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Sperm Aggregations in Female *Agkistrodon piscivorus* (Reptilia:Squamata): A Histological and Ultrastructural Investigation

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ABSTRACT Upon copulation in female *Agkistrodon piscivorus*, sperm migrate up the oviduct to sperm storage tubules (SSTs) in the posterior infundibulum. The epithelium of the SSTs is composed of ciliated and secretory cells and differs ultrastructurally from that of the epithelium lining the lumen of the posterior infundibulum. Sperm pass through an area composed primarily of ciliated cells at the opening of each gland before aligning themselves in parallel arrays with their nuclei facing an area composed primarily of secretory cells at the base of the tubules. Sperm are also found embedded inter- and intracellularly in the SSTs. The secretory vacuoles in the SSTs become highly electron dense after the start of the fall mating season along with the synthesis of lipid droplets. Histochemical analysis reveals that the alteration in secretory material density is caused by the production of neutral carbohydrates. Some sperm remain in aggregates in the nonglandular section of the posterior uterus until the time of ovulation. However, ultrastructural evidence indicates these sperm degrade before ovulation. Therefore, sperm in posterior aggregates have no role in fertilization of ovulated ova. The data presented here support the hypothesis that infundibular sperm storage is the mode that snakes utilize to sequester viable sperm until ovulation. J. Morphol. 000:000–000, 2007.

KEY WORDS: reptilia; serpentes; *Agkistrodon piscivorus*; ultrastructure; oviduct; sperm storage

When ovulation and copulation do not co-occur, sperm storage is necessary to keep sperm viable in the female oviduct until ovulation transpires, a phenomenon common in reptiles (Schuett, 1992). However, descriptions of areas of sperm storage in the female oviduct are limited, especially in squamates (lizards and snakes). Thorough ultrastructure studies in this group are restricted to a few lizards (Conner and Crews, 1980; Girling et al., 1997, 1998; Bou-Resli et al., 1981; Sever and Hamlett, 2002; Sever and Hopkins, 2004) and snakes (Halpert and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999).

In reptiles, sperm storage has undoubtedly evolved independently in different groups (Sever and Hamlett, 2002) and because of this, the mechanism of sperm storage in females seems plastic among different families, genera, and even different species of the same genera. Lizards have been shown to store sperm in the posterior infundibulum, the posterior infundibulum and vagina, and vagina alone, the latter two modes evolving secondarily (Cueller, 1966; Sever and Hamlett, 2002; Sever and Hopkins, 2004), whereas the location of sperm storage in snakes is under much debate (Siegel and Sever, 2006) and will be discussed in detail throughout this manuscript.

The aim of this study is to further the knowledge of sperm storage in female squamates by investigating areas of sperm storage in a viperid snake, *Agkistrodon piscivorus*, which we described briefly in a preliminary report (Siegel and Sever, 2006). The other oviducal histological studies concerning sperm storage in the Viperidae involve *Crotalus viridus* (Ludwig and Rahn, 1943), *Vipera aspis* (Saint-Girons, 1957, 1959), *Cerastes cerastes* (Saint-Girons, 1962a,b), and *Crotalus durrissus* (Almeida-Santos and Salomão, 1997). Snakes from other families investigated with light microscopy are from the Typhlopidae (Fox and Dessauer, 1962), Leptotyphlopidae (Fox and Dessauer, 1962), and Colubridae (Fox 1956; Fawcett et al., 1972; Hoffman and Wimsatt, 1972; Halpert et al., 1982; Aldridge, 1992; Perkins and Palmer, 1996; Sever and Ryan, 1999). In each of the species investigated, sperm storage and sperm storage receptacles were recorded. Descriptions of the ultrastructure of these areas exist only for the colubrids *Thamnophis sirtalis* (Halpert and Wimsatt, 1972), *Diadophis punctatus* (Perkins and Palmer, 1996), and *Seminatrix pygaea* (Sever and Ryan, 1999). Scanning electron microscopy (SEM) analysis was only used in the work on *Diadophis punctatus* (Perkins and Palmer, 1996) and *S. pygaea* (Sever and Ryan, 1999), whereas transmission electron microscopy (TEM) was used in the studies on *T. sirtalis* (Halpert and Wimsatt, 1972) and *S. pygaea* (Sever and Ryan, 1999).

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All of the studies mentioned above identified sperm aggregations in the posterior infundibulum except for three studies on vipers (Ludwing and Rahn, 1943; Nilson, 1981; Almeida-Santos and Salomão, 1997). Ludwig and Rahn (1943), Nilson (1981), and Almeida-Santos and Salomão (1997) recognized the posterior uterus as the site for oviducal sperm storage. However, work by Saint-Girons (1957, 1962a,b) on vipers indicated a uterine aggregation of sperm after mating and a subsequent migration of sperm to infundibular sperm storage tubules (SSTs) before ovulation. Siegel and Sever (2006) also observed this posterior aggregation during the mating season of *Agkistrodon piscivorus* and, similar to the reports of Saint Girons (1957, 1962a,b), infundibular SSTs filled with sperm shortly after mating commenced. Siegel and Sever (2006) explained this sperm aggregation in the posterior uterus as an artifact of recent mating activity. Uterine sperm aggregations are not restricted to the Viperidae and have been recorded in members of the Colubridae (Halpert et al., 1982; Perkins and Palmer, 1996; Sever and Ryan, 1999). Another hypothesis for posterior oviducal sperm aggregations is based upon a nongelatinous copulatory plug (Andrén and Nilson, 1987). Secretions from the renal sexual segment (RSS) of males cause a contraction in the uterus and thus decrease the lumen size of the posterior oviduct and trap sperm from anterior movement (Andrén and Nilson, 1987). This proposal was critically questioned in work by Stille et al. (1986) and Stille and Niklasson (1987).

Agkistrodon piscivorus is a viviparous snake common in the coastal plain of the southeastern United States and consists of three subspecies. The particular subspecies examined in this study was *A.p. leucostoma*, the Western Cottonmouth, which ranges from western Alabama to Texas, and North to Illinois and Indiana (Conant and Collins, 1998). The majority of *A. piscivorus* females have been found to be biennial breeders (Burkett, 1966; Wharton, 1966; Ford, 2002, 2004) and begin copulation in the late summer to early fall (Beyer, 1893), a phenomenon that can take up to 3 h (Allen and Swindell, 1948). Wharton (1966) states that bisexual pairs of cottonmouths can be found in every month of the year except January; however, actual copulatory events for these sightings were not observed. Like other temperate pitvipers, vitellogenesis begins in the late summer and then halts for the months of hibernation (Aldridge and Duvall, 2002). Vitellogenesis starts again in the spring, when a second mating season begins, and continues until the ovarian follicles are ready for ovulation (Burkett, 1966). Embryos can then be found in the uterus until parturition (Penn, 1943; Burkett, 1966), which takes place in the late/summer to early fall of the year following the initial summer/fall mating (Beyer, 1893; Conant, 1933; Allen and Swindell, 1948; Wharton, 1960; Funk, 1964; Burkett, 1966; Gloyd and Conant, 1990; Ford, 2002, 2004).

Agkistrodon piscivorus makes a perfect model for the first investigation with ultrastructural analysis of sperm aggregations in the oviduct of a viper because of their overall high abundance compared with the majority of North American viperids. Other vipers in the United States have been on decline for many years (Galligan, 1979; Adams et al., 1994; Rudolph and Burgdorf, 1997; Christiansen, 1998; Platt et al., 1999) whereas *A. piscivorus* still flourishes within its range. Light, TEM, and SEM were used in concert to fully examine sperm aggregations in *A. piscivorus*, following the framework provided by Sever and Ryan (1999).

MATERIALS AND METHODS

Specimens

Agkistrodon piscivorus (Lacépède, 1789) females were collected from every month of the year from three localities (see Table 1 for collection dates); the Amite River Diversion Canal (North 30°22.616/West 090°68.506, Livingston Parish, LA), Turtle Cove Environmental Research Station on Pass Manchac (North 30°29.426/West 090°35.592, Tangipahoa Parish, LA), and the private residence of Dr. Clifford Fontenot (10 km Northwest of New Albany, North 30°30.871/West 090°36.202, Livingston 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 3 days before the time of sacrifice. Water was available to these specimens ad libitum.

Specimens were sacrificed by means of a 0.2–0.5 cc intraperitoneal injection of sodium pentobarbital (1 g sodium pentabarbitol in 10% alcohol, 40% propylene glycol solution). The left side of the reproductive tract was removed and fixed in 10% neutral buffered formalin solution (NBF), for no less than 48 h, for light microscopy. The right oviduct was removed and fixed in Trump's fixative, 2.5% glutaraldehyde, and 2.5% formaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 (Electron Microscopy Sciences, Fort Worth, PA), for no less than 48 h, for examination with transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Light Microscopy Methods

Tissues fixed in NBF were rinsed for 1 h in tap water and dehydrated in a graded series of ethanol (70%, 95%, for 1 h each, 100%, two cycles for a 30 min each). Tissues were then placed in toluene (two cycles for 30 min each) and subsequently embedded in paraffin blocks for sectioning with a MR3 microtome (Research and Manufacturing Co., Tucson, AZ). Sections of 10-μm thick were cut and affixed to albumenized slides. Alternate slides were then either stained with hematoxylin–eosin (H&E) for general histological examination, Alcian blue 8GX at pH 2.5 (AB) for carboxylated glycosaminoglycans and the periodic acid-Schiff procedure for neurtral carbohydrates (PAS), or bromophenol blue (BB) for proteins. All 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 Mircosystems, Wetzlar, Germany). Images were downloaded directly to Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA), which was used for editing and printing for all methods.

