



## **Stable isotopes in mammalian research: a beginner's guide**

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## Stable isotopes in mammalian research: a beginner's guide

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We open this Special Feature on stable isotopes in mammalian research with a beginner's guide, an introduction to the novice and a refresher to the well-versed. In this guide we provide the background needed to understand the more advanced papers that follow. We describe the basic principles of isotopic fractionation and discrimination, briefly explain the processes that govern isotopic incorporation into animal tissues, list some innovative studies, and provide cautionary notes and caveats. In addition to discussing the uses of natural abundance we present the concepts and applications of enriched isotopes and the potential combination of these 2 methodologies. We end with descriptions of analytical and conceptual developments that we believe will be cardinal to the future of isotopic analyses in mammalian research.

Key words: carbon, diet, enrichment, hydrogen, incorporation, migration, mixing models, natural abundance, nitrogen, strontium

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The following manuscripts in this Special Feature describe some recent theoretical and analytical advances in the field of animal isotopic ecology. Although the authors of each paper provide background information on their topic, to understand those works a novice reader will need to become familiar with fundamental concepts and terminology. Herein, we provide an explanation of the basic chemical and physical properties of stable isotopes and describe the principles of isotopic fractionation and discrimination.

The number of studies that have successfully applied stable isotope analyses to various archeological, paleontological, and ecological questions is staggering and no single review (including books) can possibly list them all (Crawford et al. 2008; Dawson et al. 2002; Dawson and Siegwolf 2007; Hobson 1999; Kelly 2000; Martínez del Rio et al. 2009; Michener and Lajtha 2007; Newsome et al. 2010; Wolf et al. 2010). Therefore, following this general overview we concentrate our discussion on applications to animal ecology. We explain the processes that govern isotopic incorporation into animal tissues, describe some innovative studies, and provide some cautionary notes and caveats. We briefly touch on those topics that are covered in depth in the following papers and refer the reader to them as needed. We end this beginner's guide with descriptions of analytical and conceptual developments that we believe will be cardinal to the future of isotopic analyses.

### WHAT ARE STABLE ISOTOPES?

On Earth, several elements occur in more than 1 stable form (Table 1; Sulzman 2007). These forms, called isotopes, differ from each other in number of neutrons in the nucleus and thus have different atomic masses. For example, carbon occurs in 2 stable forms: the lighter,  $^{12}\text{C}$ , has 6 protons and 6 neutrons in the nucleus and thus an atomic mass of 12; the heavier,  $^{13}\text{C}$ , has 6 protons and 7 neutrons and atomic mass of 13.

Usually, the heavier stable isotopes of elements are rare (Table 1). Because all stable isotopes of the same element have the identical number of protons and electrons they are chemically equivalent (i.e., are capable of creating the same number of chemical bonds). Their behavior in chemical reactions (reaction rate and bond strength), however, varies because of their different physical properties related to atomic mass (i.e., vibrational energy of the nucleus—Sulzman 2007). These different physical properties lead to variation in the ratios of heavy to light isotopes in organic compounds.



**TABLE 1.**—Stable isotopes of several elements used in ecological studies and their relative abundance in nature (percent of atoms in a specific form = atom percent). Hydrogen, carbon, and strontium also have radioactive isotopes, which will not be discussed here. Isotopes are stable when the number of neutrons is similar to the number of protons ( $\leq 1.5$ —Sulzman 2007).

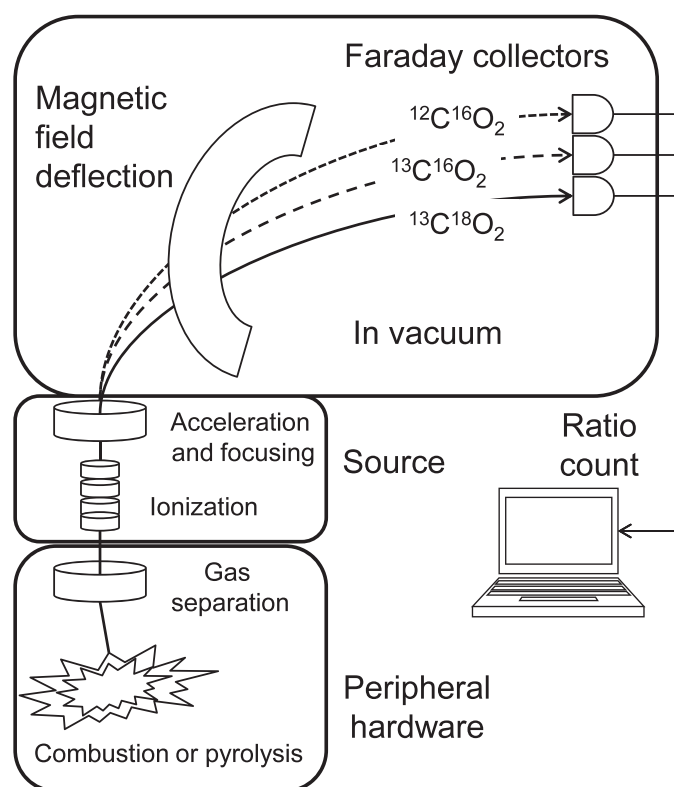
Element	Isotopes (relative abundance in atom percent)					
Hydrogen	$^1\text{H}$ (99.985)	$^2\text{H}$ (0.015) <sup>a</sup>				
Calcium	$^{40}\text{Ca}$ (96.941)	$^{42}\text{Ca}$ (0.647)	$^{43}\text{Ca}$ (0.135)	$^{44}\text{Ca}$ (2.086)	$^{46}\text{Ca}$ (0.001)	$^{48}\text{Ca}$ <sup>b</sup> (0.187)
Carbon	$^{12}\text{C}$ (98.892)	$^{13}\text{C}$ (1.108)				
Nitrogen	$^{14}\text{N}$ (99.635)	$^{15}\text{N}$ (0.365)				
Oxygen	$^{16}\text{O}$ (99.759)	$^{17}\text{O}$ (0.037)	$^{18}\text{O}$ (0.204)			
Strontium	$^{84}\text{Sr}$ (0.560)	$^{86}\text{Sr}$ (9.870)	$^{87}\text{Sr}$ (7.040)	$^{88}\text{Sr}$ (82.580)		
Sulfur	$^{32}\text{S}$ (95.016)	$^{33}\text{S}$ (0.760)	$^{34}\text{S}$ (4.210)	$^{36}\text{S}$ (0.014)		

<sup>a</sup>  $^2\text{H}$  is also called deuterium and is usually denoted as D.

<sup>b</sup> Ca has 24 isotopes of which 5 (listed here) are stable (or observationally stable) and  $^{48}\text{Ca}$  has exceedingly long half-life.

### HOW DO WE MEASURE THE RATIOS OF STABLE ISOTOPES IN ORGANIC COMPOUNDS?

The ratios of heavy to light isotopes (e.g.,  $^{13}\text{C}:^{12}\text{C}$  or  $^{15}\text{N}:^{14}\text{N}$ ) are most commonly measured with a thermal ionization mass spectrometer (known as TIMS; Fig. 1). The



**FIG. 1.**—Schematic diagram of a continuous-flow isotope-ratio mass spectrometer coupled to an elemental analyzer. Organic samples are homogenized and weighed into tin or silver cups. The samples are injected into the analyzer where organic compounds are converted to gaseous inorganic compounds such as  $\text{N}_2$ ,  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , or  $\text{SO}_2$  via combustion or pyrolysis. The gases are separated and then injected into the source of the mass spectrometer. There they are ionized and accelerated into the flight tube where a strong magnet deflects them and separates them based on mass. The resulting beams of ionized, gaseous molecules are collected at the end of the flight tube in Faraday cups; their collection creates a weak electrical current measured by the controlling computer.

mass spectrometer measures the mass of gaseous inorganic compounds such as  $\text{N}_2$ ,  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , or  $\text{SO}_2$ , so the 1st step in measuring the isotopic ratios in organic compounds requires their transformation to gases. Organic compounds analyzed for ratios of  $^{13}\text{C}:^{12}\text{C}$  or  $^{15}\text{N}:^{14}\text{N}$  are combusted to gaseous molecules with oxygen and metal catalysts (such as tin or copper) at high temperatures. This can be done either off-line ( $>900^\circ\text{C}$ ) in systems uncoupled from the mass spectrometer, or online, in systems where the sample is combusted using an elemental analyzer ( $1,600\text{--}1,800^\circ\text{C}$ ) and then automatically introduced into the mass spectrometer (Michener and Lajtha 2007; Fig. 1). Organic samples analyzed for  $^2\text{H}:^1\text{H}$  (also known as D:H) or  $^{18}\text{O}:^{16}\text{O}$  receive similar treatments (i.e., pyrolysis), although more modern procedures involve the combustion of samples in an oxygen-free environment, eliminating the need to account for the isotopic values of the combustion gas.

Once in gaseous form, the now-inorganic molecules are injected into the source of the mass spectrometer (Fig. 1). There they are ionized and accelerated into an evacuated flight tube where a strong magnet deflects and separates them based on mass (Fig. 1). The resulting beams of ionized, gaseous molecules are collected at the end of the flight tube in Faraday cups; their collection creating a weak electrical current that is measured by the controlling computer (Michener and Lajtha 2007; Fig. 1). To understand why the molecules separate based on their mass, imagine a top-performing golfer practicing a specific swing with the same golf club but with balls that vary in weight. The lighter balls will fly further than the heavier ones (for a clear illustration see Karasov and Martínez del Rio 2007).

