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Renal Sexual Segment of the Cottonmouth Snake, *Agkistrodon piscivorous* (Reptilia, Squamata, Viperidae)

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ABSTRACT The seasonal variation of the renal sexual segment (RSS) of males of the Cottonmouth snake, *Agkistrodon piscivorous*, is described using light and electron microscopy. This study is the first to describe the ultrastructure of the RSS of a viper (Viperidae) and only the fourth on a snake. Renal sexual segments from males collected February to May and from August to November are similar in appearance. The cells are eosinophilic and react with periodic acid/Schiff procedure (PAS) for neutral carbohydrates and bromphenol blue (BB) for proteins. At the ultrastructure level, the cells contain large (2 μm diameter), electron-dense secretory granules and smaller vesicles with a diffuse material, and these structures abut against the luminal border and upon clear vacuoles continuous with intercellular canaliculi. Evidence was found for both apocrine and merocrine processes of product release. In June and July, the RSS are significantly smaller in diameter, largely basophilic, and have only scattered granules that are PAS+ and BB+. Cytologically, the RSS from June to July lack electron-dense secretory granules and the smaller vesicles with diffuse material. Numerous condensing vacuoles and abundant rough endoplasmic reticulum, however, indicate that active product synthesis is occurring. This is the first report of significant seasonal variation in the histology and ultrastructure of the RSS of a snake, although such reports exist for lizards. The seasons when the RSS is most highly hypertrophied correspond to the fall and spring mating seasons of *A. piscivorous*, as determined by other studies. J. Morphol. 000:000–000, 2007.

KEY WORDS: Reptilia; Viperidae; *Agkistrodon*; sexual segment; ultrastructure

The first histological description of a squamate nephron was made on the European natricine snake *Natrix natrix* by Gampert (1866). He noted that a portion of the distal segment (the "Harncanalchen") is especially thickened, and he provided the first accurate illustration of a snake nephron. Subsequent reports by Heidenhain (1874), also on *N. natrix*, and by Tribondeau (1902), on *Coluber viridiflavus*, described the enlarged distal segment of the nephron. Regaud and Policard (1903), however, were the first to note that this segment is sexually dimorphic, being hypertrophied only in males (but see Del Conte, 1972; Sever and Hopkins, 2005). They named this region the "segment sexuel." Regaud and Policard (1903) also demonstrated that the renal sexual segment (RSS) is present in a variety of male snakes and lizards but is absent in turtles. Later work concluded that the RSS also is absent in crocodilians (Fox, 1952) but is present in amphisbaenids (Bons and Saint Girons, 1963; Saint Girons, 1972); a portion of the nephron of *Sphenodon* (Rhynchocephalia) most likely differentiates seasonally into a RSS (Gabe and Saint Girons, 1964). The reptilian clade Lepidosauria is traditionally composed of the Squamata (snakes, lizards, and amphisbaenids) and Rhynchocephalia (Pough et al., 1998). Thus, among reptiles, the presence of a RSS can be considered a synapomorphy at least for the Squamata and probably for the Lepidosauria.

The function of the RSS is still not clearly understood, but its secretions may sustain and activate sperm (Bishop, 1959; Cuellar, 1966), provide courtship pheromones (Volsøe, 1944), form copulatory plugs (Devine, 1975; Ross and Crews, 1977; Nilson and Andren, 1982), and/or have other purposes generally associated with seminal fluid (Prasad and Reddy, 1972). The only other sources of seminal fluids in squamates are the epididymis and ampulla ductus deferentis (Sever, 2004). In natricine snakes, the copulatory plug is gelatinous (Devine, 1975), but in *Viper berus* the secretion of the RSS induces contraction of "sphincter muscles" in the caudal part of the oviduct, which act mechanically to prevent sperm entering the oviduct (Nilson and Andren, 1982). Weil (1984) suggested that the RSS secretion of the snake *Nerodia sipedon* has dual functions, one of sperm transport

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and capacitation in the female reproductive tract in autumn and another related to sexual behavior in the spring.

Numerous histological studies on the RSS of squamates have been done since the first such report by Gampert (1866), and this literature has been reviewed by Saint Girons (1972) and Fox (1977). Only seven ultrastructural studies on the RSS have been done, three on snakes (Kuhnel and Kirsch, 1974; Sever et al., 2002; Krohmer, 2004) and four on lizards (Furieri and Lanzavecchia, 1959; Del Conte and Tamyo, 1973; Gabri, 1983; Sever and Hopkins, 2005).

The Viperidae is a family of venomous snakes containing 36 genera and some 259 species, and occur on all continents except Antarctica and Australia (Pough et al., 1998). The Viperidae are the sister taxon of the 1,800+ species of harmless snakes lumped into the Colubridae (Lawson et al., 2005).

Gabe (1959) first reported on the histology of the RSS of a viper, *Vipera aspis*, and Burtner et al. (1965) described the histochemistry of the RSS in the rattlesnake, *Crotalus adamanteus*. Subsequently, Saint Girons (1972) summarized observations using light microscopy on *V. aspis* and five other vipers in the genera *Bothrops, Cerastes, Crotalus, Trimeresurus, and Vipera*. Finally, Johnson et al. (1982) described the relationships among spermatogenesis, androgenic cycles, and RSS hypertrophy in the North American pitviper the Cottonmouth, *Agkistrodon piscivorous*. No ultrastructural studies on the RSS of vipers have been done. In this article, we extend the observations of Johnson et al. (1982) by describing the seasonal variation in the histochemistry and ultrastructure of the RSS of *A. piscivorous*.

MATERIALS AND METHODS

Animals and Treatment

Agkistrodon piscivorous (Lacépède) males were collected from the site of a railroad track levee along the Amite River Diversion Canal, North 30.22616/West 090.68506, Livingston Parish, LA, property owned by Dr. Clifford Fontenot, 10 km northwest of New Albany, North 30°30.871/West 090°36.202, Livingston Parish, LA, and the Turtle Cove Environmental Research Station on Pass Manchac, North 30.29426/West 090.35592, Tangipahoa Parish, LA. Specimens were housed in glass aquariums (~0.3 m × 0.6 m × 0.3 m) with locking screen lids for no more than three days before they were sacrificed. Water was available to these snakes *ad libitum*.

Individuals were sacrificed by a 0.2–0.5 ml I.P. injection of a solution of sodium pentobarbitol at a concentration of 1 g sodium pentobarbital in 10% alcohol and 40% propylene glycol. The left urogenital organs (Fig. 1) were removed from the specimens and fixed in 10% neutral buffered formalin solution (NBF) for light microscopy. The right urogenital organs were prepared for examination by TEM and SEM. The number of sexually mature specimens prepared per month was: February: 2; March: 2; April: 1; May: 2; June: 3; July: 2; August: 2; October: 2; and November: 1. Snout-vent lengths (SVL) ranged from 46.1–72.2 cm (Table 1). Protocols were approved by the Institutional Animal Care and Use Committee of Southeastern Louisiana University.

