Sample Collection Protocol for Stable Isotope Analysis

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Note that collection of blood, tissue, bone, and egg samples requires a research permit from the country in which the research is being conducted. In addition, all *Cyclura* species are CITES Appendix I listed. This means that you must have both an import and export permit when moving these samples from one country to another. The IUCN SSC Iguana Specialist Group has a blanket US CITES import permit for *Cyclura*, which can be used upon request and by authorized users. The format for obtaining CITES export permits varies and must be obtained from the respective country. *Iguana* species are CITES Appendix II listed and thus only require an export permit when moving samples, although if importing to the United States, a USFWS 3-177 importation declaration form is required. Plant material may also require specific permits and should be discussed with the appropriate in-country authorities.

Supplies

Examples of containers used for sample collection: clean scintillation vials, Eppendorf tubes, microfiber filters, Vacutainers, and Whirl-pak bags are some[5].

Background

There are many ways to study wild animal foraging ecology and habitat use patterns, yet there are drawbacks to many of these as they can be time and labor intensive and/or provide only a snapshot of an animal's most recent diet[1]. Stable carbon ($^{13}C/^{12}C$ or $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$ or $\delta^{15}N$) isotope analysis of predator and prey tissues offers a method whereby animal foraging ecology can be estimated over variable temporal scales with minimal disturbance, labor, and cost. Stable carbon isotopes from animal tissues reveal dietary carbon sources allowing for the distinction between marine and terrestrial primary production, plant and animal diet components, and C_3 and C_4 plants, among other things. Stable nitrogen isotopes from animal tissues largely reflect animal trophic position as the $\delta^{15}N$ values from organisms increase predictably with increasing trophic levels[2,3]. Stable isotope (SI) analysis can also provide a wide range of temporal data as isotopic turnover varies depending upon the protein turnover of a particular tissue.

Procedure

Always try and include any dietary item that your organism had access to and is likely to eat (e.g., an animal that eats birds has access to bird beaks, feathers, legs, and sometimes bird nests with eggs, as well as the bird's body tissues).

To prevent contamination of your sample, use aseptic techniques and clean storage containers. Keep in mind that all samples can be washed upon return to the laboratory, so perfectly clean field conditions are not necessary.

I. Plant Samples

Vascular Plants

1. Collect vascular plants in the field and keep material cool (or frozen) until processing (see pressing instructions below).

2. Living green blades, below-ground biomass, and detrital samples of vascular plants should all be considered for separate isotopic analyses as isotope values can vary among plant parts within a single plant. These types of materials should not be combined[6].

Non-vascular Plants

a. Lichens

1. Collect lichens directly into paper bags on which field collection numbers, substratum, and location can be written.
2. Some bulky lichens (e.g., *Peltigera*) will need moistening, then light pressing, as they are drying.
3. Dry lichens thoroughly as soon as they are brought back to base camp. Specimens may be spread out to dry using any available gentle heat source. If there is no heat source available, spread out the specimens in a warm, well-ventilated place until they are completely dry.
4. Once dry, place lichens in packets. The packets can be stored upright in a shoe box. Pack the packets in the box so you can easily insert two fingers between the packets and the end of the box so the specimens will not be crushed.

b. Marine Algae

1. Small specimens may be partially dried in the sun but should be placed between sheets of newspaper for further drying.
2. Large specimens may be partly dried in the sun and when they are about the consistency of leather they should be rolled up and placed in a box for further drying.
3. It is useful to document tide level (upper, middle, or low intertidal or sub-tidal), exposure to sea, currents and type of substrate data.

Figure 1. Plant press with straps[8]

Pressing the Specimens

Materials needed:

- 2 pieces of plywood 46 x 31 cm (can be solid plywood or wood grid).
- Newspapers, approximately 60 x 44 cm that are folded in half to be used as collection sheets.

- 10 - 30 sheets of cardboard cut the same size. Edges must run larger than the width of the cardboard sheet.
- 2 belts or ropes, used to tighten the press.

1. Place plants (whole) in the folded newspaper sheets and insert these between the cardboard sheets.
2. These cardboard sheets are necessary for protection of the plant specimens, as a spacer in building up the plant press, and especially, as a means for ventilating water vapor exiting the plants in the drying process and for conducting heat internally.
3. The cardboard sheets are in turn held between the outer plywood pieces (like a multi-layered sandwich). The belts, or ropes, are then used to tighten the entire "package", pressing the samples.
4. Place these packages between the two outer plywood pieces (the plant press). Add more packages until a reasonable height. Fasten two straps around the press and tighten to press the plants. The straps will need to be tightened as the plants dry (Figure 1).
5. **Plants must be protected from fungal growth** until the drying process is over. Begin the drying process of plant specimens immediately after the plants are placed in the plant press and pressure applied.
    a. If the air temperatures are high and the relative humidity low, then all that is necessary to dry the plant specimens is to place the plant press where it will receive some natural ventilation, and tighten the press straps each day (they loosen when the plants inside dry and shrink).
6. Expose press kit to a moderate heat source, but avoid excessive heat. The best place would be on a windowsill, where there is direct sun and a constant dry draft.
7. If conditions are such that little or no natural drying occurs (e.g., cool temperatures, high relative humidity), then a heat source must be applied. In all cases ventilation of the plant press is important in order to remove water vapor arising from the plant specimens, and especially to vent water vapor from the corrugations of the cardboard. The newspaper can be changed as well, but care must be taken to ensure that the label information is transferred at the same time.

