Applications of Transmission Electron Microscopy to evaluate the diversity of the male reproductive system of Neotropical bats

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Reproductive aspects of bats, especially in the Neotropics, are still poorly studied, mainly in terms of morphology and physiology. Actually the ultrastructural analysis of reproductive cells (testes and reproductive accessory glands - RAG) has shown interesting morphological variations among species of bats. In the testes, these variations are mainly related to differences in the process of the spermiogenesis, such as the acrosome formation and final morphology of the spermatozoa. Similarly, the studies show differences in the degree of lobulation, histology and ultrastructure of RAG among the families or sub-families of bats. In addition, much phylogenetic and evolutionary incongruence were observed in the Chiroptera taxa. Thus, the analysis, characterization and comparison of the ultrastructure of the spermatozoa as well as of the RAG of different species of bats, in order to find morphological characteristics that present inter-specific, inter-generic, subfamilial or familial variation, may raise important morphological data that, when assembled with other information and comparatively analyzed, may help to understand the evolutionary and phylogenetic relationships of bats.

Keywords Chiroptera; Testes; Epididymis; Prostate

1. Morpho-functional variations in male reproduction of bats

1.1 General remarks

The order Chiroptera is the second largest order of mammals, with approximately 202 genera and 1116 species widely distributed among all tropical and temperate regions, with the exception of some remote oceanic islands and Antarctica [1]. Classically, it is divided into two suborders: Megachiroptera and Microchiroptera. The first is found exclusively in the Old World and comprises a single family (Pteropodidae) with 42 genera and 186 species, while the latter is widely distributed around the globe, and includes 17 families and 930 species [2].

The suborder Megachiroptera contains the largest form of bats, known as "flying foxes", with some species of the genus Pteropus and Acerodon presenting a wingspan of 1.7 meters. In general, these bats have nails on the second finger, large and well developed eyes, no echolocation system and are, mostly, frugivores [3]. On the other hand, bats of the suborder Microchiroptera are cosmopolitan, have the extraordinary ability of echolocation, a wide variation in dietary habits, and present with the second finger devoid nails [4].

Within this wide distribution, bats inhabit a great variety of habitats and, as such, are exposed to a large variety of abiotic factors. In response to these factors, bats have evolved some unique characteristics, such as the fact that some species go into hibernation and present a process of regression of the seminiferous tubules, where only spermatogonia and Sertoli cells can be observed [5-7]. Possibly in order to adapt to this period of hibernation, many species have evolved unique characteristics, such as the prolonged storage of spermatozoa in the cauda epididymis in males and/or in the uterine cornua in females and asynchrony between periods of spermatogenesis and mating, as well as a delay in ovulation, fertilization and/or implantation of the embryo in the female reproductive tract [8-13].

Although tropical bats have morphological patterns and reproductive characteristics closer to the general pattern of mammals, some species also show unique features. These include the geographically variable reproductive pattern of Myotis nigricans, that presents in the form of a hibernating pattern in specimens in Mexico, compared to an active pattern throughout the year in Paraguay [14-15], and two peaks of intense spermatogenic activity followed by two periods of testicular regression in southeastern Brazil [16]; tropical species that do not hibernate but present testicular regression and sperm retention/storage in the cauda epididymis during their annual reproductive cycle, such as Eptesicus furinalis, Histiotus velatus, Lasiurus blossevillii and M. nigricans [16]; species that exhibit an unusual ultrastructure of spermatozoa, such as Noctilio leporinus [17]; and species that show great asynchrony between the primary (testis and epididymis) and secondary (reproductive accessory glands) reproductive functions, for example Taphozous georgianus [18].

Despite all of these unique characteristics, the reproductive aspects of bats, especially in the Neotropics, are still poorly studied with regards to morphology and physiology.
1.2 Testicular Morphology and Spermatogenesis of Neotropical bats.

Light microscopy provided some advances in the observation of testicular morphology and especially in the investigation of the process of spermatogenesis. The data show some similarity in the process of spermatogenesis in most Neotropical species of bats, with spermatogenesis being divided into seven steps and the seminiferous epithelium cycle showing uniformity [11] (**Fig. 1A**). However, some species have distinctive characteristics, such as a pattern of testicular regression where only spermatogonia and Sertoli cells can be seen in their seminiferous epithelium [16] (**Fig. 1B**), which is similar to that observed in hibernating bats.

