CHAPTER 2

An Introduction to Light Stable Isotopes for Use in Terrestrial Animal Migration Studies

Leonard I. Wassenaar

Environment Canada

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I. INTRODUCTION

Stable isotopes of the light elements (C, H, N, O, and S) are increasingly employed in terrestrial animal migration studies. The primary reason is that isotopes are powerful forensic recorders of dietary sources that can be spatially interpolated or explicitly linked to on-the-ground and large-scale patterns in the landscape and hydrosphere at a variety of scales (Hobson and Wassenaar 1997,

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Bowen *et al.* 2005b). Depending on whether the tissue of the migrating animal is biochemically fixed (feather, hair) or dynamic (blood, muscle), these isotopic dietary tracers record fundamental information about where an organism is and what it has been eating (Chapter 3). This salient feature opens a forensic door that allows us to gather new insights into one of the most confounding aspects of animal migration—their mobility.

There are stunningly few scientific tools available at our disposal to facilitate and quantify the tracking and movement of small animals over variable or large uninhabited spatial distances. In addition, the spatial scale of migration is often continental to global in scope. The focus of this volume is on one of the more newly available tools in the researchers toolbox—light stable isotope methods. Stable isotopes are not to be misconstrued as a "silver bullet" to be indiscriminately applied to answer all animal migration questions. Scientists, in practice, seek to carefully employ as many tools as possible to answer a specific research question, and one of these tools may include stable isotope methods. Further, it should be emphasized that stable isotopic investigations into the various kinds of terrestrial (and aquatic) faunal migration phenomena are relatively recent (Hobson 1999), and the accumulating literature is clear that isotopic approaches are evolving, methodologies are improving, and interpretational challenges are being actively identified and addressed. While stable isotope approaches have already illustrated remarkable successes in generating new knowledge and insight into animal migration, the limits of stable isotope tracing of migrants (from basic measurement to spatial interpretations) remain to be fully probed and tested.

Briefly, the scientific tools currently available for tracing the migratory movement of terrestrial biota fall into two general categories—*extrinsic* and *intrinsic markers* (see Chapter 1 for full listing). Stable isotopes fall into the category of intrinsic markers of dietary and spatial origin. Parlayed into everyday terms, stable isotopes enable us to determine where our migrant had its lunch by our direct or inferred knowledge of geospatial differences in the lunch menu.

Stable isotopes as intrinsic markers provide several key advantages over existing extrinsic markers. Principally, intrinsic markers do not require a subsequent recapture of the same organism to obtain a successful result because the required spatial data is recorded within each organism's tissue. As noted in Chapter 1, the success of physical tags is generally poor, and extrinsic markers are usually not feasible for small bodied migrants such as insects or small birds. Similarly, radio or satellite telemetry is technologically currently restricted to larger migrant organisms and requires subsequent electronic contact—a form of recapture. External tags and markers may also unwittingly alter or hinder normal movement behavior until miniaturization is improved.

Another comparative advantage of intrinsic markers over extrinsic markers is that they are not inherently spatially biased. A drawback to the more popular extrinsic markers (*e.g.*, tags, rings) is that the results are proportionally biased to effort at the location of marking, be it at monitoring sites, staging areas, areas within existing observational networks, or field accessibility. While this bias may not be problematic for local population interaction studies, it remains a major hurdle to our understanding of large distance animal migration. For some species of interest, extrinsic markers exclude important data from large unmonitored portions of the migratory catchment areas used by migrants (*e.g.*, boreal, far north). Intrinsic markers, therefore, hold both the potential to extend our knowledge into spatial areas where extrinsic markers are virtually impossible to deploy.

In order for light stable isotopes to function as intrinsic markers of migrant mobility, some key prerequisites must be met. The foremost, and rather obvious, requirement is that the migrant organism contains one or more of the light isotopes of interest in specific tissues. Fortunately, the light isotopes form the key atomic building blocks of the biosphere and most animal tissues; therefore, this requirement is handily met (Table 2.1). The second prerequisite is that the organism migrates between isotopically distinct landscapes or biomes (*e.g.*, Chapter 4) and retains in one or more of its tissues, permanently (*e.g.*, feather) or integrated over some period of time (*e.g.*, muscle), measurable isotopic differences that can be linked to diet at previous or current locations. This requirement is most easily met for species with discrete spatial distribution ranges that migrate seasonally across distinct isotopic landscapes.

TABLE 2.1 The approximate elemental abundances as dry weight %, stable isotope ratios of interest, and generalized isotopic ranges for bulk tissues (*e.g.*, α- or β-keratins like hair or feathers) commonly used in migratory research

Element	Weight	Isotope ratios	δ Range (‰)	Mass required (mg)
Light Isotopes				
Carbon[a]	30–40 wt.%	$^{13}C/^{12}C$	−5 to −65 (PDB)	0.2–1.5
Oxygen[b]	27–40 wt.%	$^{18}O/^{16}O$	+10 to +30 (VSMOW)	0.2–0.5
Nitrogen[a]	12–19 wt.%	$^{15}N/^{14}N$	−2 to +25 (Air)	0.5–1.5
Hydrogen[b]	6–8 wt.%	$^{2}H/^{1}H$	−250 to +90 (VSMOW)	0.1–0.4
Sulfur	5–20 wt.%	$^{34}S/^{32}S$	−20 to +30 (CDT)	1–2
Heavy Isotopes				
Strontium	<100-? ppb	$^{87}Sr/^{86}Sr$	Absolute ratios	2–30[c]

[a] Carbon and Nitrogen isotopes are generally obtained simultaneously by CF-IRMS, but need sufficient mass to obtain enough N.
[b] Hydrogen and Oxygen may be obtained simultaneously by pyrolytic methods.
[c] Dependent on the Strontium concentrations previously determined.
Keratins commonly contain the smaller amino acids such as glycine, alanine, and cysteine. Mass of sample typically required for each isotopic assay also applies to most other biological tissues like muscle, claw, and blood. The primary isotopic standards are PeeDee Belemnite (PDB), Vienna Standard Mean Ocean Water (VSMOW), atmospheric N_2 (AIR), and Canyon Diablo Triolite (CDT).

One example would be redhead ducks that migrate between higher latitude northern boreal forests and lower latitude estuarine areas of the coastal Caribbean (Hobson *et al.* 2004a). A spatial isotopic linkage also requires that the isotopic offsets (net isotope discrimination) between these seasonally used dietary landscapes and the selected tissue being measured is consistent or well known, or has been empirically measured. Of course, such ideal conditions are often not realized and may be complicated by a host of issues that will provide research opportunities and challenges for many years to come.

Most biologists and ecologists that conduct avian or terrestrial insect migration research projects do not operate a costly stable isotope laboratory facility, and so are reliant on specialized commercial, government, or university stable isotope laboratories that can analyze their samples for them on a fee-for-service basis, or by a collaborative project. This interaction will lead to queries and discussions between an isotopic analyst who may have little or no knowledge of the migration project at hand, and a biologist or ecologist who may have little or no knowledge about the intricacies of stable isotope jargon and the required analytical protocols and procedures for a successful analysis. Because of the potential for confusion or unrealistic expectations, and because stable isotope analyses may be a costly budget item, the intent of this chapter is to help bridge a discipline gap by arming the ecologist with the essential information required to ensure a good understanding of the terminology and to gain confidence that the kinds of isotopic assays sought are appropriate and useful. Practically, the types and amount of sample matrix or tissues that can be analyzed for the key light isotopes will be covered, because much of the analytical budget may be overtaken by preparative procedures that are easily done in the ecologists' laboratory by students and support staff.

From experience, a common scenario starts with a query from a researcher or graduate student who is interested in the migration ecology of a particular species. They have read published papers or viewed a presentation at a scientific meeting suggesting that stable isotopes can be used track animal migration. What follows is a dialogue among colleagues that form the outline of this chapter. What are stable isotopes? Which stable isotopes will be of use to the migration research questions for my species of interest? What sample or tissue is needed for the analysis? How do I prepare the sample? How much sample is required? Are there caveats? What kind of analytical error can I expect? How much will it cost? Why do my duplicates give different results? These are but a few of the fundamental questions and concerns that, if addressed as fully as possible from the start, will form the basis of a well-designed successful migration research project using stable isotopes.

The goal of this chapter, therefore, is to provide a practical guide for ecologists and biologists wishing to employ light stable isotopes as intrinsic markers in their terrestrial animal migration research projects. While the overall thrust of this volume is primarily on larger- and continental-scale movement of animals because of the longstanding and intractable problems associated with documenting this massive scale of movement, we must acknowledge that the scale of animal migration can be tiny indeed. One example may be the seasonal vertical migration of earthworms in soils of less than a meter. The focus here is to a give concise and practical guidance to the nonisotope specialist on obtaining the best possible stable isotope data in order to ensure that the assays are appropriately done, and as a result meaningful spatial interpretations regarding migratory movement can be made.

A. What Are the Light Stable Isotopes?

Most of the elements of the periodic table have a number of stable isotopes, that is, elements having the same number of protons but differing only in their numbers of neutrons (Criss 1999, Hoefs 2004). However, of the many elements that have stable isotopes, there are just a handful that are of immediate practical interest to studies of animal migration and ecology. These are the so-called "light isotopes" of the elements of C, N, H, O, and S. These five atomic elements comprise the primary building blocks of all components of the biosphere (plants, animals), the hydrosphere (water), and the atmosphere (gaseous N_2, O_2, H_2O). For example, we can see that these five elements, to varying proportions, overwhelmingly comprise the bulk of all animal tissues, from ~50% dry weight for C to lower levels of 5–6% dry weight for H (Table 2.1). These five elements each have two or more stable isotopes that vary widely in nature from the micro- to macroenvironmental scale. Other "heavier" elements and their isotopes may also be useful but tend to occur at trace concentrations and do not form the majority of organic tissue structure. One example of a "heavy isotope" trace element that can be extracted from tissue and that has spatial distribution patterns is the element strontium (Sr, Table 2.1).

