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SUGGESTED TECHNIQUES FOR MODERN AVIAN SYSTEMATICS

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The methodology of avian systematics at the population and near-species levels has changed profoundly in recent years. Increasingly, we see the application of quantitative analytical approaches, such as audiospectrography, reflectance spectrophotometry, multivariate morphometrics, and pigment and tissue biochemistry. However, gel electrophoresis and the histochemical staining of tissue, techniques that reveal genetic loci encoding specific proteins, have only recently been extensively used on avian material (Barrowclough 1983, Corbin 1983). Because so few studies have been conducted, there has been insufficient time and experience for a consensus to develop as to which technical procedures are most appropriate for birds. Although laboratory protocols for the electrophoretic analysis of vertebrate tissues are in general well established (Yang 1971), the literature lacks detailed information of use to the field worker on the sampling, preservation, and transport of avian tissue for electrophoretic work. Furthermore, there are possible shortcuts in laboratory procedures and experimental design that may not be widely appreciated. The present paper attempts to fill these gaps.

The advent of multidimensional and multiple character set approaches in avian systematics has also revealed certain shortcomings in standard techniques of specimen preparation. Although traditional methods of preparing skins and skeletons (Hall 1962:26–35) continue to be acceptable for many purposes, unorthodox kinds of preparations often are better suited for studies examining specific issues in modern speciation-variation research. An example of such an issue is mosaic evolution, the phenomenon of differential rates of change in different character suites (Dobzhansky et al. 1977:31). For several reasons, birds are unusually well suited for the examination of mosaic evolution. For example, avian vocalizations and plumage coloration, features important in reproductive isolation and speciation, can be quantitatively analyzed across geography. Their degree of change can easily be compared with those for morphology and structural allelic frequencies (but see Lewontin 1984).

The usual fashion, however, in which specimens are gathered and prepared results in poor material for mosaic evolutionary studies. Typically, a researcher interested in comparing geographic variation in morphology with that in song for a given species uses available museum specimens

and/or collects additional new skins from a series of populations for the morphologic analysis and records vocalizations from different individuals of those same populations. Practical considerations usually prevent gathering both morphologic and vocal data from the same birds. Nonetheless, the best data relevant to mosaic evolution will be those on character correlations within individuals, not populations. Ideally, the systematist requires thorough information on vocal behavior, morphology, coloration, and genetic variation, among other parameters. But such comprehensive data are not easily obtained, using traditional methods, for individuals. Even for morphologic characters, workers routinely gather "skin data" and "skeletal data" from separate series of specimens because during skeleton preparation the skin characters for those individuals are lost. Clearly, researchers should attempt to devise procedures that allow retrieval of maximal data for each individual sacrificed. Such a goal is also compatible with wise conservation practice. To this end, we recommend the use of unusual kinds of preparations termed "skin-skeleton" specimens, described below.

For several years we have been applying a battery of research procedures to large samples of specimens of a number of avian taxa. This experience prompts us to describe field and laboratory techniques we have derived and found useful, methods that supplement procedures already published and widely known. We recognize that other workers may already be using more effective or efficient procedures than those we describe. We encourage them to inform colleagues (including us) of any such techniques in order to facilitate the proper growth of this research arena. Finally, we call attention to areas of procedure that continue to present difficulty.

PRESERVATION IN THE FIELD OF TISSUE SAMPLES FOR ELECTROPHORETIC ANALYSIS

Interval between death of bird and tissue preservation.—To prevent protein denaturation, tissue samples should be preserved by low temperature freezing as soon as possible after death, preferably within a few hours. More rapid freezing is required if ambient temperatures are over approximately 80°F (26°C). Specimens should be kept shaded and in well-ventilated containers before processing. It is better to keep whole specimens intact than to take tissues and not to freeze them immediately. This applies to specimens stored in a normal freezer; it is preferable that such specimens not be dissected until the tissues can be transferred, either to an Ultra-cold freezer or to a liquid nitrogen refrigerator for more permanent storage. Therefore, tissue samples should not be stored in a normal freezer. Responses of tissues to freezing are discussed by Mazur (1970).

Liver and pancreas contain enzymes (proteases) that digest other proteins. If these tissues are disturbed, these proteases may be dispersed and result in rapid destruction of the enzymes, in the liver and elsewhere, of interest to the researcher. Consequently, if a bird is shot in the abdominal (liver) area, it is especially important to process the specimen and to preserve the tissues in liquid nitrogen as soon as possible.

It is not known precisely what the maximum survival interval is for any enzyme under typical field conditions. It is established, however, that proteins vary greatly in their denaturation times at a given temperature. We have seen good activity in the laboratory from tissue held at room temperature or above for up to 8 h after death. Even longer periods of retained activity are likely for certain protein systems. Thus, even road-killed specimens may harbor at least some active proteins. There is need for much experimentation and sharing of findings in this area.

