BIOLOGICAL REVIEWS
Cambridge Philosophical Society

Biol. Rev. (2009), **84**, pp. 91–111.
doi:10.1111/j.1469-185X.2008.00064.x

Isotopic ecology ten years after a call for more laboratory experiments

Carlos Martínez del Rio[1]*, Nathan Wolf[1], Scott A. Carleton[1] and Leonard Z. Gannes[2]

[1] *Department of Zoology and Physiology, University of Wyoming, Laramie, WY* 82071-3166, *USA*
[2] *Sciences and Conservation Studies, College of Santa Fe,* 1600 *St. Michael's Dr., Santa Fe, NM* 87505, *USA*

(*Received* 30 *April* 2008; *revised* 13 *October* 2008; *accepted* 17 *October* 2008)

ABSTRACT

About 10 years ago, reviews of the use of stable isotopes in animal ecology predicted explosive growth in this field and called for laboratory experiments to provide a mechanistic foundation to this growth. They identified four major areas of inquiry: (1) the dynamics of isotopic incorporation, (2) mixing models, (3) the problem of routing, and (4) trophic discrimination factors. Because these areas remain central to isotopic ecology, we use them as organising foci to review the experimental results that isotopic ecologists have collected in the intervening 10 years since the call for laboratory experiments. We also review the models that have been built to explain and organise experimental results in these areas.

Key words: $\delta^{13}C$, $\delta^{15}N$, experimental isotopic ecology, stable isotopes, trophic ecology.

CONTENTS

* Address for correspondence: E-mail: cmdelrio@uwyo.edu

I. INTRODUCTION

Stable isotope chemistry was once the domain of the earth sciences. Due to a combination of neglect and technical difficulty, its use was largely inaccessible to biologists. This situation changed about 20 years ago with development of on-line isotopic analyses and when plant physiologists and plant physiological ecologists began using these tools to discover the potential of stable isotope analyses (SIA) as tools to link processes at scales spanning from cells to ecosystems (Dawson *et al.* 2002). Animal ecologists were a little slower in adopting the method, but over the last 10-15 years they have embraced it heartily, and in this period the applications of SIA to the study of animals have grown very rapidly (Martínez del Rio & Wolf 2005). Ecologists have realised that they have a remarkable technique at their disposal and have taken advantage of it with gusto. Animal ecologists use stable isotopes in a myriad of applications that range from paleoecology to ecosystem ecology, passing through physiological and population ecology (Hobson & Wassenaar 1999; Koch 2007).

The successful adoption of the method by animal ecologists is the result of strides in three areas: (1) technological progress has made SIA widely available, and a lot cheaper; (2) ecologists have gathered large observational data sets, and have conducted many experiments that examine the boundaries of the applications of SIA; and (3) ecologists have developed models that permit the interpretation of field data and experimental results, which has motivated further observations and experiments. About 10 years ago, Gannes, O'Brien & Martínez del Rio (1997) and Gannes, Martínez del Rio & Koch (1998) assessed the state of the art in the application of stable isotopes in animal ecology. They concluded with the prediction of explosive growth in the field and with an exhortation for laboratory experiments to sustain it. Gannes *et al.* (1997) contended that the field's health depended on using experimental data to support the many assumptions needed to interpret field data, and to establish whether the assumptions that field researchers often have to make are warranted. Gannes *et al.*'s (1997) prediction was confirmed spectacularly. The use of stable isotopes in animal ecology has flourished. Their exhortation for more laboratory experiments was heeded and now the field is supported by a stronger collection of experimental studies. The literature on the application of SIAs in animal ecology has grown so much that it would be folly to attempt to review it here. Like Gannes *et al.* (1997, 1998), we will review only how experimental studies have led to advances in the application of stable isotopes in animal ecology. Our focus will not be the, often striking, insights that ecologists have gained by using stable isotopes, but rather the areas in which further experimentation can lead to progress.

The development of the application of SIA to animal ecology has been the result not only of advances in experimental research, but also of the growth in theory. The accumulation of field and experimental data has stimulated ecologists to build mathematical models that aim to explain patterns, to synthesise observations, and to generate novel predictions. The use of theory in isotopic ecology has advanced hand in hand with the accumulation of data. The purpose of this review is not only to summarise experimental results, but also to describe, and on occasion elaborate, the models that ecologists use to interpret them. We will also identify areas in which theoretical research and development are still needed.

Gannes *et al.* (1997, 1998) proposed that the successful application of SIA to animal ecology hinged on our knowledge of how rapidly and faithfully animals incorporate the isotopic composition of the resources that they use. They identified four major areas in which experimental work was needed to further this knowledge. We have labeled these areas: (1) the dynamics of isotopic incorporation, (2) mixing models, (3) isotopic routing, and (4) trophic discrimination factors. These areas remain relevant and are the organising foci of this review. In a final section we will identify novel research themes that we believe are potentially fruitful and ripe for exploration. Throughout this review, we will contend that although isotopic ecology has grown and matured, there is still much that we do not know.

II. THE DYNAMICS OF ISOTOPIC INCORPORATION

(1) Why should ecologists care about them?

Isotopic ecologists should be interested in the time course of the incorporation of the isotopic signature into an animal's tissues for two reasons: first, this information determines the time window through which they can perceive the course of changes in the isotopic composition of an animal's diet (Newsome *et al.* 2007). Second, by sampling different types of tissues in a single individual, SIA permit exploration of how an animal uses resources over a variety of temporal scales. The method therefore allows making inferences about the breadth of resources used by an individual, and to determine the contribution of intra- and inter-individual variation to the spectrum of resources used by a population (Dalerum & Angerbjörn 2005). This application is based on the observation that tissues differ in the rate at which they incorporate new materials (reviewed by Phillips & Eldrige 2006). In vertebrates, some tissues, such as liver and plasma proteins have high turnover rates, and their isotopic composition reflects integration of recent dietary inputs. Others, such as bone collagen, exhibit low incorporation rates and their isotopic composition reflects integration of dietary inputs over longer time periods (Dalerum & Angerbjörn 2005 and references therein). Some tissues such as feathers, hair, and shells are deposited in a discrete interval and because they are inert, retain the isotopic composition of resources incorporated while they were manufactured (Best & Schell 1996 & Bowen, Wassenaar & Hobson 2005).

(2) One-compartment, first-order models

The time course of isotopic incorporation is determined experimentally, most often in the laboratory, but sometimes

in the field (McIntyre & Flecker 2006). Typically a group of animals are fed a diet of known isotopic composition. After their tissues have reached a stable isotopic composition, animals are shifted to another diet with a different isotopic composition (Martínez del Rio & Anderson-Sprecher 2008). The relationship between the composition of animal's tissue and time (t) has been traditionally described by the equation:

$$\delta X_{tissue}(\mathrm{t}) = a + b\mathrm{e}^{-ct} \quad (1)$$

where the parameters a, b, and c are estimated empirically (Bearhop *et al.* 2002 and references there). Martínez del Rio and Wolf (2005) proposed the alternative form

$$\delta X_{tissue} = \delta X_{\infty} - [\delta X_{\infty} - \delta X_{tissues}(0)]e^{-\lambda t}, \quad (2)$$

In this equation $a = \delta X_{\infty}$, $b = -[\delta X_{\infty} - \delta X_{\mathrm{tissue}}(0)]$, and $c = \lambda$. The values of a and b in equation 1 are very useful to diagnose the success of an isotopic incorporation experiment. If a (or δX_{∞}) does not equal the value of the diet plus a reasonable discrimination factor ($a = \delta X_{\infty} = \delta X_{diet} + \Delta$), the experiment has not run to completion and one might question the inferences on c (or λ) that one can derive from the experiment. The non-linear routines routinely used to fit the values of equations 1 and 2 are notoriously unreliable if the data set does not include values of $\delta X_{tissue}(t)$ that are close from the function's asymptote (Bates & Watts 1988).

Equations 1 and 2 represent the behavior of a well-mixed, one-compartment system with first order kinetics. Martínez del Rio and Wolf (2005) and Olive *et al.* (2003) described similar derivations for equation 2. In systems well described by equation 2, the residence time of an element is distributed as an exponential density function of time (t):

$$f(t) = \frac{1}{\tau}e^{-\frac{t}{\tau}} \quad (3)$$

where τ is the average residence time and equals $1/\lambda$ (Martínez del Rio & Anderson-Sprecher 2008). Researchers often estimate the half-life of an element in a tissue as $t_{1/2} = \tau \ln(2) = \ln(2)/\lambda$. The half-life is the median of the residence time distribution represented in equation 3. Although in a following section we will argue that it is inappropriate to use equation 2 as a general description of isotopic incorporation in all cases (see Cerling *et al.* 2007*a*), its application has been fruitful. Perhaps because of its simplicity and the clear intuitive interpretation of its parameters, equation 2 has led ecologists to identify some of the important factors that determine isotopic incorporation rates.

(3) Why does isotopic incorporation differ among animals?

The rate at which animals incorporate the isotopic signal of their diets into their tissues varies among organisms and among the tissues within a single individual. The factors that have been recognised (or hypothesised) to influence incorporation rate are body size, growth, and protein turnover. In this section we will deal with body size and growth. We will consider protein turnover in the following section. Carleton & Martínez del Rio (2005) interpreted λ in equation 2 as the ratio of the net rate of influx of materials into a tissue ($\dot{v}$) and the size of the pool of the element in the tissue (P): $\lambda = \dot{v}/P$. Because the rate of elemental incorporation into a tissue, and hence $\dot{v}$, should be proportional to body mass (m_b) to the 3/4 power (West, Brown & Enquist 1997), and because the size of most tissues scales isometrically with body mass, λ should be proportional to $m_b^{3/4}/\ m_b = m_b^{-1/4}$. A data set on the rate of ^{13}C incorporation into the red blood cells of several bird species verified this prediction (Carleton & Martínez del Rio 2005): the fractional rate of isotopic incorporation declined with body mass to approximately the $-1/4^{\mathrm{th}}$ power. To our knowledge, this prediction has not been tested in other animal groups or other tissues. However, this result suggests that isotopic ecologists cannot use incorporation data of an animal to infer the incorporation rate of another of a different size. This cautionary note depends on the difference in mass between animals: an allometric exponent of -1/4 suggests that animals that differ in mass by a factor of 2 will differ in λ values by only 20%, whereas if they differ in mass by an order of magnitude their λ values will differ by $\approx$ 100%. Carleton & Martínez del Rio (2005) also suggest that a fruitful, and perhaps urgent, task for isotopic ecologists is to gather the data needed to construct the allometric relationships between incorporation rate and body size in a diversity of taxa and for the most widely used tissues.

An animal's body size is not the only determinant of the rate at which its tissues incorporate the isotopic composition of the diet. Fry & Arnold (1982) recognised that the value of λ is determined by both the addition of new material to the tissue ("growth") and by the replacement of material exported from the tissue as a result of catabolism ("catabolic turnover"). Hesslein, Hallard & Ramal (1993) proposed that the value of λ equals the sum of fractional net growth k_g ($k_g = m_b^{-1}[dm_b/dt]$) and catabolic turnover k_d ($\lambda = k_g + k_d$). If the animal is not growing, then λ equals catabolic turnover ($\lambda = k_d$). If the animal is growing exponentially (i.e. if k_g is approximately constant), then we can measure growth and use equation 2 to partition the contribution of net growth and catabolic turnover to λ. The term k_g can be measured as the mass specific rate of change in the size of the pool and k_d can be estimated by difference ($k_d = \lambda - k_g$). Partitioning the contribution of growth and catabolism to λ is more complicated if the animal is not growing exponentially. If we measure growth, we can find the function that describes the change of k_g in time. If we assume that k_d remains constant, we can solve

$$\delta X(t) = \delta X_{\infty} - [\delta X_{\infty} - \delta X(0)]e^{-\int_0^t (k_g(t) + k_d)dt} \quad (4)$$

to find the relative contribution of growth and catabolism to incorporation as a function of time.

If isotopic incorporation can be described adequately by equation 2, we can summarize the effects of growth and catabolism on λ as follows:

$$\lambda = \frac{1}{m_b}\left(\frac{\mathrm{d}m_b}{\mathrm{d}t}\right) + \alpha m_b^{\theta}, \quad (5)$$

where α and θ are an allometric constant and an allometric exponent, respectively. This equation states that the fractional rate of isotopic incorporation equals the sum of fractional growth rate and the allometric effect of body size on catabolic turnover. In the adults of determinate growers, the growth rate term equals 0, and therefore the allometric effect of body size should be the primary determinant of isotopic incorporation. In this case we expect both τ, the average retention time (λ^{-1}), and $t_{1/2}$, to scale with mass to the ¼ power. Because mass-specific growth rate scales with $m_b^{-1/4}$ (Peters 1983) in animals in the exponential phase of growth, both terms in equation 5 should be allometric functions of body mass with the same exponent (approximately -1/4). Because temperature has a profound effect on all metabolic processes (Gillooly *et al.* 2001), we expect the magnitude of the allometric term to be temperature dependent (Witting *et al.* 2004), and to differ between endotherms and ectotherms. Following the notation of metabolic theory (Brown *et al.* 2004), we can summarize these expectations as $\lambda \propto m_b^{-\frac{1}{4}} - \frac{E_a}{kT}$, where E_a is the "energy of activation" of metabolic processes, k is Boltzmann's constant, and T is absolute temperature (Brown *et al.* 2004).

Although the predictions embodied in equation 5 have not been tested quantitatively yet, the available data are consistent with them. Isotopic incorporation is rapid in fast-growing ectotherms. In growing fish, growth seems to account for most, if not all, of the value of λ (Jardine *et al.* 2004; Suzuki *et al.* 2005 and references therein). McIntyre & Flecker (2006) reported that incorporation rates were very similar to growth rates in snails (*Tarebia granifera*) and tadpoles (*Rana palmipes*) and Reich, Bjorndal & Martínez del Rio (2008) made the same observation in growing loggerhead turtles (*Caretta caretta*). The contribution of growth to λ in the tissues of all the ectotherms listed above was high (from 30 to 100%) relative to that reported by MacAvoy, Macko & Arneson (2005) for adult, but still growing, mice. In these endotherms, growth accounted for only $\approx$ 10% of the rate of incorporation of carbon and nitrogen. It is tempting to speculate that there is a difference in the relative contributions of growth and catabolic turnover to the rate of isotopic incorporation between endotherms (e.g. mice) and ectotherms (e.g. crustaceans, snails, fish, amphibians, and reptiles).