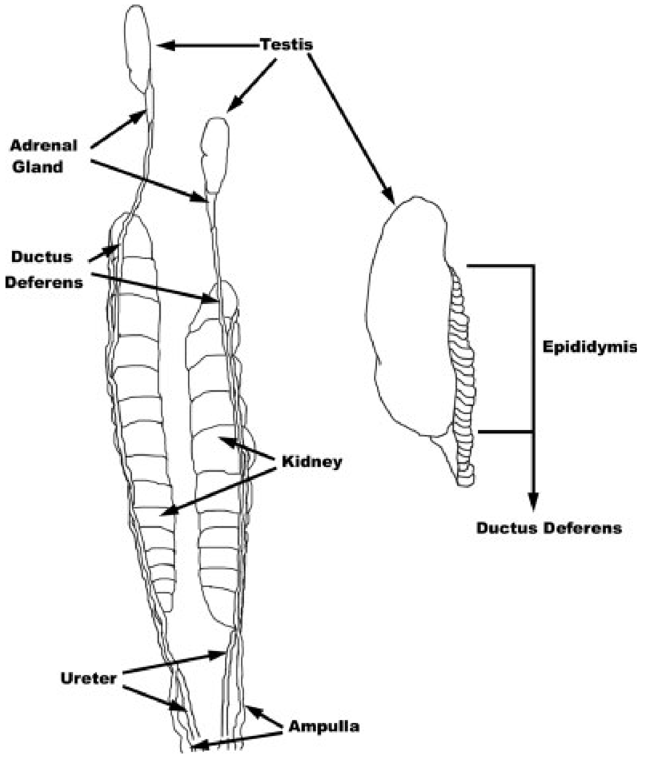

Fig. 1. *Agkistrodon piscivorous*. Outline of the gross anatomy of the male urogenital system.

Tissue Preparation for Microscopy

For all procedures, Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA) was used for editing and printing of images.

Light microscopy. Tissues fixed in NBF were rinsed for 1 h in tap water and then dehydrated by placing the tissue in a graded series of ethyl alcohol (70, 95, and 100% for an hour each). After dehydration, the tissues were embedded in paraffin blocks for sectioning with a MR3 microtome (Research and Manufacturing Co., Tucson, AZ). Sections 10-μm thick were cut and affixed to albuminized slides. Alternate slides were stained with hematoxylin-eosin (for general cytology), alcian blue 8GX at pH 2.5 (AB, for primarily carboxylated glycosaminoglycans) followed by the periodic acid-Schiff's procedure (PAS, for neutral carbohydrates and sialic acids), and bromphenol blue (BB, for proteins). Histological techniques followed Kiernan (1990). Slides were viewed under a Leica DM2000 microscope (Leica Microsystems, Wetzlar, Germany), and images were obtained via a Leica DFC420 digital camera (Leica Microsystems, Wetzlar, Germany).

TEM. Tissues fixed in Trump's solution were rinsed in deionized water and then postfixed 90 min in 2% osmium tetroxide. They were then rinsed in deionized water, dehydrated with a graded series of ethanol (70, 95, 100% for 1 h each), soaked 30 min each in 1:1 100% ethanol:propylene, followed by pure propylene oxide, and subsequently embedded in an epoxy resin (EmBed 812, Electron Microscopy Sciences, Fort Washington, PA) for ultra-thin sectioning with a RMC MT7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ). The ultra-thin sections, at 70 nm, were achieved by use of DiATOME diamond knives (DiATOME, Biel, Switzerland). Ultra-thin sections were placed on copper grids and stained with uranyl acetate and lead citrate. Grids were then viewed and photographed with a JEOL JEM 100S transmission electron microscope (JEOL USA, Peabody, MA). Kodak Film (Eastman Kodak, Rochester, NY) was used for TEM photography.

TABLE 1. Specimens examined and kidney measurements[a]

Date	SVL	Kidney length	Kidney width	RSS diameter	SE	Epithelial height	SE
25 Feb 05	64.7	74.9	6.9	103.0	3.06	32.3	1.26
23 Feb 06	52.2	61.7	6.4	133.4	4.09	27.0	1.41
13 Mar 06	46.1	54.8	6.2	146.7	3.62	59.4	2.01
28 Mar 06	48.5	52.4	7.4	133.1	2.11	56.0	1.33
7 Apr 06	50.3	52.4	7.3	140.8	2.14	59.9	1.42
24 May 06	53.6	64.9	8.4	85.7	3.31	29.4	0.75
30 May 06	54.7	59.5	8.5	131.6	3.00	49.1	1.94
14 Jun 05	73.1	84.2	10.0	89.6	3.05	28.6	1.67
15 Jun 05	59.3	72.8	7.2	128.2	2.40	37.6	2.56
27 Jun 05	69.8	79.0	11.6	111.2	3.00	38.3	0.91
18 Jul 05	62.3	60.2	6.5	79.6	2.33	30.0	1.14
27 Jul 05	64.1	54.3	7.5	78.6	2.84	28.3	1.30
12 Aug 05	60.8	64.4	7.3	133.8	2.27	51.0	1.58
17 Aug 05	61.9	68.0	9.2	184.1	3.77	69.7	1.59
17 Oct 05	61.4	72.6	8.4	155.3	1.79	54.9	1.55
17 Oct 05	72.2	85.6	10.2	156.8	3.83	54.6	2.15
22 Nov 05	56.1	64.9	7.0	159.8	4.67	50.6	1.80

[a]SVL is in cm, Kidney length and width in mm, and RSS diameter and epithelial height in μm.

SEM. Tissues were handled in the same manner for SEM as they were for TEM through the dehydration steps. The tissues were then critically point dried using a Denton DCP-1 critical point drier and sputter coated with a Denton Desk IV XLS (Denton Vacuum, Moorestown, NJ). Finally, the tissues were viewed with a Philips XL-20 scanning electron microscope (Philips Electronics N.V., Eindoven, Netherlands).

Statistics

Means, standard errors, and correlation coefficients were determined using Microsoft Office Excel 2003. Tukey's HSD method of multiple comparisons was calculated using SYSTAT v. 6.1 for Windows. A probability level of $P \leq 0.05$ was used to determine statistical significance.

RESULTS

Statistics

The Pearson's correlation coefficients between SVL and kidney length ($r = 0.825$, $P < 0.001$) and kidney width ($r = 0.675$, $P = 0.003$), and between RSS diameter and epithelial height ($r = 0.736$, $P < 0.001$) are all highly positive and significant. No significant correlation occurs between SVL and RSS diameter ($r = -0.245$, $P = 0.344$) or SVL and epithelial height ($r = -0.304$, $P = 0.235$). Seasonal variation occurs in RSS diameter and epithelial height, with hypertrophy in spring, a marked reduction in size May through July, and hypertrophy again in late summer and fall (Fig. 2).

Tukey's multiple comparison matrix (Table 2) shows that the July mean does not differ significantly from May or June but is significantly lower than all other months. Means for May and June are significantly lower than August–November but not from February–May. Means from March to April and August to November are not significantly different, but the mean from February is

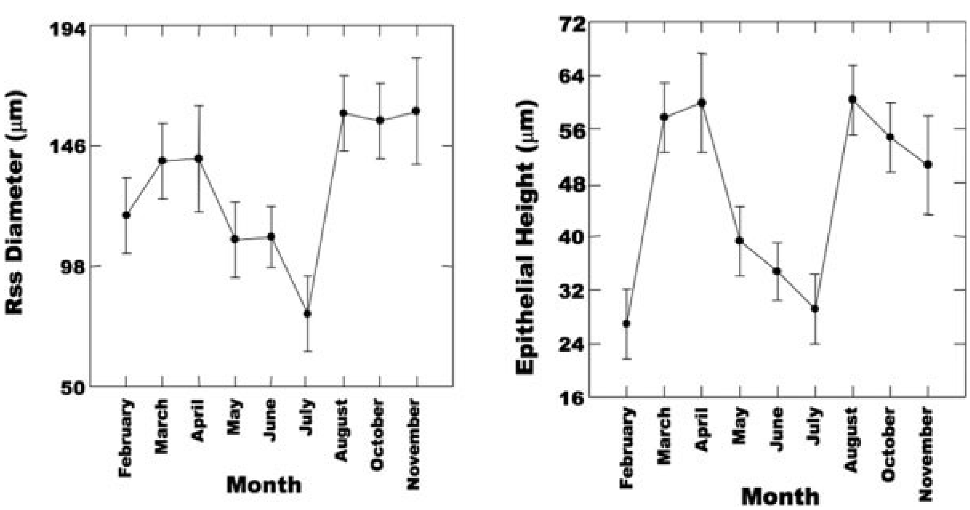

Fig. 2. Relationships between month and mean Rss diameter and mean epithelial height in male *Agkistrodon piscivorous*. Horizontal line indicates the mean, and vertical lines indicate one standard deviation.

