Marsupial Morphology of Reproduction: South America Opossum Male Model

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ABSTRACT This study aims to describe the morphology of *Didelphis* sp. male genital organs (penis, testes, epididymis, ductus deferens, prostate, and bulbourethral gland). Ten male animals were used, eight for macroscopic and light microscopy analysis, and two for scanning electron microscopy. The testes and epididymis showed similarity to other eutherian mammals. The bifid penis showed the urethra ending in the medial region where the bifurcation begins, occurring in each segment extension of the urethral groove until the beginning of the glans. Histologically, the penis consists of a cavernous and spongy body, covered by stratified squamous epithelium with loose connective tissue. The urethra was lined by transitional stratified epithelium. In the prostate, prostatic segments were found consisting of tubular glands in a radial arrangement around the urethra, coated externally by a dense connective tissue associated with a relatively thick layer of smooth muscle arranged in two layers that surround the glandular tissue. The animals had three pairs of bulbourethral glands placed at the membranous and cavernous urethra junction with descending and parallel excretory ducts ending caudally in the urethral lumen. *Microsc. Res. Tech. 76:388–397, 2013*.

INTRODUCTION

Metatherians ("marsupials") comprise one of the three major groups of modern mammals and represent the closest outgroup to the eutherian ("placental") mammals. Metatherians and eutherians diverged 180 million years ago. Although the metatherian lineage originally radiated from North America, only one extant can be found there (the Virginia opossum), whereas all other species are found in South America (including more than 65 species of opossums and shrew opossums) and Australian species, including opossums, kangaroos, koalas, and many small insectivores and carnivores (Mikkelsen et al., 2007).

The Brazilian specie, *Didelphis* sp., is considered an excellent model for reproductive experimental studies of Brazilian marsupials due to the morphological similarity or the differences in the reproductive process, particularly the embryonic development and placental organization when compared with Australian ones (Whitten, 1966; Gonçalves et al., 2009).

The *Didelphis* sp. male reproductive tract consists of one pair of testes, epididymis, and ductus deferens that opens into the anterior portion of the prostate. The membranous urethra extends from the posterior end of the prostate where the bulbourethral glands are found. The seminal vesicles are absent in this species (Whitten, 1966). The penis is internal and "S" shaped. The glans is already different in format, but in most species is bifid. The prostate gland has a tubular aspect placed around the urethra, showing three different segments and a well-defined limit entirely spread, with all glandular tissue interposed between the muscle and urethral lumen (Tyndale-Biscoe and Renfree, 1987). In marsupials, the prostate gland provides a fluid vehicle for effective sperm discharge and metabolic substrate (Howarth, 1950).

This study aims at increasing the knowledge about the morphological reproductive development and its relationship with other marsupial models for research and applications for this important group.

MATERIALS AND METHODS

Ten male animals at different ages provided by UNESP campus Araçatuba–Brazil were used for this study. The animals were euthanized by acepromazine and potassium chloride in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College in Animal Experimentation (COBEAS).

Eight animals were used for macroscopic and histological study and the other two were used for scanning electron microscopy study, amounting 10 animals.

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For gross analysis, the male genital organs were identified, measured, photographed, and subsequently fixed by intravenous infusion of formaldehyde (10%) and for ultrastructural studies, 2% of glutaraldehyde injection was made. Histological preparations were fixed and dehydrated by passing the tissue through a series of increasing alcohol concentrations (70–100%). After clearing in xylol solution, the tissue was embedded in paraffin (Paraplast Embedding Media-Paraplast Plus, Oxford Lab, US) and sectioned in microtome (LEICA® 2165). The mounted sections were stained with Hematoxylin and Eosin, Masson's Trichrome, and Picrosirius followed by mounting on the slides with coverslips (Entelan® Merck).

For scanning electron microscopy, the samples of genital organs were washed in a phosphate buffer (0.1 M), fixed with osmiun tetroxide, and dehydrated in graded series of ethanol and critical-point dried (Balzers-CPD-020). Then, the samples were examined under a scanning electron microscope (LEO-435 VP).

RESULTS

The male opossum has anatomical components of the genitourinary: kidneys, adrenal glands, ureters, bladder, vas deferens, testes, prostate, three pairs of bulbourethral gland, penis bifid, and adanal glands (Fig. 1).

The penis is positioned in the inguinal cavity (Fig. 2A) and when exposed showed itself in the form of an "S" (Fig. 2B). Surrounding the urethra is the cavernous body with a wider proximal portion divided into two parts as cavernous bulbs that extend along the penis up to glands (Fig. 2C). In microscopic studies, the penis was divided into three portions: proximal, medial, and distal. The proximal region of the penis is covered externally by keratinized stratified squamous epithelium connected to a layer highly vascularized of loose connective tissue (Figs. 2D and 2E).

The urethra is positioned in the center of penis (Fig. 3A) and the middle and distal portions are irregular and microscopically covered by keratinized stratified squamous epithelium with numerous spines (Fig. 3B). The inner face is marked by a continuous groove of urethra lined by stratified and transitional epithelium (Fig. 3C).

The testes had an oval shape and whitish color, similar to other mammals. Differences were highlighted between the tunica vaginalis (Fig. 4A) and albuginea (Fig. 4B) of young and mature animals, respectively.

Under light microscopy, observations were made of the tunica albuginea (Fig 5A) and seminiferous tubules (cross section) (Fig. 5B) bounded by flat myoid cells and separated by abundant connective tissue with clusters of Leydig cells (Fig. 5C), blood, vessels, and nerves were present. The germinal epithelium showed gametic cells at various stages of development, similar to other mammals as well as any characteristic nuclei of Sertoli cells. The spermatogonia are arranged in the basal layer of epithelium with rounded nuclei and condensed chromatin associated with nuclear membrane. The spermatocytes showed large nuclei with scattered chromatin, prominent nucleoli and abundant cytoplasm. In the apex of germinal epithelium (lumen of seminiferous tubules), slightly elongated and paired spermatids (in V-shaped) with condensed nuclei (Fig. 5D) were observed.

