

# Histomorphology of the Penis Bone (Baculum) in the Gray Long-Eared Bat *Plecotus austriacus* (Chiroptera, Vespertilionidae)

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## ABSTRACT

For the first time, the histomorphology of the penis bone of a bat (*Plecotus austriacus*) was examined in detail. From *Plecotus austriacus*, 14 whole penes and 11 isolated bacula were studied and compared to bacula of *Plecotus auritus* and *Plecotus macrobullaris*. The baculum was located on specimen microradiographs and in micro-CT images in the tip of the penis. Using serial semithin sections and surface-stained, undecalcified ground sections, the types of bone and other tissues constituting the baculum were examined by light microscopy. 3D reconstructions were generated from the serial semithin sections and from micro-CT images. The shaft and the proximal branches of the Y-shaped baculum form a tubular bone around a medullary cavity. Since the small diameter of this channel and the main lamellar bone around it resemble a Haversian canal, the baculum is equivalent to a single-osteon bone. Several oblique nutrient canals enter this medullary cavity in the shaft and branches. All ends of the baculum consist predominantly of woven bone. The collagen fiber bundles of the tunica albuginea of both corpora cavernosa insert via fibrocartilage into the woven bone of the branches. Thus, the microscopic structures support the hypothesis that the baculum functions as a stiffening element in the erect penis. In this study, several microscopic imaging techniques were evaluated for displaying the microscopic structures of the baculum. Specimen microradiography, but especially micro-CT proved to be suitable nondestructive methods for accurate and reproducible demonstration and comparison of the three-dimensional structures of the baculum in different bat species. *Anat Rec*, 293:1248–1258, 2010. © 2010 Wiley-Liss, Inc.

**Key words:** os penis; bone; osteon; ground sections; micro-radiography; x-ray microtomography; species identification; function

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## INTRODUCTION

A baculum, also called os penis, os glandis or os priapi, occurs in many different mammals, including most species of Chiroptera, Insectivora, Rodentia, Carnivora, and Primates. The name "baculum" was proposed by Thomas (1915), who was the first mammalogist to recognize its value as a taxonomic character (Burt, 1936). The baculum varies greatly among groups, but is commonly invariant within a species (Romer and Parsons, 1977; Patterson and Thaler, 1982). The penis bone is one of the most highly variable primary sex traits in male vertebrates (Tasikas et al., 2007). In bats the baculum is generally short, situated in the glans dorsal to the urethra, but can reach up to 75% of the penis length in some species (Sinha, 1976). Many hypotheses and speculations have been proposed concerning the function of the baculum.

The macromorphology of the baculum, which has been studied extensively, is a very useful distinctive trait to identify bat species. For a synopsis of the early descriptions and uses of the chiropteran baculum, see Hill and Harrison (1987) and Strelkov (1989a,b). Strelkov (1989a) has identified and hand drawn many different forms of bacula in the plecotine bats (see Fig. 3 in Strelkov, 1989a). At that time, only two species of the genus *Plecotus* were accepted, so he described the different bacula as geographical variations of both species. Each of these bacular forms could later be assigned to a different *Plecotus* species (Spitzenberger et al., 2006).

The microchiropteran genus *Plecotus* is distributed throughout the palearctic region from Ireland to Japan, including North Africa, Macaronesia, and Ethiopia (Horáček et al., 2000). It comprises 19 species, many of which are very similar to each other. *Plecotus austriacus* occurs in most parts of Europe and eastwards to the Ukraine (see Spitzenberger et al., 2006). The characters used most for species discrimination, in order of decreasing frequency, are the skeletal measurements of forearm, thumb and claw, fingers, skull, and feet; fur-color; shape of the baculum; dentition; and pigmentation of the skin on face and ears (Spitzenberger et al., 2002; Tvrtković et al., 2005).

This study was carried out with three aims:

1. To investigate the histomorphology of the baculum in *Plecotus austriacus*, and to correlate microanatomy with macromorphology by using both invasive histological techniques and noninvasive microradiography and X-ray microtomography (micro-CT).
2. To support the most plausible of the many existing theories on bacular function based on its micromorphology.
3. To show that the micromorphology of the baculum includes characters that can potentially be used for differentiating bat species.

While the primary aim is descriptive, the second and third aims above are driven by the following working hypotheses: for the second aim, the general consensus is that sexual selection is influencing bacular morphology, and therefore it must have an identifiable precise mechanical function; and for the third aim microstructural traits of the baculum can be used to distinguish closely related species.

## MATERIALS AND METHODS

### Specimens

The Mammal Collection at the Natural History Museum Vienna kindly provided all the specimens for this study. In Austria, all species of bats are protected by law. Therefore, the Natural History Museum Vienna only collects bats that are found dead and donated to the museum. Of the species *Plecotus austriacus*, 14 total penis specimens (AM 87/196, KS 08/44, NMW 34241, NMW 36137, NMW 50460, NMW 50464, NMW 50895, NMW 51137, NMW 52193, NMW 52841, NMW 52845, NMW 57250, NMW 64099, and NMW 65273), and 11 isolated bacula specimens (NMW 11490, NMW 11514, NMW 11817, NMW 25151, NMW 34418, NMW 36135, NMW 52197, NMW 52232, ZMB 42943, ZMB 9255, and ZMB 9256) were available. One specimen was available of each of the species *Plecotus auritus* (NMW 50509), and *P. macrobullaris* (NMW 34857) for micro-CT imaging (Abbreviations: Inventory numbers NMW at the Natural History Museum Vienna; AM and KS laboratory abbreviations, mammal collection NMW; ZMB, Zoological Museum Berlin).

### Specimen Preservation

The whole bats were usually stored deep-frozen after arrival at the museum, sometimes for several months. Because of the time period between death and fixation, the state of preservation was often suboptimal. When the bats were dissected, the whole penes were stored in 70% ethanol with no additional fixation (Kiernan, 1990; Kryštufek and Hrabě, 1996). Eleven of the penes were cleared in a 6% solution of potassium hydroxide (KOH), calcium-salt stained with Alizarin Red S and transferred to 100% glycerol via ascending glycerol concentrations. Then, the bacula were retrieved by dissection under a stereomicroscope, measured (Fig. 1), and further stored in glycerol solution with thymol crystals added (Kryštufek and Hrabě, 1996). The samples for this study were obtained at several different stages of this process and variously treated to prepare them for the methods detailed below.

