

## REPRODUCTIVE BIOLOGY OF *AGKISTRODON PISCIVORUS* LACÉPÈDE (SQUAMATA, SERPENTES, VIPERIDAE, CROTALINAE)

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**ABSTRACT:** Aspects of the reproductive biology of male and female *Agkistrodon piscivorus* are described using histological techniques, reviewed, and compared with historical data on *A. piscivorus*. These include anatomical description at the macro and microscopic levels, and correlation of the male and female urogenital cycles to the reproductive life history of *A. piscivorus*. New anatomical descriptions and discussion of the efferent ducts, including the ductuli efferentes, proximal and distal ductuli epididymides, ductus deferens, and ampulla ductus deferens, are also presented here at the light and electron microscopy levels. Morphology of all distinct regions of the male and female urogenital systems are discussed and compared with historical investigations on other squamates. In comparison to other snakes, *A. piscivorus* possesses some unique reproductive characters, whereas others are more conserved. In terms of the reproductive cycle, the ability of males and females to store sperm allows the dissociation of reproductive event timing between the sexes. Thus, the only event that must be coordinated between the sexes is copulation, which is proposed to occur in the fall and the spring in *A. piscivorus*. In females, the atrophy and activity of the reproductive organs (e.g., secretory activity) varies concurrently with vitellogenesis and the mating seasons. In males, spermatogenesis peaks in the summer, independent of the mating season, except in Louisiana where a spring and fall peak of spermatogenesis occur, and where the spring and fall mating seasons overlap. Renal sexual segment hypertrophy in males peaks in the fall and spring in more southern populations (Alabama and Louisiana) concurrent with fall and spring mating seasons. In Georgia, only a fall peak is observed. Secretory activity of the male excurrent ducts also peaks during times of mating activity in one population studied (Louisiana).

**Key words:** *Agkistrodon*; Histology; Morphology; Reproduction; Squamata; Ultrastructure; Viperidae.

RECENTLY, *Agkistrodon piscivorus* (Cottonmouth), a semiaquatic, viviparous, and venomous snake common throughout the southeastern United States (Conant and Collins, 1998), has been recognized as a valuable tool for studying certain aspects of the biology of snakes, and was the subject of a day-long symposium at the Joint Meeting of Ichthyologists and Herpetologists in Montreal, Canada (July, 2008). Unlike many other crotalines, *A. piscivorus* is abundant in areas of suitable habitat, and for that reason alone represents a model for studying the biology of crotaline snakes with invasive techniques (e.g., histology). We feel it timely to discuss and review historical aspects of the reproductive biology of *A. piscivorus*, particularly the reproductive anatomy and reproductive cycles (male and female), with data from recent investigations (Graham et al., 2008; Gribbins et al., 2008; Gribbins et al., in press; Sever et al., 2008; Siegel and Sever, 2006, 2008a,b). This marks

the first review of the biology of *A. piscivorus* that focuses completely on aspects of reproductive biology (a previous review focused attention on the natural history of this species; see Burkett, 1966). The following summary of historical and recent data carries great value in pinpointing future directions for studying reproduction of *A. piscivorus* and other crotalines.

Topics of this review include the gross and microscopic reproductive anatomy of male and female *Agkistrodon piscivorus*, seasonal timing of reproductive events, and variation in timing of reproductive events in different geographic populations of *A. piscivorus*. In terms of anatomy, comparisons are primarily with other snakes, although other squamates are considered where appropriate. Comparisons of reproductive cycles generally are restricted to different populations of *A. piscivorus* and to other North American crotaline snakes. Considering other investigators are currently focusing attention toward the reproductive ecology of *A. piscivorus*

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(pers. comm. N. B. Ford; E. Menzel), we leave this facet of reproductive biology out of the following review.

#### BACKGROUND

##### *Previous Anatomical Studies of Viperidae*

Few comprehensive studies, incorporating both males and females, exist on the reproductive anatomy of squamates, and of these studies viperids have been utilized in anatomical description rarely. Volsoe (1944) described the male urogenital system in *Vipera berus*, and Saint Girons (1959, 1973) described the female in *V. aspis* and *Cerastes cerastes*. Both of these in-depth tomes of literature were performed on Old World vipers from the Viperinae, the sister group to the Crotalinae, which contains all New World vipers and a few Old World species (Castoe and Parkinson, 2006). Collectively, members of the Crotalinae are termed the pitvipers. Ludwig and Rahn (1943) noted histological structure of the posterior oviduct of a crotaline (*Crotalus viridus*) in an effort to investigate copulatory adjustment and sperm transport. Other studies have described anatomical characters in crotalines in an effort to determine the timing of reproductive events by describing the testicular cycle and/or renal sexual segment (Rss) hypertrophy (e.g., Aldridge et al., 2008; Schuett et al., 2002). However, prior to this analysis, in-depth anatomical descriptions have never been compiled on all reproductive organs of a crotaline utilizing both males and females. Also, until now comparison of ultrastructural variation observed in reproductive organs to life history traits (e.g., reproductive cycles) has only been accomplished in a non-viperid, *Seminatrix pygaea* of the Colubridae (Gribbins et al., 2005; Sever et al., 2000; Sever et al., 2002; Sever and Ryan, 1999).

##### *Reproductive Cycles of North American Crotalines*

The reproductive cycles of North American crotalines have been reviewed by Schuett (1992) and Aldridge and Duvall (2002). Thus, the following is for the most part a brief synthesis of these massive works, updated with new information.

In general, female North American crotalines (*Agkistrodon*, *Crotalus*, *Sistrurus*) begin vitellogenesis in the late summer to early fall (Schuett, 1992; for complete list of *Crotalus* and *Agkistrodon* species studied see Aldridge and Duvall, 2002; *Sistrurus*, Aldridge et al. 2008). Around this time mating commences, which is evidenced by the presence of sperm in the posterior oviduct (Gloyd and Conant, 1990; Rahn, 1942; Siegel and Sever, 2006). Subsequently, a period of hibernation ensues, where the ovarian follicles of females arrest in development (Aldridge and Duvall, 2002; Aldridge et al., 2008). After hibernation, females may enter a second mating season (for a list of biannual breeders see Aldridge and Duvall, 2002), at which time follicles continue development until ovulation in the late spring (Aldridge and Duvall, 2002; Aldridge et al., 2008; Schuett, 1992). Thus, fertilization occurs in the spring and utilizes sperm from the previous fall mating season, a spring mating season, or both. All North American crotaline snakes are viviparous and carry their young until parturition in the fall (Aldridge and Duvall, 2002).

Spermatogenesis in male North American crotalines begins in the late spring–early summer (aestival; Aldridge and Duvall, 2002; Saint-Girons, 1982), coincident with an increase of testosterone (Johnson et al., 1982; Schuett et al., 1997; Schuett et al., 2002), and ends in the early fall coincident with mating and male agonistic behaviors (Schuett et al., 1997). However, secondary sexual character activity (i.e., Rss) can be hypertrophied in only the fall or in the fall and spring (Aldridge, 2002; Johnson et al., 1982) and is also androgen dependent (Prasad and Sanyal, 1969). The hypertrophy of the Rss may be linked to the mating season of temperate pitvipers (Aldridge, 2002), however, it is clear that spermatogenesis can be dissociated from mating activity due to the ability of males and females to store sperm for prolonged periods of time (Aldridge and Duvall, 2002; Schuett, 1992). This is not always the case, which can be seen in *Crotalus atrox* and *C. scutulatus* where mating and spermatogenesis overlap (Goldberg, 2007; Schuett et al., 2002; Taylor et al., 2004).

Male crotalines that mate in the spring (for a list see Aldridge and Duvall, 2002) also possess an increase in testosterone levels at this time (Schuett et al., 1997; Schuett et al., 2002; Schuett et al., 2005; Taylor et al., 2004), which likely stimulates reproductive behaviors such as mating and male–male combat (Schuett et al., 1997). However, this increase in testosterone is not as high compared to the summer–fall peak, which cues both spermatogenesis and reproductive behaviors (Schuett et al., 1997).

#### MATERIALS AND METHODS

##### *Specimens and Tissue Preparation*

The following synthesis adds new anatomical descriptions of the efferent ducts of male *Agkistrodon piscivorus*. Adult male specimens were collected in southeast Louisiana from the Amite River Diversion Canal (North 30.22616/West 090.68506), Livingston Parish (private property owned by Dr. Clifford Fontenot; North 30.871/W090.36202), and Pass Manchac (North 30.29426/West 090.35592). These were the same samples collected for use in recent studies on *A. piscivorus* testes (Gribbins et al., 2008; Gribbins et al., in press) and kidneys (Sever et al., 2008). Efferent ducts were dissected out of every male and prepared in the exact same manner for light and electron microscopy analyses as Sever et al. (2008). Sampling per month was as follows: January, one; February, two; March, two; April, two; May, two; June, five; July, two; August, two; September, one; October, six; and November, two.

##### *Microscopy Analysis*

Representative light micrographs were obtained by a combination of a Leica DM2000 microscope and Leica DFC420 digital camera (Leica Microsystems, Wetzlar, Germany). Transmission electron micrographs were obtained by a combination of a JEOL 100 transmission electron microscope and L3C CCD digital camera system (Scientific Instruments and Applications, Duluth, GA). Scanning electron micrographs were taken with a Philips XL-20 electron microscope (Philips Electronics N.V., Eindhoven, Netherlands). All digital micrographs were uploaded directly

into Adobe Illustrator and Photoshop CS for editing (Adobe Systems, San Jose, CA).

#### COMPARATIVE MORPHOLOGY: MALES

##### *Gross Morphology*

*Overview.*—When discussing the reproductive anatomy of male squamates, three main areas of interest are important to consider: (1) the testes, (2) efferent ducts (including the epididymis and ductus deferens), and (3) the kidney (Fig. 1), which produces a sexual secretion in squamates and possibly all Lepidosauria (Sever et al., 2000). From the seminiferous tubules of the testis, sperm pass sequentially through the ductuli efferentes, ductuli epididymides, ductus epididymis, ductus deferens, and the ampulla ductus deferentis. The ampulla joins with the ureter in the cloacal wall to form a urogenital duct that opens into the cloaca. The testis and the ducts associated with it are suspended from the body wall by a mesorchium, so the testis can be easily lifted and moved from side to side. The rest of the urogenital system, i.e., ductus deferens, kidney and ureter, are retroperitoneal until the ductus deferens and ureter separately enter the cloacal wall.

##### *Histology*

*Testis and germ cell morphology.*—The testis of *Agkistrodon piscivorus* is morphologically similar to that of other terrestrial amniotes (Gribbins et al., 2008). Seminiferous tubules are packaged within a thick connective tissue capsule. The tubules in transverse section have the same organization as those of other squamates (Fig. 2) and have very distinct basement membranes and a permanent population of Sertoli cells that reside on the basal lamina. Germ cells develop through the stages of spermatogenesis within cellular processes of the Sertoli cells, which compose the seminiferous epithelium of each tubule. Spermatogonia reside near the basement membrane and divide by mitosis within the basal compartment to give rise to the middle layer of cells within the seminiferous epithelium, the spermatocytes. Spermatocytes undergo both divisions of meiosis and give rise to the round spermatids of spermiogenesis. The spermatids undergo spermiogenesis within

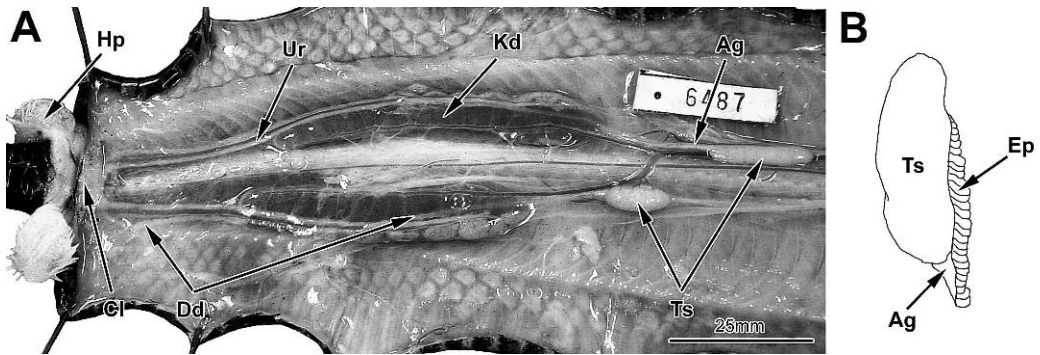


FIG. 1.—(A) Gross morphology of the male *Agkistrodon piscivorus* urogenital system. (B) Close up of testis demonstrating the association of efferent ducts to the testes. Ag, adrenal gland; Cl, cloaca; Dd, ductus deferens; Ep, epididymis; Hp, hemipene; Kd, kidney; Ts, testis; Ur, ureter.

the apical portion of the seminiferous epithelium and are shed as mature spermatozoa into the lumen of the seminiferous tubules at the completion of spermiogenesis.

The germ cell morphology is very similar to that of the colubrid *Seminatrix pygaea* (Grib-

bins et al., 2005) and other reptiles studied to date (Gribbins and Gist, 2003; Gribbins et al., 2003; Gribbins et al., 2006). The germ cells can be described and categorized according to the nomenclature established by Russell et al. (1990) for mammalian species.

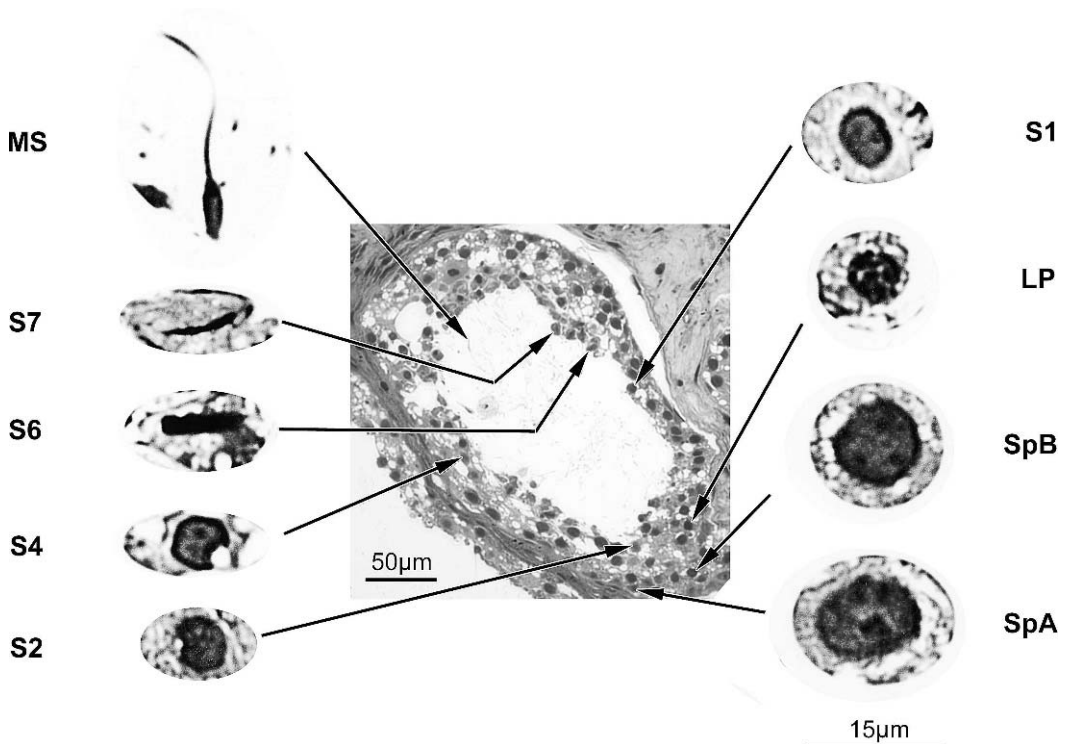


FIG. 2.—Cross section of a May seminiferous tubule from *Agkistrodon piscivorus*, and cell types represented within. Labeled cell types (in order of development): SpA, type A spermatogonia; SpB, type B spermatogonia; LP, leptotene spermatocytes; S1, step 1 spermatid; S2, step 2 spermatid; S4, step 4 spermatid; S6, step 6 spermatid; S7, step 7 spermatid, MS, Mature Spermatozoon.