All of the light isotopes have a common or abundant "light" isotope (*e.g.*, ^{12}C; 98.894%) and one or more "heavier" rare isotope of interest (*e.g.*, ^{13}C; 1.1056%; Hoefs 2004). It is the abundance ratios of these light and heavy isotopes that vary minutely in nature because of physical and chemical processes, and these variations are ultimately of interest to us. However, it is exceedingly difficult to measure absolute ratios with any great accuracy, or to determine absolute concentrations of each isotope in a sample. It was recognized nearly 60 years ago, however, that it is easier to precisely measure *relative* differences in the isotopic ratios between a pure gaseous sample (*e.g.*, CO_2, H_2, SO_2, N_2) and gaseous reference using a gas source mass spectrometer (McKinney *et al.* 1950).

The fact that *gas source* isotope ratio mass spectrometers (IRMSs) are primarily used to measure the light isotopes has two important implications. First, it means that all samples (feather, muscle, blood, claw, etc) cannot have their isotope ratios measured directly on the raw organic material or sample provided by the researcher, but the sample must first be combusted or by some means quantitatively converted to an ultrapure and appropriate gaseous analyte in order to measure its isotopic ratios relative to a calibrated reference gas of the same type. Numerous papers and books have been devoted to preparative conversions for a host of organic substrates over five decades—the most recent summaries and historical perspectives on this topic are in Volumes 1 and 2 of the *Handbook of Stable Isotope Techniques* (de Groot 2004).

Most typically for organic solid substrates, samples are quantitatively combusted using elemental analyzers (EAs; Figure 2.1) to convert samples to pure CO_2, H_2O, SO_2, or N_2 gases (Fry *et al.* 1992). While the fundamental principles of the IRMS remain virtually unchanged since the 1950s, much of the developmental in the last decade have focused on automating sample preparation in order to obtain multiple isotope assays from a single sample or new substrate, on decreasing sample size requirements, or on improving sample throughput rates while maintaining highest measurement accuracy.

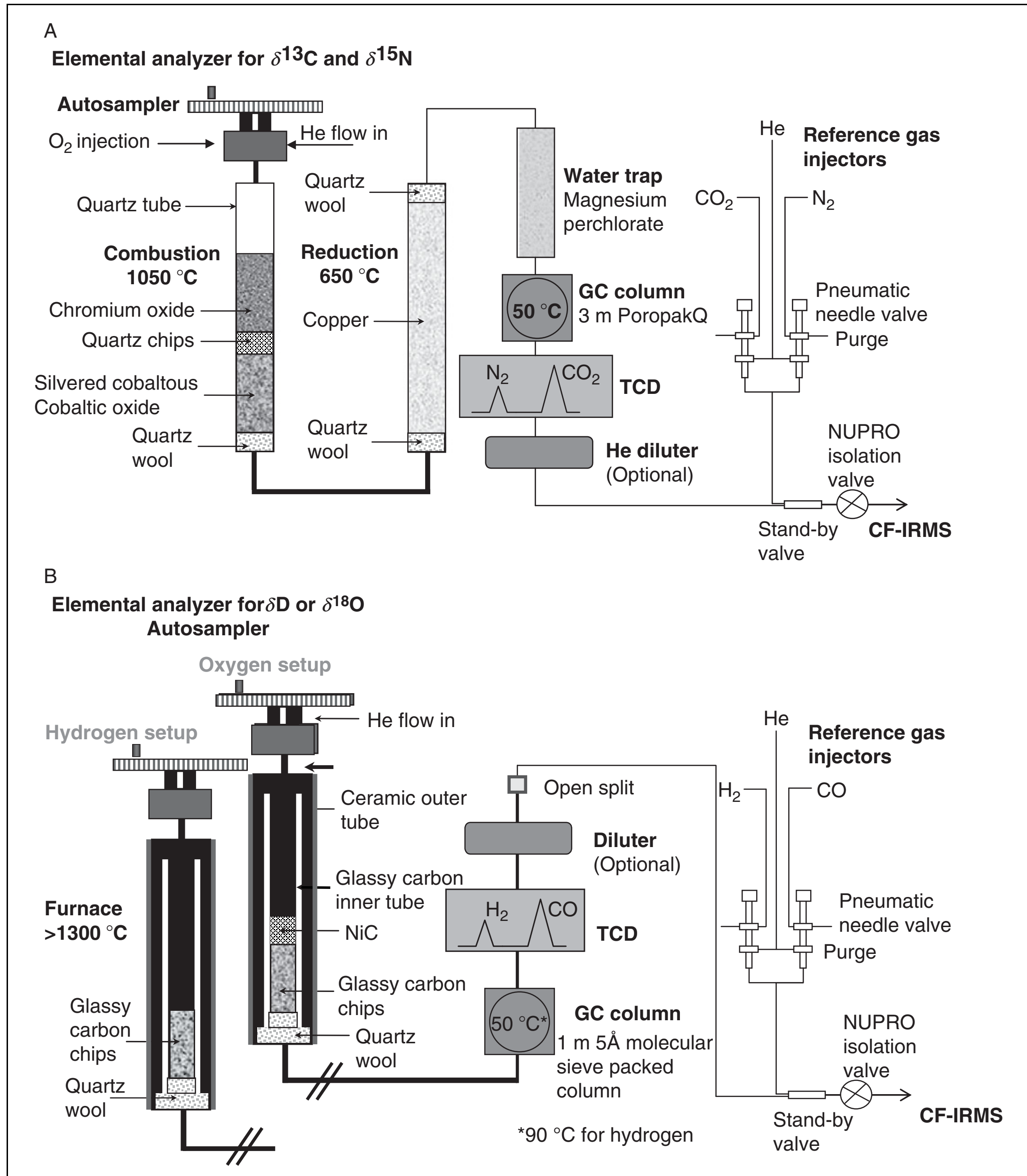

FIGURE 2.1 Typical elemental analyzer (EA) preparative interface configurations to a CF-IRMS system for (A) δ^{13}C and δ^{15}N and (B) δD or δ^{18}O in organic tissue samples (courtesy of GV Instruments).

The second implication is that stable isotopic ratio results reported back to the researcher are not provided in a readily recognized SI concentration format such as mg/Liter or µmol/g. Instead the researcher will receive a series of "δ" numbers having positive or negative values expressed as a parts per thousand (‰) relative difference to an international standard (Hoefs 2004, Fry 2006). For the uninitiated this can be confusing. But recall, because we can very precisely measure minute relative

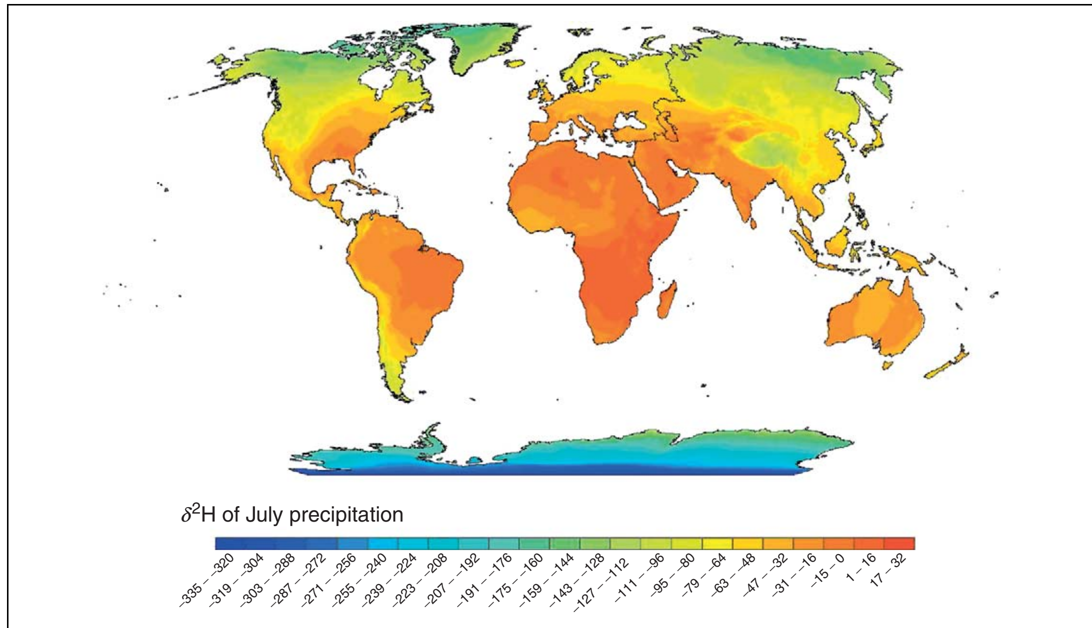

FIGURE 2.2 The average hydrogen isotopic (δD) composition of rainfall across the globe in the summertime. These large-scale and distinct isotope patterns are largely mirrored in plants and into upper-level trophic organisms and so form the basis for tracking animal movement. Vectors of seasonal migratory movement are often North-South and spanning large H isotopic gradients. The terrestrial global range of δD is over 300‰, with a measurement precision of better than $\pm$2‰. The oxygen isotopes form a similar pattern. The oceans are isotopically homogenous at $\sim$0‰ for both δD and δ^{18}O. Map courtesy of G. Bowen and may be found online at www.waterisotopes.org.

isotopic differences between two gases, an examination of the standard "δ" equation reveals what the δ data mean:

$$\delta X(‰) = \left(\frac{\text{Isotopic ratio}_{\text{sample}}}{\text{Isotopic ratio}_{\text{standard}-1}}\right) \times 1000 \tag{2.1}$$

where X on the left side is the isotopic element of interest (δ^{13}C, δ^{18}O, δD, etc). The right side of the equation in parentheses is the isotopic ratios of the sample and international standard comparatively measured by the mass spectrometer in the laboratory. The isotopic ratio is the measured ratio of the light to heavy isotopes ($^{13}C/^{12}C$, $^{18}O/^{16}O$). The measured ratio results are taken and multiplied by 1000 simply for convenience in order to transform the values primarily into whole numbers (*e.g.*, easier to work with than 5–6 decimal place numbers). Because the light isotope primary references were established arbitrarily many decades ago (these are our "zero" points), the results can be negative or positive values in parts per thousand (‰) deviations because they are always *relative to the accepted international standard* (Groning 2004). Thus, we need never be concerned about the positive or negative sign. The primary stable isotope reference materials currently used are listed in Table 2.1. Because primary references are preciously limited in quantity, most laboratories routinely employ local laboratory standards that have been carefully calibrated against primary reference materials.