In order to obtain reliable measurements of isotope ratios, one needs to follow 2 rules: avoid contamination, and prevent changes to the ratios as a result of handling. In the past when relatively large quantities of organic compounds were combusted in off-line furnaces in large evacuated glass tubes, contamination was a minor concern; a flake of dust or small air leak (introducing atmospheric  $\text{CO}_2$  and  $\text{N}_2$ ) negligibly changed the resulting isotope signature of the sample. Today, however, when we weigh less than 1 mg of the dried and homogenized sample in small tin or silver weighing cups, contamination can be a serious problem. Data presented in Table 2 demonstrate the variation one can expect when analyzing samples in duplicate. They unambiguously illustrate

**TABLE 2.**—Frequency of occurrence of discrepancy in isotopic values of samples analyzed in duplicate (number of deviant samples divided by the total [*n*]) for various sample types for carbon and nitrogen demonstrating the variation one can expect from duplicate samples. The data unambiguously illustrate the potential for generating bias when a single subsample is analyzed. Discrepancy was defined as a difference between duplicates greater than the variation among standards analyzed with each batch of samples. The variation for standards differed among facilities; at the University of Wyoming Stable Isotope Facility variation is, in general, less than 0.1‰ for carbon, 0.15‰ for nitrogen, and 2.0‰ for deuterium. Also presented are the average difference (for deviant samples only) and the maximal difference. Samples were processed following standard operating procedures of best practices (see Ben-David et al. [2012], Pauli et al. [2012], and Whiteman et al. [2012] for details) by over 50 technicians, students, and investigators in 3 different laboratories. Samples were analyzed at 5 different stable isotope facilities in the United States. For hair samples (*n* = 275), the frequency of discrepancy between duplicate samples for deuterium ( $\delta D$ ) is 0.63, the average difference is 6.89‰, and the maximal difference is 63.3‰. Samples that are more difficult to homogenize (i.e., hair and serum) are more susceptible to error as compared with samples such as bone collagen that are thoroughly homogenized during extraction. Breath samples are sensitive to air leaks.

Tissue	<i>n</i>	Carbon ( $\delta^{13}C$ )			Nitrogen ( $\delta^{15}N$ )		
		Frequency	Average difference	Maximal difference	Frequency	Average difference	Maximal difference
Blood cells	750	0.16	0.22	1.27	0.19	0.21	1.31
Bone collagen	30	0.03		0.11	0.07	0.12	0.13
Breath	115	0.40	0.20	13.58 <sup>a</sup>			
Hair	998	0.46	0.32	1.62	0.46	0.41	2.60
Muscle	265	0.20	0.33	2.56	0.34	0.25	2.50
Serum	201	0.20	0.27	1.61	0.14	0.47	2.18
Plants	610	0.40	0.24	2.10	0.55	0.34	3.45
Soil	128	0.41	0.22	0.70	0.58	0.32	2.48

<sup>a</sup> Not included in average calculation.

the potential for generating bias when a single subsample is analyzed. Also, it is clear that some types of samples (such as serum and hair, which are more difficult to homogenize) are more prone to problems than others (Table 2). For procedures used to reduce the risk of contamination see Ben-David et al. (2012), Cryan et al. (2012), and Pauli et al. (2012). Also, we recommend analyzing all samples in duplicate. If the variance of the 2 subsamples exceeds that of the laboratory standard, contamination may have occurred and its source should be tracked and eliminated.

To avoid changing the ratios of the heavy to light isotopes, we usually dry the samples at relatively low temperatures (60–70°C). At these temperatures we are less likely to cause preferential volatilization of the compounds containing the lighter isotopes. Alternatively, samples can be freeze-dried (Post et al. 2007) and then homogenized. Once weighed and submitted to the isotope facility, the built-in components of the elemental analyzer (high combustion temperatures and capillary tubing for viscous flow with helium gas) and mass spectrometer (gold or graphite plating) ensure that their internal reactions are complete and no molecules escape the count (Sulzman 2007).

## WHAT ARE STANDARDS?

By convention, we express the ratio of heavy to light isotopes in our sample in relation to an internationally set standard so that data collected from across the globe are comparable. The notation we use to describe the sample ratio as it relates to the standard is in this form:

$$\delta X = \frac{R_{\text{sample}} - R_{\text{std}}}{R_{\text{std}}} \times 1,000, \quad (1)$$

where  $\delta$  (called del) is the isotopic notation, X is the element in its heavy form (e.g., D,  $^{13}C$ , or  $^{15}N$ ), R is the ratio of heavy to light isotopes (e.g.,  $^{13}C:^{12}C$ ), and the units of measurements are in parts per thousand (‰). The international standards are Vienna Pee Dee Belemnite (VPDB;  $\delta^{13}C$ ), atmospheric nitrogen (AIR;  $\delta^{15}N$ ), Vienna Standard Mean Ocean Water (VSMOW;  $\delta D$  and  $\delta^{18}O$ ), Vienna Cañon Diablo Meteorite Troilite (VCDT;  $\delta^{34}S$ ), and United States Geological Survey *Tridacna* ( $^{87}Sr:^{86}Sr$ —Sulzman 2007). Because the stocks of some of these formal standards have been depleted or are inordinately expensive, we use other materials that have been calibrated against these formal ones as internal laboratory standards. For example, a peptone standard frequently used in our laboratory has known values of  $\delta^{13}C_{\text{std}} = -15.17\text{‰}$  and  $\delta^{15}N_{\text{std}} = 5.48\text{‰}$ . Because we analyze multiple standards with every batch of samples, we are able to monitor accuracy, repeatability, and machine linearity, all important quality-control measures.

When the ratio of heavy to light isotopes in the sample is higher than that of the formal standard ( $R_{\text{sample}} > R_{\text{std}}$ ), we call the sample enriched. When the ratio in the sample is lower than the ratio in the standard ( $R_{\text{sample}} < R_{\text{std}}$ ), the sample is depleted. Because VPDB is derived from a sedimentary limestone and contains high quantities of  $^{13}C$ , most organic samples are depleted relative to it and thus will be expressed in negative numbers (e.g., peptone  $\delta^{13}C_{\text{std}} = -15.17\text{‰}$ ). More negative values mean there are fewer  $^{13}C$  atoms in the sample (lower ratio = more depleted), less negative values mean there are more  $^{13}C$  atoms (higher ratio) and the sample is considered less depleted. When the ratio of heavy to light isotopes in inorganic or organic compounds changes as a result of the different physical properties related to their atomic mass, we denote the change with the Greek capital letter delta,  $\Delta$  ( $\Delta_{A-B}$  is the difference between 2 del values =  $\delta_A - \delta_B$ —Sulzman 2007), and call the process fractionation.

## WHAT IS FRACTIONATION?

There are 2 main types of fractionations. Equilibrium fractionation occurs when substrates and products of chemical equilibrium reactions differ in their isotope ratios because the heavier isotopes create stronger bonds with either the substrate or product. For example, the reversible exchange of oxygen between CO<sub>2</sub> and H<sub>2</sub>O molecules results in enriched CO<sub>2</sub> because <sup>18</sup>O creates stronger bonds with carbon than <sup>16</sup>O.



Kinetic fractionations, which are usually more pronounced, occur when a single type of molecule changes phase (e.g., from liquid to vapor) or when the chemical reaction is nonreversible (Sulzman 2007). For example, in colder temperatures evaporation of H<sub>2</sub>O molecules from a body of water is faster than D<sub>2</sub>O ones because the intermolecular bonds for the former are weaker (Sulzman 2007). Kinetic fractionations are the result of an interaction between bond strength and molecular velocity and are typical in evaporation, diffusion, and enzymatic processes.

Until recently, we called fractionations all changes in isotopic ratios as a result of physical or chemical processes. Now we restrict the term to changes that occur in a single reaction. When multiple (and mostly unknown) processes are involved, we call the difference between the origin compounds and the products discrimination (Cerling and Harris 1999).

## WHAT PHYSICAL AND BIOLOGICAL PROCESSES LEAD TO ISOTOPIC FRACTIONATION?

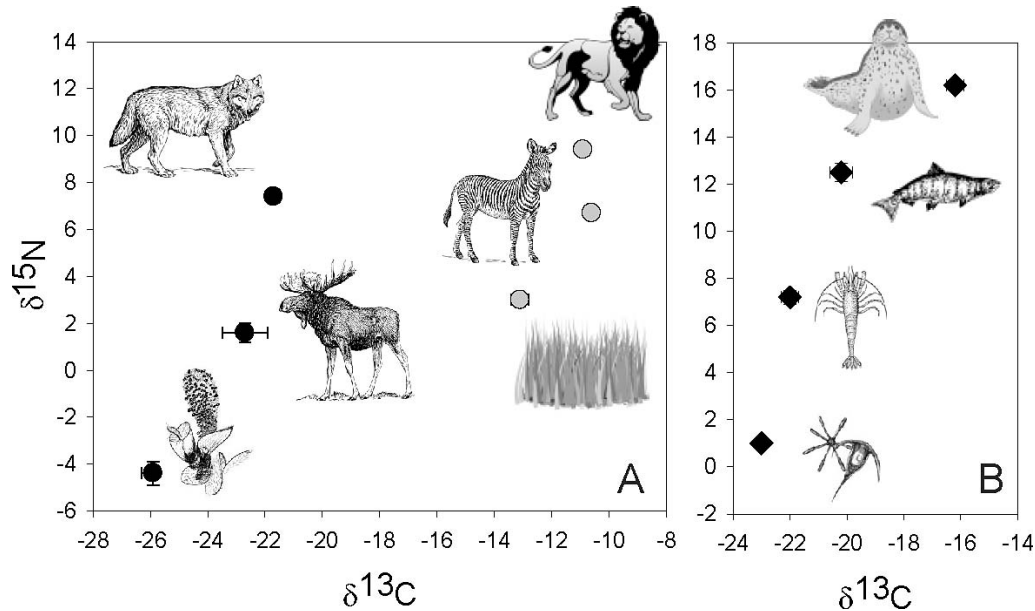
Evaporation, condensation, and diffusion are the 3 main physical mechanisms that cause fractionations between substrates and products. As such, they are all temperature dependent because temperature influences the velocity and strength of chemical bonds of molecules (Michener and Lajtha 2007; Sulzman 2007). For example, the diffusion of CO<sub>2</sub> through the stomatal pores into the leaves of plants results in carbon fractionation of about 4.4‰ ( $\Delta_{\text{air in leaf} - \text{air in atmosphere}} = \delta^{13}\text{C}_{\text{air in leaf}} - \delta^{13}\text{C}_{\text{air in atmosphere}} = (-12.4) - (-8.0) = -4.4\text{‰}$ ). Similarly, evaporation of water from the ocean results in fractionation in oxygen of about 13‰ ( $\Delta_{\text{water in ocean} - \text{water vapor}} = \delta^{18}\text{O}_{\text{water in ocean}} - \delta^{18}\text{O}_{\text{water vapor}} = (0.0) - (13.0) = -13.0\text{‰}$ ). During subsequent precipitation on land from clouds formed over the ocean (e.g., condensation), the heavier water molecules are shed 1st, creating a predictable  $\delta\text{D}$  and  $\delta^{18}\text{O}$  latitudinal gradient in precipitation on many continents (for more details see Bowen et al. [2005] and Wunder [2012]). Additional examples of physical fractionation include the enrichment of soil  $\delta^{15}\text{N}$  values with increasing depth as a result of preferential volatilization of lighter molecules of ammonium (NH<sub>4</sub><sup>+</sup>, up to 20%—Evans 2007) and leaching, or changes in <sup>87</sup>Sr:<sup>86</sup>Sr ratios as a result of weathering of rocks (Koch 2007). Indeed, one of the better known applications of stable isotope analysis is the reconstruction of prehistoric temperature records from cores collected in Greenland and Antarctica based on  $\delta^{18}\text{O}$  values

