and M. A. Katzenberg (eds.). Kluwer/Acad. Publ., Dordrecht, Netherlands, pp. 243–259.

Ambrose S. H. and Norr L. 1993. Carbon isotopic evidence for routing of dietary protein to bone collagen, and whole diet to bone apatite carbonate: Purified diet growth experiments. In: *Molecular Archaeology of Prehistoric Human Bone* J. Lambert, and G. Groupe (eds.). Springer Verlag, Berlin, pp. 1–37.

Ball P. 2002. *The Ingredients: a Guided Tour of the Elements.* Oxford Univ. Press, Oxford, UK.

Ben-David M. and D. M. Schell 2001. Mixing models in analyses of diet using multiple stable isotopes: a response. *Oecologia* **127**: 180–184.

Ben-David M. T. A., Hanley and Schell D. M. 1998. Fertilization of terrestrial vegetation by spawning Pacific salmon: the role of flooding and predator activity. *Oikos* **83**: 47–55.

Ben-David M., McColl C. J., R. Boonstra and Karels T. J. 1999. ^{15}N signatures do not reflect body condition in Arctic ground squirrels. *Can. J. Zool.* **77**: 1373–1378.

Cerling J. M., Harris J. M., and McFadden B. J. 1998. Carbon isotopes, diets of North American equids, and the evolution of North American C4 grasslands. In: *Stable Isotopes: Integration of Biological, Ecological, and Geochemical Processes* H. Griffiths (eds.). Bios Sci. Publ. Oxford, UK, pp. 363–377.

Cerling T. E. and Harris J. M. 1999. Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleontological studies. *Oecologia* **120**: 347–363.

Cherel Y., Robin J.-P., and Le Maho Y. 1988. Physiology and biochemistry of long-term fasting in birds. *Can. J. Zool.* **66**, 159–166.

DeNiro M. J. and Epstein S. 1978. Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta* **42**: 495–506.

DeNiro M. J. and Epstein S. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta* **45**: 341–351.

Dice J. F. 1987. Molecular determinants of protein half-lives in eukaryotic cells. FASEB J. **1**: 349–357.

Eggers T. and Jones T. H. 2000. You are what you eat…or are you? *Trends in Ecology and Evolution* **15**: 265-266.

Faddy M. J. and Jones M. C. 1988. Fitting time-dependent multicompartment models: a case study. *Biometrics* **44**: 587–594.

France R. L. 1995. Differentiation between littoral and pelagic food webs in lakes using stable isotopes. Limnology and Oceanography **40**: 1310–1313.

Gaebler O. H., Vitti T. G., and R. Vukmirovich 1966. Isotope effects in metabolism of 14N and 15N from unlabeled dietary proteins. *Can. J. Biochem.* **44**: 1249–1257.

Griffiths H. 1998. *Stable Isotopes: Integration of Biological, Ecological, and Geochemical Processes.* Bios Sci. Publ., Oxford, UK.

Hammer B. T., Fogel, M., and Hoering T. C. 1998. Stable carbon isotope ratios of fatty acids in seagrass and redhead ducks. *Chemical Geology* **152**: 29–41.

Hatch K. A., Pinshow B., and Speakman J. 2002. The analysis of $^{13}C/^{12}C$ rations in exhaled CO_2: Its advantages and potential application to field research to infer diet over time, and substrate metabolism in birds. *Integ. Comp. Biol.* **42**: 21–33.

Hayes J. P. and Shonkwiller J. S. 2001. Morphological indicators of body condition: useful or wishful thinking. In: *Body composition analysis of animal: a handbook of non-destructive methods* J. R. Speakman (ed.).Cambridge Univ. Press, Cambridge, UK.

Hobson K. A. 1999. Tracing origins and migration of wildlife using stable isotopes: a review. *Oecologia* **120**: 314–326.

Hobson K. A. and Clark R. G. 1992. Assesing avian diets using stable isotopes I: Turnover of 13C in tissues. *Condor* **94**: 181–188.