For example, a sample reported having a δD value of +150‰ means the sample has 150 parts per thousand (or 15%) more deuterium in it than the standard, in this case the Vienna Standard Mean Ocean Water (VSMOW) standard. If the value were negative, it simply means 150 parts per thousand less deuterium in it than VSMOW. While these isotopic concentration differences are amazingly small, it is worth noting that modern IRMSs can reliably measure isotopic ratio *differences* to the sixth decimal place, or expressed in ‰, to $\pm$0.01‰, or better. The limiting factor, and indeed most of the

analytical error, generally occurs in the preparative conversions of raw sample to the appropriate analyte gas of interest or in the sample gas handling and transfer systems.

A final term that must be briefly explained is mass-dependent *isotope fractionation*. Isotope fractionation occurs when a chemical reaction or a process results in a changing of the stable isotope ratios of the source or reactant because of the slight chemical differences arising from the subtle differences in mass. Unidirectional or irreversible isotope fractionations are known as *kinetic isotope fractionations*, whereas *equilibrium fractionations* (*e.g.*, $^{18O:16O}O_{2\ gas} \leftrightarrow {}^{18O:16O}O_{2\ dissolved}$) are chemical reactions that are reversible (Hoefs 2004). Sometimes isotope fractionation is less explicitly portrayed as "δ differences" between two or more partially related substrates. For example, in this volume, the emphasis on hydrogen isotopes as a tracer of migration is key, whereby patterns in the hydrosphere (water) are linked to those in plants and animals. While there may be consistent δ "offsets" among these three hydrogen-bearing components at each geospatial site, the water-diet-tissue "fractionations" is more accurately the "net isotopic discriminations" because every single hydrogen pool and form in those three substrates are not measured or fully known, nor do we follow all individual hydrogen atoms, but instead rely on selected analyses of bulk samples. The reader is referred to the textbooks below for exhaustive discussion of the various types of isotope fractionation occurring in nature. In short, were there no isotope fractionation in nature, all components of the geosphere, hydrosphere, atmosphere, and biosphere would have the exact same isotopic ratios and stable isotopic assays would be pointless. Fortunately, isotopic fractionations of the light isotopes in nature are widespread, diverse, and often characteristic in their magnitude and direction. This feature allows us to exploit isotopic measurements for the purposes of studying animal migration (Chapters 3–5).

The type of modern gas IRMS overwhelmingly used for biological and migration studies these days is called *continuous-flow isotope ratio mass spectrometry* (CF-IRMS), which gained widespread acceptance in the 1990s, and has been the subject of intense development since the late 1980s (Matthews and Hayes 1978). Compared to the highest precision *dual-inlet isotope ratio mass spectrometry* (DI-IRMS) developed in the 1950s, there are pros and cons to CF-IRMS assays. The primary and most attractive aspects of CF-IRMS includes much lower per-sample analytical cost, a high degree of automation by linking preparative modules such as EAs, and high sample throughput rates. The main disadvantage remains lower analytical precision, although in recent years, many CF-IRMS assays for CNHOS approach or exceed dual inlet assays simply due to automation and improvements in sample preparative handling. ^{34}S is still best done by dual inlet for the highest precision, although CF assays are constantly improving. Radiogenic Sr isotopes are conducted by solid source IRMS. The cost in US dollars of stable isotope analyses in 2007 range from as low as \$10–15 for $^{13}C + {}^{15}N$ to \$15–50 for $^{18}O/^{2}H$, \$50–100 for ^{34}S to \$100 or more for $\delta^{87}Sr$. Schematic examples of some typical CF-IRMS preparative EA systems for C, N, H, and O isotope analyses are shown in Figure 2.1.

It is beyond the scope of this chapter to exhaustively cover all aspects of stable isotope mass spectrometry and biogeochemistry. The reader is referred to some key textbooks published over the last decade that cover in detail the scope of stable isotope theory and environmental applications (Kendall and McDonnell 1998, Criss 1999, Hoefs 2004). For students and newcomers to the field of stable isotopes, a good place to start for fundamental introduction and discussion of stable isotopes in the biosphere and hydrosphere are *Stable Isotope Ecology* (Fry 2006), *Stable Isotopes in Ecology and Environmental Science* (Lajtha and Michener 2007), *Stable Isotope Geochemistry* (Sharp 2007), and *Environmental Isotopes in Hydrogeology* (Clark and Fritz 1997).

II. MATERIALS AND METHODS

In the following two sections, we will cover field sample collection and tissue subsampling and discuss each of the light isotopes, grouped in order of the type of information that can be gained that may be translated into spatial analyses useful for migratory research.

For the purposes of terrestrial animal migration research, stable H and O isotopes in the terrestrial environment may be considered "*global-spatial*" assays because the patterns of H and O isotopes on terrestrial systems are systematically controlled by global-scale hydrologic and meteorological processes that are seasonally and spatially predictable over multiyear time frames and continuously over massive geospatial scales (regional, continental, global). This salient feature provides the highest level of confidence in making spatial interpolations into areas where no long-term data or stations exist (Figure 2.2, Bowen *et al.* 2005b). Note that the oceans are global O and H reservoirs and are largely isotopically homogenous, which further allows clear marine versus terrestrial distinctions to be made.

C, N, S, and Sr isotopes may be grouped into "*local-spatial*" assays, mainly because there are no strong *a priori* reasons to predict that these isotopes vary systematically and continuously on the landscape or over very large geospatial scales (although they *could* pattern over smaller scales) to the same extent as H and O. While there are well-known spatial isotopic differences in key isotopes (*e.g.*, ^{13}C differences between C_3 and C_4 plant-dominated habitats), local variables may be strongly and unpredictably influential for C, N, and S, and for Sr include soil type, altitude, agricultural land use, industrial influences, and local geology (Hebert and Wassenaar 2001). These local-scale variations may preclude or hinder predictive continuous spatial interpolations. Ongoing and new research suggests that some larger-scale patterns in ^{13}C and ^{15}N may occur (*e.g.*, terrestrial vs marine), but this area requires further development (Chapters 4). The primary controls on Sr isotope ratios are local geology (spatially variable or discontinuous) and the ocean (isotopically homogeneous) that allows for clear marine versus terrestrial distinctions, but also terrestrial fingerprinting.

The first approach to large-scale animal migration investigations using intrinsic tracers like stable isotopes is likely to be the most fruitful by using the global-spatial assays (H and O isotopes). However, spatial resolving power and details of habitat use may be greatly improved by careful consideration and selection of a subset of local-spatial isotopes (C, N, S, and Sr) or by including other assays and data (*e.g.*, extrinsic data) and a Bayesian analysis approach. Discussions on this and multi-isotope spatial assignment approaches (and difficulties) are outlined in detail in Chapters 4 and 5 and elsewhere (Hebert and Wassenaar 2005).

A. Sample Collection and Preparation

One of the very first questions encountered by researchers using stable isotope methods for migration research involves the type of samples and subsamples that need to be collected from the organism of interest. This could be hair, claw, muscle, blood, wings, thorax, etc. There are two main categories of samples that may be defined: (1) *fixed tissues* and (2) *dynamic tissues.*

Fixed tissues are here defined as those kinds of discrete tissues or body parts that once formed are metabolically (and isotopically) inert, recording the isotopic composition of local diet at or close to the time of formation. These include the so-called "dead" tissues such as keratinous claw, nails, hair, and feathers. One of the primary and most often used fixed tissues are bird feathers—once fully formed, the vane material and rachis (stem) does not change chemically or isotopically as the organism moves away from the location of formation and along its migratory route. Key advantage of feathers is that for many species they are often grown over a very short period of time, or at one specific location. For many birds and insects, feathers or wings are often formed at the breeding site, wintering ground, or site of emergence, and so will record the isotopes of diet at that specific location. Fixed tissues are most often used for estimating net spatial vectors from the point of origin of formation.

However, there are some caveats to the use of fixed tissues that require the researcher to have a good knowledge of the ecology and physiology of the organism under study. Potential complications include slow or variably growing fixed tissues that can lead to the issue of intra- or intersample isotopic variance arising from transient dietary conditions during movement. As an illustrative example, consider human hair. Human hair forms at an average rate of ~10–15 mm per month depending on the location on the

scalp, but the hair is isotopically "fixed" once formed and therefore records information about the location of diet at the time of cellular growth. There may be some lag time before local diet is isotopically recorded in the hair, but a temporal record of diet along the length of the hair sample can be investigated and spatially linked (Hobson and Schell 1998, O'Brien and Wooller 2007).