TABLE 2. Tukey's HSD matrix of pairwise comparison possibilities among the monthly means for renal sexual segment of the kidney diameter in male Agkistrodon piscivorous

Date	Feb	Mar	Apr	May	Jun	Jul	Aug	Oct	Nov
Feb	1.00								
Mar	0.51	1.00							
Apr	0.70	1.00	1.00						
May	0.99	0.13	0.29	1.00					
Jun	0.99	0.10	0.26	1.00	1.00				
Jul	0.03	0.00	0.00	0.17	0.09	1.00			
Aug	0.03	0.66	0.87	0.00	0.00	0.00	1.00		
Oct	0.04	0.82	0.95	0.01	0.00	0.00	1.00	1.00	
Nov	0.09	0.81	0.92	0.02	0.02	0.00	1.00	1.00	1.00

The matrix consists of P values, with $P \leq 0.05$ considered significant.

significantly lower than those from August and October.

Histology and Ultrastructure of Seasonal Variation

February-May. Portions of the RSS are easily distinguished from other tubules in the kidney by their relatively larger size and staining characteristics (Fig. 3). During this period, the cytoplasm is columnar, uniformly eosinophilic, and possesses basal nuclei (Fig. 3A,B). In contrast, proximal convoluted tubules are cuboidal and basophilic (Fig. 3B). With PAS/AB, the RSS is largely PAS+, but areas of AB+ activity also occur (Fig. 3C), and the tubules react strongly for proteins with BB (Fig. 3D). Proximal convoluted tubules are AB+ and react weakly with BB.

TEM of the RSS shows the cytoplasm filled with electron-dense secretory granules (~2 μm dia), large empty vacuoles, and small vesicles containing a diffuse material (Fig. 4A). Secretory granules as well as the small vesicles abut upon the large vacuoles in some areas, and the vacuoles have connections to the intercellular canaliculi (Fig. 4B,C). The intercellular canaliculi are labyrinthine with interdigitating filamentous membranes from adjacent cells along much of their lengths (Fig. 4). Apically, the intercellular canaliculi have tight junctions at the luminal border (Fig. 4B) whereas basally, no junctional complexes exist at the interface of the intercellular canaliculi and the basal lamina (Fig. 4D). Nuceli are irregular and dark, and sit on the basal lamina or are separated from the basal lamina by sparse areas of cytoplasm (Fig. 4C,D). Rough endoplasmic reticulum (Rer) and Golgi complexes are found in the perinuclear area and are associated with condensing vacuoles that may be irregular in shape (Fig. 4C,D).

Secretory granules, vesicles, and vacuoles all can be found abutting the luminal border (Fig. 5), and apparently all three structures can be involved in release of products into the lumen. Membrane fusion, implying a merocrine process, appears evident in some sections (Fig. 5A,B), but entire secretory granules with what could be small areas of attached cytoplasm are found in other sections (Fig. 5C), indicating an apocrine process. Some sections indicate that cells that are releasing products from secretory granules alternate with others that release products from smaller vesicles (Fig. 5D).

June-July. The RSS go through a period where secretory activity is reduced and gland diameter and epithelial height is obviously decreased in histological sections (Fig. 6), supporting statistical data (Table 1, Fig. 2). The RSS are still distinguishable by being relatively larger than the proximal convoluted tubules (Fig. 6A). Hematoxylin-eosin, however, is no longer a usable way to distinguish the two types of tubules as the RSS have become decidedly basophilic, similar to the proximal convoluted tubules (Fig. 6A,B). The PAS+ and BB+ reactions in the RSS are limited to scattered granules (Fig. 6C,D). Proximal convoluted tubules do not change in appearance, and remain AB+ and have a weak reaction with bromphenol blue (Fig. 6C,D).

TEM of the June and July samples reveals marked cytological differences from spring, most notably the loss of mature, electron-dense secretory granules and the smaller secretory vesicles, and the reduction in size and number of vacuoles (Fig. 7). Nuclei are relatively larger in relation to the amount of cytoplasm, have less condensed chromatin, and although still basal, more cytoplasm occurs in the infra-nuclear regions (Fig. 7A,B). Intercellular canaliculi are still relatively wide beyond the luminal tight junctions (Fig. 7C), but the extent of interdigitating plasma membranes from adjacent cells is much reduced, especially basally, and the interface between the intercellular canaliculi and the basal lamina is wide open (Fig. 7B). Rer is abundant throughout the cells, and condensing vacuoles, partially filled with diffuse material, are abundant supranuclearly (Fig. 7C). Bundles of microfilaments are common in perinuclear areas (Fig. 7C,D). One of the most striking differences, however, is the occurrence of

