



Penile histomorphology of the neotropical bat *Eptesicus furinalis* (Chiroptera: Vespertilionidae)

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ABSTRACT

External and internal penile morphologies have evolved rapidly and divergently in many mammalian orders and are extremely useful for taxonomic studies, particularly in the recovery of phylogenetic relationships. *Eptesicus furinalis*, a Vespertilionid bat, belongs to a taxon in which species recognition can be difficult when only traditional features are employed. Therefore, any feature that may contribute to the more accurate characterization of this taxon is relevant. In this study, we describe the histomorphology of the penis and baculum of this species after analyzing serial transverse sections and three-dimensional (3D) reconstructions. The glans penis was small with no epidermal projections and had an inverted Y-shaped baculum for most of its length. Internally, the penis contained three erectile tissues: corpus cavernosum, accessory cavernous tissue, and corpus spongiosum around the urethra. The internal anatomy of the *E. furinalis* penis displayed the basic vascular mammalian pattern but had certain features that were unique to this species, such as accessory cavernous tissue located dorsal and lateral to the tunica albuginea of the corpora cavernosa, a glans penis with a cone morphology, and a marked development of spongy tissue.

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1. Introduction

Eptesicus Rafinesque, 1820 is one of five genera of Vespertilionidae located in Brazil. This genus comprises 23 species worldwide, six of which occur in Brazil: *Eptesicus andinus* Allen, 1914, *Eptesicus brasiliensis* (Desmarest, 1819), *Eptesicus chiriquinus* Tomas, 1920, *Eptesicus diminutus* Osgood, 1915, *Eptesicus furinalis* (d'Orbigny and Gervais, 1847), and *Eptesicus taddeii* Miranda et al., 2006 (Simmons, 2005; Davis and Gardner, 2007; Peracchi et al., 2011). Recognizing some of these species is simple for the largest (*E. taddeii*) and smallest (*E. diminutus*) species and when applying the morphological and morphometric variations that are generally used to distinguish taxa. However, the variation observed in other taxa, particularly *E. furinalis*, makes it difficult to distinguish sympatrically occurring species based on these characteristics. Therefore, any feature that may contribute to the more accurate characterization of taxa is relevant.

The morphology of the glans penis is a primary sex trait with significant variation in mammalian males, including bats (Matthews,

1937, 1942; Wimsatt and Kallen, 1952; Ryan, 1991a,b; Kamikawa-Miyado et al., 2005; Hooper et al., 2006). Furthermore, in several mammalian orders (Afrosoricida, Carnivora, Chiroptera, Erinaceomorpha, Primates, Soricomorpha, and Rodentia), the penis contains an os penis or baculum (Patterson and Thaler, 1982; Hosken et al., 2001; Larivière and Ferguson, 2002; Ramm, 2007), the precise function of which remains uncertain. According to Dixon (1987), Dixon (1995) and Larivière and Ferguson (2002), the baculum may facilitate sperm transfer and may stimulate the cervix, thus inducing ovulation in some species.

The variations in penile and bacular morphologies indicate a high degree of interspecific variation, which appears to be driven by sexual selection (Hosken et al., 2001; Hosken and Stockley, 2004; Ramm, 2007; Stockley et al., 2013), making the baculum a relevant taxonomic characteristic (Elder and Shanks, 1962; Patterson and Thaler, 1982; Herdina et al., 2014).

Although, the morphology of the glans penis and baculum is taxonomically important, few studies in bats have used these features to propose relationships among bat taxa (Brown, 1967; Brown et al., 1971; Ryan, 1991a,b; Jacobs et al., 2013).

Studies performed in bats have observed that the baculum length is highly variable among species and that it is positively associated with body mass in most species. However, it is absent or was lost in certain species, for example, in the Phyllostomid species

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analyzed so far (Smith and Madkour, 1980; Hosken et al., 2001). Although, there are numerous explanations for the variations observed in the length of the baculum in primates, in which elongation of the baculum has been associated with copulatory patterns, including a prolonged period of intromission and/or the maintenance of intromission during the post-ejaculatory interval (Dixon, 1987; Dixon, 1995), functional and evolutionary explanations for these variations in bats are lacking. Furthermore, despite the fact that the structures of bat penises vary considerably, structural studies of this organ are relatively scarce, and a comprehensive description from the morphological and histological perspectives is still required (Wimsatt and Kallen, 1952; Ryan, 1991a,b; Hooper et al., 2006).

