

# Preparing Amphibians as Scientific Specimens

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## Introduction

Documentation of the species recorded in biodiversity and inventory projects frequently involves preparation of voucher specimens, which are extremely important for the credibility of such studies (see Chapter 5). In addition, these specimens and their accompanying field notes are valuable sources of scientific information used in systematic, biogeographic, evolutionary, and ecological studies.

Most vouchers are preserved specimens from the study site, although photographs of individuals or tape-recorded calls of frogs are sometimes substituted. To be useful as vouchers and to facilitate identifications of species, preserved specimens must be well prepared and well documented. Poorly prepared or improperly documented specimens are of little scientific value and usually not worth the effort of preparation. In this section I review the steps involved in

the proper preparation of amphibians as scientific study specimens (for additional discussion, see Pisani 1973; Kars 1986; Simmons 1987).

## Documentation

Field notes should be written on 100% cotton rag paper with permanent ink. Most field biologists record field notes on loose-leaf paper stored in a three-ring binder. Although some people argue that loose-leaf binders are inferior to bound volumes because pages may be lost, this is not a problem if a person is careful. A loose-leaf format provides flexibility and allows for easy insertion of extra pages, maps, lists, and other important documents. Because bound books have a fixed size, they may contain too many pages for short trips and too few pages for

Roy W. Mc Diarmid Field Catalogue	Venezuela 1984
8 January 1984	Venezuela: Territorio Federal Amazonas; Neblina Basecamp, on Rio Baria; 140W; 0°50'N, 66°10'W
17081	<u>Pseudonotodes</u> sp. in leaf litter in camp, 11:40 photographed
17082	<u>Hyla sarayacuensis</u> under log in heliport clearing; afternoon photographed
9 January 84	Venezuela: Territorio Federal Amazonas; Neblina Massif, Camp II, 2.8 km NE Pico Phelps (= Neblina); 2085-2100 m; 0°49'40"N, 65°59'W
17083	<u>Hyla</u> photograph 9 (26-28)
17084	<u>Hyla</u> photograph 9 (29-32)
	both collected from the central axils of a bromeliad ( <u>Brocchinia</u> sp.?), near camp both in axil with head up - color notes - both bright green dorsally with bright yellow sides; yellow line on upper eyelid; blue green on chin & pectoral area; orange/scarlet white peritoneum; iris bright coppery tissue taken from 17084
17185	leptodactylid?
17186	eggs from mass with 17185, under moss on rock - adult gave defense posture

Figure 30. Sample page from a field notebook.

long ones. Nevertheless, I recommend against taking a bound volume with original notes into the field on a subsequent trip because of the possibility of loss. This is not a problem with a loose-leaf notebook, because original pages can be left behind. Loose-leaf pages can easily be bound into annual or trip-related volumes for permanent archives.

Preserved specimens should be accompanied by the minimum data discussed in Chapter 5: locality, date and time of collection, collector, sampling method, habitat, and other locality-related information. Other specimen-related information—such as color notes and references to photographs, tape recordings, and tissue samples—should be recorded in the field notes (Fig. 30). Each reference to a voucher specimen in the notes should include the field number and temporary field identification (e.g., frog; *Rana* sp.; small brown salamander).

The number used in the field notes is written on a field tag and attached to the specimen (see below). Most field biologists use field numbers that include their initials followed by a number (e.g., RWM 19503), but any distinct numbering scheme will suffice (simpler is better). Field tags should be relatively small and of sturdy paper stock (Karns [1986] suggested Resistall Index Bristol, 100% rag, 110 lb. wt. paper, available from University Products; see Appendix 6). Field numbers should be written on the tag in permanent ink. Printed tags with consecutive numbers and other identifiers are available commercially. Although printed tags are relatively expensive (U.S. \$20–\$100 per 1,000 tags, National Tag Company), I recommend them because they save valuable field time and prevent double numbering. Tags of cloth or plastic can be used if paper tags are not

available, but metal tags should be avoided because they sometimes corrode, and sharp edges can damage fragile specimens. Field tags should be strung with a fine white linen thread as shown in Figure 31. Colored thread should not be used because the dye may dissolve in preservative and discolor the specimens.

