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Morphological aspects and germ cells of male reproductive tract of river stingray, *Potamotrygon amandae*

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ABSTRACT. The family Potamotrygonidae are the only species of stingrays restricted to fresh water and located exclusively in South America. The objective of this research was to analyze the morphological aspects and germ cells of the male reproductive tract of *Potamotrygon amandae*. The samples were fixed in 10% formalin, and then dehydrated in an ascending ethanol series (70 to 100%). To carry out light microscopy analyses, they were embedded in paraffin, cut and stained; as for scanning electron microscopy analyses, the samples were dried, glued in metallic bases and metalized. The gross morphology consisted of the following paired organs: testis, epididymis, deferent duct, Leydig gland, seminal vesicle, clasper, and the clasper gland. Microscopically, several stages of spermatogenesis were observed in the testis, occurring in spherical follicles, similar to other stingrays. The epididymis was formed by one duct subdivided in various tubules. The deferent ducts were continuous with the epididymis, and the lumen was full of spermatozoa. The Leydig glands consisted of glandular units with eosinophilic content in the lumen of some, and the deferent ducts ran parallel to the ventral portion. The seminal vesicles possessed numerous compartments to store the sperm, with a wall similar to a hive, and the lumen was full of spermatozoa. Alcian Blue (AB) and Periodic Schiff-Acid (PAS) performed in the Leydig Gland, deferens ducts and seminal vesicle was positive only in the connective tissue, the cilia were PAS+ and the nuclei stained weakly for AB. The clasper gland was composed of unit glands and was covered with striated muscle externally. It stained very well with Periodic Schiff-Acid. The morphological aspects of the male reproductive tract of *Potamotrygon amandae* were similar to other stingrays.

Keywords: elasmobranchs; freshwater stingray; masculine tract; germ cells.

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Introduction

Stingrays are cartilaginous fishes of the class Chondrichthyes, occupying the marine environment and even fresh water (Awruch, 2013). Stingrays, in general, are back-ventral flattened, with enlarged pectoral fins in the head; they have 5 to 6 pairs of gill openings on the ventral surface.

Freshwater stingrays possess the inability to retain high serum levels of urea to compensate for water loss if exposed to a high salinity environment, and present modification of the Ampullae of Lorenzini electroreceptors, which enable it to operate in fresh water (Lovejoy, 1996). The Potamotrygonidae family are freshwater stingrays restricted to South America and five genera have been described, including *Potamotrygon* (Rizo-Fuentes et al., 2021).

The permanence over time and population of chondrichthyans depends on their reproductive strategy, meaning that their evolutionary success can be in part attributed to their reproductive adaptations, such as the pelvic fins modified into claspers that allow internal fertilization (Awruch, 2013).

The seasonality of spermatogenesis varies between species. Several studies have shown spermatocytes in different stages of spermatogenesis, with little or no variation in seasonality in elasmobranchs (Sourdaine, Gautier, & Gribouval, 2018). In *Torpedo marmorata,* spermatogenesis occurred all year long; however, in *Urobatis helleri*, the processes did not occur between March and July, and the maximum production of follicles was in the winter, in December (Sourdaine et al., 2018).

The reproductive cycle of stingrays from the genus *Potamotrygon* is related to the hydrological cycle, with seasonality playing an important role (Zaiden, Brinn, Marcon, & Urbinati, 2011). Sexual maturation can vary between freshwater stingrays, although information regarding their reproductive biology is still limited, especially microscopic morphology (Zaiden et al., 2011). This study aims to: i) describe morphological aspects of the testes, epididymides, deferens ducts, seminal vesicles, and claspers; ii) characterize the germ cells during the stages of differentiation of the spermatogenic cells. This information will allow understanding of the cells that compose the male reproductive biology of *Potamotrygon amandae* and create a reference for its reproductive biology, which can help conserve the species and encourage more studies regarding freshwater stingray biology.

Material and methods

Three *Potamotrygon amandae* specimens, provided from the research project entitled: 'Reproductive metabolic aspects of *Potamotrygon amandae* (Chondrichthyes: Myliobatiformes: Potamotrygonidae)', were used in this study, in a 'non-natural' protected area under the responsibility of PhD Professor Cristélia da Silva Ribeiro. The project is being developed at the Engineering School of Ilha Solteira (FEIS/UNESP) in the Biology and Zootechnics Departament UNESP of Ilha Solteira Institution; authorized by the SISBio: 72788-1. It has been approved by the Use of Animals Ethics Committee (CEUA/FMVZ) under the CEUAx Nº 4543010420.

The specimens were fixed and kept in 10% formaldehyde, which helped satisfactorily conserve the structures for later analyses. They were open with an incision in the cloaca, breaking the pelvic girdle until the scapular girdle through the alba line, tail-cranial direction, for visualization of the organs. The gastrointestinal tract was removed, exposing the male reproductive tract. Fragments from the cranial, medial, and caudal part of each organ were collected and identified. Processing took place in the Histological Laboratory of the Surgery Department in the Anatomy of Domestic and Wild Animals Sector at the Faculty of Veterinary Medicine and Animal Science of the *Universidade de São Paulo*.

For histological analyses, the collected fragments were fixed in 10% formalin, dehydrated in an ascending ethanol series (70 to 100%), diaphanized in xylol, and embedded in paraffin. Cuts of 5µm thickness were made in all structures in a microtome and stained using hematoxylin-eosin (HE). Alcian Blue pH 2.5 (AB) and Periodic Acid-Schiff (PAS) were used to analyze the secretions of the Leydig gland, vas deferens, seminal vesicle and clasper gland. Images were obtained using a Nikon Eclipse E-800 light microscope, located at the Advanced Center of Diagnostic Imaging - CADI- FMVZ-USP.

Samples fixed in 10% formalin were dehydrated in an ascending ethanol series in concentrations of 70, 80, 90 and 100%, dried in a LEICA Critical Pointe Dryer (CPD) 300, glued with carbon glue in metallic bases of aluminum (stub) and metalized (sputtering) with gold in a EMITECH K550. They were then analyzed and documented in a LEO 435VP Scanning Electron Microscope (SEM) at the Advanced Center of Diagnostic Imaging - CADI- FMVZ-USP.