in different ice layers (enriched layers = warmer temperatures—Barnola et al. 1987).

Because the majority of biological processes are mediated through enzymatic reactions, there are few systems (if any) that do not exhibit isotopic fractionation or discrimination (except potentially the assimilation of strontium—Koch 2007). The main regulating mechanism here is the interplay between demand and availability of the substrate. When the availability of a substrate is limited relative to demand, discrimination against the heavy form will be small and the product isotopic value will be similar to that of the substrate (small discrimination or  $\Delta$ ); when the substrate is in excess relative to demand, discrimination will be large (Montoya 2007). Of course the physical conditions relative to the enzyme optimal operating range also will affect fractionation and discrimination because temperature affects velocity and bond strength of molecules (Sulzman 2007). For example, nitrification by soil microbes creates a fractionation range from 0‰ to 35‰ in  $\delta^{15}\text{N}$  depending on substrate availability and temperature (Evans 2007).

## HOW DO PHYSICAL AND BIOLOGICAL FRACTIONATIONS MAKE STABLE ISOTOPES A USEFUL ECOLOGICAL RESEARCH TOOL?

The isotope signatures of organisms are the product of the ratios of heavy to light isotopes of the substrates they utilize and the physiological processes (i.e., enzymatic reactions) they employ in assimilating these substrates and discarding their products. The 1st described and most well known are the isotopic fractionations of carbon during photosynthesis. C<sub>3</sub> plants (i.e., those that rely on the Calvin cycle and ribulose biphosphate carboxylase [Rubisco] for CO<sub>2</sub> fixation) preferentially fix <sup>12</sup>C-bearing CO<sub>2</sub>, yielding depleted  $\delta^{13}\text{C}$  values ranging between -35‰ and -25‰ with median values around -27‰ (Marshall et al. 2007). In contrast, C<sub>4</sub> plants (i.e., those that rely on Hatch–Slack cycle and phosphoenol pyruvate carboxylase [PEP] for CO<sub>2</sub> fixation) and those that use crassulacean acid metabolism (CAM) show lower preference for the lighter isotope, resulting in values typically around -14‰ (range -15‰ to -11‰—Dawson et al. 2002; Marshall et al. 2007). This difference in  $\delta^{13}\text{C}$  values among plants provides a natural marker system to track the diets of herbivores; the difference between C<sub>3</sub> and C<sub>4</sub> plants percolates up trophic levels through consumption by herbivores and the subsequent assimilation of their tissues by predators (Fig. 2). Indeed, one of the more intriguing uses of the characteristic isotopic signatures of C<sub>3</sub> and C<sub>4</sub> plants was the reconstruction of the spread of C<sub>4</sub> plants, which are adapted to hot, arid climates, around the globe approximately 6 million years ago. Using  $\delta^{13}\text{C}$  values of tooth enamel from fossil equids (horses), Cerling et al. (1997) demonstrated that members of this group relied on C<sub>3</sub> plants in Africa, Asia, and North America until approximately 6 million years ago, when consumption of C<sub>4</sub> plants surged in Pakistan, Africa, and southern North America. Concurrently, equids in northern North America and Europe



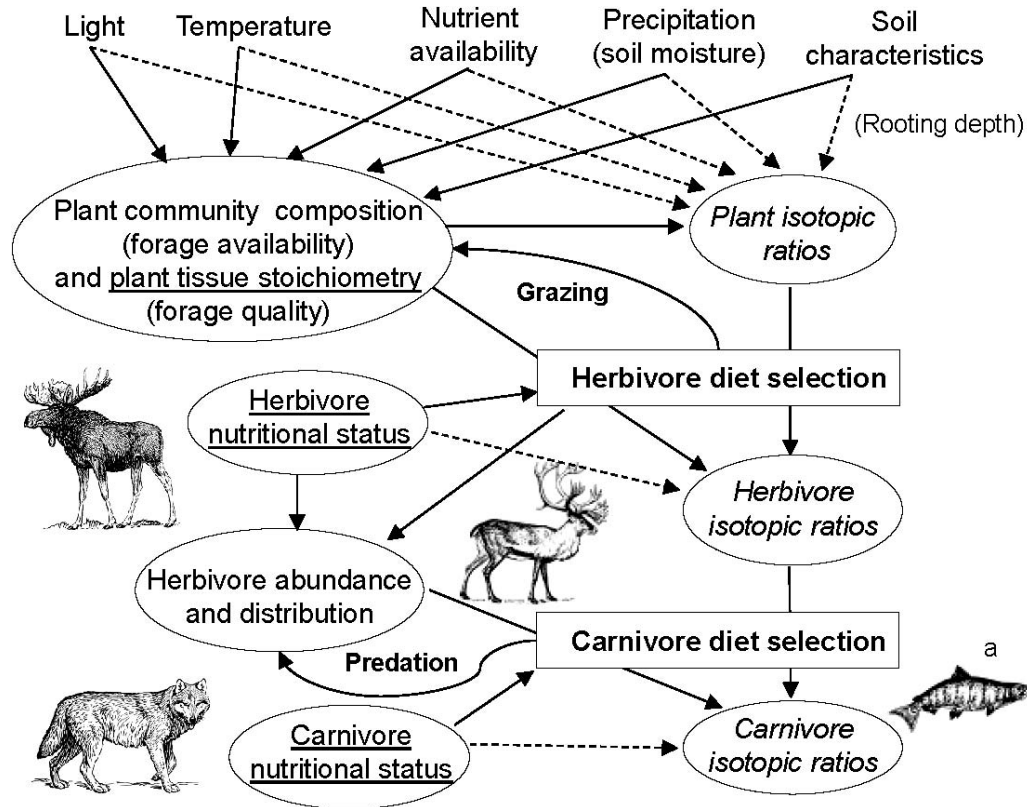
**FIG. 2.**—Illustration of trophic enrichment in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  from primary producers (plants and diatoms), to herbivores, to predators for A) terrestrial ecosystems and B) marine ecosystems. Panel A also shows differences in  $\delta^{13}\text{C}$  between food webs based on  $\text{C}_3$  (black symbols) and  $\text{C}_4$  plants (gray symbols). Values ( $\bar{X} \pm \text{SE}$ ) were adapted from the following sources: willows (*Salix*) from Ben-David et al. (2001); moose (*Alces alces*) and wolves (*Canis lupus*) from Szepanski et al. (1999); grasses from Wang et al. (2010); zebra (*Equus burchellii*) and lions (*Panthera leo*) from Codron et al. (2007); phytoplankton from Koch (2007); zooplankton from Schell et al. (1998); and pelagic fishes and harbor seals (*Phoca vitulina*) from Herreman et al. (2009).

continued feeding on  $\text{C}_3$  plants, indicating that the expansion of  $\text{C}_4$  plants was limited (as it is today) to low- and midlatitude habitats (Cerling et al. 1997).

Photosynthetic pathways are only 1 of a multitude of factors that affect isotopic signatures of primary producers and their dependent food webs (including decomposers) in terrestrial, marine, and freshwater ecosystems. For example, isotopic signatures of terrestrial  $\text{C}_3$  plants of the same species can vary depending on soil moisture and temperature because water availability affects evapotranspiration and water-use efficiency. This in turn affects photosynthetic rates, stomatal conductance, and thus isotopic discrimination. Similarly, water source (rainfall, snow pack, or ground water) will affect the  $\delta\text{D}$  and  $\delta^{18}\text{O}$  signatures in plants; rooting depths and soil microbial mineralization and nitrification rates, symbiosis with mycorrhizal fungi, and plant water-use efficiency will influence  $\delta^{15}\text{N}$  values; and the basal rock substrate will determine  $^{87}\text{Sr}:^{86}\text{Sr}$  ratios. Indeed, many of the factors that influence the composition of plant communities (species distributions and richness) and elemental composition of plant tissues or stoichiometry (C:N:P ratios) also affect the plants' isotopic signatures (Fig. 3). For example, recently we observed differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of individual soil macroinvertebrates of the same species collected in old-growth, young-growth, and clear-cut forest stands of Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*) in southeastern Alaska (Flaherty and Ben-David 2010). The differences in canopy closure induced differences in isotopic signatures reflecting the effects of light availability and water-use efficiency on photosynthetic rates of the vegetation in these 3 habitats (Flaherty and Ben-David 2010).

