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Cover Page Footnote

Collection of Wood Frogs was authorized by the Arkansas Game and Fish Commission under scientific collecting permit (Permit no. 022620199). I thank Mike Cartwright for his field assistance.

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Distal Urogenital Anatomy in the Male Wood Frog, *Lithobates sylvaticus* (Anura: Ranidae)

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Running Title: Distal Urogenital Anatomy in the Wood Frog

Abstract

I investigated the microanatomy of the distal urogenital system of the male wood frog (*Lithobates sylvaticus*) from a small sample (n = 6) collected in northern Arkansas in February 2020. Specifically, my primary objectives were as follows: 1) to focus on the histology of the paired Wolffian (urogenital) ducts caudally from the kidneys to their merging with the urodeum of the cloaca, 2) to reveal the structure of the paired seminal vesicles (sperm storage structures), and 3) to compare epithelial morphologies between the distal urogenital tract and its neighboring distal alimentary tract. This information will promote a clearer understanding of this anatomical region for a second North American ranid species and allow for comparisons to be made with other ranid frogs worldwide.

Introduction

Morphological details on the highly variable anatomy of the male anuran urogenital system have been reported by numerous authors (see reviews by Iwasawa and Michibata 1972; Hiragond and Saidapur 2000; Ogielska and Bartmańska 2009; Rheubert *et al.* 2017). In adult male anurans, the histology of the testes and kidneys has been studied much more extensively compared to the Wolffian ducts, which can transport both sperm and urine in most species. In some species, however, these urogenital end products have separate ducts (for more details on these species, see summary in Ogielska and Bartmańska 2009). At present, there remain limited detailed histological investigations on the Wolffian ducts (Bhaduri and Basu 1957; Iwasawa and Michibata 1972; Hiragond and Saidapur 2000). Also, this is especially true for the seminal vesicles (sperm storage structures), which are enlarged, glandular, sac-like outpocketings of the Wolffian ducts.

Iwasawa and Michibata (1972) provided the most comprehensive as well as in depth histological analysis on the seminal vesicles in 17 anuran species living in Japan. Of these, they examined 11 ranid species (one of these being the American bullfrog, *Lithobates catesbeianus*). They found marked seasonal differences in seminal vesicle microanatomy among most of these diverse species.

Hiragond and Saidapur (2000) reported that the Wolffian ducts were glandular along their entire length in the Indian tree frog (*Polypedates maculatus*), a common rhacophorid species of southern Asia. In a general statement, however, Ogielska and Bartmańska (2009) concluded that the posterior segments of the Wolffian ducts are more glandular than the anterior parts in most anurans. This comment was a direct reference to the glandular epithelial lining of the seminal vesicle. The glandular histology of the Wolffian ducts was not mentioned in the study by Iwasawa and Michibata (1972).

My goals in this study were to describe the histology of the Wolffian ducts (see Kardong 2015 for other terms applied to this duct) and the seminal vesicles in the wood frog, *Lithobates sylvaticus*. This ranid species has a limited breeding season (often referred to as being "explosive"), which occurs from mid-to-late winter into early spring throughout much of its range in North America (Redmer and Trauth 2005). Also, this frog is rarely found outside the reproductive season in Arkansas (Trauth *et al.* 2004).

My anatomical approach was to follow the distal portions of the Wolffian ducts caudally from the kidneys to their merging with the urodeum of the cloaca and to reveal the microanatomy of the seminal vesicles as potential sperm storage receptacles. In addition, a secondary aim was to highlight epithelial morphologies between the distal Wolffian ducts and the distal alimentary tract as these anatomical systems reside contiguous to one another in this frog.

Materials and Methods

I utilized urogenital structures from 6 male *L. sylvaticus* collected from northern Arkansas on 14 February 2020. The frogs were euthanized by submersion in a dilute chloretone solution in accordance with IACUC protocol regulations and guidelines at Arkansas State University. The urogenital tracts were removed, macrophotographed, and placed into either vials of 10% neutral buffered formalin, NBF (see below for procedures for paraffin sectioning—LM-Paraffin) or vials of 2% glutaraldehyde (GTA) solution buffered with 0.1 M sodium cacodylate at a pH of 7.2 (see below for procedures for plastic sectioning—LM-Plastic) for 2 h. For postfixation of GTA-fixed tissues, I used 1% osmium tetroxide, buffered as above, for 2 h.