Type and quantity of tissue to preserve.—We routinely analyze heart, liver, kidney, breast muscle, and blood. These tissues allow us to score consistently 40± loci per species. Other workers have examined brain, testis, eye lens, and, most recently, actively proliferating feather pulp (Marsden and May 1984). Different tissue types contain different proteins (Harris and Hopkinson 1976). Again, laboratory experimentation is necessary to determine which tissues provide scorable bands for the particular taxon being investigated. For a bird the size of a sparrow, the entire heart, liver, kidney, and approximately 1 cc of breast muscle are sufficient for a plethora of gels. For larger species, especially those that are rare or difficult to obtain, as much tissue should be saved as storage space allows.

Tubes for tissue storage.—All the necessary tissues from a small (15 g) bird will fit easily into a single nunc tube (manufacturer's specifications and address of supplier: A/S Nunc biological test tubes, screw cap, silicone washer, size 2 cc, 38 × 12.5 mm, catalogue no. UCC-76; available from Almac Cryogenics, 1108 26th St., Oakland, California 94607 and from Thomas Scientific).

If liver and muscle are saved, it may not be necessary also to save blood. The additional proteins that can be obtained from blood, two hemoglobins and several general proteins from the plasma, may not be worth the additional effort. Nevertheless, if the loci are needed, the following procedure has worked under field conditions for one of us (GFB). A small centrifuge operated off a car battery (via an AC-DC inverter) or a hand-centrifuge (e.g., Thomas Scientific No. 2506-E05) can be used to separate red cells from plasma after blood is drawn with a heparinized syringe by cardiac puncture. For most birds, small amounts of blood (less than 1 cc) are obtained from shot specimens. Consequently, small vials are necessary

for storing the separated material drawn off from the centrifuge tube using a Pasteur pipette. Snap-top, polypropylene micro-centrifuge tubes of approximately 0.25–0.5 cc capacity can be used for this purpose.

Labeling and storage of nunc tubes.—Because the writing on frosty tubes can be extremely difficult to decipher, proper labeling of specimen tubes is of extreme importance. At the very least, label the tube with the initials and field number of the collector and either the common or scientific name of the bird (if known with certainty). Because of unfortunate experiences, one of us (NKJ) prefers to duplicate the field number in large numbers in the remaining space on the tube. We often use indelible marking ("Sharpie") pens; many other suitable pens are available commercially. The practice of scratching the information on the vial with a needle or other pointed instrument should be avoided. Such etchings are easily worn away and can become extremely difficult to read.

THE TEMPORARY STORAGE OF TISSUES IN THE FIELD

Dry ice.—When field time for specimen processing is limited, dry ice (−76°C) offers the convenience of temporary preservation of whole animals. For this purpose, a thick-walled, commercially available dry ice chest is preferred over a styrofoam cooler. One hundred lb (ca. 45.4 kg) of dry ice in such a chest will be sufficient for 1 week in the field under normal conditions (R. D. Sage, pers. comm.).

Liquid nitrogen.—During extended field work, storage in liquid nitrogen is the method of choice. At least 20 models of liquid nitrogen refrigerator tanks or dewars are available that are suitable for field storage and transport of tissue. These tanks can be obtained commercially from the Cryogenic Equipment Department of Union Carbide Corporation. Technical aspects of biological storage vessels using liquid nitrogen are discussed by Gareis et al. (1969) and in the Cryogenic Equipment catalogue and other brochures distributed by Union Carbide Corporation.

Models commonly in present use vary from those with a large storage capacity (LR-50, 130 lb when full, holds 50 l) and substantial static holding time (100 days) to tanks with medium storage capacity (XR-24, 81 lb when full, holds 29 l) and long static holding time (240 days). At the Museum of Vertebrate Zoology, University of California, Berkeley, two models are especially popular. The LR-17 weighs 50 lb (22.6 kg) when full (17.4 l) and has a static holding time of 48 days. The LR-10C is smaller (33 lb or 15 kg when full) and thus holds less liquid nitrogen (10.4 l) and less storage space, but has a longer static holding time (60 days) than the LR-17.

During 1983, Union Carbide Corporation introduced several new lines of liquid nitrogen refrigerators and dewars that offer improvements in cap

and necktube design and insulation. These tanks provide longer static holding times for a given volume of nitrogen than earlier models. The HCL series of refrigerators has large storage capacities (up to 34 l) and substantial static holding times (50–200 days). The XCL series offers tanks with capacities of 3–34 l and static holding times, varying with tank size, of 27–340 days. In addition, for the shipment of small quantities of biological specimens, the "3DS Dry Shipper" is available. It offers the convenience of relatively small size (holds 3 l, 15 lb [6.8 kg] when full, 15 in [38.1 cm] high, 7.6 in [19.3 cm] in diameter) with a respectable static holding time (20 days), and a positive-closure cap. Because it contains an adsorbent, samples are kept cold and dry at cryogenic temperatures and no liquid nitrogen is available to spill or leak. In addition to the "Dry Shipper," two models from the new series of tanks are especially suitable for avian systematic work, the 18-XT (comparable to Model LR-17 mentioned above), weighing 26.8 kg when full, 18 l capacity, and with a static holding time of 200 days; and 10-XT (comparable to LR-10C), weighing 15.4 kg when full, 10 l capacity, and with a static holding time of 111 days.