Another cautionary example involves slowly growing feathers, claws, or hairs that form as an animal is enroute and ingesting diet at different geospatial locations and stopovers. This is aptly illustrated in Figure 2.3 showing hydrogen isotope data obtained along the length of a single flight feather from a bald eagle (see also intrasample issues below). The data clearly revealed distinct hydrogen isotopic patterns along the entire length of a single flight feather that can be related to southern migratory movement in North America *during* feather growth. This bald eagle had been tagged and began its flight feather growth and migratory journey in Saskatchewan, Canada (*e.g.*, the more negative δD values in older feather material near the tip) and then migrated southward into the United States during the fall (positive δD values toward the base), and was recaptured the following year in Canada long after the feather was fully grown. It is clear that as the feather was temporally extruded from the base, it gained more positive δD values as the eagle moved to more southerly locations, and essentially was recording a "net migratory track" within a single feather. However, had one not known the feather was grown enroute and had taken random subsamples and assumed they were solely indicative of natal

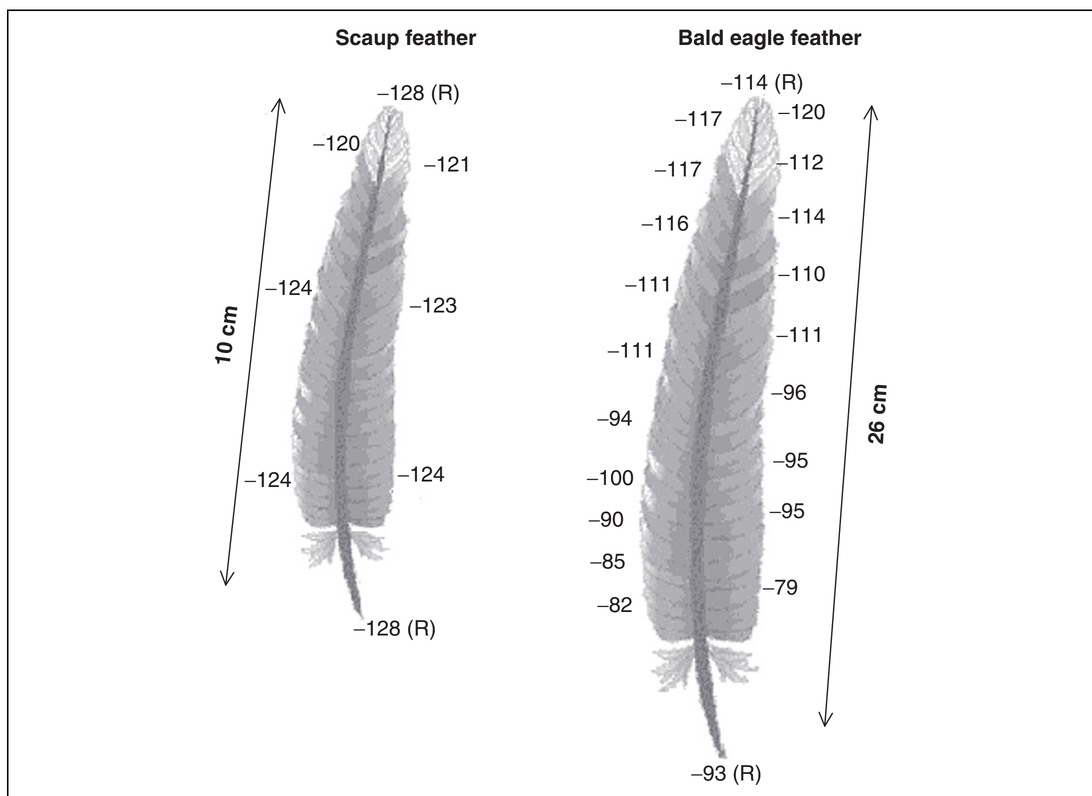

FIGURE 2.3 Illustration of measured intrasample hydrogen (δD) isotopic heterogeneity in migrating birds. Illustrated is a comparison of a flight feather from lesser scaup (*Aythya affinis*), left, that grew the entire feather at one location and a bald eagle (*Haliaetus leucocephalus*), right, that grew the feather along its southward migration. Subsamples for hydrogen isotope analyses were taken from vane material (350 μg) up and down the left and right side of a single feather, and from the top and bottom portions of the rachis (R). Note that illustrated differences in feather length are not to scale and are in centimeters from the tip to base. δD results are in percentages (VSMOW).

origin, one could have obtained highly confounding results from this individual and among eagles. Further, movement during tissue growth is another primary reason why duplicate samples taken from different parts of the same feather or tissue (often assumed by the researcher to be true repeats) can give startlingly different results. In other words, what may have been presumed to be a single fixed tissue sample (a feather) may in fact be a potentially interesting spatial "recorder."

By contrast, the intrafeather hydrogen isotope patterns from a migrant lesser scaup (*Athya affinis*) are shown in Figure 2.3. For this individual, it is known the entire flight feather was formed at the natal location in Canada. Here no significant intrasample tissue isotopic differences were observed that would indicate that this individual had moved during feather formation, despite this migrant being captured far from its natal site. In other words, this feather is an excellent candidate to assess postbreeding spatial vectors, regardless of the position on the feather where the subsample for isotope assays was taken.

Other confounding aspects of sampling may include partial molts or feather loss, differential molt times (head feathers vs breast vs flight feathers at different times and locations), or dietary switches. For fixed tissues on newborn or maternally fed animals, there is also the possibility that some of the isotopic information is obtained from the mother but was derived at another location (feeding far from the natal site or from preexisting body reserves obtained elsewhere) (Duxbury *et al.* 2003).

Another category of fixed or dynamic tissues are those preserved in museum or archaeological collections. Such collections may be attractive in order to gain historical trends information regarding migrating or even from extinct species. However, these fixed tissues may have be been preserved (*e.g.*, formalin storage), chemically treated, or dried. The researcher must therefore be vigilant to the potential isotope fractionation that may result from sample degradation or issues of contamination arising from isotopic exchange with chemicals or artifacts from applied preservatives (Hobson *et al.* 1997).

Fixed tissues seemingly provide straightforward answers. However, as shown above, fixed tissues of migrants can have a temporal (and thus spatial) component that provides the researcher with an opportunity and a dilemma. The opportunity lies in the possibility of obtaining additional temporal or comparative information about the organism's movement because longitudinal isotope transects of the tissue will faithfully record this to some degree. The dilemma lies in obtaining the correct sample for addressing the research question at hand. Have we subsampled the correct part of the tissue to address the question? Which part of a slowly growing feather should we sample? Do we sample a small piece of feather from the top or bottom? Do we grind up a hair or whole feather? It is clear from the data in Figure 2.3 that random sampling near the top or bottom of a migrating bald eagle's flight feathers could produce very puzzling results. Knowledge of the biology of the organism under study and tissue growth patterns and timings are clearly essential.

Dynamic tissues, on the other hand, are those types of metabolically active tissues that are continuously "turning over" from an ongoing dietary source (Schoenheimer *et al.* 1939). Examples of these kinds of tissues are blood, muscle, liver, etc. While some of the stable isotope literature has focused on tissue turnover (Hobson and Clark 1992), very few controlled or laboratory studies have been conducted on the dynamic tissues of migrants as a means of gaining information about migratory connectivity. Most animal tissue turnover studies have focused on ^{13}C and ^{15}N (Trueman *et al.* 2005, Chamberlain *et al.* 2006), and fewer on other light isotopes (Hobson *et al.* 2004a). It is well established that living tissues in organisms can have vastly different isotopic turnover rates, ranging from days (liver, blood) to weeks (muscle) to lifetime (bone collagen). In theory, it should be possible to exploit the comparative multiple isotopic composition of dynamic tissues both temporally and spatially (cf. Tieszen *et al.* 1983, Suzuki *et al.* 2005, Cerling *et al.* 2006) in order to gain information about migration, but this remains an area of research that is just beginning to be explored. The development of various dynamic tissue isotopic turnover models based on laboratory experiments will be an essential prerequisite to the application in migration studies (Cerling *et al.* 2007).

Another overall consideration may be whether the fixed or dynamic tissue sample of interest can be collected nonlethally. For many threatened animals, hair, nail, and feather can easily be plucked or cut

without fatally affecting the individual or its means of transport. For other species, euthanizing may be required, as in the case of small insects where entire wing sections must be taken. This may be a minor issue or could be a serious one in the case of rare or endangered species. Animal care guidelines and national regulatory guidelines must be observed when sampling.

Finally, the isotopic results obtained for both fixed and dynamic tissues will need to be related to on-the-ground spatial isotopic data (*e.g.*, isotopic base maps) in order to facilitate interpretations of geospatial movement. The assumptions and challenges in making these connections for migratory studies are fully discussed in Chapters 3–5.

In summary, the first step of capturing migrants for the purposes of applying stable isotopic assays is a major commitment and undertaking. However, equal and careful consideration must be given to the biology of the organism and to the type of tissue sample that is being collected from that individual for the purposes of stable isotope analyses. Consider carefully what kind of sample will be collected and what the results could mean. Stable isotopic assays do not deceive—it may be our understanding of the migrant's biology, biogeochemistry, or ecology that is lacking.

B. Cleaning, Storing, and Weighing

Once fixed or dynamic tissue samples have been taken from a migrant for stable isotope assays there follows the question of sample storage and preparation. The samples returned from the field may be dirty, matted, or greasy. Some degree of preparative cleaning may be required to remove external contamination. While the procedures for fixed tissues are fairly straightforward, there is ongoing debate concerning what to do with dynamic living tissues (*e.g.*, defatting, plasma separation).