Hobson K. A., Alsaukas R. T., and Clark R. G. 1993. Stable nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: Implications for isotopic analysis of diet. *Condor* **95**: 388–394.

Hobson K. A., Piatt J. F., and Pitocchelli J. 1994. Using stable isotopes to determine seabird trophic relationships. *J. Animal Ecol.* **63**: 786–798.

Hoeffs J. 1997. *Stable Isotope Geochemistry*. Springer-Verlag, New York, NY.

Jackson A. A. 1998. Salvage of urea-nitrogen in the large bowel: functional significance in metabolic control and adaptation. *Biochem. Soc. Trans.* **26**: 231–236.

Jenkins S. G., Partridge S. T., Stephenson T. R., Farley S. D., and Robbins C. T. 2001 Nitrogen and carbon isotope fractionation between mothers, neonates, and nursing offspring. *Oecologia* **129**: 336–341.

Johnson H. A., Baldwin R. L., France J., and Calvert C. C. 1999. A model of whole-body protein turnover based on leucine kinetics in rodents. *J. Nutr.* **129**: 728–739.

Johnson H. A., Baldwin R. L., Klasing K. C., and Calvert C. C. 2001. Impact of separating amino acids between plasma, extracellular and intracellular compartments on estimating protein synthesis in rodents. *Amino Acids* **20**: 389–400.

Karasov W. H. 1990. Digestion n birds: chemical and physiological determinants and ecological implications. *Studies in Avian Biology* **13**: 391–415.

Kelly J. F. 1999. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. *Can. J. Zool.* **78**: 1–27.

Koch P. L., Fogel M. L., and Tuross N. 1994. Tracing the diets of fossil animals using stable isotopes. In: *Stable isotopes in ecology and environmental science,* K. Lathja and R. H. Michener (eds.). Blackwell Sci. Publ., Oxford, UK, pp. 63–92.

Kooijman S. A. L. M. 2000. *Dynamic Energy and Mass Budgets in Biological Systems.* Cambridge Univ. Press, Cambridge, UK.

Lajtha K. and Michener R. H. 1994. *Stable Isotopes in Ecology and Environmental Science.* Blackwell Sci. Publ., New York, NY.

Lapierre H. and Lobley G. E. 2001. Nitrogen recycling in ruminants: a review. *J. Dairy Sci.* **84**: E223–236.

Levin S. A. 1992. The problem of pattern and scale in ecology. *Ecology* **73**: 1943-1967.

Lewontin R. 2000. *It ain't necessarily so: the dream of the human genome and other illusions.* New York Review Books, New York, NY.

Macko S. A. and Estep M. L. F. 1984. Microbial alteration of stable nitrogen and carbon isotopic compositions of organic matter. *Org. Geochem.* **6**: 787–790.

Macko S. A., Fogel M. L., Engel M. H., and Hare P. E. 1986. kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochim. Cosmochimica Acta* **50**: 2143–2146.

Martínez del Rio C. 1994. Nutritional ecology in nectar- and fruit-eating volant vertebrates. In: *Food and form and function of the mammalian digestive tract* D. Chivers and P. Langer (eds.). Cambridge Univ. Press, Cambridge, UK, pp. 103–127.

Mathews C. K., van Holde K. E., and Ahern K. K. 2000. *Biochemistry.* Benjamin Cummins, New York, NY.

Minagawa M. and Wada E. 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. *Geochim. Cosmochim. Acta* **48**: 1135–140.

Mook W. G. 1986. ^{13}C in atmospheric CO_2. *Netherlands J. Sea Res.* **20**: 211–223.

O'Brien D. M., Schrag D. P., and Martínez del Rio C. 2000. Allocation to reproduction in a hawkmoth: a quantitative analysis using stable isotopes. *Ecology* **81**: 2822–2831.