The seminiferous epithelium contains two morphological populations of pre-meiotic cells (Spermatogonia A and B; Fig. 3. SpA and SpB) during all months of the year. These cells are characterized by nuclei with random clumps of heterochromatin. The major morphological differences between the two types of spermatogonia are that the A type are ovoid in shape with one large nucleolus and B type are more round in shape and usually lack a prominent nucleolus. Both spermatogonial types are generally found near the basement membrane of the epithelium in association with Sertoli cells. During the spermatogenic cycle both types of spermatogonia undergo mitosis to maintain the spermatogonial population and many of the B spermatogonia divide to form pre-leptotene spermatocytes that then enter meiosis.

Meiotic cells are characterized by an increase in nuclear size and condensation of chromatin into chromosomes. Pre-leptotene through pachytene spermatocytes (Fig. 3. PL-PA) show a swift increase in nuclear size with condensation of the chromatin into visualized chromosomes.

Diplotene spermatocytes (Fig. 3. DI), metaphase I cells (Fig. 3. M1), secondary spermatocytes (Fig. 3. SS), and metaphase II cells (Fig. 3. M2) can be found together within the seminiferous epithelium throughout the active months of spermatogenesis. In diplotene spermatocytes, the nuclear membrane begins to degenerate and the almost fully condensed chromatin fibers form a tight circle just under this degenerating membrane. Metaphase 1 cells have fully condensed chromosomes that aggregate on the metaphase plate. The results of meiosis I are the secondary spermatocytes and their chromatin is dispersed randomly throughout the nucleoplasm. During Metaphase 2, chromosomes aggregate around the metaphase plate again. The only differential factor between Metaphase 1 and Metaphase 2 is that Metaphase 2 cells are slightly smaller and contain about half the amount of chromatin than Metaphase 1 cells.

Spermiogenesis can be divided into seven steps within the *Agkistrodon piscivorus* germinal epithelium based on the terminology of Russell et al. (1990) for mammalian

species. Steps of spermiogenesis are defined based on acrosomal formation, nuclear elongation, and chromosomal condensation. Step 1–4 spermatids (Fig. 3. S1–S4) represent the early round spermatids. The major trend seen during the round spermatid stage is the development of the acrosome and acrosome granule on the apex of the spermatid nucleus.

Step 5 spermatids mark the transition between round and elongating spermatids (Steps 5–7; Fig. 3. S5–S7). Elongation begins at the opposite end of the nucleus away from the acrosome creating a nucleus that is stretched on its dorsoventral plane. As elongating spermatids undergo development, they also begin to accumulate near the apical surfaces of the Sertoli cells with their tails stretching out into the lumen and their nuclear heads facing the basement membrane. As elongation proceeds, spermatids become cylindrical and roughly filiform in appearance. The width of these spermatids decreases as the DNA within the nucleus condenses into a dark uniform mass within the nucleoplasm. Once spermiogenesis is complete the mature spermatozoa (Fig. 3. MS) are released into the lumen of the seminiferous tubules where they will be transported to the excurrent ducts of the male reproductive system.

*Germ cell development strategy.*—Spermatogenic events occur during two separate seasons (spring and late summer–early fall) throughout a single calendar year within *Agkistrodon piscivorus* testes from the most southerly population studied (Louisiana; Gribbins et al., 2008). The beginning of the first wave of spermatogenesis commences in March with the presence of meiotic cells. Spermatogenesis climaxes in June (Fig. 4A) with the late stages of spermiogenesis, heavy spermiation, and mature spermatozoa flooding the lumen of the seminiferous tubules. During this time (March–June) a gradual increase in the seminiferous tubule diameter (STD; 126  $\mu\text{m}$ , 131  $\mu\text{m}$ , 150  $\mu\text{m}$ , 181  $\mu\text{m}$ ) and mean germinal epithelial height (GET; 28.2  $\mu\text{m}$ , 29.2  $\mu\text{m}$ , 38.1  $\mu\text{m}$ , 43.8  $\mu\text{m}$ ; Fig. 5 top and bottom) can be observed histologically and quantitatively (Fig. 5, superscripts STD: a–d; GET: a–c).

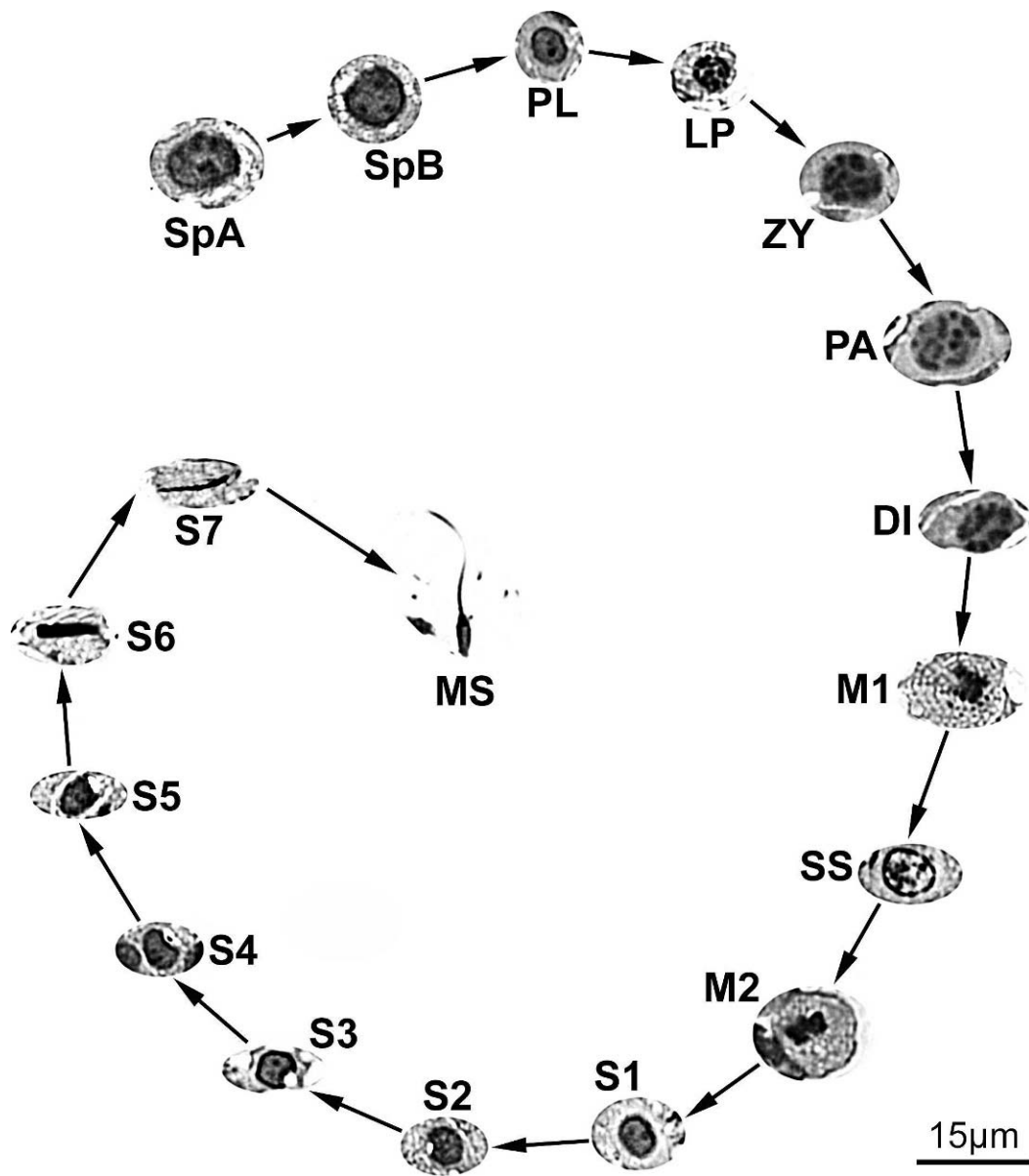


FIG. 3.—Germ cell types found within the seminiferous epithelium of *Agkistrodon piscivorus*. SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene spermatocyte; LP, leptotene spermatocyte; ZY, zygotene spermatocyte; PA, pachytene spermatocyte; DI, diplotene spermatocyte; M1, meiosis I; SS, secondary spermatocyte; M2, meiosis II; S1, step 1 spermatid; S2, step 2 spermatid; S3, step 3 spermatid; S4, step 4 spermatid; S5, step 5 spermatid; S6, step 6 spermatid; S7, step 7 spermatid; MS, mature spermatozoon.

The testes enter a quiescent period during July (Fig. 4B) in which the germinal epithelium only consists of spermatogonia A and B cells. The epithelium becomes highly vacuolated and the lumen is void of mature

spermatozoa. A significant decrease in the seminiferous tubule diameter ( $92.7\ \mu\text{m}$ ) and the epithelial height ( $20\ \mu\text{m}$ ; Fig. 5, superscripts STD: a,b and GET: a) can be observed through statistical analyses.

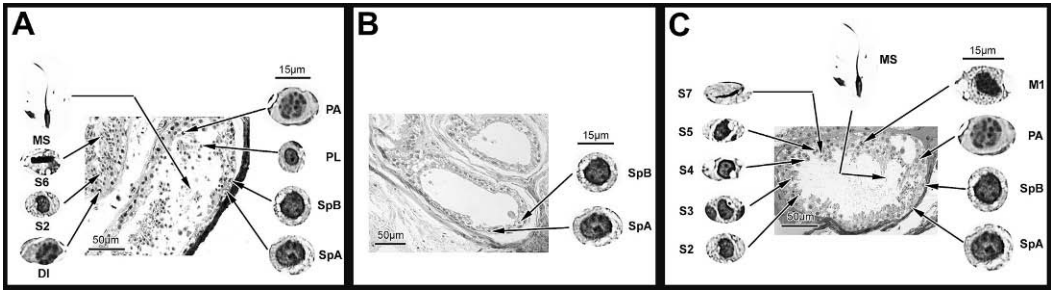


FIG. 4.—(A) Cross sectional view of June seminiferous tubule with higher magnifications of present cell types. Cell types present within the seminiferous epithelium include: SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene spermatocyte; PA, pachytene spermatocyte; DI, diplotene spermatocyte; S2, step 2 spermatid; S6, step 6 spermatid; MS, mature spermatozoa. (B) Cross sectional view of July seminiferous tubule with higher magnifications of present cell types. Cell types present within the seminiferous epithelium include: SpA, type A spermatogonia; SpB, type B spermatogonia. (C) Cross sectional view of October seminiferous tubule with higher magnifications of present cell types. Cell types present within the seminiferous epithelium include: SpA, type A spermatogonia; SpB, type B spermatogonia; PA, pachytene spermatocyte; M1, meiosis I; S2, step 2 spermatid; S3, step 3 spermatid; S4, step 4 spermatid; S5, step 5 spermatid; S7, step 7 spermatid; MS, mature spermatozoa.

Spermatogenesis commences again in late summer–early fall (August) with the presence of meiotic cells and early spermatids and continues through October. During the month of October (Fig. 4C) all germ cells within the cell cycle are represented within the epithelium of the seminiferous tubules. The lumina of the tubules are again flooded with mature spermatozoa similar to that of June. This increase in testicular activity is paralleled by an increase in seminiferous tubule diameter and epithelial height in October (STD: 215.4  $\mu$ m and GET: 41.5  $\mu$ m; Fig. 5, superscripts STD: d and GET: c).

November through February mark the second quiescent period of spermatogenesis. During these months the seminiferous tubules decrease (95  $\mu$ m, 101.25  $\mu$ m, 99.8  $\mu$ m) in size, become highly vacuolated, and only spermatogonia A and B cells are present. The lumen is once again void of mature spermatozoa. The decrease in testicular activity is shown by the decrease in tubular diameter and epithelial height similar to that observed during July (Fig. 5, superscripts STD: a,b and GET: a,b).

From the above data, two distinct events of spermatogenesis can be seen within the testes of the southeastern Louisiana population of *Agkistrodon piscivorus*. The first event begins in March with the onset of spermatogenesis and is completed in July with heavy spermiation. The two events are separated by a quiescent period in July before spermatogen-

esis commences again in August. This is evidence for the first description of biannual spermatogenesis within a North American species of Crotalinae.

Crotalines of North America typically have a single event of spermatogenesis that occurs during the late spring, peaks during summer, and ends in early fall (Aldridge, 1979; Aldridge, 1993; Aldridge, 2002; Aldridge and Brown, 1995; Aldridge et al., 2008; Goldberg, 1999a,b; Goldberg, 2000a,b,c; Goldberg and Holycross, 1999; Goldberg and Rosen, 2000; Schuett et al., 2002). Johnson et al. (1982) described the spermatogenic events of an Alabama population of *Agkistrodon piscivorus*, and the spermatogenic cycle mirrored that of a typical North American Crotalinae. Seasonal testicular histology from Graham et al. (2008) confirms this finding in a population from Georgia. The only other viperid known to exhibit biannual events in the spermatogenic cycle is *Vipera berus* (Saint-Girons, 1982), however, this is due to the culmination of spermiogenic events in the spring, not a second bout of spermatogenesis as described in Louisiana *A. piscivorus*. In general, spermatogenesis in the colubrids of North America follows a very similar pattern to typical North American crotalines with spermatogenesis commencing in the spring, reaching a peak in the summer, and concluding in the fall (for review see Aldridge et al., in press). However, one species, *Masticophis bilineatus*,

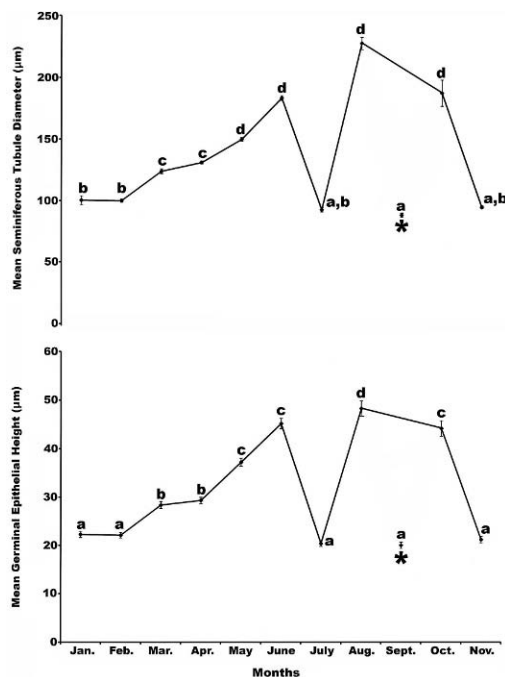


FIG. 5.—(Top) Variation in seminiferous tubule diameter and (Bottom) variation in germinal epithelial height during the annual reproductive cycle male *Agkistrodon piscivorus* from Louisiana. Values represented on this graph are means  $\pm$  1 standard error. Different superscripts indicate significant differences (Dunn's multiple range test between months). Note that September (\*) represents an aberration in the data. This specimen was caught one week after hurricane Katrina and outside of its normal habitat. Thus, spermatogenesis was halted because of stress-induced hormones (see Gribbins et al. 2008).

possesses a biannual spermatogenic cycle with distinct spermatogenic cycles occurring in both the fall and the spring (Goldberg, 1998). Two peaks also seem to occur in *Trimorphodon biscutatus* (Goldberg, 1995), however, it has been hypothesized that the spring peak is just the conclusion of the fall spermatogenic cycle as described in *V. berus*.