TABLE 1. Specimens examined, snout-vent length, follicle number and diameter (mm), and sperm abundance

Month	SVL	Follicle No.	Mean follicle diameter[c]	Maximum follicle diameter	Sperm in Avag	Sperm in Ngu	Sperm in Gu	Sperm in SSTs
June	511	10	3.3	4.2	0	0	0	0
June	591	8	4.5	6.1	0	0	0	0
July	603	10	3.8	5	+	++	0	0
July	595	10	4.2	6.1	+	++	0	0
August	639	7	4.1	6.3	0	0	0	0
August	625	8	4.9	6.7	+	++	0	0
August	473	NA	NA	NA	+	++	0	0
September	543	8	3.3	4.2	0	0	0	0
October	587	5	8.7	9.5	+	++	0	+
November	500	10	5.9	7.9	+	++	+	+
November	623	10	7.3	11.2	+	++	0	++
February	517	7	5.1	8.6	0	0	0	0
March	636	11	18.2	23.1	+	++	0	+
April	678	6	15.2	17.2	++	++	−	++
April	601	3	12.3	14	0	0	0	0
May	563	3	33.3	35.7	0	++	−	++
August[a]	NA	NA	NA	NA	0	0	0	+
October[b]	563	7	3.4	6.7	0	0	0	+
March[b]	577	10	4.2	6.7	0	0	0	+
May[b]	555	23	2.9	5.9	0	0	0	−

Avag, anterior vagina; Gu, glandular uterus; Ngu, nonglandular uterus; SSTs, sperm storage tubules; SVL, snout-vent length; (−) scant sperm; (+) sperm; (++) abundant sperm.
[a]Denotes gravid female.
[b]Denotes postpartum females.
[c]Mean follicle diameter only includes vitellogenic follicles when present.

Transmission Electron Microscopy Methods

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 oxide, 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 TEM (JEOL USA, Peabody, MA).

Scanning Electron Microscopy Methods

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 (Denton Vacuum, Moorestown, NJ) and sputter coated with a Denton Desk IV XLS (Denton Vacuum, Moorestown, NJ). Finally, the tissues were viewed with a Philips XL-20 SEM (Philips Electronics N.V., Eindoven, Netherlands).

RESULTS

Female Reproductive Cycle

Anecdotal observations of females in the field and the lab confirm all previous studies on the biennial reproductive cycle of *Agkistrodon piscivorus*. The start of the summer/fall mating season is indicated by the appearance of sperm in the posterior oviduct in July. Ovarian follicles begin reaching a noticeably larger size by October and continue growth in the spring upon surfacing from winter dormancy. Sperm are also found in the posterior oviduct of females in March, which marks the beginning of the spring mating season. Follicles reach a maximum size in May at which time reproductively active females disappear and most likely head to protective under ground cover for a gestation period lasting until parturition in late August to early September. At this time neonates are very abundant in areas with high *A. piscivorus* densities (see Table 1 for sperm presence and follicle size).

Light Microscopy

Blackburn (1998) identified three regions of the oviduct in squamate reptiles: a posterior vagina, middle uterus, and anterior infundibulum. However, in *A. piscivorus* females, we divide the oviduct into four distinct histological regions, consistent with the findings of Halpert et al. (1982) in colubrids and Siegel and Sever in viperids (2006): a posterior vagina, nonglandular uterus, glandular uterus, and an anterior infundibulum (Fig. 1). The entire vagina is greatly enlarged in viperids compared with colubrids and is referred to here as the vaginal pouch.

The vaginal pouch is characterized by a very thick muscularis and a mucosa layer with a simple columnar epithelial layer surrounding an enlarged luminal opening (Fig. 2A). The vaginal epithelium contains abundant secretory granules which stain PAS+, AB+, and BB− throughout the year. The muscularis comprises a layer of smooth longitudinal

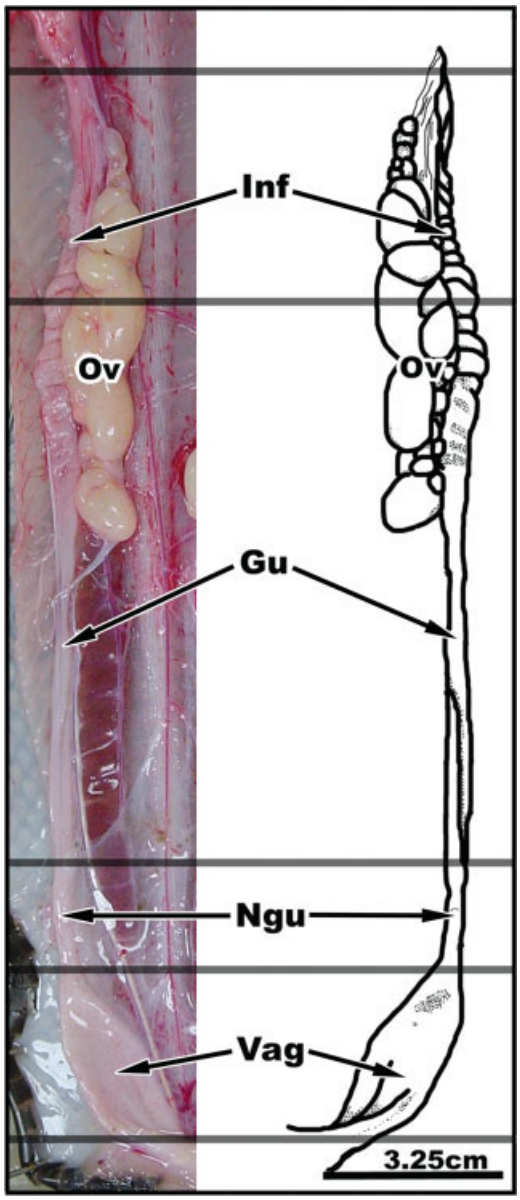

Fig. 1. The four recognized regions of the female *Agkistrodon piscivorus* oviduct discussed in this study. Gu, glandular uterus; Inf, infundibulum; Ngu, nonglandular uterus; Ov, ovary; Vag, vagina.

muscle surrounding a layer of circular muscle encompassing the mucosa (Fig. 2A). The orientation of the smooth circular and longitudinal muscle layers remains constant the entire length of the oviduct. Small amounts of sperm associated with a PAS+/BB+ material can be found occasionally at the most anterior portion of the vaginal pouch during the late summer/fall mating season and after hibernation, when a second mating season begins (Fig. 2B; Table 1). The vaginal pouch and the rest of the oviduct are connected to the body wall by a dorsal mesentery that attaches to the visceral pleuroperitoneum that surrounds the muscularis of the oviduct. Tissue-bound granulocytes are also very abundant in the mucosal layer of the vaginal pouch. These granulocytes become less abundant when moving anteriorly toward the infundibulum. The lumen of the vaginal pouch is very large and represents the largest luminal area in the entire nongravid oviduct.

The section of oviduct immediately anterior to the vaginal pouch is the nonglandular section of the uterus, which has been referred to as the furrowed section of the uterus in other studies on colubrids (Halpert et al., 1982; Aldridge, 1992). This segment is identified as uterus, because developing embryos invade this area during gravidity. Histologically, this portion of the oviduct is unique in that the exterior longitudinal muscle layer is very thick, and the mucosa has steep longitudinal folds comprising rugae (Fig. 2C). The lumen dramatically decreases in size compared with that of the lumen of the vaginal pouch and the simple columnar epithelium that lines the lumen becomes more cuboidal anteriorly through this region. This epithelial layer secretes an AB+ material throughout the year. Sperm are abundant between the rugae of this region during mating activity and can be found in variable amounts until the time of ovulation (Fig. 2D; Table 1). Occasionally sperm can be found associated with a PAS+/BB+ matrix, and tissue bound granulocytes are abundant in the lamina propria.

The glandular section of uterus is located directly anterior to the nonglandular portion. This segment of oviduct has an increased luminal area compared with that of the nonglandular uterus. Simple tubular glands formed by invaginations into the mucosa dominate this region (Fig. 2E,F), and the epithelium of the tubular glands and luminal epithelium is cuboidal. The luminal epithelium of the uterus constantly secretes an AB+ material like that of the nonglandular uterus, whereas the uterine glands secrete a PAS+/BB+ material shortly after the fall mating season ensues. Secretory activity in these glands ceases after parturition. The muscularis of the glandular uterus is a thinner layer compared with that of the nonglandular uterus and longitudinal folds cease as the luminal area increases in size compared to that of the nonglandular uterus. Small amounts of sperm can be found in the most posterior area of the glandular uterus during mating activity (Fig. 2F; Table 1).