**Fig. 1** Testicular cross-section of *Artibeus planirostris* (**A**) (Phyllostomidae) stained with hematoxylin-eosin and of *Myotis nigricans* (**B**) (Vespertilionidae) stained with toluidine blue. Note the active pattern of *A. planirostris* testes (**A**), presenting all types of germinative cells (Sg, spermatogonia; I, spermatocytes; Sd, spermatids; spermatozoa) and the regressed pattern of *M. nigricans* testes (**B**), with only Sertoli cells (S) and spermatogonia present. Scale bars: 20µm.

1.3 Epididymides of Neotropical bats.

Similarly, the epididymides present accentuated anatomical variations between the different species of bats. Species from Phyllostomidae, Noctilionidae and Molossidae families present epididymides that are anatomically similar to the normal pattern of mammals (**Fig. 2A**). On the other hand, Vespertilionidae (**Fig. 2B**) and Emballonuridae (**Fig. 2C**) have a large elongation of the caudal region that is not directly attached to the testes. The size of this elongation varied individually and was larger in some specimens than the testis itself. In emballonurid bats, the cauda epididymis remained enclosed inside the scrotum, forming a loop around the testes (**Figs. 2C**) when the testis was external, or had an elongated shape when it was internal. However, in Vespertilionidae, it extended parallel to the caudal skeleton, with its position being maintained by filaments that connected it to the caudal skeleton and by juxtapositions between the anterior and posterior inter-femural membranes (uropatagium). It was not directly attached to the testes, but, in most specimens, remained near them because both were covered with a highly pigmented fascia (black) [16].

**Fig. 2** Testicular and epididymal gross anatomy of *Artibeus planirostris* (**A**) (Phyllostomidae), *Myotis nigricans* (**B**) (Vespertilionidae) and *Peropteryx macrotis* (**C**) (Emballonuridae). Note the basic pattern of *A. planirostris* testes (**A**), presenting all three epididymal regions linked to the testes and the elongation of the cauda epididymis of *M. nigricans* (**B**) and *P. macrotis* (**C**). (Cb, caput epididymis; Cp, corpus epididymis; Cd, cauda epididymis; DD, Deferens duct; T, testes). Scale bars: 0.5cm.
1.4 Reproductive Accessory Glands of Neotropical bats.

The Reproductive Accessory Glands (RAGs) are important glands whose secretions ensure the survival, mobility and viability of sperm in male and female reproductive tracts. Upon ejaculation, spermatozoa leave the epididymis, and pass through the deferens ducts, urethra and penis. During this process, the secretions of the RAGs have a direct role in protecting and maturing the sperm, thus composing a large part of the volume of ejaculated semen [19]. Based on this importance and in combination with the large variety of reproductive characteristics that bats possess, we noted the great importance of the characterization, evaluation and comparison of the RAG patterns in different species of bats.

The RAGs of bats are composed of a glandular complex that surrounds the urethra, which is situated in the base of the urinary bladder, and two bulbourethral glands, which are located in the inguinal region, extra-abdominally, at the root of the penis [10]. In the gross anatomy, the RAGs present a great variation in composition and morphology at the familial level, varying from a compact glandular complex in Phyllostomidae (Fig. 3A) [20-22], which is similar to the human prostate, to a lobulated pattern observed in Vespertilionidae (Fig. 3B), like a rodent pattern [23].

The three-dimensional reconstruction of the RAGs shows a variation in the degree of lobulation of the glands between the different species (Fig. 4), and assists in the understanding of the dynamics of the input of the ducts in the glands (Fig. 4C). The Phyllostomidae family presents a compact pattern, there are two or three prostatic regions (a ventral, a dorsal and a dorsolateral region) in Glossophaginae, Phyllostominae and Carollinae (Fig. 4A), but there is an absence of the dorsolateral region in the Stenodermatinae subfamily (Fig. 4B). Molossidae showed an intermediate degree of lobulation, presenting a compact and well-developed ventral region and a lobulated dorsal region (Fig. 4D). In contrast, Vespertilionidae present the highest degree of lobulation, and also show the three regions (Fig. 4E).