### Microradiography

The bacula in the intact penes were first located and identified by microradiography, and microradiographs were also taken of the undecalcified ground sections. A high-resolution film (Kodak Professional SO-343<sup>TM</sup>; Eastman Kodak Company, NY) was exposed in a Cabinet X-Ray System (Faxitron Series; HP Company, Palo Alto, CA) for 20 min or 25 min at a voltage of 20 kV or 25 kV and developed under standard conditions.

### Micro-CT

X-ray microtomography scans were made of 12 unstained total penes in 70% ethanol, of one iodine-stained (Metscher, 2009) penis, fixed in buffered formalin solution (Lillie and Fullmer, 1954), and of one isolated, decalcified, and iodine-stained baculum in 100% ethanol. A high-resolution micro-CT system (model MicroXCT, Xradia, Inc., Concord, CA, USA) with either a tungsten or a rhodium source was used at a voltage of between 40 kV and 80 kV. Different magnifications gave reconstructed voxel sizes between 4.17  $\mu\text{m}$  and 0.90  $\mu\text{m}$ . Some illustrations in this article are virtual slices taken

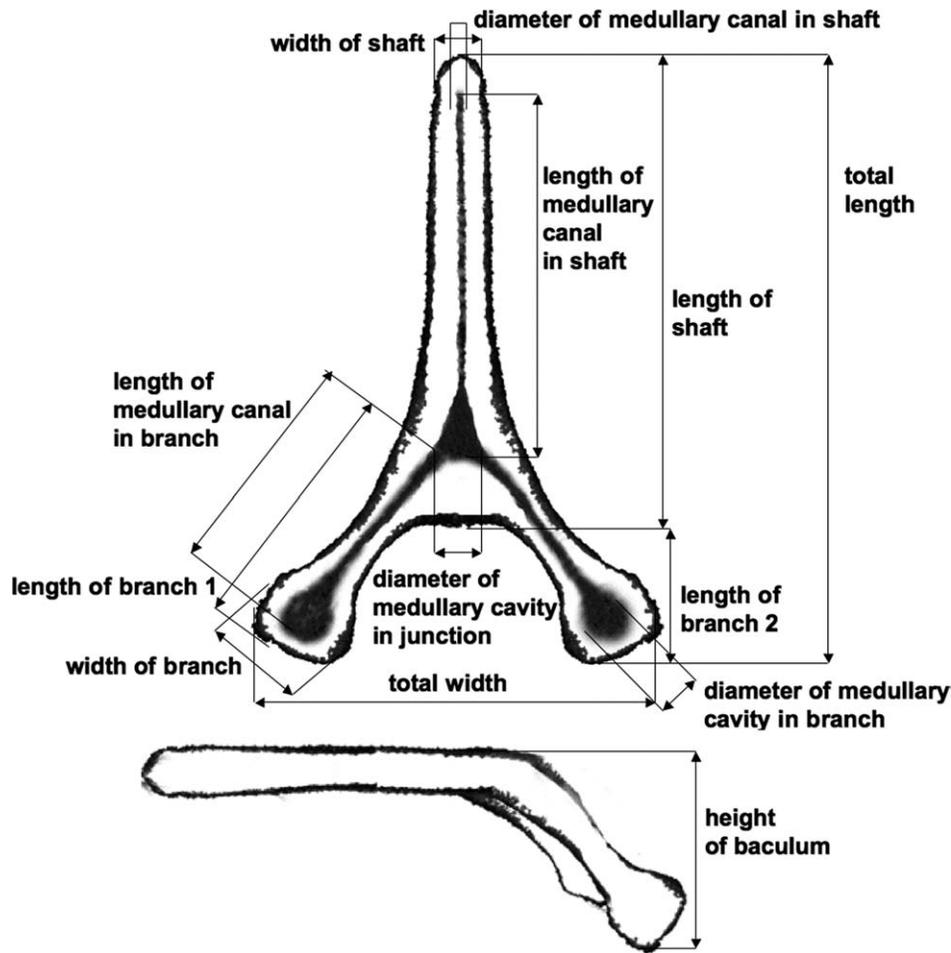


Fig. 1. Schematic drawing of a baculum with measurements as in Table 1.

from the 3D reconstructions of the micro-CT scans, made using the supplied viewing software.

### Histological Techniques

**Serial semithin sections.** Eleven isolated bacula and one intact penis were embedded in araldite resin according to Romeis (1989). The isolated bacula were prestained red with safranin O (0.5% in 80% ethanol) to make them visible inside the resin. To generate ribbons of serial semithin sections, each resin block was coated with glue (Pattex Compact<sup>TM</sup>; Henkel Central Eastern Europe, Vienna, Austria) mixed with xylol on the side facing the blade. From each isolated baculum or the one whole penis, 300–450 serial sections of 2  $\mu\text{m}$  thickness were cut with a diamond knife on a Reichert Ultracut S microtome (Leica Microsystems, Wetzlar, Germany). The section ribbons were transferred to microscope slides and stained with Richardson's stain (Richardson et al., 1960) or toluidine blue O stain (0.1% in 2.5% sodium (tetra)borate) (modified according to Romeis, 1989). Araldite resin was also used as mounting medium for the glass cover slips.

The stained sections were evaluated by light microscopy using a Nikon Microphot-FXA (Nikon Corp., Tokyo,

Japan). At 25 $\times$  to 600 $\times$  magnification, the bone and soft tissues of the penes were examined, dimensions were measured (Fig. 1 and Table 1) with an eyepiece graticule, and photographed with a digital camera (ProgRes C14, Jenoptik, Jena, Germany).

Digital microphotographs of the serial sections were imported into the reconstruction program Amira<sup>TM</sup> 3.11. (Visage Imaging, Berlin, Germany). The pictures were aligned to form an image stack. On each picture, all types of tissue were labeled manually for reconstruction by drawing the contours of the relevant structures. Based on manual image segmentation, a three-dimensional surface model was computed by the software.