Fig. 1. Photomacrography of Opossum male urogenital system: kidneys (1), adrenal glands (2), ureters (3), bladder (4), ductus deferens (5), testes (6), prostate (7), bulbourethral glands (8), bifid penis (9), rectal ampulla (10), and adanal glands (11). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The epididymis is a convoluted tube adhering along the lateral surface of testis and divided morphologically into head, body, and tail (Fig. 6A). Microscopically, the epididymis is immersed in loose connective tissue lined by simple cubic or prismatic epithelium with stereocilia, suggesting a resorption function. The simple cubic epithelium is predominant in the head of epididymis (Fig. 6B), whereas in the body and tail a monolayer of prismatic cells predominates (Figs. 6C and 6D).

The ductus deferens begins at the end of epididymal tail (Fig. 7A) crosses the pedicle base of scrotum and muscular wall, and enters the abdominal cavity surrounded by spermatic cord. In the abdominal cavity, it runs lateral to ureters and ends in the dorsal wall of the urethra (Fig. 7B). Microscopically, the ductus deferens consists on thick layer of smooth muscle covered by pseudostratified prismatic epithelium (Figs. 7C and 7D).

Macroscopically, the prostate is involved around the urethra with three distinct segments. The cranio-caudal-oriented segment lies below the neck of the bladder and is yellowish, becoming straw with tonality after fixation. The second segment is the bulk portion of

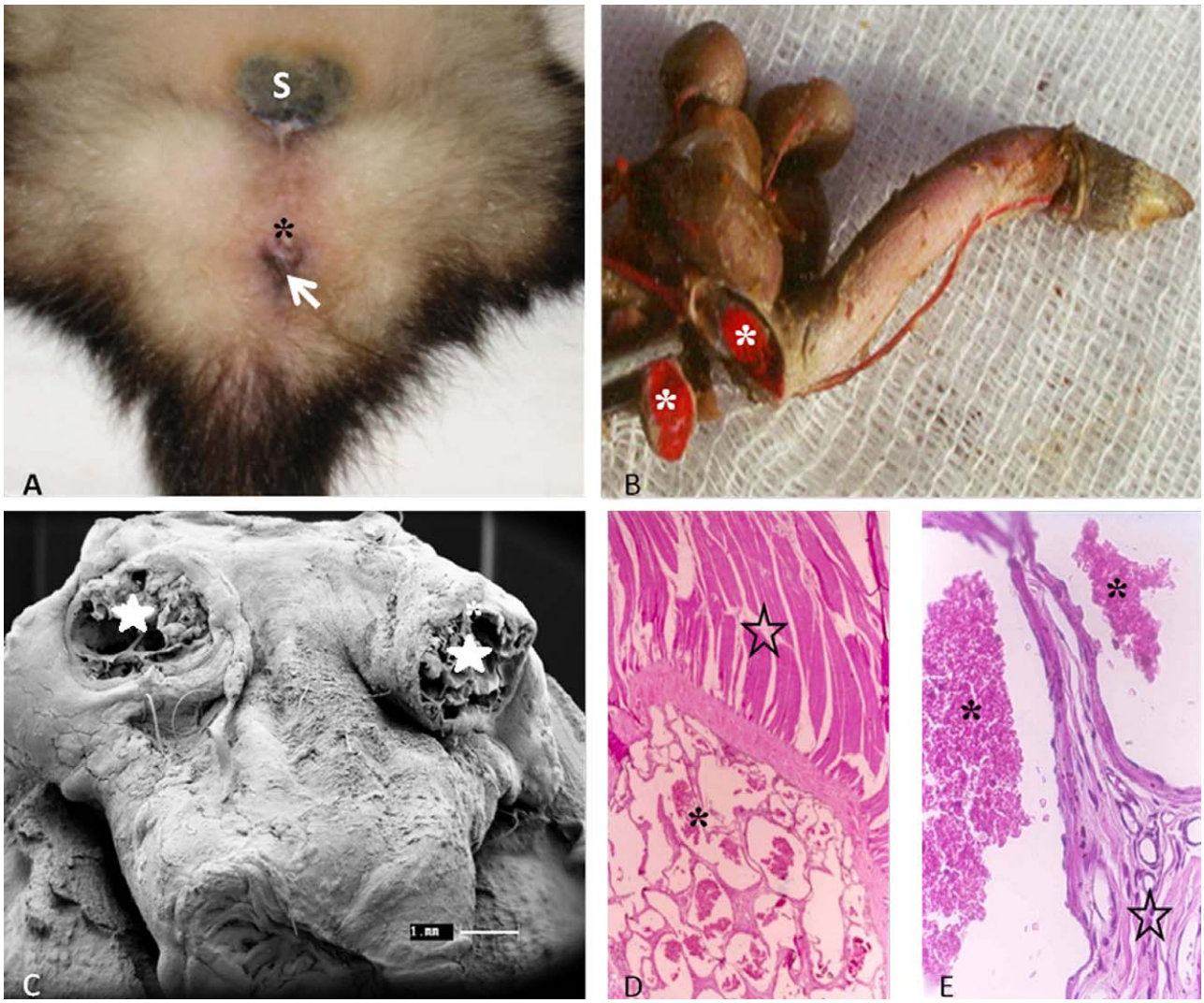

Fig. 2. Photomacrography of ventral view of penis and scrotum; penis in the inguinal cavity (*), just below the anus (arrow), **A**. The scrotum (S) is in the anterior position of penis. **B**: Photomacrography of lateral view of penis fixed in formaldehyde (10%) after red latex injection through the arterial system. Transverse section of penile bulb (*). **C**: Scanning electron micrograph of sectioned penile bulb (star). **D** and **E**: Photomicrographs of penile bulb; D: Skeletal muscle disposed externally (star) to erectile tissue (*). E: Details of erectile tissue showing connective tissue (star) and blood in the cavernous lumen (*). D: HE. ×44; E: HE. ×110. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

prostate, pinkish or brown, and extends caudally to the first segment. The longest segment that extends into the membranous urethra is greenish and darkens after fixation (Fig. 8A). Microscopic features revealed that tubular glands in a radial arrangement around the urethra are coated externally by a capsule of dense connective tissue associated with smooth muscles arranged in two layers surrounding the glandular tissue (Figs. 8B–8D) and the details of prostatic urethra were obtained through scanning electron microscopy (Figs. 8E and 8F).

