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# Histomorphology of the Distal Urogenital Ducts in the Male Northern Crawfish Frog, *Rana areolata circulosa* (Anura: Ranidae)

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Running Title: Distal Urogenital Ducts in the Northern Crawfish Frog

## Abstract

We examined the microanatomy of the distal urogenital ducts in the male Northern Crawfish Frog (*Rana areolata circulosa*) from a small sample ( $n = 5$ ) collected in February and March from central Arkansas over a 5-year period (2017–2022). Specifically, we sought information on the following structures: (1) the paired Wolffian (urogenital) ducts caudally from the kidneys to their merging with the urodeum of the cloaca, (2) the paired seminal vesicles (sperm storage structures), and (3) the paired Müllerian ducts and glands. This study provides new information on these urogenital ducts and promotes a clearer understanding of this anatomical region for this North American ranid frog.

## Introduction

Microanatomy of the highly variable male anuran urogenital system has been reported by numerous authors (see reviews by Iwasawa and Michibata 1972; Hiragond and Saidapur 2000; Ogielska and Bartmańska 2009; Rheubert *et al.* 2017). Among adult male anurans, the histomorphology of the testes and kidneys has been studied much more extensively compared to the urogenital (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; Basu 1960; 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.

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). Trauth (2022) examined the seminal vesicles in the Wood Frog (*Rana sylvatica*) and included an extensive histological description of the Wolffian ducts in this ranid species. He found that both the seminal vesicles and the Wolffian ducts possessed a glandular epithelium.

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 (*Rana catesbeiana*)). They found marked seasonal differences in seminal vesicle microanatomy among most of these diverse species.

Müllerian ducts (progenitors to oviducts in female vertebrates) are mostly lost during ontogenetic development in nearly all male vertebrate animals (Tyler 2003). In anurans, however, these ducts may persist to some degree in a very small number of males (Duellman and Trueb 1986). For example, some ranid frogs of North American may retain them in some populations but not in others of the same species (Moore 1944; Pace 1974).

As an obligate crayfish burrow dweller, the Northern Crawfish Frog has historically occupied a relatively narrow ecological niche throughout its distribution in the tall grass prairies and grasslands of the central and south-central United States (Redmer 2000; Powell *et al.* 2016; Lannoo and Stiles 2020; Kross and Willson 2022). Habitat loss and shifting climate patterns pose as major threats to the continued

existence of this species (Lannoo and Stiles 2017; Kross and Willson 2022). In Arkansas, *R. a. circulosa* occurs in only 19 of its 75 counties (Trauth *et al.* 2004; Trauth and Holt 2017). Because of their secretive nature, late winter-early spring breeding season, and current protected status by the Arkansas Game and Fish Commission, this species remains a rarity in most museum collections in the state (Trauth *et al.* 2004). Moreover, only anecdotal information exists regarding any aspect of their reproductive biology or even its natural history in Arkansas (Trauth *et al.* 1990).

Our goals in this study were to describe the histomorphology of the Wolffian ducts (see Kardong 2015 for other terms applied to this duct), seminal vesicles, and Müllerian ducts in male *R. a. circulosa*. This rare species has a restricted breeding season from late winter to early spring (Parris and Redmer 2005). Northern Crawfish Frogs are rarely encountered outside the reproductive season in Arkansas (Trauth *et al.* 2004).

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

## Materials and Methods

We utilized urogenital structures from 5 male *R. a. circulosa* collected from central Arkansas over a 5-year period (2017-2022). The frogs were euthanized by immersion in a dilute chloroform solution in accordance with IACUC protocol regulations and guidelines at Arkansas State University. The urogenital tracts were removed, macro-photographed, 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, we used 1% osmium tetroxide, buffered as above, for 2 h.