## Specimen processing

Immature and adult amphibians collected as vouchers should be maintained in plastic bags until processed. Eggs and larvae require immediate attention, and I discuss them separately. When I capture a specimen, I usually place a numbered plastic tag in the bag with it and use that number to reference associated data in a small pocket notebook. I do not write data on paper tags and place them in the bag with the specimen because these quickly become wet and illegible. As soon as possible, I transfer pertinent data for each specimen from the pocket notebook to permanent field notes and assign a field number to the specimen. The plastic field tags can be washed and reused. I try to process all specimens as quickly as possible to avoid loss of individuals and confusion of associated data. Dead amphibians generally do not preserve well and make poor voucher specimens.

In some situations specimens may be frozen for later processing. However, Scott and Aquino-Shuster (1989) showed that amphibians frozen before preservation are softer and grayer than specimens that were not frozen, often have twisted toes, and lose their epidermis. Because some of the traits affected are important for species identification (e.g., coloration, toe and webbing characters, skin texture), I strongly recommend that specimens not be frozen.



Figure 31. Numbered field tag and attached thread. Letters are the initials of the collector, and the numbers are consecutively printed ones. Note the location of the loop hitch, which holds the tag, and the knot, which holds the tag away from the animal.

## Procedures for Killing

Amphibians should be killed as quickly and humanely as possible in a way that leaves them in a relaxed condition. The most efficient method for killing adult amphibians is to immerse them in a solution of Chloretone. A Chloretone solution is made by dissolving a small amount (1 teaspoon) of hydrous chlorobutanol crystals in a liter of water. Note that chlorobutanol crystals (and hypodermic syringes) are controlled substances in some countries, and investigators may need permits to obtain them. Be-

cause chlorobutanol dissolves slowly, I often carry a small container of stock solution (95% ethyl alcohol saturated with chlorobutanol) and prepare a Chloreto solution in the field by adding a few milliliters of stock solution to 750 ml of water in a wide-mouthed liter bottle. I prepare a Chloreto solution as soon as I arrive at a field site so that it is ready when needed. Such a solution is effective for about 1 to 2 weeks (with heavy use) and then gradually loses its strength.

Species have noticeably different responses to Chloreto solutions. Some amphibians die rather quickly (5 min); others may take longer (10–15 min). The solution often increases in effectiveness, probably from the addition of various skin secretions from amphibians killed in the solution. If amphibians do not die after 20 to 30 minutes in the solution, additional chlorobutanol should be added or, preferably, a new solution prepared. Amphibians should not be left in the solution very long after they die, as they become rigid and difficult to position for fixation.

If Chloreto is not available, amphibians may be killed by drowning in warm water (43°–47°C) or in weak alcohol (15%–25%) solutions (Pisani 1973). Pithing may be effective, but it often damages the specimen, especially the skull. Benzocaine-containing gels, sold in most pharmacies as toothache medication, can be used as an alternative to Chloreto (Altig 1980). A small amount smeared on the head of an amphibian kills it within a few minutes, and the specimen is completely relaxed. Freezing is not recommended as a method for killing (Scott and Aquino-Shuster 1989).

## Preservatives

Preserving amphibians for scientific study is usually a two-step process. Initially, specimens are fixed in an appropriate preservative, and then they are transferred to an alcohol solution for storage. The most common general fixative for amphibians is formaldehyde. At room temperatures, formaldehyde is a gas; under pressure, it polymerizes to a solid called paraformaldehyde. An aqueous solution of formaldehyde is easier to handle than the gas and has been used as a histological fixative for about 100 years (Fox and Benton 1987). Formaldehyde is commercially available as a liquid that is made by dissolving formaldehyde gas in water as a 37% to 40% solution. A formaldehyde solution of this strength is equivalent to 100% formalin (formaldehyde diluted with water). One part of this full strength formalin is

diluted with nine parts water to make a 10% solution of formalin.