Following necropsy, frogs were measured (snout-vent length to the nearest in mm), fixed in 10% NBF, and later preserved in 70% ethanol. Each frog was assigned an Arkansas State University Museum of Zoology (ASUMZ) number and documented as follows (ASUMZ no. and SVL): ASUMZ 34083, 57 mm; ASUMZ 34084, 60 mm; ASUMZ 34085, 57 mm; ASUMZ 34086, 56 mm; ASUMZ 34087, 56 mm; ASUMZ 34088, 56 mm). Frogs were deposited in the herpetological collection in the Arkansas Center for Biodiversity Collections at Arkansas State University.

The urogenital systems were prepared for LM-Paraffin and LM-Plastic in the Trauth Histo-herpetology Laboratory in Morrilton, Arkansas. These histological procedures have been recently described elsewhere (Trauth 2021). In brief, following tissue fixation in 10% NBF, 3 organ masses were placed into vials of 70% ethanol and prepared for LM-Paraffin in accordance with the paraffin embedding techniques outlined in Presnell and Schreibman (1997). The methods included dehydrating tissue in increasing ethanol solutions (70 to 100%), clearing in 100% xylene, infiltrating in paraffin overnight in a paraffin oven (56°C), embedding in paraffin using plastic molds (organs positioned to yield either transverse or frontal sections), sectioning with a rotary microtome into 8 µm serial strips (affixed onto glass microscope slides coated with Haupt's adhesive prior to floating strips in 2% NBF on a slide warmer), and staining with Pollak trichrome stain for the enhancement of epithelia, connective tissues, and muscle. Cover slips were then adhered to the microscope slides with Permount© (Fisher Scientific Products).

For LM-Plastic used for epoxy-embedded organs, I transversely cut 3 urogenital tracts into equal halves, dehydrated tissue portions in a graded series of increasing ethanol solutions (50-100%), placed tissues into a 50/50% acetone/plastic mixture for overnight infiltration, and then embedded tissues in Mollenhauer's Epon-Araldite #2 (Dawes 1988). For semi-thin sectioning (approximately 1 µm in thickness) and staining, I used glass knives on an LKB Ultrotome (Type 8800) and Ladd® multiple stain (LMS), respectively.