Results

Gross morphology. The male reproductive tract of *Potamotrygon amandae* was intra-abdominally located on the dorsal portion of the body, close to the spine, and covered with a thick serosa (Figure 1a). The arrangement of the reproductive tract consisted of the following paired organs: testis, epididymis, vas deferens with the Leydig glands and seminal vesicle. The claspers of the stingrays are two separate organs located outside the abdominal cavity and used for copulation (Figure 1a).

The reproductive tract was divided in the middle by the vertebral column (Figure 1a). Each testis had a lobated surface. The epigonal organ had a smooth texture and was associated with the testis on its caudal portion. Due to its proximity, it was very difficult to differ one from another. The testis was approximately 4-6 cm in length. The afferent ducts are responsible for delivering the sperm from the testis to the epididymis; however, due to its small size and difficult dissection, the organ was not included in this research.

The epididymis was situated more cranially in the abdomen, and continuous to them it was the vas deferens, which presented a spiral format. It started very thin and ended enlarged to form the seminal vesicle (Figure 1ab). The Leydig glands embraced the vas deferens. The vesical glands were 'S'-shaped and met near the end in the middle of the abdomen. It was connected to the kidneys on the dorsal side (Figure 1b).

The clasper is a paired organ, exclusively male, located between the pelvic fins. Both claspers had an associated gland, whose function is unclear (Figure 1a). The clasper gland was in the proximal area of the clasper covered by skin. It was necessary to remove it for the visualization of the organ. The claspers glands were parallel to the clasper and the cloaca, and presented a bilobated aspect (Figure 1a).

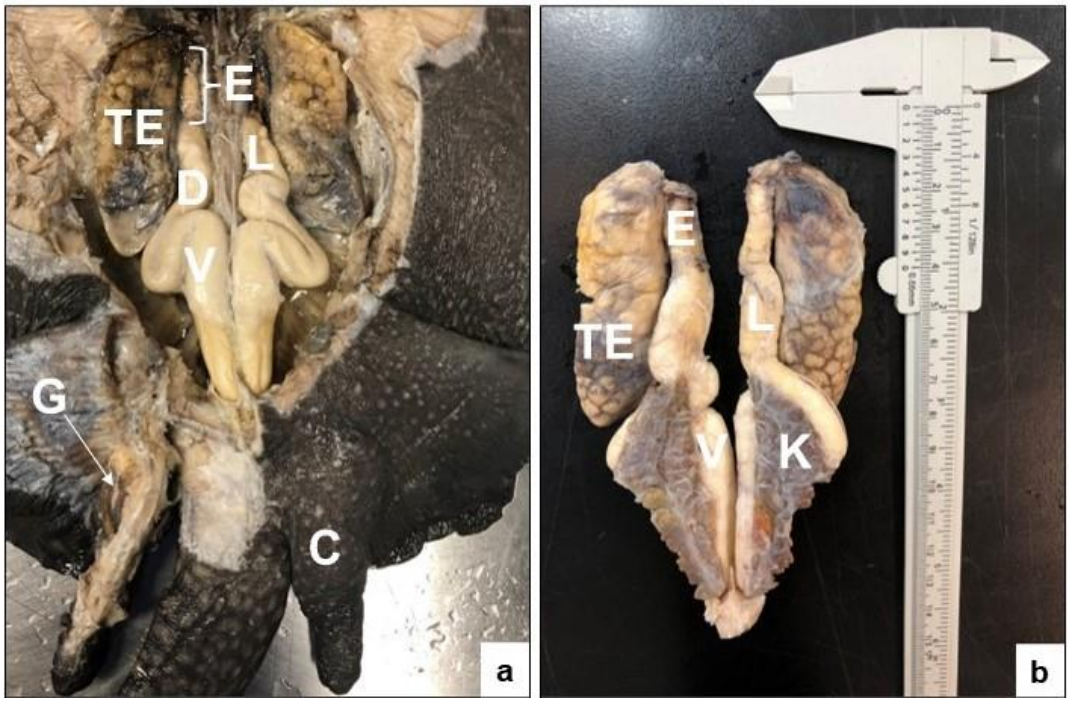

Figure 1. (a) The *Potamotrygon amandae* male reproductive tract disposed intra-abdominally with the two testes (TE), the two epididymides (E), the two deferent ducts (D) with the Leydig glands (L) adjacent, the two seminal vesicles (V) converging in the final portion, and the clasper (C) with its associated gland (G) exposed. (b) The kidneys (K) are connected to the male reproductive tract, with the seminal vesicles (V) resting on them. Other organs that can be seen are the testes (TE), epididymides (E) and the Leydig gland (L).

Microscopically

Testis. Microscopically, the testis exhibits spherical follicles where spermatogenesis takes place. Spermatogenesis occurred in testicular lobes in concentric zones from the center to the periphery, with the early stages occurring in the middle and the more advanced ones occurring near the edge of the lobe (Figure 2a). The lobes were separated by a very thin connective tissue.

The spermatogenesis stages were: spermatogonia, spermatocytes, spermatids and spermatozoa (Figure 2a). The epigonal organ, closely associated with the testis, had innumerous blood cells that could be observed near the edge (Figure 2ab).

Potamotrygon amandae had a spermatogonia section in which the cells were included in follicles and organized in layers. Some Sertoli cells were observed among the spermatogonia and presented a round elongated nuclei with a central nucleolus; blood vessels were also spotted, as well as some blood cells among the follicles (Figure 2c). The follicles were small, well defined, and a lumen was present (Figure 2c).

The primary spermatogonia presented scarce cytoplasm and were the larger cells in the spermatogenesis lineage. Their nuclei were round (some oval) and elongated (Figure 2c). The secondary spermatogonia had nuclei that were round (some oval), elongated, and smaller than the primary ones. The chromatin was lighter and more granular (Figure 2c).

The lumen became obstructed in the spermatocytes zone due to a lack of cell arrangement and an increase in their number (Figure 2d). As the mitotic division of spermatogonia progressed, the follicles increased in size, and as the cells increased in number, they decreased in size (Figure 2d). Some spermatocytes undergoing meiotic division can be spotted (Figure 2d). The Sertoli cells gradually migrated to the periphery of the follicle, and their nuclei flattened and became irregular (Figure 2d).

The primary spermatocytes were included in spermatoblasts and had round nuclei with very granular chromatin that was in different stages of condensation due to mitotic division. They also had scarce cytoplasm (Figure 2d).