A 10% solution is the standard for specimen fixation in the field. A fixed specimen retains whatever position it had when placed in the formalin. This stabilization (fixation) is effected by cross-linkage of the formalin with protein end groups in the tissue. This process stops autolysis and prevents further tissue deterioration. Although the rate of fixation varies with the size of the specimen and probably with the nature of the tissues, most small amphibians set in a few hours; larger specimens require much longer. Fixation generally is considered to be irreversible, but Pearse (cited in Taylor 1977) found that many of the cross-linking bonds are reversible with the simple process of washing; others are not. Because washing under certain situations may reverse fixation, I recommend that amphibians not be soaked in water prior to transfer to ethyl alcohol.

Because formaldehyde is a gas, a formalin solution exposed to air decreases in strength and becomes more acidic. It is especially important, therefore, to use freshly prepared 10% solutions to fix specimens. I recommend replacing formalin in trays after preserving 50 to 100 specimens of various sizes. Old formalin can be used in the field as a storage solution for specimens after fixation, or it should be properly discarded in keeping with regulations of the country where the investigator is working.

Formaldehyde oxidizes to form formic acid, which, after a time, will decalcify bone and discolor specimens. Specimens fixed in formalin that is alkaline, in contrast, tend to become transparent, or "cleared" (Taylor 1977). Because decalcification, excessive discoloration, and clearing are undesirable, most field herpetologists buffer formalin to maintain the pH as close to neutral as possible. Fox and Benton (1987) indicated that an optimum pH for formalin fixation is 7.2. Although borax has been suggested as a suitable buffer (Pisani 1973), Taylor (1977) argued against its use because specimens tend to clear in borax-buffered formalin; instead he recommended calcium carbonate. In the field I have used powdered magnesium carbonate with good results (about 1/2 teaspoon per liter of 10% formalin). To buffer formalin used in the museum, I add 4.0 g of sodium phosphate monobasic and 6.5 g of sodium phosphate dibasic (anhydrous) to each liter of 10% formalin.

In the museum, I prepare formalin with distilled water, but in the field I use whatever water is available. Field researchers should be aware, however,



that stream or pond water may be quite naturally acidic or basic, and, therefore, formalin made with such water may require additional treatment. The use of rainwater often alleviates the pH problem, but even rainwater can be acidic. If the pH of water at a field site is potentially a problem, then it may be desirable to check the pH of the solution with indicator paper. I use a pH meter in the museum.

Occasionally in remote areas, one purchases bad formalin (i.e., it does not fix animals as well as expected). I suspect that such formalin has been oxidized extensively and probably contains considerable formic acid. Workers need to be aware of this problem and to test the pH of all formalin solutions. I repeatedly examine fixed materials in the field to avoid problems of poor fixation. Formaldehyde fixation is a complicated process and can be influenced by many extraneous factors. If an investigator is obtaining the desired results, I recommend that he or she continue the process. In the absence of desired results, I recommend starting over with fresh solutions. If the problem persists, help from a chemist or a museum curator should be sought.

As most herpetologists know, formaldehyde is irritating to the eyes, upper respiratory passages, and skin. Some people develop an allergic reaction to formalin and must wear rubber gloves to prevent skin rashes. Formaldehyde also has been reported to be a potential carcinogen (Simmons 1987); thus, it should be used only in well-ventilated areas. Fortunately, most field situations fall into this category.

If formaldehyde is not available as a fixative, 70% ethyl alcohol can be used. I do not recommend other kinds of alcohol (e.g., methyl, isopropyl, rubbing). Fixatives such as formalin-acetic-alcohol (FAA), glutaraldehyde (Taylor 1977), and Bouin's solution (especially good for histological work) are more difficult to prepare but also can be used. Formalin solutions also can be prepared from paraformaldehyde (see recommendations in Huheey 1963; Pisani 1973; Saul 1981).