**Ground sections.** One whole penis was embedded in methylmethacrylate resin (Plenk, 1989).

The resin block was cut into rectangular shape on an Emco Swing<sup>TM</sup> band saw (Emco GmbH, Wr. Neudorf, Austria). Using a water-cooled diamond saw (Buehler Isomet<sup>TM</sup> low-speed saw; Evanston, IL) the resin block was cut parallel to the frontal side of the baculum. To the resulting plane, a plexiglas microscope slide was glued with instant adhesive (CA8 Pronto<sup>TM</sup>, 3M Comp., St. Paul, MN, USA). A thick section of 3000  $\mu\text{m}$  was cut and then ground (with EXAKT- Type AW 10<sup>TM</sup>) and

TABLE 1. Measurements on bacula (means  $\pm$  SD in mm)

	Stereomicroscopic Bacula in glycerol	Microscopic		
		Semithin sections	Microradiography	Micro-CT
Isolated bacula $n = 9$	$n = 9$	$n = 7$	$n = 1$	$n = 12$
Whole penes $n = 13$		$n = 1$		
Dimensions				
Total length of baculum	0.81 mm $\pm$ 0.05	–	0.85 mm	0.78 mm $\pm$ 0.05
Total width of baculum	0.55 mm $\pm$ 0.04	0.44 mm $\pm$ 0.05	0.43 mm	0.48 mm $\pm$ 0.05
Height of baculum	–	0.16 mm $\pm$ 0.03	–	0.17 mm $\pm$ 0.02
Length of shaft	0.66 mm $\pm$ 0.03	–	0.71 mm	0.65 mm $\pm$ 0.05
Width of shaft	–	0.16 mm $\pm$ 0.05	0.10–0.14 mm	0.16 mm $\pm$ 0.02
Height of shaft	–	0.06 mm $\pm$ 0.01	–	0.06 mm $\pm$ 0.01
Length of branch 1	–	–	0.22 mm	0.30 mm $\pm$ 0.03
Length of branch 2	0.16 mm $\pm$ 0.04	–	0.13 mm	0.13 mm $\pm$ 0.02
Width of branch	–	0.08 mm $\pm$ 0.02	0.09–0.12 mm	0.16 mm $\pm$ 0.02
Length of medullary canal in shaft	–	–	0.53 mm	0.41 mm $\pm$ 0.09
Diameter of medullary canal in shaft	–	0.03 mm $\pm$ 0.01	0.02 mm	0.03 mm $\pm$ 0.03
Length of medullary canal in branch	–	–	0.30 mm	0.21 mm $\pm$ 0.04
Diameter of medullary canal in branch	–	0.06 mm $\pm$ 0.01	0.02–0.04 mm	0.05 mm $\pm$ 0.01
Diameter of medullary canal in junction	–	0.05 mm $\pm$ 0.02	0.04 mm	0.23 mm $\pm$ 0.06

polished (Buehler Minimet<sup>TM</sup> Polisher; Evanston, IL) until the superficial tissue layers of the penis were exposed. Subsequently, the surface of the ground section was ground (1000-grit sandpaper, 4000-grit sandpaper) and polished (alumina polishing powder, particle size 0.3  $\mu$ m) off in sixteen 100  $\mu$ m steps until the remaining ground section was 490- $\mu$ m thick. At each step, the ground section was surface stained with either Giemsa stain (Merck, Darmstadt, Germany; (modified according to Plenk, 1989), or Sanderson's Rapid Bone Stain<sup>TM</sup> (Surgipath Medical Industries, Richmond, IL, USA) and photographed with the same equipment as above. After every fifth polishing-step, microradiographs were made as described above.

## RESULTS

### Macromorphology

The baculum is a Y-shaped bone (Fig. 1). Its shaft is straight and almost cylindrical, but flattened dorso-ventrally for most of its length. Distally, the shaft is slightly tapered, ending in a blunt tip. Proximally, the profile of the shaft is slightly convex above and concave below. There is no distinct change toward the base, just a gradual broadening of the shaft. The base (proximal end) is bifurcated into two branches which are shorter than the shaft. The branches of the base are broad and flattened, ending in blunt tips. They are curved toward the base of the penis. In dorsal view, the two branches together form a flattened arch. For macroscopic dimensions of the bacula measured in this study see Table 1.

### Microanatomy

Based on micro-CT images made after iodine staining, and on semithin microtome and ground sections, the

baculum of *Plecotus austriacus* was found to be situated in the glans penis, directly dorsal to the urethra (Fig. 2). The baculum resembles a bicycle saddle, its proximal base reaching down the sides of the urethra (Figs. 3 and 4). The distal end of the baculum is located near the tip of the glans penis and the opening of the urethra (Fig. 2).

### Histomorphology

The shaft and branches of the baculum form a tubular bone with concentric lamellae around a central medullary cavity or canal. In the shaft, this medullary canal is uniformly narrow and runs along the whole length except for the very tip (Figs. 4, 5, and 6). The canal is round to oval in cross section along the shaft and becomes slightly narrower toward the distal end. At the proximal base it widens slightly, and in a distinct crotch it splits into two canals. In the branches of the baculum, the cross section of the medullary cavity is oval and widens steadily toward the proximal tips. For microscopic dimensions of the bacula measured in this study see Table 1. Seven nutrient canals enter the medullary cavity, three of them close to each other along the shaft's axis near the distal tip of the baculum; a fourth canal enters proximally at the crotch. At the base, there are four additional nutrient canals: one in the middle of each branch, and one near the tip of each branch. The respective foramina nutritia of those seven nutrient canals are located ventrally on the shaft, ventrally in the crotch, and on the medial aspects of the branches (Figs. 4, 5, and 6).