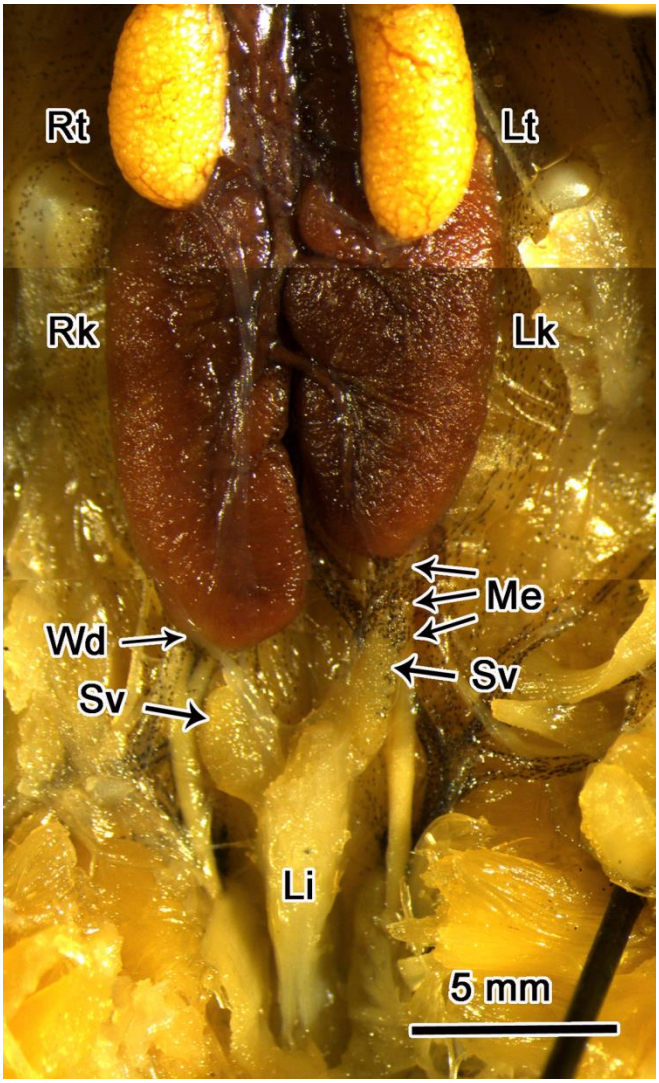

Figure 1. Macroscopic anatomy of the urogenital system (ventral view) in a male *Lithobates sylvaticus* (ASUMZ 34086). For viewing purposes, the urinary bladder and most of the alimentary tract have been removed with only the distal segment of the large intestine (Li) remaining intact. Lt, left testis; Lk, left kidney; Me, melanophore; Rt, right testis; Rk, right kidney; Sv, seminal vesicle; Wd, Wolffian duct.

For photomicroscopy, I utilized a Leica MC 120 HD camera atop a Leica DM 2000 LED compound light microscope. For the macrophotograph shown in

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Figure 1, I used a Leica M80 stereomicroscope attached to the above camera. Most descriptions of urogenital tract anatomy follow the terminology in Ogielska and Bartmańska (2009). Microscope slides are currently catalogued and housed in the Trauth Histo-herpetology Laboratory.

Results

Gross Morphology

The macroanatomy of the urogenital system of *L. sylvaticus* is typical of most male anurans (Figs. 1; 2A). The system includes paired testes, kidneys, seminal vesicles, Wolffian ducts, and a single, bilobed urinary bladder, the latter being the case in the *L. sylvaticus* (excised in Fig. 1 to allow for macroscopic viewing of the remaining system). For orientation purposes, the distal urogenital anatomy is defined here as beginning caudally from the kidneys with the anterior segments of the Wolffian ducts passing alongside the caudolateral surface of each kidney. Each duct then merges with its ipsilateral seminal vesicle immediately posterior to each kidney (best viewed in Fig. 2B). Melanophores are evident, being scattered about within the stromal connective tissue of each seminal vesicle (Figs. 1; 2A). Not shown are the Wolffian ducts posterior to the seminal vesicles as they are hidden dorsally by the distal portion of the alimentary tract.

Light Microscopy

The anteriormost region of the right Wolffian duct of ASUMZ 34087 enlarges prior to its merging with its right seminal vesicle (Fig. 2B). Here, the duct exhibits an epithelial transition from simple cuboidal (mesial surface of duct in Fig. 2C) to one containing an irregular lining of low-to-stratified columnar/cuboidal cells lying along its lateral surface (Figs. 2C; 3) while merging with the seminal vesicle. The stromal connective tissue of the Wolffian duct in this region is relatively thick and is surrounded by a loosely attached serosa (Fig. 2C).

The irregular nature of the columnar/cuboidal epithelia of the Wolffian duct (as a companion with the seminal vesicle) is also characteristic of the epithelial lining of the numerous pockets or outpocketings within the seminal vesicle (Figs. 2B – D; 3). This common epithelial feature between the two tissues is not surprising, given that the seminal vesicle develops as a diverticulum off of the Wolffian duct. An uneven, sawtooth-like morphology dominates much of the epithelial lining of these outpocketings (Fig. 2D), and a glandular component of this epithelium is evident, being typified by a thin, homogenous, apical secretory coat.

The Wolffian duct in the region lateral to the large intestine reveals its position residing dorsomedially on the surface of the seminal vesicle (Figs. 3; 4A, B). Here, the duct exhibits an additional example of the stratified columnar/cuboidal epithelium characteristic of both the duct and the seminal vesicle.

As the Wolffian ducts continue posteriorly, broad channels or canals are evident within the interior regions of the seminal vesicles (Fig. 3B). Sperm were observed within these passageways.