Under light microscopy, cross sections of the prostate showed that three regions of each segment are defined by glandular tissue. The cortical region located near the capsule is composed of the bottom of secretory tube that extends to the middle region, consisting of cubic or prismatic cells. The glandular tubes converge in the inner region in gradually thicker ducts lined by transitional stratified epithelium and opening into the prostatic urethra (Fig. 9A regions 1, 2, and 3, respectively). The prostatic urethra was pleated and coated by transitional stratified epithelium.

At the junction of the membranous and cavernous urethra the Cowper's glands were found with descending and parallel ducts that open caudally into the lumen of the urethra. Histological features of these glands were revealed to be lined by fibrous capsule of collagen fibers associated with two well-defined layers of skeletal muscles (Fig. 9B). The glandular stroma is composed of several lobes joined by narrow septa of connective tissue where the smooth muscle cells are present. In each lobe, tubular alveolar glands were found, consisting of glandular epithelium that varies according to the pair of bulbourethral glands. The first

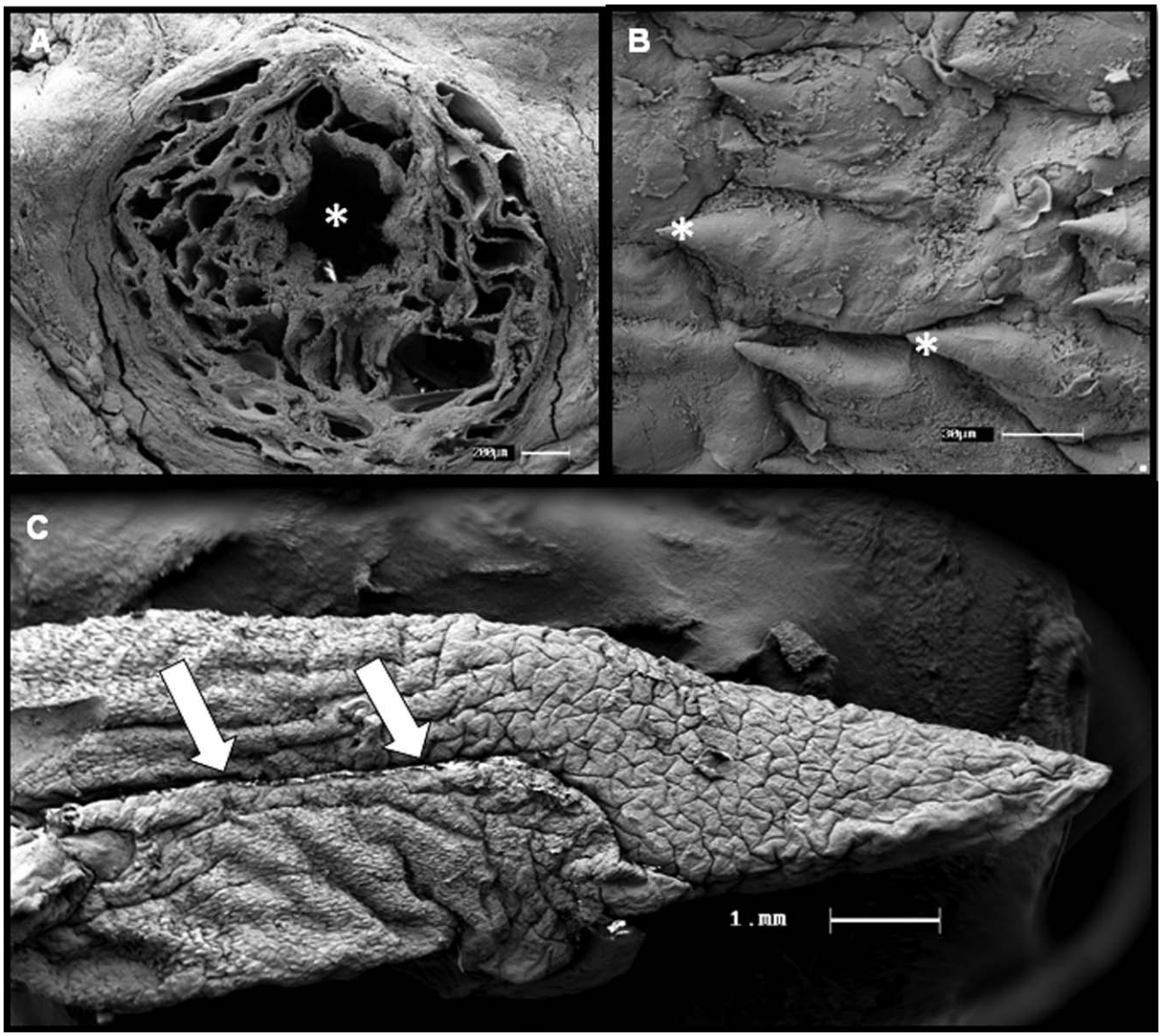

Fig. 3. Transverse section of scanning electron micrograph of the penis. **A**: Scanning electron micrograph of cavernous body surrounding the urethra (*). **B**: Scanning electron micrograph of numerous penis spines (*). **C**: Urethral groove (arrows) in the inner surface of penis.