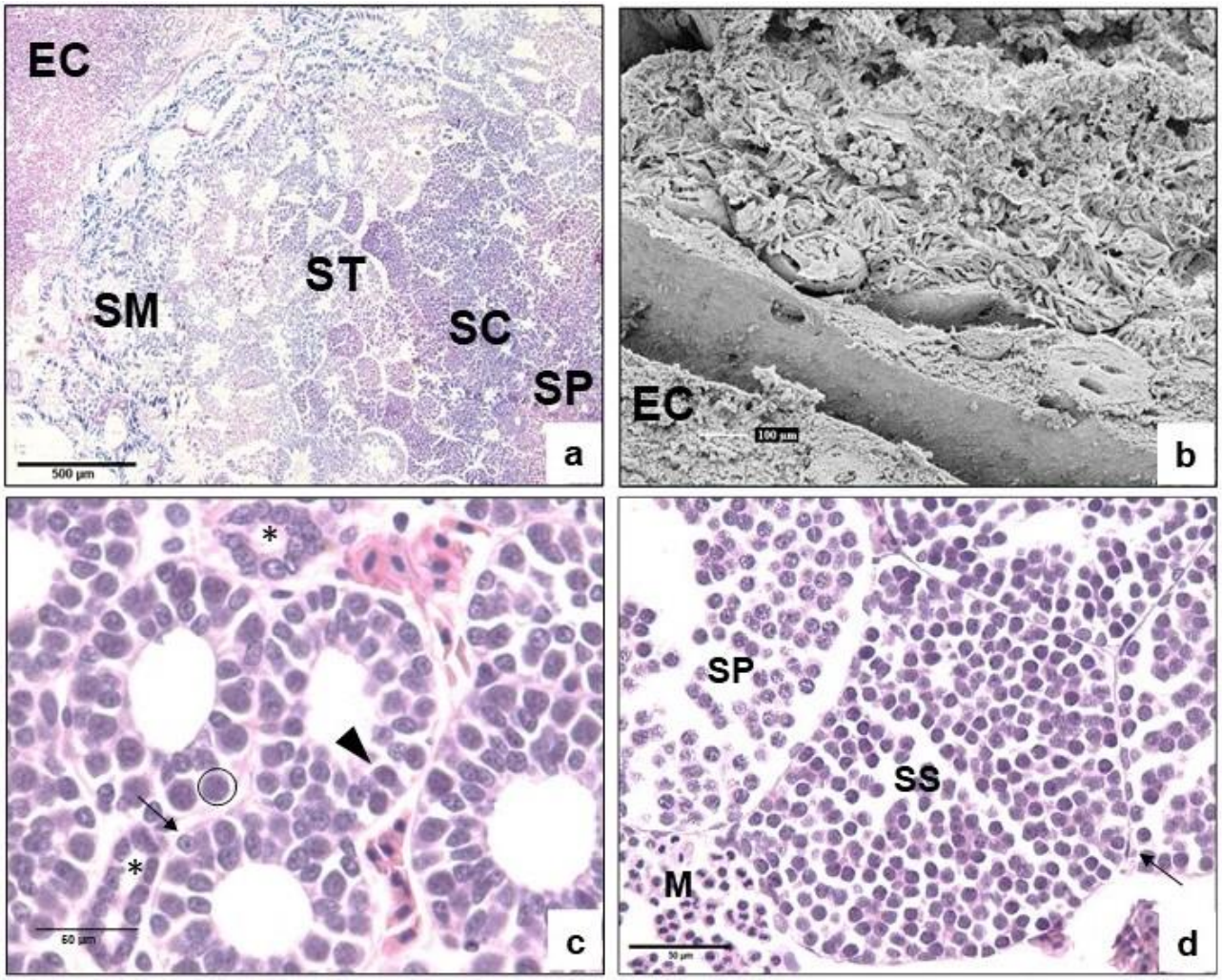

Figure 2. (a) Light micrograph of the spermatogenesis phases separated in concentric rows: spermatogonia (SP), spermatocytes (SC), spermatids (ST) and spermatozoa (SM); it is possible to visualize epigonal cells (EC). HE. (b) Scanning electron micrograph of the spermatid zone and the epigonal organ (EC) closely associated with the testis. (c) Light micrograph of the spermatogonia section where in the same follicle there are primary spermatogonia (circle) and secondary spermatogonia (arrowhead). Some Sertoli cells can be observed among the cells (arrow). Blood vessels (asterisk) can be seen among the follicles. HE. (d) Light micrograph of the spermatocytes zone with primary spermatocytes (SP), secondary spermatocytes (SS) and cells undergoing meiosis (M). The Sertoli cells (arrow) are in the periphery of the follicle with an irregular nucleus. HE.

The secondary spermatocytes were round, with smaller nuclei compared to the primary ones. The chromatin in this phase was condensed; therefore, these cells stained darker (Figure 2d).

In the spermatid zone, the spermatids begin to arrange radially and decrease in size, due to being one of the smallest in the spermatogenesis lineage; their nuclei are round or oval, with a dense chromatin (Figure 3ab). Due to the reorganization of the cells, the lumen reappears (Figure 3ab). Tail formation occurs in this phase (Figure 3ab). In the spermatozoa section the sperm is totally formed and organized in clumps (Figure 3cd). The sperm present a pointed and spiral head turned to the Sertoli cells at the membrane of the follicle, and the tails turned to the lumen at the center (Figure 3cd). A system of ducts collects the sperm, and the follicle degrades at the degeneration zone (Figure 3c).

The spermatozoa form very tight clumps that interfere with the count of in follicle cells at the end of spermatogenesis (Figure 3d). In the scanning electron micrograph, these bundles are more visible, and although the heads of the spermatozoa are spiraled, they are not as spiraled as it appears in the light microscopy.

Epididymis. The organ was organized in various tubules, each with the spermatozoa at the center of the lumen (Figure 4abcd). The epithelium was pseudostratified columnar ciliated. The cells presented round or oval nuclei, with granular chromatin and a nucleolus at the corner. The underlying connective tissue was mainly collagen fibers. Some blood vessels and blood cells were present between the tubules (Figure 4ab). The epithelium presented small folds that projected into the lumen (Figure 4bcd).