The LR-10C or 10-XT models are small enough to be transported in back packs or in pack saddles for mules. During such transport, however, it is important to maintain the tank vertically, for spillage occurs easily if the tank falls on its side. On long field trips in warm climates we have found it useful to start with two filled tanks (LR-10C). One tank is used for tissue storage; the other holds a reserve supply of liquid nitrogen. Because the first tank is opened frequently when tubes are added and frozen, the nitrogen level drops relatively quickly. Because the second tank is seldom opened, nitrogen evaporation from it is greatly reduced. When necessary, the second tank can be used to refill the first tank.

At a temperature of −320°F (−196°C), liquid nitrogen can be a dangerous substance, causing severe frostbite if it comes in contact with human skin. Instructions for its use (Form 9888-Q, Precautions and Safe Practices, Liquified Atmospheric Gases, December 1979, Linde Division, Union Carbide; see also Linde Publication F-9914), provided with the refrigerators, should be followed very carefully. For example, in the field it is important to be certain that the fluted necktube plug on the tank cap is adequately vented and free from obstructions such as frost or ice. Without proper venting, which allows the liquid to gasify directly into the atmosphere, pressure builds within the tank which then becomes, in effect, a bomb. Excessive bouncing of the tank or very humid conditions can also cause pressure increases.

Sources of liquid nitrogen.—Liquid nitrogen is available commercially in cities and, occasionally, can even be found in very remote towns. It is

a by-product of liquid oxygen production and thus is often sold by companies that supply the latter substance to welders and hospitals. In regions with active cattle and poultry industries, liquid nitrogen is used for the storage and transport of livestock semen and vaccines. College and university physics, chemistry, and agriculture departments often have supplies on hand for teaching and research purposes and will usually part with some in an emergency. The cryogenic industry, locally active in some regions, can also be a source. The Union Carbide Corporation produces a list of suppliers of liquid nitrogen in the United States.

Transport of liquid nitrogen on commercial aircraft.—Because it is a corrosive substance if it spills, liquid nitrogen is treated as a "restricted article" by airline companies. Thus, this preservative is troublesome baggage for researchers who travel by air to and from field sites, especially in foreign countries. Requirements of the United States Department of Transportation and IATA-ICAO (International Air Traffic Association-International Civil Aeronautics Organization) Dangerous Goods Regulations (24th ed.), in effect as of December 1983, are as follows:

To transport liquid nitrogen by aircraft, a form, the "Shipper's Declaration for Dangerous Goods," must be filled out in triplicate. Such forms can be obtained from airline cargo departments or from freight forwarding companies. One copy of the form must be attached to the liquid nitrogen container package and the other two copies are to be presented to the clerk of the carrier airline during check-in. The form must list the information required by the Department of Transportation Domestic Regulations (in 49-Code of Federal Regulations, Civil Aeronautics Board 82) or the International Air Traffic Association (IATA-ICAO) Restricted Articles Regulations, as shown below:

Heading on Form

"*Proper Shipping Name.*" Write "Nitrogen refrigerated liquid."

"*Class.*" Write "2 (non-flammable) UN 1977."

"*Subsidiary Risk.*" Write "NA."

"*Quantity.*" Here write the amount you ship, in liters. (Passenger planes allow up to 50 kg per package; cargo planes allow up to 500 kg per package.) Also, one must state, "Packed in cryogenic container with pressure release (vented) valve." In the appropriate place on the form, be sure to cross out the type of plane (passenger vs cargo) you are not using.

"*Additional Handling.*" Write "transitional."

"*Packing Instructions.*" Write "210." This is the section number in the IATA-ICAO 24th ed. for non-pressurized items.

In addition, the package should be labeled, (a) "This End Up," with

an appropriate arrow drawn in; (b) "Keep Upright"; (c) "Do Not Drop"; and (d) "Handle With Care." It is advisable to contact the carrier airline before arriving at the airport to inform them of your intention to ship "dangerous goods," to tell them that you are aware of the regulations governing such matters, and to stress that the liquid nitrogen is in a non-pressurized, cryogenic container. It is important to realize that the pilot has final say on what will be included as baggage or cargo on the aircraft he or she commands. We have found that the Restricted Article Department of Federal Express will often help in filling out the forms for shipments within the United States. Non-compliance with regulations governing the shipping of restricted articles can result in severe fines or imprisonment.

For international travel, if one knows of a dependable source of liquid nitrogen in the country being visited, it is a simple matter to take the empty tank as baggage, in which case no declarations need to be made. Upon returning to the United States, we have found it propitious to pour out all but a quantity of nitrogen sufficient to cover the filled nunc tubes in the bottom of the tank. This procedure significantly reduces baggage weight and the smaller quantity of nitrogen remaining (which still must be declared) usually seems less threatening to the airline desk clerk. Although tissue-filled tubes reportedly will stay frozen in the tank for up to 24 h, even after all of the nitrogen has been poured off, the real threat of mis-routed or otherwise delayed baggage dictates that at least a few liters should always be retained.