This hypothesis must be tempered by differences in the developmental stages of the endotherms and ectotherms that have been investigated. Although the mice studied by MacAvoy *et al.* (2005) were growing, they were close to their maximal size, whereas many of the studies on ectotherms have been conducted in rapidly growing young. West, Brown & Enquist (2001) have hypothesised that the fraction of energy and nutrients used for growth, relative to other functions, is more or less equal for all species at the same stage of development, as measured relative to their asymptotic mass. The relative contribution of growth to isotopic incorporation may be roughly the same in ectotherms and endotherms, provided that animals are measured at comparable developmental stages. Among vertebrates, endotherms reach their asymptotic mass in a relatively short time and then stop growing (they are "determinate growers"), whereas many (albeit not all) ectotherms continue growing for most of their lives (they are "indeterminate growers"; Sebens 1987). Therefore, in most ecological field studies growth rates will be more important determinants of isotopic incorporation in ectotherms than in endotherms.

The dominant effect of growth on the rate of isotopic incorporation in growing ectotherms has consequences for the interpretation of field isotopic measurements. Perga & Gerdeaux (2005) found that the isotopic composition of muscle in whitefish (*Coregonus lavaretus*) reflected only the isotopic composition of prey consumed in the seasons when the fish were growing. By contrast, the isotopic composition of liver tracked the isotopic composition of the diet closely throughout the year. The effects of growth on stable isotope analyses are probably a prevalent, and relatively unstudied, confounding factor in stable isotope field studies (Reich *et al.* 2008). Tieszen *et al.* (1983, p. 35) warned about an "*. . .important complication . . . is that each tissue. . .can be expected to have an isotopic memory*". The combined effect of body size and growth on incorporation rate may exacerbate this complication in large animals, such as ungulates and seals, and in the "slow" tissues that are often used to study them (bone collagen; Koch 2007). In these animals the diet ingested during growth may give collagen a long-lasting imprint, and the contribution of diets ingested after animals are fully grown may be difficult to detect.

The experimental studies that we have reviewed have revealed the profound importance of animal size, temperature, and growth for the dynamics of isotopic incorporation. These studies are useful in that they identify important factors. Unfortunately, laboratory experiments fail to capture fully all complexities of these factors in nature - including temporal variation and dependence on food abundance and quality. Field experiments that probe the effects and consequences of these factors on isotopic incorporation are needed for ecologists to interpret the results of isotopic patterns.

(4) Why does isotopic incorporation differ among tissues?

Tieszen *et al.* (1983) first recognised the importance of measuring isotopic incorporation to interpret field isotopic measurements. These authors were also the first to demonstrate variation in isotopic incorporation rate among tissues. They speculated that "*more metabolically active tissues. . .have faster turnover than less metabolically active tissues*" (p. 33), and supported this speculation with a negative correlation between oxygen consumption data measured *in vitro* and the half-life of ^{13}C in four tissues. Understandably, Tieszen *et al.*'s (1983) statement has come to be interpreted to mean that both organisms and tissues with high metabolic rate, construed narrowly as a high rate of oxygen consumption, should have fast rates of isotopic incorporation (Hobson and Clark 1992; Voigt *et al.* 2003). For

example, Klaasen, Thums & Hume (2004) measured incorporation of dietary ^{13}C and ^{15}N into the blood of long-nosed bandicoots (*Perameles nasuta*), found very low levels of incorporation, and speculated that these rates were low compared with those measured in other birds and mammals due to the low metabolic rate of marsupials (Klaasen *et al.* 2004).

However, the experimental evidence does not support this widely held assumption. Voigt *et al.* (2003) investigated the rate of dietary carbon incorporation into the tissues of nectar-feeding bats, and found that the rate of carbon incorporation in these animals was the lowest reported for a vertebrate, which is surprising in view of the exceedingly high metabolic rates of these animals. Carleton & Martínez del Rio (2005) exposed house sparrows (*Passer domesticus*) to cold temperature to increase their metabolic rate. In spite of a large increase in metabolic rate, cold exposure had no effect on ^{15}N incorporation rate, and had only a small, and biologically insignificant, effect on ^{13}C incorporation rate. Why do we find these discrepancies between experimental observations and what seems like a reasonable hypothesis?

In their original paper, Tieszen *et al.* (1983) conflated two related concepts: metabolic activity construed broadly as the collection of anabolic and catabolic processes, and metabolic rate construed narrowly as respiration rate. Although respiration rate is related to metabolism *sensu lato*, the relationship is not direct and respiration rate can be uncoupled from some (albeit not all) components of the intensity of secondary metabolism (Marsh, Marxson & Manahan 2001). This confusing conflation has been exacerbated by MacAvoy, Macko & Bassett's (2006) report of a negative correlation between mass-specific basal metabolic rate (MR/m_b) and the half-life ($t_{1/2}$) of isotopic incorporation. This correlation is a necessary consequence of the allometric dependence of both $t_{1/2}$ and MR/m_b on body mass. Because $t_{1/2}$ scales with $m_b^{1/4}$ (Carleton & Martínez del Rio 2005) and MR/m_b scales with $m_b^{-1/4}$ (West *et al.* 1997), then $t_{1/2}$ must scale with $(MR/m_b)^{-1}$.

Both Voigt *et al.* (2003) and Carleton & Martínez del Rio (2005) measured the incorporation of carbon into proteinaceous tissues (connective tissues and blood). In these tissues, respiration rate can be increased by the catabolism of endogenous non-protein substrates without increasing the rate of protein synthesis and breakdown (Marsh *et al.* 2001; Lobley 2003). Had Voigt *et al.* (2003) and Carleton and Martínez del Rio (2005) measured the turnover of lipid and carbohydrate reserves, instead of that of protein, they would have found very high incorporation rates. Indeed, Voigt & Speakman (2007) found that nectar-feeding bats incorporate dietary ^{13}C into lipid reserves very rapidly. Carleton and Martínez del Rio (2005) proposed that the term "metabolic activity" in Tieszen *et al.*'s (1983) hypothesis should be interpreted narrowly to imply synthesis and catabolism of specific tissue components. They speculated that the primary determinant of the rate of isotopic incorporation in most tissues (whose isotopic composition is typically measured after lipids are extracted, Post *et al.* 2007) is protein turnover.

Because protein turnover is a process that has been relatively well studied, we can pose two predictions: (1) the rate of isotopic incorporation into different organs/tissues should be ranked in the same order as their rate of protein turnover. Thus, structural elements (collagen, striated muscle, red blood cells) should have lower rates of isotopic incorporation than splanchnic organs (liver, viscerae, and plasma proteins; Waterlow 2006); (2) because dietary proteins are known to have a regulatory effect on protein synthesis and degradation (Lobley 2003 and reviewed by Waterlow, 2006), we expect protein intake to influence isotopic incorporation rates. Physiologists have documented increases in protein synthesis resulting from increased protein intake in a variety of vertebrates (see Tsahar *et al.* 2007).

Testing Carleton & Martínez del Rio's (2005) conjecture directly requires measuring protein turnover and isotopic incorporation concurrently. Although these measurements have not been done, there is significant support for the hypotheses' two predictions. Splanchnic organs with high rates of protein turnover such as the liver and intestine have higher rates of isotopic incorporation than collagen and muscle (see Waterlow 2006), and Tsahar *et al.* (2007) documented a 36-60% increase in the retention time of ^{15}N in blood cells and plasma when they reduced the nitrogen content of the diet of a fruit-eating bird (*Pycnonotus xanthopygos*). Tsahar *et al.* (2007) suggested that a protein-deficient diet was responsible for the unusually long ^{13}C and ^{15}N retention times in the nectar-feeding bats studied by Voigt *et al.* (2003). This hypothesis is supported by the observation that when the same bat species were fed on diets with adequate nitrogen/protein, their retention times were much lower (Mirón *et al.* 2006). Carleton & Martínez del Rio (2005) speculated that isotopic incorporation and protein turnover are linked by the two components of protein turnover: synthesis, which entails incorporation of dietary nutrients, and catabolism, which entails loss of tissue materials. Available evidence gathered so far supports this view.

(5) Isotopes and nutrient allocation

The link between isotopic incorporation and protein turnover illustrates how we can find the foundations of isotopic ecology in nutritional physiology. Recent experimental work has illustrated the other side of the coin: stable isotope analyses can open up new vistas in nutritional research. One of the most exciting applications of SIA in nutritional research has been the use of food's natural isotopic signatures to estimate the allocation of ingested nutrients to somatic and reproductive functions (Boutton *et al.* 1988). In a series of ingenious experiments, O'Brien and her collaborators have disentangled the contribution of larval and adult diet to eggs in moths and butterflies, and in the process illuminated the importance of essential amino acids in the life history of these organisms.

The methods used by these researchers are informative. O'Brien *et al.* (2000 and 2002) fed adults on nectar with a carbon isotopic composition that could be distinguished from that of the plants that they ate as larvae. From the time course of incorporation of ^{13}C from the adult diet into eggs, they estimated the fractional rate at which the pool of nutrients devoted to eggs incorporated new carbon, and the maximal percentage of carbon in eggs that was derived

from nectar (Fig. 1). Many moths and butterflies feed exclusively on nectar as adults and primarily derive carbohydrates from it. In these animals, the maximal % of carbon in eggs derived from nectar ranges from 44% to 66% (O'Brien, Boggs & Fogel 2004). O'Brien, Boggs & Fogel (2005) found that the $\delta^{13}C$ of individual indispensable amino acids in eggs was very similar to that of the essential amino acids of the larval food plant (Fig. 1). By contrast, the $\delta^{13}C$ values of dispensable amino acids resembled those of nectar. Thus, dispensable amino acids are a renewable resource that the butterflies can synthesise from carbon skeletons that they obtain from their adult diet. By contrast, when butterflies use indispensable amino acids to make eggs, they are tapping into a non-renewable, and hence finite, resource (O'Brien *et al.* 2002). Total egg production is limited by the amount of indispensable amino acids stored as larvae (O'Brien *et al.* 2004).

Indispensable amino acids appear not be a non-renewable resource in all Lepidoptera. Some butterflies in the genera *Heliconius* and *Laparus* (subfamily Heliconiini) soak pollen in the nectar that they imbibe. These butterflies live longer and are more fecund that those that rely exclusively on nectar as adults. O'Brien, Boggs & Fogel (2003) used compound-specific isotopic analyses to estimate the contribution of the nutrients that leach from pollen to the indispensable amino acids found in eggs. In *Heliconius charitonia*, they found that roughly 17% of the carbon in indispensable amino acids was derived from pollen (O'Brien *et al.* 2003). The higher longevity and fecundity of heliconine butterflies seems to be the result of the ability of these butterflies to replace some of the indispensable amino acids that they place in eggs with amino acids from pollen. Stable isotopes have revealed that the life history of butterflies and moths seems to be shaped by whether they use indispensable amino acids as renewable or non-renewable resources.

(6) One, two, how many compartments?

Ecologists have traditionally estimated isotope incorporation rates using one-compartment models that obey first-order kinetics (Martínez del Rio & Wolf 2005). By contrast, since the late 1950s, physiologists studying protein turnover typically have relied on multi-compartment models (Waterlow 2006). Ayliffe *et al.* (2004) and more recently Cerling *et al.* (2007*a*) have called for the use of multi-compartment models to describe isotopic incorporation rates. Cerling *et al.* (2007*a*, *b*) have argued that by using one-compartment models, isotopic ecologists have erroneously over-simplified a complex process. Implicit in Cerling *et al.*'s (2007*a*, *b*) contention is the observation that by using only one-compartment models, we may be biasing the estimates of how long isotopes stay in tissues, and thus the time windows of incorporation that we can assess in each tissue.

Cerling *et al.* (2007*a*) proposed a useful graphical method (the "reaction progress variable") to diagnose whether isotopic incorporation data must be described by one- or multi-compartment models. Martínez del Rio & Anderson-Sprecher (2008) extended Cerling *et al.*'s (2007*a*) method by developing statistical estimates of average retention time for isotopes in multi-compartment systems and of the uncertainty associated with these estimates. These authors also proposed the use of information theoretic criteria (such as Akaike Information Theoretic Criteria, AIC), to assess the weight of evidence in favour of one- or multi-compartment models. Tsahar *et al.* (2007) used the methods proposed by Martínez del Rio & Anderson-Sprecher (2008) to analyse the incorporation of ^{15}N into the blood cells and plasma proteins of a fruit-eating bird (*Pycnonotus xanthopygos*). They concluded that one-compartment models were better supported than two-compartment models. By contrast, Carleton *et al.* (2008) analysed the incorporation of ^{13}C into several tissues of house sparrows and found that in many (albeit not all) tissues, two-compartment models were better supported than one-compartment models (Fig. 2). More importantly, they found that using one- or two-compartment models led to different inferences: the average retention of ^{13}C estimated by the two-compartment model was systematically higher than that estimated by the one-compartment model (Fig. 2). Furthermore, the absolute difference in $\delta^{13}C$ values between tissues and diet (the tissue to diet "discrimination factor", Δ) estimated by

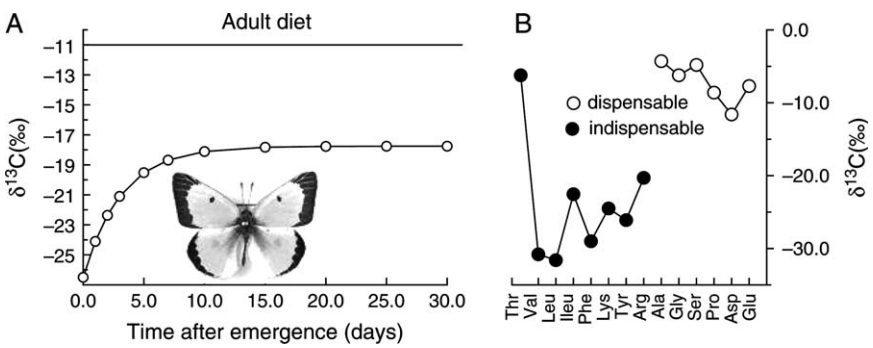

Fig. 1. The carbon isotopic composition of protein in the eggs of newly emerged *Colias eurytheme* resembled that of larval diet. The isotopic composition of eggs changed with time as more carbon from nectar was incorporated into eggs (A). However, the asymptotic $\delta^{13}C$ of eggs was intermediate between that of larval and adult diets (upper line in A). This result can be explained by the $\delta^{13}C$ of dispensable amino acids (which are derived from carbon in nectar, open symbols in B), and that of indispensable amino acids (which are derived from the larval diet, filled symbols in B). After O'Brien *et al.* (2005).