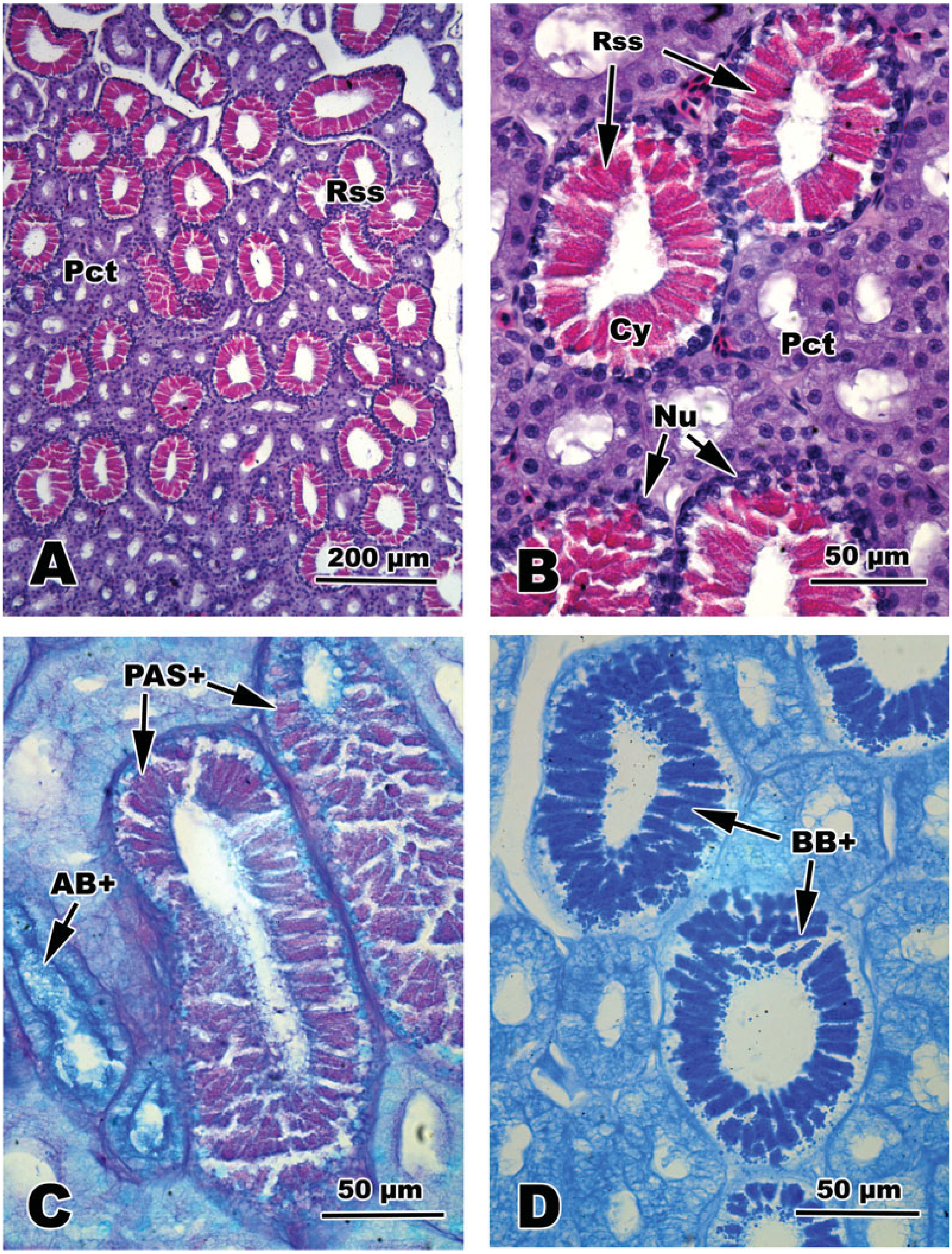

Fig. 3. *Agkistrodon piscivorous*. The kidney of a male 52.2 cm SVL collected 23 February 2006. LM. **A,B:** Overview of the relationships between portions of renal sexual segments (RSS) and the proximal convoluted tubules (Pct) stained with hematoxylin-eosin. Note that the cytoplasm of the RSS is highly eosinophilic throughout, and that nuclei are basal. Proximal convoluted tubules are uniformly basophilic. **C:** Reaction with periodic acid/Schiff procedure (PAS) and alcian blue at pH 2.5. Note that RSS are largely PAS+, although some AB+ material also occurs, whereas Pct are uniformly AB+. **D:** Reaction with bromphenol blue (BB). Note that the cytoplasm of RSS is highly BB+ whereas Pct have a weak reaction. BB+, bromphenol blue positive; Cy, cytoplasm; Nu, nucleus; Pct, proximal convoluted tubules; RSS, renal sexual segments.

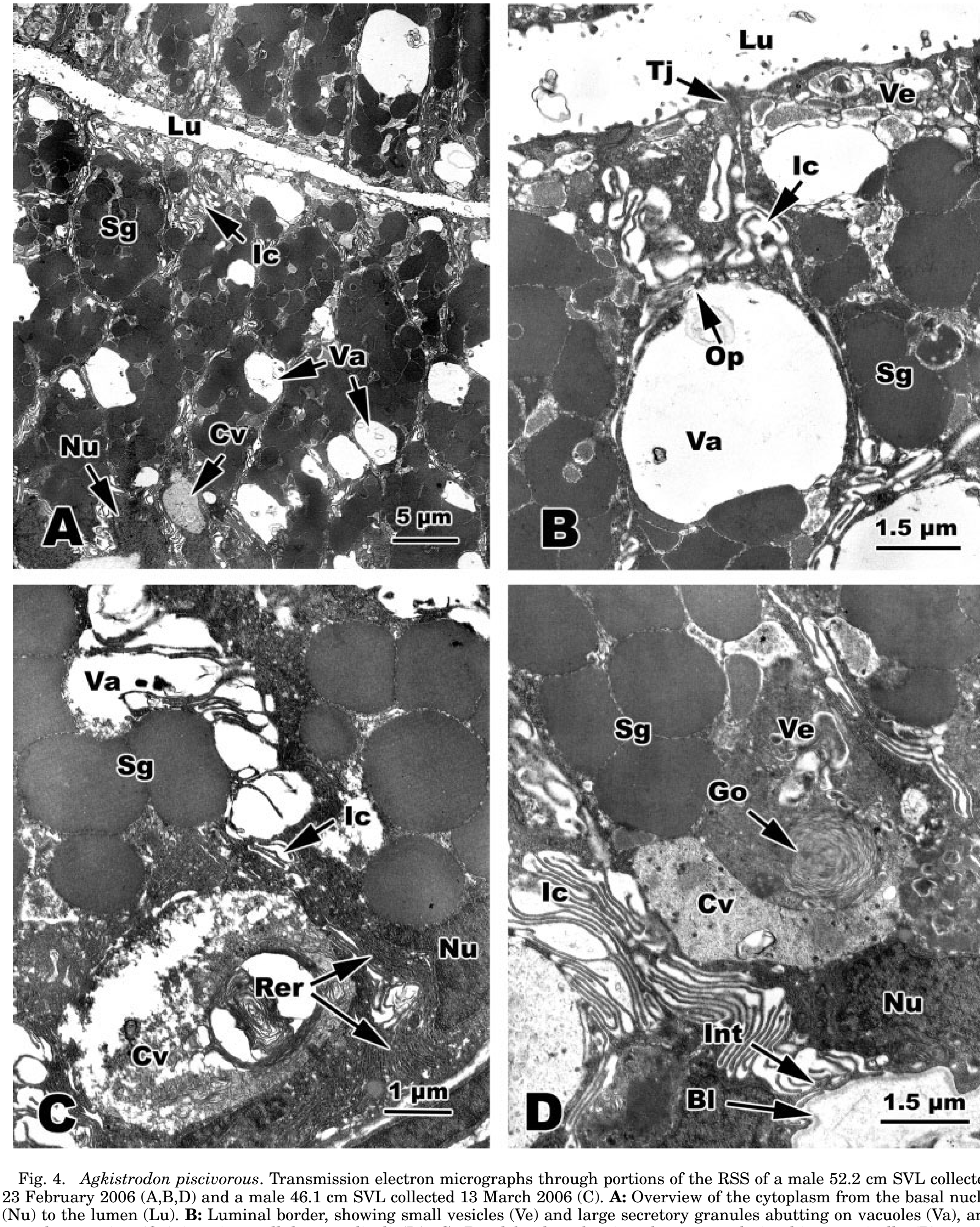

Fig. 4. *Agkistrodon piscivorous*. Transmission electron micrographs through portions of the RSS of a male 52.2 cm SVL collected 23 February 2006 (A,B,D) and a male 46.1 cm SVL collected 13 March 2006 (C). **A:** Overview of the cytoplasm from the basal nuclei (Nu) to the lumen (Lu). **B:** Luminal border, showing small vesicles (Ve) and large secretory granules abutting on vacuoles (Va), and vacuoles opening (Op) into intercellular canaliculi (Ic). **C:** Basal border, showing the same relationship as apically (B) among vacuoles, secretory granules and the intercellular canaliculi. Note abundant rough endoplasmic reticulum (Rer). **D:** Basal border, highlighting the interface (Int) between the intercellular canaliculi and the basal lamina (Bl); no junctional complexes exist. Bl, basal lamina; Cv, condensing vacuole; Go, Golgi complex; Ic, intercellular canaliculi; Int, interface; Lu, lumen; Nu, nucleus; Op, opening; Rer, rough endoplasmic reticulum; Sg, secretory granules; Tj, tight junction; Va, vacuole; Ve, vesicle.