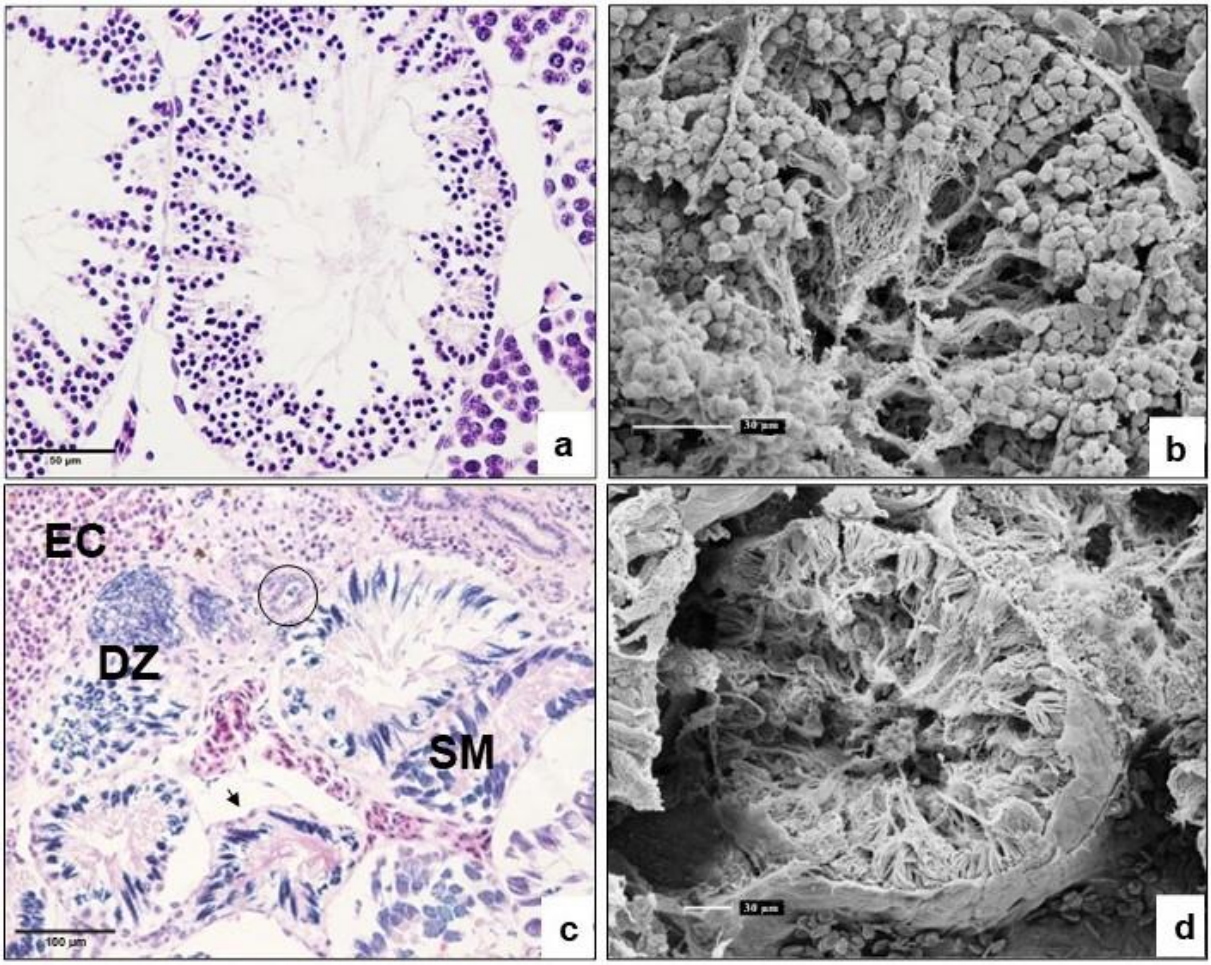

Figure 3. (a) Light micrograph of a spermatid; notice the cells organizing radially and the tail being formed. HE. (b) Scanning electron micrograph of a spermatid. (c) Light micrograph of the spermatozoa zone with mature spermatozoa (SM) and the Sertoli cells (arrow), collector duct (circle), degeneration zone (DZ) and epigonal cells (EC) associated with the testis. HE. (d) Scanning electron micrograph of mature spermatozoa showing the spermatozoa organized in very tight clumps.

Vas deferens and Leydig gland. The vas deferens had a pseudostratified columnar ciliated epithelium with a lumen full of spermatozoa. The underlying connective tissue was mainly collagen fibers, but the wall was thinner than the one in the epididymis (Figure 5a). The epithelium also had folds into the lumen (Figure 5a). Associated was the Leydig glands that consisted of several gland units with a simple columnar epithelium and some units had eosinophilic secretion in the lumen (Figure 5ab). The Leydig glands were connected to the vas deferens by a dense connective tissue (Figure 5a).

In the PAS reaction the epithelium of the organs reacted weakly, while the underlying connective tissue and the cilia in the vas deferens epithelium reacted stronger; the secretion presented in the lumen of some gland units stained strongly with PAS (Figure 6ab). In the AB stain only the connective tissue of the organs reacted; the nuclei in the cells of the Leydig gland units and in the epithelium of the vas deferens reacted poorly to AB (Figure 6cd).

Some epithelial folds containing what was probably the secretion produced by the Leydig gland were observed being released in the lumen of the vas deferens (Figure 6bc). These folds were composed of parts of the vas deferens epithelium and connective tissue. The cells presented a wide vacuole (Figure 6b).

Seminal vesicle. The seminal vesicle had a pseudostratified columnar ciliated epithelium, which created fine folds inside the duct, forming compartments (Figure 5cd). Among the spermatozoa, secretions from the ducts could be observed throughout the vas deferens and seminal vesicle (Figure 5c). The spermatozoa were found in the lumen and organized in a hive aspect in the scanning electron micrograph (Figure 5d). The performance of AB and PAS in the seminal vesicle was positive in the connective tissue (Figure 6ef). The cilia of the epithelium reacted positively with the PAS stain, and the nuclei of the epithelium reacted weakly with AB (Figure 6ef).