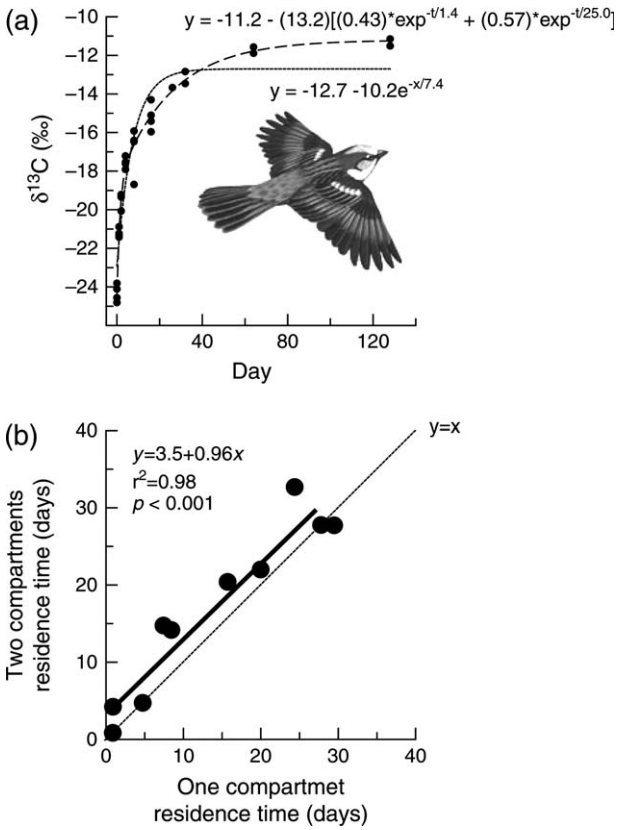

Fig. 2. (A) The pattern of incorporation of ^{13}C into the liver of house sparrows (*P. domesticus*) was best described by a two (dashed curve, AICc = 39.9) than by a one-compartment (dotted curve, AICc =83.1) model. (B) In a variety of tissues of *P. domesticus*, the average retention time estimated by two-compartment models was consistently higher (by about 3.5 days) than that estimated by one-compartment models (b). After Carleton *et al.* (2008).

two-compartment models was smaller than that that estimated by the one-compartment model (Carleton *et al.* 2008). Martínez del Rio & Anderson-Sprecher (2008) have emphasised the important observation that multi-compartment models are convenient simplified statistical descriptions of complex phenomena and pointed that one cannot infer from these models the number of "real" isotopic compartments in an organism. Indeed, O'Brien *et al.* (2002) have shown that isotopic incorporation patterns vary greatly among individual amino acids! The results of compound-specific isotopic analyses open new vistas and remind us that the results of "bulk" isotopic analyses are the results of the statistical averages of a myriad of metabolic transformations of organic compounds.

The use of multi-compartment models in isotopic incorporation studies is a relatively recent development. Consequently, we do not know yet if the application of these models will force us to reconsider the inferences hard won with studies that used one-compartment models. Because these patterns are strong, we venture that their general trends will be robust to model structure. However, details might change (Fig. 2). A re-analysis of the myriad isotopic incorporation studies following the guidelines of Cerling *et al.* (2007*a*) and Martínez del Rio & Anderson-Sprecher (2008) should be an important priority in isotopic ecology. Recognising the possibility that a data set is described by many, rather than by only one compartment has implications for the design of experiments. Many isotopic incorporation experiments have sampled tissues at intervals that increase in length following a 2^n geometrical sequence rule (e.g. sapling at 1, 2, 4, 16,. . ., days, e.g. Carleton *et al.* 2008). Cerling *et al.* (2007*b*) worry that this sampling schedule can prevent analyses from detecting compartments and recommend a sampling protocol that follows a $2^{n/2}$ rule that places strong emphasis on compartments with short residence times (i.e. 1.4, 2, 2.8, 4, 5.6, . . ., h). This is a sensible recommendation that must be tempered only by the objectives of the researcher (including the ecologically relevant time windows that researchers might be interested in probing) and the cost of an experiment. For the duration of any experiment, Cerling *et al.*'s (2007*b*) proposed schedule includes twice as many sampling dates as the 2^n schedule.

III. MIXING MODELS

(1) Mixing models

Many problems in isotopic ecology demand that we estimate the contribution of different sources to the tissues of an animal. Mixing models are the tool of choice for this purpose, and isotopic ecologists have made strides in the development of these models. Briefly, in a mixing model we attempt to estimate the fractional contribution of an isotopic source to a tissue from the isotopic composition of the tissue and from the isotopic composition of the dietary sources. The simplest mixing model is of the form:

$$\delta_T = f_A\delta_A + f_B\delta_B \quad (6)$$

$$1 = f_A + f_B, \quad (7)$$

where δ_T is the isotopic composition of an animal's tissue, δ_A and δ_B are the isotopic compositions of sources A and B, and f_A and f_B are their relative fractional contributions (Philips 2001). Equation 6 and 7 can be generalized to a linear system of N equations in N unknowns that allows estimating the contribution of N sources if one measures the composition of N-1 isotopes in a tissue. Most studies rely on two isotopes (^{13}C and ^{15}N), and thus:

$$\delta^{13}C_T = f_A\delta^{13}C_A + f_B\delta^{13}C_B + f_C\delta^{13}C_C \quad (8)$$

$$\delta^{15}N_T = f_A\delta^{15}N_A + f_B\delta^{15}N_B + f_C\delta^{15}N_C \quad (9)$$

$$1 = f_A + f_B + f_C. \quad (10)$$

Phillips & Gregg (2001, 2003) have developed methods to (1) estimate the uncertainty in source proportions (f_i) based on the variance in the isotopic composition of the sources,

and (2) to estimate the contribution of different sources when the number of sources is equal or larger than the number of isotopes measured. Three user-friendly algorithms loosely based on Phillips and Gregg's (2003) methods are available online: Isosource (http://www.epa.gov/naaujydh/pages/models/stableIsotopes/isosource/isosource.htm) and SISUS/Stable Isotope Sourcing Using Sampling (http://statacumen.com/sisus/), and SIAR (http://cran.r-project.org/web/packages/siar/index.html). Equations 8, 9, and 10 describe a perfectly constrained model with a single solution. The computer packages used to solve mixing models also allow finding the set of feasible solutions when there are more unknowns than equations (the system is underconstrained). In these cases, there is an infinite number of solutions and the algorithms estimate the set of source proportions that satisfies the mixing model.

We emphasise that f_i estimates the contribution of source i to the isotopic composition of a tissue. It does not estimate the fraction of source i in the animal's diet (see Martínez del Rio & Wolf 2005). Estimating the fractional contribution of a source in an animal diet requires additional data and remains tricky. Using equations 8-10 to estimate contributions of different sources to diet assumes (1) that the elemental composition (i.e. the C:N ratio) of all the diets is equal, (2) that the efficiency with which each element in each source is assimilated is the same, 3) that there is no tissue to diet discrimination, and (4) that there is no isotopic routing. The variation among sources in elemental ratios and in assimilation efficiency can be addressed relatively easily with concentration-dependent mixing models (Phillips & Koch 2002) and by adding an assimilation efficiency term to the models (Martínez del Rio & Wolf 2005). Although both Isosource and SISUS can address these complications, it is fundamentally important that users of these programs recognize the assumptions that they are making when using canned analyses programs. For example, an isotopic analysis of an omnivore that relies on both plants and animals must recognise the complications posed by all the assumptions listed above. Conversely, isotopic analyses of strict carnivores satisfy the four assumptions listed above much more easily. As Gannes *et al.* (1997) recognised, using mixing models properly demands some knowledge of the nutritional biology of the animals studied.

(2) Tissue to diet discrimination: a neglected complication in the analysis of mixing models

The term "tissue to diet discrimination" (usually denoted by Δ) refers to the difference in isotopic composition between a tissue and diet (i.e. $\Delta = \delta_{tissue}-\delta_{diet}$). If discrimination factors are measured experimentally, we can include them in a mixing model:

$$\delta_T = f_A(\delta_A + \Delta_A) + f_B(\delta_B + \Delta_B) \quad (11)$$

$$1 = f_A + f_B. \quad (12)$$

Unfortunately, discrimination factors vary among species, among tissues within a single species, and among diets (e.g. McCutchan *et al.* 2003). Discrimination factors are not often measured experimentally in field studies that rely on mixing models. Thus, it is common to use discrimination factors from the literature. Researchers use an idiosyncratic collection of procedures to account for discrimination factors. Sometimes researchers use the average value from large reviews. For example, because 3.4‰ is the average $\Delta^{15}N_{tissue\text{-}diet}$ value reported in several reviews (Post 2002 and references there), this number is frequently used as a discrimination factor. Because average discrimination factors differ somewhat among reviews, other values are used as well. Some researchers use Δ values from related (i.e. in the same animal class) species fed on similar diets, but others use values from unrelated species fed on different diets (reviewed by Caut, Angulo & Courchamp 2008*b*).

This variety of approaches begs the question: how big an error can we make in the estimation of source proportions if we use the wrong discrimination factor? For the simplest case exemplified by equations 11–12 , and assuming that Δ_A and Δ_B are equal ($\Delta_A=\Delta_B=\Delta$), the difference between the real value (f_A) and the estimated one ($f_A^*(\Delta^*)$) is given by:

$$f_A^*(\Delta^*) - f_A = \frac{\Delta - \Delta^*}{\delta_A - \delta_B}, \quad (13)$$

where Δ^* is the discrimination factor derived from the literature. Equation 13 reveals the, perhaps evident, observation that errors in the estimation of the fractional contribution of a source are smaller when the isotopic difference between the sources is large. Equation 13 is the simplest possible case. Many studies rely on two isotopes to estimate the proportional contribution of three or more sources (see equations 8–10). In such cases there are six possible unknown Δ values for three diets, greatly increasing the potential errors that result from using erroneous discrimination factors.

Caut, Angulo, & Courchamp (2008*a*) illustrated with an experiment the errors that can be committed by using discrimination factors from the literature. They designed three diets with similar elemental composition, but with contrasting isotopic values. They fed a group of rats on these diets to estimate discrimination factors. They fed another group of rats on a mixture of these diets, measured the isotopic composition of the rats' tissues, and used mixing models to estimate the fractional contribution of each diet. Caut *et al.* (2008*a*) found that the models worked best when they used discrimination factors estimated experimentally. When they used values from the literature, the estimated source proportions differed considerably from the real values.

What can ecologists interested in using mixing models do? The best alternative is to do experiments and estimate discrimination factors for each of the sources of interest (Haramis *et al.* 2001). This alternative is often unrealistic and ecologists might have to rely on values from the literature. In this case, it is wise to rely on a range of Δ values for related species fed on similar diets and measured on the same tissues. Even in this case, a sensitivity analysis that examines the effect of variation in Δ is necessary. For the simplest mixing models with two sources and one isotope, the value of a source proportion depends only on

two Δ values and the sensitivity analysis can be performed easily by applying the following function:

$$f_A = \frac{\delta_{tissue} - (\delta_B + \Delta_B)}{(\delta_A + \Delta_A) - (\delta_B + \Delta_B)}. \quad (14)$$

The values of f_A for the range of possible Δ_A and Δ_B values can be represented visually in a 3D plot with f_A as the dependent variable. To our knowledge, the computational tools to do a sensitivity analysis for the 2-isotope, 3-source case have not been developed.

So far, we have emphasised potential errors that result from using erroneous discrimination factors. Using Δ values measured experimentally is better, but the use of these values in mixing models is not without problems. Discrimination factors are measured with some variation, and this variation will propagate when the mixing model is solved to obtain source proportions. To our knowledge, the current methods to solve mixing models do not account for the variation associated with discrimination factors measured experimentally. Because mixing models are used very frequently, finding out the effect of uncertainty in discrimination factors on the estimation of source proportions is an area in which theoretical progress is needed. In the future, studies that use discrimination factors in mixing models should be accompanied by discussion about how variation in their value, or errors in their estimation, contribute to uncertainty in the calculation of source proportions.

IV. ISOTOPIC ROUTING

Mixing models in all their guises make an assumption that should give pause to nutritional ecologists. They assume that assimilated nutrients are disassembled into their elemental components and that these elements are then reassembled into the molecules that make up tissues. Van der Merwe (1982) called this unrealistic assumption the "scrambled egg" premise. Consider carbon: the building blocks that animals use to manufacture tissues are not carbon atoms, but the carbon skeletons of a myriad of molecules. These carbon skeletons are conserved to various degrees from assimilation to the manufacture and breakdown of macromolecules. For example, the amino acid pool in an animal at any given moment is comprised of indispensable amino acids whose carbon skeletons come from the diet and indispensable amino acids whose carbon skeletons come from either the diet or that are manufactured endogenously from bits and pieces of other macromolecules (Bequette 2003). Anthropologists and palaeontologists have observed that the bone collagen and bone apatite of omnivores sometimes have contrasting isotopic signatures (Ambrose & Norr 1993). The explanation that they give to this difference is that collagen represents the protein component of diet whereas apatite represents the carbohydrates and lipids ingested and used to fuel metabolism (the carbon in apatite comes from CO_2; Gannes *et al.* 1998). Schwarcz (1991) christened the differential allocation of isotopically distinct dietary components to different tissues "isotopic routing".

The isotopic routing conjecture highlights the quandary faced by isotopic ecologists that work with omnivores that often ingest diets in which carbohydrates (and sometimes lipids) are derived from one dietary source and protein is derived from another. They may find that using different tissues for isotopic analyses to reconstruct an animal's diet might give different answers (Voigt *et al.* 2008). Worse, using a single type of tissue might give the wrong answer (Fig. 3, Podlesak & McWilliams 2006). Good examples of animals in which isotopic routing might be prevalent are nectar- and fruit-eating animals, and most animals that include both animals and plants in their diet. Although isotopic routing has been recognised as an interesting phenomenon for a long time (reviewed by Ambrose & Norr 1993), it has

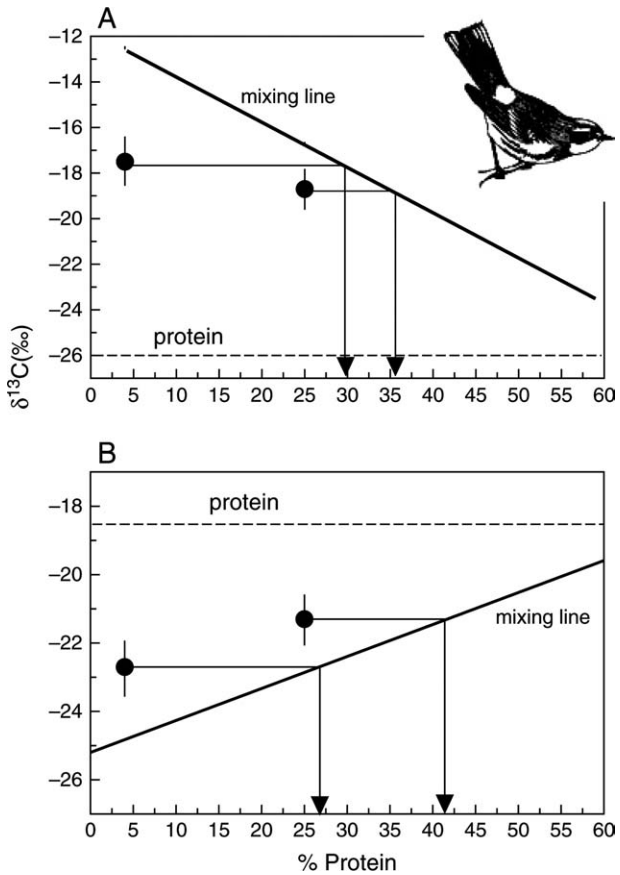

Fig. 3. Podlesak & McWilliams (2006) fed yellow-rumped warblers (*Dendroica coronata*) on diets that differed in protein content (4% and 25%). They also varied the $\delta^{13}C$ of protein and lipids and carbohydrates. (A) Results for birds fed diets with "C3" protein and "C4" carbohydrates and lipids. (B) birds fed with "C4" protein and "C3" carbohydrates and lipids. The $\delta^{13}C$ of plasma proteins was intermediate between that of dietary protein and that of diet (represented by the mixing line). Consequently, a mixing model overestimates the contribution of the protein source to the bird's diet (the arrows point to the estimated fractional contribution).

received relatively scanty recent attention from both theoreticians and experimenters.

