Biochemical techniques have opened up new sources of data with relevance to many questions in ecological and evolutionary biology. Currently, molecular analytical techniques range from protein assays to determination of DNA sequences. Not all current molecular techniques require frozen tissues, but frozen tissues, if properly sampled and stored, are suitable for most analyses. For this reason, we recommend collection and storage of frozen tissues. If it is impossible to freeze tissues in the field, certain tissue-specific preservatives can be used to provide material for microcomplement fixation and DNA analysis (Dessauer et al. 1990:29–30).

Freezing tissues in the field

Two materials can be used to freeze tissues in the field: dry ice (solid CO₂) and liquid nitrogen. Tissues frozen in ice (solid water) are not suitable for most standard biochemical assays of proteins or DNA.

Dry ice can be stored in a sealed plastic bag in a Styrofoam chest or box, but even so, it evaporates quickly (about 2–3 kg/day). Maximum field time for dry ice, using a very large portable ice chest, is about a week. Dry ice generally is available from industrial chemical supply firms or frozen food (ice cream) distributors.

Liquid nitrogen tanks or refrigerators (essentially large stainless steel vacuum flasks, about U.S. $500; see Appendix 6) are used in the field for freezing and storing tissues. The common term among users for this type of container is tank or, rarely, Dewar flask; however, the vendor’s term is refrigerator. Tanks come in a variety of sizes with capacities of 3 to 34 liters. The largest hold enough liquid nitrogen to keep tissues frozen for at least several months in the field. A tank containing liquid nitrogen must be kept upright at all times and under shade as much as possible. Liquid nitrogen is not generally available, but
it often can be found in larger industrial cities, because it has various industrial and agricultural applications. Liquid nitrogen generally is less readily available than dry ice; some vendors require 48 hours to fill a tank to capacity.

Both dry ice and liquid nitrogen (in its refrigerator) are legally transportable, including by air. However, many airline personnel are reluctant to allow shipment of liquid nitrogen. Patience on the part of the shipper is required. Copies of the appropriate pages from the most recent International Air Transport Association regulations (Anonymous 1991b), to which all airlines adhere, are often helpful in demonstrating that liquid nitrogen can be shipped safely and legally. A useful strategy, if there is a good mass of tissues and if it is certain that the tank will be in transit for no more than 24 hours, is to empty the tank of liquid nitrogen just prior to return by air. An alternative is to add dry ice pellets after pouring off the liquid nitrogen (before going to the airport, as the addition of dry ice results in hissing sounds and fog). Tissues in a tank with dry ice will remain frozen for 3 to 4 days. In the laboratory, tissues should be maintained either in liquid nitrogen or in an ultracold freezer (constant temperature of at least \(-70^\circ\) C).

Protocol for preparing tissue samples

First, the amphibian to be sampled is killed by anesthesia (see Appendices I and 4) or by pithing before tissues are removed. Next, tissues are collected separately for each individual. Tissues are removed as quickly as possible under as cool conditions as possible (shade) to avoid degradation, especially when small animals are processed in hot, dry weather.

Liver, heart, kidney, muscle (abdominal or thigh), and, for large amphibians, blood are sampled (the tissues analyzed will depend on the research question). The abdominal cavity is opened, and the organs are pulled from the body and cut into small chunks that will fit in cryotubes (e.g., Nunc tubes). Two tubes (up to 2 cc total) of tissue are prepared if the animal is large enough and time permits. Muscle and all viscera are sampled from small individuals (the stomach and intestine should be opened and cleaned, and the gall blad-

der either removed or not broken). We do not recommend freezing the entire specimen. Whole frozen amphibians make poor voucher specimens when preserved, and if the entire specimen is consumed in analysis, there is no voucher. If, because of time constraints, it is necessary to freeze entire specimens, other specimens of the species from the same population should be preserved as vouchers.

Tissues are placed either in commercially available screw-top plastic tubes designed for ultracold temperatures or in aluminum foil. We recommend plastic tubes. Mixing of different tissues from the same individual within tubes is acceptable for most analytical procedures. An air space should always be left in the tube for expansion of the tissue when it freezes.

Each tissue sample is labeled with a field number that is the same as the number of the voucher specimen from which the tissues were taken. A label with the specimen field number is also placed inside the tube (written in India ink, preferably, or in pencil); the label must be dry before the tissue is added. In addition, the field number (and, if space is available, brief locality information) should be written on the outside of the dry tube before the tissue is added. Ink that neither decays at cold temperatures nor smears when moist must be used on the outside of the tube (not India ink); a pen such as the Sharpie by Sanford works well.

After each specimen is sampled, scissors, forceps, all other equipment, and all working surfaces should be cleaned thoroughly. Material from one individual must not be contaminated with tissues from another individual or with blood or skin from the investigator. This precaution is especially critical for DNA analysis, because current replication techniques (e.g., polymerase chain reaction) can amplify tiny amounts of DNA.

Finally, the carcass is prepared as a voucher specimen (see Appendix 4) with the same number as the tissue sample and is deposited in a museum. For more detailed guidelines on collection and storage of tissues, see Dessauer et al. (1990). Tissue depositories are listed in Dessauer and Haffner (1984).

Sample size

The number of specimens to be sampled from each locality depends on the research question. For
example, a single individual may be adequate for a phylogenetic question using DNA sequencing techniques, but 30 individuals per population may be needed to study interpopulational variation using electrophoretic or mitochondrial restriction site techniques (for more explicit guidelines, see Baverstock and Moritz 1990). However, most researchers using molecular techniques subscribe to the idea that one specimen is better than none.

We strongly encourage persons who are taking tissues to collect samples from amphibians other than those in which they have a direct research interest. Such sampling is particularly important if rarely encountered species are collected or remote areas are visited.

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