Following necropsy and removal of ducts, frogs were measured (snout-vent length to the nearest in mm[SVL]), 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., SVL, and date of collection): ASUMZ 33611, 85 mm, 28 February 2017; ASUMZ 33746, 75 mm, 20 February 2018; ASUMZ 33749, 88 mm, 20 February 2018; ASUMZ 33748, 88 mm, 27 March 2018; ASUMZ 34143, 120 mm, and 6 March 2022. 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 Histoherpétology Laboratory in Morrilton, Arkansas. These histological procedures have been recently described elsewhere (Trauth 2022). In brief, following tissue fixation in 10% NBF, 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 Schreiber (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 (PTS) 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 tissues, urogenital tracts were cut transversely into equal halves or cut frontally in their entirety, dehydrated in a graded series of increasing ethanol solutions (50-100%), placed into a 50/50% acetone/plastic mixture for overnight infiltration, and then embedded in Mollenhauer's Epon-Araldite #2 (Dawes 1988). For semi-thin sectioning (ca. 1 µm in thickness), glass knives were used on an LKB Ultratome (Type 8800); Ladd® multiple stain (LMS) was used to stain tissues.

For photomicroscopy, we utilized a Leica MC 120 HD camera atop a Leica DM 2000 LED compound light microscope. For the macro-photograph shown in Figure 1, a Leica M80 stereomicroscope attached to the above camera was used. Most descriptions of urogenital tract anatomy followed the terminology in Ogińska and Bartmańska (2009), Pewhorn *et al.* (2016), and Trauth (2022). Microscope slides are currently catalogued and housed in the Trauth Histoherpétology Laboratory.

## Distal Urogenital Ducts in the Northern Crawfish Frog

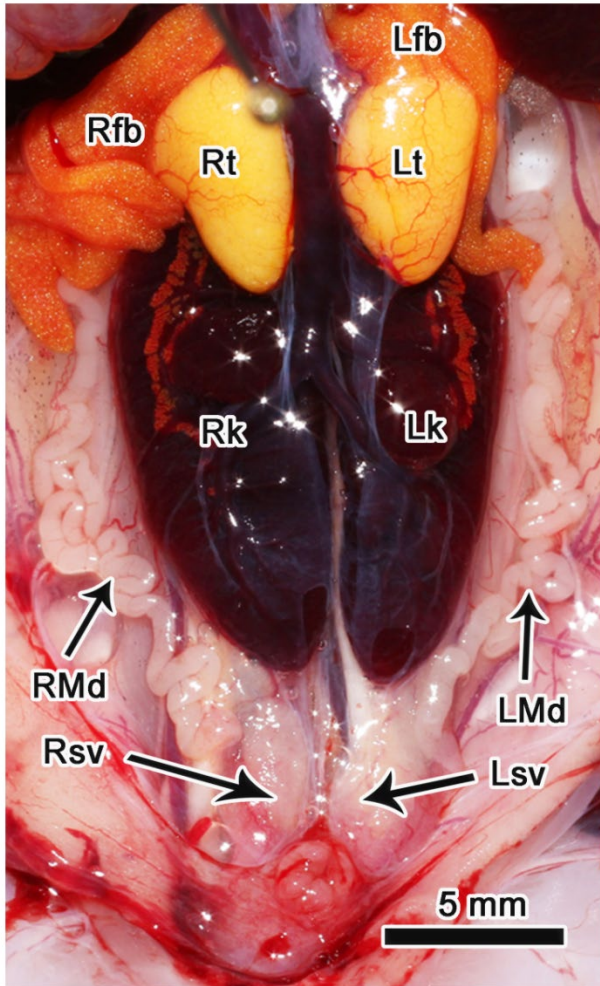


Figure 1. Macroscopic anatomy of the urogenital system (ventral view) in a male *Rana areolata circulosa* (ASUMZ 34143). For viewing purposes, the urinary bladder and the alimentary tract have been removed. Lfb, left fat body; Rfb, right fat body; Lt, left testis; Lk, left kidney; Rt, right testis; Rk, right kidney; Rsv, right seminal vesicle; Lsv, left seminal vesicle; RMd, right Müllerian duct; LMd, left Müllerian duct.