Isotopic variations are not unique to terrestrial systems. Although most primary producers in aquatic ecosystems rely on Rubisco photosynthetic pathways, large differences in  $\delta^{13}\text{C}$  exist between intertidal and pelagic oceanic systems, largely because of differences in temperature, levels of dissolved  $\text{CO}_2$ , phytoplankton growth rates (Michener and Kaufman 2007), and whether the system is fueled by phototrophs or chemotrophs (Van Dover 2007). Similarly, these 2 systems differ in  $\delta^{15}\text{N}$  values. Although intertidal and nearshore systems derive much of their nitrogen from terrestrial runoff or nitrogen fixation, which are usually depleted in  $^{15}\text{N}$ , pelagic systems largely assimilate subsurface  $\text{NO}_3^-$ , which is usually enriched with  $^{15}\text{N}$ . In addition,  $\delta^{34}\text{S}$  varies (up to 40‰) between estuarine and deep-ocean habitats as a result of the uptake of sulfides in the former and sulfates in the latter (Michener and Kaufman 2007). Upwelling, currents, tides, and to some extent algal blooms influence the spatial distribution of primary producers and consumers in the ocean resulting in isotopically heterogeneous seascapes (Clementz 2012; Lee et al. 2005; Montoya 2007).

Similarly, freshwater ecosystems are characterized by large variations in  $\delta\text{D}$ ,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^{18}\text{O}$ , and to some extent  $\delta^{34}\text{S}$ . Water source, hydrology, and temperature (through evaporation) will determine the values of  $\delta\text{D}$  and  $\delta^{18}\text{O}$ . In situ photosynthesis (by aquatic autotrophs [also known as autochthonous sources]) compared to allochthonous inputs from surrounding terrestrial plants will affect  $\delta^{13}\text{C}$  in streams and lakes. Similarly, the extent of local nitrogen fixation relative to inputs from precipitation and leaching from the surrounding watershed will determine the variation in  $\delta^{15}\text{N}$ . Indeed, in aquatic systems temporal changes in stable isotope values can happen exceedingly fast; all it takes is 1 large rainstorm (McGuire and McDonnell 2007).



**FIG. 3.**—Interactions between processes that influence stable isotope ratios of herbivores and carnivores, showing biochemical, physiological (underlined), and behavioral (in rectangles) processes. Solid lines represent ecological interactions; dotted lines represent factors affecting diffusion rates and enzymatic reactions (i.e., photosynthesis, nutrient routing, and nutrient recycling). A single isotopic value obtained from tissue of a carnivore is the emergent property of multiple ecological, behavioral, and physiological processes of various ecosystem components. Modified from Ben-David et al. (2001). (a) Effects of marine subsidies on wolf diets and ungulate population dynamics are described in Adams et al. (2010) and summarized in the text.

These naturally created spatial and temporal variations in the abundance of heavy and light isotopes in all ecosystems on Earth is in essence a marker system that allows us to track the flow of nutrients, species interactions, trophic relations, animal diets, and animal migrations (Dawson and Siegwolf 2007; Hobson 2007; Martínez del Rio et al. 2009; Post 2002; Schell et al. 1989, 1998). It is for that reason that we refer to most stable isotope studies as natural abundance studies, not to be confused with studies that use artificially enriched isotopic compounds as tracers (see Pauli et al. 2012). We call isotopically heterogeneous landscapes and seascapes “isoscapes.”

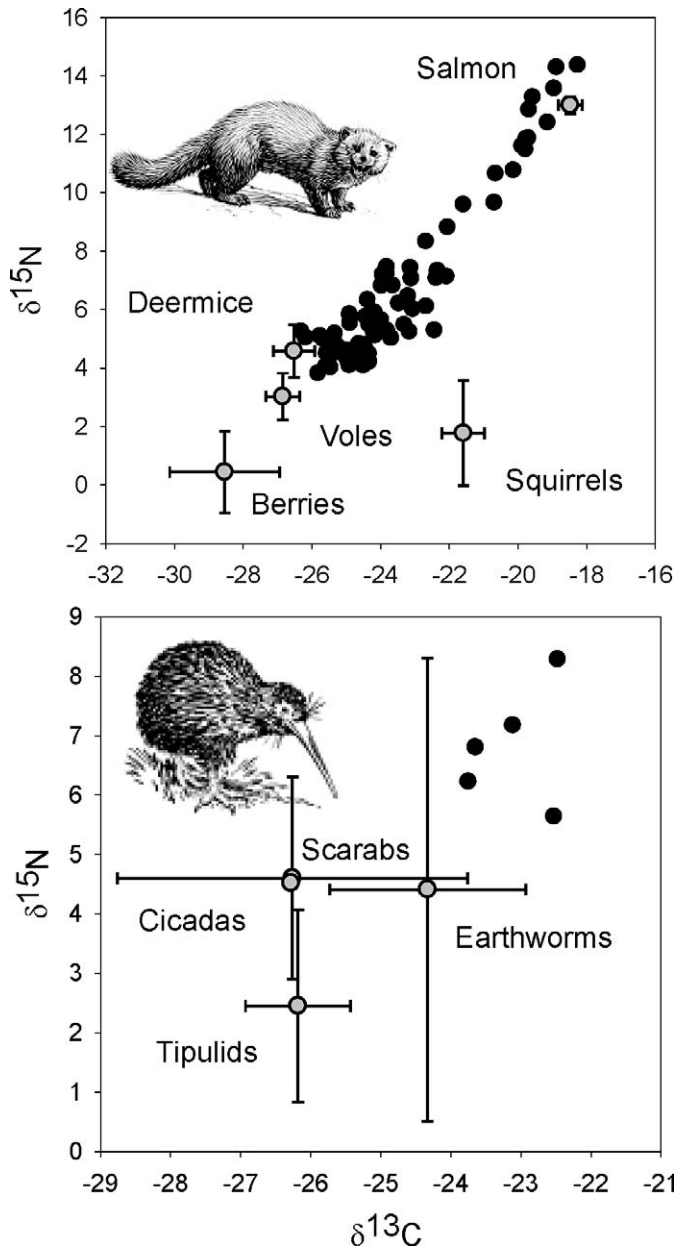
### HOW DO WE QUANTIFY NUTRIENT FLOWS, SPECIES INTERACTIONS, TROPHIC RELATIONS, AND ANIMAL DIETS WITH NATURAL ABUNDANCE STABLE ISOTOPE ANALYSES?

Briefly, nutrient flows in ecosystems result from consumption of some organisms by others, which then discard unused nutrients via respiration and excrements. The excrements, and in many cases the carcasses, of consumers are later decomposed. Or in other words, nutrient flows, species interactions, trophic relations, and animal diets are all different expressions

of consumption, assimilation, excretion, and decomposition. As we mentioned above, the isotopic signatures of organisms reflect the ratios of heavy to light isotopes of the substrates they use (i.e., what they consumed), plus some added discrimination factor because of the physiological processes they employ in assimilating these substrates and discarding their products.

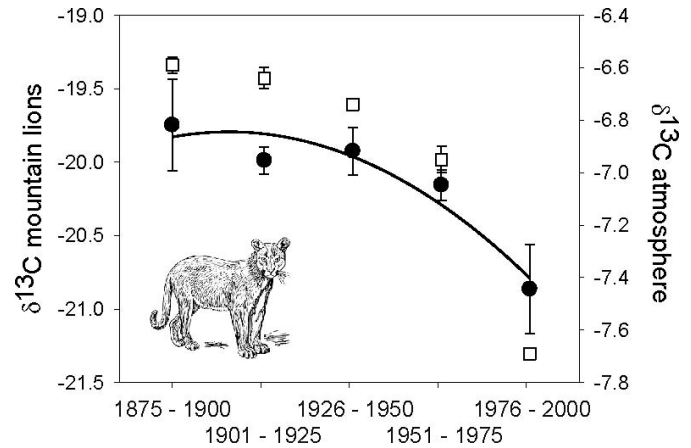
As a 1st step in assessing trophic relations and animal diets we describe the underlying isoscape by measuring the isotope ratios of all potential foods and verify that they are isotopically unique (Rosing et al. 1998; Fig. 4). For large sample sizes and normally distributed data, we recommend the use of multivariate analysis of variance with the isotopes in question as the dependent variables and dietary sources as the grouping variable (Flaherty et al. 2010; Stewart et al. 2003). For small sample sizes we recommend the K nearest-neighbor randomization test described by Rosing et al. (1998). This test appears to have high power even with small sample sizes and comparatively low displacement, and has been used in various mammalian studies.

In longitudinal studies, it is necessary to account for atmospheric depletion in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  through time (Long et al. 2005; Schell 2001). Anthropogenic inputs of carbon and nitrogen from burning of fossil fuels have resulted in measurable changes to atmospheric values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$



**FIG. 4.**—Values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of individual American martens (*Martes Americana*; black circles) and their foods ( $\bar{X} \pm 95\%$  confidence intervals; gray symbols—top panel), and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  individual kiwis (*Apteryx australis*; black symbols) and their potential foods (gray symbols—bottom panel). The distribution and variance of marten foods allows for determination of the diet of these mustelids with linear mixing models (Phillips 2012). In contrast, because of high variation and large overlap in isotopic signatures of food items, the diet of kiwis cannot be estimated with stable isotope analysis. Samples of kiwis and foods were collected by B. Taborsky (University of Bern, Switzerland) following methods described by Taborsky and Taborsky (1991) and analyzed by M. Ben-David following methods described in Ben-David et al. (1997). Data for martens are from Ben-David et al. (1997).