In light of the importance of studying these traits and the difficulty of distinguishing between *Eptesicus* taxa, the present study aimed to characterize the penis histomorphologically and characterize the baculum of the vespertilionid bat *E. furinalis* morphologically.

2. Materials and methods

2.1. Specimens, aging, and licenses

The penises of nine fresh adult *E. furinalis* specimens were used for the morphological and histological analyses. The specimens were collected in northwest São Paulo state, Brazil (São José do Rio Preto: 49W 220 4500 20S 490 1100), with authorization from the Brazilian institution responsible for wild animal care (Instituto Brasileiro do Meio Ambiente, IBAMA, Process: 21707-1). The study proposal was approved by the Institutional Ethics Committee for Animal Experimentation (Document: Protocol. 013/2009CEEA/IBILCE-UNESP). The animals were deposited in the Chiroptera collection (DZSJRP-UNESP).

The glans and bacular morphologies were analyzed in five adult males preserved in alcohol in the Chiroptera Collection of São Paulo State University (DZSJRP).

The bats were aged as adults based on body weight, complete ossification of the metacarpal-phalangeal epiphyses, and degree of tooth wear (De Knegt et al., 2005).

2.2. Animal processing, baculum visualization, and slide preparation

The fresh animals were killed by cervical dislocation. The penis was then removed and fixed in buffered paraformaldehyde fixative solution for 48 h at room temperature, dehydrated in a series of ethanol (50–100%), clarified in xylene, embedded in paraffin and sectioned (5 μ m in a Leica RM 2155 microtome). Hematoxylin-eosin was used for the general characterization of the penis and to distinguish cell types, and Masson Tushmann's blue was used to highlight collagen (blue) and smooth muscle (orange).

The slides with the stained sections were examined and photographed using a light microscope (Zeiss Axio Imager-A2) and an image scanner (Olympus BX-VCB) coupled with capture and image analysis software (Axiovision Rel 4.8 and VS-ASW, respectively) to observe the components of the penile tissue structure and the position and shape of the glans.

A three-dimensional reconstruction of the penis was obtained by cross-sectioning the whole length of the penis (5 μ m) and staining it with hematoxylin-eosin. Images were captured and processed using VS-ASW and Reconstruct (Fiala 2005 version 1.0.9.9) software. After alignment, the main penile structures (urethra, baculum, corpora cavernosa, and corpus spongiosum) were delineated in each image and then processed to obtain a threshold for each interface section to generate a three-dimensional (3D) model.

Before using the penis for diaphonization, the body length (from the frontal end of the rostrum to the anal region) and the penis length (from the distal opening of the proximal urethra to the crura penis) were measured with a pair of digital calipers (Mitutoyo) to a precision of 0.01 mm. The penises of the specimens preserved in alcohol were removed by dissecting proximally to body attachment and separating the penile body from the crura penis. They were then placed in a neutralized solution of saturated sodium borate and cleared and softened in an alkaline solution of 2.5% trypsin in 1% KOH for at least two hours. The prepuce was then removed to expose the glans, and the penis was transferred to a staining solution of Red Alizarin 5% (Taylor, 1967) for 2 h to identify the bone structure (baculum). The penises were subsequently preserved in a 100% glycerol solution.

The diaphonized penises were analyzed under a Leica MZ 16 stereomicroscope coupled with a Leica DFC 295 digital camera and capture and image analysis software (Leica Application Suite—LAS, Version 3.8). After the images were obtained, the glans penis was dissected to isolate the baculum. The baculum was photographed, and length (from base to apex) and width (of the more basal region) measurements were recorded. The ratios between the baculum and penis length and between the baculum length and baculum width were calculated.

The descriptions and orientation of the baculum in the glans refer to an unerect and cranially directed organ from a ventral urethral perspective.

To compare the results with published data for mammalian species, we adopted the terminology suggested by Hooper (1958) and Smith and Madkour (1980) to identify the glans, which indicates that the glans is the distal end of the penis after the glans-prepuce junction.