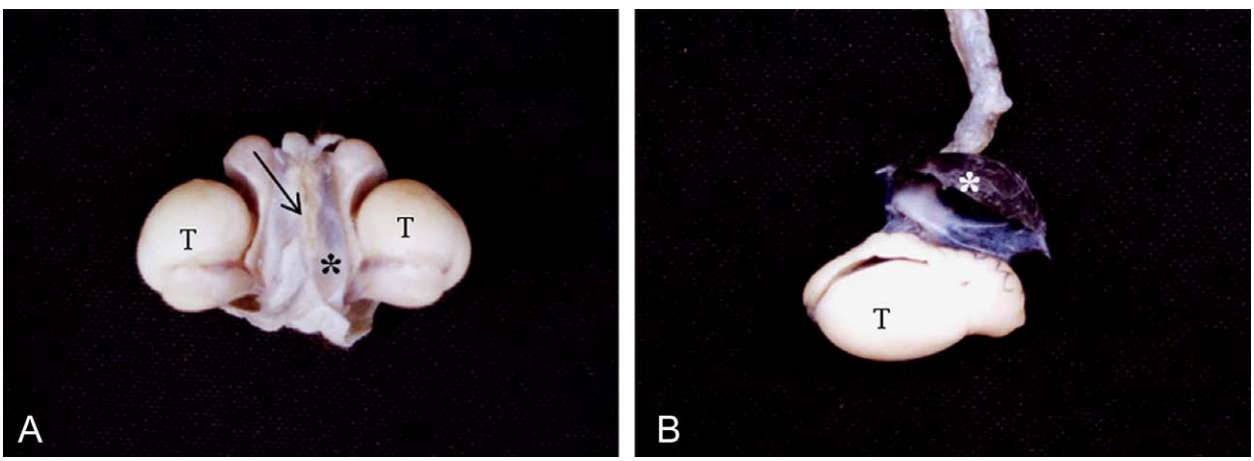

Fig. 4. Photomacrography of gross morphology of testis (T). **A**: Tunica vaginalis of young animal (*) and septum of testis (arrow). **B**: Dark tunica albuginea of adult animal (*). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

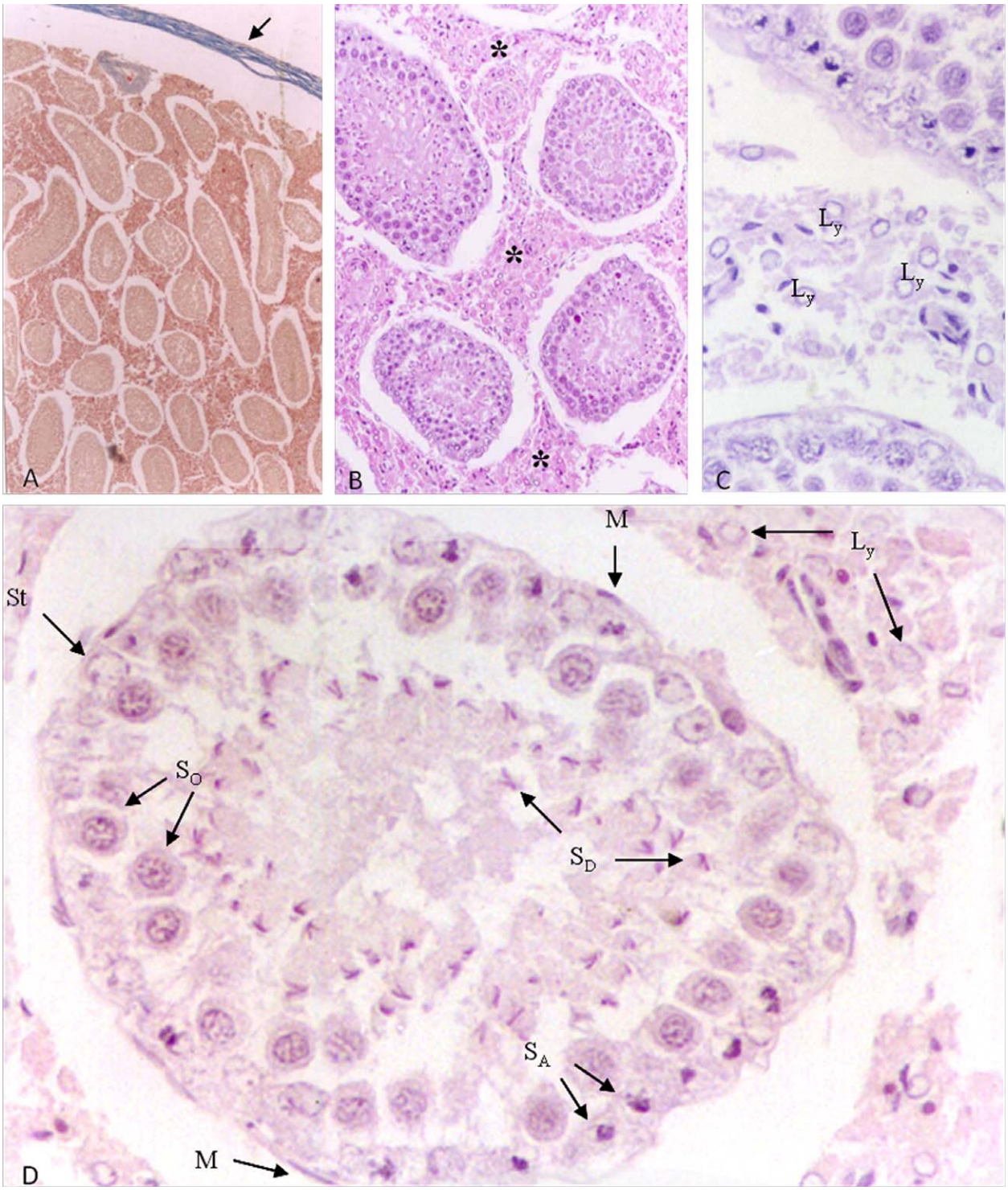

Fig. 5. (**A–D**) Photomicrographs of the testis. (A–C) Transverse section of seminiferous tubules with tunica albuginea (arrow), immersed in the interstitial tissue (*), and Leydig cells (Ly), respectively. (5D) Cross section of seminiferou tubule: peritubular myoid cells (M), Sertoli cell (St), Leydig cells (Ly), and Germ Cell Lineage: spermatogonia (SA), spermatocytes (So) and spermatids (SD). (A): Masson's Trichrome Staining. (B–D) HE. ×44, ×176, ×280, and ×440. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pair is lined by simple prismatic mucinous epithelium with basal nuclei and rounded shaped secretions in the lumen, suggesting corpus amylaceous. The middle pair showed well-developed muscular tunics surrounding the glandular stroma (Fig. 9C). The other pair showed the glandular epithelium consisting of single layer of