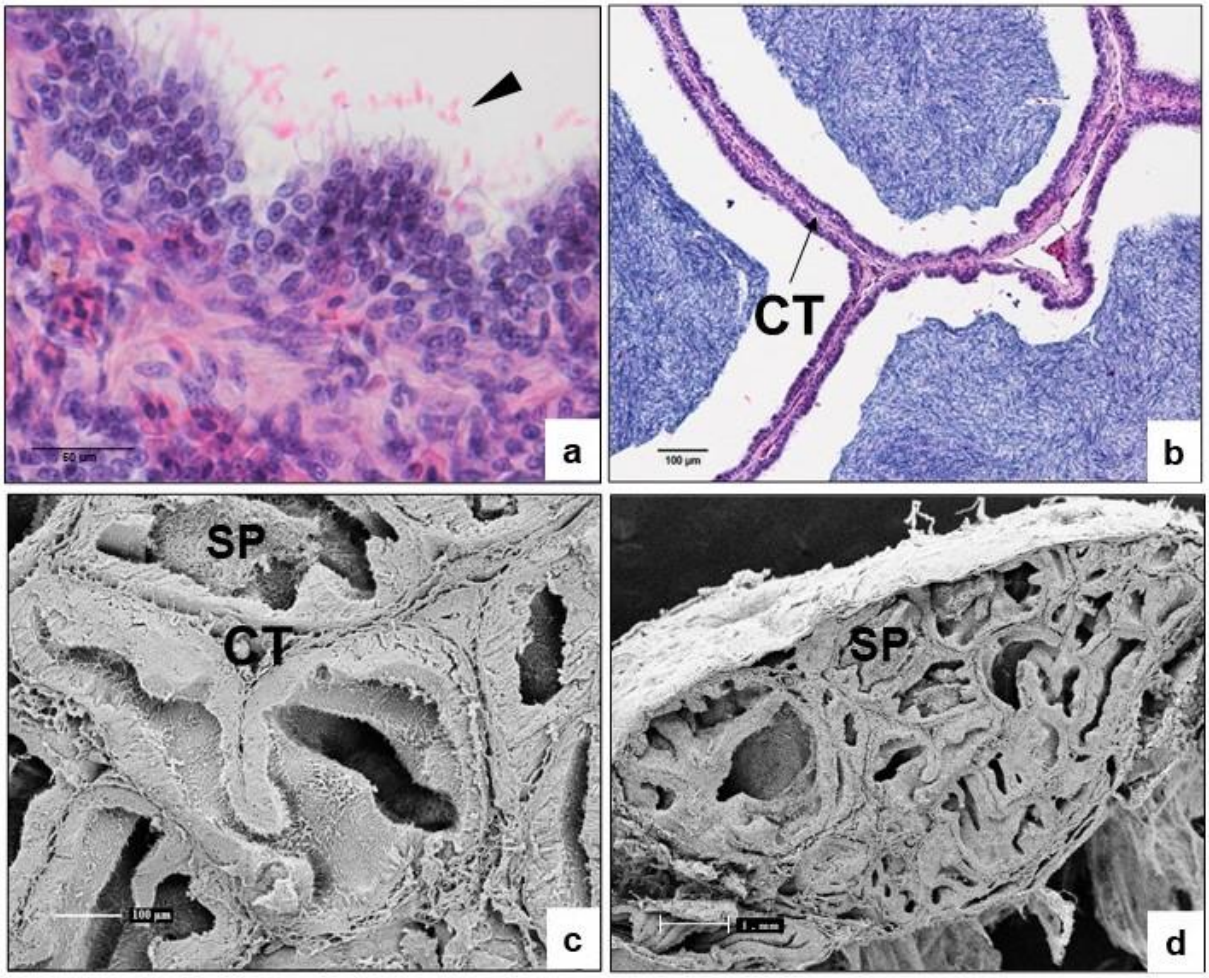

Figure 4. (a) Light micrograph of the epididymis showing the epithelium and the cilia (arrowhead) in detail. HE. (b) Light micrograph of the epididymis; notice the lumen full of spermatozoa and the tubules separated by connective tissue (CT). HE. (c) Scanning electron micrograph of a section of the epididymis with several tubules separated by connective tissue (CT). A lumen with spermatozoa (SP) can be seen. (d) Scanning electron micrograph of the epididymis showing the various tubules that compose the organs and some lumens full of spermatozoa (SP).

Clasper gland. The clasper gland was formed by gland units with a simple columnar epithelium, some nuclei were basal and others apical (Figure 7ab). The lumen had just a few secretions (Figure 7a). The glandular acini were arranged radially, and all discharged its content in the papilla, a duct in the longitudinal region with some secretion in it (Figure 7c). The glandular units and secretion stained a strong pink with PAS and was negative for AB (Figure 7cd). Externally, the gland was coated with striated muscle that was arranged in two ways: the inner layer in a circular arrangement and the outer layer in a longitudinal one (Figure 7a).

Discussion

The configuration of the male reproductive tract of *Potamotrygon amandae* was like other stingrays, such as *Himantura signifier* (Chatchavalvanich, Thongpan, & Nakai, 2005) and *Potamotrygon magdalenae* (Pedreros-Sierra & Ramírez- Pinilla, 2014). The epigonal organ is associated with the dorsal surface of the testis in *Potamotrygon* cf. *histrix* (Zaiden et al., 2011), and although also located on the dorsal face of the testis, did not cover the entire surface, only the caudal portion of the organ.

Hamlett, Reardon, Clark and Walker (2002) described the reproductive tract connected to the urinary tract with the seminal vesicle resting in the kidney and Pedreros-Sierra and Ramírez-Pinilla (2014) described the seminal vesicle on the ventral surface of the kidneys, which also occurs in *Potamotrygon amandae*.

In male *Potamotrygon amandae*, spermatogenesis occurred in zones in concentric rows. The same pattern was described in other rays (Stanley, 1966; Chatchavalvanich et al., 2005; Zaiden et al., 2011; Pedreros-Sierra & Ramírez-Pinilla, 2014). In *Himantura signifier* (Chatchavalvanich et al., 2005), the parenchyma of the testis was divided in lobes separated by connective tissue, as in *Potamotrygon amandae*.