3. Results

The *E. furinalis* penis showed the basic internal structure of a mammalian vascular penis, with a corpus spongiosum around the urethra and two paired corpora cavernosa. The penis had a thick, vascularized and glandular prepuce and a reduced glans, which was restricted to the more terminal portion of the penis (distal quarter), with an absence of epidermal projections (Figs. 1 A–C and 3 A and B).

The corpora cavernosa constituted the largest mass of the corpus of the penis and were arranged dorsally, extending through the full length of the penis. The corpora cavernosa were surrounded by the tunica albuginea and separated by a septum along the penile length, only merging in the most apical portion. The corpora cavernosa had a circular shape in the more proximal portions of the penis and widened into a semi-lunar shape in the more terminal portions, immediately prior to the fusion of the septum of the tunica albuginea (Figs. 1 A–D and 3 A and B). The tunica albuginea was composed of a thick and well-defined layer of non-patterned, dense connective tissue and smooth muscle fibers (Figs. 1 A–D and Fig. 3B).

The corpus spongiosum, located in the ventral groove formed by the cavernous bodies, was a smaller mass of erectile tissue restricted around the urethra (Figs. 1 A–C and 2 C and D). The corpus spongiosum was also surrounded by a tunica albuginea with a similar constitution to that observed in the cavernosa, although, it was thinner.

The urethra was composed of a transitional stratified epithelium enclosed in a layer of connective tissue and smooth muscle cells (Figs. 1 A–D, 2 C and D, and 3 A and B), occurred throughout the penis body length, opened ventrally (urinary meatus) early in the

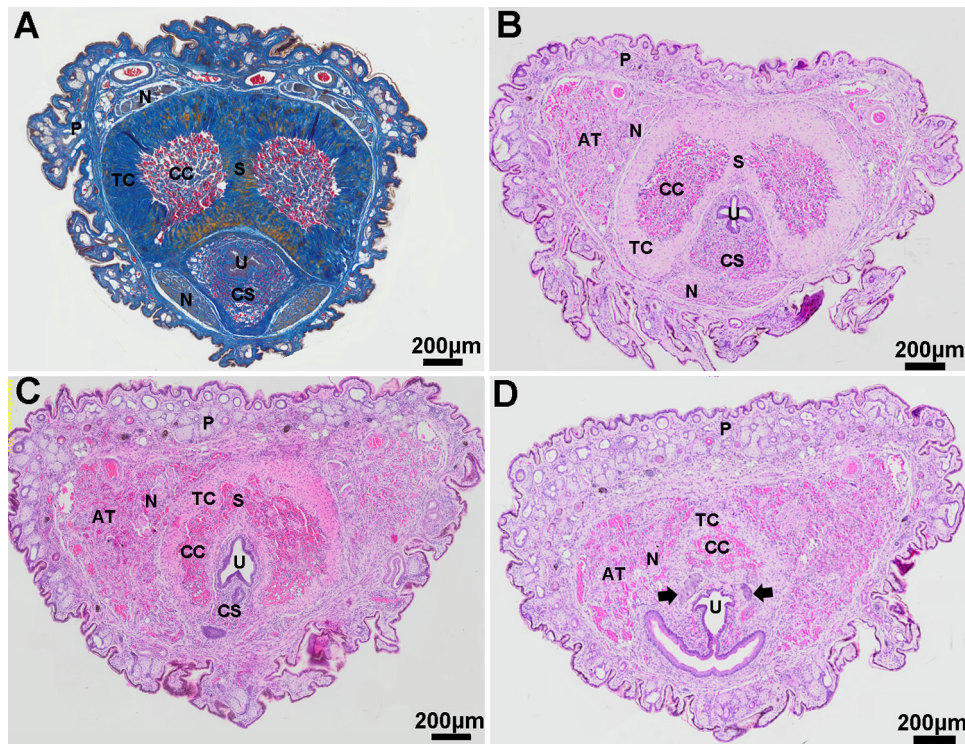


Fig. 1. Penile sections of *Eptesicus furinalis* from base (A) to apical (D) region stained with Masson Thusmann's blue (A) and hematoxylin-eosin (B–D). P: prepuce; N: nerve; TC: tunica albuginea; CC: corpus cavernosum; S: septum of the corpus cavernosum; CS: corpus spongiosum; U: urethra; and AT: accessory cavernous tissue. In D, the arrows indicate the ends of the baculum lobes.