The infundibulum consists of a very thin muscularis and mucosal layer and is dominated by abundant infundibular glands throughout. The luminal epithelium of this area is simple cuboidal, and the lumen decreases dramatically in size compared with that of the uterus. The most posterior portion of the infundibulum contains unique infundibular glands that are larger than those found more anteriorly (Fig. 2G). These glands contain abundant

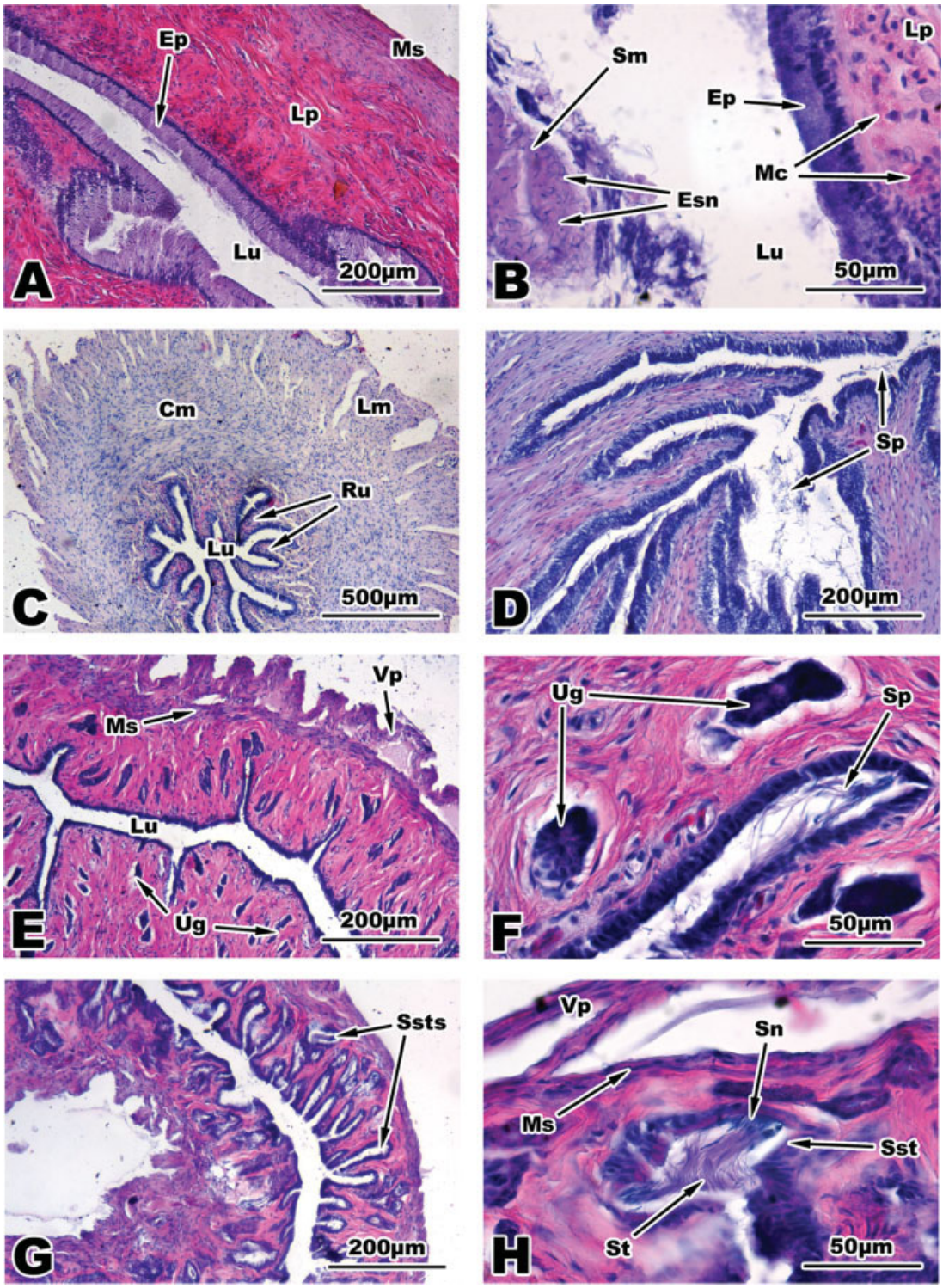

Fig. 2. Histological analysis of the oviduct in female *Agkistrodon piscivorus* (stained with H&E). **A:** Transverse section of an April female showing the histology of the vaginal pouch. **B:** Sagittal section of the vaginal pouch of a July female illustrating sperm imbedded in an eosinophilic secretory material. **C:** Transverse section through a June female's nonglandular uterus revealing unique characters of the region. **D:** The presence of sperm in a sagittal section of the nonglandular uterus from a May female. **E:** Transverse section of a November female revealing the histology of the glandular uterus. **F:** The presence of sperm in the most posterior portion of the glandular uterus in a transverse section from a female sacrificed in November. **G:** Sagittal section of the posterior infundibulum of a November specimen demonstrating the presence of numerous sperm receptacles. **H:** Higher magnification of **G** showing the orientation of sperm in a sperm receptacle. Cm, circular muscle; Ep, epithelium; Esn, embedded sperm nuclei; Lm, longitudinal muscle; Lp, lamina propria; Lu, lumen; Mc, mast cells; Ms, muscularis; Ru, rugae; Sm, secretory material; Sn, sperm nuclei; Sp, sperm; St, sperm tail; Ug, uterine gland; Vp, visceral pleuriperotoneum.

amounts of sperm before the onset of the late summer/fall mating season in preovulatory, postovulatory, and in postpartum specimens (Fig. 2H; Table 1). Shortly after the beginning of the late summer/fall mating season, these sperm storage tubules (SSTs) begin secreting a PAS+ material coincident with the presence of sperm, which can be observed until ovulation. The SSTs also secrete a BB+ material that is not associated with the reproductive cycle or the presence of sperm. The luminal epithelial cells secrete an AB+ material throughout the year until the month of ovulation (May) in which the secretions become PAS+. The tubules are not empty of sperm until the onset of the next mating season; however, the secretions in the SSTs cease after ovulation.

The cytological analysis above indicates two areas of abundant sperm aggregations; the SSTs of the posterior infundibulum and the lumen of the nonglandular uterus, in both of which sperm aggregates from the onset of mating until at least the timing of ovulation. Even though sperm can be found at the anterior vagina and the most posterior portion of the glandular uterus, the concentrations of sperm in these regions are minute and are likely to be artifacts of mating activity and anterior sperm migration. The remainder of this investigation will focus on the SSTs of the posterior infundibulum and the nonglandular uterus. A full ultrastructural investigation of the entire oviduct of *Agkistrodon piscivorus* including seasonal variation will be explored in future work.

Electron Microscopy

Posterior infundibulum. Sperm in the posterior infundibulum enter simple tubular glands, SSTs, which are occasionally branched and are lined with a simple layer of cuboidal epithelium. This cuboidal epithelium of the SSTs is consistent with that of the epithelium lining the lumen in that it is composed of ciliated and secretory cells (Fig. 3A) with basally located nuclei that generally have sparse, scattered chromatin (Fig. 3B). Apically, the epithelial cells are joined by tight junctions followed by desmosomes (Fig. 3B). Secretory cells of the posterior infundibulum are covered by microvilli, contain scattered mitochondria, and Golgi complexes can be viewed in a paranuclear position in the epithelial cells (Fig. 3B). The ciliated cells of this region are filled with small mitochondria in association with basal bodies anchoring cilia. The SSTs have some obvious ultrastructural characters that distinguish them from just being invaginations of the luminal epithelium. One of these specializations is the presence of smooth endoplasmic reticulum and basally positioned lipid droplets in the secretory epithelial cells shortly after the fall mating season has begun (Fig. 3C). The distribution of ciliated and secretory cells is also more structured in SSTs compared with that of the random orientation of these cell types in the epithelium lining the lumen. The opening of a SST is composed primarily of ciliated cells (Fig. 4A) and the concentration of these ciliated cells decreases (Fig. 4B) when moving to the terminal end of the gland, where secretory cells dominate (Fig. 4C).

Sperm enter each SST through a densely ciliated region and then orient with their nuclei facing the secretory cells found at the distal end of the gland (Fig. 4C). As sperm move from the entrance of a SST to the base, they become more ordered and are aligned in parallel arrays next to the secretory vacuoles (Figs. 4C, 5A). Sperm are associated with a carrier matrix material (Fig. 5A), which occasionally contains cellular debris. Product release is merocrine in the SSTs and all other secretory cells in the infundibulum. The secretory material in the SSTs and luminal secretory vacuoles is electron lucent in the fall and becomes electron dense in the spring after mating, concordant with the appearance of numerous Golgi complexes and mitochondria. The histochemistry of these secretions, however, does not change from the fall to the spring, except for the luminal epithelium, which becomes increasingly PAS+ in the spring before ovulation (AB+ the rest of the year). No secretory activity is evident in the SSTs when sperm are not present; however, secretory activity in the epithelium lining the lumen occurs throughout the nonreproductive and reproductive season.

Not only can sperm be found oriented neatly in SSTs in the fall and spring, sperm can also be found embedded within the epithelium (Fig. 5B,C). This can occur by sperm embedding inter- or intracellularly. Intercellular embedment of sperm occurs in complex, interdigitating canaliculi between cell membranes (Fig. 5B). Sperm can be found in these intercellular canaliculi as well as spaces between the basal lamina and the epithelium. Intracellular embedment occurs by sperm directly entering into the epithelial cells (Fig. 5C). Intracellular embedment was only observed occurring in secretory cells of SSTs. Sperm were never found embedded in the epithelium lining the lumen of the infundibulum.