Figure 2. Plant press dryer[9]

8. Store samples in a dry, warm environment.

Note: The body of the dryer consists of an open-ended plywood (Figure 2) or wooden box (with or without a bottom) measuring 46 cm long, 35-60 cm wide and at least 30 cm high. To allow

for proper air circulation, the dryer should have a small air vent cut near the base (or should be raised slightly off the ground, if the bottom of the dryer is absent). A 100-watt bulb is placed in the bottom of the dryer (on an aluminum pie plate or fire resistant pad). The press is placed on edge, length-wise, across the dryer's open top, with cardboard placed alongside to block air spaces. This will cause the warm air within the dryer to be forced through the press. Depending on the turgidity of the plant samples, the collection sheets will dry in 24 - 48 hours[7,8].

II. Animal Prey Items

1. Make sure to collect any and all possible dietary components.
2. Always try and include any dietary item that your organism had access to, and had likely eaten. Collecting a muscle sample from your animal prey of interest is generally sufficient, but if that is not possible, then collect feathers, egg shells, egg albumen, fur, legs, etc. Handle and process the animal dietary items as you would the tissues from your focal predator species (see: *'Animal Tissue Samples'*, below).
3. All animal tissues should be kept frozen, or dried and kept in a dry, cool environment.

III. Animal Blood Samples

Plasma: reflects diet a few days previous and up to the time the tissue was collected.
Red Blood Cells: reflects diet ingested *up to two months previo*us and up to the time of tissue collection.

1. Collect 200 µl blood in a chemical free Vacutainer or cryovial.
2. If you use a Vacutainer with no additive, spin them as soon as you can, preferably within 2 hours.
3. Once centrifuged, the clear portion from these samples will be serum, which is slightly different from plasma in composition (it contains certain proteins that act as clotting factors), but should not be different in stable isotope values.
4. Regardless of the Vacutainer used, spin the blood in a centrifuge for 5 minutes at high speed.
5. Blood samples should be separated immediately after centrifuging.

a. Use a pipette and clean tip to transfer the clear portion off the top of the spun blood sample and store the plasma or serum in capped, labeled cryovials. Carefully take as much plasma from the top of the sample without disturbing the packed red cells beneath. If you can't get all the plasma or serum out, you can leave it and discard it with the pipette after sampling the red blood cells.

6. All samples should remain in the freezer until ready for isotope preparation.
7. If you don't have access to a freezer, then pipette the plasma/serum onto a combusted glass fiber filter paper and immediately fold the paper loosely in half and store in a glass scintillation vial or plastic cryovial. Do the same with the red blood cells. If they are clotted, then dump the red cells onto the paper and gently fold the paper together and put into the vial.
8. If centrifuging is not possible, drip whole blood on a single glass microfiber filter, placed in a glass vial, let dry, and store until analysis.

9. Leave the vials, with their lids just resting on the tops, so that they will be exposed to the air and dry. If possible, leave them in a window with warm sun on them so they will dry faster.
10. Once dried, safely store vials until you can get it back to the lab for analysis. The plasma/serum will look like a tannish stain on the filter paper and that is what is analyzed later.
11. Tissue may be stored dried in a dry, cool environment, or frozen.

IV. Animal Scat Samples

Scat: reflects very recent diet.

1. Collect samples, either from ground or directly when excreted and place in glass cryovials.
2. Remove any external litter (leaves, sand, rocks, etc.).
3. Freeze-dry if possible.
4. Place in drying oven at 120˚C for 48 hours to kill any remaining bacteria.
5. Store in dry, cool environment before analysis.
6. Another option:
    a. Place collected scat sample in new, clean paper bag.
    b. Fold and dog-ear the bag end.
    c. Place in dry environment (preferably on a windowsill - exposed to direct sunlight and air).

V. Animal Tissue Samples

Muscle: reflects diet up to two months previous and up to the time of tissue collection.

1. Collect tissue and/or skin from live or dead animal.
2. Freeze at -20° C (a normal food freezer works fine) until ready to analyze.
3. If there is no available freezer, oven dry samples at 40˚C overnight. Tissue may be stored dried at room temperature in combusted vials.
4. If neither are available, separate different tissues into individual combusted vials.
5. Leave the vials, with their lids just resting on the tops, so that they will be exposed to the air and dry.
6. If possible, leave them in a window with warm sun on them so they will dry faster[6].

Skin: reflects diet ingested several months previous and up to molting/sampling; if skin is collected from an animal that molts regularly, the skin will reflect the diet of the animal ingested while it was growing new skin during its molting time.

- Molted reptile skin:
    1. dry, bag, and keep in dry environment
- Skin on carcass:
    1. Remove any other tissue.
    2. Follow 'Animal Tissue: Muscle' protocol.
    3. Keep in dry environment.

Bone: bone growth rings can reflect annual diet trends throughout the animal's lifetime and whole bone reflects the diet of the animal across its entire life.

1. If found dry, place in Whirl-pak or paper bag
2. If harvested, remove tissue (which can be bagged separately, following 'Animal Tissue: Muscle' protocol, and used in your study).
3. Rinse any residue left, dry and bag (Whirl-pak or paper bag).
4. Keep in dry environment.

References

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