Martínez del Rio & Wolf (2005) incorporated routing into a mixing model for ^{13}C. Here we present a simplified graphical version of this model. For simplicity, we assume that the animal ingests two dietary sources, one (source 1 with δ_1) that only contains protein and another one (source 2 with δ_2) that contains only carbohydrates. We assume that these diets provide other essential macronutrients (vitamins, indispensable fatty acids, …, etc,), but that the amounts of these materials contribute little to the overall isotopic composition of the diet. The carbon isotopic composition of the tissue (δ_T) can be given by the sum of two terms: one that represents the fraction of carbon (f_e) contributed by indispensable amino acids ($f_e\delta_1$), and one that represents the weighted sum of the contribution of dispensable amino acids from diet ($p(C)\ \delta_1$) and the dispensable amino acids synthesised endogenously from carbon derived from source 2 ($(1\text{-}p(C))\ \delta_2$):

$$\delta T = f_C\delta_1 + (1 - f_C)(p(C)\delta_1 + (1 - p(C))\delta_1). \quad (15)$$

The term p(C) in equation 11 represents the fractional contribution of source 1 to the carbon in dispensable amino acids. It is reasonable to assume that this fraction depends on the fraction of the total assimilated carbon derived from source 1 (denoted by C). We hypothesize that p grows with C (i.e. $dp/dC > 0$) in such a way that at low C values most of the carbon in dispensable amino acids should come from source 2 ($p \approx 0$), whereas at high C values most of the carbon in dispensable amino acids should come from source 1 ($p \approx 1$). The steepness with which p increases with C depends on the match between the amino acid profile of source 1 and that of the tissue (Fig. 4). In Fig. 4 we also have assumed that protein quality determines the shape of $p(C)$. This simple model predicts that δ_T will be consistently higher than the value expected from a mixing model. Using a mixing model to estimate dietary contributions will overestimate the contribution of source 1 to the animal's diet. The predictions of the model are consistent with the results of Ambrose & Norr (1993) and Podlesak & McWilliams (2006) for collagen and blood, in laboratory mice and yellow-rumped wablers, respectively.

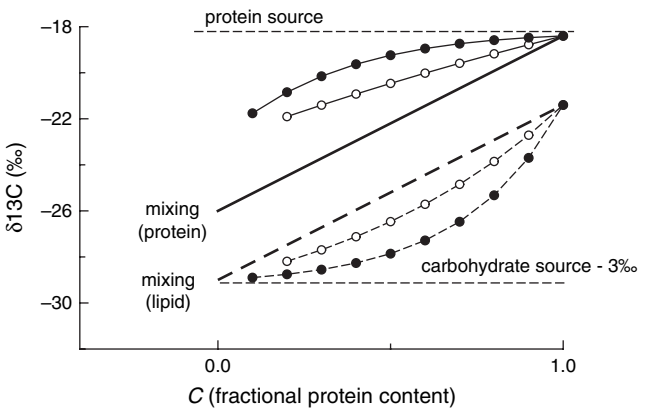

Fig. 4. A simple model of isotopic routing predicts that on diets in which sources with contrasting $\delta^{13}C$ are comprised primarily of protein or carbohydrate, the $\delta^{13}C$ of protein in tissues (solid curves) will be intermediate between that predicted by a mixing model (solid line) and that of the protein source (dashed horizontal line). The model also predicts that the isotopic composition of lipid will be intermediate between that of a mixing line (dashed line, accounting for the -3‰ fractionation resulting from the synthesis of lipid from carbohydrate) and the $\delta^{13}C$ of the carbohydrates source minus 3‰.

Equation 15 predicts the carbon isotopic composition of a proteinaceous tissue. What might the isotopic composition of the lipid or the CO_2 exhaled by this animal be? If the amino acid profile of source 1 matches that of the animal's tissues (i.e. if its protein efficiency ratio, or PRE, is high; Bodwell 1989) and if C is small, we expect a very small contribution of this source to the molecules catabolised for energy or allocated to energy storage. However, if the amino acid profile of source 1 differs from that of the animal's tissues (i.e. if its PRE is low), then we expect those amino acids that are not used for protein synthesis to be used as fuel. This qualitative prediction suggests that the isotopic composition of the carbon of lipid, and exhaled CO_2 (and hence the carbon in apatite), will represent a mixture of sources 1 and 2 except when the animal's intake of source 1 is low and the PRE of this source is high (Fig. 4). In both cases, using a mixing model that does not account for routing will underestimate the contribution of source 1 to the animal's diet.

The simple model depicted in Fig. 4 suggests that the notion that the $\delta^{13}C$ of collagen reflects that of dietary protein, whereas that of apatite represents that of non-protein components might be too simplistic. It is more likely that the isotopic composition of both will represent mixtures, and that the relative contributions of the alternative sources will depend on the diet's protein content and on the quality of ingested protein. This is an area in which we can apply the tools of nutritional physiology to solve an important question in isotopic ecology. We need theoretical research to determine the functional form of $p(C)$ in equation 15 and experiments to test the conjectures in Fig. 4.

V. TROPHIC DISCRIMINATION FACTORS

(1) Fractionation, discrimination, trophic spacing: what are we talking about?

In the previous section we referred to the difference in the isotopic composition between an animal's tissue and its diet as the discrimination factor and used Δ to denote it. What we have chosen to call discrimination factor also has been referred to by the following alternatives: fractionation factor, fractionation, apparent fractionation, enrichment, and trophic enrichment (Cerling & Harris 1999). Martínez del Rio & Wolf (2005) argued for the use of the term discrimination factor for $\Delta = \delta_{tissue}\text{-}\delta_{diet}$. They suggested that the term fractionation should be restricted to the equilibrium and kinetic effects that cause differences between

reactants and products in chemical reactions. They contended that the differences in isotopic composition between a tissue and the diet from which it is derived result from a plethora of possible processes, including fractionation during metabolic transformations but also isotopic routing. Thus, using fractionation confuses a pattern with only one of the processes that create it.

If we adopt the metaphorical perspective of ecological stoichiometry and view organisms as collections of elements akin to molecules (Sterner & Elser 2002), and we envision assimilation and excretion as the chemical reactions in which they participate, then the term "trophic fractionation" does not seem inadequate. The appropriate use of the term depends on restricting the use of trophic fractionation to $\Delta X_{\text{trophic}} = \delta X_{\text{whole body}} - \delta X_{\text{diet}}$, where X is the isotope under study. This stoichiometric interpretation is consistent with fractionation as the effect of all physiological processes that lead to differences between an animal and its diet. Because isotopic routing is certainly one of the factors that lead to differences between tissues and diet, the term "fractionation" appears inappropriate for the difference between diet and one tissue.

It is probably wise for isotopic ecologists to adopt a uniform terminology. We suggest not using the term enrichment. A tissue can have a more positive (enriched) or more negative (depleted) δ value than the diet. To avoid ambiguity we propose adopting discrimination factor for a tissue, and in studies that rely on whole body isotopic composition, we propose using the term trophic fractionation as defined above.

(2) On the uses of isotopic discrimination

In previous sections we have emphasised the potentially serious problems that tissue to diet discrimination poses for the use of mixing models in dietary reconstruction. The observation that the isotopic composition of an animal's tissues differs from that of their diet has been very useful. In an important early paper, DeNiro & Epstein (1981) noted that animal tissues were enriched in ^{15}N relative to their diets (i.e. $\Delta^{15}\text{N} = \delta^{15}\text{N}_{\text{tissues}} - \delta^{15}\text{N}_{\text{diet}} > 0$). This observation led to the conjecture that the content of ^{15}N in animal tissues is biomagnified along the length of a food chain (Post 2002). This conjecture allows ecologists to use δ^{15}N to estimate an animal's trophic level (*TL*) using the following equation devised by Vander Zanden, Cabana & Rasmussen (1997) and modified by Post (2002):

$$TL = \lambda + \frac{\left(\delta^{15}N_c - \delta^{15}N_{base}\right)}{\Delta_n} \qquad (16)$$

where, $\delta^{15}N_c$ is the nitrogen isotopic composition of the consumer, $\delta^{15}N_{\text{base}}$ is that of the food base, λ is the trophic level of the base (λ=1 if the base is primary producers), and Δ_n is a robust estimate of the *average* increase in Δ^{15}N per trophic level (Post 2002). The application of equation 16 has been very fruitful. Among other applications, equation 16 has been used productively to discover the factors that determine food chain length in lakes (Vander Zanden *et al.* 1999) and to conduct global comparisons of food chains (Vander Zanden & Fetzer 2007). Equation 16 has also been remarkably successful in ecotoxicological studies that use TL to predict the concentration of contaminants that bioaccumulate in consumers' tissues (reviewed by Kidd, Jardine & Fisk 2006).

In a previous section we argued that using the wrong estimated discrimination can lead to large errors in the estimation of fractional source contributions. Equation 16, also relies on an estimated value of Δ^{15}N. How can we then explain its many successful applications? There are two possible reasons: first, it may be that variable values of Δ^{15}N will become averaged over multiple trophic levels and potentially over the various sources of a consumer (Vander Zanden & Rasmussen 2001). Second, Vander Zanden & Rasmussen (2001) suggest that Δ_n is more variable for herbivores (primary consumers) than for carnivores. Therefore, using primary consumers (i.e. $\lambda = 2$) as a baseline might reduce error in the estimation of TL. Like all models, equation 16 must be tested. Vander Zanden *et al.* (1997) found a positive correlation ($r^2 = 0.78$, $N = 7$) between the average trophic positions of freshwater fish estimated using δ^{15}N values and that estimated by gut content analyses. To our knowledge, although equation 16 is frequently used in terrestrial systems, it has not been cross-validated yet.

(3) Why does ^{15}N bioaccumulate?

The prevalent use of δ^{15}N values in the study of food chains begs the question, why does ^{15}N bioaccumulate? The answer to this question is important because answering it holds the key to understanding why nitrogen discrimination factors vary, and hence can help us predict their value. The most widespread explanation for bioaccumulation of toxicants along a food chain is that absorption is higher than elimination (Karasov & Martínez del Rio 2007). Thus, if the same explanation applies to ^{15}N, Δ^{15}N should have a positive value if animals retain ^{15}N preferentially over ^{14}N (Martínez del Rio & Wolf 2005). Available evidence supports this observation. The materials excreted by the animals that have been measured tend to be isotopically lighter than tissues (reviewed by Tibbets, Wheeless & Martínez del Rio 2007). For example, Tibbets *et al.* (2007) found that the frass produced by insect larvae was strongly depleted in ^{15}N relative to both the insect's diet and tissues. Adults emerging from pupae were further enriched in ^{15}N, and their enrichment appeared to be the result of the production of the isotopically light metabolic waste products of metamorphosis (Tibbets *et al.* 2007).

Sponheimer *et al.* (2003) questioned the ^{15}N preferential excretion hypothesis. They measured δ^{15}N in llamas (*Lama glama*) and found that although urine was significantly depleted in ^{15}N relative to diet, there were no significant differences between the δ^{15}N of feed and the weighted average of the δ^{15}N of urine and faeces ("excreta"). They concluded that ^{14}N is not preferentially excreted. Unfortunately, Sponheimer *et al.* (2003) did not measure the isotopic composition of the llama's tissues. By definition, at steady state the isotopic composition of dietary inputs should equal that of outputs (Martínez del Rio & Wolf 2005). Thus, finding that excreta are more depleted in ^{15}N than diet is

a sufficient condition for a positive $\Delta^{15}N$ value, but it is not a necessary one. However, a positive $\Delta^{15}N$ demands that excreta are depleted in ^{15}N relative to the animal's body, and in all cases measured this seems to be the case (Tsahar *et al.* 2007).

In an attempt to give theoretical coherence to the physiological factors that might influence $\Delta^{15}N$, Olive *et al.* (2003) and Martínez del Rio & Wolf (2005) constructed isotopic mass balance models. These two models are complementary, rather than alternative and differ only in the need for more empirical data to parameterise Olive *et al.*'s (2003) model. Olive *et al.*'s (2003) model is particularly useful to investigate discrimination in animals for which a great deal of data are available, whereas Martínez del Rio & Wolf's (2005) model makes less precise, but more general, predictions. Specifically, Martínez del Rio & Wolf's (2005) model predicts that: (1) $\Delta^{15}N$ should decrease with increased protein quality in diet; 2) $\Delta^{15}N$ should increase with diet's protein content; (3) $\Delta^{15}N$ should decrease with the efficiency of nitrogen deposition measured as the ratio between protein assimilation and protein loss; and (4) $\Delta^{15}N$ should increase with fasting time.

What is the evidence for or against the predictions listed above? Prediction (1) is supported by Robbins, Felicetti & Sponheimer (2005) report of a highly significant interspecific negative correlation between $\Delta^{15}N$ and the diet's protein value. Robbins *et al.* (2005) is a comparative study. To our knowledge there are no experimental tests of this prediction yet. Prediction (2) has mixed support: two studies that varied protein intake and measured the isotopic composition of animal tissues show patterns that are consistent with the predictions of Martínez del Rio & Wolf's(2005) model. Pearson *et al.* (2003) found a positive linear relationship between $\Delta^{15}N_{\text{body-diet}}$ in yellow-rumped warblers (*Dendroica coronata*) and Focken (2001) found an increase in $\Delta^{15}N_{\text{body-diet}}$ with increased protein intake in Nile tilapia (Orechromis niloticus). By contrast, Tsahar *et al.* (2007) found lower $\Delta^{15}N$ in fruit-eating birds fed on diets with higher protein content, and Robbins *et al.* (2003) found no effect of protein content in their comparative study. These two studies suffer from the shortcoming that they did not vary protein content independently of protein quality. In Tsahar *et al.*'s (2007) study, a fruit-based diet was enriched with casein, and thus protein quality probably increased with protein content. In Robbins *et al.*'s (2005) study protein content and quality covaried.