### Results

#### Gross Morphology

The macroanatomy of the urogenital system of *R. a. circulosa* is typical of most male anurans (Fig. 1), except for the presence of enlarged Müllerian ducts. The system consists of paired testes, kidneys, seminal vesicles, Wolffian ducts, and a single, bilobed urinary bladder (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 Wolffian duct then merges with its ipsilateral seminal vesicle immediately posterior to each kidney. (Not shown are the Wolffian ducts themselves as they are hidden from view in their dorsal position by the seminal vesicles.) Well-developed opaque Müllerian ducts reside lateral to the kidneys. The Wolffian ducts and the Müllerian ducts terminate upon joining the urodeum of the cloaca.

#### Light Microscopy

Histologically, the distal urogenital ducts and the seminal vesicles are revealed in a series of cranial-to-caudal transverse sections beginning near the caudal region of the kidneys (Fig. 2A). Here, both Müllerian ducts appear as mostly oblong structures that exhibit slit-like, longitudinal lumina. A glandular epithelial mucosa is also present, which is dominated by numerous tubular (Müllerian) glands (for gland morphology, see Figs. 4 and 5). The right Müllerian duct reveals itself as three noticeable sections due to looping of the duct (as shown in Fig. 1). The Müllerian ducts on both sides lie lateral to and separated from the small, sac-like Wolffian ducts and diminutive segments of the seminal vesicles by medial mesentery attachments (e.g., see mesentery with respect to the left ducts in Fig. 2A).

Approximately 500  $\mu\text{m}$  posterior to the kidney (post-kidney), the left seminal vesicle (only left side in view) has now become greatly expanded (Fig. 2B), flattened, and contains numerous blind diverticula or pouches resulting in a larger structure in comparison to the left Müllerian duct. In addition, the tubular nature of the Müllerian glands in this region of the left Müllerian duct is revealed (Fig. 2C). The left Wolffian duct has also increased in size and has broadened into an oblong sac-like channel atop the seminal vesicle (Fig. 2B).

At a distance of ca. 1.5 mm post-kidney (Fig. 2C) a transition in the glandular epithelium of the Müllerian glands is apparent, sharply changing from mucosal-like tubules (Fig. 2C, D) to a total absence of tubules altogether (see arrows in Figs. 2D, 5E). At a point ca. 2.5 mm post-kidney (Fig. 2E), the left Müllerian duct exhibits an expansive and irregularly folded mucosal epithelium. Also, this duct has now lost its medial mesentery and has moved into close proximity with its ipsilateral seminal vesicle. Both ducts and the seminal vesicle are now surrounded by a common layer of loose connective tissue.