**Ultrastructure of spermiogenesis.**—Spermatid morphology in *Agkistrodon piscivorus* testes (Gribbins et al., in press) is very similar to that observed within the colubrid *Sematrix pygaea* (Gribbins, unpublished). A number of recent studies have focused on some aspects of spermiogenesis (Al-Dokhi, 2004, 2005; Al-Dokhi et al., 2004, 2005a,b; Dehlawi and Ismail, 1994; Hondo et al., 1994; Ismail et al., 1995) or ultrastructure of the spermat-

zoon (Al-Dokhi et al., 2007; Oliver et al., 1996; Tourmente et al. 2006, 2008) within Serpentes. To our knowledge, *A. piscivorus* represents the first snake species to have its entire spermiogenic cycle described ultrastructurally. Furthermore, there are only two papers dealing with ultrastructure within the male testis of crotalines and they primarily cover the morphology of the spermatozoon (Cunha et al., 2008; Tourmente et al., 2008), with no information on spermiogenesis.

Spermatid morphological changes during spermiogenesis within *Agkistrodon piscivorus* are very similar to those seen within other squamates studied to date, such as *Scincella lateralis* (Gribbins et al., 2007; Gribbins et al., in press). However, subtle differences exist between *S. lateralis* and other squamate testes during the morphogenesis of spermatids when compared to *A. piscivorus*. For brevity, we will focus here on the more interesting differences observed in the ultrastructure of spermatids and mature spermatozoa. Figure 6 shows the round (A and B) and early elongate stages (C) of spermiogenesis in *A. piscivorus*. The developing acrosome and juxtapositioned Golgi apparatus are seen within *A. piscivorus* round spermatids similar to that described for other squamates. However, the development of the acrosome granule early in acrosome development in a centralized position within the acrosome vesicle, prior to contact with the nuclear apex, seems to be unique to *A. piscivorus* spermatids. In *S. lateralis* the granule forms later (based on vesicle size), and the granule is located against the acrosome vesicle membrane. The later events of acrosome development in round spermatids within *A. piscivorus* (Fig. 6C) are similar to that of *S. lateralis*. One major difference between *S. lateralis* and *A. piscivorus* elongate stages is the large and prominent peripheral fibers associated with microtubule doublets 3 and 8 (Fig. 7F,G) in cross sections of the developing flagella within the midpiece of *A. piscivorus* round and elongating spermatids (Fig. 6C), which is a similar trait found in mature spermatozoa of *Crotalus durissus* (Cunha et al., 2008). These fibers have also been described in other squamate spermatozoa (Jamieson, 1999; Oliver et al., 1996; Tourmente et al., 2008). Unique to *A.*

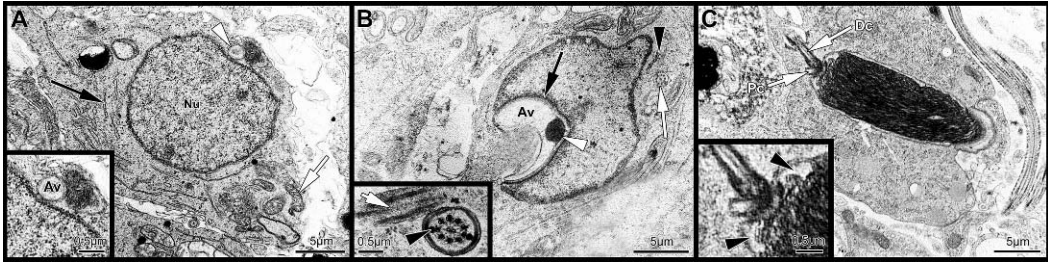


FIG. 6.—Round and early elongate spermatids undergoing acrosome development during the early stages of spermiogenesis within the seminiferous epithelium of *Agkistrodon piscivorus*. (A) The acrosomal vesicle (white arrowhead) is juxtapositioned to the apical portion of the nucleus (Nu). The vesicle is in the early phase of growth. The cytoplasm of the spermatid has numerous mitochondria (white arrow) and many layers of endoplasmic reticulum (black arrow). **Insert:** Shows that the vesicle (AV) has not quite made contact with the nuclear membrane. (B) Very late-stage round spermatid exhibiting a deep indented acrosome (AV) and a prominent acrosomal granule (white arrowhead). An accumulation of dark staining proteins are lining the nuclear membrane side of the subacrosomal space (black arrow). The caudal portion of the nucleus has begun elongation and the proximal centriole (black arrowhead) can be seen in sagittal section and the growing flagellum is shown in transverse section (white arrow) near the caudal end of the nucleus. **Insert:** Shows the proximal centrioles (white arrow) and transverse flagella in greater detail. The flagellum has two opposing protein plaques/dense bodies (black arrowhead) juxtapositioned to the conserved 9 + 2 microtubule core. (C) The development of the flagella is prominent in sagittal sections of elongates showing the presence of both the proximal (Pc) and distal (Dc) centrioles arranged in a perpendicular fashion. The distal centriole begins to elongate to form the flagellum. **Insert:** Large open nucleoplasmic vesicles (black arrowheads) devoid of chromatin can be seen lateral to the developing flagellum.

*piscivorus* spermiogenesis is the large open nucleoplasmic vesicles on either side the developing flagella in early elongating spermatids (Fig. 6C, inset). Another major difference between *A. piscivorus* elongating spermatids and those of other squamates studied to date is the accumulation of a thick band of dense staining material inside of the outer acrosomal membrane (Figs. 6C, 7D) during the development of the late stage spermatids (Fig. 7A,B). This may be a synapomorphy of Crotalinae; however, more species within Serpentes need to be studied before this suggestion can be tested robustly. The acrosome complex of late elongates is very similar in *A. piscivorus* to that described for other squamates except for the absence of the perpendicular part of the manchette (Fig. 7E), which has also been described in *S. lateralis* (Gribbins et al., 2007). Dense bodies within the midpiece of *A. piscivorus* sperm are scattered and not as abundant as those described in some lizards, a trait also observed in another crotaline, *C. durissus* (Cunha et al., 2008).

**Morphology of the efferent ducts.**—Except in the region of the efferent ducts, the outer seminiferous tubules are bordered by a layer of collagen fibers and the thin, superficial

visceral pleuroperitoneum (Fig. 8A). In the region of the excurrent ducts, the tunica propria of the serosa splits, becomes thickened and invested with smooth muscle, blood vessels and more fibrous connective tissue (Fig. 8A,B). Distal ends of seminiferous tubules as well as the ductuli efferentes, ductuli epididymides, and the ductus epididymis are encased in this capsule, along with the adrenal gland (Fig. 8B).

The ductuli epididymides can be found along the entire medial side of the testis. The ductus epididymis and adrenal gland appear at the start of the posterior two-thirds of the testis. Ductuli efferentes are short tubules that branch off the distal ends of seminiferous tubules that protrude into the epididymal sheath (Fig. 8C). The seminiferous tubules narrow abruptly into the ductuli efferentes, which are composed of simple cuboidal, basophilic epithelium and lack cilia or a secretory product (Fig. 8D).

The proximal and distal ductuli epididymides differ histologically from one another and from the epididymis (Fig. 9A,B). The distal ductuli epididymides empty into the epididymis along its entire length.

The proximal ductuli epididymides (Pde) are up to 100  $\mu$ m in diameter, somewhat

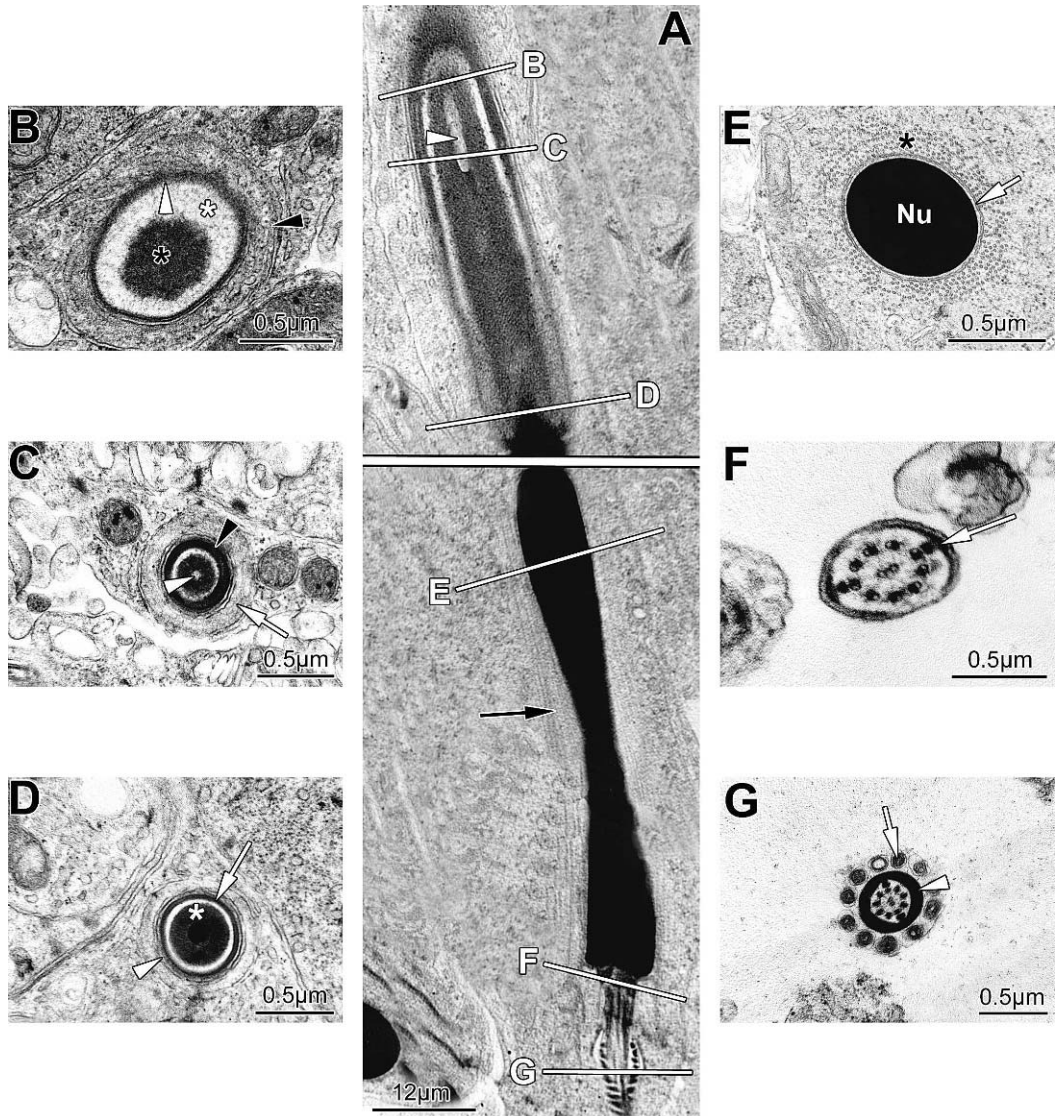


FIG. 7.—(A) Sagittal section of *Agkistrodon piscivorus* elongate spermatid in the termination phase of spermiogenesis with all three major parts of the spermatid visible (acrosome, nucleus, and flagella; white line through the middle of A denotes that two separate micrographs were combined to obtain this image). Lines and represented letters show approximately where transverse sections (CS) occurred within spermatids at or near the same stage of development as A in order to obtain cross sections B–G. Note the well-developed parallel microtubules of the manchette (**black arrow**) running along side the nucleus and the perforatorium (**white arrowhead**). (B) CS through the cranial subacrosomal space and acrosomal vesicle. Granulated protein layer of subacrosomal space (**white \***), acrosomal vesicle (**black \***), protein accumulation on the inside of the outer acrosomal membrane (**white arrowhead**), perforatorium, and acrosomal vesicle. The **white arrowhead** points to the perforatorium, which is surrounded by the granulated proteins of the subacrosomal space. The white circular region around the subacrosomal space is the acrosomal vesicle, which again has protein accumulations under its outer membrane (**black arrowhead**). Also note the numerous Sertoli cell membrane layers surrounding the entire acrosomal complex (**white arrowhead**). (C) CS through the subacrosomal space, perforatorium, and acrosomal vesicle. The **white \*** labels the subacrosomal space. Within the middle of this space is the conical point of the nucleus in CS. Also present are the protein accumulation under the outer membrane of the acrosome (**white arrow**) and the Sertoli cell membrane layers (**white arrowhead**). (E) CS through nucleus proper. Nucleus (Nu), Manchette (\*), inner single circular microtubule layer (**white arrow**). (F) CS through the proximal neck of the flagella. Axoneme is nicely represented with 9 pairs of doublet microtubules and a microtubule triplet within the center of the axoneme. Attached protein blacks within the axoneme (**white arrow**). This CS represents a transition zone between the basal plate and the proximal neck. (G) CS through the midpiece. Dense fibrous sheath/ring (**white arrowhead**), mitochondria (**white arrow**).