To our knowledge, prediction (3) has not been examined experimentally. However, it can be argued that the efficiency of nitrogen utilisation is correlated with age. Non-growing animals are in neutral nitrogen balance, and hence the ratio of input to output is ≈ 1. In growing animals the ratio of nitrogen input to output is lower than 1. Thus, a corollary of prediction (3) is that $\Delta^{15}N$ should be lower in growing than in non-growing animals. Several studies have reported variation (and lack of variation) in $\delta^{15}N$ with age. Minagawa & Wada (1984) reported invariant $\delta^{15}N$ with size, and hence presumably with age, in two species of clams. However, although the clams differed almost four fold in length, they maintained relatively constant fractional growth of the nitrogen pool (Fig. 3 in Minagawa & Wada 1984). Ambrose (2000) reported no effects of age on $\Delta^{15}N_{\text{body-diet}}$ in rats but he measured the isotopic composition as a function of age only in rats older than 100 days. At this age rats are not growing at high rates and hence the effect of growth on $\Delta^{15}N_{\text{body-diet}}$ should be small. Several studies have compared $\Delta^{15}N_{\text{body-diet}}$ between mammalian young feeding on milk and their mothers (reviewed by Jenkins *et al.* 2001). Jenkins *et al.* (2001) found that although mothers were significantly enriched in ^{15}N relative to their diets (average $\Delta^{15}N_{\text{body-diet}}$, $= 4.1$ ‰), growing nursing offspring were only very slightly enriched in ^{15}N relative to milk. In a widely cited study, Hobson, Alisauskas & Clark (1993) fed Japanese quail (*Coturnix japonica*) on diets of identical nutrient and isotopic composition but at different rations. One of the rations allowed the quail to grow, whereas the other was sufficient only to allow birds to maintain mass but not to increase it. As predicted by the model, growing birds were depleted in ^{15}N (by from 0.5 to 2‰ depending on tissue) relative to the birds that just maintained body mass. Again, in support of Martínez del Rio & Wolf's (2005) prediction Fantle *et al.* (1999) found a strong negative relationship between $\Delta^{15}N_{\text{body-diet}}$ and growth rate in juvenile blue crabs *Callinectes sapidus*. Gaye-Siesseger *et al.* (2003, 2004) and Trueman, McGill & Guyard (2005) also documented highly significant negative relationships between $\Delta^{15}N_{\text{body-diet}}$ and the rate of protein gain in three fish species (*Oreochromis niloticus*, *Cyprinus carpio*, and *Salmo salar*).

Ponsard & Averuch (1999) reached a different conclusion about the effect of growth on $\delta^{15}N$ than that predicted by Martínez del Rio & Wolf (2005). They concluded that *"...growing animals should show the same $\delta^{15}N$ values as those of adults if the total nitrogen that they assimilate during their growth is large compared to total nitrogen content in their adult body..."* (p. 1308). In essence, Ponsard & Averuch (1999) argued that the $\Delta^{15}N$ of growing animals will be the same as that of adults if the integrated ratio of protein retained in tissues to protein intake, is small. But is this ratio always small? Their argument is based on data on rodents (Ponsard & Averuch 1999). A large body of knowledge on the correlates of growth and allocation casts a shadow on their (1999) extrapolation from rodents to the animal kingdom. The conversion of food's nitrogen into tissue nitrogen varies by more than an order of magnitude among species depending on a variety of factors including metabolic rate (Steyermark 2002 and references therein), food's stoichiometric composition (C:N ratios, see Chapter 5 in Sterner & Elser 2002), and life history characteristics (Rochette *et al.* 2000).

Finally, Martínez del Rio & Wolf's (2005) model predicts that $\Delta^{15}N$ should increase with fasting time. This is a reasonable hypothesis that has been posed many times (see Gannes *et al.* 1997, 1998) but that has received mixed support. Of 8 studies on the effect of fasting on invertebrates, five found a significant enrichment in ^{15}N and three found no effect (Fig. 5). Because there are fewer fasting studies in vertebrates, the patterns are less clear. Hobson *et al.* (1993) found significant increases in $\delta^{15}N$ in fasting snow geese (*Chen caerulescens*) that lost $\approx 50\%$ of their body mass. In fasting spawning salmon (*Salmo salar*), only the

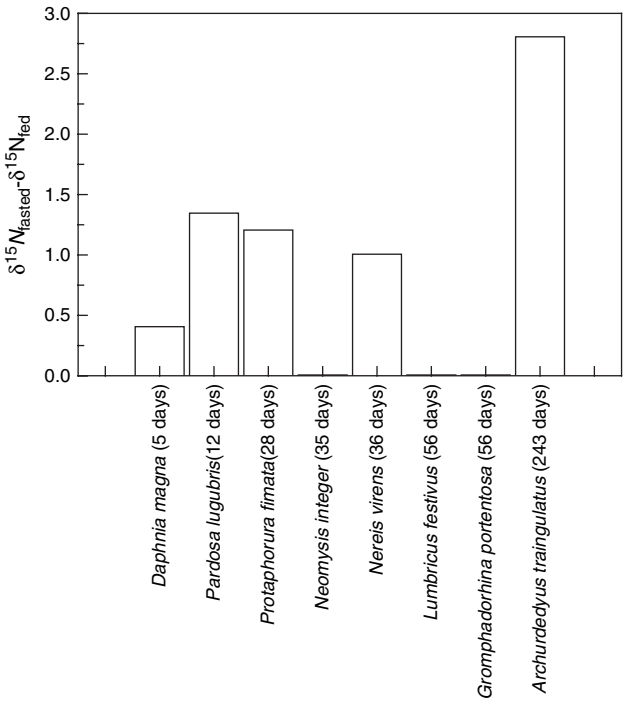

Fig. 5. Five out of eight studies on the effects of fasting in invertebrates revealed a significant ^{15}N enrichment in tissues ($\delta^{15}N_{fasted}$) relative to the values before the fast ($\delta^{15}N_{fed}$). Fast durations are given in parentheses after species names. Data are from Adams & Sterner (2000), Boag *et al.* (2006), Gorokhova & Hansson (1999), Haubert *et al.* (2005), McCue (2008), Oelbermann & Sechu (2002), Olive *et al.* (2003), Schmidt *et al.* (1999).

liver became significantly enriched in ^{15}N in post-spawning kelts relative to pre-spawning adults (Doucett *et al.* 1999). Castillo & Hatch (2007) fasted two species of lizards (*Anolis carolinensis* and *Uta stansburiana*) for 14 days and found that the tail muscles were not enriched in $\delta^{15}N$ relative to those of fed animals. However, they found that the $\delta^{15}N$ of excreta increased significantly from the beginning to the end of the fast. McCue (2007) also found a significant increase in $\delta^{15}N$ in excreta during a 24-week fast in rattlesnakes (*Crotalus atrox*), but no significant change in whole-body $\delta^{15}N$.

What are we to make of these results? We suspect that the failure to observe a consistent enrichment in whole-body $\delta^{15}N$ with fasting is because Martínez del Rio & Wolf's (2005) model is incomplete. This model is a "scrambled eggs" model (*sensu* van der Merwe 1982) that assumes a single well-mixed protein pool. This assumption is incorrect. In fasting animals not all organs lose nitrogen to the same degree and in the same way (e.g. Doucett *et al.* 1999). For example, the splanchnic organs of small migratory birds, reptiles, and hibernating mammals account for a small fraction of total body protein, but contribute predominantly to total protein loss during fasting (Karasov & Pinshow 1998). Although those organs might become enriched in $\delta^{15}N$ during fasting, their contribution to total body ^{15}N enrichment might be masked by that of organs such as muscle whose protein mass is maintained. Assuming that splanchnic organs represent $\approx 10\%$ of all body protein (Karasov & Pinshow 1998), an increase in their $\delta^{15}N$ of 4‰ will lead to an increase of only ≈ 0.4‰ in body protein.

Protein is lost in a tissue during a fast by a series of processes. First, protein is broken down by proteases into its component amino acids, these amino acids are then deaminated *in situ*, or exported to other organs (Caloin 2004). De- and transamination, should lead to ^{15}N-depleted nitrogenated by-products (ammonia, urea, and uric acid) and a remaining pool of enriched amino acids that can then be incorporated into proteins (Macko *et al.* 1986, 1987). Therefore, mass loss in an organ does not guarantee ^{15}N enrichment during a fast. Some organs such as muscle reduce their rate of protein synthesis during a fast (Waterlow 2006). Because these organs do not incorporate residual enriched amino acids, we should not expect them to become enriched. Other organs, such as liver retain high rates of protein synthesis during a fast (Waterlow 2006). They presumably manufacture protein from the ^{15}N-enriched pool of amino acids that remains from protein catabolism. Thus, we predict that the organs that will become enriched during a fast are those that maintain significant synthesis. Experimental tests of this hypothesis require measuring the isotopic composition of tissues with contrasting rates of synthesis and catabolism during prolonged fasts.

Fasting animals conserve protein when they fast, especially if they have adequate reserves (Caloin 2004). Thus, it is likely that animals do become enriched in ^{15}N when they fast, but that the enrichment is difficult to detect in their whole body protein or in organs and tissues that are conserved during a fast and in organs that do not maintain anabolic capacity. The increase in $\delta^{15}N$ of excreta observed in fasting reptiles by Castillo & Hatch (2007) and McCue (2007) may reflect the progressive enrichment of the organs that lose significant amounts of protein/nitrogen during a fast, but that also maintain protein synthesis. This hypothesis remains to be tested.

(4) Why do tissues differ in $\Delta^{13}C$?

Because a variety of tissues including claws, scales, blood, feathers, and hair, can be sampled relatively non-invasively, ecologists can make a surprisingly large number of inferences about an animal without harming it. Using these tissues to make ecological inferences requires that we recognise a potential complication: tissue to diet discrimination factors differ among tissues (McCutchan *et al.* 2003). The variation among tissues is sometimes very large. For example, Reich *et al.* (2008) in a controlled feeding study on loggerhead turtles (*Caretta caretta*), found that $\delta^{13}C$ varied from 0.9 ‰ to 2.6‰. Reich *et al.*'s (2008) study is not exceptional, many studies report differences in $\Delta^{13}C$ among tissues (McCutchan *et al.* 2003). What are the factors that determine the value of $\Delta^{13}C$? Lipid content and amino acid composition are two important candidates. Lipid synthesis is accompanied by depletion in ^{13}C of the resulting fatty acids relative to the precursor substrates (DeNiro & Epstein

1977). Thus, some of the variation in $\Delta^{13}C$ is explained by tissue's lipid content (Post *et al.* 2007). To reduce this variation, Post *et al.* (2007) recommend either analysing extracted lipids, or using data on the samples' %C and/or C:N ratios to correct for the effect of lipid content on $\Delta^{13}C$.

Lipids are not the only factor that can cause differences in $\delta^{13}C$ values among tissues. The value of $\Delta^{13}C$ and $\Delta^{15}N$ in tissue protein is determined by the tissue's amino acid composition and by the $\delta^{13}C$ of these amino acids. The $\delta^{13}C$ values of amino acids of primary producers can range widely. O'Brien *et al.* (2005) reported differences of over 20‰ among the $\delta^{13}C$ values of indispensable amino acids in the foliage of several plant species. They found that the $\delta^{13}C$ values of the essential amino acids in larval food plants were an excellent predictor of the $\delta^{13}C$ values of the essential amino acids in the eggs of butterflies and moths. By contrast, because non-essentials were synthesized from carbon derived from adult food, their $\delta^{13}C$ value was much more homogeneous and reflected the $\delta^{13}C$ value of adult diet. In O'Brien *et al.*'s (2005) studies we can predict the $\delta^{13}C$ value of eggs from their amino acid composition and from the $\delta^{13}C$ value of the indispensable amino acids in food plants.

The research of O'Brien *et al.* (2005) represents an extreme case of "molecular isotopic routing" in which the indispensable amino acids in a tissue are derived exclusively from one source, whereas the dispensable amino acids are derived from another. If, as suggested by Fig. 4, isotopic routing depends on the diet's protein content, and if the animal is feeding on mixed diets, then we should expect the isotopic signals of indispensable amino acids to reflect a mixture of the isotopic signature of bulk diet resulting from *de novo* synthesis and of amino acids incorporated directly from protein sources (Howland *et al.* 2003). Howland *et al.* (2003) found that the carbon isotopic composition of individual dispensable amino acids in pig (*Sus scrofa*) collagen was better predicted by the isotopic composition of bulk diet than by the composition of the individual amino acids in the diet. Surprisingly, Howland *et al.* (2003) only found a tight correlation between the $\delta^{13}C$ value of dietary indispensable amino acids and those in collagen for phenylalanine and leucine. The $\delta^{13}C$ value of other dietary indispensable amino acids was a poor predictor of the $\delta^{13}C$ value of those in collagen. This is a disturbing result with no adequate explanation. Perhaps not surprisingly, Howland *et al.* (2003) were able to predict the isotopic composition of collagen from a mass balance model that includes the amino acid composition of collagen and the $\delta^{13}C$ value of each individual amino acid in this tissue. The challenge for isotopic ecologists should be more ambitious. We must be able to predict the isotopic composition of a tissue from its amino acid composition and from the isotopic composition and content of amino acids in the animal's diet.

(5) Why do tissues differ in $\Delta^{15}N$?

Tissues can differ greatly in $\delta^{15}N$ values, and hence in $\Delta^{15}N_{\text{tissue-diet}}$. In the controlled feeding study of sea turtles reported above, $\delta^{15}N$ values varied among tissues from -0.64‰ to 1.65‰ (Reich *et al.* 2008). It is likely that the difference in $\delta^{15}N$ values among tissues can be explained by their amino acid content and by the isotopic composition of individual amino acids. $\delta^{15}N$ values vary among the amino acids of primary producers, and this variation seems to be amplified by the metabolic processes of consumers (Fig. 6; McClelland & Montoya, 2002, Popp *et al.* 2007). The range in $\delta^{15}N$ values among amino acids appears to increase with trophic level. The $\delta^{15}N$ of amino acids in animal tissues seems to have a roughly bimodal distribution (Fig. 6). Popp *et al.* (2007) called the relatively ^{15}N-enriched amino acids "trophic", and the relatively ^{15}N-depleted ones, "source".