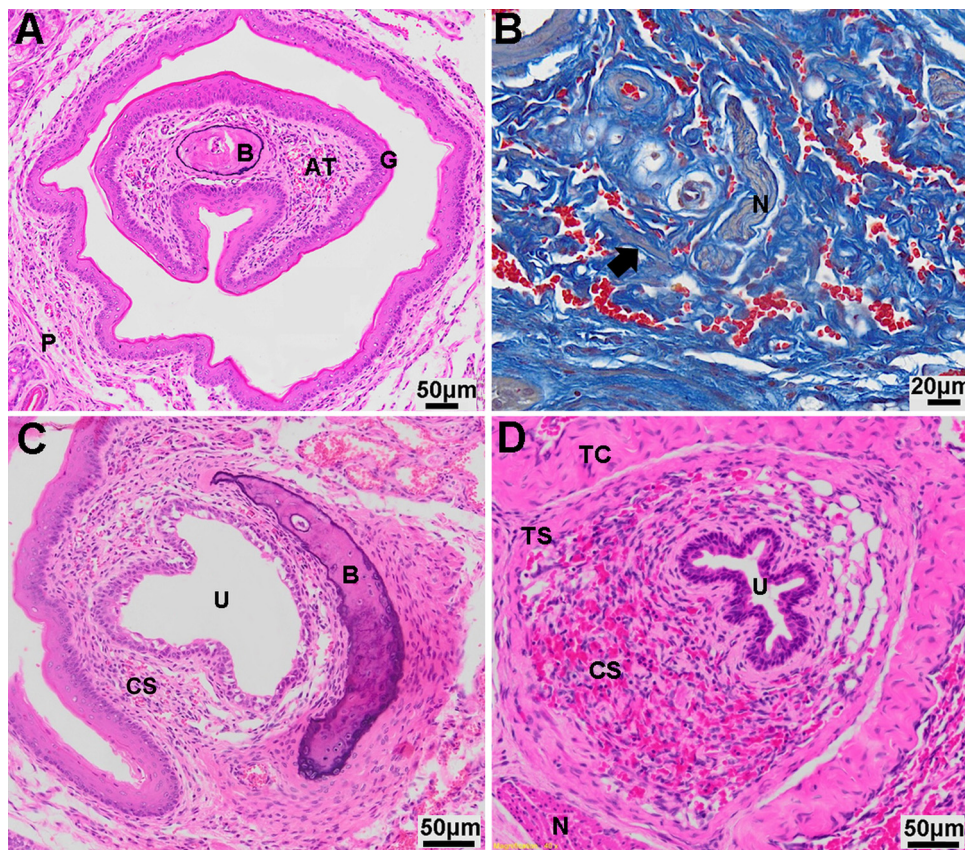


Fig. 2. Penile sections of *Eptesicus furinalis* stained with hematoxylin-eosin (A, C and D) and Masson Tushmann's blue (B). A: a glans penis showing the distal region of the baculum, B: the accessory cavernous tissue (the arrow indicates smooth muscle cells), C: the distal region of glans showing the median region of baculum and, D: the corpus spongiosum. AT: accessory cavernous tissue; B: baculum; CS: corpus spongiosum; G: glans; N: nerve; P: prepuce; TC: tunica albuginea of the corpus cavernosum; TS: tunica albuginea of the corpus spongiosum; and U: urethra.