NOTES ON ELECTROPHORETIC METHODS

The notes to follow assume that the researcher has at least a beginner's familiarity with standard electrophoretic methods, such as those outlined by Yang (1971) and Harris and Hopkinson (1976). Many of the following procedures, however, are either not explicitly described in the literature or are in very scattered publications not usually read by avian systematists. Moreover, because each laboratory evolves certain local techniques appropriate to its particular needs, the source of a given protocol is sometimes unclear. Therefore, we acknowledge that some of the methods we describe were devised by others, especially Suh Y. Yang, Monica M. Frelow, and Richard D. Sage, at the Laboratory of Evolutionary Genetics, Museum of Vertebrate Zoology.

Preparation of whole-tissue extracts.—Thaw tissue samples and keep on ice (4°C). Always preserve some unground tissue and return it to the Ultra-cold freezer for storage. Once tissue is ground, its survival time is significantly lessened. Furthermore, repeated thawing and refreezing of tissue extracts is the primary cause of inactivity in enzymes. Mince ap-

TABLE 1
ELECTROPHORETIC CONDITIONS USED AT THE MUSEUM OF VERTEBRATE ZOOLOGY

Gel type[a]	Electrode buffer[a]	Volts	h	Tissue	Loci[a,b,c]
LiOH, pH 8.2	LiOH, pH 8.1	300	3	liver[d]	LGG; LA-1, 2; GDA; LAP; EST-1, 4; AB-1, 2, 3, 4
Tris Maleic, pH 7.4	Tris Maleic, pH 7.4	100	4	liver[d]	6-PGD, SOD-1, 2; G-6-PDH
Poulik, pH 8.7	Borate, pH 8.2	250	3	muscle	GPI; CK-1, 2; LDH-1, 2
Tris Citrate II, pH 8.0	Tris Citrate II, pH 8.0	130	4	muscle	ADA; MPI; GPD
				liver[d]	ICD-1, 2; PGM; GLUD; GR; GPT; ADH; SDH; ACON-1, 2; EAP; GOT-1, 2; NP; ME; MDH-1, 2

[a] Gel type, electrode buffer, and recipes for specific protein assays are described by Selander et al. (1971) and Harris and Hopkinson (1976).
[b] Abbreviations for loci follow Harris and Hopkinson (1976).
[c] Many loci are scorable on several gel/buffer types and with several tissues.
[d] All loci scorable with liver tissue are also scorable in kidney tissue. Occasionally, loci that yield indistinct bands in liver can be scored more clearly with kidney; thus, the reason for saving both kinds of tissue.

proximately 0.5 cm × 0.5 cm tissue with a razor blade on a glass plate, combine it with an equal volume of de-ionized water, and put the mixture into a labeled centrifuge tube. Some workers (e.g., Nakanishi et al. 1969) use a buffer solution in this process. This amount of tissue extract, once centrifuged (at 18,000 RPM for 20 min), will be sufficient for running at least 15–20 gels per individual. For some very concentrated enzymes, the extract can be diluted. Initially, prepare extracts of each tissue type separately for a few individuals and determine tissue and gel/buffer specificity of each protein to be assayed. Once optimal tissue/gel specificity is determined it will be possible to mince tissue types together to prepare the tissue extracts, thereby reducing the time and effort involved in preparing extracts.

Gel/buffer combinations and staining.—Experimentation reveals which loci are best scored on which gel/buffer combinations. See Barrowclough and Corbin (1978), Yang and Patton (1981), and Harris and Hopkinson (1976) for some starting points. In Table 1, we list the electrophoretic conditions in common use at the Museum of Vertebrate Zoology, University of California, Berkeley. These conditions have proved to be suitable for the analysis of over 2800 birds of 40 genera and 10 families. It is desirable to examine a locus on several gel types (e.g., Coyne et al. 1979, Aquadro and Avise 1982). However, we attempt to minimize the number of gel types needed to complete a survey. Our standard protocol for birds is eight gels for 40± loci.

Often a given slice of a gel can be stained for several enzymes. For example, we use a number of ultraviolet protein assays (e.g., EAP, EST-D, GSR). These gel slices can be rinsed with de-ionized water and treated with a visual stain for another protein. Several peptidases are routinely surveyed in birds and other vertebrates. The recipes for these assays differ only in the substrates used (e.g., leucylalanine, leucylglycyl-glycine). First, determine if the protein products of these peptidases have different mobilities on the gel. If so, then two substrates can be used at once in the same assay, resulting in multiple peptidases being scored on the same slice.

Always strictly follow the safety precautions given by the manufacturers of chemicals, such as those on the use of gloves and masks. It is important to note that some routinely used reagents are known or suspected carcinogens (e.g., L-LEUCYL-β-NAPHTHYL-AMIDE HCl, used in the stain for the LAP locus; o-DIANISIDINE, used in the stains for all peptidases; and α-NAPHTHYL ACID PHOSPHATE, used in the stain for acid phosphatase [AcPH]; M. M. Frelow, pers. comm.).