In the shaft and the branches, particularly the inner surface of the medullary canal, the tubular bone appears lamellar like an osteon. Here, the small, elongated, and oval shaped osteocyte lacunae are arranged between the

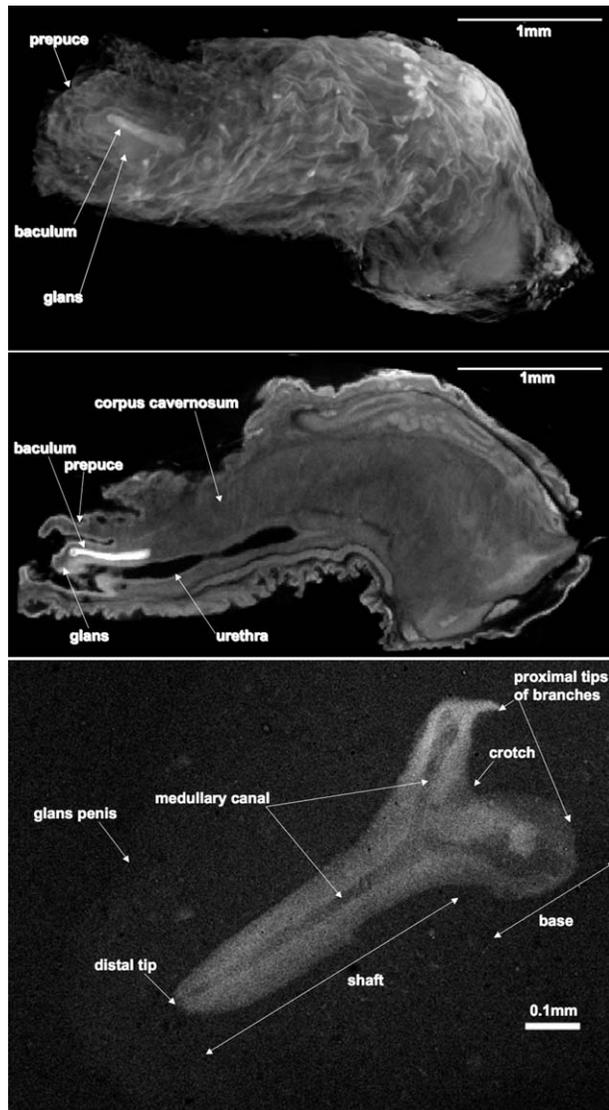


Fig. 2. Top: Volume rendering from micro-CT images of an iodine-stained *Plecotus austriacus* penis (KS 08/44; tungsten source, 79 kV, 43  $\mu$ A, cubic voxel size: 4.17  $\mu$ m). Middle: Virtual longitudinal section of the same micro-CT scan, showing the radiodense baculum. Bottom: Film microradiograph of a *Plecotus austriacus* baculum in an unstained total penis specimen (NMW 51137).

lamellae. All three ends of the baculum consist primarily of woven bone. Here, the osteocyte lacunae are larger, more rounded, and more numerous than in the shaft, closer together and randomly oriented (Figs. 4 and 5). Zones of bone remodeling, marked by reversal cementing lines, were found between woven bone and the inner lamellar layer in the tubular bone of the branches (Figs. 4 and 5).

The intact penis specimens of *Plecotus austriacus* showed that the tips of the branches of the baculum are linked to the corpora cavernosa (Figs. 2, 4, and 5). While the medial portions of the tunicae albugineae of the two corpora cavernosa merge in the middle, the peripheral portions of the tunicae continue into the periosteum of the baculum. Thus, the corpora cavernosa and the bacu-

lum form a unit enveloped by a continuous fibrous structure. Chondroid cells are scattered between the collagen fiber bundles of the tunica albuginea, where they insert into the woven bone of the branches (Fig. 5). In this fibrocartilaginous area, mineralization fronts can be seen between the bone and the tunica albuginea (Fig. 5).

### Bacular Morphology Traits for Species Identification

Comparison of micro-CT volume renderings of bacula from *Plecotus austriacus* with a baculum of *Plecotus auritus* and *Plecotus macrobullaris* (see Fig. 7) revealed a different outer shape and diameter of the canals in all three species. Also, the number and distribution of nutrient canals seems to differ between species. In the bacula from two adult *Plecotus austriacus*, a different canal pattern was found (see Fig. 8). The bacula from juvenile bats of all three species appear to differ according to age in all these aspects.

### Problems and Merits of the Applied Methods

The specimens used in this study were originally preserved for only macromorphological examination and measurements. The oldest of them had been preserved in ethanol since 1965. Alcohol fixes and preserves tissues by protein coagulation, dehydration, and antimicrobial action, but is generally acknowledged as a poor substitute for reagents with cross-linking and other fixation properties (Kiernan, 1990). Also, fixation often took place long after the death of the bat. At the Mammal Collection of the Natural History Museum Vienna, bat specimens are kept either in ethanol, or as dry skins and/or skeletons, depending on their state of preservation at acquisition. For the Museum's purposes, deep-freezing the animals until they can be processed is the best solution.

Unfortunately, deep-frozen tissue is less appropriate for histological methods. According to Plenk (1986), immediate postmortem or post-sacrifice dissection is always preferable to cryopreservation. This is because, especially in larger specimens, adequate cellular and soft tissue preservation cannot be achieved even by rapid deep-freezing; subsequent fixation and penetration with the embedding medium is always impaired in previously frozen specimens.

Even though storage in glycerin with thymol is the standard method (Dingerkus and Uhler, 1977; Kryštufek and Hrabě, 1996; Narotsky and Rogers, 2000; Bhudoye et al., 2001), the thymol failed to prevent the growth of mold on the samples. In addition, some of the specimens were found to be decalcified, or partially decalcified at the margins. As thymol is a weak organic acid in solution (Haug, 1999), this could account for decalcification.

The mineralization of the baculum allows for radiographic imaging. Specimen microradiographs are a simple way for depicting and measuring the baculum and the medullary cavity, but radiographic projection images can give an impression of the three-dimensional structure of the mineralized bone only if they are taken in different orientations. Micro-CT proved to be a more complete solution (see also Neues and Epple, 2008), providing not only projection radiographs with down to 0.90  $\mu$ m pixel size but also data to automatically create virtual sections and 3D-reconstructions. Both the