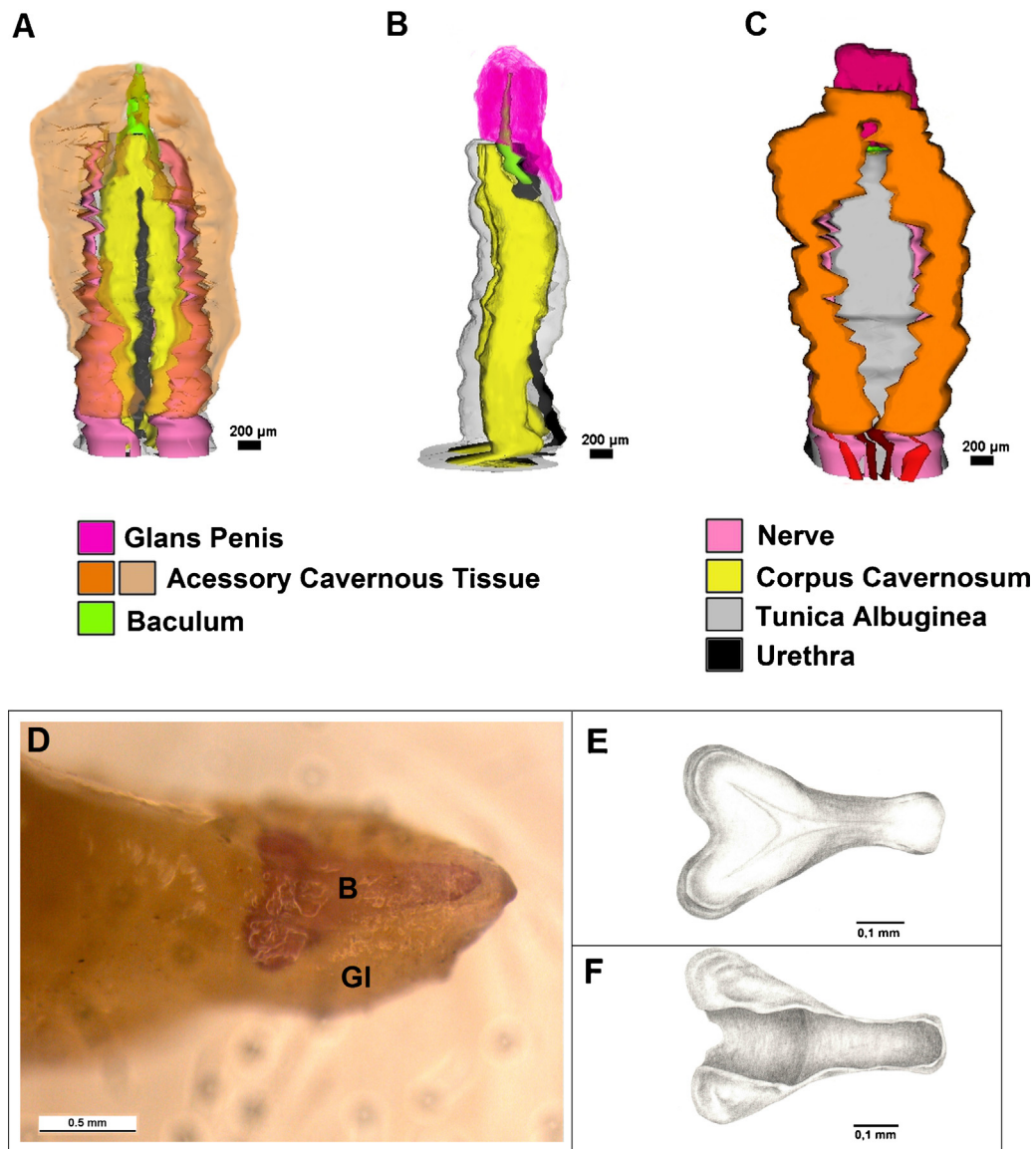


Fig. 3. Three-dimensional reconstructions of the penis of *E. furinalis* from ventral (A), lateral (B) and dorsal (C) perspectives, with a diaphanized glans showing the baculum inside (D) and a schematic representation of the baculum from dorsal (E) and ventral (F) perspectives. Note the greatest proportion of the accessory tissue in (A) and (C) and the location of the baculum in the glans penis in (B).

proximal region of the penis bone and was not observed in the glans penis (Fig. 3B).

