PROTOCOL OF FEMUR EXTRACTION FROM BATS IN FLUID-PRESERVED COLLECTIONS

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Abstract.—The anatomy of appendicular bones has been demonstrated to be informative for taxonomic, paleontological, and functional studies of bats. Its use in such studies, however, is limited by the small number of skeletons available for this taxonomic group in scientific collections. Here we describe a protocol for the extraction of the femur from fluid-preserved bats. This protocol was successfully tested on a large sample, including 58 species in 43 genera and 9 families (a total of 183 specimens), and is minimally invasive, requiring only two incisions in the leg at disarticulation points (knee and coxofemoral joints). This method provides material for appendicular anatomical research with minimal damage to external morphology.

Key words.—Chiroptera, fluid-preserved specimens, femur extraction, bat morphology, osteology.

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Introduction

A taxonomic collection is an ordered set of preserved specimens prepared for scientific study (Martins 1994). In the case of mammals, three main preparation methods have been used: "skin and skull," "fluid," and "skeleton" (Yates et al. 1996). Bats are increasingly represented in collections (e.g., Dunnum et al. 2018) and are frequently preserved as "fluid" specimens (i.e., fixation of the entire animal in 10% buffered formalin and storage in 70% ethanol). As emphasized by Simmons and Voss (2009), although fur color fades in fluid preservatives, eventually hampering the identification of banding patterns, fluid preservation has several advantages over the traditional "skin and skull" preparation, including faster execution and better preservation of external morphology. Not only external morphology but all internal structures are conserved in fluid-preserved specimens, maximizing their usefulness for subsequent studies (Simmons and Voss 2009).

Fluid-preserved bats are (or at least should be) always fixed with their mouth open, allowing examination of dental morphology without the need of skull extraction (Simmons and Voss 2009). Furthermore, the procedure of keeping the specimen with the mouth open greatly facilitates skull removal (Simmons and Voss 2009) in case a more detailed dental analysis is required, or if skull structures need to be examined—both common practices in taxonomic studies of bats (e.g., Gardner 2008). On the other hand, few postcranial skeletal preparations are available in collections for examination of the complete bat skeleton, and no formal procedure describing bone removal from fluid-preserved bats has been published. Skeleton preparation is a lengthy process that in many cases involves significant damage to pelage, musculature, and internal organs (Simmons and Voss 2009). Thus, a

protocol that allows the removal of specific bones without compromising the entire specimen is needed.

The anatomy of the bat femur has long been used for research in taxonomy, paleontology, and functional morphology (e.g., Vaughan 1959, Swartz 1997), helping to clarify evolutionary trends in this group (Simmons et al. 2008, Swartz and Middleton 2008, Louzada et al. 2019). Considering the breadth of comparative studies that would benefit from an increase in the number of available taxa and specimens, a technique allowing the extraction of this bone from fluid-preserved material with minimum damage would be useful. Here we describe a protocol for the extraction of the femur of fluid-preserved bats. This protocol was tested on a sample of 183 femora removed from specimens belonging to 9 families, 43 genera, and 58 species of bats (see Table 1) stored in the fluid collection of Museu Nacional (MN, Rio de Janeiro, Brazil). Nomenclature of bones and ligaments followed König and Liebich (2016), and values of body weight (g) were obtained from Reis et al. (2017).