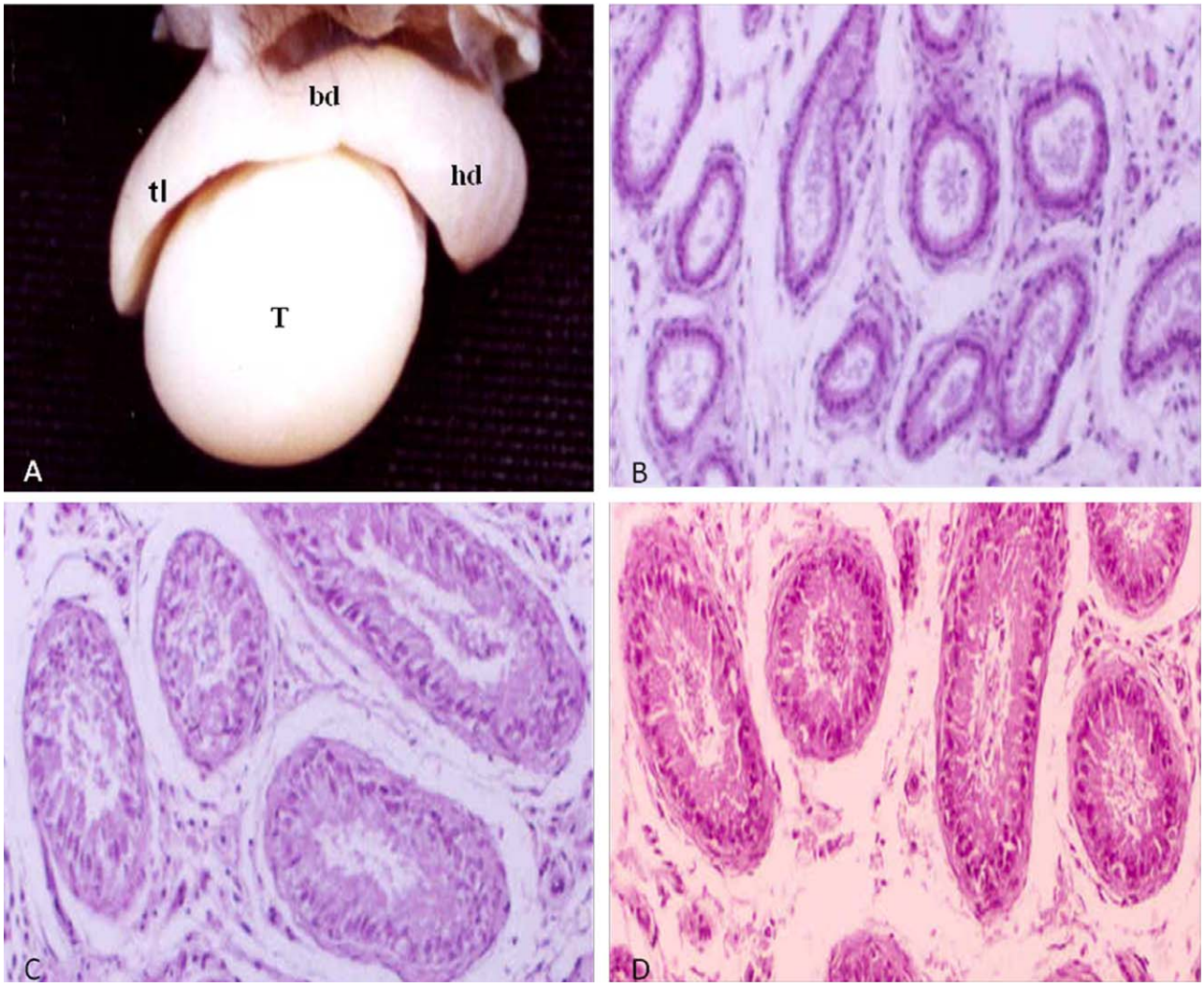

Fig. 6. Morphology of epididymis. Photomacrography (**A**) of the head (hd), body (bd), and tail (tl) of epididymis adhered to the testis (T). **B–D**: Cross section photomicrographs of head, body and tail respectively. (B–D): HE. ×110. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cuboidal cells and amorphous material in the lumen similar acidophilic colloid.

The blood supply of pelvic viscera is made through the common iliac artery, from which the external and internal iliac arteries arise. Conversely, the venous drainage is provided by the iliac vein that flows into the caudal vena cava. The accessory glands are supplied by the external iliac artery through a prostatic branch that falls within the first and second segment of prostate, continuing parallel to the whole prostatic until the region of bulbourethral glands. Three or four branches supply the bulbourethral glands and an artery that nourishes the bulb cavernous muscle. Then, a dorsal arterial branch nourishes the body and glans of penis, parallel placed in the double-shaped, termed the dorsal artery of penis, with the veins running along the arteries having the same nomenclature.

A further vascular source is through the femoral artery and femoral veins, which are essential to the erection process, without any relationship of its branches with the penis and accessory glands, only with bulbospongiosus muscles. The vascular source is issued through the femoral artery and femoral veins are essential to the erection process, without any relationship of its branches with the penis and accessory glands, only with bulbospongiosus muscles.