O'Brien D. M., Fogel M. L., and Boggs C. L. 2002. Renewable and non-renewable resources: amino acid turnover and allocation to reproduction in lepidoptera. *Proc. Natl. Acad. Sci. USA* **99**: 4413–4418.

Peterson B. J., and Fry B. 1987. Stable isotopes in ecosystem studies. *Ann. Rev. Ecol. Syst.* **18**: 293–320.

Phillips D. L. 2001. Mixing models in analyses of diet using multiple stable isotopes: a critique. *Oecologia* **127**: 166–170.

Phillips D. L, and P. L. Koch 2002. Incorporating concentration dependence in stable isotope mixing models. *Oecologia* **130**:114–125.

Post D. M. 2002. Using stable isotopes to estimate trophc position: models, methods, and assumption. *Ecology* **83**: 703–718.

Pritchard G. T. and Robbins C. T. 1990. Digestive and metabolic efficiencies of grizzly and black bears. *Can J. Zool.* **68**: 1645–1651.

Raju M. R. D., Vijayalakshmi K., and Karuna K. M. 1993. Influence of dietary protein on aminotransfrases and phosphatases in rat tissues. *J. Food Sci. Tech.* **30**: 183–186.

Reeds P. J. and Beckett P. R. 1996. Protein and amino acids. In: *Present knowledge in nutrition* E. E. Ziegler and L. J. Filer (eds.). ILSI Press, Washington DC, pp. 67–86.

Richardson S. M. and McSween H. Y. 1989. *Geochemistry: pathways and processes.* Prentice Hall, New York, NY.

Robbins C. T. 1993. *Wildlife Feeding and Nutrition.* Acad. Press, New York, NY.

Robbins C. T. Hilderbrand G. V., and Farley S. D. 2002. Incorporating concentration dependence in stable isotope mixing models: a response to Phillips and Koch. *Oecologia* **133**: 10–13.

Robinson D. 2001. $\delta^{15}N$ as an integrator of the nitrogen cycle. *Trends Ecol. Evol.* **16**: 153–162.

Schmidt O., Scrimgeour C. M., and Curry J. 1999. Carbon and nitrogen isotope ratios in body tissue and mucus of feeding and fasting earthworms *(Lumbricus festivus). Oecologia* **118**: 9–15.

Schwilch R., Grattarola A., Spina F., and Jenni L. 2002. Protein loss during long-distance migratory flight in passerine birds: adaptation and constraint *J. Exp. Biol.* **205**: 687–695.

Steele K. W. and Daniel R. M. 1978. Fractionation of nitrogen isotopes by animals: A further complication to the use of variation in the natural abundance of ^{15}N for tracer studies. *J. Agric. Sci.* **90**: 7–9.

Tieszen L. L., Boutton T. W., Tesdahl K. G., and Slade N. A. 1983. Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet. *Oecologia* **57**: 32–37.

Tieszen L. L. and Fagre T. 1993. Effect of diet quality and composition on the isotopic composition of respiratory CO_2, bone collagen, boapatite, and soft tissues. In: *Molecular Archaeology of Prehistoric Human Bone* J. Lambert, and G. Groupe (eds.). Springer Verlag, Berlin, pp. 123–135

Van Soest P. 1994. *Nutritional Ecology of the Ruminant.* Cornell Univ. Press, Ithaca, NY.

Weatherhead P. J. and Brown G. P. 1996. Measurement versus estimation of condition in snakes. *Can. J. Zool.* **74**: 1617–1621.

Wolf B. O. and Martínez del Rio C. 2000. Use of saguaro fruit by White-winged doves: isotopic evidence of a tight ecological association. *Oecologia* **124**: 536–543.

Young V. R. and Marcini J. S. 1990. Mechanisms and nutritional significance of metabolic responses to altered intakes of protein and amino acids, with reference to nutritional adaptation in humans. *Amer. J. Clin. Nutr.* **51**: 270–289.