Our protein assays, like those of other workers, consist of several types: ultraviolet, "aqueous," and agar-overlay. In most instances we photograph stained gels of the latter two types, unless all of the individuals on a gel are monomorphic. Gels stained in a non-agar medium, except for UV assays, are dried with a paper towel, wrapped in plastic, and stored in a cold room for future reference. For agar-overlays, we make a filter paper "print." Within a few days after stopping the reaction with acetic acid, we drain off the remaining acetic acid, lay a piece of filter paper over the gel, and invert the box. This causes the gel, agar, and filter paper to drop out onto a paper towel, with the gel on top and the filter paper on the bottom. Because the bands are in the agar, the gel can be removed and discarded. Within 1 or 2 days, the bands will transfer to the filter paper as the agar evaporates and dries. The filter paper is then taped to a large index card, labeled, and saved for future reference (see Fig. 1).

Design of electrophoretic experiments and studies.—Electrophoretic experiments should be designed for maximal efficiency and economy. In our laboratory we put 18–20 individuals (a set) on a single gel. Rather than do 20 individuals for all loci (40 $\pm$ 5), it is much easier to run at one time multiple sets of individuals for a lesser number of loci. For example, we often run three sets of individuals on two gel types (i.e., six gels at a time). This procedure greatly reduces the number of protein assays to be prepared per experiment. The protein assay recipes then should be tripled and can each be prepared in a single flask. From each individual vial we dip two wicks, one for placement into each of the two gel types. The individuals must be ordered identically on the protocols (list of spec-

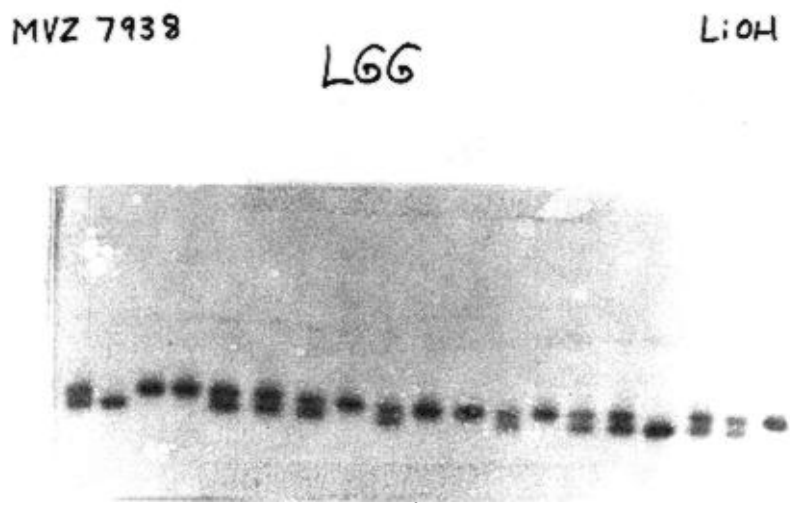

FIG. 1. Photograph of an agar-overlay preparation for LGG. Genotypes of 19 individuals of the Solitary Vireo (*Vireo solitarius*) are shown. From left to right the genotypes of the first four individuals are as follows: MS heterozygote, S homozygote, M homozygote, M homozygote, etc.

imens being run) for each gel type. That is, if we examine sets "a"–"c," using LiOH and TC 8 gels (three gels of each type), we load set "a," on LiOH and TC 8 gels at the same time.

In a large geographic survey, we often first examine a few birds from each population for a total of at least 45 loci. Loci showing sufficient variability either within or among samples are surveyed for all remaining individuals. However, for questions such as paternity analysis or selective neutrality of allozymes (e.g., Zink and Winkler 1983, Barrowclough et al. 1984), every individual must be scored for every locus. Sarich (1977), Nei (1978), and Gorman and Renzi (1979) discuss the number of individuals and loci needed for electrophoretic studies. In general, it is preferable to examine as many loci as possible, rather than as many individuals as possible. Different questions require, however, different sampling methods.

Computer routines for electrophoretic data analysis.—The proper analysis of electrophoretic data involves the equations of population genetics (e.g., Crow and Kimura 1970, Hartl 1981). A program package, BIOSYS (Swofford and Selander 1981), performs most calculations routinely used in electrophoretic studies. Other types of calculations can be found in

numerous papers in the literature (see summaries in Powell 1975, Nevo 1978, and the extensive bibliography in Smith et al. 1982).

"SKIN-SKELETON" PREPARATIONS

The importance of skin-skeleton preparations for phenetic studies was first recognized in the late 1960s and early 1970s by D. M. Power and R. F. Johnston at the University of Kansas and then generally implemented by J. C. Barlow and J. D. Rising at the Royal Ontario Museum in Toronto. With the development of biochemical methods for the study of geographic variation, it has now become of interest to compare patterns of variation of different sets of characters from the same individual specimens, to see if there is geographic concordance of varying attributes of a bird's phenotype and genotype. Thus, many avian systematists now require plumage, skeletal, and tissue data from each specimen. This need has led to the further development of non-traditional methods of preparing specimens in the field.