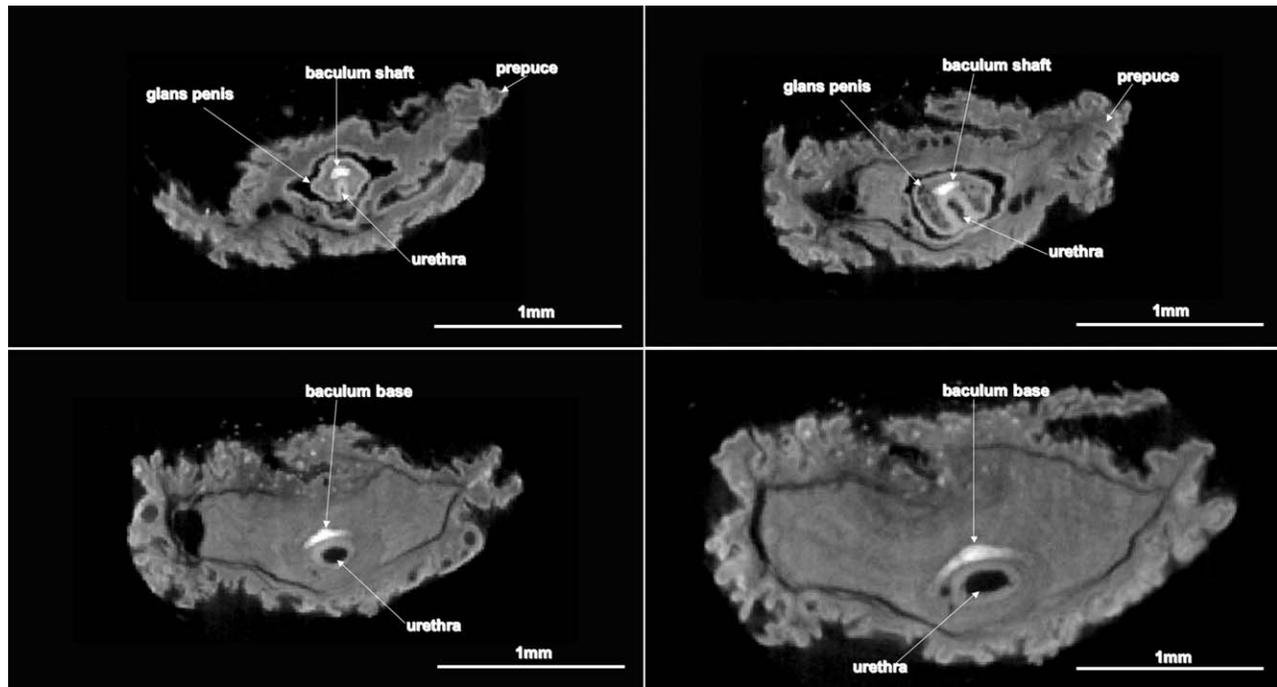


Fig. 3. Virtual cross sections, from distal (top left) to proximal (bottom right), of the micro-CT scan of an iodine-stained *Plecotus austriacus* penis (KS 08/44; tungsten source, 79 kV, 43  $\mu$ A, cubic voxel size: 4.17  $\mu$ m).

radiographic methods are nondestructive and obviously well suited for accurate and reproducible imaging and measurements, which can be used for comparison of the three-dimensional structures of the baculum in different bat species. The drawback of these methods is that they only work with undecalcified samples. A solution to this problem is the whole specimen staining with radiodense contrast media such as iodine solution (Metscher, 2009). 3D reconstruction from semithin serial sections is destructive and much more time consuming but can also use decalcified bone samples, and it can be used to show only selected histological details.

The retrieval of the bacula by dissection under the stereomicroscope also allowed for microscopic measurements. The differences in dimensions measured on isolated bacula in glycerin and those measured on radiographic images and microscopic sections could either be due to intraspecific variation, or to residual tissue attached to the bacula.

## DISCUSSION

Microscopic investigation of the baculum in *Plecotus austriacus* showed that the penis bone contains a continuous central medullary cavity or canal, which is entered by several nutrient canals. The shaft and branches of the baculum form a lamellar bone, which we interpret as a single-osteon bone. The typical dimensions of an osteon concur with the measurements of the baculum in *Plecotus austriacus*: According to Knese (1979 and citations therein), the length of an osteon can vary between 3 and 40 mm, the diameter between 154 and 418  $\mu$ m, and the diameter of its Haversian canal between 20 and 300  $\mu$ m. Long bones of this single-osteon type are also

found in other vertebrates, for example the femur of the Japanese fire-bellied salamander (*Cynops pyrrhogaster*) (Urschitz, 1982). Demeter and Mátyás (1928) describe the whole femur cross section in other amphibians (e.g. *Rana esculenta*) as representing a single large osteon with visible nutrient canals.

The distal end of the bacular shaft and especially the proximal ends of the two branches consist of woven bone with a fibrocartilaginous insertion of the tunica albuginea. In this study of the adult *Plecotus austriacus*, the tunica albuginea of the corpora cavernosa continues into a tubular bone without any remaining evidence of chondral ossification. However, reversal cementing lines between the outer woven bone and the inner lamellar bone point to previous remodeling processes. The occurrence of chondroid cells between inserting fibers in the woven bone appears to be a functional adaptation to biomechanical needs, similar to tendinous insertions at joints. Matthews (1942) already reported that the bacula of some African species of bats are fused with the distal end of the corpora cavernosa. He also found that, in *Hipposideros caffer*, the corpora cavernosa are fused throughout the length of the penis: at their distal end, the tunica albuginea is continuous, through a region of cartilage, with the baculum. Crichton and Krutzsch (2000) reported that the baculum of bats is typically attached to the distal end of the partially fused corpora cavernosa with its two branches. Generally, in the mammalian penis the baculum (when present) is closely associated with the distal portion of the corpora cavernosa (Smith and Madkour, 1980). Wimsatt and Kallen (1952) state that the fetal baculum might be derived from the corpora cavernosa, because the tunica albuginea transforms imperceptibly into the periosteum of the baculum.