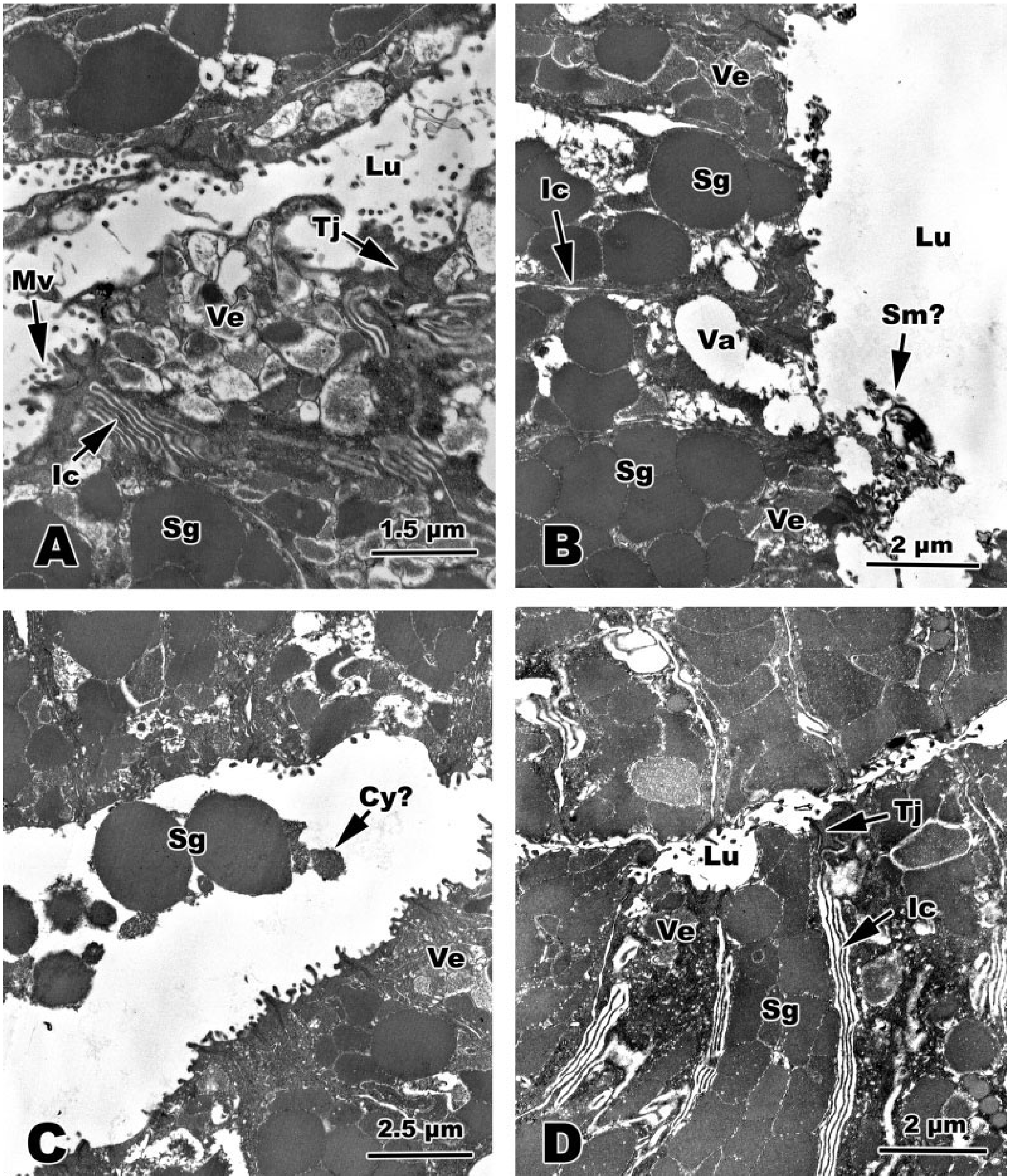

Fig. 5. *Agkistrodon piscivorous*. Transmission electron micrographs through portions of the RSS of a male 52.2 cm SVL collected 23 February 2006 (A), a male 46.1 cm SVL collected 13 March 2006 (B,C), and a male 54.7 cm SVL collected 30 May 2006. **A:** Apical border, showing small vesicles (Ve) filled with diffuse material abutting the lumen. **B:** Apical border, showing vesicles, secretory granules (Sg), and vacuoles (Va) along the apical border with what could be secretory material (Sm?) released into the lumen. **C:** Apical border, showing secretory granules in the lumen with what could be attached pieces of cytoplasm (Cy?). **D:** Apical region, showing alternate cells with either small vesicles or secretory granules along the luminal border. Cy?, cytoplasm; Ic, intercellular canaliculi; Lu, lumen; Mv, microvilli; Sg, secretroy granules; Sm?; secretory material; Tj, tight junction; Va, vacuole; Ve, vesicle.

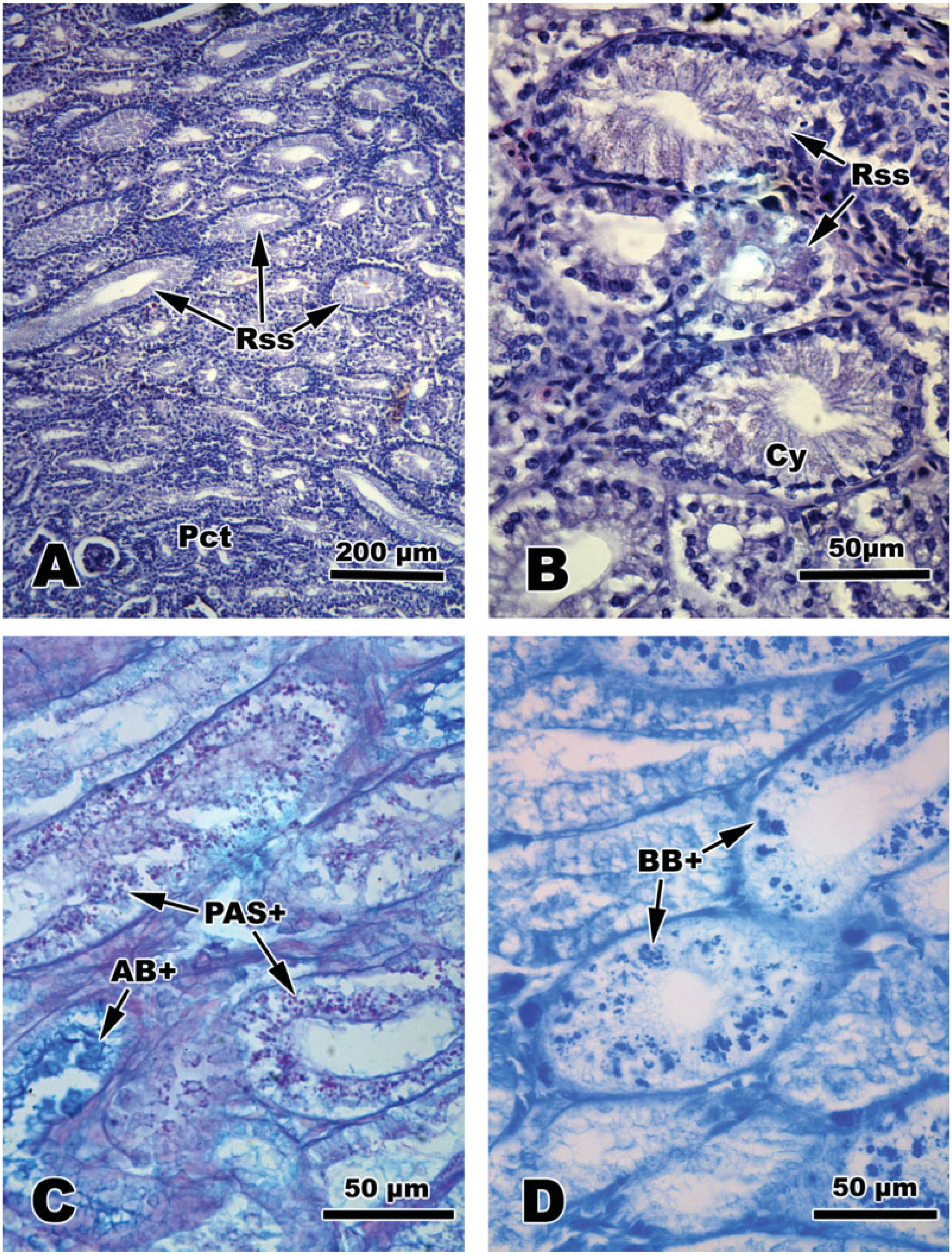

Fig. 6. *Agkistrodon piscivorous*. Light micrographs through the kidney of a male 62.3 cm SVL collected 18 July 2005. **A,B:** Overview of the relationships between portions of the Renal sexual segment (RSS) and proximal convoluted tubules (Pct) stained with hematoxylin-eosin. Note that the cytoplasm of the RSS is now decidedly basophilic and reacts similar to that of the Pct. **C:** Reaction with periodic acid/Schiff procedure (PAS) and alcian blue at pH 2.5. Note that RSS contain some PAS+ granules and that some AB+ material also occurs. Pct are uniformly AB+ and similar to the condition shown in Figure 3. **D:** Reaction with bromphenol blue (BB). Similar to the PAS reaction, a BB+ reaction in the RSS is limited to clusters of granules. BB+, bromphenol blue positive; Cy, cytoplasm; Pct, proximal convoluted tubules; RSS, renal sexual segments.