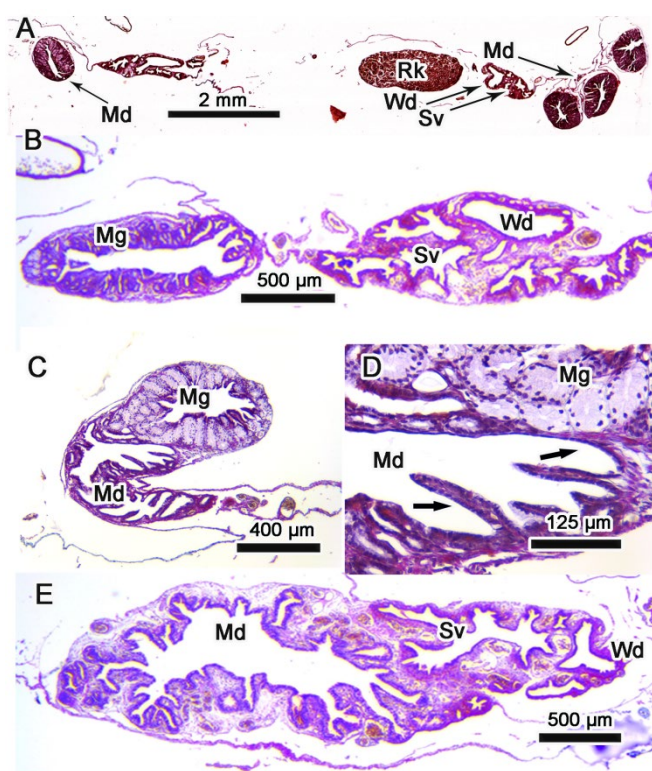


Figure 2. Light micrographs (LM-Paraffin) of transverse sections of urogenital structures in a male *Rana areolata circulosa* (ASUMZ 34143). A. Section through the caudal region of the kidneys showing the Müllerian ducts (Md), the Wolffian ducts (Wd), and the seminal vesicles (Sv). B. Left side only view of above structures reveals tubular Müllerian glands (Mg) and an oblong Wolffian duct atop an enlarging seminal vesicle. C-D. Transition of Müllerian duct anatomy from a poorly staining mucosal-like glandular epithelium to a highly folded epithelium devoid of glands (see arrows; see Fig. 5). E. Merging of Müllerian duct with its ipsilateral seminal vesicle. Wolffian duct lies medial to seminal vesicle. Rk, right kidney. See text for further explanation of figure.

At a point ca. 3.5 mm post-kidney (Fig. 3—a continuation of Fig. 2), both Müllerian ducts and both Wolffian ducts are now in view. The more cranial seminal vesicles are no longer visible. The Wolffian ducts now reside in a dorsomedial position relative to the greatly expanded and non-glandular Müllerian ducts (Fig. 3A). Both sets of ducts lie dorsal to the emerging large intestine. Layers of smooth muscle and loose connective tissue now separate these two systems. As the dorsal wall of the large intestine becomes more visible (Fig. 3B), the Müllerian ducts begin to move medially, displacing the loose connective tissue lying between them. In doing so, these ducts gradually become more cylindrical (Fig. 3C) in shape. Both Wolffian ducts remain in their dorsomedial position and retain their oblong configuration.

At 6.5 mm post-kidney (Fig. 3D), the Müllerian ducts have descended ventrally within two prominent, parallel urodeal ridges and now occupy the dorsal wall of the urodaeum. The large intestine has transitioned into the coprodeum, the anteriormost chamber of the cloaca. The coprodeal chamber has now shifted into a ventral position beneath the newly emerging and expanding urodeum. Before dumping their contents into the urodeal chamber, Müllerian ducts become greatly reduced in size, have lost their highly folded epithelial surfaces, and appear as thin-walled cylindrical channels (Fig. 3E) at the distal ends of the urogenital papillae. Likewise, the Wolffian ducts have also narrowed their diameters and have followed the Müllerian ducts into urogenital papillae.

