Chapter 3
Practical Considerations

3.1 Sample Collection and Tissue Preparation

In assessing the value of stable isotopes for tracing animal migration the O and H isotopes in the environment are “global spatial” in their resolution as the isotope are related to hydrological and meteorological processes that are seasonally and spatially predictable over many years on a regional, continental and global scale. This enables interpolation into regions where no long term data are in existence (Bowen et al. 2005). Isotopes of C, N, and S are local spatial in character as they do not vary over the landscape as H and O. investigations into large scale migrations is therefore most fruitful using H and O isotopes, while the resolving power at the local habitat level can be improved with data on the local spatial isotopes.

Two types of samples that can be used for analysis; the first type is fixed tissue consisting of body material that once formed is metabolically and therefore by extension, isotopically inert. This material will give an indication of the isotopic composition pertaining in the local environment at the time that the tissue was formed. Tissues of this type include keratinous material of claws, nails, hair and feathers. Bird feathers are particularly well-suited for use in tracking migration as the fully formed feather vanes and rachis do not change chemically or isotopically as the bird moves away from the site of feather formation, on its migration pathway. The feather is often grown over a short period of time at a specific site such as a breeding site or wintering ground and so will retain the isotopes present in the diet at that location. It is essential that the researcher has adequate knowledge of the ecology and physiology of the birds being studied. For instance, slowly growing feathers or claws that grow as an animal migrates, ingesting different diets en-route means that a growing feather will record a variation in isotope as it progresses. In bald eagles where flight feathers grow as they migrate from the northern to the southern USA, there is intrasample variation where the feather show a negative δH value in the oldest part of the feather at the wing tip, while at the base of the feather the δH values are more positive. In this case the feather is not equivalent to a fixed

The original version of this chapter was revised: The chapter was inadvertently published with materials that were reproduced or modified without permission for the table headings in Table 3.1 which has been updated now. The correction to this chapter is available at https://doi.org/10.1007/978-3-319-28298-5_4

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tissue and provides instead a dynamic spatial recorder. In the case of a migratory bird in which the feather is formed at the natal location there would be no significant intrasample differences in δH that would indicate where the bird had moved, even though captured far from the region in which the feather was formed. Archived or museum material is also a potential source of investigation for stable isotope analysis, but care must be taken in using such material if it has been preserved or contaminated in some way whilst in storage.

The second type of sample material is dynamic tissue, metabolically active tissues in which the stable isotope composition reflects the current, active dietary habitat. These tissues are the blood, muscle, liver etc. There have been few studies using such tissues to plot migratory connectivity. Most studies have concentrated on measuring $\delta^{13}C$ and $\delta^{15}N$. The turnover rate for isotopes in different tissues varies from a few days in liver and blood, to weeks in muscle to a lifetime in bone collagen.

An important consideration is whether the sample can be obtained non-lethally by plucking or cutting feathers, nails or hair so as not to inconvenience the animal. Animal care guidelines should always be exercised when sampling live animal in the field. Collecting a sample for analysis is a major undertaking and it is essential that due consideration has been given for the animal's biology and ecology to ensure that appropriate samples are collected.

3.1.1 Questions of Sample Heterogeneity

For birds, flight feathers or contour feathers can be sampled for isotopes, but to if replicate samples are obtained what extent would isotopic values vary from the isotopic range that defines the bird's spatial distribution? There will be individual and population level intersample isotopic heterogeneity. There could be minor variation in values between contour feathers grown by an individual at a single location. There would as also be intersample isotopic variation for the same feathers obtained from a population of birds that grew the feathers at the same location. This sort of heterogeneity is usually much less than the large scale geospatial isotopic patterns. Intrasample isotopic heterogeneity is the variance seen in a single discrete sample; an example of this is as mentioned above for the bald eagle.

3.1.2 Preparation of Feather Samples

Samples collected from the field are likely to be dirty and greasy. Dirt can be removed by washing in distilled water but surface oils on feathers will need to be removed by organic solvents since the composition of H in the oils differs from that contained in the feather keratin, thereby giving false data on isotope composition.