DISCUSSION

Through gross and microscopic analyses, it was observed that the *Didelphis* sp. male genital consists of a pair of testes, epididymis, spermatic cord, and ductus deferens; the prostate was divided in three distinct segments, and the bifid penis and the absence of seminal vesicle gland was replaced by three pairs of bulbourethral glands, thus supporting the findings of Sweet (1907) and Nogueira et al. (2004).

The gross anatomy of the testis showed them to be small, rounded whitish, placed in the inguinal region, and covered by scrotum, anterior to penis as was

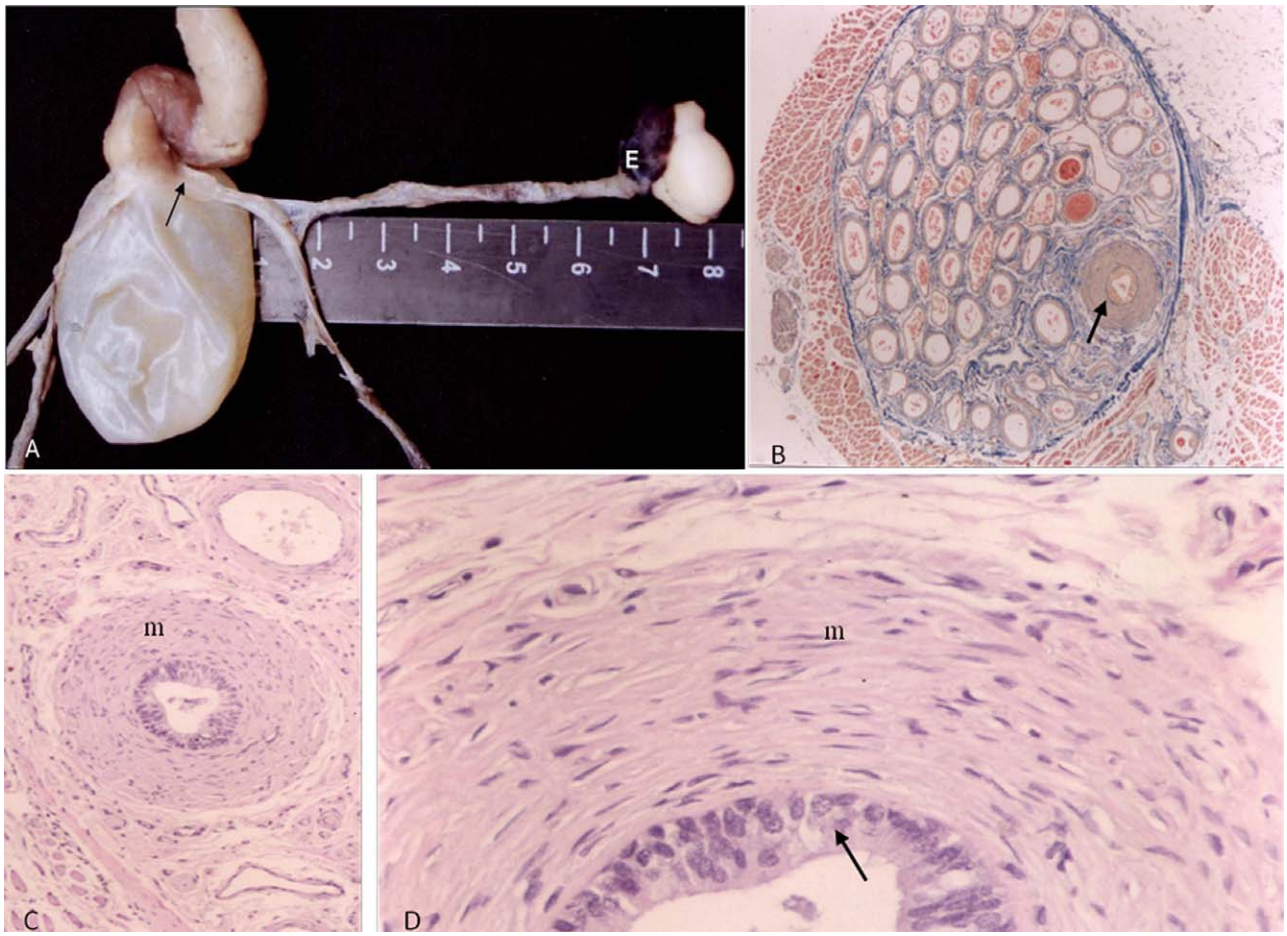

Fig. 7. Spermatic cord and ductus deferens photograph and photomicrographs. **A**: Ductus deferens emerging at the tail of the epididymis (**E**); the final portion at the fundus of bladder (arrow). **B**: Spermatic cord photomicrograph with ductus deferens (arrow). **C** and **D**: Cross section photomicrograph of ductus deferens; thick layer of muscle (M) and the inner lining epithelium (arrow). (B): Masson's Trichrome Staining. ×44; (C and D): HE. ×110 and ×440. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

described by several investigators (Renfree et al., 2001; Nogueira et al., 2004).

The epididymis are also whitish surrounding the testes and provide a set that is macroscopically similar to other eutherian mammals. Carneiro et al. (2010) described the epididymis of the ocelot, proving to be very similar to marsupials, being formed by convolutions of the epididymal duct attached to the lateral surface of the testis, being divided into head, body, and tail.

The microscopic features of the testis reveled a germinal epithelium consisting of characteristic nuclei of Sertoli cells and gametic cells traditionally observed in other mammals; however, the paired spermatids through the head and the large amount of Leydig cells and testicular interstitial tissue are typical of this specie and are similar to findings in domestic cats (Silva et al., 2009), in collared peccary and lipped peccary (Sonner et al., 2004), in pigs (Avelar et al., 2010), and in ocelots (Sarti et al., 2011).