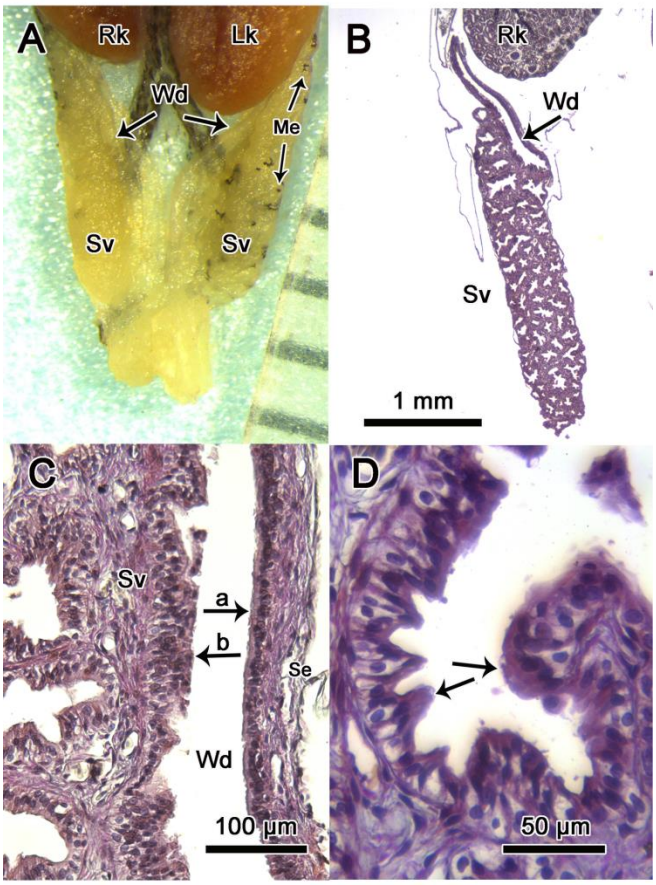

Figure 2. Macroscopic image of urogenital structures (A) and light micrographs (B – D) of the right seminal vesicle and right Wolffian duct in a male *Lithobates sylvaticus* (ASUMZ 34087; LM-Paraffin). B. Frontal section of the anterior segment of the right Wd lying adjacent to the seminal vesicle as seen in A. C. Segment of Wd of B revealing a duel epithelial mucosa: a mesial, low columnar/cuboidal epithelium (arrow a) in contrast to a lateral intricate complex of stratified columnar/cuboidal epithelial cells associated with the adjoining Sv (arrow b); Se, serosa. D. Secretory mucosal epithelium of an outpocketing within the Sv revealing sawtooth-like projections comprised of basal round cells and low columnar cells. Arrows point to thin apical layer of secretory material. Abbreviations are the same as in Figure 1. Scale bar in A in mm.