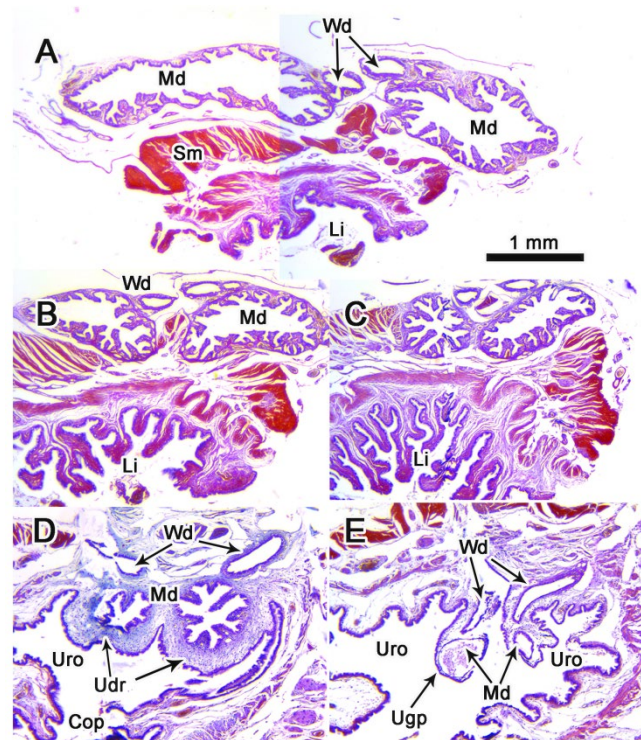


Figure 3. Continuation of Fig. 2. A. Müllerian ducts lie adjacent to their greatly reduced Wolffian ducts. B and C. Müllerian ducts and Wolffian ducts move medially to lie dorsal to the large intestine (Li). D. Müllerian ducts move ventral to the Wolffian ducts; urodeal ridges (Udr) occupy dorsal wall of urodeum (Uro). E. Each Müllerian duct moves into the distal end of a urogenital papilla (Ugp) and is followed by each Wolffian duct. Cop, coprodeum. Abbreviations are the same as in previous figures. See text for further explanation of figure.

The cellular complexity of the Müllerian glands is best exemplified in the Müllerian ducts in the region of the lower kidney (Fig. 4A). Müllerian glands are basically simple straight tubular exocrine glands (Fig. 4D). Their serous-type cells (Fig. 4E) release their



## Distal Urogenital Ducts in the Northern Crawfish Frog

secretions into a longitudinal central lumen. Numerous lumina then dump their contents into a common central channel, which in this case, is the lumen of the Müllerian duct. Pronounced secretory activity is

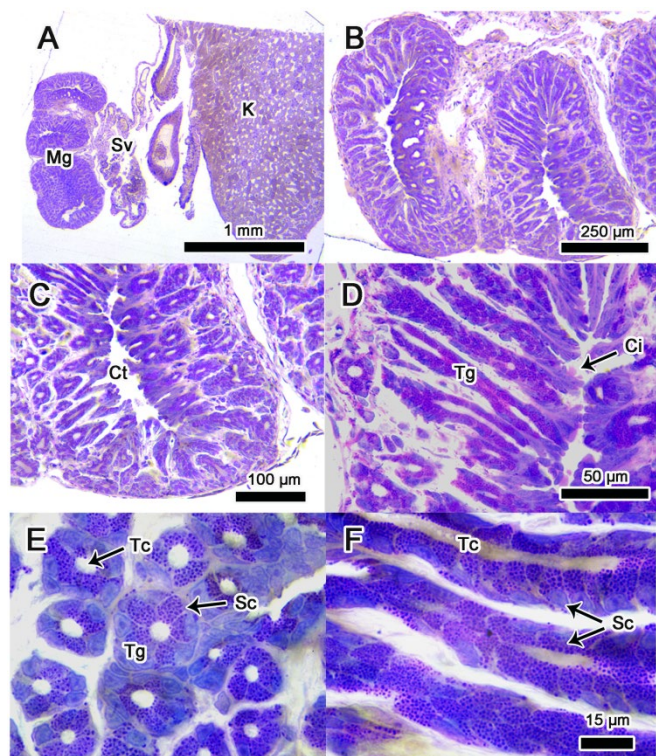


Figure 4. Light micrographs (LM-Plastic) of transverse sections of Müllerian glands of a Müllerian duct in a male *Rana areolata circulosa* (ASUMZ 33746) in the region of the kidneys. A-F. Increasingly higher magnifications reveal preponderance of secretory cells within the simple tubular (Müllerian) glands. Ci, cilia; Ct, central tube; Sc, secretory cell containing zymogen granules; Tc, tubular canal; Tg, tubular gland. Scale bar in F the same for E.

clearly visible within the cells of the glandular epithelium (Fig. 4E, F) in this region of the Müllerian duct as conspicuous zymogen granules occupy the supranuclear cytoplasm of these cuboidal cells. In addition, ciliated cells can be seen scattered about along the luminal margin (Fig. 4F).

Secretory activity appears to wane within the Müllerian ducts as one proceeds in a posterior direction possibly due to a change in glandular morphology (e.g., note anatomical differences between Fig. 2B, E; see also Fig. 7). A dramatic transition in the cytoplasmic staining properties of the tubular glands occurs (Fig. 5). The staining properties of these cells indicate a shift from primarily serous-type cells with a high affinity for hematoxylin (Fig. 5A), a component of PTS to mucous-type cells (Fig. 5E) that show little to no affinity for hematoxylin. Along with this

cytoplasmic transition are a reduction in thickness of the mucosal epithelium and a concomitant increase in the size of the central lumina. Eventually, the configuration of the mucosal epithelium loses its glandular properties and becomes a shrunken epithelium characterized by irregular longitudinal folds and no secretory cells (Fig. 5F).