Methods

Hydration Process

The first step is the skin hydration process. Remove the specimens from fluid storage, allow the excess alcohol to drain, and then submerge in a container with 40% ethanol. The time each specimen needs to be soaked in this stage varies according to size: small bats (3.5–20 g), such as *Furipterus horrens* and *Natalus macrourus*, should be immersed for 15–20 min, medium-sized bats (20–50 g), such as *Molossus rufus* and *Chiroderma villosum*, for 30–40 min, and larger bats (>50 g), such as *Noctilio leporinus* and *Chrotopterus auritus*, for 40–50 min. The number of bats immersed in a single container should be calculated according to their weight: a maximum of 100 g of bats for each liter of 40% ethanol (e.g., two 50-g specimens of *Noctilio leporinus* immersed in 1 L of ethanol). This step helps to rehydrate the specimen and soften the leg articulations (Simmons 2014). Because excessive exposure to dilute ethanol solution may result in soft tissue deterioration (Neto et al. 2015), check specimens for flexibility every 10 to 15 min depending on size. After the soaking time is completed, remove the specimen from the bath and rotate the femoral joints at the pelvis and knee, making circular and top-down movements, respectively. If the joints are still rigid, return the specimen to the 40% ethanol bath until joints become flexible enough to rotate the leg. Once the joints are flexible, blot out excess moisture from the pelage to facilitate handling of the skin.

Femoral Extraction

The femoral extraction is carried out with the specimen on its back in a dissecting tray. Two incisions will be made, one at the knee joint and another at the coxofemoral joint (Fig. 1a). For the first incision at the knee, make a slight transverse incision with a scalpel between the femur and tibia in order to disarticulate the femur. Because the skin in this region is very thin and the muscles and tendons are easily dissected, use small scalpel blades (no. 12 or no. 15) and make the incision with care to avoid damaging the bones. The second transverse incision is a superficial cut made from the lateral margin of the pelvic region toward the tail or uropatagium area. Because of the considerable musculature in this region, especially in molossids, use fine scissors to dissect the muscles surrounding the proximal region of the femur and locate the head of the femur within the pelvic joint. Take care to avoid damaging the bone. Slit the ligament (ligamentum capitis ossis femoris) joining the head of the femur to the inner wall of the acetabulum and detach the femur from the pelvis.

Table 1. Fluid-preserved specimens analyzed in this study (Museum number: MN, Museu Nacional, Rio de Janeiro, Brazil; ALP, Adriano Lúcio Peracchi Collection, Universidade Federal Rural do Rio de Janeiro, donated to Museu Nacional).

Taxon	Museum number
Thyropteridae (1 genus, 1 species)	
Thyroptera tricolor	ALP 2912, 2914, 2920
Furipteridae (1 genus, 1 species)	
Furipterus horrens	MN 36053, 36287, 78115, 78116, 78117
Noctilionidae (1 genus, 2 species)	
Noctilio albiventris	MN 64141, 64144, 64145, 64152
Noctilio leporinus	MN 71319, 71321, 71577, 71580
Mormoopidae (1 genus, 2 species)	
Pteronotus gymnonotus	MN 68061, 68073, 68074, 68085
Pteronotus parnellii	MN 80542, 80553, 80564, 80569, 80623
Phyllostomidae (7 subfamilies)	
Micronycterinae (1 genus, 3 species)	
Micronycteris megalotis	MN 36158, 36159
Micronycteris microtis	MN 80534, 80573
Micronycteris sanborni	MN 75194, 79755, 80572
Desmodontinae (2 genera, 2 species)	
Diaemus youngii	MN 71029, 71037, 71379, 77875, 79877
Diphylla ecaudata	MN 68033, 68034, 68035, 68036
Lonchorhininae (1 genus, 1 species)	
Lonchorhina aurita	MN 79798, 79802, 80540, 80541
Phyllostominae (9 genera, 12 species)	
Chrotopterus auratus	MN 70862
Gardnerycteris crenulatum	MN 71390, 71391, 75189, 80583
Lophostoma brasiliense	MN 80495
Lophostoma carrikeri	MN 71404
Lophostoma silvicola	MN 71351, 71486
Macrophyllum macrophyllum	MN 70599, 70600, 70601, 70662
Mimon bennettii	MN 79816, 79827, 79891, 80537
Phylloderma stenops	MN 70594, 70861
Phyllostomus discolor	MN 67723, 67724, 67725
Phyllostomus elongatus	MN 70545, 70546
Tonatia saurophila	MN 70248, 70860
Trachops cirrhosus	MN 71426, 71346, 71469, 80536
Lonchophyllinae (1 genus, 1 species)	
Lonchophylla dekeyseri	MN 80497, 80525, 80563
Rhinophyllinae (1 genus, 2 species)	
Rhinophylla fischerae	MN 70252, 70254, 70255, 70323
Rhinophylla pumilio	MN 70808, 70809, 70811, 70845
Stenodermatinae (6 genera, 7 species)	
Chiroderma villosum	MN 64518, 64519, 71370, 71372
Mesophylla macconnelli	MN 71372
Pygoderma bilabiatum	MN 81269, 81275
Sturnira lilium	MN 36189, 36192, 36313, 36314
Uroderma bilobatum	MN 70877, 70881, 70888, 70918
Uroderma magnirostrum	MN 70302, 70655, 70885
Vampyriscus bidens	MN 70908, 70909, 70910, 70921
Emballonuridae (4 genera, 4 species)	
Diclidurus isabella	MN 70449, 70644
Peropteryx macrotis	MN 79767, 79768, 79797, 79809
Rhynchonycteris naso	MN 70428, 70429, 70431
Saccopteryx bilineata	MN 70445, 70611, 70914, 71594