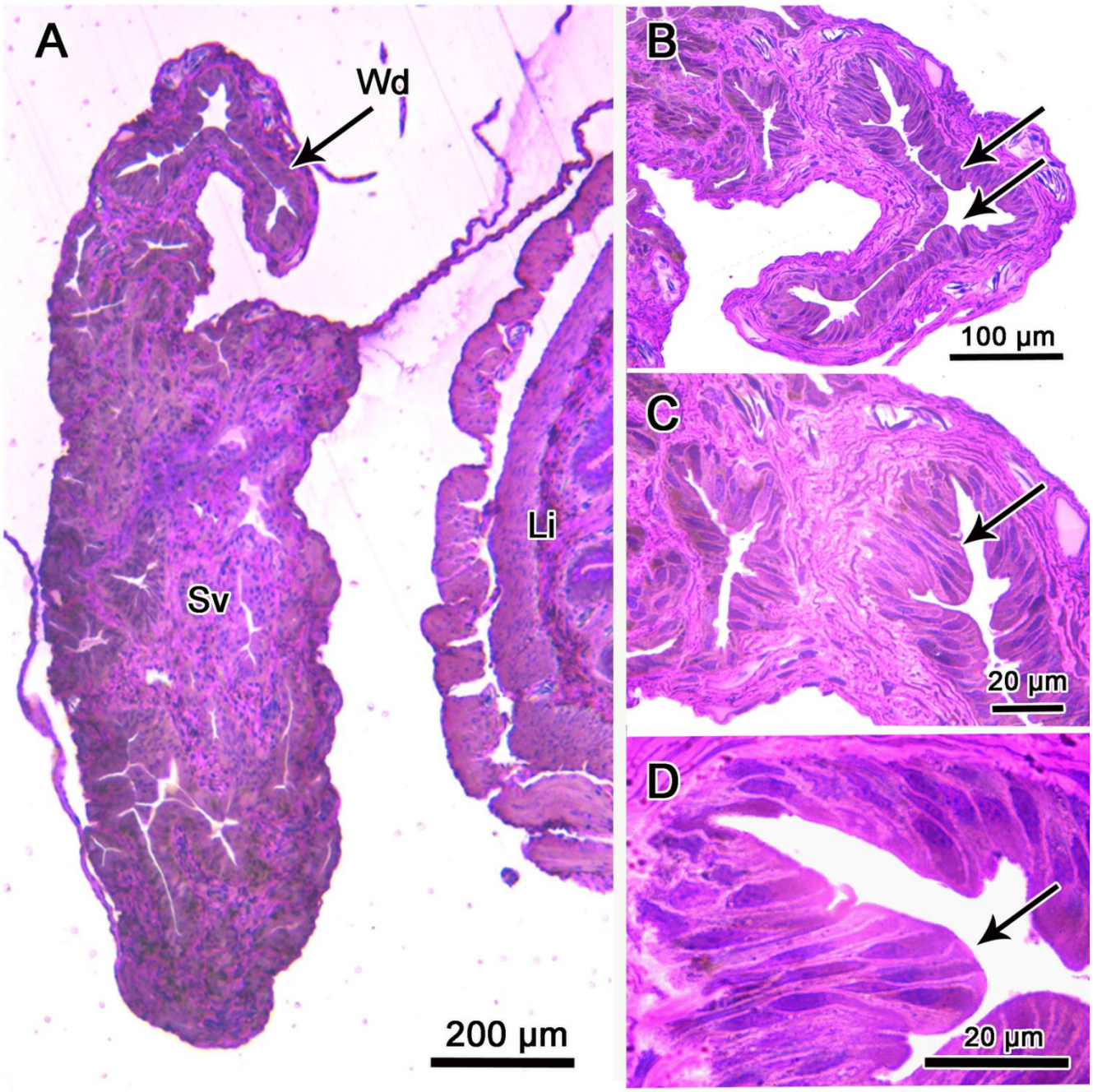

Figure 3. Light micrographs (LM-Plastic) of transverse sections of the Wolffian duct and seminal vesicle in a male *Lithobates sylvaticus* (ASUMZ 34084). A. Wolffian duct lying dorsally atop the seminal vesicle, which is positioned lateral to the large intestine of the alimentary tract. B and C. Arrows point to the stratified columnar epithelium of the Wolffian duct. D. Magnification of the stratified columnar epithelium (arrow) shown in C. Abbreviations are the same as in previous figures.

Posterior to the seminal vesicles, the Wolffian ducts begin a regressive move, becoming narrower in circumference as they shift their positions more medially to reside dorsal and embedded within the stromal connective tissue of the large intestine (Fig. 4C – H). Eventually, the ducts descend ventrally, each occupying an anatomical location directly above paired urogenital papillae of the urodeum (Fig. 4E – H).

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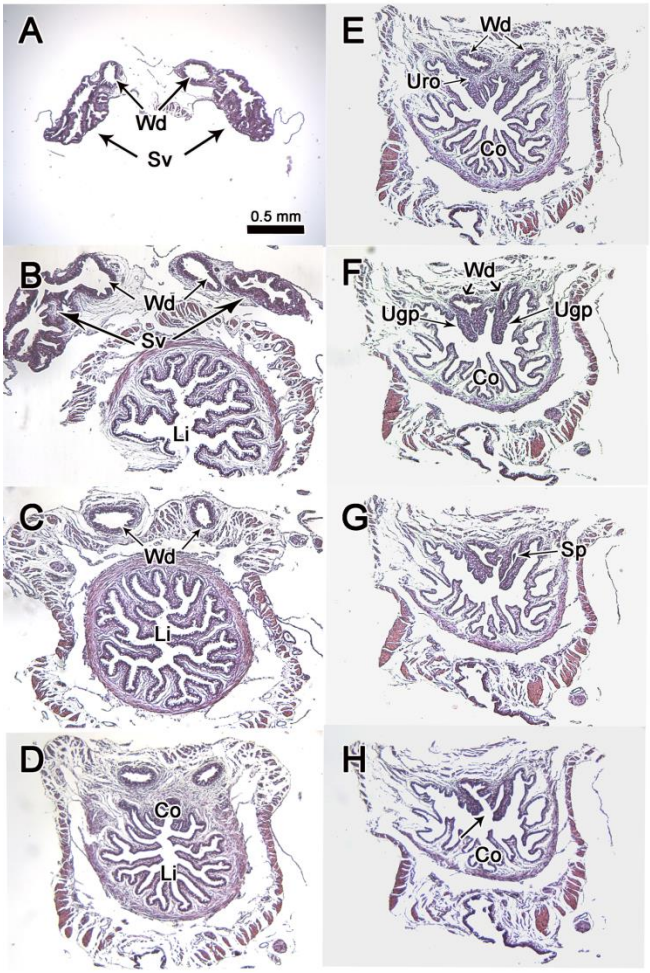