Two pairs of major nerves, namely, the upper and lower nerves, were observed on the sides of the spongiosum tissue of the urethra (ventral) and in the upper portion of the tunica albuginea of the corpora cavernosa (dorsal) (Figs. 1 A–D and Fig. 3 A). A third type of erectile tissue, the accessory cavernous tissue, was observed dorsal and bilateral to the tunica albuginea of the corpora cavernosa, and it was not associated with a surrounding tunica delimiting its contours (Figs. 1 B–D, Fig. 2B, and 3 A and C). The erectile constitution of this tissue was confirmed by staining with Masson Tushmann's blue, which identified the smooth muscle cells surrounding the venous trabeculae (Fig. 2B).

The accessory tissue was not present in the whole length of the penis; instead, it occurred from approximately the midline of the penis body to the glans. It appeared in the midline of the dorsal face, increased in size and spread to more lateral portions of the penis and then decreased in size as it approached more distal regions of the penis, where it constituted the glans and the prepuce (3 A and C). This tissue was responsible for the characteristic enlargement of

the penis when viewed externally under stereoscopic microscopy (not illustrated) and in the 3D model (Fig. 3A).

Inside of the glans, there was a small bone structure, known as the baculum or os penis, which was located dorsal to the urethra (3 D–F). This bone occurred from the distal portion of the urethra into the middle portion of the glans (Fig. 1D, 2 A and C, and 3 A and B). The baculum had a triangular morphology or an inverted Y-shape. Its apex was rounded and represented the more terminal portion of a short shaft. The base (proximal portion) was wide and projected in two lobes because of a bifurcation in this region, which distinguished the proximal and distal regions. The baculum showed a ventral concavity, but the tip was not deflected downward. When viewed laterally, the baculum showed a slightly convex dorsal portion and a concave ventral portion, through which the urethra passed. A medullary canal extended through the inside of the baculum.

As expected, small variations of the lengths of the major axes (Table 1) were observed, with mean values of 0.63 mm (variation: 0.47–0.72 mm) and 0.46 mm (variation: 0.31–0.59 mm) (Table 1).

The values of the ratios calculated among the body, penis, and baculum lengths showed that there is a direct relationship between the size of the animal and the size of the penis; however, this relationship was not observed for the baculum. Moreover, despite the variations observed in both axes, when the ratio between the length and width of the baculum was calculated, the values were observed to be very similar. These data describe the morphology of the baculum of this species.

4. Discussion

In a study of the morphology of the glans penis in nine species of African bats belonging to five different families of Chiroptera (Emballonuridae, Hipposidaridae, Megadermatidae, Nycteridae, and Vespertilionidae), Matthews (1937, 1942) reported the occurrence of two basic patterns: one with an elongated glans, a complex structure, and a thin and retractable prepuce, and the other with a smaller glans, a simpler (less elaborate and less complex) structure, and a thick and vascular prepuce. Similar to other vespertilionids analyzed by Matthews, *E. furinalis* fits in the latter category.

The penis anatomy of *E. furinalis* resembles to the other species of bats analyzed with three types of erectile tissues: the corpora cavernosa, the corpus spongiosum, and the accessory cavernous tissue. However, these tissues varied in the proportions of their representation in the penis body when compared to those of other bat species.

The cavernous tissue is well developed throughout the length of the penis shaft, but it does not extend into the glans. The literature indicates that the absence of cavernous tissue in the glans, reduction of the corpus cavernosum, and reduction or absence of the baculum are derived conditions in bats and may be accompanied by an increase in the spongy body, as observed in the human penis (Smith and Madkour, 1980).

Although the corpus spongiosum in *E. furinalis* has a smaller mass than the cavernous body, it is relatively well developed when compared to those of taxa of other families of Chiroptera, for example, Molossidae (Ryan, 1991a,b), where it is reduced.

The third erectile tissue, the accessory cavernous tissue, which is an addition to the corpora cavernosa and corpus spongiosum, despite its denomination, i.e., cavernous, seems to exhibit no relationship or homology with the cavernous tissue.