A stark contrast in epithelial morphology is evident between the two urogenital ducts and the seminal vesicle as shown in a contiguous configuration of these structures (Fig. 6). As expected, the mucosal epithelium of the Wolffian duct and the seminal vesicle are quite similar to one another (Fig. 6A) because the seminal vesicle is an outgrowth of the Wolffian duct. They are lined with a simple-to-stratified columnar

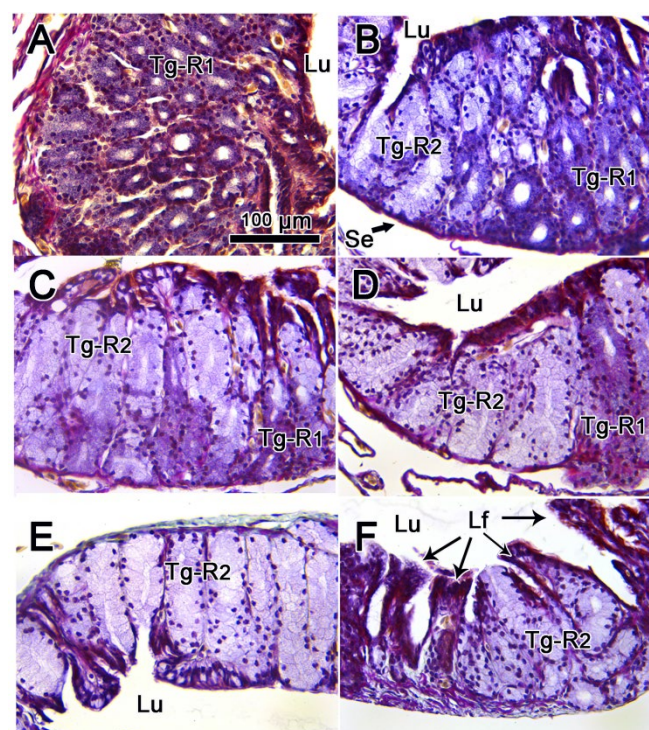


Figure 5. Light micrographs (LM-Paraffin) of successive transverse sections of the Müllerian duct post-kidney of a male *Rana areolata circulosa* (ASUMZ 34143) showing transitional regions in Müllerian gland morphology. A. Section of tubular glands designated as region 1 (Tg-R1). B-D. Region 1 giving way to an increasing presence of glands in region 2 (Tg-R2). E. Only Tg-R2 glands are present. F. Tg-R2 glands diminishing in number as longitudinal folds (Lf) lacking a glandular mucosa begin to dominate. Se, serosa; Lu, lumen. Scale bar in A the same for B-F.

epithelium, which consequently, varies greatly in thickness throughout their anatomical surfaces. Numerous small crypts exhibiting uneven depths form a pocketed epithelium (Fig. 6B). No ciliated cells were observed in either the seminal vesicle or the Wolffian



duct. In contrast, the epithelium in this post-glandular region of the Müllerian duct is lined with a continuous layer of ciliated columnar cells (see arrow in Fig. 6A) interspersed with support cells (Fig. 6C). The presence of sperm was scant within the seminal vesicle and the Wolffian duct in the males examined in this study, although the testes of all males examined were undergoing spermiogenesis (as seen in Fig. 7).

An overview examination of the histomorphology of the urogenital system in *R. a. circulosa* (Fig. 7) reveals relationships among all of the component anatomical structures examined in this study. The prominence of the seminal vesicle (as first viewed in Fig. 2B, E) can be clearly observed embedded between the caudal segments of the Wolffian duct and Müllerian duct. Also, the overall span of the glandular portion of the Müllerian duct versus its non-glandular region (caudal segment) is apparent.