Table 3.1 Procedure for preparing feather samples for δD stable isotope analysis. Reproduced with Permission from Wassenaar (2008)

Procedure
1. In order to remove surface oils clean feathers in 2:1 v/v chloroform/methanol by soaking for 24 h and rinsing twice in same mixture. Air-dry in a fume hood for at least 48 h
2. Cut off a small amount of feather vein, not the central rachis, for analysis. Always cut samples from same region on different feather samples to ensure consistency (e.g. sample at tip). Cut feathers using stainless steel scissors and forceps
3. Clean instruments using methanol and tissue wipes and allow to dry. Do not use acetone
4. Clean and calibrate microbalance
5. Tare a silver capsule* handling it only with instruments, remove and set on a metal surface. Use the smallest size that will safely hold a sample (3.5 × 5.0 mm)
6. Transfer a small amount of feather to capsule using forceps. Reweigh and add or subtract material until the target sample weight of 350 ± 10 μg is achieved. Reference materials must be weighed to a comparable elemental mass as the samples
7. Seal the capsule by crimping the end shut with straight edge forceps and then fold down tightly as if rolling down a bag. Use forceps to gently compact the capsule into a small tight cube or ball. There should be no loose sides, stray edges or feathers poking out. Flattened samples can jam the autosampler
8. Record sample weight and sample name in an Excel spreadsheet conformed in a 96-well ELISA template format. Put capsule in appropriate position in ELISA plate. Clean all instruments with methanol and tissue wipes after completing each sample. Put lid on ELISA plate and secure with rubber bands or tape to ensure samples do not jump out of wells in later handling. Record sample name etc. and weight and its position in the ELISA plate. Transfer information to an isotope laboratory submission form

*Silver Capsules must be used for analysis of $\delta^{18}O$ and δD. They can be obtained from: (1) Silver capsules for solid samples, 3.5 × 5 mm, 041066 240-054-00; Costech Analytical Technologies Inc.; 26074 Avenue Hall, Suite 14; Valencia, CA 91355; USA or (2) D2003 – Silver Capsules Pressed 3.5 × 5 mm; OEM Parts: E12050, 041066, SC1082; Elemental Microanalysis; 1 Hameldown Road; Okehampton, EX20 1UB; UK

Samples collected for stable isotopic analysis must be stored properly both before and after preparation to ensure there is no degradation. Many laboratories can provide a service but if the researcher wishes to reduce costs then cleaning and weighing can be undertaken. This is not to be undertaken lightly as accurate weighing to ±10 μg is required using analytical microbalances. A recommended procedure for cleaning and preparing feather samples for analysis is detailed in Table 3.1.

In order to be successful, the comparative equilibration approach for SIA relies on the long term availability of keratin and organic tissue working standards because it is essential that there is rigid QA and comparability of results between laboratories to ensure accuracy of results. Although there are some primary, certified organic standards – e.g. IAEA-CH-7 which has been checked for isotopic homogeneity and has a recommended $\delta^{13}C$ value determined by international calibration – they do not contain exchangeable hydrogen and are not suitable reference material for calibrating δD in feathers. Three keratin standards have been prepared at the laboratories of Environment Canada, Saskatoon, Saskatchewan, Canada from cryogenically ground and isotopically homogenized chicken feathers (CFS), cow hoof (CHS) and bow-

head whale baleen (BWB-II). They were calibrated according to Wassenaar and Hobson (2003). CFS was obtained from a single batch of 2 kg of chicken feathers; CHS was prepared from a hoof of a cow obtained from an abattoir and BWB-II was obtained from the University of Alaska. All of the keratins were solvent cleaned using methanol/chloroform, air-dried and cryogenically homogenized to about 0.5 kg. They were passed through <100 μm sieves and further homogenized to ensure isotopic homogeneity at the 100 μg level. These standards were sufficient to last for several years of use in a single laboratory. The results for δD were CFS, −147 ± 5 %, (VMSOW), CHS, 187 ± 2 % (VMSOW) and BWB-II, −108 ± 4 % (VMSOW).