(Lee et al. 2005; Long et al. 2005; Schell 2001). For example, using museum specimens of mountain lions (*Puma concolor*), collected between 1893 and 1995 in California, Long et al. (2005) found a temporal decrease in  $\delta^{13}\text{C}$  values in bone



**FIG. 5.**—Values of  $\delta^{13}\text{C}$  ( $\bar{X} \pm \text{SE}$ ) from bone collagen of mountain lions (*Puma concolor*) harvested in California between 1893 and 1995 (black circles) and for atmospheric  $\text{CO}_2$  for the same 25-year periods (open squares). Superficially, the overall decline of 1.2‰ in mountain lion values could have been interpreted as a dietary change. The change, however, was more likely caused by a depletion of atmospheric  $\delta^{13}\text{C}$  values, which percolated through the food web. Data modified from Long et al. (2005).

collagen. This decrease was unexpected given the recent expansion of wild pigs (*Sus scrofa*) in California (Waithman et al. 1999). Indeed, if mountain lions were decreasing their consumption of deer (*Odocoileus hemionus*) in favor of predation on the expanding omnivorous wild pigs,  $\delta^{13}\text{C}$  values should have increased. However, once a correction of atmospheric depletion in  $\delta^{13}\text{C}$  was applied, no temporal change in isotopic values for mountain lions was detected (Long et al. 2005; Fig. 5). Similar correction of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  was necessary to properly assess dietary changes through time of individual bowhead whales (*Balaena mysticetus*—Lee et al. 2005). These examples illustrate the importance of accounting for temporal changes in isoscapes, whether they occur naturally or are human-induced.

Following the evaluation of the isoscape (in terms of uniqueness of components and temporal changes), one needs to account for trophic discrimination factors (i.e., the effects of assimilation and excretion) before launching into the investigation of nutrient flows, species interactions, trophic relations, and animal diets (Newsome et al. 2012; Phillips 2012). Since the early application of stable isotope analysis to archeological, paleontological, and ecological questions, a large and growing volume of literature has been dedicated to the quantification of discrimination factors (see Koch [2007], Martínez del Río et al. [2009], and references therein). More often than not we have used a 1‰ discrimination for  $\delta^{13}\text{C}$  and 3‰ for  $\delta^{15}\text{N}$  as originally proposed by DeNiro and Epstein (1978, 1981). We too have used these “magic” numbers in our recent study of foraging ecology of northern flying squirrels (*Glaucomys sabrinus*—Flaherty et al. 2010). Nonetheless, the recent flurry of controlled experiments and meta-analyses suggests that these discrimination factors may not be universal (Kelly and Martínez del Río 2010; Martínez del Río et al. 2009; Vanderklift and Ponsard 2003). We now know that



the range of  $\delta^{13}\text{C}$  discrimination in soft tissues for animals of the same species can vary from  $-1\%$  to  $5\%$  and for  $\delta^{15}\text{N}$  from  $-1\%$  to  $8\%$  (Barnes et al. 2007; Ben-David et al. 2012; Kelly and Martínez del Rio 2010). Similar variations in discrimination factors have been described for tissues such as bone collagen, apatite (as in tooth enamel), and dentin (Koch 2007).

### WHAT DETERMINES ISOTOPIC DISCRIMINATION FACTORS IN ANIMAL TISSUES?

It turns out that the incorporation of dietary isotopic signatures into consumer tissues is complicated. Signatures are dependent on the size of the animal; its age; nutritional status (Fig. 3); whether it is a herbivore, omnivore, or carnivore; the tissue sampled; the macronutrient composition of the diet (carbohydrates, amino acids, and fatty acids); and assimilation efficiency (Martínez del Rio and Carleton 2012; Martínez del Rio and Wolf 2005). All these factors affect not only the rate of isotopic incorporation but also the discrimination between diet and consumer tissues even for the same species (Ben-David et al. 2012; Robbins et al. 2010; Whiteman et al. 2012). For example, adult herbivores consuming a nitrogen-poor diet will incorporate the  $\delta^{15}\text{N}$  signature of the diet at a relatively slow rate because they will be recycling much of their body nitrogen stores (Martínez del Rio and Carleton 2012; Martínez del Rio and Wolf 2005). In contrast, young, growing animals (or those that have indeterminate growth patterns) feeding on a high-protein diet will exhibit fast incorporation of dietary isotopic values and will have a smaller  $\Delta_{\text{body-diet}}$  (Martínez del Rio and Carleton 2012; Martínez del Rio et al. 2009), at least in those tissues that have a fast growth rate (such as muscle—Carleton et al. 2008). For a thorough discussion of isotopic incorporation please see Martínez del Rio and Carleton (2012).

The complexity of isotopic incorporation as revealed from the controlled studies listed above is only part of our problem when attempting to reconstruct diets from stable isotope analysis. In nature, animals do not cleanly switch from 1 isotopically distinct diet to another over a period long enough to allow for full incorporation. In fact, even animals that exhibit high levels of dietary specialization consume multiple foods that vary temporally in macronutrient composition and isotopic ratios (Fig. 3). Or else they feed on the same organisms but in different habitats with different underlying isoscapes (Flaherty and Ben-David 2010). In fact, dietary changes exhibited by animals occur on a much faster schedule than their tissue turnover rates, at least for most of their tissues (Carleton et al. 2008). The newly developed mixing models, designed to convert the isotopic ratios of consumers and their foods to dietary contributions (Phillips 2012), attempt to account for stoichiometry (C:N ratios) and allow for variable discrimination factors, assimilation efficiencies, and variation in isotopic values of different foods (Parnell et al. 2010; Phillips 2012; Ward et al. 2010). Nonetheless, although they are a great improvement on previous tools (Phillips 2012),

these models still fall short of capturing the dynamic nature of isotopic incorporation. Thus, quantification of animal diets with stable isotope analysis may be problematic. For newly developed analytical tools that may reduce some of these problems, see Newsome et al. (2012).

### CAN WE QUANTIFY TROPHIC RELATIONS WITH STABLE ISOTOPE ANALYSIS?

The commonly used  $1\%$  for  $\delta^{13}\text{C}$  and  $3\%$  for  $\delta^{15}\text{N}$  discrimination factors (DeNiro and Epstein 1978, 1981) have been used extensively to assess trophic positions and food-web interactions (Layman et al. 2007; Post 2002), evaluate the functional role of organisms, estimate energy flows through ecological communities (Dunton et al. 1989; Post 2002), describe anthropogenic alterations to food webs (Pauly et al. 1998), as well as quantify the contribution of marine resources to terrestrial ecosystems (Helfield and Naiman 2001). Trophic position is usually estimated from  $\delta^{15}\text{N}$  using the equation:

$$\text{trophic position} = \lambda + \frac{\delta^{15}\text{N}_{\text{secondary consumer}} - \delta^{15}\text{N}_{\text{base}}}{\Delta_n}, \quad (3)$$

where  $\lambda$  is the trophic position of the organism used to estimate  $\delta^{15}\text{N}$  base (i.e.,  $\lambda = 1$  for primary producers—Martínez del Rio and Wolf 2005), and  $\Delta$  is the trophic discrimination—usually  $3.4\%$  (Martínez del Rio and Wolf 2005). Post (2002), in an exhaustive study of lakes, demonstrated that on average trophic discrimination is indistinguishable from  $3.4\%$ , but the variation surrounding this value is large. Because there is no possibility to address this variation with equation 3, estimates of trophic position, functional role of organisms, and anthropogenic alterations to food webs may be biased.

Things are further complicated when sources of nitrogen (and carbon) are numerous, although Post (2002) describes how equation 3 can be expanded to account for 2 nitrogen sources (1 with a fraction  $\alpha$  and the other with a fraction  $1 - \alpha$ ). In that model the  $\delta^{15}\text{N}_{\text{base}}$  is decomposed to  $\delta^{15}\text{N}_{\text{base-1}} \times \alpha$  and  $\delta^{15}\text{N}_{\text{base-2}} \times (1 - \alpha)$ . Regardless, equation 3 becomes invalid when isotopic values are spatially and temporally heterogeneous, and where trophic discrimination deviates from  $3.4\%$ . As we described above, these 2 caveats are prevalent in animal studies. Therefore, we recommend interpreting results from such exercises with caution.

### WHAT ARE THE BENEFITS OF USING STABLE ISOTOPE ANALYSIS IN DIETARY AND TROPHIC STUDIES?

First, stable isotope analysis is the only option we have to study the foraging ecology of extinct animals and, in many cases, diets of marine mammals (Koch 2007; Newsome et al. 2010). Indeed, how else could Feranec and MacFadden (2006) evaluate resource partitioning among ungulates in  $\text{C}_3$ -dominated communities from the Miocene? Or Matheus (1995) determine that the short-face bear (*Arctodus simus*) was highly carnivorous, likely scavenging carcasses of Pleistocene herbivores killed by other predators? Or Drago

et al. (2010) find that foraging location of female South American sea lions (*Otaria flavescens*) influenced the growth rate of their pups? See Clementz (2012) for other intriguing and insightful examples on marine mammal and paleontological studies including the use of calcium isotopes.

Second, stable isotope analysis has been successfully applied to systems where the problems associated with incorporation rate and variable discriminations could not significantly alter the conclusions. For example, Ben-David et al. (1997) used repeated sampling of multiple individual martens (*Martes americana*) to assess the effects of small mammal availability on the consumption of salmon (*Oncorhynchus*). In that system, the intragroup variation in isotopic values of possible prey was much lower than intergroup variation, reducing the effect of that variance component. The sampling schedule corresponded to the availability of salmon in the system (approximately every 4 months), and the turnover rate of the tissue sampled also was in agreement (red blood cells in a mammal the size of marten have a life span of 70–80 days [Ben-David et al. 2012]). The repeated sampling of multiple individuals that changed their foraging strategies concurrent with changes in small mammal availability provided a baseline for interpreting the overall data; that is, when small mammals were abundant all martens had similar and nearly uniform  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values that could not be generated if these animals were consuming salmon. Finally, both main dietary items (small mammals and salmon) had similar C:N ratios, reducing the potential effects of macronutrient composition (Ben-David et al. 1997).