Nonglandular uterus. The epithelium of the nonglandular section of uterus also consists of a simple layer of alternating secretory cells and ciliated cells (Fig. 6A). Ciliated cells in this region contain apically positioned euchromatic nuclei (Fig. 6A) and are otherwise ultrastructurally identical to those in the posterior infundibulum. The secretory cells contain secretory vacuoles varying in electron density and basally located euchromatic nuclei (Fig. 6A). Golgi complexes are paranuclear, and mitochondria are scattered throughout the cytoplasm of the secretory cells. Complex intercellular canaliculi exist between both ciliated and secretory cells with tight junctions apically

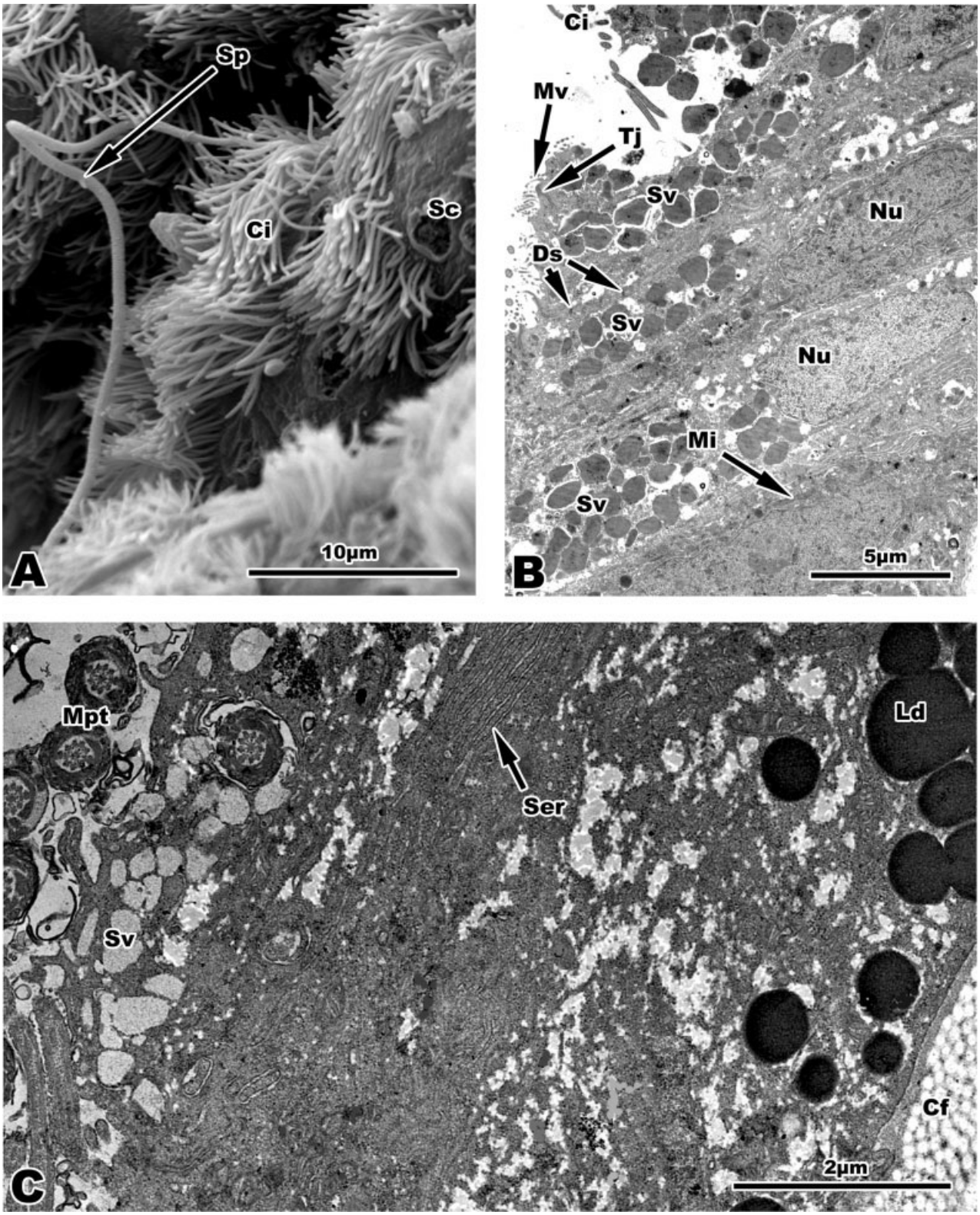

Fig. 3. Ultrastructure of the epithelium lining the lumen and the SSTs of the posterior infundibulum in female *Agkistrodon piscivorus*. **A:** The epithelium lining the lumen showing alternating ciliated and secretory cell and sperm in an October female. SEM. **B:** The epithelium lining the lumen in a May female exhibiting ultrastructure and cytoplasmic contents. TEM. **C:** Epithelial cells in a SST of an October female showing differing cytoplasmic contents compared with the epithelium lining the lumen. TEM. Ci, cilia; Ds, desmosomes; Ld, lipid droplets; Mi, mitochondria; Mpt, middle piece of sperm tail; Mv, microvilli; Nu, nucleus; Sc, secretory cell; Ser, smooth endoplasmic reticulum; Sp, sperm; Sv, secretory vacuoles; Tj, tight junction.

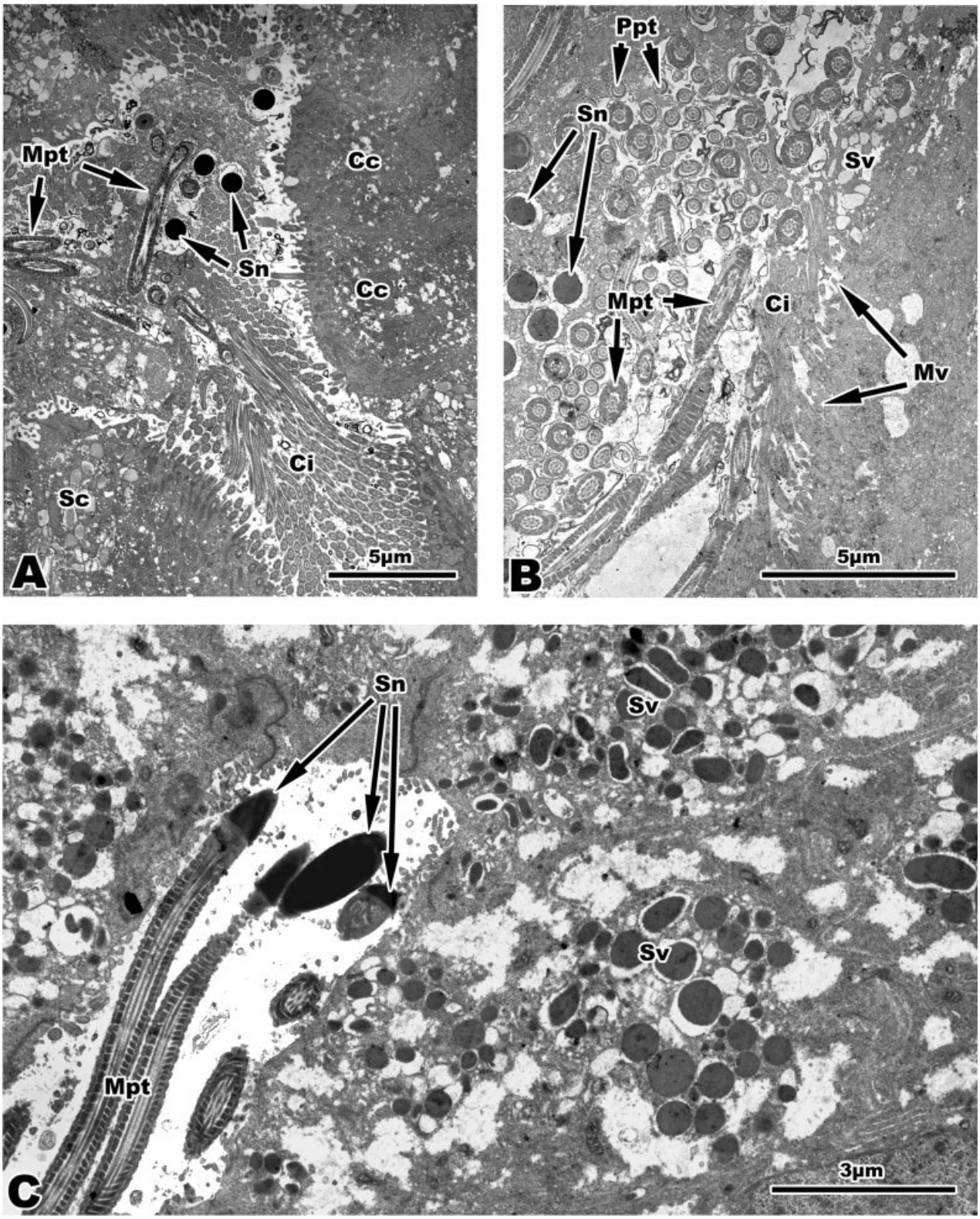

Fig. 4. Ultrastructure of the SSTs of female *Agkistrodon piscivorus*. **A:** Proximal portion of a SST (in terms of lumen) from an October female illustrating the high density of cilia at this region and unordered alignment of sperm. **B:** Middle portion of a SST in an October specimen showing the decrease in ciliated cells. **C:** SST of a May female showing ordered alignment of sperm at the most distal portion (in terms of the lumen) of the SST with sperm nuclei facing secretory cells, which dominate this area. Cc, ciliated cell; Ci, cilia; Sc, secretory cell; Mpt, middle piece of the tail; Mv, microvilli; Ppt, principle piece of the tail, Sn, sperm nuclei.