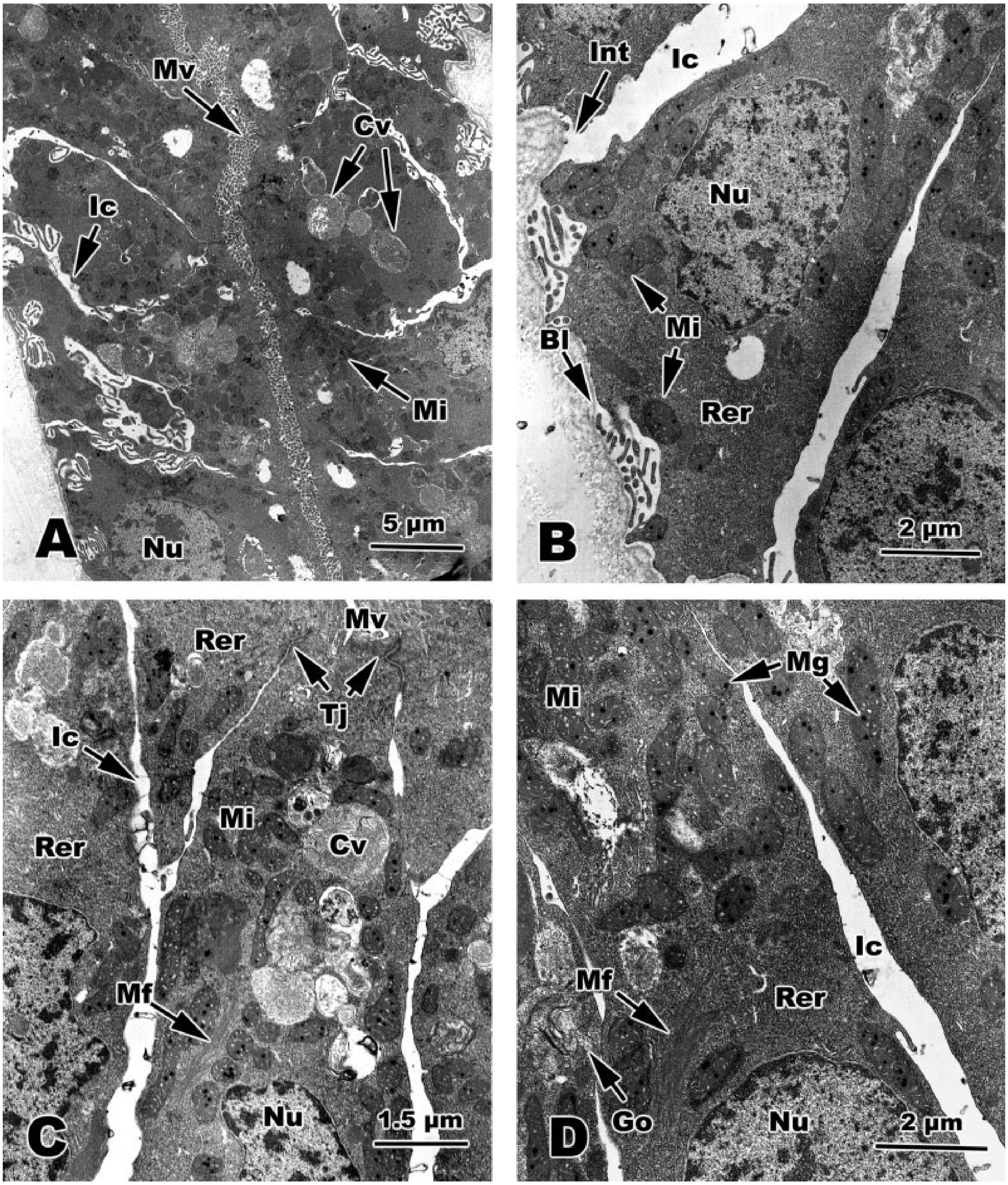

Fig. 7. *Agkistrodon piscivorous*. Transmission electron micrographs through portions of the RSS of a male 62.3 cm SVL collected 18 July 2005. **A:** Sagittal section through a RSS. **B:** Basal epithelium. Note especially the lack of any barrier (Int) between the interceluular canaliculus (Ic) and the basal lamina (Bl). The intercellular canaliculi also lack the interdigitating epithelial projections conspicuous in spring (see Fig. 3). **C:** Apical cytoplasm. **D:** Supranuclear cytoplasm. Bl, basal lamina; Cv, condensing vacuole; Go, Golgi complex; Ic, intercellular canaliculus; Mf, microfilaments; Mg, mitochondrial granules; Mi, mitochondria; Mv, microvilli; Nu, nucleus; Rer, rough endoplasmic reticulum; Tj, tight junction.

numerous mitochondria throughout the cells, but especially in the supranuclear regions (Fig. 7). These mitochondria are characterized by possession of numerous granules interspersed among the cristae (Fig. 7D).

August-November. The RSS of specimens from these months resemble those from specimens collected from February to May (Fig. 8). The cytoplasm is once again uniformly eosinophilic (Fig. 8A), an intense BB+ reaction occurs (Fig. 8B,C), and the cytoplasm is largely PAS+ with some areas of AB+ material (Fig. 8D). The only variation noted was in the two October specimens, one of which had a BB+ reaction limited to the apical cytoplasm (Fig. 8B), whereas the other had a reaction throughout the entire cytoplasm (Fig. 8C), which was typical of other males sampled from this period. We cannot resist speculating that the former specimen had been actively involved in mating and had reduced its stores of secretory material. TEM images of specimens collected from August to November revealed no difference from those of the males collected from February to May.

Scanning Electron Microscopy. SEM confirms the major changes that occur in cytology of the RSS in June and July from other periods of the annual cycle. In Figure 9A, the apical cytoplasm of a specimen from June is devoid of secretory material, and globules in the remaining cytoplasm could be condensing vacuoles as labeled in Figure 7. In a November specimen (Fig. 9B), mature secretory granules are obvious throughout the cytoplasm, as typical of specimens collected in spring and fall.

DISCUSSION

Histochemical results on 73 species of squamates representing 19 families were reported by Saint Girons (1972). He found that the RSS of squamates is always rich in protein secretion, but that the PAS/AB reaction is quite variable and seems to have no phyletic significance.

Johnson et al. (1982) found that hypertrophy of the RSS of *Agkistrodon piscivorous* from Alabama coincided with elevations in plasma testosterone levels, and the tubules attained maximum diameters in March and September. Thus, their results agree with ours that hypertrophy occurs in spring and fall, and that diameters decrease in early summer. Maximal development of the RSS has been related to increased androgen levels in other squamates as well (Bishop, 1959; Pandha and Thapliyal, 1964; Misra et al., 1965; Prasad and Sanyal, 1969; Krohmer, 1986). Our results also agree with our observations on peaks of mating activity (Siegel and Sever, 2008), although as Johnson et al. (1982) reported, males have sperm in the ductus deferens year around.