It is essential that isotopic working standards for δD in keratinous materials are produced to provide for the long-term needs of migration and ecological research. Such materials would need to be: (i) in sufficient quantities to allow their use by many different laboratories working on stable isotopes for up to 10 years; (ii) be certified for δD homogeneity to <100 μg and (iii) be certified for an isotopic range of at least 200 %. This need for standardization will become more important as studies on stable isotope for migration and other reasons increases to become a mainstream analytical tool.

3.2 Other Stable Isotopes for Migration Research

Stable oxygen isotopes $\delta^{18}O$ could also be used for migration research, especially as keratins have no exchangeable oxygen so there is no need for comparative equilibration procedures. The disadvantage is that the $\delta^{18}O$ range for tissues is small (approx. 15‰) and the analytical error for measurements are high so that precision regarding geospatial information is lost. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes can also be used in migration studies for local-spatial analysis, to refine populations or indicate habitat type.

3.2.1 Sampling Instructions for Water $\delta^{18}O$ and $\delta^{2}H$ – Rivers/Lakes/Groundwater

Materials

Twenty five to sixty milliliter of HDPE (plastic) bottles with caps. Only a few ml of water is required for the analysis in the laboratory, but very small bottles are awkward for field staff to clearly write labels on so for logistical reasons use bottles of a capacity of 2 ml. Do not use glass bottles since they can shatter in transport. Evaporation and leakage during storage must be avoided. This can occur by mishandling or from loose caps.

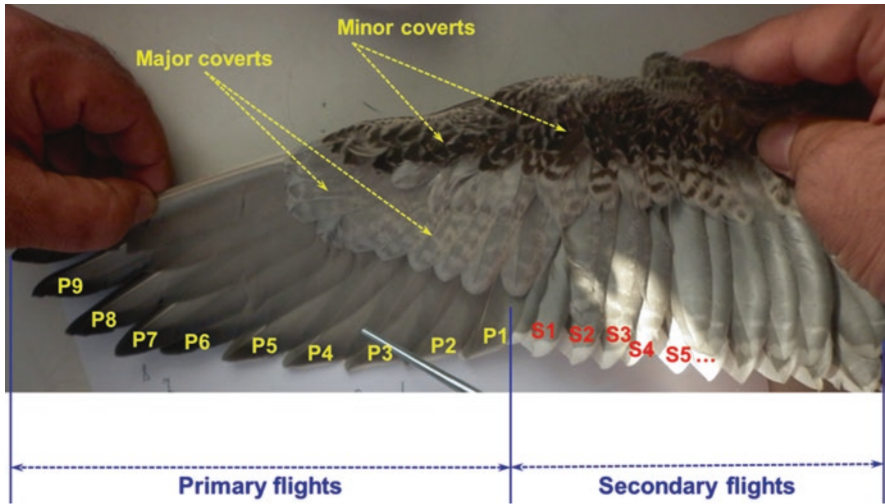

Fig. 3.1 Nomenclature of wing feathers of birds (Sample from the CRP D32030)

Method

Fill sample bottle with raw river, ground or lake water to overflowing. Cap tightly. Before sampling ensure the sample bottle is clearly and labeled with site or station, and dated using permanent ink. Ensure label does not smear. Do not freeze. Store at room temperature. No preservation is required. Samples should be shipped ground or air with adequate protection (box with foam chips, bubble wrap) and sent to the isotope laboratory of choice.

3.2.2 Sampling Instructions for Bird Feathers for Use in SIA

Materials

Steel laboratory tweezers.
Steel Scissors
Paper Envelope – Sealable paper envelopes are best to use for storage of bird feathers. Stored feathers should not be wet. Sealable plastic bags can be used as well.
Long wing feathers: Cut long feather from wing (Fig. 3.1) with pair of scissors (preferably P1 or P2 feathers).
Down feathers: Pluck down feathers (5–10) with tweezers from wing, or breast.
If possible check bird body for Blood keels (feathers in growth) (Fig. 3.2): Pluck blood keels (down feathers in growth) with tweezers from breast, or cut one large blood keel (large feather in growth) with pair of scissors from wing. Feathers

Fig. 3.2 Blood keel feathers in growth

should be dry. No visible infestation of feather inhabiting parasites. Put all feathers from one individual bird in the same envelope. Close, seal and label the envelope. Note which samples it contains (large feather, down feathers, blood keel(s); bird species, [if possible also: sex, growths status (juvenile, adult)], date and location of sampling, name of person sampling. The samples can be stored at room temperature. No preservation is required. They should be shipped by ground or air with adequate protection (bubble wrap envelope) and sent to the isotope laboratory of choice.