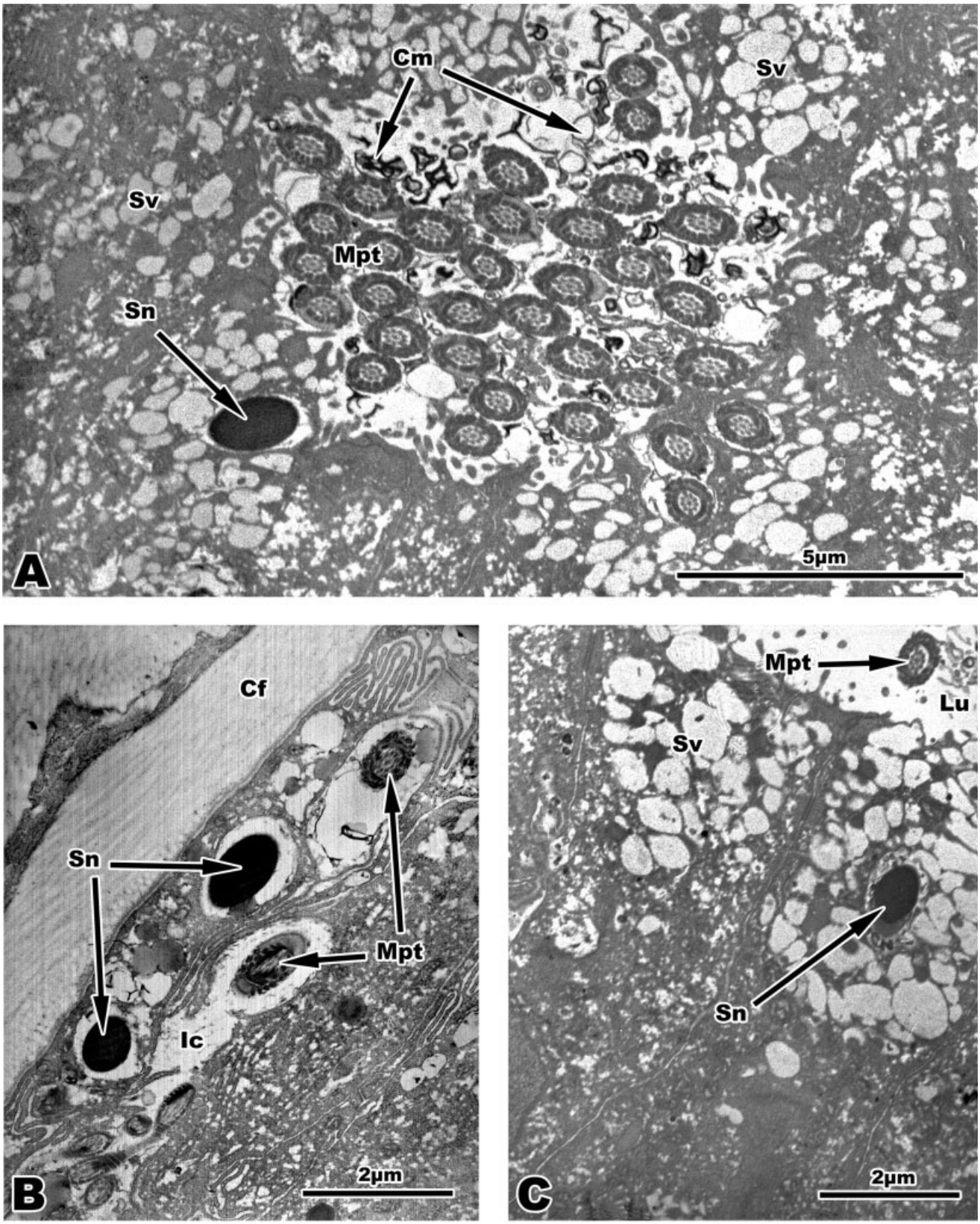

Fig. 5. Ultrastructure of sperm orientation and embedment in SSTs in female *Agkistrodon piscivorus*. **A:** Parallel array of sperm associated with a carrier matrix in a SST of an October specimen. **B:** Intercellular embedment of sperm in an October female. **C:** Intracellular embedment of sperm in an October female. Cf, collagen fiber; Cm, carrier matrix; Ic, intercellular canaliculi; Lu, lumen; Mpt, middle piece of sperm tail; Sn, sperm nuclei; Sv, secretory vacuoles.

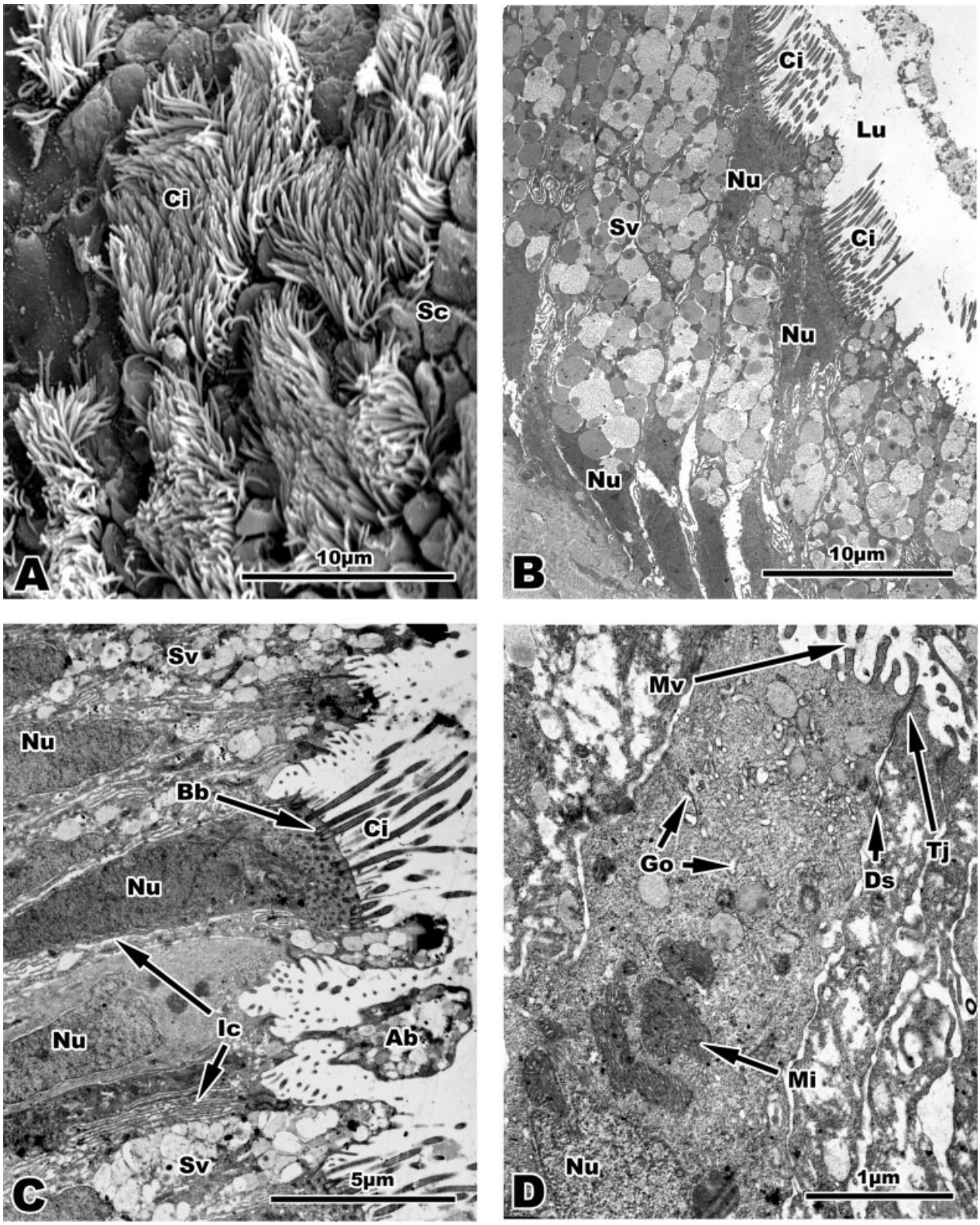

Fig. 6. General ultrastructure and seasonal variation in the nonglandular uterus of female *Agkistrodon piscivorus*. **A:** The surface of the epithelium in an October female showing alternating ciliated and secretory cells. SEM. **B:** Overview of the epithelium lining the lumen in an October female exhibiting cell composition and cytoplasmic contents. TEM. **C:** Increased secretory activity of the nonglandular uterus in spring females (May female shown). **D:** Abundance of synthetic organelles in secretory cells of the epithelium in a May female. TEM. Ab, apocrine bleb; Bb, basal bodies; Ci, cilia; Ds, desmosome; Go, Golgi complex; Ic, intercellular canaliculi; Lu, lumen; Mi, mitochondria; Mv, microvilli; Nu, nucleus; Sc, secretory cell; Sv, secretory vacuoles; Tight junction.

and desmosomes connecting these two different cell types. Plasma-like cells filled with rough endoplasmic reticulum and large mitochondria invade the intercellular canaliculi in this region, and mast cells are abundant in the lamina propria. The longitudinal folds (rugae) of this region, which make up the majority of the lining of the lumen, are not differentiated along their lengths. The secretory cells of the luminal epithelium are similar in regard to ultrastructure all year round, and the only significant variation throughout the reproductive season occurs in the spring when the secretion rate increases. The increase in secretory activity is determined by the occurrence of secretory cells with varying amounts of secretory material, and cells actively producing apocrine blebs (Fig. 6C). Cells with decreased amount of secretory vacuoles exhibit enlarged mitochondria and well defined Golgi complexes, which indicates rapid secretory product synthesis (Fig. 6D). Before and during the start of the fall mating season some epithelial cells contain lipofuscin while others can be found desquamating from the basal lamina.