In our opinion, the principal strength of stable isotope analysis is the ability to investigate the responses of individuals to environmental conditions (such as habitat and food availability, competition, predation, and predation risk), and ultimately to explore how the responses of individuals influence fitness components (i.e., reproductive success and survival), emerging population dynamics, and community and ecosystem processes (Flaherty and Ben-David 2010). Exploring such complex ecological interactions spanning multiple levels of organization is only possible with stable isotope analyses. For example, Darimont et al. (2007), using stable isotope analysis on hair collected from wolf (*Canis lupus*) scats, observed that in coastal British Columbia, individual black-tailed deer (*Odocoileus hemionus columbianus*) with isotopic signatures indicative of foraging in high-quality stands of cedar (*Thuja plicata*) and hemlock (*Tsuga heterophylla*) were more likely to be killed by wolves than conspecifics foraging in lower-quality habitats. In this clever application of stable isotope analysis, Darimont et al. (2007) verified that isotopic values of hairs of live deer can be unambiguously assigned to the various forest stands. Here too, the temporal scope of the study was appropriate (deer using these different habitats molt at the same time), the effects of macronutrient composition were low (keratin is an inert tissue and varies little in composition among individuals), and the underlying isoscape (isotopic signatures and variance of all potential foods) was well characterized.

Other examples highlighting the ability to track individuals include the study by Ben-David et al. (2004). Using stable isotope analysis on blood and hair, the authors described the trade-off between meeting the nutritional requirements of lactation and avoiding the risk of infanticide in female brown bears (*Ursus arctos*). The authors demonstrated that many females with cubs-of-the-year avoided salmon streams, likely to reduce interactions with other bears, some of which could potentially be infanticidal (Ben-David et al. 2004). The implications of individual variation in diet also were described by Yeakel et al. (2009), who quantified the predation on humans by the infamous man-eating lions (*Panthera leo*) of Tsavo, Kenya. Isotopic values of hair collected from the 2 lions differed in their overlap of available prey, indicating that only 1 of the males in that coalition fed largely on the unfortunate railroad workers in 1898 (Yeakel et al. 2009). In another study, Wolf et al. (2002) combined  $\delta^{13}\text{C}$  and  $\delta\text{D}$  values to demonstrate that in the Sonoran Desert the seasonally abundant saguaro fruit (*Carnegiea gigantea*) contributed about 90% of the carbon budget of the white-winged dove (*Zenaida asiatica*) but only 50% for the closely related mourning dove (*Z. macroura*). The former species also derived most of its body water from saguaro, whereas the latter did not, indicating that a common resource can satisfy different needs of similar consumers. Again, without stable isotopes such investigations would not have been possible.

Similarly, tying the responses of individuals to emerging population dynamics and community and ecosystem processes can only be achieved with stable isotope analyses. For example, Adams et al. (2010) have recently shown that the number of wolves in the northwestern region of Denali National Park was elevated because of the availability of salmon (i.e., salmon subsidies), which led to 3 times higher predation rates on moose (*Alces alces*) and caribou (*Rangifer tarandus*). The authors concluded that consumption of salmon by wolves likely contributed to the 78% lower ungulate densities observed in that region of the park compared with adjacent areas (Adams et al. 2010). In the Neotropical forests of Trinidad, Sagers et al. (2000) assessed the costs and benefits of mutualism between *Cecropia* trees and *Azteca* ants. They found that although only 18% of carbon in worker ants was derived from *Cecropia*, approximately 93% of the nitrogen in host trees was derived from ant excrements and debris. Similarly, Fox-Dobbs et al. (2010) recently showed that nitrogen derived from fixation in *Acacia drepanolobium* trees is higher away from termite mounds compared with trees growing near mounds. Also, the trees growing near termite mounds preferentially used soil-derived nitrogen sources rather than investing in nitrogen fixation (Fox-Dobbs et al. 2010). Finally, Crait and Ben-David (2007) showed that in Yellowstone Lake, a significant proportion of nitrogen assimilated by riparian vegetation was provided through the predation on cutthroat trout (*Oncorhynchus clarki bouvieri*) by river otters (*Lontra canadensis*) and subsequent transport of nutrients to their latrines. The authors postulated that after the invasion of lake trout (*Salvelinus namaycush*), decline of

cutthroat trout and their mammalian predators will disrupt the transport of nutrients from the lake to its surrounding watershed.

In all these studies, conclusions were drawn only after careful consideration of the limitations of the approach. Indeed, a review of the literature will reveal that well-designed isotopic studies can yield interesting and valid conclusions. This rule applies not only to dietary and trophic studies but should also be followed by those employing stable isotope analyses to study animal migrations.

### HOW DO WE MEASURE ANIMAL MIGRATIONS WITH "NATURAL ABUNDANCE" STABLE ISOTOPE ANALYSES?

One of the 1st studies to use an isotopic seascape to track animal movements was conducted by Schell et al. (1988) on bowhead whales. Using radiocarbon ( $^{14}\text{C}$ ) data to determine age of sections of baleen, together with  $\delta^{13}\text{C}$  values of the same sections, and comparing baleen isotopic ratios to those of zooplankton in the Bering, Chukchi, and Beaufort seas, Schell et al. (1988) were able to reconstruct the annual migrations of these elusive animals. Using the same concept, Chamberlain et al. (1997), Hobson and Wassenaar (1997), and Hobson et al. (1999) pioneered the investigation of migration of terrestrial animals based on  $\delta\text{D}$  values in keratin. Similar to baleen, fur and feathers are largely inert tissues that record the isotopic values of assimilated nutrients at the time of growth (e.g., during molt). Because  $\delta\text{D}$  and  $\delta^{18}\text{O}$  values vary with latitude and altitude (recall the temperature effects on precipitation), assigning individuals to specific areas where molt occurred (i.e., geographic area of origin) can be achieved (Cryan et al. 2012; Hobson 1999, 2007). Similarly, because basal rock composition and weathering create variation in  $^{87}\text{Sr}:^{86}\text{Sr}$  ratios on the landscape, their incorporation into animal tissues can be used to track movements. For example, using  $^{87}\text{Sr}:^{86}\text{Sr}$  in fossil tooth enamel, Hoppe and Koch (2007) described long-range movements of mastodons (*Mammut*) in Florida during the Pleistocene, but observed relatively short distances traveled by mammoths (*Mammuthus*).

Similar to dietary and trophic studies, the application of stable isotope analysis to investigate migration will require careful planning and clear understanding of the physiological and ecological processes that influence isotopic incorporation into fur, feather, teeth, or baleen. The incorporation of hydrogen (and thus D) and oxygen (and thus  $^{18}\text{O}$ ) into animal tissues is significantly more complex than that of carbon and nitrogen. In essence, carbon and nitrogen are largely assimilated by animals from 1 source—their diet (or in some cases from the excreta of the microbial gut flora [see Greller 2010; Whiteman et al. 2012]). In contrast, hydrogen and oxygen can be derived from drinking water, the water contents of the diet, the skeletons of the macronutrients of the diet, as well as from molecular exchange (both during life as well as after death or shedding) with atmospheric gases (McKechnie et al. 2004; Wolf 2011). Each of these sources in turn can be variable. Although we are careful to quantify only nonexchangeable hydrogen in feathers

and fur (and thus can assume atmospheric contributions are negligible [Wassenaar and Hobson 2000]), the daunting number of source contributions makes inferences from  $\delta\text{D}$  and  $\delta^{18}\text{O}$  difficult. Indeed, in a series of elegant, controlled studies, Wolf (2011) demonstrated that large individual variation in incorporation of hydrogen and oxygen may mask any environmentally generated patterns in isotopic signatures.

The observed individual variation in controlled experiments is further complicated by processes of habitat use and diet selection of individual animals within a given geographic area (Fig. 3; Wunder 2012), processes that increase variation at the population level. Imagine the mourning doves from the Sonoran Desert (Wolf et al. 2002). The  $\delta\text{D}$  signatures in their feathers were derived from a combination of hydrogen atoms found in the seeds of saguaro, other seeds they consumed, the water in the saguaro fruit, and water they found in pools, creeks, or irrigation canals (S. A. Carleton, New Mexico State University, pers. comm.). In each bird this combination can vary based on the amount of water or saguaro they consumed. Thus, if members of the same population use different water sources and vary in diet composition, the intrapopulation variation may exceed that derived from latitude. Under such conditions misassignment of individuals to a specific geographic area where feathers or fur have been grown will be more likely than not. Indeed, Rocque et al. (2006, 2009), using  $\delta\text{D}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$ , were able to correctly assign 80% of summer- and winter-grown feathers of American golden-plovers (*Pluvialis dominica*) and Pacific golden-plovers (*P. fulva*) nesting in Alaska, but only 41% of feathers to origin of growth on a continental scale (North America—summer, and South America and Southeast Asia—winter).

Unfortunately, studying migration with stable isotope analysis may be hampered by another key problem—the high variation in isotopic values of available water at any given sampling location coupled with paucity of data for vast geographic areas. In creating isotopic maps, researchers such as Bowen et al. (2005) interpolate data collected from few stations to the larger landscape based on latitude, elevation, temperature, and amount of precipitation. These precipitation maps have limited accuracy in North America, and for continents such as Europe, Africa, South America, and Australia they are virtually uninformative. These fundamental problems may only be solved by increasing the number of sampling stations globally. Because of the inherent problems with incorporation of hydrogen and oxygen into animal tissues and the low accuracy of isotopic maps, assignment of individuals to specific areas required the development of new analytical tools. Wunder and Norris (2008) were 1st to develop Bayesian probability density surfaces, which account for some of the uncertainty and yield probabilistic assignments of individuals to geographic areas (Wunder 2010, 2012). For a full discussion of this methodology and its applications see Wunder (2012).

Despite the problems we discussed above, several authors have designed and executed some high-quality studies. The spectacular migration of monarch butterflies (*Danaus plexippus*) was described by Wassenaar and Hobson (1998).