3.2.3 *Sampling Wild Birds in the Field*

A comprehensive guide and strategy for sampling wild birds is given by Whitworth et al. (2007) and that manual should be referred to in dealing with HPAI (http://www.fao.org/docrep/010/a1521e/a1521e00.htm). The following section highlights a few relevant points.

On arrival at the site, evaluate the extent of the mortality, if appropriate, including number of birds, species directly involved, other wildlife or domestic animals involved, and geographic range of mortality. This information should be recorded in a Sick or Dead Bird Sample Collection Log. In addition to preparing for animal sample collection, also consider collection of other environmental samples including water, soil, vegetation or other elements from which it might be possible to identify HPAI virus. If it is possible to get a GPS locations that characterize the extent of the die-off area, this is preferable to a general verbal description.

Wear the appropriate level of personal protective equipment, based on the situation being investigated. Try to minimize direct contact with dead birds and always keep animals away from the face, wear vinyl or latex gloves if handling a dead bird.

The best method for collecting a dead bird is to invert a plastic bag around your gloved hand and then surround the animal with the bag so that the bird is not directly touched. Seal the bag tightly (double bag if required for strength and cleanliness) and clearly and indelibly label the bag with an Animal Identification Number (which must match the number entered in the Sick or Dead Bird Sample Collection Log), species, date, time and location. If more than one species has been affected, collect several specimens of each for diagnosis. In general, carcasses of birds that have been dead for less than 24 h (fresh carcasses) are sufficiently adequate (moribund or viraemic birds are best) for diagnostic purposes. In colder climates, carcasses may last in relatively good condition for longer periods of time; in warm climates, carcasses will decompose faster.

When possible, fresh carcasses should be refrigerated (NOT frozen); a decomposing carcass is desiccated, bloated, green, foul smelling and has feathers that pull out easily. To increase diagnostic value, fresh carcasses must be transferred to the appropriate veterinary or pathology facility and examined as soon as possible. In field settings and/or far from appropriate diagnostic facilities, collect samples on site and place them in an ice chest or cooler. Keep carcasses away from refrigerators used for animal or human food.

3.2.4 Sampling Strategy for H5N1

For each affected species, select up to 3 birds that have most recently died (less than 24 h) or more if practical, up to 3 sick birds (suffering respiratory, neurologic or gastrointestinal disease or moribund) and up to 3 apparently healthy birds in direct contact with currently sick birds. If possible, also conduct a survey of other live birds that share the same habitat (cloacal swabs and/or tracheal swabs only). Priority should be given to birds that share wetlands with affected birds since the main mode of transmission of AI virus is probably faecal contamination of water, shores or banks. It is best to collect as many carcasses as possible and to place them in a central location for processing. Removal of dead birds from the site may also help prevent secondary contamination of scavengers or the environment. It is very important to complete the Sick or Dead Bird Sample Collection Log as carcasses are being collected and processed. If possible, try to collect and examine sick animals as well as newly dead birds, making sure that you have the appropriate permits to capture live samples. If there are too many dead to be able to individually bag and label, try to bag or examine well preserved animals that will be most useful for diagnostic purposes, and keep these separate from decomposing carcasses. If possible, transport carcasses (in sealed bags) in a space well separated from the occupants of the vehicle. If you are working in a remote area, you may have to perform field necropsies on site.

Recommended ornithological information to be collected during surveillance programmes or field assessment of wild bird mortality events.