The light microscopy brought great insights into the morphology of these glands, showing similarities as well as differences between families and subfamilies (Fig. 5). The epithelium of the ventral gland was different from any other prostatic epithelium, despite presenting an undefined epithelium; in bats it is highly conserved (Fig. 5B-G-J-N). The ventral region possesses this atypical epithelium, with an irregular luminal interface. This epithelium contains secretory cells with a basal nucleus, and sporadic basal cells. The lumen presents numerous secretory vesicles of distinct sizes.

Fig. 3 Gross anatomy of the glandular complex of Artibeus planirostris (Phyllostomidae) (A) and Myotis nigricans (Vespertilionidae) (B). Note the more compact glandular complex of A. planirostris in relation to the lobulated glandular complex of M. nigricans. (Bl, Bladder; D, dorsal prostate; DL, dorsolateral prostate; Ur, urethra; V, ventral prostate).

Fig. 4 Three-dimensional reconstruction of the glandular complex of Neotropical bats. A-C, Phyllostomidae family. D, Molossidae family. E, Vespertilionidae family. A, Lateral view of the glandular complex of Phyllostomus discolor (Phyllostominae). B, Lateral view of the glandular complex and ducts (C) of Artibeus planirostris (Stenodermatinae). D, Caudal view of the glandular complex of Molossus molossus (Molossidae). E, Ventral view of the glandular complex of Myotis nigricans (Vespertilionidae). Legend: Black, deferens ducts; Blue, dorsolateral prostate; Dark green, region of paraurethral glands; Light green, dorsal prostate; Pink, tissue of urethra; Purple, region of ducts; Red, muscle of the urethra; Yellow, ventral prostate. Scale bars: 1 mm.
The dorsolateral region presents some differences between the families, with the epithelium found in two forms: pseudostratified columnar in Phyllostomidae-Phyllostominae (Fig. 5C) and simple cubic in Vespertilionidae (Fig. 5O). The dorsal region does not exhibit structural differences between families, but consists of simple columnar epithelia in which there is also the presence of secretory and basal cells (Fig. 5D-G-K-M).

The bulbourethral glands are an oval-shaped acinar gland. The morphology of this gland is conserved in all of the bats that were studied. It was partially surrounded by muscular tissue and presented a columnar epithelium with basal nuclei (Fig. 5H). The secretion of these glands was granular and PAS-positive (asterisks, detail Fig. 5H).
Many features have been clarified in the analyses described above, but the different characteristics displayed by these glands still deserved a closer look, such as the new structure of the ventral gland and more details of the other glands.

2. Contributions of Transmission Electron Microscopy to evaluate and characterize the diversity of the male reproductive system of bats

Actually, the ultrastructural analysis of reproductive cells (testes, epididymides and reproductive accessory glands) has provided more detailed information and shown interesting morphological variations among different species of bats.

2.1 Ultrastructural Characteristics of Testicular Cells and Spermatogenesis of Neotropical bats.

In the testes, the variations are mainly related to differences in the process of spermogenesis, such as acrosome formation and the final morphology of the spermatozoa. Analyzing three different Neotropical families of bats, three different patterns of acrosome formation were observed: 1) Acrosomes formed by the junction of the proacrosomal vesicles containing electron-dense granules within them (Fig. 6A); 2) Acrosomes formed only by the junction of electron-lucid proacrosomal vesicles (Fig. 6B); 3) Acrosomes formed by the junction of two different types of proacrosomal vesicles, an electron-dense and another electron-lucid (Fig. 6C-D). This difference seems to be correlated with the formation of the perforatorium, where the electron-dense material possibly corresponds to the basic substance of its mount. Thus, the first case corresponds to the pattern found in many mammals that have the perforatorium, which is possibly an ancestral trait, the second to animals that do not have the perforatorium, by loss or simply because they never had one, and the third to animals that have a different form of formation, maybe an apomorphy.