## Fixation

Once an amphibian is dead and relaxed, it can be fixed in a preserving tray in 10% formalin. Typically, I use a plastic refrigerator tray (33 × 21 × 6 cm) with a tightly fitted lid. I line the bottom with a white paper towel soaked with 10% formalin (dye from colored towels will discolor specimens). Each specimen is positioned in the tray in a way that will

facilitate measurement and examination of key features on preserved specimens and that also will allow for more-effective storage and hence long-term maintenance of the specimens. I position frogs so that their limbs are drawn in next to the body and flexed in a natural way; fingers and toes are straightened and spread to display webbing (Fig. 32). I do not recommend the positioning illustrated by Pisani (1973: plate 1) or Karns (1986: fig. 24), in which one or both hind limbs of a frog are extended posteriorly. In my experience extended limbs easily become tangled with other specimens and tags as animals are removed from a storage jar. Often the epidermis is scraped excessively and the hind feet damaged. From a practical perspective, frogs with extended limbs require more storage space (fewer frogs per container). They also require closer monitoring; the toes and webbing on the hind feet are particularly subject to desiccation and may be the first structures exposed to air as the alcohol level in the jar drops.

Most salamanders are laid out straight with the limbs pointing forward parallel to the body. The hands and feet are arranged with palmar surfaces down and toes spread, and the tail is straight (Fig. 32). Caecilians are preserved with the body straight or in a flat loop of a size appropriate for the containers used in the collection (Fig. 32). I preserve caecilians with their mouths held open by a small stick or piece of paper towel; this practice facilitates later examination of tooth arrangement.

Formalin penetrates the body cavity of small amphibians rather quickly, so injection is not necessary. Large frogs (adults of many species of *Bufo*, *Rana*, and *Leptodactylus*), salamanders (sirenids, cryptobranchids, and adults of larger species of *Ambystoma*), and caecilians may require injections of formalin into the body cavity and larger muscle masses. Care should be taken not to distend the body by injecting too much formalin and not to introduce air into the body cavity. Frogs distended with air float in the hardening solution, so some areas of the body are not covered with fixative. It also is important to keep track of individuals in the tray so that the correct field tag can be associated with the appropriate individual after it has hardened. I sometimes lay the tag across the specimen in the tray. Once the floor of the tray is covered with specimens, I blanket them with a second paper towel wet with formalin and carefully fill the tray with formalin to about one third its depth.

After a few hours, most specimens will have hardened enough to maintain their shape. This is the