The following three descriptions of specimen preparation techniques permit one to take a complete range of both skin and skeletal measurements and tissue from the same individual. Each method has its advantages and disadvantages and we do not necessarily recommend one over the others. One of us (NKJ) prefers method 1; another (RMZ), method 2. Method 3 has been in use for many years by J. C. Barlow and J. D. Rising and their colleagues at the Royal Ontario Museum and University of Toronto. We emphasize that these detailed descriptions are of methods the authors have found to be suitable and efficient; other workers may develop variant procedures that are equally effective, or better, for their purposes.

Method 1.—In this procedure, the resulting specimen is a roughed out skeleton that retains its rectrices, primaries and secondaries, and their coverts, and the ramphotheca of the bill. The specimens are dried in the typical study skin posture (Fig. 2). All routine measurements (bill length, wing length, tarsus length, etc.) are taken from the specimen before it is prepared as a complete skeleton.

One distinct advantage of this preparation over the standard study skin is that the mandibles remain in perfect alignment. Such is rarely the case for study skins which have the mandibles tied by thread. A high proportion of museum specimens of many small birds have their lower mandibles improperly seated in the upper mandibles; thus, bill depth measurements are invalid. And, flat-billed small birds (e.g., many Tyrannidae) frequently have their relatively soft bills crushed by over-zealous preparators during the thread-tying procedure.

The steps for the preparation of specimens according to method 1 are

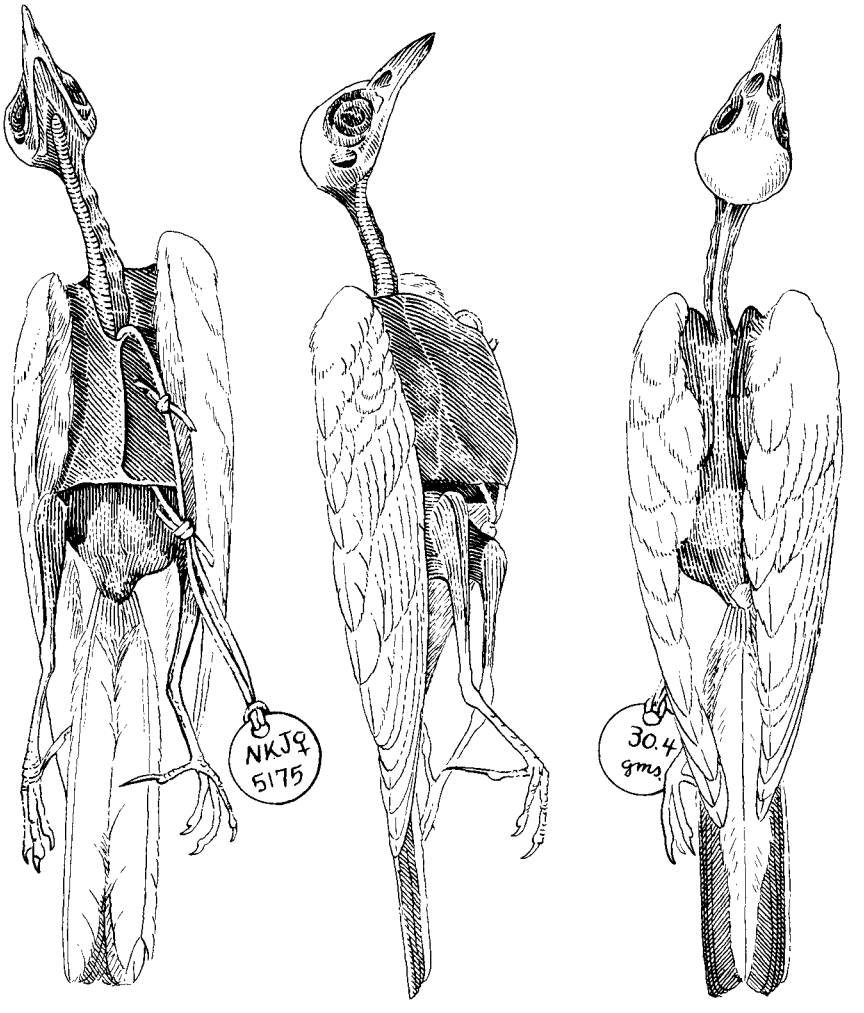

FIG. 2. Ventral, lateral, and dorsal views of a skin-skeleton preparation of a Horned Lark (*Eremophila alpestris*), specimen NKJ 5175, resulting from method 1. Sketch made directly from a dried specimen ready for measurement of skin features prior to being cleaned as a complete skeleton. A completed specimen tag (not shown) should be attached to the tibiotarsus. Note the position of attachment of the "skull tag."

as follows: make catalogue entry, label nunc tube, weigh specimen, and examine it for evidence of molt, ectoparasites, and external signs of breeding (incubation patch, cloacal protuberance). Fill out a stringed tag with collector's initials, field number, and sex symbol on one side, body weight

on the other and record data in field catalogue. "Skull tags," of high rag-content paper which is resistant to damage by immersion or dermestid beetles, the tags commonly used by mammalogists are suitable for this purpose. Limber specimen, first by rotating the head around the long axis of the body and then by gently extending each wing forward and then perpendicularly to body. Strip the skin from the head, starting from the ventral base of the neck, and pull skin toward the bill in successive strips. Note and record in catalogue the condition of the cranium with regard to relative pneumatization. Strip skin from the body: (a) pinch the patagium and pull skin off toward the body; (b) strip skin from humerus so as to leave this bone bare of feathers but retain all secondaries and their coverts and all primaries and their coverts intact on the wing in their natural positions; and (c) strip skin from body and from legs, proceeding caudally. If legs are broken take special precaution not to tear away the legs with the strips of skin and feathers.