For fixed tissues (hair, nail, claw), the sample cleaning procedure is uncomplicated. Ideally, the first goal would be to obtain the cleanest possible sample material directly from the animal. If there is dirt or other foreign adherents, samples may be washed in distilled water and air dried. Where there are natural oils on the fixed tissue (*e.g.*, oil on hair, feathers), samples should be further cleansed using a volatile solvent mixture. Here solvent cleansing is strongly recommended to remove surface oils because the carbon and hydrogen isotopic composition of oils and waxes can be markedly different (usually more negative) from pure keratinous tissue, and so could impart a disproportionate degree of isotopic variance. A recommended procedure for preparation of fixed tissues for light stable isotopes is outlined in Table 2.2. Note that natural oils on feathers typically do not contain S or N and so solvent cleaning may not be needed for these specific isotopic assays. For any trace elements (*e.g.*, Sr isotopes), sample cleaning and contamination of tissues is of major concern because contamination of the sample from dust and handling may be especially problematic (Font *et al.* 2007).

For dynamic tissues, the approach to cleaning procedures is less clear. A long-standing debate revolves around the fact that solvent or acid washes may change the isotopic composition of the bulk living tissue by selectively removing fatty acids or amino acids, and thereby changing the isotopic composition (CNS) of the bulk sample (Pinnegar and Polunin 1999, Sotiropoulos *et al.* 2004, Post *et al.* 2007). Opinions range from no cleaning, to solvent cleaning, to the use of C/N ratios to correct for fat content or empirical correction models. Currently there is no definitive one-size-fits-all cleaning procedure or correction approach that applies to all dynamic tissues. Nevertheless, the removal of lipids is highly preferable for fixed tissues given the potential variance in lipid $\delta^{13}C$ and δD values (Sessions *et al.* 1999). By removing fatty acids and lipids, we are assured that the isotopic analysis is conducted on only proteins.

Samples taken for stable isotopic analyses should be properly stored before and after preparative procedures in order to prevent degradation (and possible isotope fractionation). Many fixed tissue samples can be air or freeze-dried and stored in glass or plastic vials or paper envelopes at room temperature. For living tissues, samples can be stored frozen (−40 °C) to avoid decomposition, or freeze dried. If properly prepared and preserved, stable isotopic integrity over time will not be an issue.

TABLE 2.2 Recommended standard procedure for cleaning and weighing of "fixed" tissue for stable isotopic analysis

Working materials required: Analytical microbalance, cleaned tissue samples, clean culture tray(s), weighing utensils, methanol, Kimwipes, tray template, tape, marker, silver, or tin capsules

1. Obtain a clean 96 position plastic culture tray (Elisa Plate) and print out an Excel sample template.
2. Ensure feathers have been previously solvent cleaned [2:1 (v/v) chloroform/methanol 24 hour soak and 2× rinse] to remove surface oils. Air dry feathers in fume hood (>48 hours).
3. Cut off a small amount of feather vane (not rachis) material for analysis—always cut samples from the same location on different feather samples if possible for consistency (*e.g.*, sample near tip). Feather pieces can be cut using small stainless steel surgical scissors.
4. Clean weighing utensils using methanol and Kimwipes™ and allow to dry. Do not use acetone!
5. Make sure the microbalance is clean and calibrated. Ensure that the doors are closed when taring and weighing.
6. Tare a silver capsule,[a] handling only with tweezers, remove, and set on a clean metal surface. Use the smallest available capsule that will safely contain the sample (*e.g.*, 3.5 × 5.0 mm).
7. Using a spatula or tweezers, transfer a small amount of feather material into the capsule.
8. Reweigh, and continue adding or removing feather material until the target sample weight of 350 μg ± 10 μg is obtained.[b] This will take practice to get the feel of an appropriate amount. With practice, samples should take less than 5 min each to weigh out. Ensure the microbalance is accurate and stay within stated weight tolerance to avoid mass spectrometer mass-dependent source nonlinearity effects. Keratin or references must be weighed to the comparable elemental mass as the samples.
9. To seal the capsule, crimp the top of the capsule shut with a pair of straight edge tweezers and then fold tightly (as if folding down from the top of a paper bag). Then use the edge of the tweezers (use of two tweezers helps) to gently compact the capsule into a small, tight cube or ball. There should be no stray edges, loose sides, or feather material poking out. Flattened samples (rather than cube/ball-shaped) or capsules with stray or loose edges can jam our autosampler, cross-contaminate samples, and ruin an analysis.
10. Record final sample weight and sample name in spreadsheet. Place the sample capsule in the 96 position tray and record the weight on the tray template. Clean all utensils lightly with Kimwipes and methanol after completing each sample, air dry briefly. Secure the lid of the culture sample tray with rubber bands and masking tape and label the tray when done. Ensure samples cannot "jump" cells when the Elisa lid is properly closed (some brands of trays allow this).
11. Record sample name and weights (in milligrams) for each sample in the appropriate tray and its position (*e.g.*, tray 1, position A5). When completed, transfer this information to appropriate isotope laboratory sample submission form.
12. Use 3.5 × 5.0 mm silver or tin capsules designed for elemental isotope analysis. Suggested suppliers are Costech (1–800–524–7219) and Elemental Microanalysis (1–800–659–9885).

[a] Silver capsules must be used for δD and δ^{18}O analyses, tin capsules for δ^{13}C, δ^{15}N, and δ^{34}S.
[b] Consult isotope analyst or laboratory being used for the specific mass to be used for each isotope. The example of feathers for C, H, N, O, and S assays is used, but it also applies to claw, hair, etc.

One exception noted above is the use of preservatives—it is not recommended to store samples in formalin as this has been shown to affect carbon and nitrogen isotope ratios (Hobson *et al.* 1997, Edwards *et al.* 2002). Formalin storage is a common problem for many museum specimens. If short-term solvent storage is required, instead use a 70% ethanol mixture.

C. Intra- and Intersample Heterogeneity

One question immediately facing the researcher in the field is the issue of which sample should be collected from the migrant organism for stable isotopic assays. For insects, the entire animal may need to be euthanized and taken. For nonlethal sampling of small and large birds or bats, there are a host of potential samples that can be considered. Fixed-tissue options include flight or contour feathers, hair, and nails. Dynamic-tissue options are typically blood, muscle, liver, and fats.

Questions have been raised regarding the issue of inter- and intrasample isotope heterogeneity (Wassenaar and Hobson 2006), where replicates of subsamples of feathers, hair, or other tissues may produce a range of C, H, N, O, and S isotopic values that could exceed the expected isotopic range that defines the organisms spatial distribution (Lott and Smith 2006). Studies have revealed that there are significant C, N, and H isotope differences among bulk tissue types (*e.g.*, blood, muscle, nails, feathers, hair) within a single individual (Tieszen *et al.* 1983, Mazerolle and Hobson 2005). Stable isotopic differences may be amplified at molecular levels if specific biochemical fractions (*e.g.*, lipids, amino acids) are further isolated (Teece and Vogel 2004). All of these intra- and intertissue isotopic differences arise from differential biochemical isotopic discriminations and from temporal changes in dietary sources that occur over the course of tissue biosynthesis (Phillips and Eldridge 2006). As noted above, each tissue type contains some shorter or longer term temporal record of diet (and likely each with differential diet-tissue isotopic fractionations) at a location or from many locations.

The researcher must be aware of both individual and population level *intersample isotopic heterogeneity.* There are two concerns. The first is the measurable C, H, N, O, and S isotopic differences that occur among the same tissues on the same animal. For example, due to inherent natural variance in isotope fractionations occurring during biochemical synthesis of tissues, we might expect there to be some minor isotopic variance, for example, among contour feathers grown by an individual at a single location. Second, we can expect intersample isotopic variance for the same feathers from a population of local birds that grew feathers at the same location. Intersample isotopic heterogeneity of both kinds are always greater than the instrumental analytical error for C, H, N, O, and S, and ideally would be much less than large-scale isotopic patterns in order to make geospatial interpretations (see Chapter 5).

Intrasample isotopic heterogeneity can be defined as the isotopic variance at the molecular (or microgram scale for our purposes here) level within a single discrete sample. This was illustrated above using δD and the eagle feather in Figure 2.3. Intrasample isotopic heterogeneity may be close to, but is always greater than, the instrumental analytical error for C, H, N, O, and S. A general rule-of-thumb is that large and slowly growing tissues will likely contain a greater degree of intrasample isotopic variance that will be amplified if the animal is moving across large spatial distances.

Several experiments with feathers and hair have shown that the overall level of δD variance associated with measurements strictly due to internal metabolic processes and laboratory methodology is of the order of ±3‰ (Bowen *et al.* 2005a, Wassenaar and Hobson 2006). Indeed, this is very close to the analytical error inherent in CF-IRMS measurements for δD (±2‰). Comparable results are expected for other light isotopes. However, for most animals within- and among-tissue variance due to ecological considerations can greatly decrease the power of spatial resolution. In general, subsamples from selected tissues among individuals should be taken at the same location in order to represent, as best as possible, identical periods of growth.

Another issue that is commonly encountered in stable isotopic assays in migration studies is the problem of "outliers." For example, a researcher may submit a suite of feather samples for stable isotopic analysis from a local population of birds, and discover that all values are tightly clustered, except for a couple of extreme isotopic outliers. There are only two possible explanations, either the outliers are correct and must be explained or the data are faulty. The first thing the researcher should do is contact the laboratory to double check if any of the individual outlier analyses were faulty. A diligent analyst will welcome the query, even though the researcher may feel they are questioning the laboratory's integrity. Highly automated (100s of samples per day) IRMS systems are never foolproof and can have unexpected hardware glitches and mistakes in data handling. Fortunately, this is generally a rare occurrence. Nevertheless, the analyst will check the sample and standards QA/QC. If all seems to be OK, it may be worth requesting repeats for specific outliers (or by including subsample repeats in the first place). Once the outliers are confirmed to be correct, the researcher is left to ponder the ecological significance of the outliers (*e.g.*, recruitment of immigrants?).