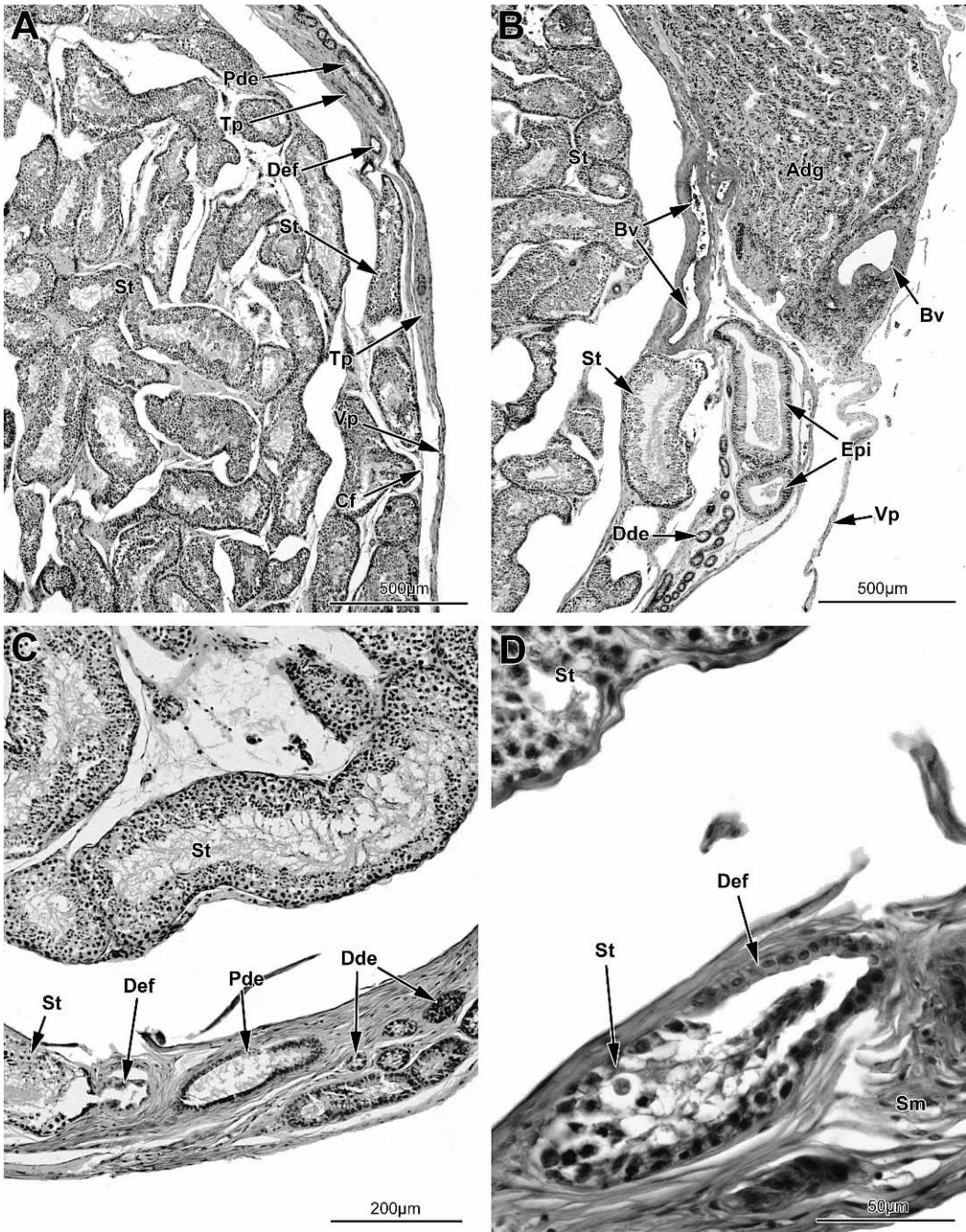


FIG. 8.—Light micrographs of transverse sections through the testis and proximal efferent ducts of a 57.6 cm SVL male *Agkistrodon piscivorus* collected 2 October. (A) Medial testis showing transition of superficial lining associated with the region of the efferent ducts. (B) Relationship between the seminiferous tubules, efferent ducts, and the adrenal gland. (C) Relation of seminiferous tubules, ductuli efferentes, and ductuli epididymides. (D) Transition between seminiferous tubules and ductuli efferentes. Adg, adrenal gland; Bv, blood vessel; Cf, collagen fibers; Dde, distal ductuli epididymides; Def, ductuli efferentes; Pde, proximal ductuli epididymides; St, seminiferous tubules; Tp, tunica propria; Vp, visceral pleuropéritoneum.

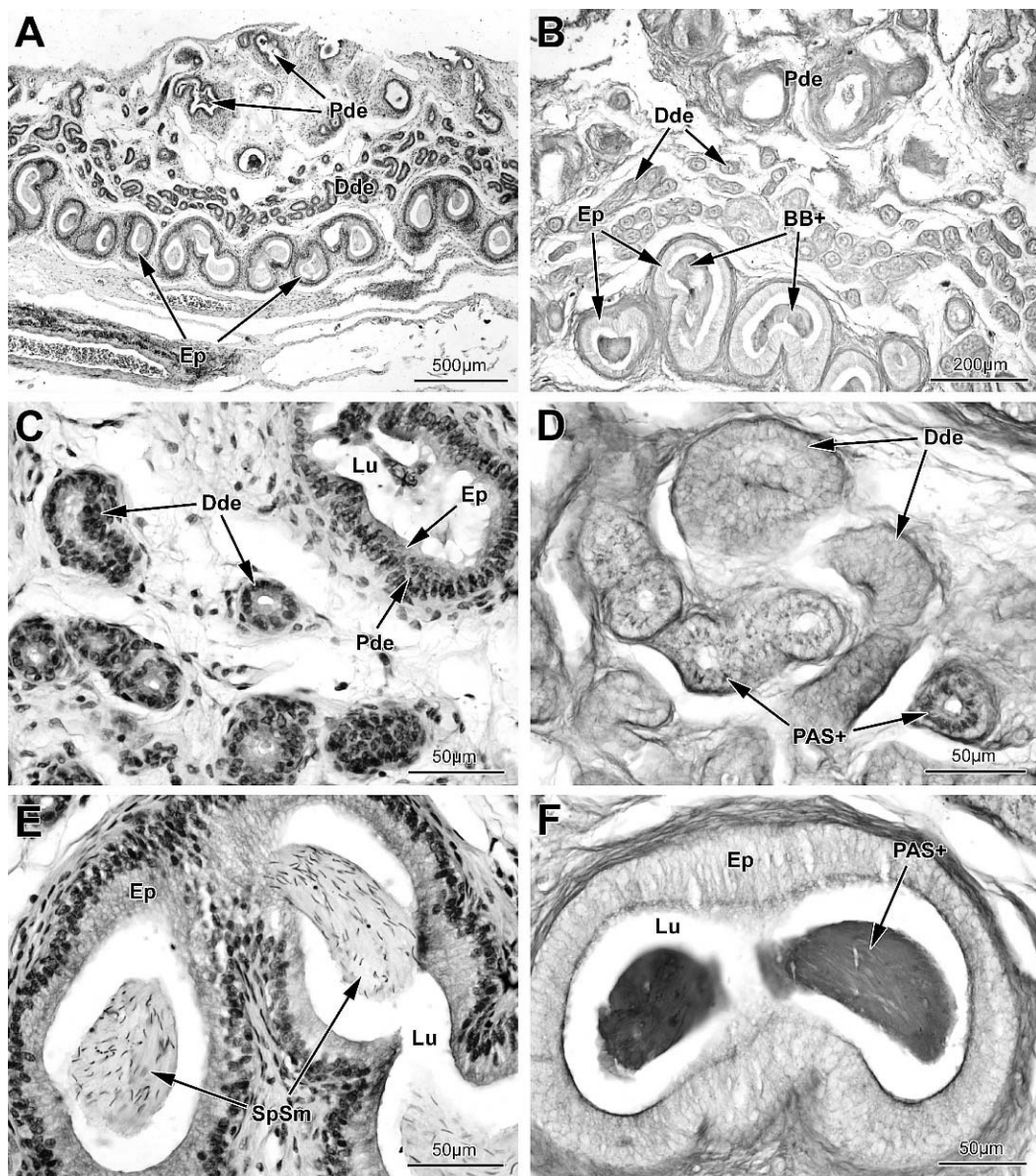


FIG. 9.—Light micrographs of the proximal efferent ducts associated with the testis of a 46.1 cm SVL male *Agkistrodon piscivorus* collected 13 March. (A) Overview stained with hematoxylin-eosin. (B) Overview stained with bromphenol blue. (C) Detail of histology of the ductuli epididymides stained with hematoxylin-eosin. (D) Distal ductuli epididymides stained with PAS and alcian blue. (E) Detail of ductus epididymis stained with hematoxylin-eosin. (F) Ductus epididymis stained with PAS. BB+, bromphenol blue positive; Dde, distal ductuli epididymides; Ep, epithelium; Lu, lumen; PAS+, periodic acid-Schiff's reagent positive; Pde, proximal ductuli epididymides; SpSm, sperm in secretory material.

irregular in shape, and in March–April and August–November, the epithelium is simple columnar. The basal nuclei have their long axes oriented with that of the cells, and the

apical cytoplasm is basophilic, although the basophilia is not as intense as for nuclei (Fig. 9C). The epithelium is reduced to cuboidal in a specimen collected 23 February

and one collected 27 June. Neither the epithelium or luminal material other than sperm react with periodic acid-Schiff's (PAS), alcian blue, or bromphenol blue. Sperm from a spermiation event apparently pass through these tubules quickly as sperm occur only in a specimen collected 14 June and one collected 12 August.

The distal ductuli epididymides (Dde) are smaller and more numerous than the Pde, with a diameter of 35–50  $\mu\text{m}$ . The epithelium is cuboidal with the long axes of the basal nuclei oriented with the basement membrane, and the cytoplasm, like that of the Pde, is basophilic (Fig. 9C). PAS+ granules (Fig. 9D) occur in specimens from every month except for a specimen collected on 27 June, in which the Dde, like the Pde, is highly regressed. Bromphenol blue positive material is also scattered through the cytoplasm of the Dde. Fluoculent material, possibly in vacuoles, occurs in the cytoplasm of specimens collected 30 May, 14 June, and 12 August. Sperm are found in specimens collected 24 May, 17 August, 17 October, and 22 November.

The ductus epididymis is the largest of the proximal efferent ducts, with a diameter of 150–250  $\mu\text{m}$ . The epithelium appears pseudostratified with columnar principle cells like those of the Pde and scattered basal cells. The apical cytoplasm of the basal cells is once again basophilic, but secretory material in the lumen is decidedly eosinophilic (Fig. 9E). The secretory material in the lumen is also strongly PAS positive (Fig. 9F). Luminal secretory material is found in specimens collected 13 March, 28 March, 24 May, 30 May, 18 June, and 22 November, and sperm are associated with the secretory mass on 13 March, 28 March, and 17 August (Fig. 9E). A scant amount of sperm is found in the epididymis of a male collected 23 February and which lacks secretory material in the lumen. The cytoplasm of glands with secretory material in the lumen did not respond generally to histochemical tests, although a scattered, diffuse reaction to PAS, alcian blue, and bromphenol blue is detectable. The individual collected 27 June has a completely regressed epididymis, but in the others, the epithelium generally seems hypertrophied. Vacuoles of flocculent material occur in the epithelium of a specimen

collected 12 August, which also possesses such vacuoles in the Dde.

The ductus deferens is the continuation of the epididymis posterior to the testis, and passes retroperitoneally with the ureter toward the cloaca. The ductus deferens is formed into short tight loops (not coils) as it passes caudally. The demarcation between epididymis and ductus deferens is gradual, with the lumen of the ductus becoming wider, from 200–300  $\mu\text{m}$  in diameter, and the epithelium becoming lower. Other studies have reported that the epithelium of the squamate ductus deferens is pseudostratified but that is difficult to verify by light microscopy (Fig. 10A,B). The epithelium of the principle cells usually is cuboidal with a scant amount of basophilic cytoplasm, although columnar principle cells are found in specimens collected 18 June, 27 June, 27 July, and 17 October. Copious sperm occur in the ductuli deferentia of every specimen examined. Secretory material associated with the sperm mass is eosinophilic, bromphenol blue positive, and PAS positive (Fig. 10B–D), and these reactions are most intense in areas around the border of the sperm mass that lack sperm. In the latter areas the secretory material is globular. The bromphenol blue reaction is less intense than for smooth muscle encasing the ductus deferens (Fig. 10C). Scattered reactions to alcian blue occur around the sperm mass in specimens collected 13 March, 30 May, 27 June, 27 July, and 17 October. Numerous vacuoles are found between the apical cytoplasm and the luminal secretory material in a specimen collected 24 May. The cytoplasm of the ductus deferens usually seems rather unreactive to histochemical tests (Fig. 10C–D), but in all seasons PAS positive, alcian blue positive, and bromphenol positive material is located in the apical cytoplasm. Transmission electron microscopy (TEM) reveals the presence in the apical cytoplasm of secretory vacuoles containing a diffuse material, characteristic of carbohydrates (Fig. 10E). Scanning electron microscopy shows the tight packing of sperm (Fig. 10F).

The ampulla consists of the most distal 6–10 mm of the ductus deferens retroperitoneal in the coelom, and another segment of similar

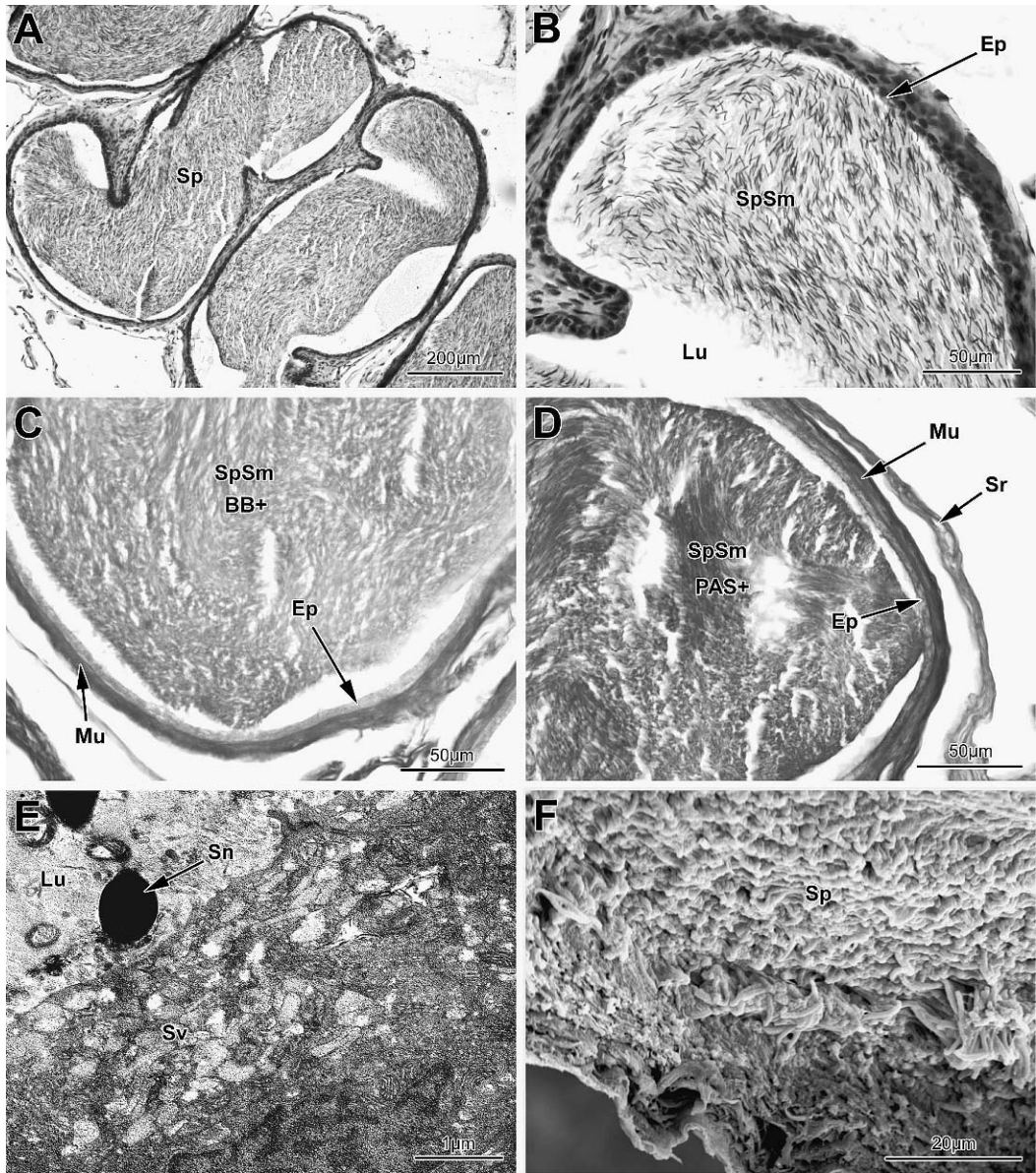


FIG. 10.—Micrographs of the ductus deferens of a 46.1 cm SVL male *Agkistrodon piscivorus* collected 13 March. (A) Light micrograph overview stained with hematoxylin-eosin. (B) Light micrograph detail of sperm in secretory material stained with hematoxylin-eosin. (C) Light micrograph stained with bromphenol blue. (D) Light micrograph stained with PAS and alcian blue. (E) Transmission electron micrograph of luminal border. (F) Scanning electron micrograph showing sperm packed in the lumen. BB+, bromphenol blue positive; Ep, epithelium; Lu, lumen; Mu, muscularis; PAS+, periodic acid-Schiff's reagent positive; SpSm, sperm in secretory material; Sn, sperm nucleus; Sp, sperm; Sr, serosa; Sv, secretory vacuoles.