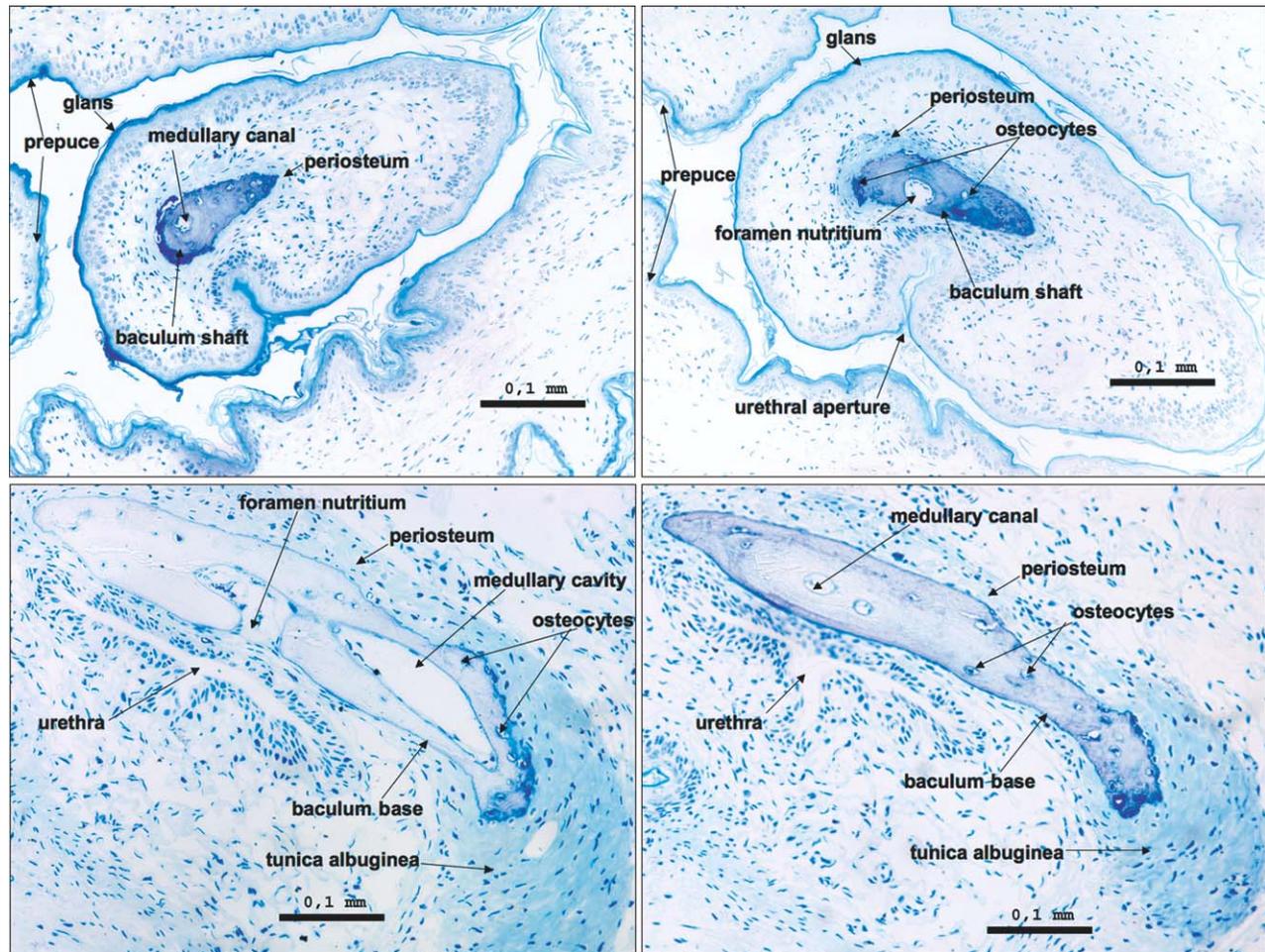


Fig. 4. Semithin (2  $\mu\text{m}$ ) oblique cross sections, from distal (top left) to proximal (bottom right), of a *Plecotus austriacus* penis tip, stained with toluidine blue O.

This would imply membranous ossification. On the other hand, according to Kelly (2000), the baculum and the corpus cavernosum originate from the same mesenchymal mass in the embryonic penis in mice (Glucksmann et al., 1976) and rats (Murakami and Mizuno, 1984), but the two structures develop separately, and the fetal baculum has a cartilaginous anlage (Smirnov and Tsytulina, 2003).

The microanatomy of the plecotine baculum and penis implies a mechanical function of the baculum. Apparently, it forms a functional unit with the corpora cavernosa, enveloped in a common fibrous structure. Even though the microanatomy of the *Rattus norvegicus* penis is different, the interpretation of Kelly (2000) concerning force transfers between baculum and corpus cavernosum seems to be similar. The penis of *Rattus norvegicus* contains only one corpus cavernosum, the distal end of which envelops the flared proximal end of the baculum. She suggests that bending as well as compressive forces on the tip of the penis during intromission are transferred to the tensile wall of the single corpus cavernosum via the baculum in rats. The single-rod baculum is pressed into the proximal end of the corpus cavernosum, with the resulting compression increasing the hydro-

static pressure inside the corpus cavernosum. Because of inelasticity of the collagen fibers in the tunica albuginea, this will further increase the stiffness of the corpus cavernosum and thereby the flexural stiffness of the entire penis. The layer of fibrocartilage between the baculum and corpus cavernosum could serve as a flexible joint similar to the joints in the hyoid (Kelly, 2000).

In contrast, the Y-shaped baculum in plecotine bats seems to be the mechanical solution for transferring and distributing the above-mentioned forces equally to and from the two corpora cavernosa. The hydrostatic stiffness of the filled corpora cavernosa can continue into the tip of the glans via the stiffness of the penis bone, as they are enveloped in a common fibrous structure to form a structural unit. The concave surface of the baculum's distal tip in plecotine bats also suggests a function in keeping the distal orifice of the urethra open during copulation. Finally, the fibrocartilage at the insertion of the tunica albuginea into the baculum indicates alternating shearing forces on this joint-like interface during erection and copulation. The function of fibrocartilage at tendon-bone attachments (entheses) seems to be very similar in humans (Benjamin et al., 1986) and other vertebrates (Kelly, 2000).