Sperm are found in the nonglandular section of uterus in nonorderly, luminally located, tangled masses (Fig. 7A,B). Even though complex intercellular canaliculi exist between ciliated and secretory cells of this region, no sperm are ever found embedded inter- or intracellularly. Ultrastructural analysis also confirms the presence of large amounts of secretory material and cellular debris associated with the sperm in the nonglandular uterus (Fig. 7C,D). This secretory material consists of cellular material including cell organelles and nuclei (Fig. 7D).

Ultrastructurally, sperm in the posterior aggregations look identical to those found in the infundibulum. However, a late spring, preovulatory female, showed unusual myelinic figures abundant in intercellular canaliculi and on the surface of the epithelium of this region (Fig. 8A). Upon closer investigation, the majority of sperm in the nonglandular uterus during this time period possess thinning fibrous sheaths, unusual axonemes, and are covered with myelinic figures (Fig. 8B,C).

DISCUSSION

The Posterior Infundibulum and Sperm Storage Tubules

Sperm storage in infundibular SSTs is considered the ancestral state of sperm storage in squamates (Sever and Hamlett, 2002). Ultrastructurally and cytologically, SSTs in the viperid *Agkistrodon piscivorus* are similar to those observed in colubrids previously studied histologically and cytologically (Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999). Hypertrophy occurs in the infundibular SSTs in postcopulatory, previtellogenic specimens, and secretions staining PAS+ for neutral buffered carbohydrates are abundant with the arrival of sperm. These conditions also occur in the colubrids previously investigated (Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999). The SSTs in *A. piscivorus* also secrete a BB+ material (for proteins) at different times of the year; however, this secretion does not seem to be activated by the presence of sperm. In nonreproductive females, the SSTs are inactive.

Similar to the SSTs in *S. pygaea* (Sever and Ryan, 1999), those in *Agkistrodon piscivorus* are simple tubular glands that are composed of epithelial cells that are not significantly differentiated from the luminal epithelium. However, in *A. piscivorus*, abundance of secretory cells at the distal portion of the SSTs does seem to increase whereas ciliated cells tend to dominate the ducts leading to the secretory portions of the glands. This condition also occurs in SSTs of the colubrid *Thamnophis sirtalis* (Hoffman and Wimsatt, 1972) and in sperm receptacles in some lizards (Bou-Resli et al., 1981; Sever and Hopkins, 2004).

Sperm orientation within the SSTs of *Agkistrodon piscivorus* is similar to that observed in the snakes *Thamnophis sirtalis* and *Diadophis punctatus* where sperm are aligned with their nuclei facing the distal, secretory portion of a SST (Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996), which was also observed in the lizards *Acanthodactylus scutellatus* and *Scincella laterale* (Bou-Resli et al. 1981; Sever and Hopkins, 2004). These observations differ from those observations on *S. pygaea*, in which the opposite orientation (sperm nuclei facing the lumen) was noted by Sever and Ryan (1999). In *A. piscivorus*, embedded sperm were found intra- and intercellularly, the latter having also been found in *Thamnophis sirtalis* (Hoffman and Wimsatt, 1972) in the distally located secretory portion of a SST. However, spermiophagy (the intercellular phagocytosis of sperm) was not observed, and the fertilization capability of the embedded sperm is unknown. Sever and Brizzi (1998) proposed that embedded sperm never escape and therefore have no input toward fertilization in plethodontid salamanders. Sever and Hopkins (2004) found embedded sperm in secretory cells of SSTs in *Scincella laterale* that were closely associated with primary lysosomes, which could indicate imminent phagocytosis.

After ovulation, sperm in *Diadophis punctatus* disappear from SSTs (Perkins and Palmer, 1996) whereas in *Agkistrodon piscivorus*, sperm remain through gravidity, after parturition, and up to the beginning of the next reproductive season (Siegel and Sever, 2006). This phenomenon could indicate that SSTs in vipers have not evolved a mechanism to evacuate sperm and thus rely on degradation of sperm because of their natural aging. The occurrence of a biennial breeding cycle in *A. piscivorus*

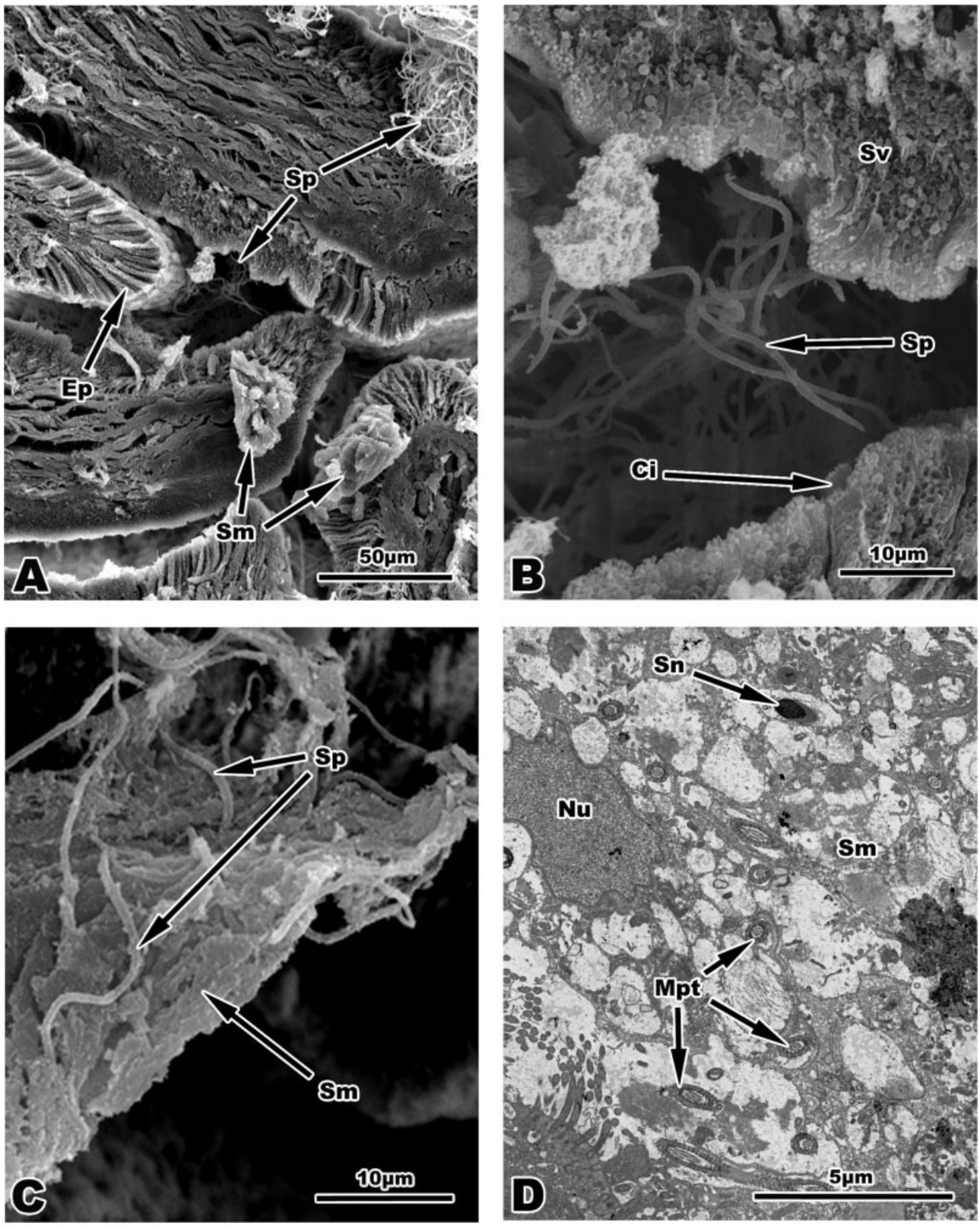

Fig. 7. Sperm presence in the nonglandular uterus of *Agkistrodon piscivorus* females. **A:** Overview of a transverse cut through the nonglandular uterus in an August female. SEM. **B:** Higher magnification of **A** showing sperm orientation in the nonglandular uterus. **C:** Higher magnification of **A** demonstrating sperm relationship to secretory material. **D:** Sperm relationship to secretory material in an October female. TEM. Ci, cilia; Ep, epithelium; Mpt, middle piece of tail; Nu, nucleus; Sm, secretory material; Sn, sperm nuclei; Sp, sperm; Sv, secretory vacuoles.