length that passes through the cloacal walls before joining with the urethra. Externally, the coelomic ampulla is discerned by a straightening of the loops from the ductus

deferens. Internally, the ampulla is characterized by irregular folded epithelial walls (Fig. 11A,B) until the final intramural one-fourth when the epithelium becomes regular

again as the junction with the ureter is approached. The cytoplasm of the anterior three-fourths of the ampulla is characterized by numerous vacuoles, and the vacuoles also occur between the apices of the epithelial cells and the secretory material encasing the sperm mass (Fig. 11C,D). Sperm occur in the ampulla throughout the year, although sperm are scant in October and November specimens. The eosinophilic secretory material is elaborated from the apical cytoplasm (Fig. 11C). Throughout the year, luminal material is intensely PAS positive, and small PAS positive granules are scattered throughout the epithelium (Fig. 11D). Positive reactions with alcian blue and with bromphenol blue are also observed in the cytoplasm and luminal material, but these reactions are not as strong and pervasive as those for PAS. TEM confirms the presence of apical secretory vacuoles containing a diffuse substance and a dense matrix containing sperm in the lumen (Fig. 11F). The uniformity of the secretory matrix is revealed by scanning electron microscopy (Fig. 11F).

*Discussion of the efferent ducts.*—The proximal efferent ducts show a regression in activity in June and July, although the ductus deferens and ampulla show less seasonal variation. The periods (spring and fall) when the proximal ducts are most highly hypertrophied correspond to the fall and spring mating seasons of *Agkistrodon piscivorus*.

The two most comprehensive histological studies on the efferent ducts of snakes were by Volsøe (1944) and Fox (1952). In *Vipera berus*, and apparently *Natrix natrix*, Volsøe (1944) indicated that the ductuli efferentes consist of squamous epithelium and the proximal ductuli epididymides have columnar epithelium that has “irregular flames of long cilia.” At certain seasons the proximal ductuli epididymides were recorded to be filled with eosinophilic secretion granules. Volsøe (1944) also noted that proximal ductuli epididymides narrow without bifurcation into the distal ductuli epididymides, which are longer, more cuboidal, generally ciliated, and lack secretory granules. Fox (1952) reported similar results for *Thamnophis elegans*.

These observations are at odds with our observations on *Agkistrodon piscivorus*. The

ductuli efferentes of *A. piscivorus* are clearly cuboidal, not squamous. Cilia appear to be present in the ductuli epididymides, but we await the results from ultrastructural work to confirm their presence and cytology. The distal segment is secretory, not the proximal. Finally, the distal portion clearly has more tubules than the proximal portion, indicating division of the proximal ductuli epididymides into smaller units.

In lizards, the few studies that have been done of the ductuli epididymides indicate that they arise from an extra-testicular rete testis and open into a large sinus inside the epididymis (Akbarsha et al., 2007; Averal et al., 1992; Fox, 1977). Production of secretory material is not mentioned, but the efferent ductules of *Sitana ponticeriana* are described as spermiophagic.

Volsøe (1944) found that at “certain seasons” the epithelium of the epididymis of *Vipera berus* contains numerous small granules which become blackened with osmium tetroxide, which is an indicator of lipids. Fox (1952) stated the columnar epithelium of the epididymis of *Thamnophis elegans* is “probably secretory” during certain seasons of the year. Dufare and Saint Girons (1984) examined the ductus epididymides of 89 species of squamates, including 35 species in seven families of snakes. They found five types of secretory activity in the main duct of the epididymis. Type 1 consists of large secretory granules with a chromophilic central core surrounded by a chromophobic vacuole, and this type characterizes the Lacertidae. Types 2–4 show decreasing size and density of secretory products, and Type 5 demonstrates no secretory activity. Type 5 characterized all species of snakes that were examined, including *V. berus*.

We found limited evidence of secretory activity in the ductus epididymis of *Agkistrodon piscivorus*. The scattered, diffuse reaction to carbohydrate and protein stains detected, however, does indicate that an ultrastructural examination might reveal product synthesis.

Neither Volsøe (1944) nor Fox (1952) reported secretory activity in the ductus deferens. The ductus deferens is the storage organ for sperm in male squamates, but the epithelium has been characterized as non-

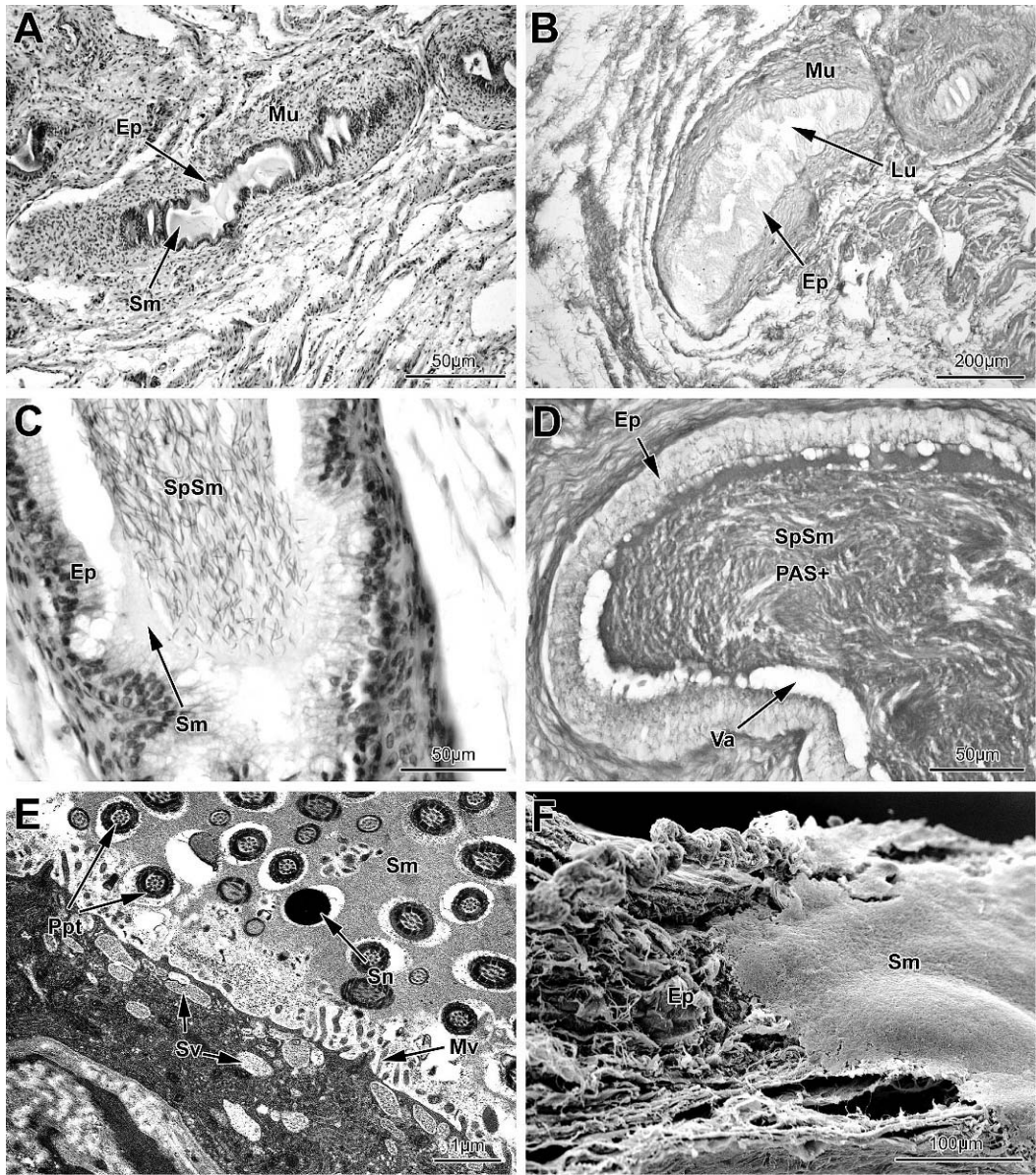


FIG. 11.—Micrographs of the ampulla ductus deferentis of a 46.1 cm SVL male *Agkistrodon piscivorus* collected 13 March (A,B,E,F), a 48.5 cm specimen collected 28 March (C), and 53.6 cm specimen collected 24 May (D). (A) Light micrograph overview stained with hematoxylin-eosin. (B) Light micrograph overview stained with bromphenol blue. (C) Light micrograph detail of sperm in secretory material stained with hematoxylin-eosin. (D) Light micrograph detail stained with PAS and alcian blue. (E) Transmission electron micrograph of luminal border. (F) Scanning electron micrograph showing layer of secretory material in the lumen. Ep, epithelium; Lu, lumen; Mu, muscularis; Mv, microvilli; PAS+, periodic acid-Schiff's reagent positive; Ppt, principle piece of the tail; SpSm, sperm in secretory material; Sm, secretory material; Sn, sperm nucleus; Sp, sperm; Sr, serosa; Sv, secretory vacuoles; Va, vacuoles.

secretory (Fox, 1977). Our histochemical and ultrastructural results on the ductus deferens of *Agkistrodon piscivorus* appear to be the first observations on secretory activity in the squamate ductus deferens.

Volsøe (1944) noted that the ductus deferens straightens before entering the cloacal wall, but stated that the ampulla ductus deferentis occurs in the ureter. The area indicated in his drawing is posterior to the junction of the ureter and ductus deferens, which is actually the urogenital duct. This area is widened, and apparently was his criterion for considering this area the ampulla. The urogenital ducts on either side unite in *Vipera berus*, although their lumina remain separate. Fox (1952) followed the interpretation of Volsøe (1944).

The occurrence of an ampulla ductus deferentis consisting of the posterior end of the ductus deferens has been documented histologically in two species of lizards, *Calotes versicolor* (Akbarsha and Meeran, 1995) and *Sitana ponticeriana* (Akbarsha et al., 2005), and one species of snake, *Seminatrix pygaea* (Sever, 2004). Our results clearly indicate that an ampulla also occurs in the ductus deferens of *Agkistrodon piscivorus*. Sever (2004) found no evidence of secretory activity in the ampulla of *S. pygaea*, but Akbarsha et al. (2005) found that the ampulla of *S. ponticeriana* is divided into glandular and storage portions, and the storage portion was involved in endocytosis and phagocytosis of dead sperm. We found no evidence of spermiophagy, but the anterior three-fourths of the ampulla of *A. piscivorus* is involved in production of a carbohydrate secretion.

**Renal sexual segment.**—The renal sexual segment (Rss) is an enlargement of the distal convoluted tubule of the kidney in male snakes and lizards, and may include the collecting ducts and ureter as well. The secretions of the Rss may sustain and activate sperm (Bishop, 1959; Cuellar, 1966), provide courtship pheromones (Volsøe, 1944), form copulatory plugs (Devine, 1975; Nilson and Andrén, 1982; Ross and Crews, 1977), and/or have other purposes generally associated with seminal fluid (Prasad and Reddy, 1972). Several female lizards are also known to possess a hypertrophy of the nephron similar

to males (Sever and Hopkins, 2005). 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 Sever et al. (2002, 2008) and Sever and Hopkins (2005). Among reptiles, the presence of a Rss can be considered a synapomorphy at least for Squamata and probably for Lepidosauria. Sever et al. (2008) studied the histology and ultrastructure of the Rss of *Agkistrodon piscivorus*. This study was the first to describe the ultrastructure of the Rss in a family of snakes other than the Colubridae.

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 early fall (Fig. 12A). Portions of the Rss are easily distinguished from other tubules in the kidney by their relatively larger size and staining characteristics. When hypertrophied, the epithelium is columnar, uniformly eosinophilic, and possesses basal nuclei (Fig. 12B). In contrast, proximal convoluted tubules are cuboidal and basophilic. Rss tubules react strongly for proteins with bromphenol blue (Fig. 12C) and are largely PAS positive (Fig. 12D) but areas of alcian blue activity also occur. Proximal convoluted tubules are alcian blue positive and react weakly with bromphenol blue.

Transmission electron microscopy 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. 12E). 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. Rough endoplasmic reticulum and Golgi complexes are found in the perinuclear area and are associated with condensing vacuoles that may be irregular in shape. Release of the secretory products could involve both apocrine and merocrine processes. Scanning electron microscopy reveals how secretory granules fill the cytoplasm of active Rss (Fig. 12F).