Interpretation of the isotopic data was possible because of the extensive laboratory and field-rearing experiments by Hobson et al. (1999) that identified the underlying isoscapes and correct discrimination factors across the natal range of these long-range invertebrate migrants. Lott et al. (2003) accounted for the high variation in  $\delta D$  values by adding analysis of sulfur isotopes to hydrogen analyses. In that study, the authors used values of  $\delta^{34}S$  to account for coastal compared with inland foraging of various raptors (Lott et al. 2003).

Similarly, Dugger et al. (2004), using  $\delta D$  values in feathers of birds captured over 15 years in Gunica Forest in Puerto Rico, documented a link between rainfall on the breeding grounds in the eastern United States and apparent survival of ovenbirds (*Seiurus aurocapilla*). In that study, the authors assigned individuals to a continent-scale geographic area, avoiding issues with small-scale variation. With a combination of  $\delta D$  and  $\delta^{13}C$  measurements in muscle tissues and eggs of redhead ducks (*Aythya americana*), Hobson et al. (2004) observed that these individuals relied mainly on dietary lipids and proteins for egg production. The authors postulated that unlike capital breeders (i.e., those that use nutrients stored on the wintering grounds for egg production), redheads used endogenous reserves to satisfy the energy requirements of the hen (Hobson et al. 2004). More recently, with a clever use of the altitudinal variation in  $\delta D$ , Boyle et al. (2011) described the trade-off between reproductive success and survival for male white-ruffed manakins (*Corapipo altera*) in Costa Rica. In that system, males that remained at high-elevation lekking areas during the nonbreeding season were able to maintain or increase their social status and thus increase mating opportunities. This benefit was outweighed by lower survival during severe rainstorms likely causing males in lower body condition to migrate to lower elevations (Boyle et al. 2011).

A common theme in all these studies, which we advocated above, was the investigation of individual responses to environmental conditions and the influence of these responses on fitness components. In addition, the underlying isoscape was sufficiently variable or well-documented to override the problems of individual variation, and the turnover rate of the tissues corresponded with the sampling schedule. As is clear from these studies, here too, the successful use of stable isotope analysis is dependent on careful design and implementation. In cases where the underlying variation in natural abundance is uninformative or where individual variation may mask landscape-level patterns, animal migration and dispersal could potentially be traced with artificially enriched isotopic labeling.

### WHAT ARE ARTIFICIALLY ENRICHED STABLE ISOTOPES?

Technological advances in chemistry facilitated the production of organic compounds that are partially composed of heavy isotopes of particular elements. For example, a quick visit to the Web site of 1 of the large suppliers of stable isotopes will reveal long lists of compounds from ammonium to amino acids that have 1 or all of their carbons in the form of

$^{13}C$ , their nitrogen in the form  $^{15}N$ , or all hydrogen atoms in the form of D. For example, to study fertilizer uptake in crops one can purchase potassium nitrate that has 60% of all nitrogen atoms in the form of  $^{15}N$  (known as 60 atom percent) or the same compound with 98% of all atoms as  $^{15}N$  (or 98 atom percent).

Traditionally, enriched isotopes have been used in agricultural and biomedical research mostly to investigate the effects of fertilizers on crop yields (e.g., Chalk et al. 2010; Harmsen and Moraghan 1988) or to explore the dynamics of metabolic diseases (e.g., Schwarz et al. 2003). In animal studies, enriched stable isotopes have been used for decades to assess body condition ( $\delta D$ -labeled water—Nagy 1988) and measure field metabolic rate (doubly labeled water with  $\delta D$  and  $\delta^{18}O$ —Speakman 1997). More recently, enriched stable isotopes were applied to assess oxidation rate of different dietary macronutrients in several species including house sparrows (*Passer domesticus*—McCue et al. 2010), to trace the sources of nutrients used to metabolize prey in pythons (*Python regius*—Starck et al. 2004), or to explore the function of urea transporters in hibernating hind-gut fermenters (Wyoming ground squirrel [*Urocyon elegans*]—Greller 2010). In addition, enriched isotopic tracers have been used to track the flux of nutrients in aquatic ecosystems (Hall and Tank 2003) and to quantify dispersal of aquatic invertebrates (Macneale et al. 2004; Wanner et al. 2006) and seeds of plants (Carlo et al. 2009). For an application of isotopic labeling in the study of mammalian dispersal see Pauli et al. (2009) and Pauli et al. (2012).

### HOW DO WE ESTIMATE BODY COMPOSITION (CONDITION) AND FIELD METABOLIC RATES WITH ENRICHED STABLE ISOTOPES?

Water dilution methods estimate water flux, body composition, energy metabolism, and field metabolic rates using water artificially enriched with D and  $^{18}O$ . Estimation of body composition ( $\delta D$ ) and field metabolic rates ( $\delta D$  and  $\delta^{18}O$  or doubly labeled water) both rely on the concept that after an injection into an animal the artificially enriched water will be diluted in the body pool and then slowly cleared at a constant rate (Nagy 1988; Speakman 1997). The rate of decline of  $\delta D$  in the body water provides a measure of the size of the body water pool and water flux (Nagy 1988). Because fat tissues are hydrophobic, calculating percentage fat is possible based on estimates of the body water pool and body mass (Hilderbrand et al. 1998). The rate of decline of both  $\delta D$  and  $\delta^{18}O$  provides a measure of  $CO_2$  production because the decline of  $\delta^{18}O$  is influenced by the rate of water clearance and also by the rate of exchange with  $CO_2$  (see equation 2; Nagy 1988; Speakman 1997; Fig. 6).  $CO_2$  production is then converted to oxygen consumption, an estimate of metabolic rate (Speakman 1997), although this conversion will depend on the substrate oxidized (Whiteman et al. [2012] discuss respiratory exchange ratio).

To estimate body composition and field metabolic rates, an initial blood sample is taken to measure background isotope levels. A mixture of D and  $^{18}O$  enriched water is then injected

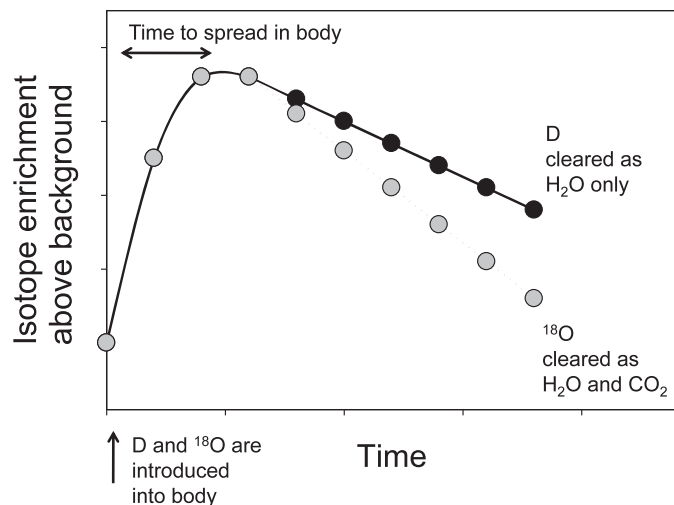


FIG. 6.—Hypothetical illustration of changes in isotopic enrichment of D (or  $^2\text{H}$ ) and  $^{18}\text{O}$  in the body water of an animal injected with doubly labeled water. Rate of decline of  $\delta\text{D}$  in the body water provides a measure of the size of the body water pool and water flux. Rate of decline of both  $\delta\text{D}$  and  $\delta^{18}\text{O}$  provides a measure of  $\text{CO}_2$  production because the decline of  $\delta^{18}\text{O}$  is influenced by the rate of water clearance and also by the rate of exchange with  $\text{CO}_2$  (Nagy 1988; Speakman 1997).

intravenously, subcutaneously, or into the peritoneal cavity (Speakman 1997). After equilibration (Hilderbrand et al. 1998; Speakman 1997), another blood sample is collected and  $\delta\text{D}$  values are determined via mass spectrometry. Repeated blood sampling over a period of several days or weeks and estimation of both  $\delta\text{D}$  and  $\delta^{18}\text{O}$  yields the values of  $\text{CO}_2$  production (Speakman 1997). Water dilution methods have been validated over the years in a variety of vertebrates, including mammals (Nagy 1988). Some recent applications include comparisons of body condition and field metabolic rates of 2 species of phytophagous lemurs (ring-tailed lemurs [*Lemur catta*] and brown lemurs [*Eulemur*]) in Madagascar by Simmen et al. (2010). These authors demonstrated that the low energy output in these primates is largely a function of low basal metabolic rate (Simmen et al. 2010). Using doubly labeled water, Zub et al. (2011) explored the effects of body size on energy balance in least weasels (*Mustela nivalis*). The authors showed that energetic constraints lead to intraspecific spatial segregation among males, with larger individuals inhabiting areas occupied by larger prey (Zub et al. 2011).