Some amino acids appear to retain approximately the same nitrogen isotopic composition of food, whereas others become enriched in ^{15}N by the animal's metabolism. The reasons for why the N in some amino acids is protected from isotopic enrichment are unknown. They are probably complex and likely depend on (1) how freely each amino acid exchanges nitrogen with others, and (2) on the details of the synthesis and catabolism of each amino acid. Note that we have not included whether the amino acids are dispensable or indispensable as a potential criterion for ^{15}N-enrichment. Both of these types can be found among the ^{15}N-enriched and ^{15}N-depleted amino acids (Fig. 6). It appears that the dispensable and N-promiscuous amino acids involved in the transport and movement of nitrogen (alanine and glutamic acid) tend to be ^{15}N-enriched, whereas those that are essential and not easily transaminated (phenylalanine and threonine, theronine was not measure in the study on Tuna in Fig. 6) tend to be relatively ^{15}N-depleted. The $\delta^{15}N$ value of some amino acids is befuddling. Both proline and serine receive their nitrogen from glutamate (a ^{15}N-enriched amino acid) during synthesis (Bequette 2003). However proline is a highly

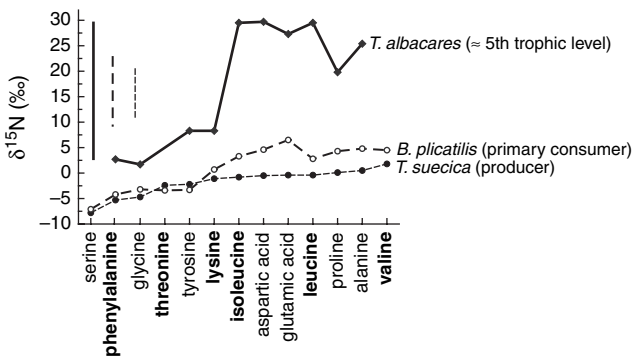

Fig. 6. Amino acids can differ dramatically in $\delta^{15}N$ within a single individual and tissue. The range in $\delta^{15}N$ values within an organism/tissue (illustrated by vertical bars) appears to increase with trophic level. Some amino acids ("source" amino acids) appear to have consistently low $\delta^{15}N$ values, whereas others appear to be consistently ^{15}N-enriched ("trophic" amino acids). The values for the rotifer *Brachionus plicatillis* (analyses of whole-body) and the alga *Tetraselmis suecica* (analysis of whole body) are from organisms cultured in the laboratory by McClelland & Montoya (2002). Popp *et al.* (2007) collected the data for the muscle of wild caught yellowfin tuna (*Thunnus albacares*). Amino acids in bold are indispensable.

^{15}N-enriched amino acid, whereas serine is ^{15}N-depleted (Fig. 6, serine was not measured in the study on Tuna in Fig. 6). Understanding the processes that led to variation in δ^{15}N values among amino acids will take time and the devotion of biochemically minded isotopic ecologists.

The heterogeneity in δ^{15}N values among amino acids within a tissue not only allows us to explain variation in nitrogen isotopic composition among tissues, it also suggests that we might be able to estimate an animal's trophic position from information contained within its tissues. McClelland & Montoya (2002) proposed using

$$TL = 1 + \frac{\left(\left[\delta^{15}N_{\text{glutamic acid}} - \delta^{15}N_{\text{glycine}}\right] - 4‰\right)}{7}, \quad (17)$$

where 4‰ is the difference in δ^{15}N values between glutamic acid and phenylalanine in the algae at the base of the food chain, as an "internal" index of trophic level. Recall that glutamic acid is a trophic, whereas phenylalanine is a source amino acid. McClelland and Montoya (2002) found that $\Delta^{15}N_{\text{glutamic acid-phenylalanine}} \approx 7‰$. Therefore, Popp *et al.* (2007) suggested using the following modifications of equation 13:

$$TL = 1 + \frac{\left(\delta^{15}N_{\text{glutamic acid}} - \delta^{15}N_{\text{glycine}}\right)}{7} \quad (18)$$

and

$$TL = 1 + \frac{\left(\bar{\delta}^{15}N_{\text{trophic}} - \bar{\delta}^{15}N_{source}\right)}{7}, \quad (19)$$

where $\bar{\delta}^{15}N_{trophic}$ is the average δ^{15}N of the trophic amino acids and $\bar{\delta}^{15}N_{source}$is the average δ^{15}N of the source amino acids. The denominator of the second factor in equation 18 should be modified to account for the difference in δ^{15}N between glutamic acid and glycine at the base of the food chain ($\Delta^{15}N_{\text{base glutamic acid-glycine}}$) as ($\delta^{15}N_{\text{glutamic acid}}$-$\delta^{15}N_{\text{glycine}}$)-$\Delta^{15}N_{\text{base glutamic acid-glycine}}$ (B. Popp, personal communication). Popp *et al.* (2007) use glycine as a source amino acid because their analytical methods give accurate δ^{15}N values. Popp *et al.* (2007) compared the TL estimates using these equations with those obtained using equation 16 and found roughly comparable results. McCarthy *et al.* (2007) used an equation similar to equation 19 to estimate the trophic levels in plankton and particulate matter in the central pacific ocean. We hasten to add that the assumption that 7‰ represents a valid average increase in $\Delta^{15}N_{\text{glutamate-phenylalanine}}$ or in $\Delta^{15}N_{\text{trophic-source}}$ per trophic level in all systems remains to be tested. We can derive a measure of confidence from a study by Hare *et al.* (1991). We estimated $\bar{\delta}^{15}N_{trophic} - \bar{\delta}^{15}N_{source}$ from their data and found that the value was $\approx 6‰$, which is not far from the 7‰ used in equations 17–19 (data from Table 4 in Hare *et al.* 1991). However, in spite of this result we believe that it is unwise to derive a parameter that can be applied generally from just a couple of studies.

In an informative study, Schmidt *et al.* (2004) compared the δ^{15}N of reproductive males and females of Antarctic krill (*Euphausia superba*). They found that females had more negative whole-body bulk δ^{15}N than males. They also found that within each sex, the δ^{15}N of abdominal muscle was higher than that of the digestive gland. These differences in δ^{15}N were the result of both differences in amino acid composition and in differences in isotopic composition among amino acids. Curiously, they were also the result of inter-sexual differences in δ^{15}N values between the same amino acids. The trophic amino acids in females tended to be more depleted in ^{15}N than those in males, especially in the digestive gland. Source amino acids differed less in δ^{15}N value among tissues than trophic amino acids and did not differ between males and females.

The results of Schmidt *et al.*'s (2004) study hold two cautionary lessons. (1) If we apply equation 18 to data on the isotopic digestive gland, we would infer that males have a much higher trophic level than females (Table 1). If we apply it to data from the whole body, we would infer that the difference in trophic level between males and females is much lower. The inferences that we make using TL equations can be tissue-dependent. (2) Schmidt *et al.* (2004) relied on several lines of evidence to infer that male and female krill feed on the same trophic level. Thus, they concluded that differences in δ^{15}N values between the trophic amino acids of males and females were the result of differences in amino acid metabolism. We still need theory, more field observations, and more experiments to find out how much δ^{15}N values vary among amino acids and to identify the factors that shape this variation. Although there is much potential in the use of compound specific ^{15}N analyses to estimate trophic level, and although these measurements have significant advantages over bulk analyses, isotopic ecologists must still do a significant amount of experimental work to use these methods confidently.

VI. *QUO VADIS* EXPERIMENTAL ISOTOPIC ECOLOGY?

(1) Crucial role of technology

Science seems to advance as a result of the vigorous interaction between (a) observations and experiments, (b) theory, and (c) technological advances (National Research Council 2007). Isotopic ecology seems to be a prime

Table 1. Estimation of trophic level (TL) in tissues of *Euphasia superba* using the δ^{15}N of glutamate and phenylanine. TL was estimated as $TL = 1 + \frac{(\delta^{15}N_{\text{glutamic acid}} - \delta^{15}N_{\text{phenylaline}})}{7}$

	$\delta^{15}N_{\text{glutamic acid}}$	$\delta^{15}N_{\text{phenylalanine}}$	TL
Digestive Gland			
Male	15.5‰	3.5‰	2.7
Female	7.7‰	3.9‰	1.5
Whole body			
Male	13.2‰	-1.4‰	3.1
Female	12.1‰	-2.5‰	3.1

example of progress through technology. It is unlikely that isotopic ecology would have progressed as rapidly if continuous flow isotope ratio mass spectrometers (CF-IRMS) had not replaced dual inlet ones for routine isotopic measurements. For some uses (analysis of water and gas samples) faster methods that rely on laser absorption spectroscopy and that do not require consumables might soon replace conventional mass spectrometry (Lis, Wassenaar & Hendry 2008). In previous paragraphs we have emphasised the potential of compound specific analyses to answer a variety of questions in isotopic ecology. Unfortunately, relative to bulk measurements, compound specific analyses remain time consuming and expensive.

For example, analysing the $\delta^{13}C$ values of amino acids in 10 samples requires about two days of off-line preparation (including hydrolyses and derivatisation) and each sample requires about an hour on line as the derivatised amino acids are separated by gas chromatography and then analyzed in a CF-IRMS (Evershed *et al.* 2007 & Teece & Fogel 2004). By contrast, it takes a few minutes to weigh a sample for bulk analyses, and less than 10 minutes to analyse it in a CF-IRMS. On the positive side, a single sample yields data for 15 different compounds when analysed with amino-acid-specific methods. Fry (2006) daydreams of a hand-held isotope scanner for bulk analyses. We yearn for the simple, fast, cheap, and accurate protocols that will make compound-specific analyses accessible to isotopic ecologists. To use bulk isotopic analyses confidently and effectively in ecological research, we need mechanistic understanding of the biochemical factors that underpin isotopic signals. This understanding will come from experimentation and the use of compound-specific analyses.

(2) Other isotopes

In this review we have emphasised carbon and nitrogen because these two elements, have received most of the attention of experimentalists. δ^2H and $\delta^{18}O$ values exhibit predictable patterns over the Earth's surface, and they have received enormous amounts of attention by researchers interested in finding the site of origin of animals (reviewed by Rubenstein & Hobson 2004 and Bowen *et al.* 2005). However, most of this attention has been phenomenological. Few experimental studies inform the inferences of the huge data sets already generated by field observational research. A recent study highlights the need for experimental research: Doucett *et al.* (2007) found large differences in δ^2H values between aquatic and terrestrial plants. The difference in δ^2H values between these two types of plants was large ($\approx 100‰$), with aquatic plants having more ^2H-depleted isotopic compositions than terrestrial plants. Doucett *et al.* (2007) also measured the δ^2H values in aquatic insects and fish and used simple mixing models to estimate the contribution of autochtonous (aquatic) and allochtonous (terrestrial) sources to the diets of these animals. To be able to use mixing models they had to make two assumptions: (1) $\Delta^2H_{\text{tissues-diet}} = 0$, and (2) the contribution of hydrogen (H) body water to the hydrogen bound in the organic compounds of tissues is negligible. How confident can we be in these two assumptions?

Not very - as Doucett *et al.* (2007) admit. The reason is that the incorporation of hydrogen (H) into biomolecules is a complex process that we are just beginning to understand. We know little about whether there is fractionation during the synthesis of biomolecules from precursors and body water, and very little about the relative contribution of hydrogen in body water and precursor dietary nutrients to the hydrogen bound to biomolecules. Birchall *et al.* (2005) reported large differences in δ^2H values between the collagen of carnivores and herbivores, and assumed that these differences were the result of a trophic/biomagnification effect. This effect is plausible, but has so far, not been documented in a controlled feeding study. Estimating this putative biomagnification effect requires that diet and preformed water have the same δ^2H value.

Hydrogen in an animal's biomolecules can come from two potential sources: (1) the H in dietary nutrients, and (2) the H in body water. The body-water pool, in turn, is derived from free ("pre-formed") water that animals ingest with food or that they drink, and water produced during the catabolism of carbohydrates, proteins, and lipids ("metabolic water"). The relative contributions of all these sources are likely to vary among animals (Karasov & Martínez del Rio 2007). Furthermore, it is probably incorrect to assume that the D and ^{18}O composition of water inside and outside of cells within an organism are the same. Kreuser-Martin *et al.* (2003 & 2005) found that in rapidly reproducing *bacterial* cells (*Escherichia coli* and *Bacillus subtilis*) metabolic water accounts for more than 50% of total intracellular water. By contrast, in non-growing cells medium water accounts for over 75% of intracellular water. The δD values of fatty acids differed between growing and non-growing cells and reflected that of intracellular water. Although it is unknown if these results apply to animals, they suggest that experimentalists cannot assume that the isotopic composition of extracellular water can be used as a surrogate for that of water within cells -where synthesis of organic molecules takes place. They also suggest that growth and metabolic activity might play a role in determining the relative contribution of pre-formed and metabolic water for the isotopic composition of animal tissues and biomolecules.

Isotopic ecologists should probably abandon the hope that we will be able to assign relative fractional contributions of these sources that can be applied to all animals in all situations. Instead, we believe that we will be able to develop rules of the form: "in aquatic/terrestrial animals/tissues with such and such metabolic characteristics (e.g. rapidly growing ectotherms), the contribution of dietary water to H bound to protein/lipid will range from x to y%". Deriving these rules of thumb will require extracting patterns from field data (Whitledge, Johnson & Martinez 2006), devising theory to explain these patterns (Kohn 1996), and then conducting experiments to test the hypotheses generated by models. The study by Podlesak *et al.* (2008) on the turnover and incorporation of ^{18}O and ^2H into the body water and tissues of woodrats (*Neotoma* spp.) is a fine example of the combination of theory and experiments needed to explain δ^2H and $\delta^{18}O$ variation in animal tissues. Hobson *et al.* (1999) state that interpreting

this variation demands a "*good understanding of the physiological ecology of study animals*" (p. 8003). We echo this comment.