In summary, the researcher must carefully consider which sample is the most likely to satisfy the requirements of answering, wholly or partially, the migration research question at hand. Even after

careful selection and analysis of stable isotopes, a critical scrutiny of the results is warranted. This scrutiny will require knowledge of the biology of the species and may require some experimentation with the species under study, for example, to better quantify diet-tissue isotope fractionation. It is considered unwise to assume and extrapolate the experimental findings on intrasample isotopic heterogeneity among completely different species, as is aptly illustrated in Figure 2.3, although it has been noted that for many insectivorous passerines and waterfowl, the water-dietary-tissue fractionations for δD are remarkably uniform (Clark *et al.* 2006).

D. Sample Weighing

All samples submitted for stable isotope measurements require some form of sample processing, subsampling, and analytical weighing prior to their analysis. Many laboratories can provide this service and the client need not be overly concerned about the technical details. However, given the important cautions noted above regarding intrasample heterogeneity, clear and explicit instructions regarding exactly where subsamples need to be taken (*e.g.*, where on flight feathers) may have to be provided to the laboratory in order to avoid confusing results. Finally, a major cost savings and considerable improvement in sample turnaround time can usually be achieved if the researcher conducts the sample preparation and weighing. This should be done in full consultation with the stable isotope laboratory that will be employed.

Target weights for samples are typically grouped by the type of isotopic assay that is requested. For researchers that wish to conduct preparative weighing they will be requested by the laboratory to use a (costly) analytical microbalance. These balances are found in specialty analytical research laboratories and are capable of weighing samples to a readability of $\pm$0.001 mg. For each isotope, the mass of sample required will depend on the sensitivity of the instrument and the mass of the element in the sample.

Accurate weighing to the required laboratory target weight for the isotope of interest is absolutely essential. The main reason being that for isotopic assays by CF-IRMS, there is often a dependence of the δ result on sample mass because of differential gas pressures in the source of the IRMS, as demonstrated in Figure 2.4. This is commonly known as "source linearity," and is quantified in many laboratories for each gas species. For C + N, this mass dependency is typically low and forgiving. However, for isotopes like δD, this mass dependency can be significant, and easily up to 10‰ per 100 μg of sample in a positive or negative direction depending on the IRMS instrument. This means an accuracy of weighing to $\pm$10 μg is required to reduce this potential source of variance to below IRMS analytical error. Careless weighing (or poorly calibrated balances) is one of the first causes to consider for high variance in replicated results within and among laboratories. Hence, the researcher must enquire and adhere closely to the sample weight guidelines provided by the laboratory. If the selected laboratory has not established mass dependencies for their isotopic results, this is definitely the kind of QA/QC is worth asking about before doing the work. An example of the masses typically required for stable isotope assays are listed in Table 2.1.

III. GLOBAL-SPATIAL ISOTOPES

A. Stable-Hydrogen Isotopes

Stable-hydrogen isotope (δD) measurements are typically among the first to be considered when isotope assays for spatial analyses in migration studies are required (Hobson and Wassenaar 1997, Bowen *et al.* 2005b). Hydrogen isotopes have been shown to be especially robust in migration research

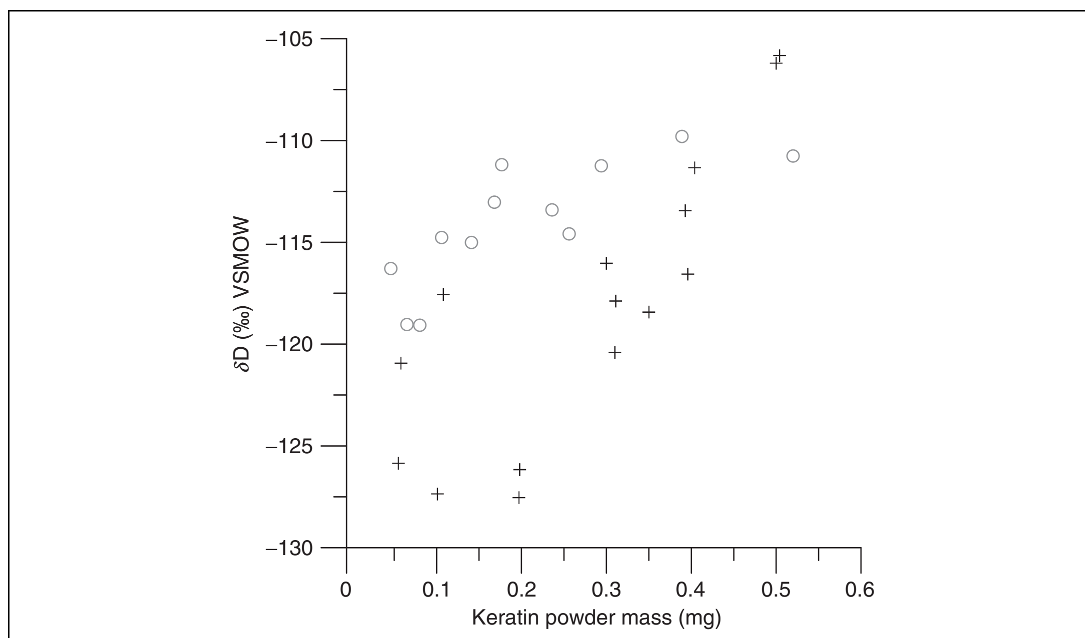

FIGURE 2.4 The dependence of δD results on mass from two different isotope ratio mass spectrometers at two different laboratories on isotopically homogenous keratin powder. Weighing error alone can result in δD changing at a rate of up to 10‰ per 100 μg of sample, well beyond acceptable error. From these data it is clear that sample and references must together be precisely and consistently weighted to a target weight ±10 μg.

applications (Wassenaar and Hobson 1998, Hobson 2002). There are several important and practical reasons why this is the case. First, there is extensive variation in δD (and δ^{18}O) in nature between marine and terrestrial biomes, and across and among continents as a result of climatic and meteorological processes (Dansgaard 1964), and from equilibrium and kinetic isotope effects (Cormie *et al.* 1994, Hoefs 1997). These variations and patterns (which also apply to δ^{18}O) are driven by well-known global meteorological processes and the equilibrium and kinetic isotope effects that occur spatially and that are strongly dependent on key environmental variables (temperature, elevation, rainout, prevailing source of moisture). In short, what this means is that for most continents, there are clear and predictable long-term geospatial isotopic patterns in the global H_2O cycle that are available to be translated into plants via primary productivity (Figure 2.2, see Chapter 4). Finally, the hydrogen isotope range in nature is large (~500‰), and compared to analytical error (less than ±2‰) gives by far the best signal to noise ratio of all of the light stable isotopes. In fact, δD has the sensitivity to resolve geospatial origins by a factor of three to five times better than δ^{18}O due to this factor alone.

B. The Problem of Hydrogen Isotope Exchange

Unfortunately, δD isotope analyses of organic substrates are more complicated than δ^{13}C and δ^{15}N because of the problem of uncontrolled hydrogen isotopic exchange between "labile" organic hydrogen in the sample matrix and ambient atmospheric water vapor (Schimmelmann 1991, Wassenaar and Hobson 2000b, Sessions and Hayes 2005). Although most of the hydrogen in fixed and dynamic tissues are not exchangeable and bound to carbon (C–H), a significant fraction of the total hydrogen, mainly in the form of –COOH, –NH_2, and –SH functional groups, readily exchanges hydrogen atoms with ambient water vapor (Schimmelmann *et al.* 1999). For proteins and keratins, the proportion of

exchangeable hydrogen can be on the order of 15–25% or more of the total hydrogen (Wassenaar and Hobson 2000b, Bowen *et al.* 2005a), depending on temperature and how much of the exchangeable hydrogen is "exposed" by sample tissue grinding versus using whole sample pieces. Stable-hydrogen isotopic measurements of organic samples, however, can only be made on the total hydrogen in the sample. Therefore, unless there is some accounting and correcting for the exchangeable proportion of hydrogen, δD results will be incomparable among different laboratories where δD in local moisture is isotopically different and locally as the air changes seasonally.

C. The Comparative Equilibration Approach Facilitates δD Analyses

In the past, there was little consensus in the way isotope laboratories prepared, measured, and corrected for uncontrolled hydrogen isotopic exchange in organic samples. Nitration procedures aimed at replacing exchangeable hydrogen are useful for few substrates (cellulose, chitin) and require extensive processing of individual samples (Schimmelmann and DeNiro 1986). Nitration was not applicable to keratins and other dynamic and fixed tissues. Steam equilibration with H_2O of known isotopic composition was successful when dual-inlet assays were prevalent, but this method was extremely slow (10 samples per day) and expensive due to high degree of labor by technicians. Several years ago the "comparative equilibration" approach using CF-IRMS was proposed for δD analyses of organic materials for migration research (Wassenaar and Hobson 2003). The key benefits of this approach are comparable results among laboratories coupled with ease of application and rapid and automated sample throughput (200 samples per day), as increasingly demanded by the large numbers of samples typically generated in migration studies.

The comparative equilibration approach takes advantage of the fact that hydrogen isotope exchange between ambient laboratory air moisture and the exchangeable hydrogen in keratins is fast, and will reach full equilibrium by ~96 hours at room temperatures, even when samples are fully weighed and prepared in their silver capsules (Wassenaar and Hobson 2000a, Bowen *et al.* 2005a). The comparative aspect means the inclusion of precalibrated keratin working standards (see below) along with all unknown tissue samples. As the ambient moisture changes its δD, both keratinous samples and references will equilibrate in identical fashion. These "comparatively equilibrated" keratin standards and unknowns are then isolated from the atmosphere (using a zero-blank autosampler) and analyzed together in a single analysis session. This approach follows the principle of identical treatment for stable isotope analyses, whereby samples and working standards are not only identical in their chemical composition, but go through exactly the same preparation and analysis steps (Werner and Brand 2001).