In June and July, the Rss go through a period where secretory activity is reduced and gland diameter and epithelial height is obvi-

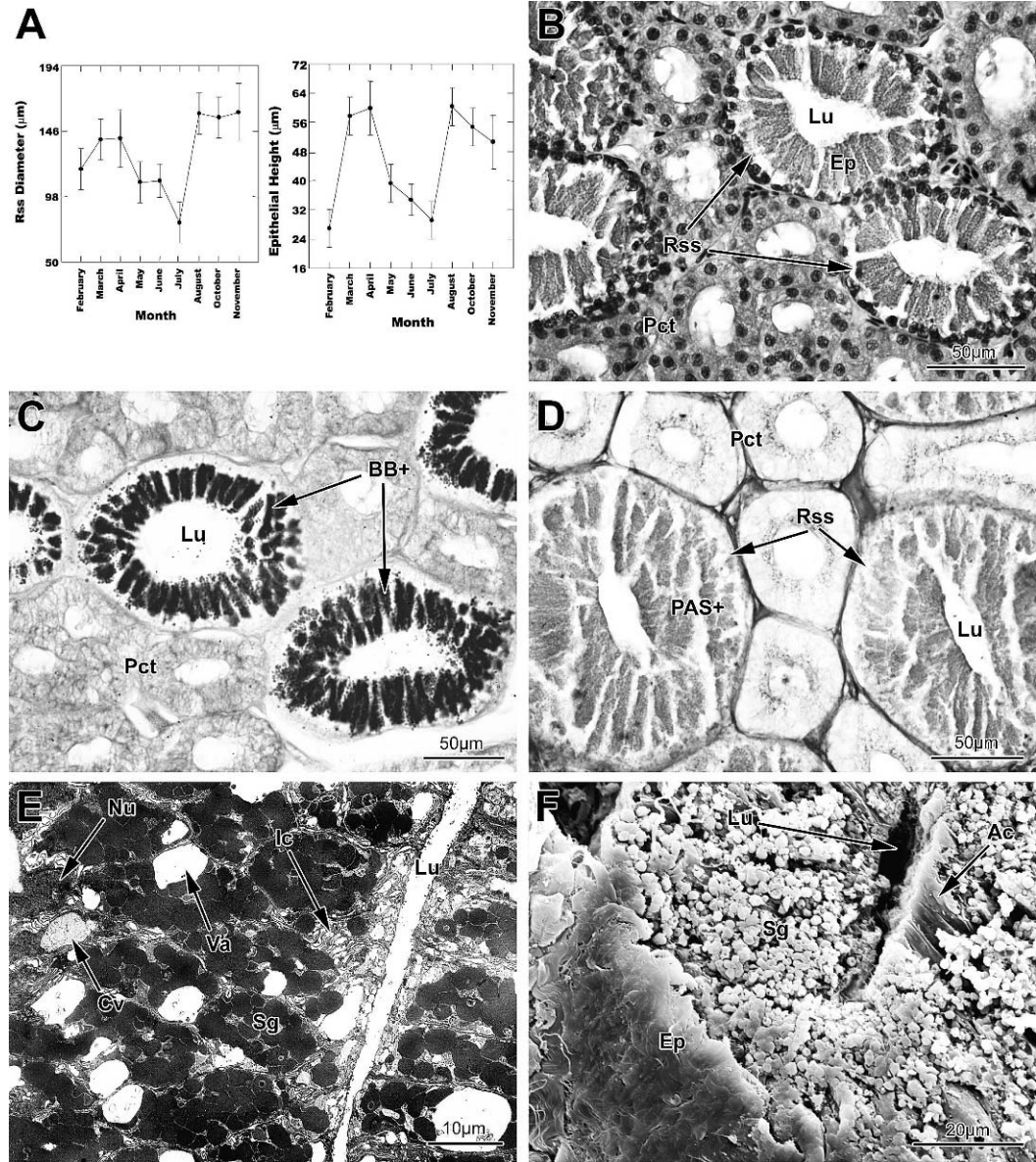


FIG. 12.—Data and micrographs that concern the renal sexual segment (Rss) of male *Agkistrodon piscivorus*. Specimens in the micrographs include a 52.2 cm SVL male collected 23 February (B,C,E), a 46.1 SVL individual collected 13 March (D), and a 56.1 cm specimen collected 22 November (F). (A) Seasonal variation in the diameter and epithelial height of the Rss; error bars represent standard variation. (B) Light micrograph overview of Rss stained with hematoxylin-eosin. (C) Light micrograph overview of Rss stained with bromphenol blue. (D) Light micrograph overview stained with PAS and alcian blue. (E) Transmission electron micrograph of luminal border. (F) Scanning electron micrograph showing a tubule packed with secretory granules. Ac, apical cytoplasm; BB+, bromphenol blue positive; Cv, condensing vacuole; Ep, epithelium; Ic, intercellular canaliculi; Lu, lumen; Nu, nucleus; PAS+, periodic acid-Schiff's reagent; Pct, proximal convoluted tubules; Rss, renal sexual segment; Sg, secretory granules; Va, vacuoles.

ously decreased. The Rss epithelium is decidedly basophilic, and the positive PAS and bromphenol blue reactions are limited to scattered granules. TEM of the June and July samples reveals the loss of mature, electron-dense secretory granules and smaller secretory vesicles, and the reduction in size and number of vacuoles.

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 been reported (Fox, 1977; Sever et al., 2002). Johnson et al. (1982) reported that granules are present in the epithelial cells of the Rss in Alabama *Agkistrodon piscivorus* in April and in the lumina from March through October. Although some PAS positive and bromphenol blue positive granules were present in our specimens from June and July, secretory activity is dramatically reduced from other times of the year.

#### COMPARATIVE MORPHOLOGY: FEMALES

##### *Gross Morphology*

The gross female reproductive morphology of *Serpentes* was reviewed by Blackburn (1998). Figure 13 depicts the undissected and dissected anatomy of female *Agkistrodon piscivorus*. The oviduct is positioned ventral (when intestine is full with fecal waste) or lateral (when intestine is emptied) to the intestines within the body wall (Fig. 13A; a specimen with a full intestine is depicted here). The anterior regions are positioned dorsally to the ventral abdominal vein, which is associated with fat at its periphery (Fig. 13A). After removal of the ventral abdominal vein and fat, the anterior portions of the oviduct can be observed clearly (Fig. 13B), and four regions can be discerned (Fig. 13B): an enlarged posterior region termed the vaginal pouch (Ludwig and Rahn, 1943), a narrow tubular region termed the non-glandular uterus, a slightly enlarged and sometimes-folded region termed the glandular uterus, and a highly folded anterior region termed the infundibulum. Each oviduct is suspended in the body cavity by the dorsal

mesentery. The ovaries, filled with small or enlarged macrolecithal follicles depending on reproductive condition (non-reproductive depiction in Fig. 13B), lie lateral to their respective oviduct (Fig. 13B).

##### *Histology (Reviewed from Siegel and Sever, 2008a,b)*

*Infundibulum and sperm storage tubules.*—The most anterior oviductal region in *A. piscivorus* is the infundibulum that opens anteriorly into the body cavity through the ostium. The infundibulum possesses a thin mucosa with a simple squamous epithelium (Fig. 14A). Small invaginations into the lamina propria form simple tubular glands in the infundibular region (Fig. 14A). Ultrastructurally these glands are undifferentiated from the epithelium lining the lumen. Ciliated cells are observed sporadically throughout the epithelium with secretory cells that produce mainly lipid materials. Ciliated cells possess basally positioned nuclei and are packed with apically positioned mitochondria interacting with basal bodies anchoring the cilia. Lipoid material produced in the secretory cells is either diffuse and unorganized or tightly packed into denser lipid droplets. Smooth endoplasmic reticulum is abundant in the cytoplasm of these secretory epithelial cells. In *Seminatrix pygaea*, another snake investigated with ultrastructural analysis, only organized lipid material was produced by the infundibulum along with protein packed secretory vacuoles (Sever et al., 2000). As with the epithelium of the entire oviduct, epithelial cells are adhered apically by tight junctions, followed basally by desmosomes. Two thin muscle layers encompass the mucosa, an interior circular muscle and exterior longitudinal muscle. As in other squamates investigated (for review see Blackburn, 1998) this muscularis externa is continuous throughout the whole length of the oviduct. The entire oviduct and its muscular layers are encompassed by a layer of visceral pleuroperitoneum, which communicates with the parietal pleuroperitoneum lining the dorsal body wall via the dorsal mesentery (for review on mesenteries in snakes see Blackburn, 1998).

The most posterior tubular glands of the infundibulum possess a wider terminal por-

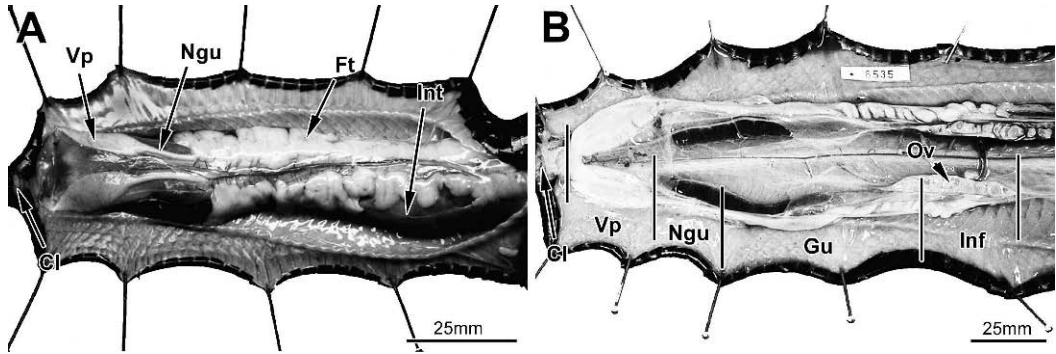


FIG. 13.—Gross morphology of the female *Agkistrodon piscivorus* genital system. (A) Undissected anatomy. (B) Dissected anatomy. Cl, cloaca; Ft, fat; Gu, glandular uterus; Inf, infundibulum; Int, intestine; Ngu, non-glandular uterus; Ov, ovary; Vp, vaginal pouch.

tion and invade deeper into the lamina propria (Fig. 14B). These glands are sperm storage tubules. Distally, the glands are comprised of numerous ciliated cells; terminally, however, these ciliated cells decrease in concentration and secretory cells take over the majority of the epithelium (Fig. 14C). When sperm are present in sperm storage tubules they align themselves in parallel arrays with their nuclei facing the terminal ends of the glands (Fig. 14C). This condition is consistent with what is observed in the snakes *Thamnophis sirtalis* (Halpert et al., 1982; Hoffman and Wimsatt, 1972) and *Diadophis punctatus* (Perkins and Palmer, 1996), and the lizards *Acanthodactylus scutellatus* (Bou-Resli et al., 1981) and *Scincella lateralis* (Sever and Hopkins, 2004). However, one snake species investigated, *S. pygaea*, exhibits sperm with nuclei facing the openings of the sperm storage tubules (Sever and Ryan, 1999).

Infundibular sperm storage tubules were also observed in the snakes *Thamnophis elegans* (Fox, 1956), *Vipera aspis* (Saint-Girons, 1957, 1959), *Cerastes cerastes* (Saint-Girons, 1962a,b), multiple members of the families Typhlopidae and Leptotyphlopidae (Fox and Dessauer, 1962), and *Tantilla coronata* (Aldridge, 1992). In studies where histochemical analysis was conducted, the secretory material produced by infundibular sperm storage tubules was always positive for neutral carbohydrates (Hoffman and Wimsatt, 1972; Perkins and Palmer, 1996; Sever and Ryan, 1999), including sperm storage tubules of *A. piscivorus*. *Thamnophis sirtalis parietalis*

studied by Halpert et al. (1982) also possessed neutral carbohydrate secretions in the infundibular sperm storage tubules. However, it is unclear whether or not the epithelium of the tubules is synthesizing this material. Structure of sperm storage tubules was typically described as simple to complex tubular except in *Tantilla coronata* where sperm storage tubules are more alveolar (Aldridge, 1992).

**Glandular uterus.**—The glandular uterus is characterized by having a thicker muscularis and lamina propria than the infundibulum, and scattered invaginations of the epithelium lining the lumen forming simple tubular glands (Fig. 14D,E). The simple cuboidal epithelium of the mucosa (Fig. 14E) secretes an acidic mucoid substance whereas the uterine glands secrete a protein along their whole length. Ultrastructurally, the epithelium bordering the lumen of the uterus is composed primarily of secretory cells that produce an electron lucent material released apocrinely. Ciliated cells are intermixed randomly with the secretory cells. The glands of the epithelium are composed strictly of secretory cells (Fig. 14F). Cells of this epithelium are packed with an electron dense secretory material (Fig. 14F) released by a merocrine type secretory mode. During synthesis and release of the protein material produced by these glands synthetic organelles, including mitochondria and rough endoplasmic reticulum, are abundant throughout the cell and perinuclearly respectively.

The only other snake uterus that was investigated thoroughly with ultrastructural

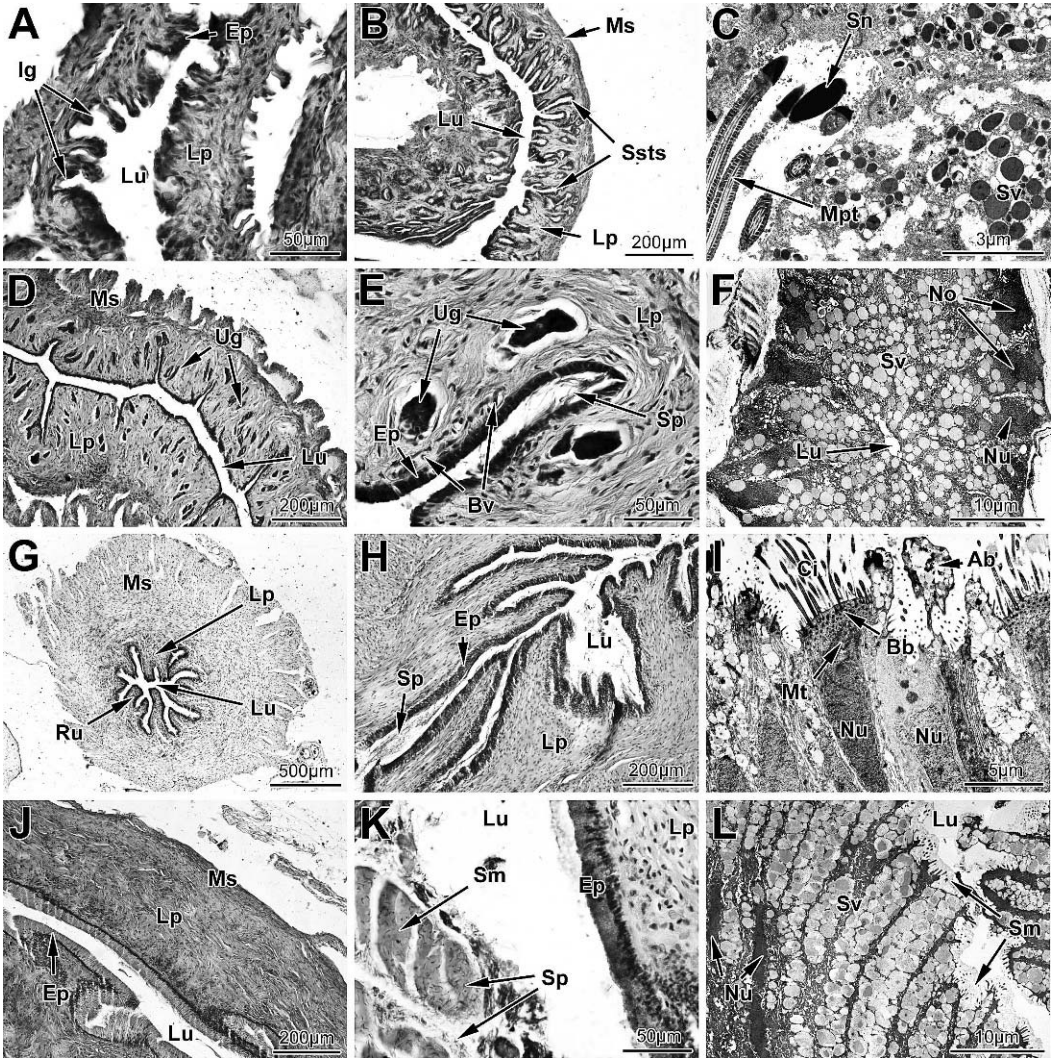


FIG. 14.—Histology of female *Agkistrodon piscivorus* genital system. (A) Anterior infundibulum of a specimen from November. (B) Sperm storage region of the posterior infundibulum of an November specimen. (C) TEM micrograph of sperm at the terminal end of a sperm storage tubule in a May female. (D) Overview of the glandular uterus in a November specimen. (E) High magnification of D showing structural components of the glandular uterus. (F) TEM micrograph of cellular components in the epithelium of a uterine gland from an April female. (G) Histological overview of the non-glandular uterus from a June specimen. (H) Sperm in the lumen of the non-glandular uterus of a May female. (I) Cellular components of the epithelium lining the lumen of the non-glandular uterus in a May female. (J) Histological overview of the vaginal pouch in an April specimen. (K) Sperm associated with eosinophilic secretory material in the vaginal pouch of a July female. (L) Cellular components of the epithelium lining the lumen of the vaginal pouch in a March female. Ab, apocrine bleb; Bb, basal bodies; Bv, blood vessel; Ep, epithelium; Ig, infundibular gland; Lp, lamina propria; Lu, lumen; Mpt, mid principle piece of the sperm tail; Ms, muscularis; Mt, mitochondria; No, nucleolus; Nu, nucleus; Ru, rugae; Sn, sperm nucleus; Sp, sperm; Ssts, sperm storage tubules; Sv, secretory vacuole; Ug, uterine gland.