With the tips of skinning scissors, cut through the body wall just posterior to the last rib and posterior to the caudal rim of the sternum. With the belly up, arch specimen in the hand to expose the internal organs. With curved forceps, pinch the heart away from its major blood vessels and place it near the labeled nunc tube. Sever the esophagus a few millimeters above its attachment to the stomach and place of attachment of the liver. Seize the posterior stub of the esophagus with forceps and gently pull the viscera out of the body cavity in one mass (while looking for the gonads from the left side) and sever the large intestine near the cloaca. This operation must be done with care so as not to disturb the placement of the gonads. Determine the sex of the specimen, measure the gonads, and record these data in field catalogue. Remove the lobes of liver from the visceral mass and place the liver next to the heart. Examine and record contents of stomach. Remove lungs from body cavity.

With tips of forceps, break through membrane lying between the arms of the furcula. This procedure leaves a hole, between the furcula and the neck, through which the string of the skull tag eventually passes. With curved forceps, lift entire kidney out of the body cavity and place it next to heart and liver. Cut away the breast muscle mass on both sides by incisions first running anteriorly along the keel of the sternum and sides of the furcula and then along the rib cage from the base of the keel to the humerus, where it meets the other incision. Great care must be taken not to cut through either the keel of the sternum or the arms of the furcula. Save as much undamaged breast muscle as desired; a piece 12 mm × 12 mm × 6 mm is suitable for a small nunc tube. Place the piece of breast muscle near the other three tissues from the same specimen.

Place the four types of tissue into the labeled nunc tube in the following

order: (a) heart, which fits well into the rounded bottom of the tube; (b) liver; (c) breast muscle; and (d) kidney. The exact sequence of storage is not crucial, but it is helpful if the sequence of each preparator is known to the laboratory worker who will analyze the tissue. Liver and kidney tissue can be difficult to distinguish when frozen and, thus, should be kept separate in the tube. Some workers prefer to store each tissue type in a separate tube; in general we have not found this procedure to be useful. Avoid packing tissue tightly into the nunc tube because overly-crowded tubes easily shatter when placed in liquid nitrogen.

Enter sex symbol on skull tag and complete catalogue entry. Insert one string of the skull tag into the body cavity and out through the opening between the furcula and the neck. Tie the skull tag with two overlapping square knots. Do not tie the tag tightly around the sternum and furcula because the soft and flexible keel of the sternum and arms of the furcula may be crushed and/or distorted and may dry in unnatural positions. Firmly attach completed specimen tag to the tibiotarsus using two overlapping square knots. Arrange specimen for drying by folding wings along sides of body as in an ordinary study skin. Place specimen in a well-ventilated but insect-proof container. Clean instruments before next specimen is started to avoid mixing fragments of tissue and blood from different individual birds.

Method 2.—This method describes the preparation of a complete study skin and partial skeleton from the same individual. Many details overlap with method 1. One important difference is that in this procedure the tissue samples are removed before the specimen is prepared. First, weigh specimens, label them plus nunc tubes, and record data in field catalogue. Slit the ventral skin covering the abdomen and separate the skin from the body, as in the preparation of a typical study specimen (see Hall 1962: 26–35). Make another ventral slit into the body cavity, thereby exposing the internal organs. Remove liver and heart samples through this slit. Remove gizzard and intestines. Avoid damaging gonads, which can be difficult to find on non-reproductive individuals, and avoid soiling the feathers surrounding the ventral opening. Stomach contents can be preserved at this time. Remove kidney sample and then push skin up slightly on body, exposing enough pectoral muscle for a sample. Place tissue samples in labeled nunc tube as soon as they are taken from the body. Determine sex and record condition of the gonads. Put sawdust or cornmeal in and around opening to prevent soiling of feathers (for skin preparations only). Secure label to specimen and set it aside. Once eviscerated, specimens can be prepared as skins or skeletons, either immediately or up to several days after removal of tissues, especially if the birds are kept cool. This procedure allows tissue samples to be taken from up to a dozen individuals in a relatively short time. On short autumn or winter days,

extraction of tissue from all specimens as described permits one to spend as much time as possible afield during daylight hours; specimen preparation can be completed after dark.