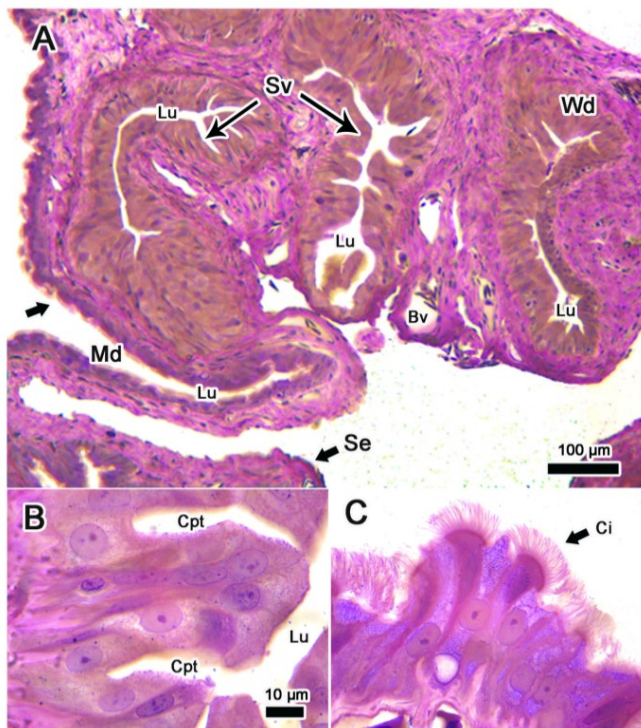


Figure 6. Light micrographs (LM-Plastic) of the Müllerian duct, the Wolffian duct, and the seminal vesicle of a male *Rana areolata circulosa* (ASUMZ 33746). A. Image shows all three anatomical structures. B. High magnification of two crypts (Cpt) within a portion of the seminal vesicle. C. High magnification of the ciliated columnar epithelium (Ci; also see arrow in A) of the Müllerian duct (post-glandular region). Bv, blood vessel. Abbreviations the same as in previous figures.

## Discussion

The most conspicuous anatomical structures of the distal urogenital system in reproductively active male

*R. a. circulosa* are the prominent Müllerian ducts and the linear-directed, sac-like seminal vesicles. On the other hand, the Wolffian ducts remain mostly ovoid in shape throughout their entire lengths, except for segments in the region of the seminal vesicles.

As a whole, comparative histology between the seminal vesicles and Wolffian ducts with other ranid frogs reveals distinctive similarities in these structures.