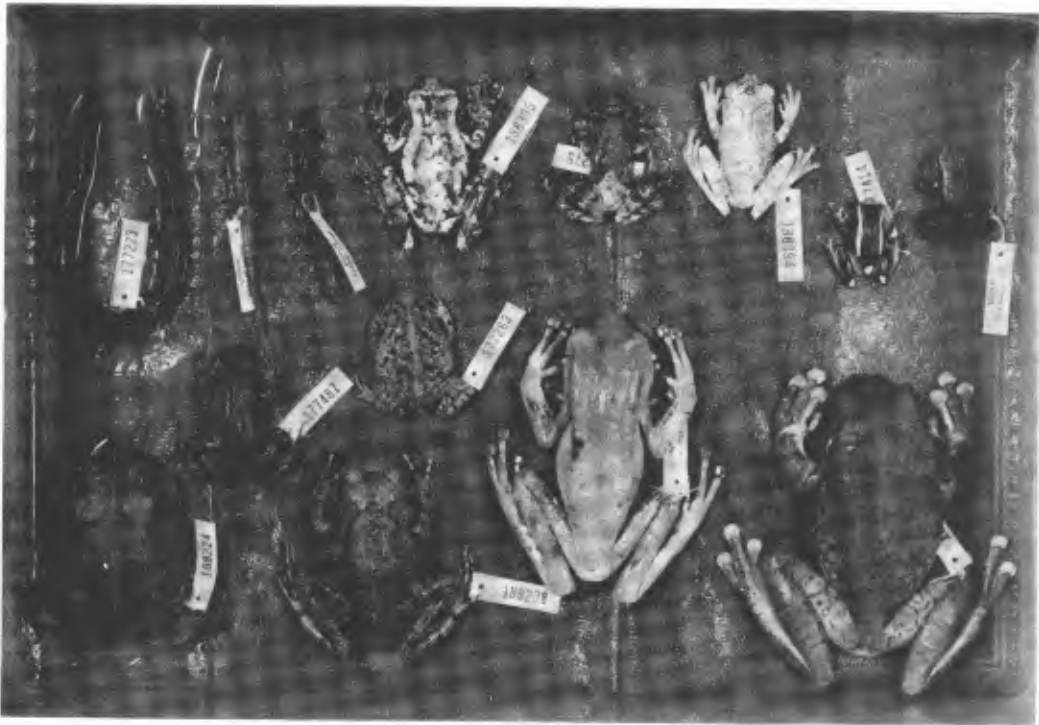


Figure 32. Specimens of frogs, salamanders, and a caecilian preserved in the recommended postures (see text for explanation).

time to attach the field tag. Some herpetologists prefer to attach tags to specimens prior to positioning them in the hardening tray to ensure that tags and specimens do not get mixed. I have found, however, that specimens are more difficult to position for hardening with the tag attached. The tag is tied with a square knot above the knee on the right rear leg of frogs and large salamanders, and around the neck of small salamanders and caecilians; ends of the thread are trimmed. The specimens are transferred to a hardening jar, where they remain immersed in 10% formalin for at least 4 days or, preferably, for the remainder of the field season.

### Eggs and Larvae

Amphibian eggs and larvae usually require special treatment. They are easily damaged, especially during collecting, if not handled carefully. As a result, when collecting eggs or tadpoles I usually preserve some in the field and take others back to camp alive.

I place single, short strings or small clumps of aquatic eggs directly into small bottles or vials of freshly prepared 10% buffered formalin that I carry with me in the field for that purpose. I usually place terrestrial eggs, eggs adhering to leaves, and larger egg masses (e.g., those of some species of *Ambystoma*), into plastic bags and carefully transport them back to the work area for examination, rearing (see below), and preservation in suitably sized containers. I put some larvae directly into formalin as they are collected, and carry others back to camp in plastic bags. Because eggs and larvae, especially of anurans, contain much more water than adults contain, they seemingly require a larger volume or slightly stronger concentration of fixative initially than do adults. I sometimes carry a small amount of pure formalin into the field to meet such needs.

On returning to camp or the lab, I routinely sort and transfer all preserved eggs and larvae to larger containers of fresh 10% formalin. During the sorting process, I identify larval morphotypes, write color notes, and take photographs. At this time I also decide whether to maintain some of the live eggs until

hatching or to rear some of the live tadpoles through metamorphosis. I often preserve a few eggs from a clutch and place the remainder in an appropriate container to continue their development. With larvae, I often return to the site and collect additional specimens for rearing (see below). After sorting the specimens, I write field notes and assign tags to the larval samples. Tadpoles that have been in an adequate volume of fresh formalin for 24 hours are well fixed and can be transferred with their field tags to smaller vials for storage and eventual transport. A tag should never be tied to a tadpole or to a small salamander or caecilian larva; tags may be attached to larger salamander and caecilian larvae if tagging does not damage the specimen. I prefer to sort samples into morphotypes in the field and tag them accordingly. However, if storage space or tags are limited, all specimens collected at a single site on a single day can be maintained as a single sample. Samples collected at different localities or sites or on different days must not be mixed.

Because the eggs and larvae of many species are unknown, efforts should be made to associate each with its respective adult form. If time permits, an investigator can obtain and rear eggs from known adults or can rear unknown larvae through metamorphosis in the field. Aliquot samples representative of the eggs and larvae from a single known sample can be preserved at appropriate intervals during development. Such developmental series are very useful in understanding the ecology of species and may contribute significantly to investigations of the evolution of morphological traits in amphibians.