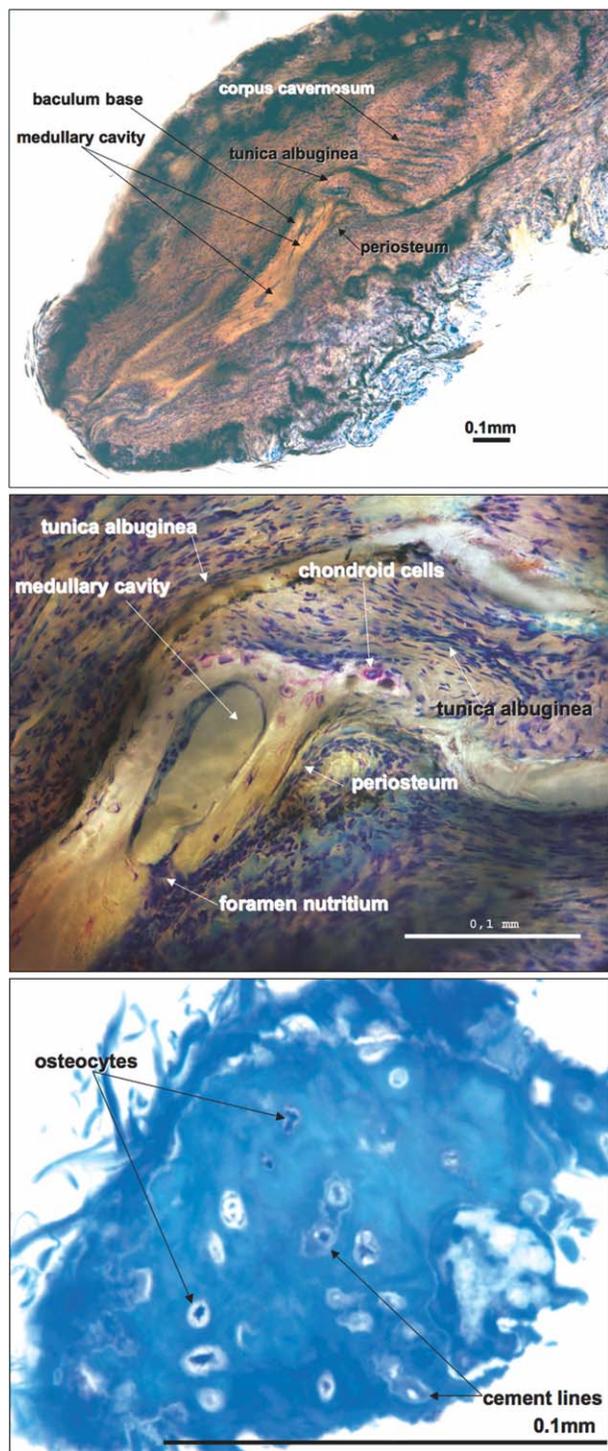


Fig. 5. Top: Undecalcified ground section (longitudinal) of a *Plecotus austriacus* penis tip, Giemsa stained. Middle: Enlarged detail of the same ground section, Giemsa stained. Bottom: Corresponding detail of a semithin (2 μm) cross-section of a *Plecotus austriacus* baculum, stained with toluidine blue O.

The function of the variable shapes of the baculum in various species may be different and the precise mechanical function of the baculum in bats has yet to be

determined. According to Dyck et al. (2004), the mammalian baculum probably has multiple overlapping functions. It functions during intromission indirectly, by affecting penile shape or providing mechanical support. The baculum could also protect the urethra from compression, enable protracted copulations, stimulate the female reproductive tract, provide information about male size or quality during intromission, or facilitate reproductive isolation. For the evolution of the baculum, three hypotheses have been put forward: the vaginal friction hypothesis (Long and Frank, 1968), the prolonged intromission hypothesis (Dixson, 1987, 1995; Dixson and Anderson, 2004) and the induced ovulation hypothesis (Eberhard, 1985). Larivière and Ferguson (2002) give a synopsis of those three hypotheses.

The macroscopic findings of this study are consistent with those of Spitzenberger et al. (2002), who stated that the baculum of the holotype of *Plecotus "microdon-tus"* (= *P. macrobullaris*) clearly differs in shape from that of *Plecotus auritus* and less clearly from *Plecotus austriacus*. Kiefer and von Helversen (2004) reported that the form of the baculum of *Plecotus macrobullaris* is intermediate between those of *Plecotus auritus* and *Plecotus austriacus*.

In contrast, macroscopic plus microscopic results of the present study—especially 3D micromorphology revealed by micro-CT—show much more similarity between the bacula of *Plecotus macrobullaris* and *Plecotus austriacus* than between either of them and *Plecotus auritus*. In *Plecotus auritus*, the baculum is noticeably more slender than those of *Plecotus macrobullaris* and *Plecotus austriacus* (see Fig. 7). *Plecotus auritus* also has the least saddle-like and least curved baculum in lateral view of the three species. In *Plecotus macrobullaris*, the base is much bulkier than in the other two species. The arch between the branches is more arcuated in *Plecotus macrobullaris* than in *Plecotus austriacus*, almost like an inverted letter U. Light microscopy showed a narrow medullary cavity in the baculum of all three species, which is narrowest in proportion to the baculum cross section in *Plecotus auritus*. In *Plecotus macrobullaris*, the cavity is a little wider than in *Plecotus austriacus* in both the shaft and branches. The difference is especially striking at the junction, where the canals from the branches meet with the shaft's canal. *Plecotus macrobullaris* has a very wide junctional cavity, encased by a narrow bone shell. In *Plecotus auritus*, the medullary canal does not widen at the junction and is thus encased by a thicker bone shell there than in shaft and branches. *Plecotus austriacus* shows an intermediate situation: the medullary canal is slightly widened at the junction.

The genus *Plecotus* (long-eared bats) contains many cryptic species, whose existence was revealed mainly by genetic studies in the last few years. To discriminate all *Plecotus* species morphometrically, many characters and measurements have been successfully used by Spitzenberger et al. (2006). Craniometric variables were used in multivariate analyses (discriminant analyses and UPGMA) to separate the taxa (Spitzenberger et al., 2006). Apart from species discrimination, the mammalian baculum has also been used for age determination (Callery, 1951; Elder, 1951; Patterson and Thaeler, 1982; Smirnov and Tsytsulina, 2003; Dyck et al., 2004) and as phylogenetic trait (for a synopsis see Hill and Harrison,