### CAN WE COMBINE NATURAL ABUNDANCE AND ENRICHED STABLE ISOTOPE STUDIES?

Until recently, few studies have combined natural abundance with tracer studies. This separation within the field is rather surprising given the potential utility of combining these methods. For example, Hilderbrand et al. (1999) demonstrated that brown bears that consumed meat on the Kenai Peninsula, Alaska, had accumulated more fat deposits than individuals that largely consumed vegetation. Meat consumption was quantified using  $\delta^{15}\text{N}$ , whereas fat deposits were quantified with deuterium-labeled water dilution methods. Similarly,

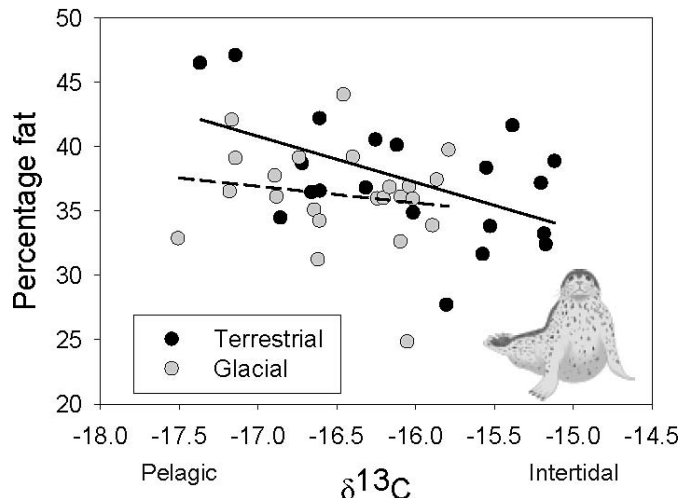
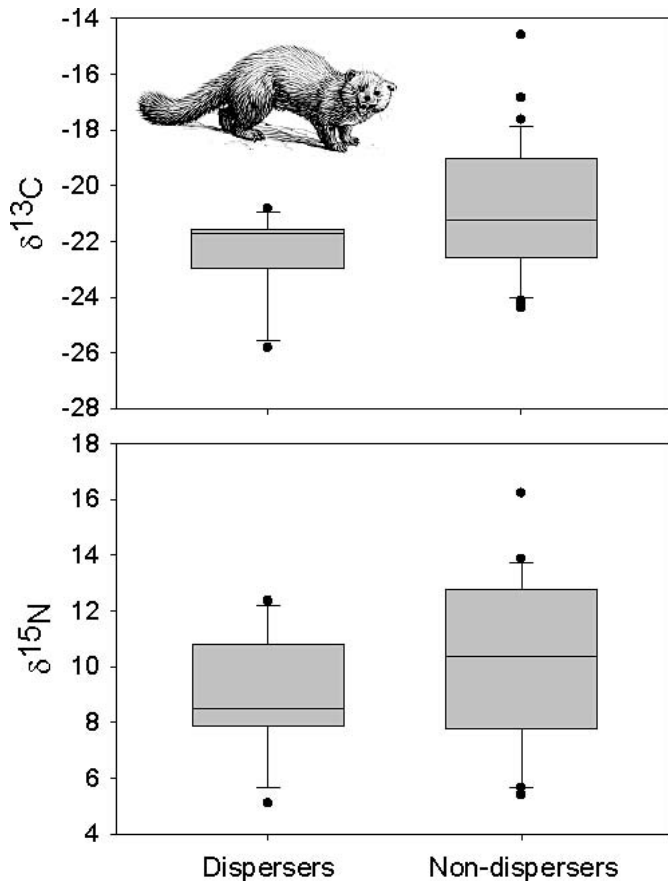


FIG. 7.—An example of a combination of natural abundance and artificially enriched stable isotope analyses in dietary studies. In this study, diet of harbor seals (*Phoca vitulina*) was assessed with natural abundance  $\delta^{13}\text{C}$  values of serum and related to resulting body condition (presented as percentage fat) as assessed by  $\delta\text{D}$  water dilution methods (Blundell et al. 2011). Results suggest that for seals that used terrestrial haul outs (black symbols), individuals that fed on intertidal fishes achieve lower levels of body condition than those that foraged on pelagic fishes ( $R^2 = 0.28$ ,  $P = 0.017$ ). No such relation occurred in seals that hauled out on glacial ice floes (gray symbols). Adapted from Blundell et al. (2011).

Blundell et al. (2011) used natural abundance  $\delta^{13}\text{C}$  values to determine the diets of harbor seals (*Phoca vitulina*) captured at glacial and terrestrial haul-out sites in Glacier Bay National Park, Alaska, and concurrently used deuterium-labeled water to assess the body condition of these individuals (Fig. 7). Thus, these authors were able to investigate the effects of diet selection on body condition of free-ranging mammals by combining natural abundance and artificially enriched stable isotopes (Blundell et al. 2011; Hilderbrand et al. 1999).

Another application would be the investigation of effects of diet selection on the probability that an individual will engage in dispersal. For example, using  $\delta\text{D}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  labeling by Pauli et al. (2012), we identified martens (*Martes caurina*) on Admiralty Island, Alaska, that dispersed from their original trapping location. Then, using natural abundance signatures of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , we determined that nondispersers were more likely to switch from feeding on small mammals to consuming salmon (Fig. 8). Or in other words, martens that were less likely to switch diets were more likely to disperse (Fig. 8).

As these examples demonstrate, by combining natural abundance and artificially enriched isotopic analyses it will be possible to explore the effects of field metabolic rate on diet selection, the effects of diet selection on body condition, the effects of body condition on the assimilation efficiency of exogenous nutrients, or the relation between dietary specialization and dispersal. In addition to the advantages offered by combining these 2 methods, recent innovations in mass spectrometry will likely change the face of the field of isotopic ecology beyond recognition.



**FIG. 8.**—An example of a combination of natural abundance and artificially enriched stable isotope analyses in migration and dispersal studies. Pacific martens (*Martes caurina*) on Admiralty Island, Alaska, were marked with  $\delta$ D-,  $\delta^{13}\text{C}$ -, and  $\delta^{15}\text{N}$ -labeled bait and designated as either dispersers or nondispersers based on the location of sample collection relative to the forest stand where bait was offered (Pauli et al. 2012). Dispersers had lower  $\delta^{13}\text{C}$  values ( $P = 0.03$ ) and marginally lower  $\delta^{15}\text{N}$  ( $P = 0.09$ ) than nondispersers. For details on methods and assessment of species differences in dispersal power as estimated from isotopic labeling, see Pauli et al. (2012).

### HOW WOULD INNOVATION CHANGE THE FUTURE OF ISOTOPIC ANALYSES?

The 1st innovation, providing accurate data of stable isotope ratios in minutes rather than hours, involves use of an inductively coupled plasma–mass spectrometer (often referred by its acronym, ICP-MS—Becker 2002) in which ionization of the molecules is done via excitation of argon gas rather than heating. The acceleration and collection of the ionized molecules also differs from use of a TIMS although the general principles are similar (details are given in the Web site Elemental Analysis Inc. [www.elementalanalysis.com/services/inductively-coupled-plasma-icp/; accessed 20 February 2011] and others). In this method, the main source of error is introduced from interfering elements. For example, for Sr, krypton (Kr) or rubidium (Rb) can have interfering effects. This error, however, can be estimated and corrected for (Barnett-Johnson et al. 2005). In addition to high precision, use of an ICP-MS coupled with another innovation—laser

ablation—can significantly reduce sample processing time and allow sampling of minute quantities of organic materials (Barnett-Johnson et al. 2005). In laser ablation organic compounds are vaporized via irradiation with laser beams and directly introduced into the ICP-MS or via gas chromatography into a TIMS. Laser ablation coupled with use of either an ICP-MS or a TIMS has been successfully used to measure isotopic ratios of Sr in fish otoliths,  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  of fossil tooth enamel (Cerling and Sharp 1996), and several different elements (such as calcium) and  $\delta^{13}\text{C}$  in tree rings (Garbe-Schonberg et al. 1997; Hoffmann et al. 1994; Schulze et al. 2004). It is easy to imagine the application of such technology to investigate seasonal and annual changes in animal diets and movements from tissues such as teeth, hooves, horns, and baleen. We expect that as the cost associated with these methods is reduced they will become the predominant ones used by researchers in the field.

Another innovation that will reduce much of the difficulties in estimating animal diets with stable isotope ratios is the analysis of signatures of individual macronutrients, or what is known as compound-specific isotopic analysis. In compound-specific isotopic analyses, fatty acids, amino acids, and, to a lesser extent, carbohydrates in organic materials are separated with liquid or gas chromatography, combusted, and the resulting gasses are introduced into the mass spectrometer (Evershed et al. 2007). By obtaining the  $\delta\text{D}$ ,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , or  $\delta^{34}\text{S}$  values of essential and nonessential compounds we can gain better understanding of dietary contributions, because the same compounds (e.g., glycine or linoleic acid) from various sources can have different isotopic values (Evershed et al. 2007; Fogel and Tuross 2003).

For example, using  $\delta^{13}\text{C}$  values in essential fatty acids from milk residues collected from archeological pottery fragments, Evershed et al. (2008) were able to determine that the earliest dates of milk use were linked to herding of cattle (*Bos primigenius*) in the Near East and not to that of goats (*Capra hircus*), sheep (*Ovis aries*), or pigs. Isotopic analysis of amino acids in blood of penguin chicks from 4 species provided clear distinction of trophic position (Lorrain et al. 2009). For northern and southern rockhopper penguins (*Eudyptes chrysocome*), the authors were able to demonstrate differences in foraging locations based on differences in  $\delta^{15}\text{N}$  values of phenylalanine (phe) and their respective trophic levels based on the difference between  $\delta^{15}\text{N}$  of glutamic acid (glu) and phenylalanine (or  $\Delta_{\text{glu-phe}}$ —Lorrain et al. 2009). Using  $\delta^{13}\text{C}$ , Newsome et al. (2011) demonstrated that the signatures of indispensable (or essential) amino acids in Nile tilapia (*Oreochromis niloticus*) fed low-protein diets resembled that of the carbohydrates they consumed, a pattern that was consistent with assimilation of indispensable amino acids produced by microbial gut flora. Finally, Larsen et al. (2009) have shown that bacteria, fungi, and plants produce essential amino acid with distinct isotopic signatures that can be tracked in insects that consume them. Because lipid contents and amino acid composition of the diet can affect incorporation rates, discrimination factors, and routing of macronutrients (Ben-David et al. 2012), compound-specific stable isotope

analysis may reduce many of the ambiguities associated with reconstruction of animal diets.

To fully understand the state of the isotopic ecology field you, the reader, will need to continue studying the following papers in this Special Feature. As is clear, we only glossed over the topics covered in those works. Also, we hope that by reading this beginner's guide you will be able to evaluate other contributions and carefully design your own isotopic studies. We would like to emphasize that despite the problems and cautionary notes we alluded to throughout this beginner's guide, we believe that the future of isotopic analyses is bright, because in many cases the pitfalls we encountered along this research path are paving the road to the development of robust and reliable tools for the investigation of mammalian ecology with stable isotopes.

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