Containers suitable for rearing tadpoles can be set up in camp, and tadpoles of specific morphotypes can be reared. I usually maintain each sample (species) separately in water taken from its habitat and, if possible, feed the tadpoles natural-occurring food. I use artificial food (e.g., tropical fish food, trout chow, rabbit pellets) if natural foods are not easily obtainable. Periodic sampling of tadpoles from the same aquatic habitat sometimes is more efficient than rearing them. Tadpoles sampled from populations maintained artificially in the field or laboratory should be given separate numbers and cross-referenced in field notes to the field number of the original sample. Date, time of sampling, and rearing conditions (e.g., container size, temperature, food) for each sample should be recorded.

## Special preparations

Sometimes it is desirable to prepare specimens for osteological study. Because of distortion from drying, cleared and stained preparations (see below) are preferable for most species of amphibians. However, dry preparations of larger species are suitable and sometimes preferred. Simmons (1986) described a method for making osteological preparations from alcoholic specimens. I recommend his approach, although it is time-consuming. If a specimen is prepared as a dry skeleton in the field, the selected individual should be weighed and measured before it is put into Chlorotone. Skinning should follow the steps outlined by Simmons (1986), except that no parts of the skeleton should be disassociated. The sex of the specimen should be determined when the animal is eviscerated, and stomach contents should be recorded. If the latter seem interesting, they should be preserved and referenced with the same field number as the specimen.

To make an osteological specimen, the preparator removes most of the muscle mass, being careful not to cut the pectoral girdle or hyoid apparatus, and attaches a field tag to the leg once it is mostly free of tissue. The specimen is then wrapped into a compact but loose ball with string and hung in a dry place, preferably within a screened enclosure or cage to discourage flies and animals that may carry off a carcass. If the specimen does not dry quickly, it can be immersed in alcohol for a few hours and dried again. It should not be put in formalin, because dermestid beetles, which are used to clean skeletal material, will not eat formalin-treated carcasses. Putting the carcass in a small cheesecloth bag will help to exclude flies and to maintain all skeletal elements in a single package. Once a carcass is thoroughly dry, it can be packed in its cheesecloth bag for shipment back to the museum. Simmons (1987) provided many references to techniques for preparing skeletons and cleared and stained specimens.

## Packing and shipping

At the end of a field season or work at a site, all prepared specimens should be checked to see that field tags are attached and that no problems with associated data remain. If live material is to be taken to the next site or back to the museum for continued rearing (a job that is sometimes difficult but potentially



worth the effort), then a sample should be preserved at the last possible moment to ensure that representative material at that stage of development is available for study.

Preserved specimens are sorted by shape, size, and method of preparation. Dry skeletal preparations should be packed in dry containers (small cans or durable cardboard boxes) with lots of padding. Vials containing eggs and larvae are grouped by size and wrapped with packing material or strips of paper towel. Vials of similar size are packed tightly in plastic jars, cans, or other appropriate containers with paper or cardboard dividers, as necessary. I pack vials in sealable containers (cans or plastic jars) rather than in cardboard boxes because of the possibility of leaks or breakage during transport. If cardboard boxes are used, they should be sealed in plastic bags. Wet specimens should be loosely wrapped in paper towels or cheesecloth for protection and easier packing. Many small packages, each containing a few specimens, seem to be better than fewer, larger packages. If the collecting site is near the laboratory, so that collections can be returned from the field by car, then transporting wrapped specimens in trays with formalin works well if the trays are kept flat. Small specimens can be left in formalin in sealed containers.