Table 1. Continued

Taxon	Museum number
Natalidae (1 genus, 1 species)	
Natalus macrourus	MN 67863, 67868, 67873, 81448
Molossidae (8 genera, 12 species)	
Cynomops planirostris	MN 70276, 70287, 70288, 70301
Eumops glaucinus	MN 71472
Eumops perotis	MN 64370, 64747, 64750, 64787, 64789, 71287
Molossops temminckii	MN 71350, 71355, 71415, 71418, 71421, 71434
Molossus currentium	MN 71560, 71563
Molossus molossus	MN 71561, 71562, 71564
Molossus pretiosus	MN 71551, 71552, 71566
Molossus rufus	MN 71568, 79894
Neoplatymops mattogrossensis	MN 36029, 36030, 36031, 37322
Nyctinomops macrotis	MN 49583, 49584, 49586, 49587, 49588, 49594
Promops nasutus	MN 64677, 64678, 64746, 64762, 64763, 71480
Tadarida brasiliensis	MN 6562, 6564, 6565, 6566)
Vespertilionidae (4 genera, 7 species)	
Eptesicus brasiliensis	MN 71417
Histiotus velatus	MN 46491
Lasiurus blossevillii	MN 71304, 71459
Lasiurus ega	MN 70593
Myotis nigricans	MN 71530, 71532
Myotis riparius	MN 71311, 71589
Myotis simus	MN 71451, 71458

Carefully pull the detached femur out through the incision made in the area of the pelvis, minimizing damage to the skin (Fig. 1b). In the femoral region, the skin easily detaches from the leg musculature so that extraction does not require special technique. Once the femur is removed, set it aside in a labeled container for further cleaning and submerge the bat specimen in 70% ethanol. To correct for dilution, check the concentration periodically with an alcoholometer and do several rinses with 70% ethanol. Once the ethanol concentration has stabilized at 70%, return the specimen to the original container for storage.

Cleaning Process

The next step involves cleaning the extracted femur using both mechanical cleaning and treatment with larval hide beetles (*Dermestes maculatus* DeGeer, 1774). Remove excess musculature from the femur with scissors or tweezers, again taking care not to damage the bone, and dry the femur under a lamp for a day. This latter procedure is important to allow residual ethanol to evaporate before the material is introduced to the dermestid larvae. Place the dry femur with a resistant tag (we used Rotex© adhesive tape) in a container with two to four dermestid larvae. The time these beetles take to clean femora varies from two to five days, so check the bone daily during this phase, moistening slightly with water whenever necessary. Remove the cleaned femur from the dermestid colony and wash, dry, and store in a labeled container for further study (Fig. 1c).