(3) Coda: a renewed call for laboratory experiments and for the development of theory

Perhaps not surprisingly, the number of observational field studies that apply stable isotopes to ecological problems far surpasses the number of experimental studies that aim to clarify the mechanisms that explain the patterns that isotopic ecologists find. For example, in the Fifth International Conference on Applications of Stable Isotope Techniques to Ecological Studies (Belfast, UK, 2006) only approximately 10% of the presentations, and 5% of the posters were experimental (C. Martínez del Rio unpublished data). It is unclear what the optimal ratio of observational/theoretical/experimental research is for a discipline. However, it is evident that progress in isotopic ecology has come about from the vigorous interaction among these three modes of inquiry. Ten years ago, Gannes *et al.* (1997, 1998) identified some of the areas that could be fruitfully explored by experimentally minded isotopic ecologists. Although stable isotopes have become firmly established as tools for animal ecologists, many questions about their use still remain, and most of these questions can only be resolved experimentally. We hope that this review has identified how much progress has been made in 10 years, but also how much remains to be done. Hence, we must end it with a renewed call for experimentation. Because experiments and observations are most efficient at answering questions when informed by theory (National Research Council 2007), we add to our call an exhortation for the development of theoretical models.

VII CONCLUSIONS

(1) The dynamics of isotopic incorporation into animal tissues are measured experimentally and are most often described by one-compartment models with first-order kinetics. These experiments and models have revealed that the rate of isotopic incorporation depends on tissue growth and catabolic turnover. They also depend allometrically on the organism's body mass. Differences in isotopic incorporation rates among tissues seem to be explained by inter-tissue variation in protein turnover. A recent theoretical development in isotopic incorporation studies is the application of multi-compartment models. The inferences derived from these models can differ from those derived from one-compartment models.

(2) Isotopic ecologists use a variety of mixing models of varying degrees of complexity and realism to find out the contribution of isotopic sources to the elements in an organism's tissues. Theoreticians have devised computer programs to apply these mixing models. The use of mixing models demands the use of accurate tissue to diet discrimination factors. These are rarely measured experimentally and error in them can lead to inaccurate estimation of source proportions.

(3) Mixing models assume that assimilated nutrients are disassembled into their elemental components and that these elements are reassembled into biomolecules. This assumption is unrealistic: for example, carbon skeletons are conserved to various degrees from assimilation to the manufacture and breakdown of macromolecules and routed differentially into tissues. There are both theoretical reasons and empirical evidence for isotopic routing. Isotopic routing is an area that isotopic ecologists have so far neglected in their experimental and modeling research.

(4) Ecologists use the difference in $\delta^{15}N$ between an animal's tissues and its diet ($\Delta^{15}N$) to diagnose trophic levels. Isotopic ecologists are just beginning to understand why ^{15}N biomagnifies along trophic chains, and to explore the factors that determine the degree of ^{15}N biomagnification. We examine evidence for or against the following 4 hypotheses: (1) $\Delta^{15}N$ should decrease with increased protein quality in diet; (2) $\Delta^{15}N$ should increase with diet's protein content; (3) $\Delta^{15}N$ should decrease with the efficiency of nitrogen deposition measured as the ratio between protein assimilation and protein loss; and (4) $\Delta^{15}N$ should increase with fasting time.

(5) The protein in the tissues within a single animal differ in $\delta^{13}C$ and $\delta^{15}N$ because they differ in amino acid composition and because amino acids vary in ^{13}C and ^{15}N content. The range in $\delta^{15}N$ among amino acids appears to increase with trophic level. Some amino acids appear to retain approximately the same nitrogen isotopic composition of food, whereas others become enriched in ^{15}N by the animal's metabolism. The inter-amino acid variation in $\delta^{15}N$ allows devising an "internal" index of trophic level. The reasons why the N in some amino acids is protected from isotopic enrichment, whereas that of others is subject of ^{15}N biomagnification are unknown.

IX. ACKNOWLEDGEMENTS

The section on the nomenclature of discrimination stems from a useful discussion with David Post. The manuscript benefited from the detailed and insightful comments of Kena Fox-Dobbs, Seth Newsome, Brian Popp, and Christian Voigt. Research on stable isotopes in our laboratory has been funded by NSF (IBN-0114016). This review is dedicated to the 1991-1994 Princeton Isotope Group (Paul Koch, Diane O'Brien, and Dan Schrag).

X. REFERENCES

Adams, S. A. & Sterner, R. W. (2000). The effect of dietary nitrogen content on trophic level ^{15}N enrichment. *Limnology and Oceanography* **45**, 601–607.

Ambrose, S. H. (2000). Controlled diet and climate experiments on nitrogen isotope ratios of rat bone collagen, hair, and muscle. *Biogeochemical Approaches to Paleodietary Analysis* (eds. S. H. Ambrose & M. A. Katzenberg), pp. 243–259. Kluwer Academic/Plenum, New York, United States.

Ambrose, S. H., & 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. *Molecular Archeology of Prehistoric Human Bone* (eds. J. Lambert & G. Grupe), pp. 1–37. Springer-Verlag, Berlin, Germany.

Ayliffe, L. K., Cerling, T. E., Robinson, T., West, A. G., Sponheimer M., Passey, B. H., Roeder, B., Dearing, M. D. & Ehleringer, J. R. (2004). Turnover of carbon isotopes in tail hair and breath CO_2 of horses fed on an isotopically varied diet. *Oecologia* **139**, 11–22.

Bates, D. M. & Watts, D. G. (1988) Nonlinear regression analalysis and its applications. Wiley, New York, N.Y.

Bearhop, S., Waldron, S., Votier, S. C. & Furness, R. W. (2002). Factors that influence assimilation rates and fractionation of nitrogen and carbon stable isotopes in avian blood and feathers. *Physiological and Biochemical Zoology* **75**, 451–458.

Bequette, B. J. (2003). Amino acid metabolism in animals: An overview. *Amino Acids in Animal Nutrition* (ed. J. P. F D'Mello), pp. 87–101. CABI Publishing, Wallingford.

Best, P. B. & Schell, D. M. (1996). Stable isotopes in southern right whale (*Eubalaena australis*) baleen as indicators of seasonal movements, feeding and growth. *Mar. Biol.* **124**, 483–494

Birchall, J., O'Connell, T. C., Heaton, T.H.E. & Hedges, R. E. M. (2005). Hydrogen isotope ratios in animal body protein reflect trophic level. *Journal of Animal Ecology* **74**, 877–881.

Boag, B., Neilson, R. & Scrimgeour, C. M. (2006). The effect of starvation on the planarian *Arthurdendyus triangulates* as measured by stable isotopes. *Biology and Fertility of Soils* **43**, 267–270.

Bodwell, C. E. (1989). A collaborative study of methods of protein evaluation: introductory paper. *Plant Foods for Human Nutrition* **39**, 3–11.

Boutton, T. W., Tyrrell, H. F., Patterson, B. W., Varga, G. A. & Klein, P. D. (1988). Carbon kinetics of milk formation in Holstein cows in late lactation. *Journal of Animal Science* **66**, 2636–2645.

Bowen, G. J., Wassenaar, L. I. & Hobson, K. A. (2005). Global application of stable hydrogen and oxygen isotopes to wildlife forensics. *Oecologia* **143**, 337–348.

Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M., & West, G. B. (2004). Toward a metabolic theory of ecology. *Ecology* **85**, 1771–1789.

Caloin, M. (2004). Modeling of lipid and protein depletion during total starvation. *American Journal of Physiology* **287**, E790–E798.

Carleton, S. A. & Martínez del Rio, C. (2005). The effect of cold-induced increased metabolic rate on the rate of ^{13}C and ^{15}N incorporation in house sparrows (*Passer domesticus*). *Oecologia* **144**, 226–232.

Carleton, S. A., Kelly, L., Anderson-Sprecher, R. & Martínez del Rio, C. (2008) Should we use one or multi-compartment models to describe 13C incorporation into animal tissues? *Rapid communications in Mass Spectrometry* **22**: 3008–3014.

Castillo, L. P. & Hatch, K. A. (2007). Fasting increases $\delta^{15}N$-values in the uric acid of *Anolis carolinensis* and *Uta stansburiana* as measured by non-destructive sampling. *Rapid Communications in Mass Spectrometry* **21**, 125–4128.

Caut, S., Angulo, E., & Courchamp, F. (2008a). Caution on isotopic model use for analyses of consumer diet. *Canadian Journal of Zoology* **86**, 438–445.

Caut, S., Angulo, E., & Courchamp, F. (2008b). Discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$) in an omnivorous consumer: effect of diet isotopic ratio. *Functional Ecology* **22**, 255–263.

Cerling, T. E., Ayliffe, L. K., Dearing, M. D., Ehleringer, J. R., Passey, B. H., Podlesack, D. W., Torregrossa, A. & West, A. G. (2007a). Determining biological tissue turnover using stable isotopes: the reaction progress variable. *Oecologia* **151**, 175–189.

Cerling, T. E., Bowen, G. J., Ehleringer, J. R. & Sponheimer, M. (2007b). The reaction progress variable and isotope turnover in biological systems. *Stable Isotopes as Indicators of Ecological Change* (eds. T. E. Dawson & R. T. W. Siegwolf), pp. 163–171. Academic Press, New York, NY.

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

Dalerum, F. & Angerbjörn, A. (2005). Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia* **144**, 647–658

Dawson, T. E., Mabelli, S., Plamboeck, A. H., Templer, P. H. & Tu, K. P. (2002). Stable isotopes in plant ecology. *Annual Review of Ecology and Systematics* **33**, 507–559.

DeNiro, M. J., & Epstein, S. (1977). Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* **197**, 261–263

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

Doucett, R. R., Booth, R. K., Power, G. & McKinley, V. (1999). Effects of the spawning migration on the nutritional status of anadromous Atlantic salmon (*Salmo salar*): insights from stable-isotope analysis. *Canadian Journal of Fisheries and Aquatic Sciences* **56**, 2172–2180.

Doucett, R. R., Marks, J. C., Blinn, D. W., Caron, M. & Hungate, B. A. (2007). Measuring terrestrial subsidies to aquatic food webs using stable isotopes of hydrogen. *Ecology* **88**, 1587–1592

Evershed, R. P., Bull, I. D., Corr, L. T., Crossman, Z. M., van Dongen, B. E., Evans C., Jim, S., Mottram, H., Mukherjee, A. J. & Pancost, R. D. (2007). Compound-specific stable isotope analysis in ecological research. *Stable Isotopes in Ecology and Environmental Science 2nd Edition* (eds. R. Michener & K. Lajtha), pp. 480–540. Blackwell, London.

Fantle, M. S., Dittel, A. I., Schwalm, S. M., Epifanio, C. E. & Fogel, M. L. (1999). A food web analysis of the juvenile blue crab *Callinectes sapidus* using stable isotopes in whole animals and individual amino acids. *Oecologia* **120**, 416–426.

Focken, U. (2001). Stable isotopes in animal ecology: the effect of ration size on the trophic shift of C and N isotopes between feed and carcass. *Isotopes in Environmental and Health Studies* **37**, 199–211.

Fry, B. (2006). *Stable Isotope Ecology*. Springer, Berlin.

Fry, B. & Arnold, C. (1982). Rapid $^{13}C/^{12}C$ turnover during growth of brown shrimp (*Penaeus aztecus*). *Oecologia* **54**, 200–20.

Gannes, L. Z., Martínez del Rio, C. & Koch, P. (1998). Natural abundance variation in stable isotopes and their uses in animal physiological ecology. *Comparative Biochemistry and Physiology* **119A**, 725–737.

Gannes, L. Z., O'Brien, D. & Martínez del Rio, C. (1997). Stable isotopes in animal ecology: assumptions, caveats, and a call for laboratory experiments. *Ecology* **78**, 1271–1276.

Gaye-Siesseger, J., Focken, U., Abel, H. & Becker, K. (2003). Feeding level and diet quality influence trophic shift of C and N isotopes in Nile tilapia (*Oreochromis niloticus* (L.)). *Isotopes in Environmental and Health Studies* **39**, 125–134.

Gaye-Siesseger, J., Focken, U., Muetzel, S., Abel, H. & Becker, K. (2004). Feeding level and individual metabolic rate affect $\delta^{13}C$ and $\delta^{15}N$ values in carp: implications for food web studies. *Oecologia* **138**, 175–183.

Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., & Charnov, E. L. (2001). Effects of Size and Temperature on Metabolic Rate. *Science* **293**, 2248–2251.

Gorokhova, E. & Hansson, S. (1999). An experimental study on variation in stable carbon and nitrogen isotope fractionation during growth of *Mysis mixta* and *Neomysis integer*. *Canadian Journal of Fisheries and Aquatic Science* **56**, 2203–2210.

Haramis, G. M., Jorde, D. G., Macko, S. A. & Walker J. L. (2001). Stable isotope analysis of canvasback winter diet in upper Chesapeake Bay. *Auk* **118**, 1008–1017.

Hare, P. E., Fogel, M. L., Stafford, T. W., Mitchell, A. D. & Hoering T. C. (1991). The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. *Journal of Archaeological Science* **18**: 277–292.

Haubert, D., Langel, R., Scheu, S. & Ruess L. (2005). Effects of food quality, starvation and life stage on stable isotope fractionation in Collembola. *Pedobiologia* **49**, 229–237.

Hesslein, R. H., Hallard, K. A. & Ramal, P. (1993). Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$. *Canadian Journal of Fisheries and Aquatic Science* **50**, 2071–2076.

Hobson, K. A., Alisauskas, R. T. & 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., Atwell, L. & Wassenaar L. I. (1999). Influence of drinking water and diet on the stable-hydrogen isotope ratios of animal tissues. *Proceedings of the National Academy of Sciences* **96**, 8003–8006.

Hobson, K. A. & Clark, R. G. (1992). Assessing avian diets using stable isotopes I: Turnover of ^{13}C in tissues. *Condor* **94**, 181–188.

Hobson, K. A. & Wassenaar L. I. (1999). Stable isotope ecology: an introduction. *Oecologia* **120**, 312–313.

Howland, M. R., Corr, L. T., Young, S. M. M., Jones, V., Jim, S., van der Merwe, N. J., Mitchell, A. D. & Evershed, R. P. (2003). Expression of the dietary isotope signal in the compound specific $\delta^{13}C$ values of pig bone lipids and amino acids. *International Journal of Osteoarchaeology* **13**, 54–65.

Jardine, T. D., McLatchy, D. L., Fairchild, W. L., Cujack, R. A. & Brown, S. B. (2004). Rapid carbon turnover during growth of Atlantic salmon (*Salmo salar*) in salt water, and evidence for reduced food consumption by growth stunts. *Hydrobiologia* **527**, 63–75.

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

Karasov, W. H. & Martínez del Rio, C. (2007). *Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins*. Princeton University Press, Princeton.

Karasov, W. H. & Pinshow, B. (1998). Changes in lean mass and in organs of nutrient assimilation in a long-distance migrant at a springtime stopover site. *Physiological Zoology* **71**, 435–448.

Kidd, K., Jardine, T., & Fisk, A. (2006). Uses and abuses of stable isotopes in ecotoxicology. *Society of Environmental Toxicology and Chemistry Globe* **7**, 28–30

Klaasen, M., Thums, M. & Hume I. D. (2004). Effects of diet change on carbon and nitrogen stable-isotope ratios in blood cells and plasma of the long-nosed bandicoot (*Perameles nasuta*). *Australian Journal of Zoology* **52**, 635–647.