If collections have to be shipped from the field site to the laboratory or taken as baggage on an airplane, then different packing is required. Weight and leakage are primary considerations, and plastic containers are much better than glass ones. Wet specimens should be wrapped in cheesecloth or paper towels and placed in a plastic bag. When the bag is about one third full of one or more equal-sized bundles, just enough formalin to soak the packages should be added, and the bag tied securely or closed with a rubber band. After the bag is checked for leaks, it is placed in a second bag that also is tied. Bags of comparable size can be packed snugly in plastic jars, preserving trays, or other sealable containers, which are then taped shut. These containers can be packed into shipping boxes or fiberboard drums designed for shipping wet materials. An address label should be placed on the inside as well as on the outside of each shipping container. Sometimes I hand-carry small containers of fragile specimens or vials of eggs and larvae. I have carried half-liter plastic jars filled with small frogs and salamanders packed between layers of cheesecloth wet with formalin, with good success. The formalin in these containers can be replaced with 70% ethyl alco-

hol for transport on an airplane. If I know that a trip is going to be short (< 12 hr), and I am hand-carrying specimens, I sometimes replace the formalin in vials with water. This ensures that formalin will not be spilled during transport. I refill the vials with fresh 10% formalin *immediately* on reaching my destination. Larvae need to be shipped in vials or similar containers with some liquid and should never be wrapped in cheesecloth. With careful packing and common sense, important materials will arrive unharmed.

## Field equipment and supplies

One or more good-quality headlamps, along with batteries and replacement bulbs, are essential for nighttime survey work. I rely on a plastic headlamp with four D-size batteries. I prefer D-cells over a single 6-volt battery because D-cells generally are more readily available and easier to pack. Some people use rechargeable D-cells, but in my experience, they do not last very long and do not provide the service that an alkaline cell gives. Another option is the more powerful, rechargeable miner's lamp with a wet-cell (motorcycle type) battery. A miner's lamp gives a brighter beam and, though initially more expensive, may be less expensive in the end, because the batteries can be recharged hundreds of times. However, like nickel-cadmium dry cells, the batteries require electricity or solar panels for recharging; also they are hard to pack and burdensome to maintain during long periods of disuse.

The following list of equipment and supplies is provided as an aid to preparing for fieldwork. Sources for these supplies can be found in Appendix 6.

**Collecting Equipment.** Headlamp and batteries; replacement bulbs; small flashlight and batteries; assorted plastic bags; cloth bags; temporary plastic field tags; thermometers; compass; altimeter; machete and file; pocket knife; insect repellent.

**Equipment for Observing, Studying, and Measuring.** Measuring tape; marking flag; calipers; plastic rulers (10- and 30-cm); spring scales (10-, 100-, and 500-g); small scissors; hand lens; camera and film; tape recorder, microphone, batteries, and tape; binoculars.

**Equipment for Recording Data.** Notebook and waterproof paper; small pocket notebook; pens



and ink; pencils; Sharpie indelible-ink marking pen; field tags; waterproof paper for duplicate tags; thread; scissors (for paper); cigarette lighter; candles for light when writing.

**Equipment for Larval Sampling.** Dipnets (large and small); spare net bags; small-mesh strainers; assorted vials and small jars; large-gauge pipette; rearing containers (plastic food containers with lids); plastic bags; artificial food (trout chow, tropical fish flakes); plastic spoon.

**Preserving Equipment.** Forceps (long and needle-nosed); dissecting scissors; scalpel and blades; single-edged razor blades; needle and thread; preserving trays; wide-mouth jar for Chloretone; sy-

ringes (1-, 10-, and 30-cc); syringe needles (various sizes); hardening and storage jars; paper towels.

**Chemicals.** Formalin (full strength); buffer (magnesium carbonate); chlorobutanol or Chloretone (saturated solution); alcohol (full-strength ethanol).

**Containers.** Assorted plastic jars (0.5- to 3.2-liter); shipping containers; bucket or Liquipak (a watertight fiber drum for shipping wet materials).

**Packing Supplies.** Fiber tape; string; rubber bands; cheesecloth; heavy plastic bags; scissors (for cheesecloth); paper towels; mailing labels; Sharpie indelible-ink pen.