In addition to these differences in the formation of the acrosome, currently, the observation and analysis of the ultrastructure of spermatozoa have been used as an important tool in the identification and classification of the taxa of various groups of organisms, from amphibians [24-25], reptiles [26-27] and birds [28-29] to some groups of mammals [30] and even within Chiroptera [17, 31].

As a highly specialized cell, it is expected that the morphology of the spermatozoa is specific for each species, or at least that is similar between closely related species, and thus structural variations may possibly indicate taxonomical differences.
Despite the scarcity of studies on the ultrastructure of spermatozoa in bats, these few studies, when correlated, show interesting morphological variations, where three morphological characteristics appear to vary in different families, and are possibly inter-specific: (1) the morphology of the outer dense fibers, (2) the presence and morphology of the perforatorium and (3) the morphology of the spermatozoa head.

The morphology of the outer dense fibers divides the few bats that have already been ultrastructurally studied in at least two groups, one with the fibers 1, 5 and 6 larger than the others and the other where 1, 5, 6 and 9 are larger. The first group includes only the Neotropical species *P. lineatus* - Phyllostomidae [12] (Fig. 7D) and *M. molossus* - Molossidae [13] (Fig. 7E) and the second includes species of the Rhinolophidae [32-34], Miniopteridae [34], Pteropodidae [31] and Vespertilionidae [6, 35, 36] families (Fig. 7F).

The perforatorium is either absent or under-developed in Vespertilionidae (Fig. 7C) [35] and Noctilionidae [17], under-developed in Rhinolophidae [32-33] and well-developed in Molossidae (Fig. 7B) [13, 37] and *P. lineatus* - Phyllostomidae (Fig. 7A) [12].

The unusual morphologies in the spermatozoa head are also typical in some families of bats, such as the unusually large, flat and eccentrically placed, folded accordion-shaped head typical of the Noctilionidae family [17], and the wave-form projections of the acrosome in Molossidae (Fig. 7B, arrows) [13, 37].

Analyzing these data it was observed that the morphology of spermatozoa in bats may be an interesting parameter to be characterized in different species, and then analyzed, compared and used in phylogenetic analyses and evolution. Thus, further investigations of the ultrastructure of spermatozoa of other species in these families, as well as those from other families, are both interesting and necessary.

2.2 Epididymides of Neotropical bats.

Despite the anatomical variation observed in the epididymides among species of bats, differing from the testes, ultrastructural accentuated differences were not observed yet (Fig. 8).
2.3 Reproductive Accessory Glands of Neotropical bats.

As previously described, the order Chiroptera presents a wide variation in RAGs, for example the degree of lobulation, which can be between families and/or subfamilies. It was also shown that there is a ventral prostate in bats which appears to be unique and highly conserved in bats. Transmission electron microscopy is of paramount importance to further clarify the morphology of this gland complex and to also provide new parameters to compare the families.

The ventral prostate showed an epithelium composed of secretory cells. The cells were observed to adhere firmly to the basal lamina, had irregular nuclei with condensed chromatin clusters dispersed throughout the nucleus and numerous secretory vesicles in their cytoplasm (Fig. 9A). The observations in MET indicate that it produced and stored a great amount of highly electron-dense vesicles that were later released into the lumen (Fig. 9B). Apparently, the cell bursts its cytoplasm, releasing a large amount of cytoplasm into the lumen, along with the vesicles, leaving only the nucleus in the basal region (Fig. 9C). Other nuclei stand out from the basal lamina and are thrown into the lumen with the secretion (Fig. 9D).

These ultrastructural features were of paramount importance to understand why large morphological differences are found in this region in the same species. While there is evidence that this gland has holocrina-type secretions, little is understand about the plasticity of this gland, and it is thought that these differences are related to the reproductive status of the animal analyzed.