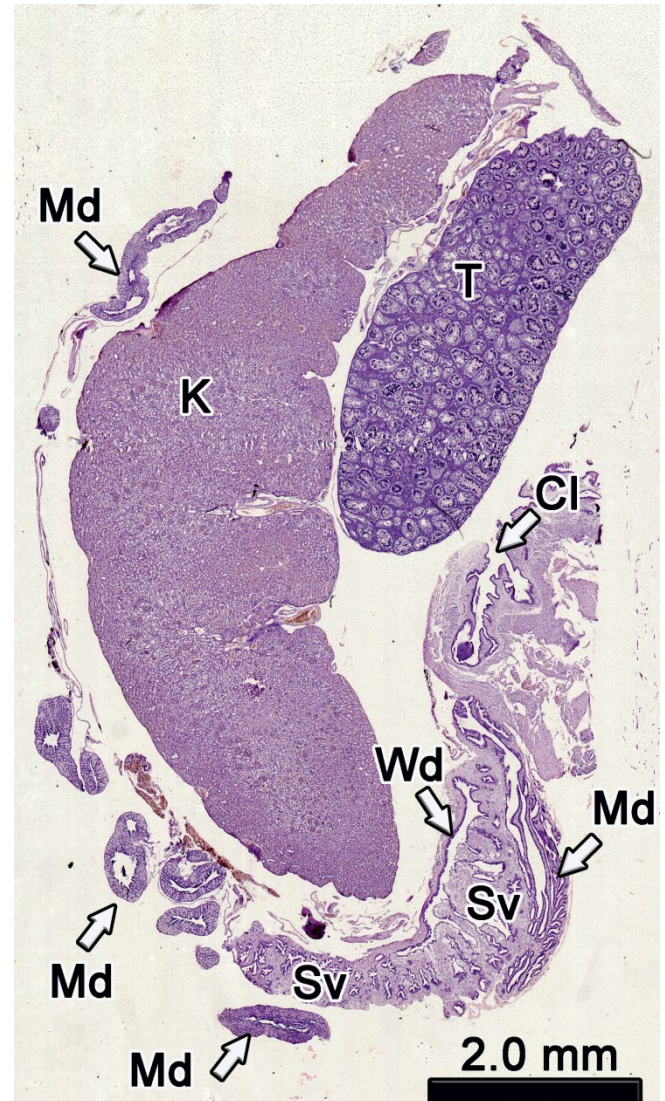


Figure 7. Light micrograph (LM-Paraffin) of a frontal section of the right side of the entire urogenital system (distal portion swung cranially and medially for sectioning convenience) of a male *Rana areolata circulosa* (ASUMZ 33748). Abbreviations the same as in previous figures.

For example, in general, many of the ranid frogs studied by Iwasawa and Michibata (1972) possessed seminal vesicles whose mucosal epithelia were lined with low columnar cells; however, some were lined with a cuboidal epithelia. Moreover, many ducts also

## Distal Urogenital Ducts in the Northern Crawfish Frog

bore numerous outpocketings, and some contained villus-like projections in the mucosal walls of the Wolffian ducts. Although quite variable due to the seasonal nature of their frog collections these authors consistently found sperm within the seminal vesicles during each frog's reproductive season.

We also found structural similarities in the seminal vesicles and Wolffian ducts of *R. a. circulosa* with those of the *R. sylvatica* as reported by Trauth (2022). However, one major difference pertained to the partitioning of the cloacal chambers. A common urodeal/coprodeal chamber was present in the *R. sylvatica* in the region of the urogenital papillae, whereas in *R. a. circulosa* these two chambers formed separate entities. Incidentally, both species possessed well developed urogenital papillae within the urodeum, although *R. a. circulosa* also possessed well developed urodeal ridges which were lacking in *R. sylvatica*.

The Müllerian ducts pose a slightly different challenge regarding any comments on comparative histomorphology or common functionality in male anurans. According to most authors, very few male anurans possess these ducts (Pace 1974; Duellman and Trueb 1986; Tyler 2003), and no histological studies have addressed these ducts since a study by Johnston and Gillies (1919) on an Australian hyloid frog. Yet, these ducts are well developed and have been well studied in male caecilians (worm-like amphibians) from the early works by Wake (1968, 1970, 1972, 1981) to a more recent study by Pewhorn *et al.* (2016). It would be misleading to draw any direct inferences from histological similarities between the Müllerian ducts of male caecilians and those of a single male anuran. Granted, a common histomorphology is present and includes a glandular region (*sensu stricta*, the *pars convoluta* portion of the female anuran oviduct—see Tyler 2003) consisting of simple tubular glands and the duct lumen that exhibits a ciliated epithelium. A probable function of secretions from Müllerian glands in male caecilians, as put forth by many authors, is to nourish spermatozoa and become a component of the semen (Wake 1981; Pewhorn *et al.* 2016, and others). The same function for the glandular secretions in *R. a. circulosa* could possibly be true. The non-glandular caudal region of the Müllerian ducts in *R. a. circulosa* (*sensu stricta*, *pars utera* or uterus = ovisac, as in female anuran oviducts—see Tyler 2003) contains a ciliated epithelium, and this common feature with male caecilians could serve to pass along glandular secretions to the cloaca for reproductive functions in this frog.

In conclusion, additional investigations on the Wolffian ducts and seminal vesicles in other North American ranid frogs should greatly increase an understanding of the comparative urogenital anatomy among the other species. Moreover, the discovery of additional male anurans that possess Müllerian ducts would be rewarding.

### Acknowledgments

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