techniques is that of *Seminatrix pygaea* (Sever et al., 2000). The uterine glands in this snake were basically undifferentiated invaginations of the epithelium lining the lumen (Sever et

al., 2000), whereas in *Agkistrodon piscivorus* the uterine glands are highly differentiated from the luminal border. With the shift from oviparity to viviparity, the activity and number

of uterine glands is hypothesized to decrease (Blackburn, 1998). Considering uterine glands are responsible for eggshell formation (Palmer et al., 1993), the act of not producing an eggshell in viviparous species would tend to support this hypothesis. However, it is clear that in some snake species, including *A. piscivorus*, uterine glands may still play a major roll in reproduction. Because viviparity has evolved multiple times independently in squamates (Blackburn, 1985; Blackburn, 1999; Shine, 1985), uterine gland morphology and activity would vary between taxa that have independently evolved live-bearing capabilities. Uterine gland variation, and association with parity mode has currently not been investigated in snakes, however, Girling et al. (1998) demonstrated ultrastructural variation in lizards that possessed differing modes of parity. Interestingly, uterine glands of the lizard *Saltuarius wyberba* are most similar in terms of ultrastructure to uterine glands of *A. piscivorus* (Girling et al., 1998). However, this lizard is oviparous and produces a parchment-type eggshell (Girling et al., 1998). Other studies on squamate uteri that demonstrate unique uterine morphology due to parity mode shift include those of Girling (2002) and Thompson et al. (2006).

Unfortunately, in our two-year collecting efforts, pregnant *Agkistrodon piscivorus* were not obtained. Thus, discussion of the pregnant uterus is limited to light microscopy from a museum specimen and from other historical studies. It appears that in late gestation uterine glands are still actively synthesizing and releasing secretory material in *A. piscivorus*. This is evidenced by the presence of intensely staining protein granules in the glandular uterus in late August. Thus, uterine gland activity most likely continues throughout gestation. As reviewed by Blackburn (1998) in a variety of squamates, the pregnant uterus of *A. piscivorus* is highly reduced in terms of the oviducal lining (e.g., muscularis thickness, lamina propria thickness, and height of epithelial cells). Similar to a colubrid (*Thamnophis sirtalis*; Hoffman, 1970) and another crotaline (*Crotalus viridis*; Rahn, 1942), the uterus of *A. piscivorus* becomes highly vascularized during pregnancy. However, no statistical analyses were conducted to

assess this quantitatively, and as Blackburn (1998) states, the quantification of vascularization in the uterus from histological sections is difficult.

Microscopy of fetal membranes and uterus/extra-embryonic membrane association were not conducted in our investigations on reproductive morphology in *Agkistrodon piscivorus* due to the lack of pregnant specimens. However, recent literature is available on the structure and evolution of placentation/viviparity in snakes, particularly thamnophiines. For information regarding these topics we refer readers to Blackburn (1998) and Blackburn and Flemming (2008).

*Non-glandular uterus.*—The non-glandular uterus, sometimes called the vaginal tube or segment (Saint-Girons, 1973), anterior vagina (Hoffman and Wimsatt, 1972), or furrowed portion of the uterus (Aldridge, 1992; Halpert et al., 1982), was previously described in the Colubridae and Viperidae. We term this region the non-glandular uterus because like the glandular uterus, embryos invade this region during embryogenesis. However, unlike the glandular uterus, no tubular glands can be observed in the lamina propria of this region (Fig. 14G). In *Agkistrodon piscivorus* this oviducal segment possesses a very thick muscularis, a thin lamina propria compared to the glandular uterus (Fig. 14G,H), and a simple cuboidal epithelium that becomes columnar when moving posteriorly down the oviduct (Fig. 14H). The thick exterior layer of longitudinal muscle creates the longitudinal folds that are observed grossly (Fig. 14G). The majority of the epithelium is composed of ciliated cells, and secretory cells can be observed scattered throughout (Fig. 14I) with acidic mucous secretions in the apical portion of the cells. Ultrastructurally, the secretions are packed in electron lucent vacuoles and are released via an apocrine type secretion (Fig. 14I). During times of high secretory activity, mitochondria and Golgi bodies are observed apical to the nucleus in cells that have recently secreted their product. Nuclei of ciliated and secretory epithelial cells are located in a basal position (Fig. 14I). This region of the oviduct contains numerous mast cells in the lamina propria.

To our knowledge, the only other snake where a similar region has been described ultrastructurally has been *Seminatrix pygaea* (Sever et al., 2000). In that study, this region was termed the vagina. However, after a close look at the similarities of these regions, it is probable that the non-glandular uterus in *Agkistrodon piscivorus* and vagina in *S. pygaea* are in fact homologous. As mentioned above, Halpert et al. (1982) also described this region of the oviduct using basic histology in *Thamnophis sirtalis*. They believed that secretions from the non-glandular uterus interacted with sperm during mating and facilitated transport of sperm anteriorly up the oviduct. This is not likely in *A. piscivorus* because sperm were never associated with acidic mucoid secretions like those from the epithelium of the non-glandular uterus while in the female reproductive tract. The secretions associated with sperm observed in the female oviduct were more similar to those observed in the secretory epithelium of the Rss in males (Sever et al., 2008). We question whether the secretory epithelium of the non-glandular uterus in *T. sirtalis* could really be producing a carrier matrix for sperm.

**Vagina.**—The vagina in *Agkistrodon piscivorus*, and all viperids, is an interesting oviductal region. Histologically, no comparable region has been described. This region of the oviduct possesses a single layer of high columnar epithelial cells that are primarily secretory in function (Fig. 14J,K,L). Ciliated cells are occasionally interspersed with the majority secretory cells that produce an electron lucent material with an electron dense core. This material is composed of acidic and neutral carbohydrates and coats the apical surface of the epithelium after secretion in a merocrine manner. Around the basally positioned nuclei (Fig. 14L) there exists large electron dense lipid droplets. Between adjacent epithelial cells, and the epithelium and the basal lamina, exist large vacuolization's of the intercellular canaliculi. Plasma-like cells are often found inhabiting these vacuoles. These plasma-like cells are not the only immune cells inhabiting this region of the oviduct, for like the non-glandular uterus, mast cells are found in very high concentrations in the wide lamina propria (Fig. 14J,K)

of the vaginal pouch. The muscularis of the vaginal pouches is thick compared to all regions of the oviduct except the non-glandular uterus (Fig. 14J).

It is clear through literature searches (Gabe and Saint-Girons, 1965; Sánchez-Martínez, 2007), personal investigation by D. S. Siegel on other snake families, and conversations with R. D. Aldridge, that the vaginal pouch of vipers is actually what is traditionally called the urodeum in other colubroid snakes. Thus, the vaginal pouches are actually the anterior-most portion of the cloaca. Anatomically, this is an interesting discovery because like other vertebrates that possess a true vagina, what is traditionally called the vagina of vipers is derived from cloacal tissue, instead of coelomic mesothelial tissues, which gives rise to the infundibulum and uterus (Raynaud and Pieau, 1985). Therefore, in a sense, vipers have a "true" vagina. Functionally, the purpose of a highly extended urodeum like that seen in vipers is unknown. However, electron and light micrographs of the vaginal pouch epithelium reveals similarities to the vaginal epithelium of some mammals (e.g., the microvillus, columnar, mucin secretory epithelium of rodents; Lamb et al., 1978). It is unclear if the squamate urodeum is capable of tactile function, or if the hemipenis of vipers protrude into the pouches; however, Pope (1941) describes the male hemipene reaching the opening of the oviduct (i.e., protruding through the urodeum) in a colubrid and Cope (1898) actually termed the cloaca the vagina because of its function in accepting a copulatory organ. Like Ludwig and Rahn (1943), we hypothesize that the hemipenes of male vipers actually extend into the vaginal pouches of their conspecific females. Thus, vipers may actually possess a correctly defined anatomical and functional vagina.

**Oviductal regions.**—From an extensive review of literature and histological examination of snakes and lizards Blackburn (1998) simplified the squamate oviduct into three regions: (1) a posterior vagina, (2) middle uterus, and (3) anterior infundibulum. The region termed the non-glandular portion of the uterus in *Agkistrodon piscivorus* (Siegel and Sever 2008a) is undoubtedly homologous with the region Blackburn (1998) terms the

vagina, as he discusses in his lengthy review. This was based on similar histology and position of this oviducal region. Thus, the terminology for the oviduct in Viperidae varies from Blackburn (1998) because of the presence of an extension of the cloaca (vaginal pouches; see above), which may be a synapomorphy of Viperidae. In conclusion, the oviducts of squamates all appear to have three homologous regions (posterior, middle, and anterior), however, variation within and between these regions occurs (e.g., uterine gland morphology, Girling et al., 1998; sperm storage receptacle morphology and location, Eckstut et al., 2009).

#### REPRODUCTIVE CYCLE

In reviewing the reproductive cycle of *Agkistrodon piscivorus* it is necessary to consider males and females individually because of the dissociation of the timing of reproductive events. This dissociation creates a model in which the only event that has to be coordinated between males and females is copulation. Although copulation is obviously an integral part of the reproductive cycle, published accounts of actual copulation events are lacking. Beyer (1893) provides evidence of a male and female in coitus in the spring; however, only the female was captured. Positive identification of the conspecific as a male was not achieved. Wharton (1966) notes bisexual pairing of *A. piscivorus* in almost all months of the year and suggests *A. piscivorus* could be continually mating throughout the active seasons. Siegel and Sever (2006, 2008a) note sperm in the posterior portion of the female reproductive tract (vaginal pouch and non-glandular uterus) in the late summer–fall and spring before ovulation has occurred. These data suggest a biannual mating season, at least in Louisiana populations of *A. piscivorus*, and are consistent with data presented for *A. piscivorus* reviewed by Aldridge and Duvall (2002).

#### Males

To this date, four studies have tracked reproductive events in male *Agkistrodon piscivorus* by utilizing histology of the testis and Rss (Graham et al., 2008; Gribbins et al., 2008; Johnson et al., 1982; Sever et al., 2008).

Sever et al. (2008) and Gribbins et al. (2008) studied specimens from the most southern distribution, using snakes from southeastern Louisiana. The most northern study was conducted by Graham et al. (2008) at the confluence of Morning Creek with the Flint River in Georgia, while Johnson et al. (1982) investigated a slightly more southern population in Perry County, Alabama.

*Testicular cycle.*—In the population of male *Agkistrodon piscivorus* investigated in Louisiana, development of seminiferous tubule epithelia can be observed early in the spring after hibernation and continues until July (Figs. 4,5). During this time period, all meiotic stages of spermatocyte advancement, spermiogenesis, and spermiation can be observed in the testes. Subsequent to a quiescent period in mid-summer (July; Figs. 4,5), the development of the seminiferous tubules reoccurs and a second term of sperm formation can be observed (Figs. 4,5; see *Germ cell development strategy* for in-depth review). The histology and data analysis robustly supports that male *A. piscivorus* in Louisiana possess a biannual spermatogenic cycle. To our knowledge, this is the first time this has been reported in a North American crotaline.

Johnson et al. (1982) and Graham (2008) reported that male *Agkistrodon piscivorus* are only spermatogenic in the summer–fall time period in Alabama and Georgia respectively. This fits what has also been described for all other temperate pitvipers in North America (for review see Aldridge and Duvall, 2002).

*Rss cycle.*—Similar to the spermatogenic cycle of male *Agkistrodon piscivorus* from Louisiana, two peaks of major secretory material synthesis and release can be observed in the Rss; one in the spring and one in the later summer–fall (Sever et al., 2008; see above). A spring and late summer–fall peak of Rss activity was also described by Johnson et al. (1982) from male *A. piscivorus* in Alabama. In Georgia a significant peak of activity, based on Rss diameter was not observed (Graham et al., 2008). However, a noticeable non-significant peak was observed in the late summer–fall (Graham et al., 2008). We believe that this represents one peak of major Rss activity in male *A. piscivorus* from Georgia, and that statistical significance would be revealed if

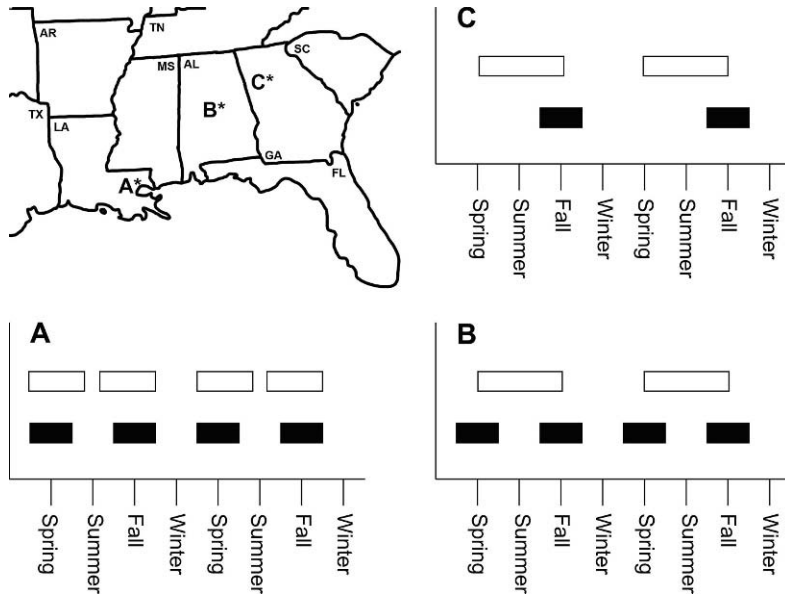


FIG. 15.—Geographical variation in male secondary sexual characteristics and sperm formation in *Agkistrodon piscivorus* from Louisiana (A), Alabama (B), and Georgia (C). White rectangles represent seasonality of spermatogenesis. Black rectangles represent seasonality of major secretory activity in the renal sexual segment.

epithelial height was used for monthly comparison instead of tubular diameter. Tubular diameter varies less from month to month whereas epithelial height is the more important indicator of Rss activity (e.g., directly measuring the amount of secretory material produced in the Rss).