Dorsolateral and dorsal regions have differences in accordance with the ultrastructural study. In Phyllostominae, the epithelium of dorsolateral region is simple columnar, composed of secretory and basal cells. The secretory cells are supported in the basal lamina and have a spherical nucleus with the chromatin associated with the nuclear envelope. In the cytoplasm the presence of granules and small secretory electron-lucid vesicles was noticed, and the limits between the lateral membranes were well-defined. The basal cell has an elongated, electron-dense nucleus, which fills almost the entire cytoplasm (Fig. 9E). In Carollinae, this same region shows simple cubic epithelia, where the secretory cell nucleus possess a large electron-density and a very small amount of cytoplasm. In the lumen fewer electron-dense secretion vesicles are observed (Fig. 9F). Vespertilionidae already has a columnar epithelium, with elongated nuclei. Its apex is provided by microvilli (Fig. 9G).

The dorsal region of Phyllostominae (Fig. 9H) and Carollinae (Fig. 9I) are very similar, and both have simple columnar epithelium, however the secretory cells differ. In Phyllostominae, these cells have a spherical nucleus with dispersed chromatin. The cytoplasm is filled with secretory granules and the limits between the membranes are clearly visible. In the apical cytoplasm, specializations of the plasmatic membrane are noted, the microvilli are long and numerous structures, and the observed secretion is granular and electron-dense (Fig. 9H). In Carollinae, the secretory cells exhibit a spherical nucleus but with concentrated chromatin. The cytoplasm contains electron-lucid vesicles, and apical microvilli are also present, but are less numerous (light arrows). The lumen is filled by a homogeneous electron-dense discharge (Fig. 9I). In Vespertilionidae, an epithelium that differs from other species is noted. These have no apex with microvilli and their cytoplasm has a large amount of endoplasmic reticulum (Fig. 9J).
Fig. 9 Electron micrographs of the prostatic and bulbourethral cells of Neotropical Bats. A-B-C-D. Ventral prostate, this region is similar in the studied species. Images of Stenodermatinae (*Artibeus planirostris*). E-F-G. Dorsolateral prostate: note the differences in epithelium: E. Phyllostominae (*Phyllostomus discolor*), F. Carolininae (*Carollia perspicillata*), G. Vespertilionidae (*Miotys nigricans*). H-I-J. Dorsal prostate: note the similarity of Phyllostominae (H, *Phyllostomus discolor*) and Carolininae (I, *Carollia perspicillata*) subfamilies, and the difference of both compared to Vespertilionidae (J, *Miotys nigricans*). K-L-M. Bulbourethral gland: this gland is similar in the studied species. Images of Glossophaginae (*Glossophaga soricina*). Bc, basal cell; bl, basal lamina; L, lumen; M, microvilli; N, nucleus; s, secretion; sc, secretory cell. Scale bar: 1000nm.
The bulbourethral gland showed no differences between the families analyzed by light microscopy. It presented a columnar epithelium, with basal nuclei (Fig. 9K). Their cells are filled with a large amount of droplets of secretion, which present variable electron-densities (Fig. 9L). These vesicles are released at the apex of the epithelia, which present small projections of the cell membrane (Fig. 9M).

3. Perspectives

There are many gaps in our knowledge of the male reproductive system of Chiroptera. Only a few taxa have been substantially examined up to now, mainly from an ultrastructural viewpoint. The light microscopic morphology of the testes and RAGs remains poorly known and the ultrastructures were examined and correlated with their reproductive cycle in few species of bat.

In addition, many phylogenetic and evolutionary incongruences were observed in the Chiroptera taxa, with the relationships within the order and between the different families and subfamilies still poorly understood. Thus, the analysis, characterization and comparison of the ultrastructure of the spermatozoa of different species of bats, in order to find morphological characteristics that present inter-specific, inter-generic, subfamilial or familial variation was performed. This may raise important morphological data that, when assembled with other information (morphological, genetic, cytogenetic, biochemical, molecular, etc) and comparatively analyzed, may help in understanding the evolutionary and phylogenetic relationships of bats.

As discussed above, bats have significant variations in the composition and morphology of RAGs, with the variation appearing to be linked to the phylogeny of the group. The anatomical, microscopic and ultrastructural characterization of these glands is the first step to unravel the mysteries guarded by these animals. For future research, the importance of characterizing these glands with regards to the manner refined is highlighted, in order to detect similarities and differences between species, and can contribute to reproductive features in phylogenetic analyses. It is also emphasized that a tool needs to be refined, that includes efficient transmission electron microscopy.

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