Figure 4. Light micrographs (LM-Paraffin) of a series of cranial-to-caudal transverse sections of the distal urogenital system (DUGS) of a male *Lithobates sylvaticus* (ASUMZ 34086) revealing relationship between the DUGS and the distal portion of the alimentary tract (DAT). A. Wolffian ducts and seminal vesicles immediately posterior to the kidneys. B, C. Structures in A seen lying dorsal to the large intestine. D. Intestinal transition of DAT into the coprodeum (Co) of the cloaca (see epithelia in Fig. 5B, C). E. Positioning of the Wolffian ducts dorsal to the urodeum (Uro). F. Wolffian ducts move ventrally into the urodeum to become established within urogenital papillae (Ugp). G. Sperm (Sp) are present in the Ugp of the left Wd. H. The two Ugp unite to form a common urogenital orifice (arrow). Abbreviations are the same as in previous figures. Scale bar in A the same for B – H.

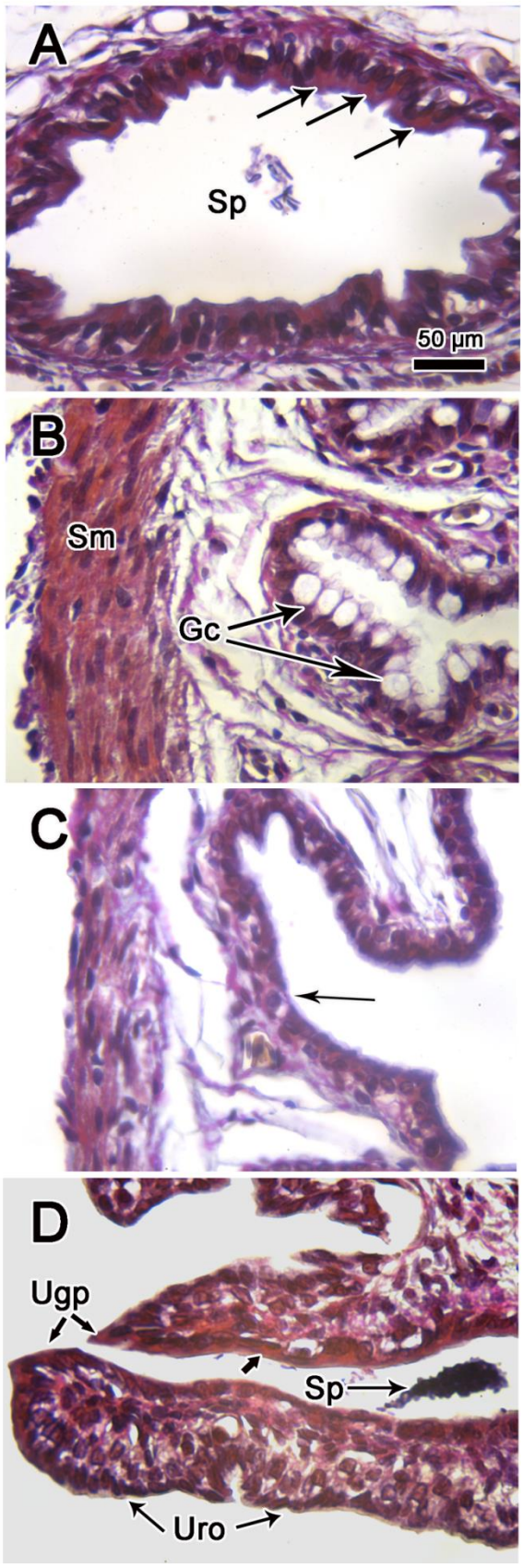

Figure 5. Light micrographs (LM-Paraffin) of the mucosal epithelia of the DUGS and DAT obtained from Figure 4. A. Arrows point to the highly secretory columnar epithelium of the Wd (caudad to seminal vesicle); Sp reside in the lumen. B. The presence of numerous goblet cells (Gc) within the simple columnar epithelium of the Li. Sm, smooth muscle. C. The lack of Gc and a low columnar epithelium (arrow) reveal a transition to the coprodeum. D. Transitional epithelial lining (arrow) of low stratified columnar/cuboidal cells present in urodeum. Sp are present in the neck of the Ugp (as seen in Fig. 4G). Abbreviations are the same as in previous figures. Scale bar in A the same for B – D.

An examination of the distal segment of the alimentary tract reveals a distinctive change in its epithelium from the one characteristically found in the large intestine into one found in the coprodeum, the anteriormost chamber of the cloaca (Figs. 4; 5). Typically, the large intestine exhibits a simple columnar epithelium containing numerous mucus-secreting goblet cells (Fig. 5B) and absorptive cells. During its transition into the coprodeal chamber, the goblet cells become fewer in number, and the inner wall of the large intestine is superseded, being replaced by a more dorsal coprodeum, whose epithelial lining exhibits low columnar cells and contains no goblet cells (Fig. 5C). Eventually, the epithelial lining of the coprodeum dominates the entire ventral wall (Fig. 4E – H) as the urodeum gradually appears dorsally (Fig. 4E). The urodeum possesses a distinctive transitional lining of low stratified cuboidal cells (Fig. 5D). The two urogenital papillae (Fig. 4F – H), the most prominent features of the urodeum, appear to share a common urogenital orifice (Fig. 4H). In addition, the stromal connective tissue of the urodeum was noticeably more densely packed compared to the coprodeum, giving the stroma of this cloacal chamber a greater affinity (staining intensity) for Pollak stain.