The pigmentation of the tunica vaginalis has been reported in several species of marsupials (Finkel, 1945; Biggers, 1966; Heddle and Guile, 1970). Biggers (1966) was the first study to suggest that the high concentration of melanin is related to testicular thermoregulation, which should be lower than the body in sexually mature animals. Our data emphasizes this hypothesis since the young and sexually inactive animals showed whitish tunica vaginalis while the adults showed high pigmentation.

The ducts deferens were found lateral to ureters, entering into the dorsal wall of the urethra near the bladder, similar to American and Australian *Didelphis* (Chase, 1939). Histologically, they consist of several layers of smooth muscle lined by a pseudostratified prismatic epithelium similar in coati to that reported by Franciolli (2007). However, Nogueira et al. (1977) described similar histological features in *Didelphis azarae*, with discordance on the inner covering epithelia, which were claimed to be simple prismatic.

In a normal anatomical position, the penis is entirely positioned in the inguinal cavity, posterior to scrotum and, when exposed, showed an "S" shaped. The glands penis is bifid occurring in each segment, and the extension of urethral groove results is also described by Cowper (1704) and Tyndale-Biscoe and

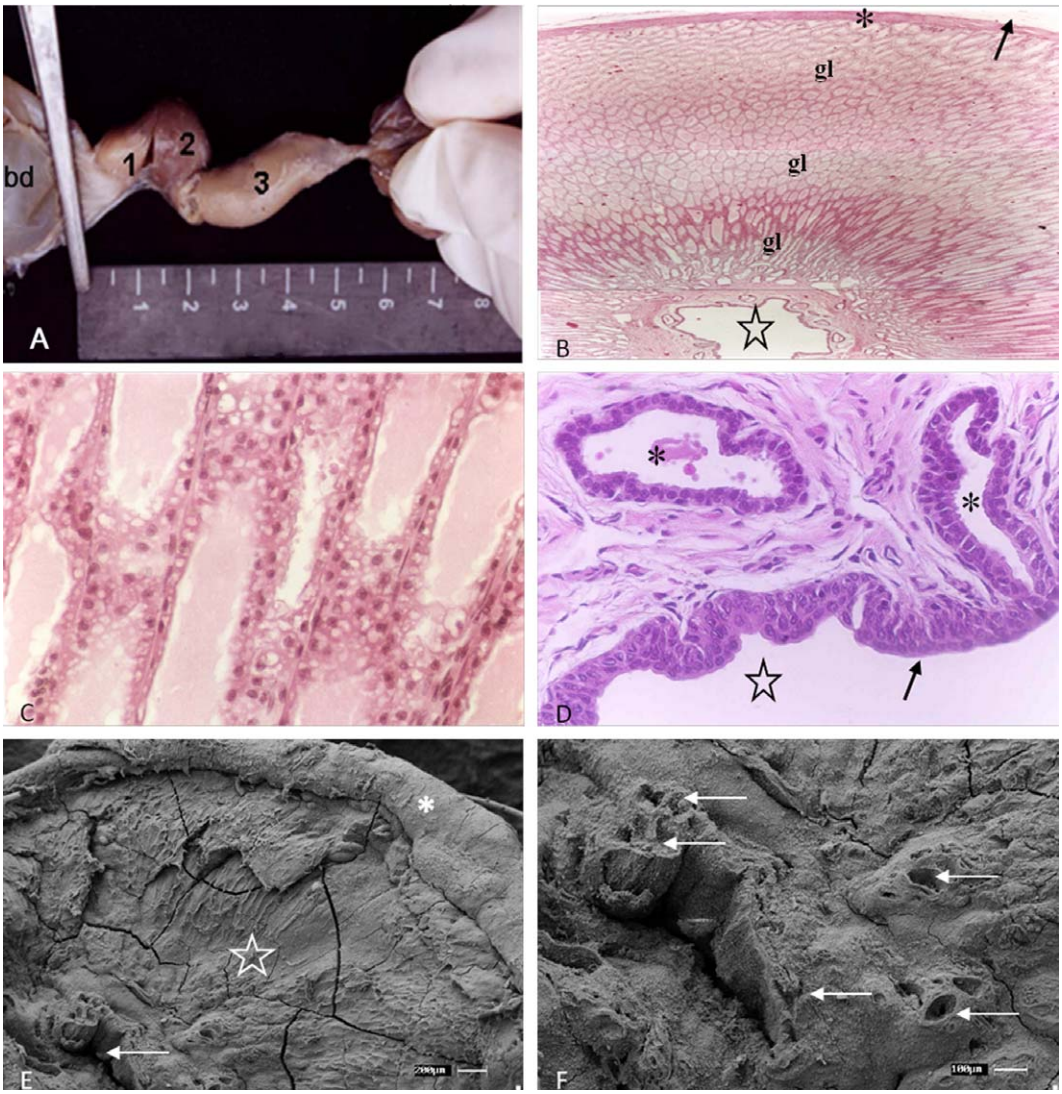

Fig. 8. The prostate photograph, photomicrograph and electromicrograph. **A**: Ventrolateral view of prostatic segments (1, 2, and 3) and bladder (bd). **B**: Assembled photomicrographs showing the connective capsule (arrow) connected to the muscle layer (*), glandular epithelium (gl) which open in the prostatic urethra (star); **C**: Details of glandular epithelium, see the bottom of the tubular glands. **D**: Thicker ducts (*) lined by transitional epithelium (arrow), opening into the prostatic urethra (star). **E**: Cross section of prostate; the fibrous capsule (*) glandular parenchyma (star) and prostatic urethra (arrow). **F**: Details of the prostatic urethra and small openings excretory ducts (arrows). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Renfree (1987). In microscopic findings, the cavernous and spongy body throughout the length of penis and the presence of spicules in the glands were observed.