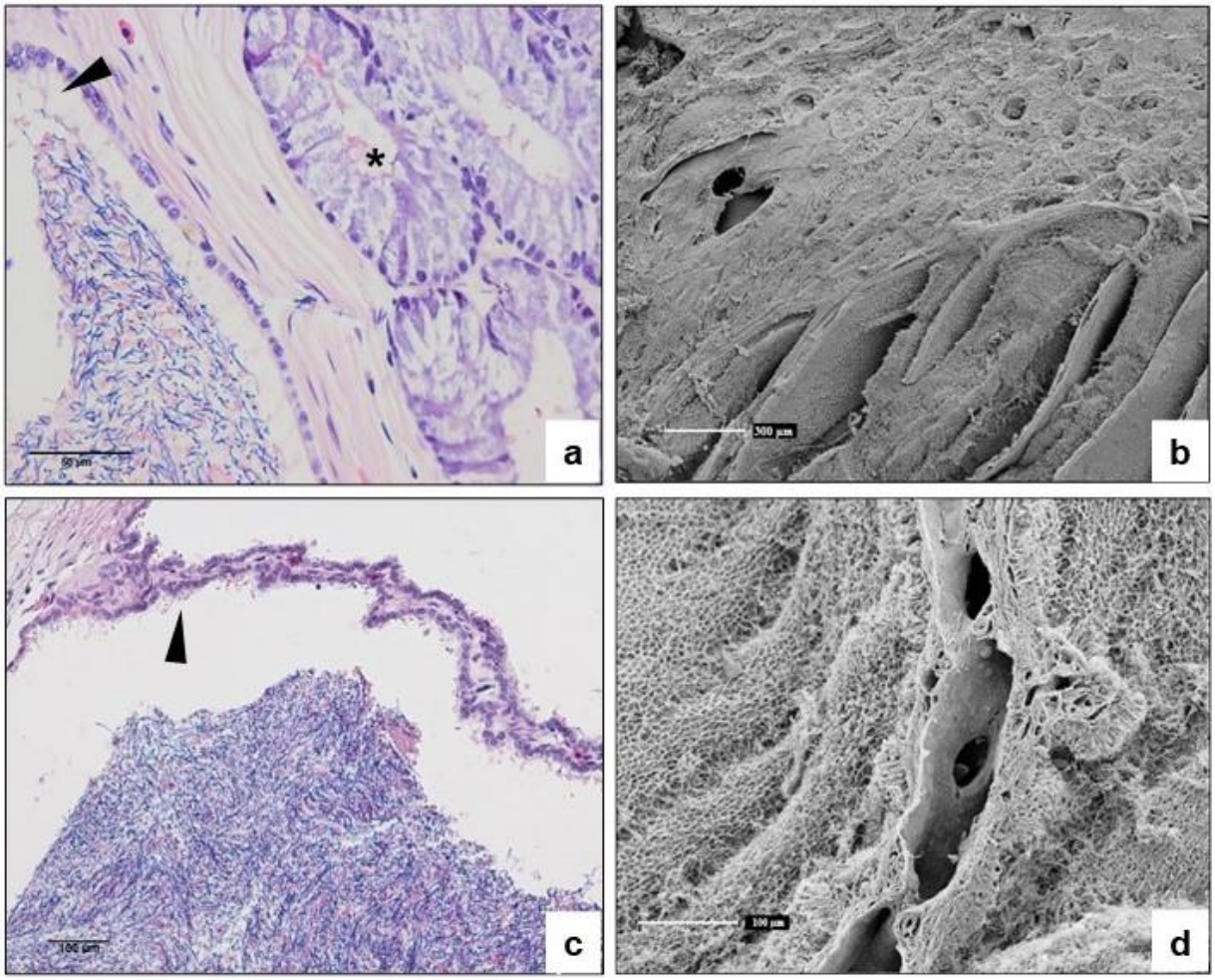

Figure 5. (a) Light micrograph of the deferent duct. The cilia (arrowhead) in the epithelium and the Leydig gland are present. The dense connective tissue connecting the deferent duct and Leydig gland can be seen. Some gland units have an eosinophilic secretion in the lumen (asterisk). HE. (b) Scanning electron micrograph of the deferent duct with spermatozoa in the lumen and connected to the Leydig gland. (c) Light micrograph of the seminal vesicle with a ciliated epithelium (arrowhead) and spermatozoa in the lumen. HE. (d) Scanning electron micrograph of the epithelium of the seminal vesicle with a hive aspect.

The scanning electron micrograph shows that the follicles are spherical with a membrane surrounding each one, which corroborates findings of Stanley (1966).

Some stingrays have a germinal papilla (Chatchavalvanich et al., 2005; Zaiden et al., 2011; Pedreros-Sierra & Ramírez-Pinilla, 2014), which was not observed in this study, although this does not mean that *Potamotrygon amandae* does not have one, since the germinal papilla can easily be missed, according to Chatchavalvanich et al. (2005).

The lining of the epididymis was a pseudostratified columnar ciliated epithelium, as in *Himantura signifier* (Chatchavalvanich et al., 2005) and *Potamotrygon* cf. *histrix* (Zaiden et al., 2011). Pedreros-Sierra and Ramírez-Pinilla (2014) described a change in the epithelium throughout the organ in *Potamotrygon magdalenae*, although it was the only stingray described with this pattern. The epididymis is responsible for transporting and producing a secretion that may nurture and mature the semen; thus, the ciliated epithelium and the folds help with the transport and nutrition (Chatchavalvanich et al, 2005).

The pseudostratified columnar ciliated epithelium presented in the male ducts corroborates the findings of Chatchavalvanich et al. (2005) and Spieler, Fahy, Sherman, Sulikowski, & Quinn (2013). Like *Himantura signifer* (Chatchavalvanich et al., 2005), the epithelium did not have a smooth muscle layer. Chatchavalvanich et al. (2005) suggested that due to this, the sperm is transported through the male duct via cilia activity.

The Leydig gland was similar to that described in other stingrays, with some gland units having secretory content in the lumen. Chatchavalvanich et al. (2005) suggested that the secretion of the gland plays a role in sperm aggregation.

Hamlett et al. (2002), Chatchavalvanich et al. (2005) and Pedreros-Sierra and Ramírez- Pinilla (2014) described folds of the epithelium into the lumen in the epididymis, vas deferens and seminal vesicle, much

like *Potamotrygon amandae*, which presented the same partitions. This seems to increase the area for nutrient exchange and physical support (Chatchavalvanich et al., 2005).