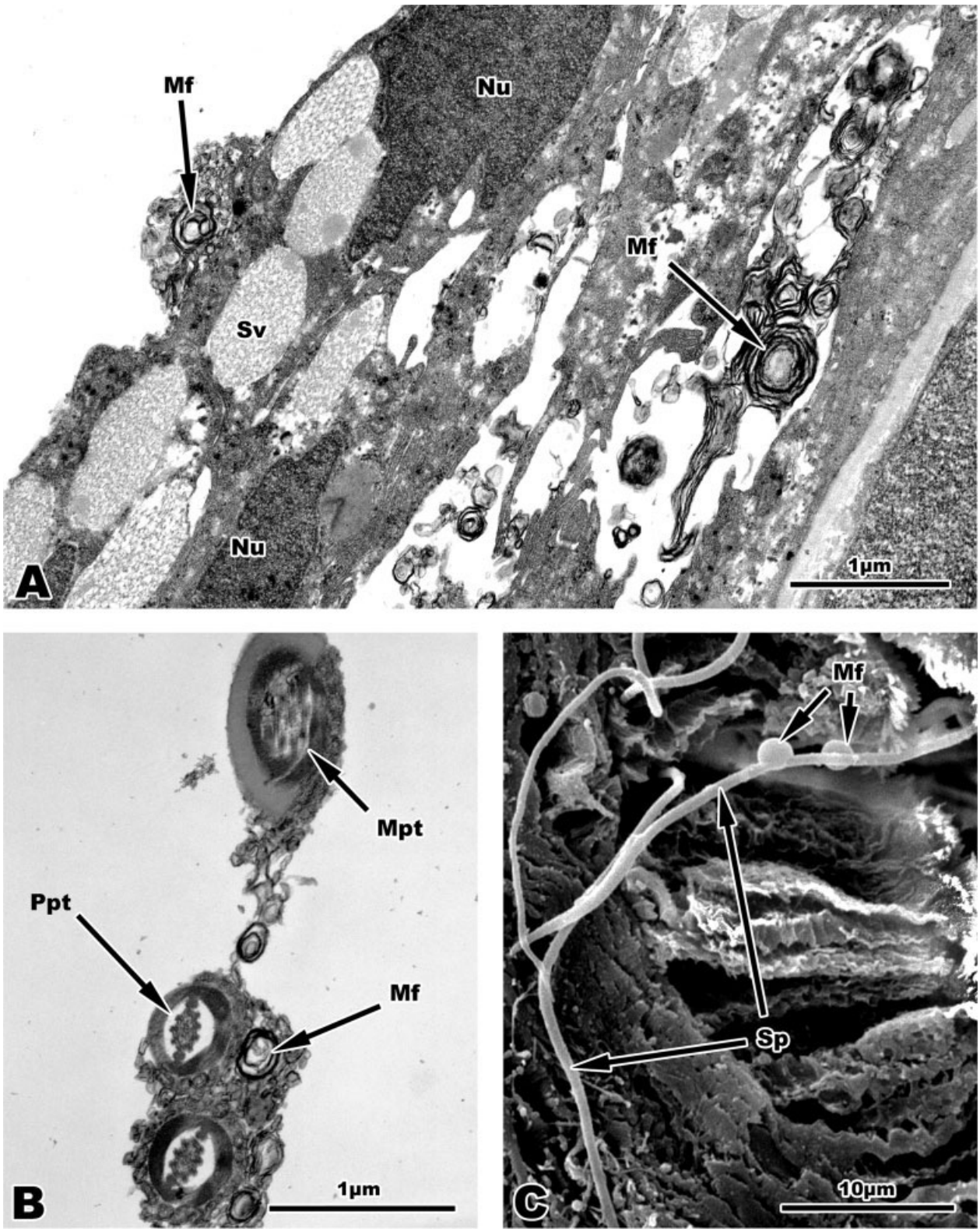

Fig. 8. Sperm degradation in the nonglandular uterus in the spring time of female *Agkistrodon piscivorus*. **A:** The presence of myelinic figures intercellularly and on the surface of the epithelium in a May female. TEM. **B:** Myelinic figures associated with sperm in a May female. TEM. **C:** SEM of **B** indicating sperm association with myelinic figures. Mpt, middle piece of sperm tail; My, myelinic figures; Nu, nucleus; Ppt, principle piece of sperm tail; Sp, sperm; Sv, secretory vacuoles.

females, and thus an extended period between reproductive activities, adds support to this hypothesis because a longer period is present before new sperm need to fill the SSTs.

The result of sperm degrading in other portions of the oviduct not containing SSTs and the eventual degradation of sperm in SSTs not actively producing a secretory product, without the presence of spermiophagy, leads us to believe that SSTs in *Agkistrodon piscivorus* may not only function in sperm protection (Fox, 1956; Aldridge, 1992; Hoffman and Wimsatt, 1972; Sever and Ryan, 1999) but also function in supplying sperm with nutrition. This hypothesis contradicts earlier beliefs that SSTs in snakes are functioning only in mechanical protection of sperm (Aldridge, 1992). Although not proven in snakes, the possibility of sperm receptacles providing nourishment has been hypothesized to occur in studies on salamanders (Dent, 1970) and lizards (Cuellar, 1966), and one study on snakes leaned toward the possibility of sperm nourishment in SSTs (Hoffman and Wimsatt, 1972). Sever (2003), however, has rejected the notion that sperm stored by female salamanders are nourished or even need nourishment. The idea that secretions in sperm receptacles function in the attraction of sperm was also presented in work on salamanders by Dent (1970).

SSTs in snakes have not been found to be bordered by contractile elements (Hoffman and Wimsatt, 1972; Blackburn, 1998; Sever and Ryan, 1999), and thus contraction of SSTs does not expel sperm into the lumen for fertilization and instead the pressure from ovulated eggs functions in ovum–sperm association (Fox, 1956; Hoffman and Wimsatt, 1972; Sever and Ryan, 1999). The finding of no contractile elements around SSTs in snakes was confirmed in *Agkistrodon piscivorus*. If pressure causes the interaction between sperm and ovum, the orientation of sperm in the SSTs would not matter, so the reason for differing sperm orientations between *A. piscivorus* and *S. pygaea* needs further investigation. Hoffman and Wimsatt (1972) suggest ciliary action might aid in the expulsion of sperm from SSTs. The observations presented here of high concentrations of ciliated cells at the opening of SSTs support the hypothesis of cilia involvement in moving sperm. However, this high concentration of cilia could be instrumental in propelling sperm into the SSTs and not just expulsion.

In conclusion, SSTs in snakes are found in the posterior infundibulum (Fox, 1956; Saint-Girons, 1957, 1959, 1962a,b, 1973; Fox and Dessauer, 1962; Fawcett et al., 1972; Hoffman and Wimsatt, 1972; Halpert, et al., 1982; Aldridge, 1992; Perkins and Palmer, 1996; Blackburn, 1998; Sever and Ryan, 1999; Sever and Hamlett, 2002; Siegel and Sever, 2006) and secrete a PAS+ material indicating neutral carbohydrates during sperm presence (Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999). Although the exact function of these carbohydrate secretions is unknown, their high densities at the time sperm enter a SST leads us to believe that they function either as an attractant to sperm, a source of nutritional supplement, or both. Other secretions (proteins and lipids) are also synthesized in SSTs of snakes (Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999); however, their presence does not seem to mirror the presence of sperm and thus their function is unknown in *Agkistrodon piscivorus*. The SSTs, themselves, seem to be variable in gland type between and within families, with tubular and alveolar glands being identified in colubrids and vipers (Aldridge, 1992; Perkins and Palmer, 1996; Sever and Ryan, 1999; Aldridge, personal communication). Further investigation, with the use of cytological, ultrastructural, and physiological techniques, in even more varieties of snakes is required to describe the complete function and the evolutionary trends of SSTs in snakes. Historical studies on SSTs in snakes, however, along with analysis presented here, leads us to believe that sperm storage location might not be as variable in snakes as is in lizards, but the degree of specialization of SSTs could be variable within and between squamate families.

The Nonglandular Uterus

Sperm aggregations in the nonglandular section of the uterus have been recorded in viperid snakes many times (Ludwig and Rahn, 1943; Saint-Girons, 1957, 1962a,b; Almeida-Santos and Salomão, 1997; Siegel and Sever, 2006). However, the cause and function of these aggregations are not well known. Almedia-Santos and Salomão (1997, 2002) hypothesized that this area is a site of sperm storage in viperids where sperm are held by means of utero-muscular contractions until the timing of ovulation. However, Siegel and Sever (2006) propose that this region is not the primary site of sperm storage in viperids because of their identification of anterior infundibular SSTs in the viperids *A. piscivorus*, *Sistrurus miliarius*, and possibly *Crotalus durissus*.

The results of the ultrastructural study presented here support the hypothesis proposed by Siegel and Sever (2006). Although sperm are found in the posterior oviduct before ovulation, seasonal variation of the epithelium lining the lumen does not exhibit specialization with the presence of sperm and sperm begin undergoing degradation before ovulation. The phenomenon of active sperm degradation in areas other than that of specific sperm storage receptacles is not a new observation and has been recorded in higher level organisms, such as bats, in which sperm are actively phagocytosed in the posterior oviduct while sperm are stored in receptacles of the utero-tubal junction

(Mōri and Uchida, 1980). No sperm phagocytosis or association with macrophages was observed in this study, so the means by which these sperm are degrading remain unknown. However, in a study on salamanders, myelinic figures were also associated with sperm while spermiophagy was occurring (Sever and Kloepfer, 1993). Even though spermiophagy was not observed in preovulatory female *A. piscivorus* from early May, the likelihood that it is occurring at some level cannot be ruled out. With this being the case, sperm found in the posterior region of the oviduct would have no input toward fertilization in a given reproductive year and therefore, the posterior region of the oviduct should not be referred to as an area of sperm storage but should instead be described as an area of sperm presence.