Conclusions

This protocol was successfully applied on bat specimens fixed in formalin and stored for decades in 70% ethanol (the oldest specimen dates from 1942), demonstrating its

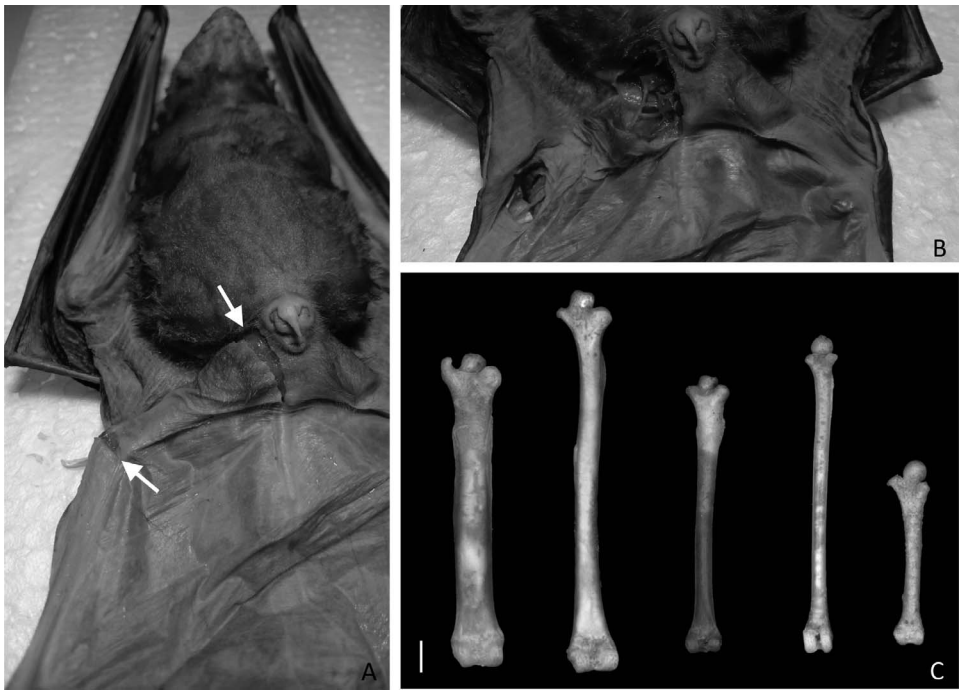

Figure 1. Fluid-preserved specimen of *Noctilio leporinus* (MN 71321) showing the pelvic and knee incisions (arrows) for subsequent femur extraction (A) and the hindlimb region after the bone extraction (B). Note that the plagiopatagium and the tail membrane were not damaged. (C) Cleaned femora of *Diaemus youngii* (MN 71037), *Phyllostomus discolor* (MN 67724), *Sturnira lilium* (MN 36192), *Lasiurus blossevillii* (MN 72304), and *Cynomops planirostris* (MN 70276), respectively. Scale bar: 2 mm.

effectiveness for the extensive fluid-preserved material available in scientific collections (e.g., Louzada et al. 2019). Based on our experience, no special treatment (e.g., Nicholson and Dana 2010) is necessary before introducing the material to dermestids for bone cleaning. However, such alternative treatments may be necessary for specimens stored for long periods in formalin, which is not a recommended practice in mammalian collections (Meeuse 1965, Martin et al. 2011). Residual formalin may not only prevent dermestids from feeding on the carcasses (McDiarmid 1994) but may also adversely affect these beetles and their larvae (Nicholson and Dana 2010). Our protocol also allows the study of an underutilized part of the skeleton, the femur, without causing excessive damage to the fluid-preserved specimen, allowing for future studies of external morphology. It is important, however, to limit the removal of the femur to only one side of the specimen, preserving the other bone *in situ* for studies of muscle attachment or for imaging. Although our focus has been on the femur, the technique described here can also be adapted for use on other appendicular bones.

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