Koch, P. L. (2007). Isotopic study of the biology of modern and fossil vertebrates. *Stable Isotopes in Ecology and Environmental Science, 2nd Edition* (eds. R. Michener & K. Lajtha), pp. 99–154. Blackwell Publishing, Boston.

Kohn M. J. (1996). Predicting animal $\delta^{18}O$: accounting for diet and physiological adaptation. *Geochimica et Cosmochimica Acta* **60**, 4811–4829.

Kreuser-Martin, H. W., Ehleringer, J. R. & Hegg, E. L. (2005) Oxygen isotopes indicate most intracellular water in log-phase Escherichia coli is derived from metabolism. *Proceedings of the National Academy of Sciences* **102**, 17337–17341.

Kreuser-Martin, H. W., Lott, M. J., Dorigan, J. & Ehleringer, J. R. (2003). Microbe forensics: Oxygen and hydrogen stable isotope ratios in Bacilus subtilis cells and spores. *Proceedings of the National Academy of Sciences* **100**, 815–819.

Lis, G., Wassenaar, L. I. & Hendry, M. J. (2008). High-precision laser spectroscopy D-H and ^{18}O-^{16}O measurements of micro-liter natural water samples. *Analytical Chemistry* **80**, 287–293.

Lobley, G. E. (2003). Protein turnover - what does it mean for animal production. *Canadian Journal of Animal Science* **83**, 327–340

MacAvoy, S. E., Macko, S. A. & Arneson, L. S. (2005). Growth versus metabolic tissue replacement in mouse tissues determined by stable carbon and nitrogen isotope analysis. *Canadian Journal of Zoology* **83**, 631–641.

MacAvoy, S. E., Macko, S. A. & Bassett, E. (2006). Correlation of metabolism with tissue carbon and nitrogen turnover rate in small mammals. *Oecologia* **150**, 190–201.

Macko, S. A., Fogel Estep, M. L., Engel, M. H. & Hare, P. E. (1986). Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica et Cosmochimica Acta* **50**, 2143–2153.

Macko, S. A., Fogel Estep, M. L., Hare, P. E. & Hoering, T. C. (1987). Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chemical Geology* **65**, 79–87.

Marsh, A. G., Maxson, R. E. & Manahan, D. T. (2001). High macromolecular synthesis with low metabolic cost in Antarctic sea urchin embryos. *Science* **291**, 1950–1952.

Martínez del Rio, C. & Anderson-Sprecher, R. (2008). Beyond the reaction-progress variable: the meaning and significance of isotopic incorporation data. *Oecologia* **156**, 765–772.

Martínez del Rio, C. & Wolf, B. O. (2005). Mass balance models for animal isotopic ecology. *Physiological and Ecological Adaptations to Feeding in Vertebrates* (eds. M. A. Starck & T. Wang), pp. 141–174. Science Publishers, Enfield, New Hampshire.

McCarthy, M. D., Benner, R., Lee, C. & Fogel, M. L. (2007). Amino acid nitrogen isotopic fractionation patterns as indicators of heterotrophy in plankton, particulate, and dissolved organic matter. *Geochimica et Cosmochimica Acta* **71**, 4727–4744.

McClelland, J. W. & Montoya, J. P. (2002). Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. *Ecology* **83**, 2173–2180.

McCue, M. D. (2007). Western diamondback rattlesnakes demonstrate physiological and biochemical strategies for tolerating prolonged starvation. *Physiological and Biochemical Zoology* **80**, 25–34.

McCue, M. D. (2008). Endogenous and Environmental Factors Influence the Dietary Fractionation of ^{13}C and ^{15}N in Hissing Cockroaches *Gromphadorhina portentosa*. *Physiological and Biochemical Zoology* **81**, 14–24.

McCutchan J. H. Jr., Lewis, W. M. Jr., Kendall, C. & McGrath, C. C. (2003). Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* **102**, 378–390.

McIntyre P. B. & Flecker A. S. (2006). Rapid turnover of tissue nitrogen of primary consumers in tropical freshwaters. *Oecologia* **148**, 12–21.

Minagawa, M. & Wada, E. (1984). Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. *Geochimica et Cosmochimica Acta* **48**, 1135–140.

Mirón, L. L. M., Herrera, L. G. M., Ramírez, N. P. & Hobson, K. A. (2006). Effect of diet quality on carbon and nitrogen turnover and isotopic discrimination in blood of a New World nectarivorous bat. *Journal of Experimental Biology* **209**, 541–548.

National Research Council. (2007). *The Role of Theory in Advancing 21-st Century Biology*. The National Academies Press, Washington D.C.

Newsome, S., Martínez del Rio, C., Phillips, D. L. & Bearhop, S. (2007). A niche for isotopic ecology. *Frontiers in Ecology and the Environment* **5**, 429–436.

O'Brien, D. M, Boggs, C. L. & Fogel, M. L. (2003). Pollen feeding in *Heliconius charitonius*: isotopic evidence for essential amino acid transfer from pollen to eggs. *Proceedings of the Royal Society B* **270**, 2631–2636.

O'Brien, D. M, Boggs, C. L. & Fogel, M. L. (2004). Making eggs from nectar: the role of life history and dietary carbon turnover in butterfly reproductive resource allocation. *Oikos* **105**, 279–291.

O'Brien, D. M., Boggs, C. L. & Fogel, M. L. (2005). The amino acids used in reproduction by butterflies: a comparative study of dietary sources using compound specific stable isotope analysis. *Physiological Biochemistry and Zoology* **78**, 819–827.

O'Brien, D. M., Fogel, M. L. & Boggs, C. L. (2002). Renewable and non-renewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. *Proceedings of the National Academy of Sciences* **99**, 4413–4418.

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

Oelbermann, K. & Sechu, S. (2002). Stable isotope enrichment ($\delta^{15}N$ and $\delta^{13}C$) in a generalist predator (*Pardosa lugubris*, Araneae: Lycosidae): effects of prey quality. *Oecologia* **130**, 337–344.

Olive, P. J. W., Pinnegar, J. K., Polunin, N. V. C., Richards, G. & Welch. R. (2003). Isotope trophic-step fractionation: a dynamic equilibrium model. *Journal of Animal Ecology* **72**, 608–617.

Pearson, S. F., Levey, D. J., Greenberg, C. H. & Martínez del Rio, C. (2003). Effects of elemental composition on the incorporation of dietary nitrogen and carbon and isotopic signatures in an omnivorous songbird. *Oecologia* **135**, 516–523.

Perga, M. E. & Gerdeaux D. (2005). "Are fish what they eat" all year round? *Oecologia* **144**, 598–606

Peters, R. H. (1983). *The Ecological Implications of Body Size*. Cambridge University Press, Cambridge.

Phillips, D. L. & Eldrige, P. M. (2006). Estimating the timing of diet shifts using stable isotopes. *Oecologia* **147**, 195–203.

Phillips, D. L., & Gregg, J. W. (2001). Uncertainty in source partitioning using stable isotopes. *Oecologia* **127**, 171–179.

Phillips, D. L. & Gregg, J. W. (2003). Source partitioning using stable isotopes: coping with too many sources. *Oecologia* **136**, 261–269.

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

Podlesak, D. W. & McWilliams, S. R. (2006). Metabolic routing of dietary nutrients in birds: effects of diet quality and macronutrient composition revealed using stable isotopes. *Physiological Biochemistry and Zoology* **70**, 534–549.

Podlesak, D. W., Torregrossa, A. M., Ehleringer, J. R., Dearing, M. D., Benjamin, H. & Cerling, T. E. (2008). Turnover of oxygen and hydrogen isotopes in the body water, CO_2, hair, and enamel of a small mammal. *Geochimica et Cosmochimica Acta* **72**, 19–35

Ponsard, S. & Averuch, P. (1999). Should growing and adult animals fed on the same diet show different $\delta^{15}N$ values. *Rapid Communications in Mass Spectrometry* **13**, 1305–1310.

Popp, B. N., Graham, B. S., Olson, R. J., Hannides, C. C. S., Lott, M., Lopez-Ibarra, G. & Galvan-Magana, F. (2007). Insight into the trophic ecology of yellowfin tuna, *Thunnus albacares*, from compound-specific nitrogen isotope analysis of proteinaceous amino acids. *Isotopes as Tracers in Ecological Change* (eds. T. Dawson & R. Seigwolf), pp. 173–190. Elsevier, New York.

Post, D. M. (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology* **83**, 703–718

Post, D. M., Layman, C. A., Arrington, D. A., Takimoto, G., Quattrochi, J. & Montaña, C. G. (2007). Getting to the fat of the matter: models, methods, and assumptions for dealing with lipids in stable isotope analysis. *Oecologia* **152**, 179–189.

Reich, K. J., Bjorndal, K. A. & Martínez del Rio, C. (2008). Effects of growth and tissue type on the kinetics of ^{13}C and ^{15}N incorporation in a rapidly growing ectotherm. *Oecologia* **155**, 651–658.

Robbins, C. T., Felicetti, L. A. & Sponheimer, M. (2005). The effects dietary protein quality on nitrogen isotope discrimination in mammals and birds. *Oecologia* **144**, 534–540.

Rochette, M. J., Comillon, P. A., Sabatier, R. & Pontique, D. (2000). Comparative analysis of phylogenetic and fishing effects in life history patterns in teleost fishes. *Oikos* **91**, 255–270.

Rubenstein, D. R. & Hobson, K. A. (2004). From birds to butterflies: animal movement patterns and stable isotopes. *Trends in Ecology and Evolution* **19**, 256–263.

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

Schmidt, K., McClelland, J. W., Mente, E., Montoya, J. P., Atkinson, A. & Voss, M. (2004). Trophic-level interpretation based on delta N-15 values: Implications of tissue-specific fractionation and amino acid composition. *Marine Ecology Progress Series* **266**, 43–58

Schwarcz, H. P. (1991). Some theoretical aspects of isotope paleodiet studies. *Journal of Archaeological Science* **18**, 261–275.

Sebens, K. P. (1987). The ecology of indeterminate growth in animals. *Annual Review of Ecology and Systematics* **18**, 371–407.

Sponheimer, M., Robinson, T., Roeder, B., Ayliffe, L., Passey, B., Cerling, T., Dearing, D. & Ehleringer, J. (2003). An Experimental Study of Nitrogen Flux in Llamas: Is ^{14}N Preferentially Excreted? *Journal of Archaeological Science* **30**, 1649–1655.

Sterner, R. W. & Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton University Press, Princeton.

Steyermark, A. C. (2002). A high standard metabolic rate constrains juvenile growth. *Zoology* **105**, 147–151.

Suzuki K. W, Kasai A., Nakayama K., & Tanaka M. (2005). Differential isotopic enrichment and half life among tissues in Japanese temperate bass (*Lateolabrax japonicus*) juveniles: impli-

cations for analyzing migration. *Canadian Journal of Fisheries and Aquatic Sciences* **62**, 671–678.

Teece, M. A. & Fogel, M. L. (2004). Preparation of ecological and biochemical samples for isotope analysis. *Handbook of Stable Isotope Analytical Techniques*, Volume-I (ed. P. A. De Groot), pp. 178–208. Elsevier, New York.

Tibbets, T., Wheeless, L. A. & Martínez del Rio, C. (2007). Isotopic enrichment without change in diet: an ontogenetic shift in $\delta^{15}N$ during insect metamorphosis. *Functional Ecology* **22**, 109–113.

Tieszen, L. L., Boutton, T. W., Tesdahl, K. G. & 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.

Trueman, C. N., McGill, R. A. R. & Guyard P. H. (2005). The effect of growth rate on tissue-diet isotopic spacing in rapidly growing animals. An experimental study with Atlantic salmon (*Salmo salar*). *Rapid Communications in Mass Spectrometry* **19**, 3239–3247

Tsahar, E., Wolf, N., Ishaki, I., Arad, Z. & Martínez del Rio, C. (2007). Diet influences the rate of ^{15}N incorporation in blood cells and plasma of yellow-vented bulbuls (*Pycnonotus xanthopygos*). *Journal of Experimental Biology* **211**, 459–465.

Van der Merwe, N. J. (1982). Carbon isotopes, photosynthesis, and archaeology. *American Scientist* **70**, 209–215.

Vander Zanden, M. J., & Fetzer, W. W. (2007). Global patterns of aquatic food chain length. *Oikos* **116**, 1378–1388.

Vander Zanden, M. J., Cabana, G. & Rasmussen, J. B. (1997). Comparing trophic position of freshwater littoral fish species using stable nitrogen isotopes ($\delta^{15}N$) and literature dietary data. *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 1142–1158.

Vander Zanden, M. J., Lester, N. P., Shuter, B. J. & Rasmussen, J. B. (1999). Patterns of food chain length in lakes: a stable isotope study. *Am. Nat.* **154**, 406–416.

Vander Zanden, M. J. & Rasmussen, J. B. (2001). Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: Implications for aquatic food web studies. *Limnology and Oceanography* **46**, 2061–2066.

Voigt, C., Katja, R., Michener, R. & Speakman, J. (2008). Nutrient routing in omnivorous animals tracked by stable carbon isotopes in tissue and exhaled breath. *Oecologia* (in press).

Voigt, C. C., Matt, F., Michener, R. & Kunz, T. H. (2003). Low turnover rates of carbon isotopes in tissues of two nectar-feeding bat species. *Journal of Experimental Biology* **206**, 1419–1427.

Voigt, C. C. & Speakman, J. R. (2007). Nectar-feeding bats fuel their high metabolism directly with exogenous carbohydrates. *Functional Ecology* **21**: 913–921.

Waterlow, J. C. (2006). *Protein Turnover.* CABI Publishing, Wallingford.

West, G. B., Brown, J. H., & Enquist B. J. (1997). A general model for the origin of allometric scaling laws in biology. *Science* **276**, 122–126.

West, G. B., Brown, J. H. & Enquist, B. J. (2001). A general model for ontogenetic growth. *Nature* **413**, 628–631.

Whitledge, G. W., Johnson, B. M. & Martinez, P. J. (2006). Stable hydrogen isotopic composition of fishes reflects that of their environment. *Canadian Journal of Fisheries and Aquatic Sciences* **63**, 1746–1751

Witting, D. A., Chambers, R. C., Bosley, K. L. & Wainright, S. C. (2004). Experimental evaluation of ontogenetic diet transitions in summer flounder (*Paralichtys dentatus*), using stable isotopes as diet tracers. *Canadian Journal of Fisheries and Aquatic Sciences* **61**, 2069–2084.