Glycosaminoglycan secretions indicated by AB+ histochemistry were the only secretions observed in the nonglandular uterus, so specialization of secretions for creating an environment suitable for sperm quiescence (usually indicated by PAS+ secretions in snakes; Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999) does not occur. Also, seasonal variation in the ultrastructure of the nonglandular uterus does not mirror the presence of sperm. That observation and the fact that sperm degrade in this region of the oviduct before ovulation lead us to believe that the nonglandular uterus is not capable of viably storing sperm in female *A. piscivorus*. Thus, no snakes that have been studied possess sperm storage in any other area than the ancestral location of the posterior infundibulum (Sever and Hamlett, 2002). Sperm storage in the posterior infundibulum is logical because ovulated eggs pass the infundibular region before entering or exiting any other region of the oviduct. Therefore, the posterior oviduct of viperids (and all other snakes investigated) did not evolve sperm storage because SSTs were already present in the posterior infundibulum. Of course, this leads to more questions than answers because lizards, many of which possess infundibular sperm storage (Saint-Girons, 1962a,b, 1973; Cuellar, 1966; Schaefer and Roeding, 1973; Bou-Resli, 1981; Adams and Cooper, 1988; Kumari et al., 1990; Murphy-Walker and Haley, 1996), have secondarily evolved sperm storage in other regions of the oviduct (Sever and Hamlett, 2002), the posterior oviduct being one of them (Bou-Resli et al., 1981; Sever and Hamlett, 2002; Sever and Hopkins, 2004). In ultrastructural investigations of sperm storage in lizards, sperm are stored in shallow vaginal crypts in *Acanthodactylus scutellatus* (Bou-Resli et al., 1981), tubulo-alveolar glands in *Anolis sagrei* (Sever and Hamlett, 2002), and between the rugae of the vagina in *Scincella laterale* (Sever and Hopkins, 2004).

Halpert et al. (1982) describes a region similar to the nonglandular uterus (referred to as the furrowed region of the oviduct) in *Thamnophis sirtalis* and propose that the formation of a carrier matrix occurs here. Apparently the epithelial cells shed, interact with sperm, and help facilitate the movement of sperm to anterior SSTs. Although we found desquamating of epithelial cells before and during the beginning of the fall mating season of *A. piscivorus*, the carrier matrix surrounding sperm is histochemically different from that of the epithelial cells lining the lumen of the nonglandular uterus. Whereas the secretory material that makes up the carrier matrix around the sperm stains PAS+ and BB+, the epithelium of the nonglandular uterus stains only AB+. We hypothesize that this material is supplied from male *A. piscivorus*, and previous studies on the kidneys of male *Seminatrix pygaea* show a similar PAS+ and BB+ material produced in the renal sexual segment of this species (Sever et al., 2002). Also, desquamation of epithelium lining the lumen of the nonglandular uterus does not occur in the spring time, when a second mating season occurs before ovulation.

If sperm storage and carrier matrix formation are not the functions of the nonglandular uterus, what is the function of this highly unique area? Studies have shown that this area is highly contractile (Nilson and Andrén, 1982; Almeida-Santos and Salomão, 1997, 2002) and Nilson and Andrén (1982) propose that secretions from the renal sexual segment in male *Vipera berus* cause contraction of the posterior oviduct and limit further mating activity from other males. This idea was rejected by Stille et al. (1986, 1987) because of the high occurrence of multiple paternity in this species, a phenomenon shown to occur in many snake species (Schuett and Gillingham, 1986; Stille and Niklasson, 1986; Stille and Niklasson, 1987; Höggren and Tegelström, 1995).

We propose that this area functions only to help evacuate offspring during parturition, by means of enhanced contractile capability (evidenced by the thick musculature), and lubrication of the oviduct (evidenced by this regions typical AB+ mucous secretions). The enlarged vaginal pouch, a synapomorphy of Viperidae (Ludwig and Rahn, 1943), also creates the possibility of foreign invader entry to the oviduct. The high concentration of antiinflammatory cells (plasma-like cells and mast cells) in the lamina propria of the nonglandular uterus undoubtedly helps to aid against this occurrence. Mast cells have been recorded in the oviducts of other snakes (Sottovia-Filho, 1973, 1974; Blackburn, 1998); however, this is the first investigation that has observed plasma-like cell invasion in the intercellular canaliculi of the epithelium in the oviduct.

The marked decrease in luminal volume between the vaginal pouch and the nonglandular uterus (Fig. 9A,B) creates a gigantic bottleneck in the oviduct of *Agkistrodon piscivorus* and other

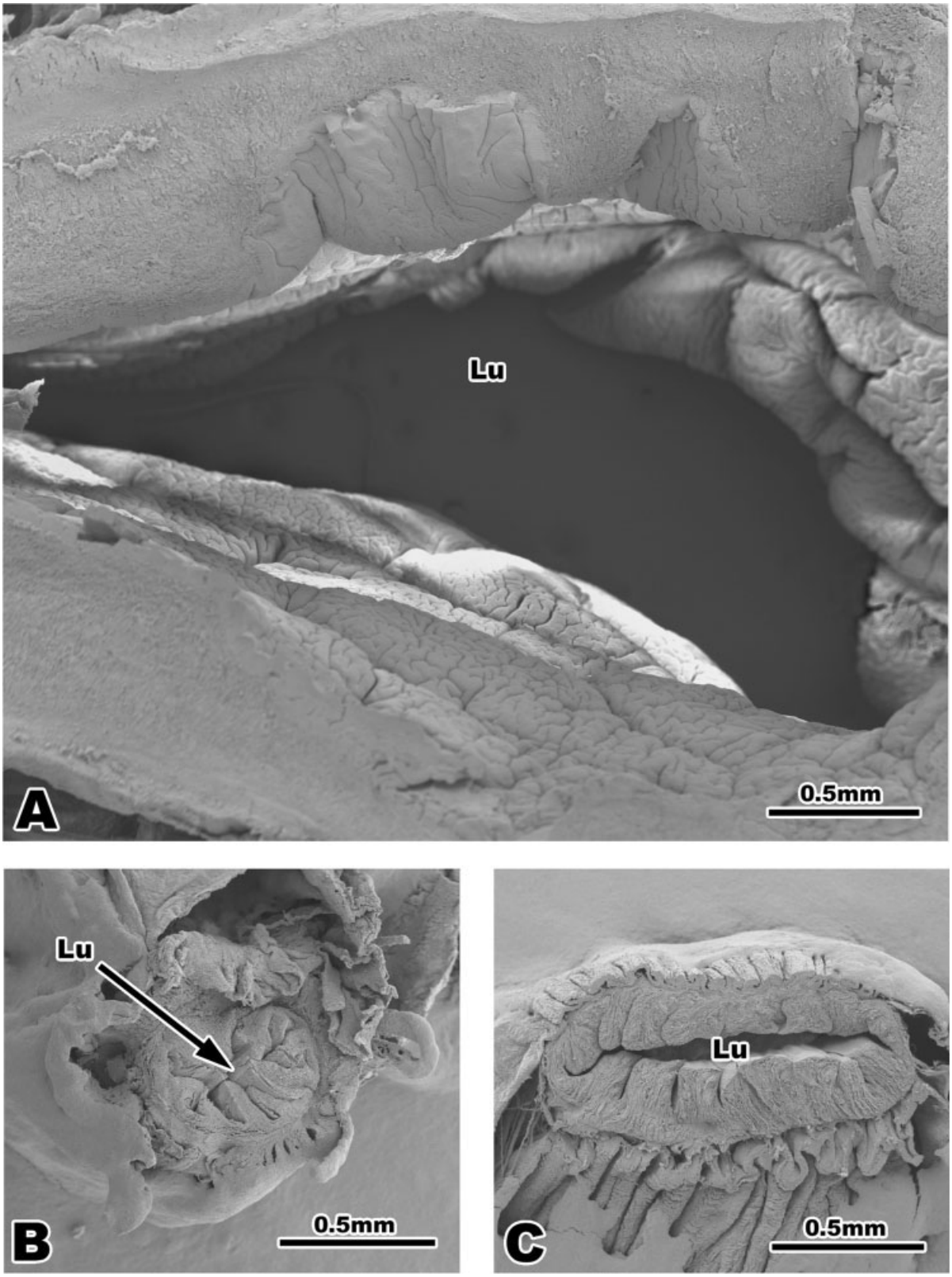

Fig. 9. Morphological bottleneck created by the enlarged vaginal pouch in viperids. Low magnification SEM. **A:** Transverse section of the vaginal pouch in a May specimen (note large luminal orifice). **B:** Transverse section of the nonglandular uterus in the same specimen as A (note the decreased luminal area). **C:** Transverse section of the glandular uterus in the same specimen as A and B (note the increased luminal area compared to that of B). Lu, lumen.

vipers. Karr and Pitnick (1999) believe "the female reproductive tract in many species is extraordinarily complex both morphologically-with tortuous pathways, blind-ended sperm storage tubules, elaborate muscles and highly developed sensory epithelia-and physiologically", which could aid in sperm selection. The evolution of this bottleneck could be an example of this with the most superior sperm breaking through the morphological bottleneck (Fig. 9C). These superior sperm would then find SSTs functioning in viable sperm quiescence in the posterior infundibulum while inferior sperm meet eventual degradation in the nonglandular uterus. Additional studies on sperm quality in the two areas of sperm aggregations in the snake oviduct could prove useful in determining if sperm selection is occurring in the female oviduct of snakes.

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