In most laboratories, stable-hydrogen isotope measurements on organic tissues are currently performed on H_2 derived from high-temperature flash pyrolysis and by CF-IRMS (Figure 2.1B). Pure H_2 is used as the sample analysis gas and the isotopic reference gas. A high-temperature EA and autosampler is used to pyrolyze samples to a single pulse of H_2 gas (and N_2 and CO gas). The pyrolysis column consists of a ceramic tube partially filled with glassy carbon chips held at 1250–1350 °C, followed by a molecular sieve GC column at 80–100 °C. The GC column is used to resolve the sample H_2 from N_2 and CO. All δD results are reported in units of per mil (‰) relative to the VSMOW-SLAP standard scale using previously calibrated keratin or organic working standards.

D. The Problem of Organic Reference Materials for δD

The success of the comparative equilibration approach for δD assays relies on the long-term availability and widespread distribution of previously (steam) calibrated keratin and organic tissue working standards. The reason is that intercomparability of results among laboratories and studies is critical for ensuring quality and accuracy of results. While a number of primary and certified organic standards

currently exist (IAEA CH-7, etc), these certified primary reference materials do not contain exchangeable hydrogen, and so are not suitable reference materials for calibrating samples that do have exchangeable hydrogen.

For this reason, three keratin laboratory working standards were previously prepared and distributed among a number of laboratories. These keratins were composed of cryogenically ground and isotopically homogenized chicken feather (CFS), cow hoof (CHS), and bowhead whale baleen (BWB-II) that were calibrated to account for exchangeable hydrogen using an off-line steam equilibration and dual inlet assays as outlined by Wassenaar and Hobson (2003). CFS was obtained from a single batch of chicken feathers from a poultry processing operation located in Wynyard, Saskatchewan, Canada. Approximately 2 kg of feathers were obtained and processed. The CHS (~0.5 kg) was obtained by cutting hoof from a single cow carcass at an abattoir in Saskatoon, Canada. The BWB-II was a powdered whale baleen obtained from Professor Don Schell (retired) from the University of Alaska. All of these keratins were solvent cleansed (2:1 chloroform:methanol solution), air-dried, and cryogenically homogenized to large quantities (>0.5 kg). Sieving to the <100 μm fraction and further blending was required to ensure isotopic homogeneity at the 100 μg level. Sufficient standards were prepared to ensure years of use in a single laboratory. The following results for δD are accepted: CFS = −147 ± 5‰ (VSMOW), CHS = −187 ± 2 (VSMOW), and BWB-II = −108 ± 4‰ (VSMOW). It should be emphasized that these keratins are not primary isotopic reference materials for organic δD analyses. They are a rapidly dwindling supply of unofficial laboratory working standards to be used for comparative equilibration.

Concerted efforts are urgently needed to produce isotopic working standards for δD of keratinous and other organic matrices of ecological interest that are not only suitable for comparative equilibration, but will be available for the long-term need of migration and ecological research. Currently, the range of encountered δD values for keratins in nature greatly exceeds the CHS-CFS-BWB calibration range. This will require a global search and preparation of at least three keratin and organic working standards that meet the following criteria: (1) sufficient quantities to meet the demand of many stable isotope laboratories internationally for at least 10 years, (2) certified stable isotopic homogeneity for δD at the <100 μg level, and (3) a δD isotopic range of at least 200‰, spanning from about +50‰ to −200‰. At the time of this writing, no certified or internationally accepted organic standards currently exist for δD for matrices that have exchangeable hydrogen. A number of isotope laboratories have attempted to widen availability by grounding the results of bulk commercial keratin powders and or other types of local or broadly available keratinous standards to the BWB-CFS-CHS scale (*e.g.*, Table 2.3), or by producing internal working keratin standards based on their own calibration curves. However, this problem has largely been addressed on an ad hoc basis for the short-term needs of a few laboratories or researchers for specific projects. This problem of standardization for organic matrices

TABLE 2.3 Laboratory intercomparison of δD results for powdered keratin samples run over many months using the comparative equilibration approach

	Laboratory 1 mean	Laboratory 1 SD	*n*	Laboratory 2 mean	Laboratory 2 SD	*n*
Moose hair	−163.5	2.1	84	−164.7	2.4	54
Vole hair	−106.2	2.3	85	−105.1	2.6	21
Human hair (IAEA-085)	−70	2.4	84	−68.7	2.6	51
Keratin (Spectrum)	−117.2	1.9	103	−116.1	2.7	106

The results reveal excellent repeatability and comparability of results among latitudinally separated laboratories. All samples were treated as unknowns and normalized using CHS, CFS, and BWB keratin laboratory standards. (Courtesy T. Jardine and R. Doucette, unpublished data.)

with exchangeable hydrogen will become pressing in the near future as interest in using δD in migration and forensic research diversifies and increases, and the demand by new laboratories increases and isotopic approaches move out of the realm of academics to become a mainstream tool for decision makers. Further, directly comparable results will be required as data from numerous migration studies accumulate over time and the potential for meta-analysis becomes possible. The problems of reference materials for organic-H samples currently remain a formidable and outstanding challenge.

A number of laboratory intercomparison tests on discrete samples and homogenized keratin powders have been conducted using the comparative equilibration approach. The data reveal that laboratories can consistently achieve comparable results on homogenized standards to within an acceptable error of ±2‰ for δD. Another example of excellent agreement obtained by using comparative equilibration is shown in Figure 2.5. Here independent laboratory intercomparison of δD assays was made on single feathers from 18 individual songbirds using the comparative equilibration procedure approach. All laboratories used the keratin laboratory standards described below. However, no attempt was made here to homogenize the feathers (*e.g.*, subsamples were cut from the same feather at each laboratory with no consideration of location on the feather), and because the samples were not examined for isotopic heterogeneity the average δD range per sample was 7‰, which is remarkably good. An excellent example of a long-term test from two laboratories using the comparative equilibration approach for δD is shown in Table 2.3.

E. Stable Oxygen Isotopes

Stable-oxygen isotopes ($\delta^{18}O$) may also be considered for global-spatial analyses in migration studies for precisely the same reasons as δD noted above (Hobson *et al.* 2004b, Bowen *et al.* 2005b). A key advantage to $\delta^{18}O$ is that organic materials like keratins have no exchangeable oxygen, circumventing the problem and need for comparative equilibration approaches. The main disadvantage, however, is that the $\delta^{18}O$ range for tissues in nature is relatively small (~15‰ range) and the analytical error by CF-RIMS methods currently remains comparatively high (±0.5‰), resulting in a lowered precision in resolving geospatial information. However, there is still a paucity of $\delta^{18}O$ data on migrant tissues, and the use of oxygen isotopes, especially in concert with hydrogen isotopes, remains promising, but largely unexplored.

In most modern laboratories, stable oxygen isotope measurements on organic tissues are performed on CO derived from high-temperature pyrolysis and by CF-IRMS (Figure 2.1B). Pure CO is used as the

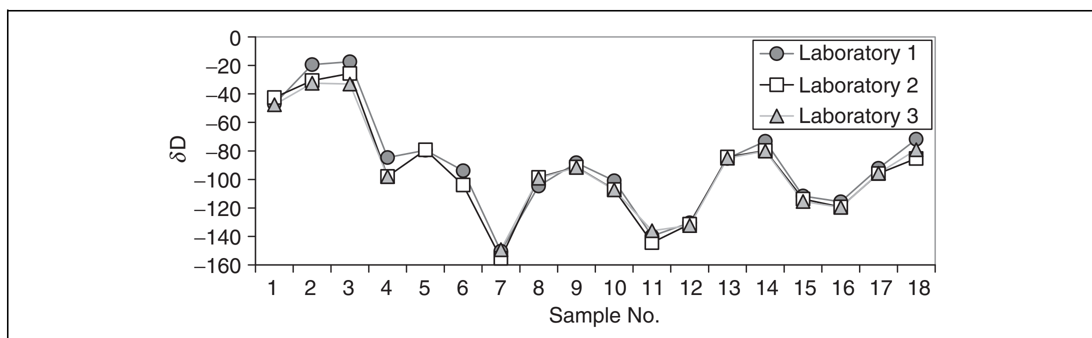

FIGURE 2.5 Independent laboratory intercomparison of δD assays on 18 individual songbird feathers using the comparative equilibration procedure approach. No attempt was made to homogenize the feathers (*e.g.*, subsamples were cut from the same feather at each laboratory with no consideration of location on the feather), and because the samples were not screened for heterogeneity, the average δD range per sample was 7‰ (additional data from T. Jardine and R. Doucett).

sample analysis gas and the isotopic reference gas. A high-temperature EA and autosampler is used to automatically pyrolyze samples to a single pulse of CO gas (and N_2 and H_2 gas). The pyrolysis column consists of a ceramic tube and glassy carbon tube insert, filled to the hot zone with glassy carbon chips held at >1350 °C, followed by a molecular sieve GC column at 40–60 °C. The GC column is used to resolve the sample CO from H_2 and N_2. All $\delta^{18}O$ results are reported in units of per mil (‰) relative to the VSMOW-SLAP standard scale using two newly available primary and certified organic reference materials (IAEA Benzoic Acid Standards). These primary standards have $\delta^{18}O$ values of +23.1‰ VSMOW (IAEA-601) and +71.4‰ VSMOW (IAEA-604). It should be noted that the $\delta^{18}O$ of biological tissues from migrants range between +10 and +20‰, below this primary reference calibration range, and while this calibration is highly linear, the development of keratin references for $\delta^{18}O$ spanning the natural range is encouraged.