Discussion

The most prominent anatomical structures of the distal urogenital system in *L. sylvaticus* are the paired seminal vesicles. These sperm storage structures were similar to those found in *L. catesbeianus*, which were macro-photographed as well as histologically examined by Iwasawa and Michibata (1972). These authors also noted the presence of "black pigments" (i.e., melanophores) scattered about within the outer surfaces of the seminal vesicles. This pigmentation was also present in the seminal vesicles of *L. sylvaticus*.

Sperm were observed within the seminal vesicles (Fig. 4B), the posterior region of the Wolffian duct (Figs. 4D, G; 5A), and within urogenital papillae (Figs. 4G; 5D) in *L. sylvaticus*. These findings regarding the ubiquitous presence of sperm within the distal urogenital structures in this species were an anticipated outcome of this study. However, it is doubtful if long-term sperm storage within the distal urogenital system (specifically the seminal vesicles) could serve a functional role for this ranid frog, which has a limited breeding season. The presence of a secretory material, as observed on the surfaces of epithelial linings of the seminal vesicles (Fig. 2D) and the Wolffian ducts (Fig. 5A), supports the premise that sperm could receive some nutritional benefit from these secretions.

In conclusion, given that *L. sylvaticus* occurs well into northern climes in North America, a necessity for sperm storage during extreme weather conditions could prove advantageous for this species in some regions within its range. Also, the ability to store sperm would be especially significant in species that have an extended or a biannual breeding season, such as in the southern leopard frog, *Lithobates sphenocephalus* (McCallum *et al*. 2004). Additional investigations on the seminal vesicles in other North American ranid frogs should prove helpful in understanding whether long-term sperm storage exists in other species.

Acknowledgments

Collection of wood frogs was authorized by the Arkansas Game and Fish Commission under a scientific collecting permit (permit no. 022620199). I thank M. Cartwright for his field assistance.

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