Studies on lizards to date have reported that the RSS is hypertrophied only during periods of sexual activity and cannot be distinguished from adjacent tubular regions during sexual quiescence (Fox, 1977; Gabri, 1983; Sever and Hopkins, 2005). In snakes, complete regression has not previously been reported (Fox, 1977; Sever et al., 2002). Johnson et al. (1982) reported that granules were present in the epithelial cells of the RSS of their specimens in April and in the lumina from March through October. Although some PAS+ and BB+ granules were present in our specimens from June and July, secretory activity was dramatically reduced from other times of the year. The reduction in secretory activity we found was best illustrated by the ultrastructural differences.

Only seven ultrastructural studies have previously been done on the RSS of squamates. Furieri and Lanzavecchia (1959) were concerned only with the effects of castration on the ergastoplasm and Golgi complexes of the RSS of the lacertid lizard, *Lacerta sicula*. Shortly after castration, the characteristic structure of these organelles is no longer recognizable. Del Conte and Tamyo (1973) compared the RSS of males of the teiid lizard, *Cnemidophorus lemniscatus*, to a homologous area that undergoes some differentiation in females. They stated that this species has continuous sexual activity, but used only three specimens of each sex. In comparison to our results on *Agkistrodon piscivorous,* their results are of interest because *C. lemniscatus* is the only other squamate reported to have secretory granules of different sizes and opacities and numerous large clear vacuoles. Other similarities are few; the RSS of *C. lemniscatus* involves the collecting ducts rather than the distal convoluted tubules, and the release of secretion involves both holocrine and merocrine processes.

Gabri (1983) examined captives of the lacertid *Podacris taurica* kept under temperatures mimicking natural conditions. In *P. taurica* the RSS also involves only the collecting ducts, and distinct seasonal variation occurs. During the mating season, a protein secretion is produced, but the product changes to a mucous secretion during the period of seasonal inactivity. The intercellular canaliculi of *P. taurica* are described as wide and labyrinthine, which is similar to the condition in *Agkistrodon piscivorous*, and not reported in other squamates.

Sever and Hopkins (2005) studied seasonal variation at the light and electron-microscopy level of the RSS in the North American skink, *Scincella laterale*. This was the first study to examine wild-caught lizards collected throughout the entire active season. *Scincella laterale* has a single breeding season from March to May during which the RSS are highly hypertrophied. Cytologically, the active tubules consist of columnar cells with numerous apical, electron-dense secretory granules that are released by an apocrine process. After the

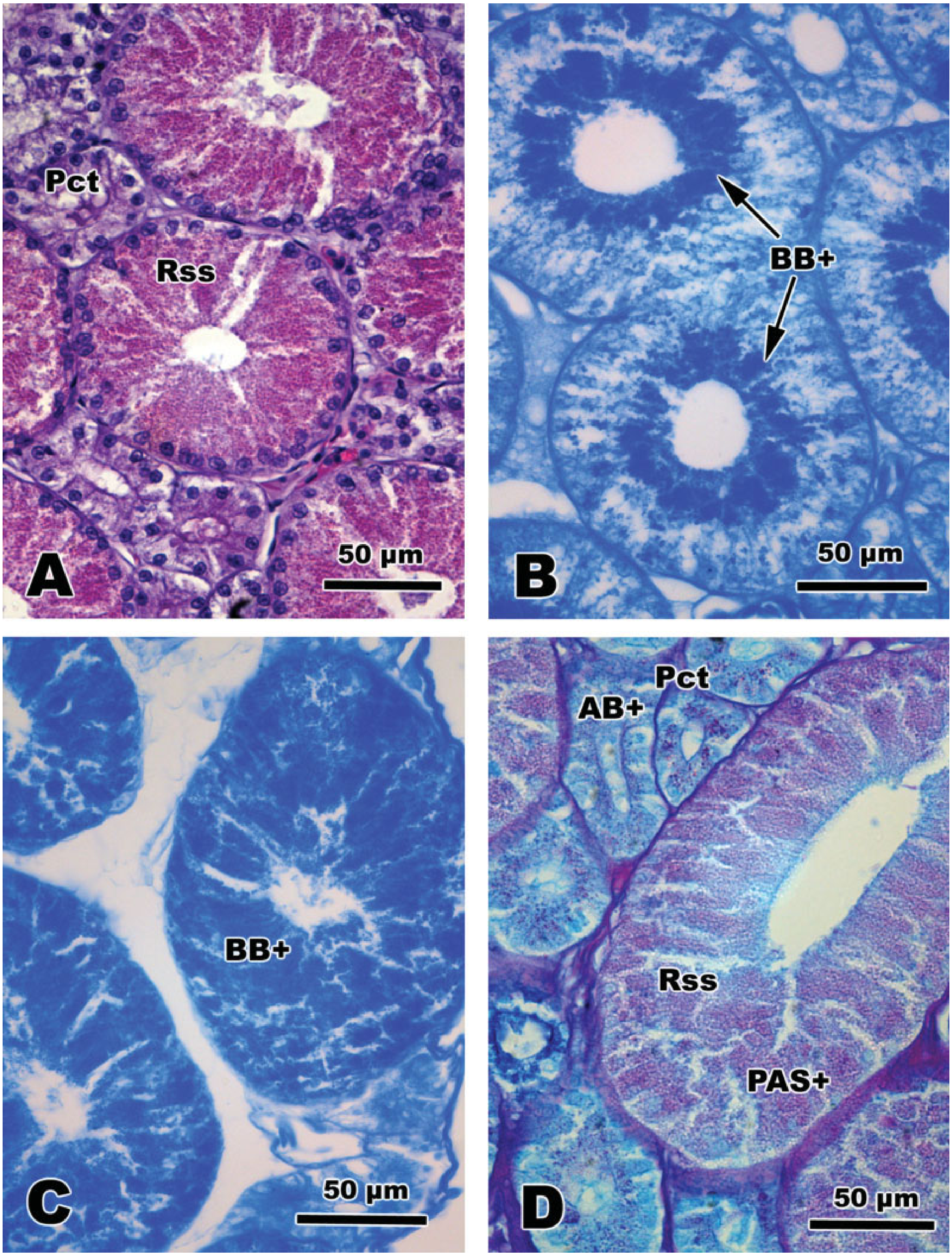

Fig. 8. *Agkistrodon piscivorous*. Light micrographs through the kidneys of a male 60.8 cm SVL collected 12 August 2005 (A), males 61.4 cm SVL (B) and 72.2 cm (C) collected 17 October 2005, and a male 56.1 cm SVL collected 22 November 2005 (D). **A:** Kidney from October specimen stained with hematoxylin- eosin. The cytoplasm of the renal sexual segments (RSS) are highly eosinophilic as in spring (Fig. 3). **B,C:** October specimens stained with bromphenol blue. Note variation in extent of intense BB+ staining. **D:** Kidney from November specimen treated with periodic acid/Schiff procedure and stained with alcian blue at pH 2.5. As in spring (Fig. 3), the RSS are generally PAS+ with some AB+ substance, and Pct stain AB+ as they do consistently all year. AB+, alcian blue positive; BB+, bromphenol blue positive; PAS+, periodic acid/Schiff's reagent positive; Pct, proximal convoluted tubules; RSS, renal sexual segments.