The reader may have noticed that the CF-IRMS analytical methods for H_2 and CO described above are virtually identical, and because H_2 is produced during the same thermochemical reduction reaction as for CO, preparative systems have been tweaked in order to obtain $\delta^{18}O$ and δD on a single tissue sample. While this appears to be an attractive option, the primary challenge is lowered sample throughput and a more complicated analytical setup involving multiple reference gases and IRMS peak jumping. The analysis time for dedicated δD assays is rapid at about 3 min per sample (20 samples/hour), but when combined to obtain both $\delta^{18}O$ and δD, the analysis rate decreases to 8–12 min per sample. Given that $\delta^{18}O$ is expected to provide correlative results to δD with lowered precision, this approach may be questioned as to its practical utility. However, it should be noted that additional information may be obtained from $\delta^{18}O$ because there are more oxygen sources (air O_2, H_2O, dietary O) and sinks (H_2O, CO_2) in biological dietary systems compared to hydrogen. This additional complexity (and associated isotope fractionations) may prove to be of use in the future with more research.

IV. LOCAL-SPATIAL ISOTOPES

A. Stable Carbon and Nitrogen Isotopes

Stable-carbon and -nitrogen isotope assays are considered local-spatial analyses, but may be very useful in further delineating migratory populations or indicating type of habitat (see Chapter 1). There may also be larger-scale spatial patterns (Chapter 4). The analysis of carbon and nitrogen isotopes are almost universally considered routine among stable isotopes laboratories.

The analyses are composed of coupled $\delta^{13}C$ and $\delta^{15}N$ isotope measurements on the same organic tissue sample performed on CO_2 and N_2 derived from flash combustion and by CF-IRMS (Figure 2.1A). Pure CO_2 and N_2 are used as the sample analysis gas and the isotopic reference gas. A standard EA and autosampler is used to quantitatively combust samples to a pulse of CO_2 and N_2 gas (combustion H_2O is scrubbed out with a trap). The oxidation column consists of a quartz tube partially filled to the hot zone with chromium oxide held at 1050 °C, followed by reduction column filled with copper (to reduce NO_x to N_2) held at 600–800 °C, and then a packed GC column at 35–50 °C. The GC column with a thermal conductivity detector is used to quantify and resolve CO_2 from N_2. Isotope peak jumping is used on the IRMS to switch between nitrogen and carbon isotopes. All $\delta^{13}C$ results are reported in units of per mil (‰) relative to the PDB using newly available primary certified organic reference materials (L-glutamic acids). These standards have $\delta^{13}C$ values of +37.6‰ VPDB (USGS-41) and −26.4‰ PDB (USGS-40) and $\delta^{15}N$ values of +47.6‰ (AIR) and −4.5‰ (AIR), respectively. The standards have similar C/N ratios as proteinaceous tissues, and fully span the isotopic range encountered for migrant tissues in nature.

B. Stable Sulfur Isotopes

Stable sulfur isotope analyses for use in migration research are still relatively rare, and can also be grouped into the local-spatial analyses category. The range of $\delta^{34}S$ in nature is very large (spanning > 150‰), although it should be noted that the $\delta^{34}S$ of the seawater sulfate pool in the oceans is essentially invariant (+21‰ VCDT), and that terrestrial systems tend to have negative $\delta^{34}S$ compositions, making S isotopes useful for detecting or distinguishing between marine and terrestrial dietary sources.

For the tissues of interest in migratory organisms, sulfur is primarily in the form of S-bearing amino acids (*e.g.*, cysteine). As mentioned previously, the best precision $\delta^{34}S$ analyses are still made using conventional dual-inlet IRMS. This requires a lengthy and costly preparative process that involves oxidative and quantitative conversion of total S in the tissue sample to an appropriate analyte (*e.g.*, $BaSO_4$, Ag_2S). These matrices are then converted to SO_2 or SF_6 gas for analyses on a gas source dual-inlet IRMS (Mayer and Krouse 2004).

More recently, CF-IRMS methods have been developed, with the advantage of lower cost and higher sample throughput. Similarly to C + N, tissue samples that have been converted to $BaSO_4$ or Ag_2S are combusted in an EA, and the SO_2 produced is chemically purified and separated from CO_2 and N_2 using a GC column (Mayer and Krouse 2004). This analysis is made on $BaSO_4$ or Ag_2S and so still requires preparative sample conversions. The precision for this method is about ±0.2‰, and requires about 15 min per sample (<100 μg S).

There is also immense interest in the direct EA combustion of tissue and keratin samples to SO_2 without conversion to $BaSO_4$ or Ag_2S. This approach is achievable but is complicated by high C:S ratios of most samples that generate comparatively large amounts of CO_2 and H_2O and subsequent separation of the undesired combustion gases by GC. However, recent innovations and adaptations have enabled concurrent C + N + S assays (Fry 2007). The general consensus is that direct combustion and analysis is only feasible where samples have >0.1 wt.% S (requiring 2–5 mg of keratin), and the resulting $\delta^{34}S$ precisions will be on the order of ±0.5‰, although this may be sufficient for some studies. Analytical vigilance is required because the $\delta^{18}O$ of the SO_2 produced changes as reagents are depleted, requiring dynamic oxygen corrections to obtain correct $\delta^{34}S$ values (Fry *et al.* 2002).

A further complication is that keratin working standards for organic-S are currently nonexistent, and large discrepancies occur when comparing inorganic standards to organic samples. Ideally, a set of keratin (*e.g.*, cysteine) or organic S standards would need to be developed that had been previously converted to $BaSO_4$ or Ag_2S and measured by highest precision dual-inlet analyses, and that span the natural $\delta^{34}S$ range. However, given the large sample size requirements (2–5 mg) and the need to include many references within a CF-IRMS autorun, it is likely that calibration and organic reference material development will remain within the realm of a few specialized individual laboratories.

C. Stable Isotopes of Trace Elements: $^{87}Sr/^{86}Sr$

Another type of local-spatial stable isotope analysis that has been used in animal migration research is the isotopes of the trace element strontium ($^{87}Sr/^{86}Sr$) (Chamberlain *et al.* 1997). One of the key advantages of "heavy isotopes" compared to the light stable isotopes of the previous sections is that there is usually little or no isotopic fractionation from geologic sources through the food web and into tissues (Blum *et al.* 2001). Hence, Sr isotopes among all species in local food webs are expected to show fidelity to the $^{87}Sr/^{86}Sr$ ratios of the underlying bedrock or soil. Thus, $^{87}Sr/^{86}Sr$ variations in and among landscapes and continents may be distinctive, but these can be highly variable at small scales in areas of complex geology and are not *a priori* suitable for continuous interpolation (Chapter 4). As with S, the $^{87}Sr/^{86}Sr$ distinctions between terrestrial and marine environments are very clear (Kennedy *et al.* 2005).

Hence, similarly to the other local-spatial isotopes, Sr isotopes are best used in conjunction with δD or $\delta^{18}O$ or other light isotopes in a multiisotope approach.

One important difference between the light isotopes and Sr isotopes in fixed tissues like feathers is that Sr occurs as a trace element at exceedingly low concentrations (*e.g.*, <20 μg Sr/g of feather) (Font *et al.* 2007). The only exception is calcium-bearing tissues that contain much higher Sr concentrations (*e.g.*, bones, teeth, otoliths) (Blum *et al.* 2001). For noncalcium-bearing tissues, this means the potential for extraneous contamination (*e.g.*, entrained dust, handling, background) is extremely high and must be quantified and requires rigorous cleaning and QA/QC procedures (Font *et al.* 2007). There is no standardized agreement on how this should be done.

Further, trace Sr concentrations means that up to 25 mg of sample may be required. All samples will require prescreening for total Sr content by inductively coupled-plasma mass spectrometry before preparative procedures for isolating Sr are begun. Further, extensive wet chemical or microwave digestions and selective ion chromatography are required to isolate Sr for isotopic analysis. Difficulties arise with organic samples in being able to fully extract all available Sr. Sr isotopic ratios are determined using thermal ionization mass spectrometry and using the NBS-987 standard. Similar to S, no organic-Sr isotope standards exist.

The issue of intrasample variability has not been rigorously tested for Sr isotopes, although differences in concentrations and isotope ratios between the rachis and vane of individual feathers have been reported (Font *et al.* 2007), although these differences were comparatively smaller than potential geospatial differences. From the preceding section, this could be a major issue for slowly growing tissues of birds or animals that are moving among areas having variable $^{87}Sr/^{86}Sr$, especially given the large sample requirements. For example, when we consider that feathers of small birds only weigh 10–20 mg, or hair strands 1 mg or less, sample tissue pooling may be required. The example of the eagle feather heterogeneity and the implications for confounding Sr isotope interpretations regarding spatial interpretations are critical to consider.

Finally, Sr isotope analyses are costly in comparison to the light isotopes. Current costs do not include the costs of prescreening for Sr concentration nor the labor involved in clean-laboratory wet chemical digestions. This, and given the large sample size requirements, it is likely that Sr isotope assays may remain a specialized assay for projects where the value of the anticipated outcomes exceeds the analytical cost considerations.

V. CONCLUSIONS

In this chapter, some of the fundamental and practical aspects of stable isotope analyses for use in animal migration research have been outlined for those interested in applying these isotopic tracers to research questions. The researcher is strongly encouraged to be discriminating and critical in the application of stable isotopes, and to carefully consider all angles of sampling and selection of isotopes to answer the research question at hand. Only when utmost confidence in the stable isotope analyses is fully assured, then the researcher can move on to the task of making the kinds of spatial interpretations outlined in the subsequent chapters.

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