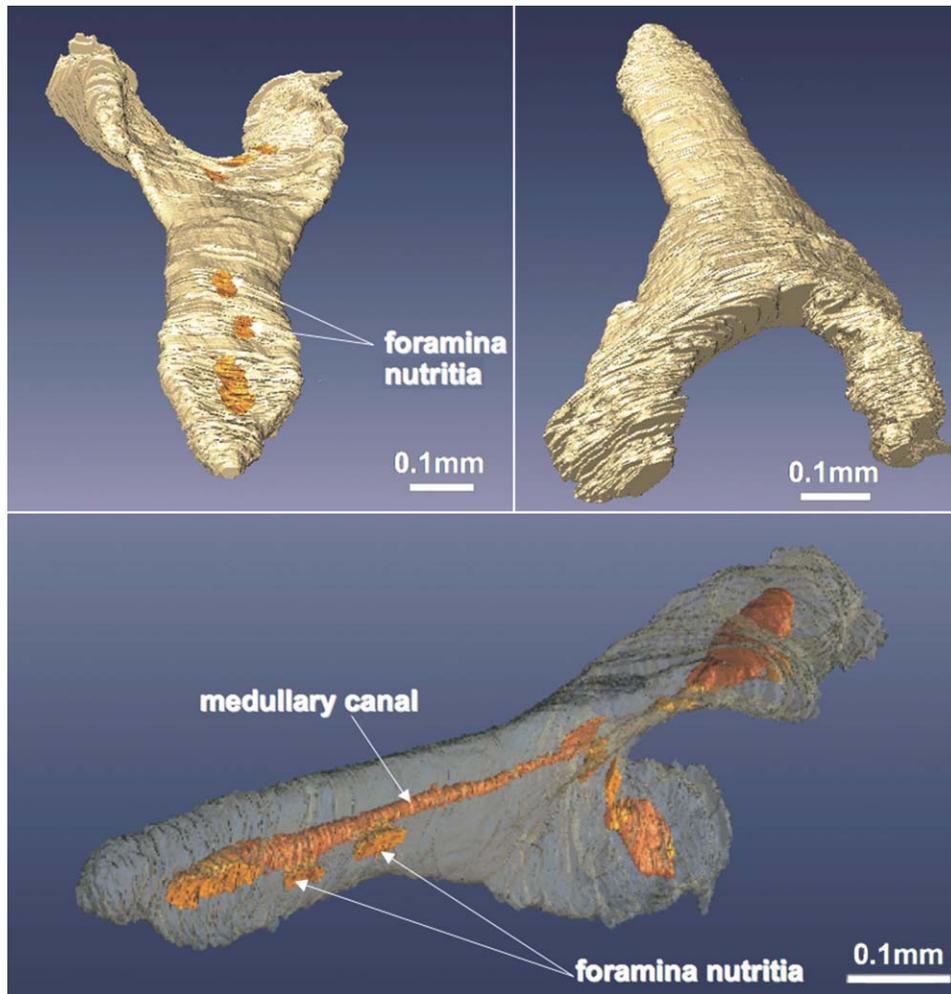


Fig. 6. 3D surface reconstruction from serial semithin ( $2\ \mu\text{m}$ ) cross sections of a *Plecotus austriacus* baculum, showing the medullary canal, foramina, and nutritial canals in orange.

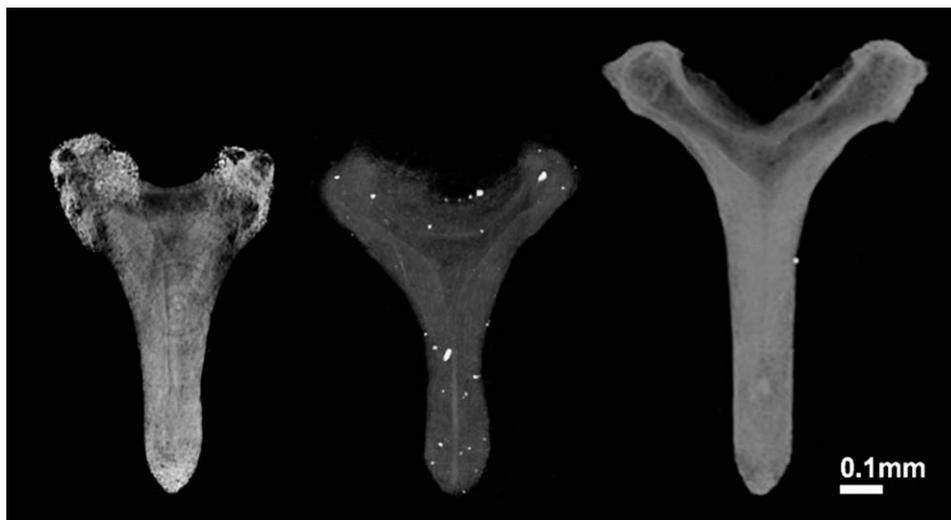


Fig. 7. Volume renderings from micro-CT images of unstained bacula of *Plecotus austriacus* (left) and *Plecotus auritus* (right), and of a decalcified, iodine-stained baculum of *Plecotus macrobullaris* (middle). *P. austriacus* (NMW 34241): tungsten source, 80 kV, 100  $\mu\text{A}$ ,

cubic voxel size:  $0.90\ \mu\text{m}$ . *P. macrobullaris* (NMW 34857) tungsten source, 40 kV, 200  $\mu\text{A}$ , cubic voxel size:  $1.0\ \mu\text{m}$ . *P. auritus* (NMW 50509): tungsten source, 60 kV, 66  $\mu\text{A}$ , cubic voxel size:  $1.49\ \mu\text{m}$ .



Fig. 8. Volume rendering from micro-CT images of an unstained *Plecotus austriacus* penis (NMW 52193; rhodium source, 77 kV, 47  $\mu$ A, cubic voxel size: 1.86  $\mu$ m), showing a baculum with deviating bone canals (outlined in orange).

1987; Frost and Timm, 1992). Newer studies caution that the penis bone could be an unreliable trait for phylogenetic studies (Kearney et al., 2002; Baryshnikov et al., 2003). Clearly, more research on different species is needed, and micro-CT in combination with histomorphology of bones could be valuable tools for studying ontogeny and phylogeny.

In addition to destructive histological evaluation, specimen microradiography, but micro-CT in particular proved to be suitable nondestructive methods for accurate and reproducible demonstration, measurement and comparison of the three-dimensional structures of the baculum in different bat species.

This study confirms that macroscopic details and dimensions along with histomorphology of the baculum can be used for bat species identification. Both these methods have been successfully applied to differentiate *Plecotus austriacus*, *P. auritus* and *P. macrobullaris* (Herdina, 2008).

Future work with these techniques on larger sample sizes in a greater number of species will also provide more insight into potential intraspecific variation in bacular microanatomy. Based on the specimens used in this study, the number and distribution of the bone canals seems to be fairly consistent in adult males. We did, however, find in the micro-CT scans of two bats differing bone canal patterns. These two individuals will be further studied histologically to find possible reasons for

this intraspecific variation. Also, depending on age, the shape of the baculum seems to be quite variable in juvenile bats (Smirnov and Tsytsulina, 2003), which warrants further micromorphological studies.

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