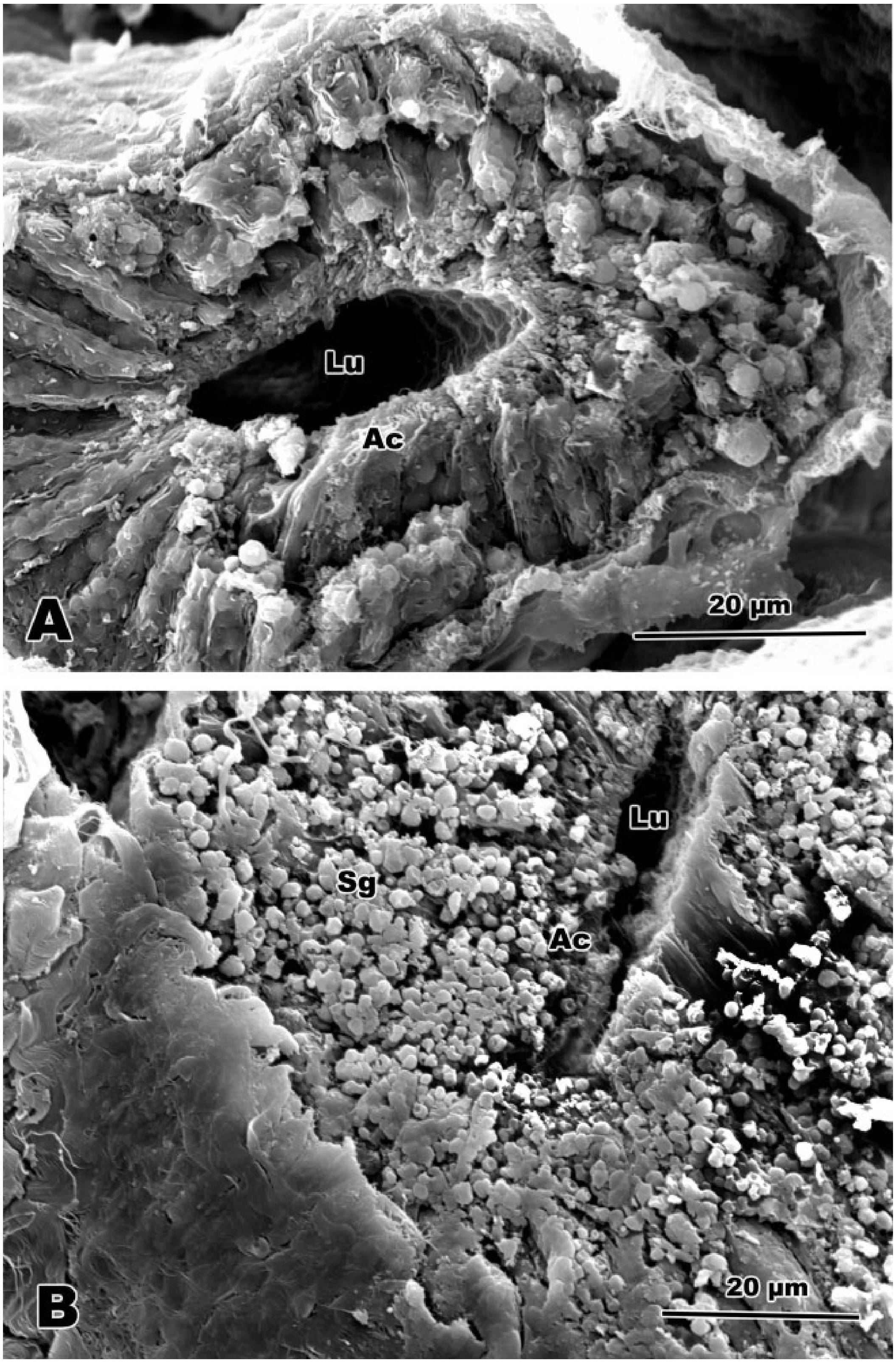

Fig. 9. *Agkistrodon piscivorous*. RSS of a male 73.1 cm SVL collected 14 June 2005. (**A**) and a male 56.1 cm SVL collected 22 November 2005. (**B**) SEM. Ac, apical cytoplasm; Lu, lumen; Sg, secretory granules.

mating season, the RSS undergoes regression and the electron-dense granules are replaced by a mucoid secretion that characterizes more proximal portions of the nephric tubules throughout the year.

Thus, the cytolological changes found in *Podacris taurica* and *Scincella laterale* significantly differ from those noted in *Agkistrodon piscivorous*. In *A. piscivorous*, the electron-dense secretory granules are absent during the period of regression, but mucoid substances do not take their place. Instead, active production of new secretory granules is occurring in *A. piscivorous* in anticipation of the late summer-fall mating season, which is lacking in *P. taurica* and *S. laterale*.

The first ultrastructural study on a snake was by Kuhnel and Krisch (1974) on *Natrix natrix*. All of Kuhnel and Krisch's specimens were sacrificed in 1 month (November), and they did not describe the reproductive condition of their specimens. They report the presence of large secretory granules with a wide range of opacities, but these granules seem to be of one type in various stages of maturity. Contractile cells with "long thin filaments" were found basal to the RSS, and unmyelinated axons were noted between the epithelial cells and these contractile elements. Like other authors aside from Kuhnel and Krisch (1974), we observed only collagen fibers basal to the RSS in our specimens, and nervous tissue was not conspicuous. Product release was merocrine in *N. natrix*.

Sever et al. (2002) provided the first study at the ultrastructural level of seasonal variation in the RSS of the kidney of a snake, the natricine *Seminatrix pygaea*. They found significant variation in the diameters of RSS in which the diameter is at its peak in March, declines gradually in spring and summer, and begins increasing once more in late summer and fall. Mating occurs only in spring. Despite the variation in diameter, the RSS of *S. pygaea* does not go through an extended period of inactivity, but shows a cycle of synthesis and secretion that can be related to the spermatogenic cycle and mating activity. The synthesis of secretory product is initiated with the onset of spermatogenic activity in the spring and culminates with completion of spermiation in the fall. Apocrine release of the product, however, occurs in a premating period in March when the testes are inactive. Sever et al. (2002) proposed that product release during this premating period is probably necessary to provide time for the passage of the products down the ureter in order to mix with sperm during mating later in spring.

Krohmer (2004) studied seasonal variation in ultrastructure of secretory activity in *Nerodia sipedon,* like *Natrix natrix* and *Seminatrix pygaea*, an aquatic snake in the colubrid subfamily Natricinae. Following the initial hypertrophy at maturity, this snake maintains a level of RSS hypertrophy throughout the year. However, variations in the appearance and makeup of the secretory vacuoles provide an identifiable and quantifiable seasonal pattern that can be correlated with the concentration of plasma androgens. When plasma androgens are high after hibernation, the RSS are filled with solid granules, and the granules become more diffuse as androgen levels decrease. At this time the secretions may be utilized. When androgen synthesis increases later in the summer, solid granules return. Instead of an apocrine mode of secretion, Krohmer (2004) suggested that granules are converted from solid to diffuse and secreted into the cytoplasm, from which they moved into the lumen by merocrine secretion or diffusion. A similar process may be occurring in *Agkistrodon piscivorous*; that is, the small vesicles with diffuse material may arise from the larger, electron dense secretory granules, although we found no direct evidence of such a transformation.

In summary, the other two snakes that have been studied differ greatly from *Agkistrodon piscivorous* and from each other in details of the secretory cycle of the RSS. The main difference, that *Agkistrodon piscivorous* has two peaks of secretory activity whereas the other species have just one, is obviously related to periods of mating activity. *Agkistrodon piscivorous* has peaks of mating activity in spring and late summer/fall, and *Nerodia sipedon* and *Seminatrix pygaea* have mating activity only in spring, when release of RSS products occurs.

We are impressed by the diverse cytology of the RSS discovered so far in the few species that have been examined. Because the RSS occurs, as far as known, in all male squamates (some 7,800 species), the RSS is considered most parsimoniously as an ancestral character for the group, whose origins date from the Middle Jurassic (Pough et al., 1998). As the various clades diverged since that time, one could expect that the cytology of the RSS evolved in concert with differing species specific reproductive adaptations. The challenge in comparative morphology is to uncover the correlations between structure, function and phylogeny, and the RSS of squamates offers fertile ground for further explorations in these regards.

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