Although, the origin and function of this tissue have not been widely studied in Chiroptera, according to Wimsatt and Kallen (1952), who identified a cavernous accessory tissue in the vespertilionid *Myotis lucifugus lucifugus* (Le Conte, 1831) this accessory tissue may have undergone a specialized and independent development. These authors reported that the presence of this structure in adults may suggest that its ontogenetic and evolutionary origin was similar to that observed in some insectivores, in which it arose as a result of the independent vascularization of the subcutaneous tissues that surrounded the erectile bodies through the branches of the penile dorsal arteries. Our results do not enable us to support this theory because we did not evaluate the physiology of the tissue. However, based on the proportion and anatomy of the penis of

E. furinalis, we can infer that among the possible functions for this structure in the bat penis is the triggering of swelling in the terminal region of the glans, which would only occur after the intromission of the penis inside the female vagina. Thus, this tissue could perform a function similar to that of a dog's bulbus glandis and thus delay the erection and allow the bats to remain in copulation for a longer period.

The accessory tissue is not a characteristic observed in all bat taxa, and its presence has been interpreted as a primitive trait within the group (Smith and Madkour, 1980), similar to the epidermal projections that cover the glans in some species of Chiroptera and Primates (Ryan, 1991a,b; Dixson, 2012).

Epidermal projections have been observed in many mammalian orders, including some previously analyzed bats (Vamburkar, 1958; Phoenix et al., 1976; Ryan, 1991a,b). The function of these projections has not been fully elucidated, but the literature suggests a possible relationship with the removal of copulatory plugs, the stimulation of the reproductive tract of females, or in prolonging copulation in molossids bats (Sachs et al., 1984; Ryan, 1991a,b; Dixson, 2012). Although, *E. furinalis* does not exhibit these specializations, similar to other species of vespertilionids, a similar function can be attributed to the thick foreskin and accessory cavernous tissue observed in this family.

Although, Vespertilionidae includes the largest number of species of Chiroptera, and the baculum was present in most of them, few studies have examined it under a histomorphological perspective (Wimsatt and Kallen, 1952; Hosken et al., 2001; Benda et al., 2004; Herdina et al., 2010, 2014).

Similar to other, previously analyzed species of *Eptesicus* (Hamilton Jr, 1949; Brown et al., 1971; Heller and Volleth, 1984; Hill and Harrison, 1987; Mies et al., 1996) Brown et al., 1971; Heller and Volleth, 1984; Hill and Harrison, 1987; Mies et al., 1996), the specimens of *E. furinalis* studied here presented a baculum with the characteristic morphology of this genus: a more or less triangular shape, similar to an arrow or an inverted-Y, with a medullary canal inside, which is a characteristic that has not been previously reported in the literature. Despite this generalization, there are variations in the different morphological descriptions related to the length, width and lobe character of the base, ventral concavity, and degree of vertical flexure of the base or of the more distal part.

We observed variations in the baculum morphometry between individuals, but because of the small number of specimens, it was not possible to determine the extent of this variation. However, our results show that there was a direct relationship between animal size and penile length but not between penile size and baculum length.

The baculum of the specimens of *E. furinalis* analyzed in the present study were morphologically similar to the descriptions and schematic representations of *E. furinalis* presented by Hill and Harrison (1987) but different from those presented by Mies et al. (1996), Heller and Volleth (1984), and Brown et al. (1971). The differences occurred in terms of both morphology and size, which was lower in our study.

However, some similarity was observed between *E. furinalis* with *Eptesicus fuscus* (Palisot de Beauvois, 1796) and *E. brasiliensis* but not between *E. furinalis* and *E. andinus* (synonyms *E. brasiliensis andinus*) (Hamilton-Jr, 1949; Heller and Volleth, 1984; Hill and Harrison, 1987), which occur in Brazil.

Although, the presence of a baculum is taxonomically important, little is known about its anatomical relationship with the corpus of the penis or its origin and functional importance during copulation (Kelly, 2000).

The literature indicates that the baculum originated through the ossification of the distal portion of the corpora cavernosa (Smith and Madkour, 1980). A more complex origin is possible, as reported by Glucksmann et al. (1976) and Murakami and Mizuno (1986),

Table 1

Values in millimeters of body length (BoL), penile length (PL), baculum length (BcL), and baculum width (BcW), as well as the BcL/BoL and BcL/BcW ratios.