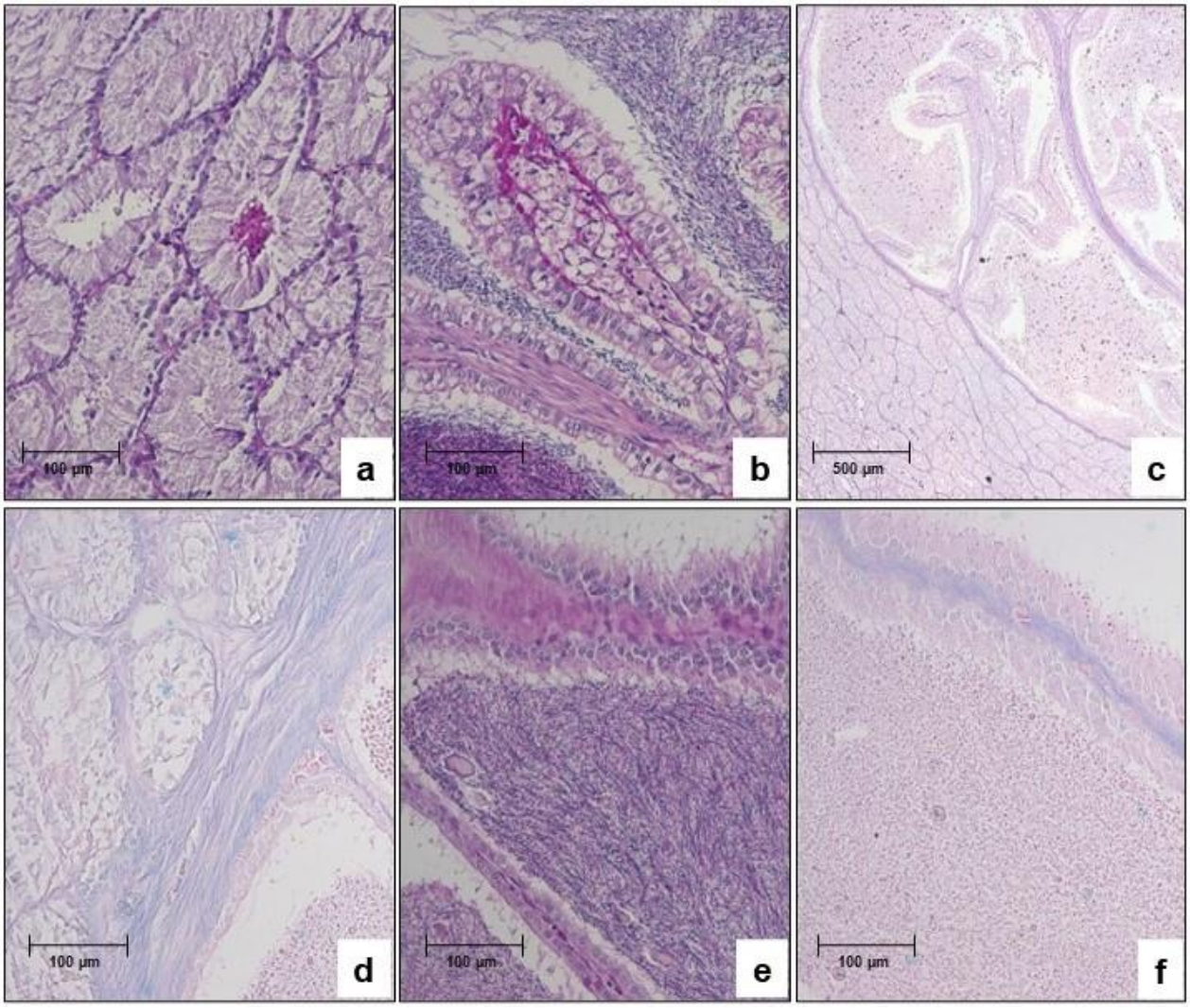

Figure 6. Light micrograph. (a) Gland units of the Leydig gland; a gland unit with secretion in the lumen can be observed and the affinity for PAS stain. PAS. (b) Vas deferens and the epithelial fold projecting into the lumen and the underlying connective tissue stained strongly with PAS. PAS. (c) Vas deferens with the epithelial folds and the Leydig gland associated. AB. (d) The underlying connective tissue that stained well with AB, the nuclei of the Leydig gland units, and vas deferens epithelium that stained weakly with AB can be observed. AB. (e) Section of the seminal vesicle. The connective tissue and the cilia stained strongly with PAS. PAS. (f) Section of the seminal vesicle. With AB the connective tissue was positively stained, and the nuclei of the epithelium was moderately stained. AB.

Although the folds presented in *Potamotrygon magdalenae* (Pedreros-Sierra and Ramírez-Pinilla, 2014) vas deferens were microscopically similar to the ones found in *Potamotrygon amandae*, the black ray had some folds that were detached from the epithelium and in the center of the lumen, suggesting that this is how the spermatozoa are nurtured through this organ. These types of epithelial folds were found only in the vas deferens and, according to Pedreros-Sierra and Ramírez-Pinilla (2014), they are only visualized in active mature males, suggesting that the subjects of this study were sexually mature and active.

Pedreros-Sierra and Ramírez-Pinilla (2014), Hamlett et al. (2002), and Pratt Jr. and Tanaka (1994) reported sperm aggregates in the male ducts, while *Potamotrygon amandae* did not present this sperm arrangement like *Himantura signifer* (Chatchavalvanich et al., 2005).

The spermatozoa in the seminal vesicle had a wall that was organized in a hive aspect in the scanning electron micrograph, which was not described in other stingrays. This may occur to increase the surface, facilitating physical support and nutrient exchanges. More studies are required.

The positive reaction of the cilia, present in the seminal vesicle and vas deferens, connective tissue and secretion of the Leydig gland to PAS staining indicate the presence of neutral glycoproteins in these regions. The AB-positive reaction of the connective tissue indicates the presence of acid glycoproteins, and the weak

reaction of the nuclei in the epithelium of the seminal vesicle, vas deferens and Leydig gland points out that there is some amount of acid glycoproteins. In *Sympterygia acuta* and *Sympterygia bonapartii* (Basallo, Varela Jr., & Oddone, 2018), the connective tissue was PAS+ and AB+ like *Potamotrygon amandae*; however, some secretions from the male reproductive tract were AB+ and/or PAS+, different from *Potamotrygon amandae*, in which the spermatozoa did not stain for AB or PAS.