All birds from which samples are taken should be identified to species. Where clearly distinguishable subspecies or discrete populations exist, as for some geese, this information should also be collected and reported. Age and sex should be recorded wherever possible. Close collaboration with ornithologists in the capture and sampling of live birds not only facilitates identification of birds but also gives the opportunity to collect additional information on the sampled live birds (such as weight, age, sex and condition), which are important to developing a better understanding of viral ecology and epidemiology. Standard protocols exist for the collection of such data through national ringing schemes [details of which are available for Europe, for example, via EURING (www.EURING.org)]. Recording individual ring numbers in the reporting spreadsheet provides a means of accessing these data for future analysis. To provide an audit of identification, it is highly desirable that a clear digital photograph is taken of each sampled bird (especially those found dead and/or not identified by ornithologists) and stored at least until confirmation of laboratory tests. In order to facilitate identification of bird species (which can sometime vary in quite minor plumage details, especially at certain times of the year), photographs should be taken according to the guidance given in Sect. 3.2. In the event of positive results, further examination of such photos can provide additional information on the age and sex of the bird, in addition to proving the identity of the species beyond doubt and thus allowing the case to be correctly put into context. To facilitate this, each individual bird should be given a code that is used on the cloacal and oropharyngeal swabs taken, and this code should be on a piece of card that is visible in each photograph taken.

Especially related to sampling in the vicinity of outbreaks, it is desirable to collect a range of contextual information so as to better understand the viral epidemiology of H5N1 HPAI in wild bird populations. Such information should include:

- date of sampling, clear locational and descriptive data about the catching site, ideally GPS coordinates, including habitat description (e.g., lake, river, village pond, fish farm, etc.) and distance to human settlement, agricultural land, and poultry farms; it may also be useful to include details about the season and relate this information to the natural behaviour/cycle of the affected birds, e.g., moulting, premigration, during migration, etc.;
- record of the numbers of each species of other live birds in the sampling area that were not sampled;
- if available, records of bird movements (arrivals/departures) that occurred at the sampling site prior to the sampling;
- assessment of the numbers of each species of live bird in the sampling area that were not sampled but that were showing signs of ill health; and
- given that birds of some species (such as Mallards-Anas platyrhynchos) can occur either as wild birds that are able to move between sites or occur in a feral state, habituated to foods provided by humans, distinguishing between these categories would be useful. Sometimes the presence of unusual plumage patterns, indicating domestication, is useful in this respect.

Taking photographs of dead birds for identification purposes

Different bird species are identified by differing characteristics, so it is difficult to provide universal guidance applicable in all situations. However, the following is a minimum standard that should be followed. All wild birds collected for analysis for HPAI should have digital photographs taken as soon as possible after collection. The bird should fully fill the photograph and wherever possible include a ruler or other scale measure. Each photograph should be taken at the highest resolution possible and if the camera has a 'date stamp' feature then this should be enabled so that the image is saved with a time reference – this may help verify the sequence of images taken at a site on a day. Images should be downloaded to a computer as soon as possible and information about location and date added to the file properties.

Photographs should be taken of:

- The whole bird, dorsal side, with one wing stretched out and tail spread and visible;
- The head in profile clearly showing the beak;
- Close-up photos of the tips of wing feathers, as these can often determine whether the bird is an adult or a juvenile (bird in its first year); and
- Ideally photographs of both dorsal and ventral views of the bird, as photos of the upper and under surfaces of the wing and spread tail will facilitate aging and sexing of birds

Any ventral photographs should show the legs and feet (since leg color is often an important species diagnostic). If any rings (metal or plastic) are present on the legs, these should be photographed *in situ* as well as recording ring details. Any conspicuous markings/patterns should also be photographed.

At certain times of the year, such as late summer (July–August in the northern hemisphere) many waterbirds, especially ducks and geese, undergo moult and can be especially difficult to identify by non-specialists. At such times clear photographs are especially important to aid identification of (duck) carcasses. The patch of colour on the open wing (called the "speculum") is often especially useful. The identification of young gulls at any time of the year is also difficult and typically they will also need to be photographed and identified by specialists.

Photographs should be retained, linked to an individual specimen, at least until laboratory tests are returned as negative for avian influenza. A unique code or reference number that is the same as the code or reference number of any samples taken from the birds should be visible in each photograph so as to link samples and photographs. Photographs can be used immediately if identification of the species of bird is in any doubt, and for subsequent checking of the identification if necessary.

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