Standard methods (Hall 1962) of study skin preparation are then followed, except: (1) dissect out and set aside one tibiotarsus. This requires everting the skin over the juncture of the tibiotarsus and tarsometatarsus, whereas normally one stops before this point. Replace the tibiotarsus with a wooden stick of equivalent length, and wind an appropriate amount of cotton on it to simulate the normal leg; (2) when the wings are reached in the skinning process, use a scalpel to dissect out an ulna from one wing and a radius from the other wing; set these bones aside with the tibiotarsus if they become detached from their connections to the humerus. This procedure necessitates "stripping" the secondaries from the ulna, a practice which otherwise should be avoided because it distorts the natural alignment of the secondaries on the finished skin. At this point, one usually ties together the distal ends of the ulnae or the humeri, so that the wings are more firmly attached to the rest of the skin. However, in this procedure, place a thread with a slip knot at one end around the distal end of the ulna and tie it to the opposing distal end of the radius, on the other side of the body, at a distance about equal to the distance between the heads of the humeri in the intact body. In the event that the ulna and radius must be taken from the same side, the wings can be tied together by running a threaded needle through the skin of the inverted wing near the distal end of the ulna, i.e., near the junction of the ulna/radius and carpometacarpus. (3) Continue with standard procedures by working the skin over the head, but remove the eyes and set them aside with the excised tibiotarsus. With a knife, sever the skull (in cross section) near the anterior part of the orbits; however, do not cut the tongue and hyoid apparatus; the latter elements are left with the trunk skeleton. This allows the distal one-half of the skull to be preserved. The sharpened end of a stick, with an amount of cotton that approximates the size and shape of the body, is then seated firmly into the base of the upper mandible. Complete preparation of the study skin. Although much of the skull has been removed, the skin shows no apparent ill effects. The cotton eyes can be made somewhat larger than usual to support the skin in the absence of the cranium. Using fine thread, loosely wrap the trunk skeleton and the leg bones, after placing the eyes (and wing bones if detached) inside the body cavity. Attach a label bearing the same field number as that on the nunc tube and skin. Allow skeleton to dry out of sunlight.

Method 3.—As a further variant, it is possible to preserve a study skin with a partial skeleton, in which complete leg and wing skeletal elements are left in the skin on only one side (Barlow and Flood 1983). J. D. Rising (in litt., 17 May 1984) has kindly supplied the following information on

this method: as in other methods, the specimen is weighed, selected measurements are taken, etc. Then, the bird is skinned out over one side. Distal-most bone elements are left in the appendages on one side (viz. carpometacarpus; foot and tarsometatarsus, and perhaps one-half of the tibiotarsus). If the bird has been shot, the preparer may elect to leave "broken" elements on the skin-side of the specimen; it is even possible to leave the wing from one side with the skin and the leg from the other.

As soon as the skin is removed from the carcass, tissue samples can be removed and frozen. The skin can then be prepared as a conventional study skin, minus one leg and one wing, and the bill, or prepared as a flat skin. In the field, generally, the skin is salted later to be washed and cleaned in an organic solvent in the lab. The bill is with the skeletal part of the specimen. The mandibles will remain in perfect alignment and can be measured at any time prior to sending the skeleton to be cleaned. This method gives a study skin, nearly complete skeleton, and opportunity to easily remove tissues, assess gonads and stomach contents, and is fast. An average skinner can do five to eight birds an hour.

With experience, certain of these procedures can be combined or modified to suit the individual needs of the investigator. For example, tissues can be taken from all specimens prior to preparation regardless of the method used to prepare skins and skeleton. It might be desirable in some instances to measure the wing and tail in the field prior to preparations of specimens as complete skeletons. One of us (GFB) preserves a complete skeleton and a partial flat skin (minus wings) from each individual; the latter are used for studies of dorsal, flank, head, and throat coloration of juncos.

These techniques all share a common goal—to preserve as much material as possible from each specimen, given time constraints. Considering the prevalence of anti-collecting attitudes and widespread habitat destruction, it is difficult to justify the preservation of only study skins, as was historically prevalent. At the least, a "trunk" skeleton should be saved along with the standard study skin, whenever possible.

SUMMARY

We discuss several procedures suitable for the needs of modern systematic ornithology, with emphasis on electrophoresis and specimen preparation. Specifically, we describe: (a) methods for the sampling, preservation, and transport of tissue in liquid nitrogen; (b) available liquid nitrogen storage vessels; (c) regulations governing the transport of liquid nitrogen aboard commercial aircraft; (d) techniques useful in the preparation of whole-tissue extracts, gel/buffer combinations and stains, and the design of electrophoretic studies; and (e) methods for the preparation of "skin-skeletons," specimens that allow all routine skin and skeletal measurements and tissue to be taken from the same individual, thereby maximizing the informational content of every specimen.

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ADDENDUM

While the present paper was in press, the following articles appeared, each of which contains information relevant to one or more of the topics we discuss:

BUTH, D. G. 1984. The application of electrophoretic data in systematic studies. Ann. Rev. Ecol. Syst. 15:501–522.

DESSAUER, H. C. AND M. S. HAFNER (compilers and editors). 1984. Collections of frozen tissues: Value, management, field and laboratory procedures, and directory of existing collections. The Association of Systematics Collections, Univ. Kansas, Lawrence, Kansas.

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