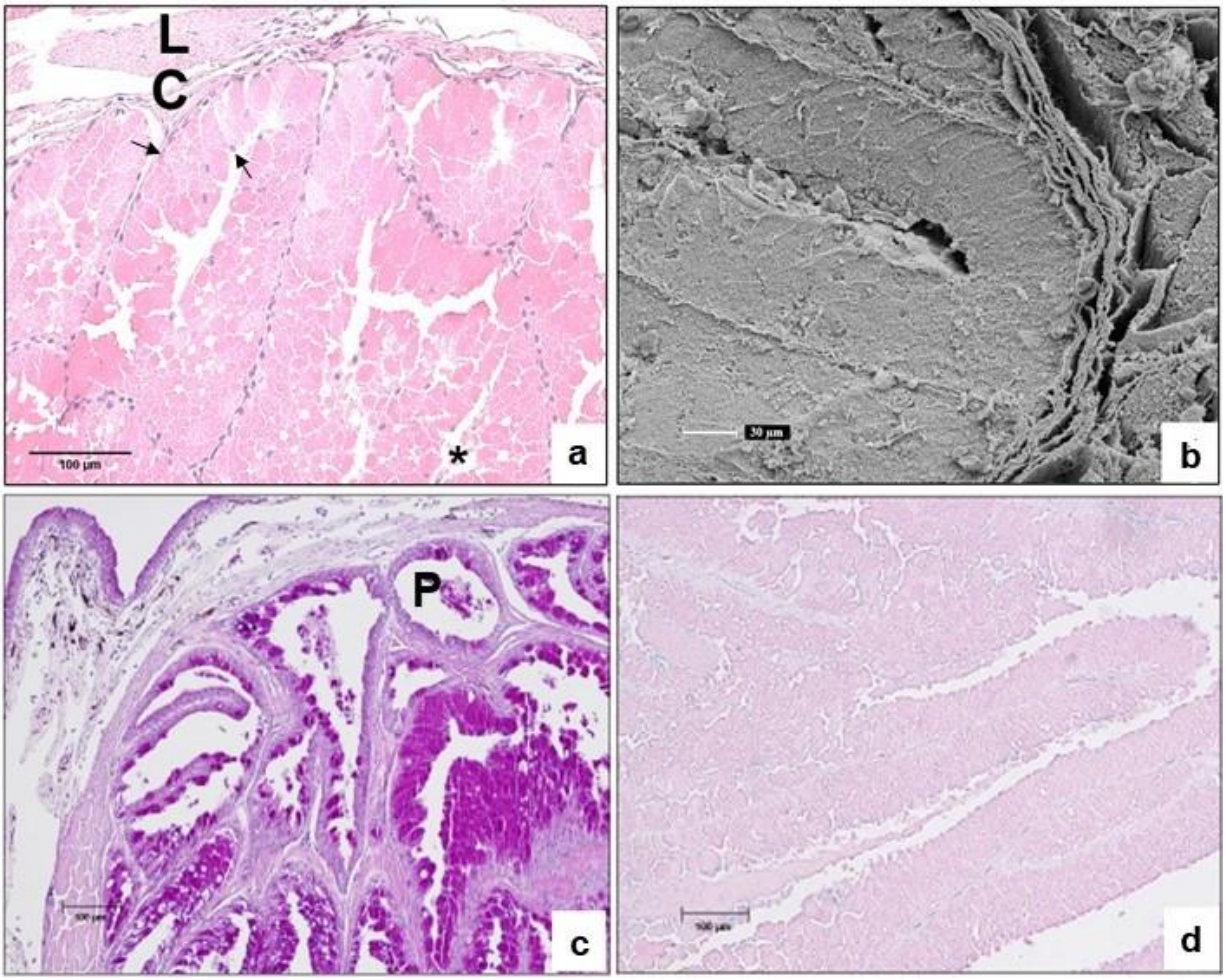

Figure 7. (a) Light micrograph of the clasper gland with its gland units. The nuclei are in the apical and basal portion (arrow); there is a gland unit with eosinophilic secretion in the lumen (asterisk); externally the gland is surrounded with striated muscle in a circular (C) and longitudinal (L) arrangement. HE (b) Scanning electron micrograph of a gland unit of the clasper gland. (c) Light micrograph of a section of the clasper gland positively stained with PAS. The papilla (P) that collects the secretion in the longitudinal region. PAS. (d) Light micrograph of the clasper gland that did not stain for AB. AB.

The clasper gland was formed by gland units, and the configuration of the epithelium was similar to *Potamotrygon magdalenae* (Anaya-López & Ramírez-Pinilla, 2017) and *Urolophus jamaicensis* (LaMarca, 1964). The gland is responsible for producing and secreting a viscous white fluid that is involved in internal fertilization (Anaya-López & Ramírez-Pinilla, 2017). The lumen of the individual tubules was expanded, and Piercy, Gelsleichter, & Snelson Jr. (2006) reported that this expansion only occurred during the mating season in *Dasyatis sabina*, when the lumen was full of secretary fluids, meaning that *Potamotrygon amandae* has a reproductive tract that functions during the whole year, or that the species was in its reproductive cycle.

In *Urolophus jamaicensis*'s (LaMarca, 1964), *Potamotrygon magdalenae* (Anaya-López & Ramírez-Pinilla, 2017) and *Dasyatis sabina* (Piercy et al., 2006), the clasper gland epithelium and secretion were strongly PAS-positive, validating the findings in *Potamotrygon amandae*, indicating the presence of neutral glycoproteins in the organ. The AB stain performed in *Urolophus jamaicensis* (LaMarca, 1964) indicated that the end cell secretion had a strong affinity for the stain and the major cell secretion had a weak affinity; the *Potamotrygon magdalenae* (Anaya-López & Ramírez-Pinilla, 2017) clasper gland in mature males was negative for AB pH 2.5, similar to *Potamotrygon amandae*. Anaya-López and Ramírez-Pinilla (2017) also performed AB pH 1.0, which was positive in *Potamotrygon magdalenae*. In this study, such histochemical analyses were not performed.

Conclusion

It can be concluded that although freshwater stingrays present morphological and physiological differences in some systems when compared to sea rays, there is little morphological difference in the male reproductive tract, being mostly physiological to adapt to the environment in which they are inserted (thermoregulation, osmolarity, reproductive seasonality, etc.). The components of the male reproductive tract play an important role in guaranteeing reproduction and, spermatogenesis and spermiogenesis, which can be observed in distinct stages in the testicles, and later storage, with several compartments for sperm storage. These adaptations allow the river stingray to reproduce.

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