The prostate was revealed be divided into three different segments (in color, size, and shape); however, the microscopic constitution is identical. It consists basically of tubular glands in a radial arrangement around the urethra, coated externally by a dense connective tissue associated with a relatively thick layer of smooth muscle, similar to Tyndale-Biscoe et al.'s (1987) and Martinelli et al.'s (1990) findings.

According to the reports of Fernandez et al. (2010), in capybaras, the prostate has secretory epithelium that is a pseudostratified columnar type, with some portions formed by simple columnar epithelium. The cylindrical cells are high and basophilic cytoplasm, rounded nuclei with clear chromatin, and evident nucleoli and basal cells that are sparse, have clear cytoplasm, and have a small nucleus.

According to the first macroscopic trials, four pairs of bulbourethral glands were expected. Through histological studies, only three pairs were confirmed, and placed between them was a pair of cavernous bulbs that continue with the cavernous body of penis, as observed by Chase (1939).

The gross morphology of the testes is similar to other eutherian mamals, thus confirming the importance of this Brazilian specie as a reproductive model. However, the spermatids are paired through the head and there is a large amount of Leydig cells and testicular interstitial tissue. The epididymis is macroscopically and microscopically similar to other mammals. The

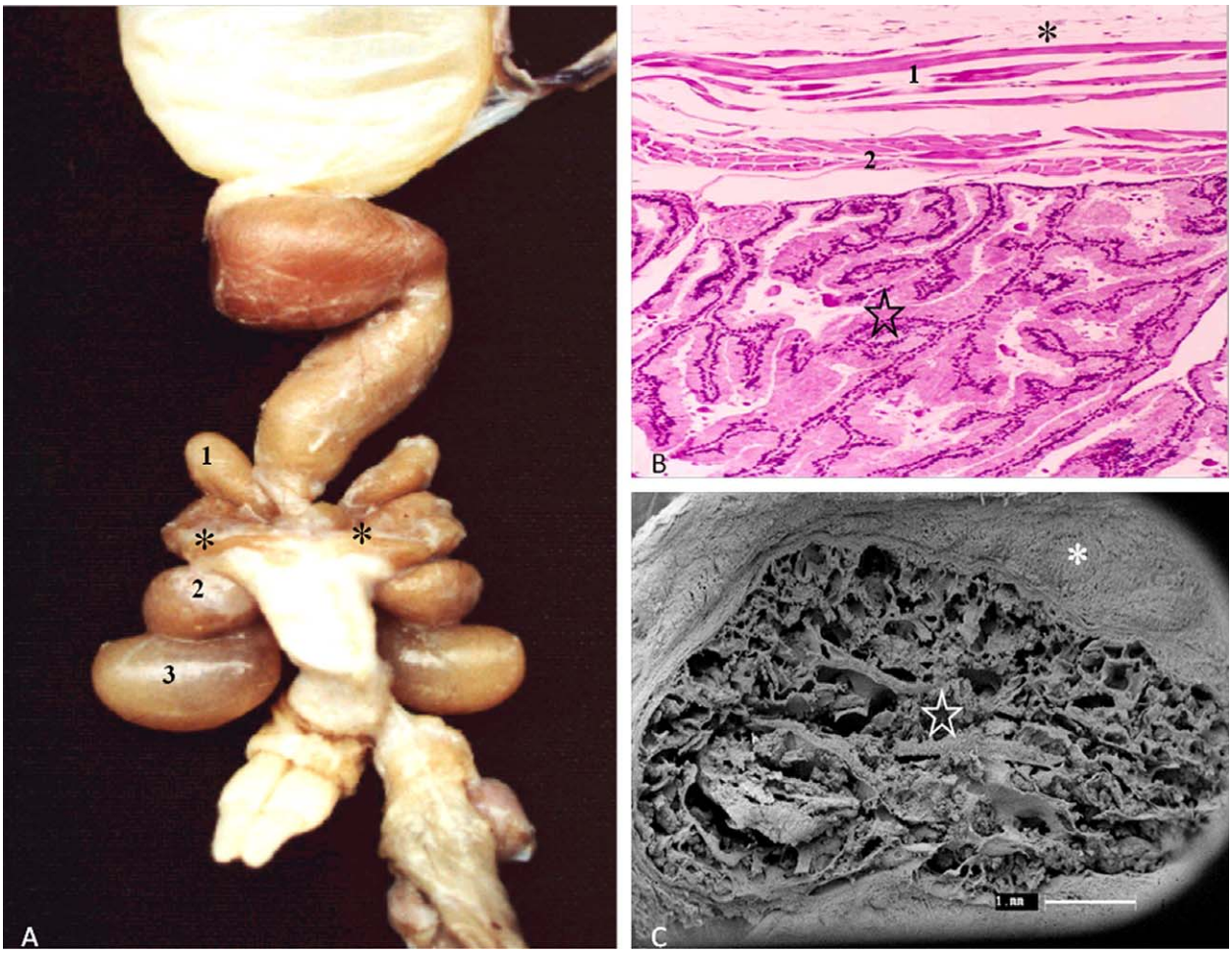

Fig. 9. The bulbourethral gland morphology. **A**: Pairs of bulbourethral glands (1, 2, and 3) and penile cavernous bulbs (*). **B**: The first bulbourethral gland; connective capsule (*); layers of skeletal muscle (1 and 2) and glandular epithelium (star); **C**: The second bulbourethral gland scanning electromicrograph showing the thick muscular tunica (*) and glandular parenchyma (star). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

penis is bifid, entirely positioned in the inguinal cavity, and occurring in each segment extension of the urethral groove. The ductus deferens is similar to other animals. It begins at the end of epididymal tail, enters the abdominal cavity surrounded by spermatic cord, and opens in the dorsal wall of the abdominal urethra. The prostate is rather large and divided into three distinct segments, basically composed of tubular glands in a radial arrangement around the urethra. The seminal vesicles glands are absent in this species. Three pairs of bulbourethral glands were described with an identical microscopic constitution.

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