Specimen	BoL	PL	BcL	BcW	BcL/BoL	BcL/BcW
DZSJRP 16914	49.90	4.61	0.69	0.44	0.15	1.6
DZSJRP 14308	46.93	3.88	0.72	0.5	0.18	1.4
DZSJRP 11970	44.69	3.82	0.63	0.45	0.16	1.4
DZSJRP 12964	48.04	4.39	0.47	0.31	0.11	1.5
DZSJRP 14429	53.53	4.0	0.63	0.59	0.16	1.1

who indicated that these two structures share the same mass of mesenchymal tissue, wherein the progenitor cells of the corpus cavernosum penis and the os penis require the presence of epithelium to differentiate during the embryonic development of the penis in mice.

Researchers have discussed the possible functions of the baculum, but some proposed theories have not been supported. Such theories include the following: that the ossicle would only be an artifact derived from pleiotropy (Burt, 1936) and thus would have no significant importance in copulation; that the baculum has a direct role in copulation and provides an additional support to the penis (Dixon, 1987, 1995); that the baculum facilitates the intromission of the penis, thus inducing the opening of the vagina (Long and Frank, 1968); that it stimulates the female during copulation, induces ovulation or prepares the uterus for implantation (Patterson and Thaeler, 1982); and that, in species with elongated bacula, the baculum extends or maintains intromission after ejaculation has occurred (Dixon, 1987).

Larivière and Ferguson (2002) tested the last hypothesis by analyzing species of North American carnivores, but found little supporting evidence. However, after analyzing 315 species of carnivores, primates, and bats, Dixon et al. (2004) confirmed that elongated bacula characterize those species in which a single prolonged intromission occurs.

Unfortunately, data regarding the intromission duration in bats are rare. Nevertheless, the recent study of Herdina et al. (2015), which analyzed three species of vespertilionid bats, *Nyctalus noctula* (Schreber, 1774), *Pipistrellus pipistrellus* (Schreber, 1774), and *Pipistrellus nathusii* (Keyserling and Blasius, 1839) using a novel approach combining postmortem manipulation and three-dimensional (3D) imaging, provided evidence that the baculum probably performs two different roles during erection: protecting the urethral opening from compression during erection and forming a functional unit with the corpora cavernosa to provide stiffness within the glans. While further studies testing other hypotheses remain lacking, the true function of the baculum in bats will remain unknown and may be a combination of these previously suggested hypotheses or may be unrelated.

Our data showed that the baculum in *E. furinalis* is interposed between two erectile tissues, the spongiosum and the accessory of the urethra, near the urethral opening. This finding supports the hypothesis that one of the functions of the baculum is to protect the urethra from compression during intromission (Dixon, 1987; Dixon, 1995; Dixon et al., 2004; Herdina et al., 2015). Furthermore, we cannot fail to mention that many of the species that have a baculum store sperm for prolonged periods. Thus, it is likely that this genital morphology and the occurrence of a baculum favor this phenomenon. Nonetheless, additional research into the functions of this tissue is necessary.

Regardless of the function of the baculum in different mammalian species, the results of this study indicate that the internal anatomy of the *E. furinalis* penis exhibited one of the basic mammalian patterns, the vascular type, and shared some features with other taxa in its family, such as *Myotis lucifugus* and *Plecotus austriacus* (Fischer, 1829) (Wimsatt and Kallen, 1952; Herdina et al., 2010). However, the penis had some features that were not apparent in other species, such as developed accessory cavernous tissue, which was located dorsal and lateral to the tunica albuginea of the corpora cavernosa; a glans penis with a cone morphology; the marked development of spongiosum tissue; and a baculum with a length of approximately 0.63 mm and an inverted-Y shape.

Based on all of these findings, the results of the present study examining the penis morphology of *E. furinalis* reinforce the importance of these structures as an important taxonomic feature in bat species and indicate that *E. furinalis* has both ancestral and derived characteristics.

Accession numbers of the animals analyzed

Histological analysis: DZSJRP 1406, DZSJRP 1342, DZSJRP 1341, DZSJRP 1306 DZSJRP,;1; 1337 DZSJRP,;1; 1345 DZSJRP 1410, DZSJRP 18,116 DZSJRP 16,935, DZSJRP 1399 (juvenile), DZSJRP 1364 (juvenile); morphological analysis: DZSJRP 11,970, DZSJRP 12,964, DZSJRP 14,308, DZSJRP 14,429, DZSJRP 16,914.

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