*Geographic variation in reproductive events.*—

From the above analysis, a trend can be observed while moving northeast from populations of *Agkistrodon piscivorus* in southeastern Louisiana. In northerly populations primary and secondary sexual character activity in males is pushed to one end of the active season, in this case, summer and fall respectively. *Agkistrodon piscivorus* males from southeastern Louisiana exhibit biannual reproductive events, the most northern population studied to date in Georgia has annual reproductive events, and the population in Alabama between these two extremes is transitory (Fig. 15). Increases in the production of material in the excurrent ducts (see above) also coincide with the mating seasons (fall and spring) in Louisiana *A. piscivorus*. However, no data is available from the Alabama and Georgia populations for comparison.

In general, spermatogenesis and Rss hypertrophy are androgen dependent. Thus, variation in the timing of male reproductive events presents two very interesting questions:

- (1) Are two peaks of testosterone necessary for biannual spermatogenesis observed in the Louisiana population of *Agkistrodon piscivorus*? Fall and spring peaks of testosterone have been recorded in other North American crotalines, *Crotalus atrox* (Schuett et al., 2005; Taylor et al., 2004) and *C. scutulatus* (Schuett et al., 2002), that do not have biannual spermatogenesis. Typically, spring testosterone peaks have been interpreted as increases in testosterone to benefit only mating activity (Schuett et al., 2005; Taylor et al., 2004), and thus, the testosterone peaks in the spring are not as high as those observed in the summer-fall (Schuett et al., 1997; Taylor et al., 2004) when spermatogenesis is occurring. However, in Louisiana *A. piscivorus* spring testosterone peaks (if observed) may also be involved in stimulating spermatogenesis and therefore may be as high as those observed in the fall.

Interestingly, *A. piscivorus*, which has been recorded as a biannual breeder (although published accounts of mating activity are lacking; see Graham et al., 2008, and above), does not have a spring peak of testosterone in the Alabama and Georgia populations studied (Graham et al., 2008; Johnson et al., 1982). This supports the hypothesis that *A. piscivorus* may not be a biannual breeder in all portions of its range (Graham et al., 2008).

- (2) Given that the Rss is androgen dependent (Prasad and Sanyal, 1969), how is spring hypertrophy maintained without an increase in testosterone in the Alabama population of *Agkistrodon piscivorus* and many other North American crotalines that exhibit biannual Rss hypertrophy (Aldridge and Duvall, 2002)? A hypothesis for this is that testosterone is effective in stimulating Rss secretion synthesis, however, it is not necessary for maintenance after initial stimulation (Aldridge, personal communication). This also could account for the lack of a testosterone peak in the spring in biannually reproducing populations.

Traditionally the *Agkistrodon piscivorus* complex is made up of three subspecies (Gloyd and Conant, 1990): an eastern variety (*A. p. piscivorus*), a western variety (*A. p. leucostoma*), and a Florida variety (*A. p. conanti*). However recent molecular data and phylogeographic analysis of the *A. piscivorus* complex reveal that the only independently evolving lineages in the complex are the continental members (*A. p. leucostoma* and *A. p. piscivorus*) and the Florida member (*A. p. conanti*; Guiher and Burbrink, 2008; Douglas et al., 2009). Considering the variation in reproductive timing reviewed above (from the southwest to the northeast) is within the genetically similar continental members, it is hypothesized that variation in reproductive attributes is due to local adaptation, and not evolutionary history. Out of the four reproductive cycle patterns described for North American crotalines (Aldridge and Duvall, 2002), it appears that males from the *A. piscivorus* complex are utilizing the "Temperate Zone Primitive Pattern" in Louisiana

(Spring and fall activity), "Temperate Zone Derived Pattern" in Georgia (summer-fall activity), and a pattern that falls somewhere in between the former two in Alabama (Fig. 15). However, no previous patterns described a biannual spermatogenic cycle as seen in Louisiana *A. piscivorus* in North American crotalines. Similar results have been presented on another North American crotaline, *Crotalus oreganus*, where the timing of reproductive events is variable across the range of genetically similar populations (Aldridge, 2002; Ashton and de Queiroz, 2001; Pook et al., 2000).

### Females

**Vitellogenesis.**—As in most North American crotaline snakes (for review see Aldridge and Duvall, 2002), vitellogenesis commences in the later summer-early fall in *Agkistrodon piscivorus* (Siegel and Sever, 2008b). This pattern is observed by the noticeable increase in follicular size beginning around October in Louisiana *A. piscivorus*. Follicles cease further enlargement during winter hibernation in Louisiana females, which lasts from approximately late November to late February. Follicular growth recommences in spring after emergences from over-winter hibernacula. This is consistent with observations from ten species of North American crotalines (for review see Aldridge and Duvall, 2002). However, Taylor et al. (2004) and Schuett et al. (2004) note a caveat to this pattern with data that they propose support vitellogenesis commencing in the spring in a population of *Crotalus atrox* in Arizona. Aldridge et al. (2008) review this controversy and propose that too few specimens may have been examined from the fall in the *C. atrox* studied to make the above conclusions.

Although data are lacking for hormonal control of vitellogenesis in *Agkistrodon piscivorus*, Bonnet et al. (1994; 2001), Schuett et al. (2004), and Taylor et al. (2004) provide some insight from studies on *Vipera aspis* (Viperinae) and *Crotalus atrox* respectively. These studies found the progress of vitellogenesis is concurrent with elevated estradiol levels. Vitellogenesis terminates with a decrease in estradiol and a subsequent increase in progesterone.

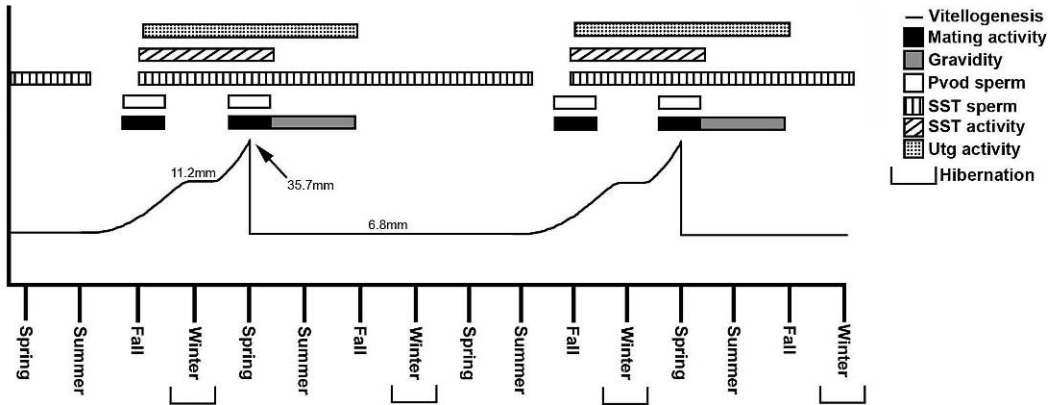


FIG. 16.—The oviducal cycle of female *Agkistrodon piscivorus* and its relation to the vitellogenic cycle. Measurements are equal to the greatest follicular diameter during different stages of the vitellogenic cycle. Pvoid, sperm present in the posterior oviduct; SST sperm, sperm present in the sperm storage tubules; SST activity, secretory activity of sperm storage tubules; Utg, secretory activity of uterine glands.

**Oviducal cycle.**—The glandular uterus and sperm storage tubules are the only two regions of the oviduct that exhibit dramatic seasonal changes in female *Agkistrodon piscivorus* (Siegel and Sever, 2008b). This is similar to data published for a colubrid, *Seminatrix pygaea* (Sever et al., 2000). The vaginal and anterior infundibular portion of the *A. piscivorus* oviduct exhibits no seasonal variation. Figure 16 graphically depicts the entire oviducal cycle of *A. piscivorus* females.

Initial follicular growth in *Agkistrodon piscivorus* occurs in the fall (approximately mid-September to October), coincident with the fall mating season (evidenced by sperm presence in the posterior oviduct of females). During this time secretory activity increases in the epithelium of the non-glandular uterus and the epithelium lining the lumen of the glandular uterus. Concurrent to the increase of secretory activity of the epithelium lining the lumen of the mid-oviduct, simple tubular glands of the glandular uterus and posterior infundibulum (sperm storage tubules) begin synthesizing secretory material that was previously not observed. While the epithelium lining the lumen of the uterine regions increases the synthesis and release of mucus (Fig. 14I), tubular glands of the glandular uterus produce an electron dense protein (Fig. 14F). Synthetic organelles (rough endoplasmic reticulum, Golgi bodies) also increase in abundance in the epithelial cells at this time

(Fig. 14F,I). Sperm storage tubules synthesize an electron lucent neutral carbohydrate during this stage of the oviducal cycle. These observations are consistent with what occurs in the infundibulum and uterine regions of *Seminatrix pygaea*, except that uterine glands of the *S. pygaea* uterus exhibit minor seasonal changes in terms of secretory activity (Sever et al., 2000). Secretory material synthesis and release continues in the oviduct and varies little until the spring. After hibernation the material synthesized by sperm storage tubules becomes electron dense (Fig. 14C), an occurrence that has not been noted in any other investigations on snakes.

The next dramatic change in the oviducal cycle does not occur until ovulation, after final follicular growth and the spring mating season. At this time the synthesis of secretions in the sperm storage tubules ceases. In other snakes investigated (*Thamnophis sirtalis* and *Seminatrix pygaea*), the lack of secretory activity coincides with the disappearance of sperm (e.g., Halpert et al., 1982; Sever and Ryan, 1999). However, residual amounts of sperm remain in *Agkistrodon piscivorus* (see next section).

Uterine glands continue to remain active in *Agkistrodon piscivorus* until parturition. At this time they regress in activity and size in comparison to their previous state prior to vitellogenesis. Parturition, which occurs in the later summer–early fall in *A. piscivorus* (Allen

and Swindell, 1948; Beyer, 1893; Burkett, 1966; Conant, 1933; Ford, 2002; Ford et al., 2004; Funk, 1964; Gloyd and Conant, 1990; Wharton, 1960), marks the end of the oviducal cycle in a reproductive year. Offspring number ranges from 5–20 (Dundee and Rossman, 1989). Female *A. piscivorus* is hypothesized to breed less than annually (Wharton, 1966), as is hypothesized for many temperate crotaline snakes (Klauber, 1972), and thus activity in the oviduct does not increase again until at least the fall of the subsequent year. Collection data on Louisiana *A. piscivorus* is consistent with this hypothesis with approximately half of the females being reproductive in a given year.

*Vipera aspis* (Bonnet et al., 1994, 2001) and *Crotalus atrox* (Schuett et al., 2004; Taylor et al. 2004) provide insight on the hormonal control of oviducal activity and parturition in viviparous vipers. In both viperid species gestation is concurrent with high levels of progesterone, which is undoubtedly the proximate stimulus for oviducal development towards gestation. At the timing of parturition, progesterone levels decrease, however, Bonnet et al. (2001) notes that exogenous supplementation of progesterone before parturition does not delay birth. Thus, the decrease of progesterone alone is not the proximate stimulus to parturition (Bonnet et al., 2001).

*Sperm transport and storage.*—The occurrence of sperm storage in North American crotalines, and the potential costs and benefits was reviewed extensively by Schuett (1992). Sperm transport and storage in colubrids was described by Fox (1956) and Halpert et al. (1982), and was reviewed in viperines by Saint-Girons (1973). In those studies and the recent investigations on sperm transport and storage in *Agkistrodon piscivorus* (Siegel and Sever, 2008a), sperm were found to be located in the posterior uterus shortly after copulation. However, histology of the mid and anterior oviduct revealed that sperm were migrating from the posterior uterus to infundibular sperm storage tubules. Sperm storage in the posterior uterus is thus hypothesized to be an artifact of mating activity. Because sperm seem to remain in the posterior uterus for an extended period of time (up to the time of ovulation), two sites of sperm storage in

snakes could be proposed (Eckstut et al., 2009). In *A. piscivorus*, however, sperm in the posterior uterus were undergoing degradation shortly before ovulation, which supports the hypothesis of sperm presence because of mating activity. Since the posterior uterus has such a small luminal diameter compared to the rest of the oviduct (and the highly enlarged vaginal pouch), it may be possible that a morphological bottleneck causes some sperm to get stuck in this area. We also suggest that some sperm are inherently poor and cannot migrate anteriorly. Nilson and Andr  n (1982) reported in *Vipera berus* that the posterior uterus forms a non-gelatinous copulatory plug preventing the migration of sperm from subsequent matings to infundibular sperm storage tubules by uterine contraction shortly after copulation. They proposed and provided evidence, that secretions from the renal sexual segment caused this uterine contraction.

As mentioned above, in *Thamnophis sirtalis* and *Seminatrix pygaea* sperm are evacuated from sperm storage tubules after ovulation. However, in *Agkistrodon piscivorus* females this is not the case (Siegel and Sever, 2008a). Sperm remain in the sperm storage tubules through pregnancy, the inactive season, and all the way to the month before mating activity in the subsequent mating season. At this point the sperm disappear. No evidence for how this occurs was observed. This presents an interesting question of the function of secretions from sperm storage tubules. Because secretory activity in sperm storage tubules only occurs during vitellogenesis, and thus the mating seasons, we believe that this fact provides evidence that sperm storage tubule secretions may function in only attracting sperm. However, evidence that supports the continued viability of sperm in sperm storage tubules after ovulation and cessation of secretory activity is needed to support this hypothesis.

Almeida-Santos and Salom  o (1997, 2002) proposed that contractions of the uterus trap sperm in the posterior region of the oviduct after mating in all temperate pitvipers. However, from the above information, it is clear that sperm in all snakes investigated, including *Agkistrodon piscivorus*, which was included in the work by Almeida-Santos and Salom  o

(2002), migrate anteriorly shortly after mating. Therefore, we believe this phenomenon observed by Almeida-Santos and Salomão (1997, 2002) serves another function and could be similar to the non-gelatinous copulatory plug reported by Andrén and Nilson (1987) in *Vipera berus*.

One study suggested that secretions from the posterior uterus interacted with sperm and may facilitate sperm migration anteriorly to infundibular sperm storage tubules (Halpert et al., 1982). However, in *Agkistrodon piscivorus* secretions from the epithelium lining the lumen of the posterior uterus produced only mucous, no proteins (Siegel and Sever, 2008a). Sperm in this region were associated with glycoproteins similar in appearance to those synthesized in the renal sexual segment of the male kidney in *A. piscivorus* and other snakes investigated (Sever et al., 2002; Sever et al., 2008). Considering the paucity of secretory material produced in the efferent ducts of male *A. piscivorus*, we believe the majority of seminal fluid associated with sperm in the female oviduct comes from the Rss of males.

#### Overlap of Male and Female Reproductive Cycles

As stated above, the reproductive cycles of male and female North American crotalines are clearer when thought of individually because of the dissociation of reproductive events that can occur because of the ability of males and females to store sperm (Schuett, 1992). However, in Fig. 17 (corroboration of Fig. 15 and 16) we present a graph that depicts the association of the male and female reproductive cycles in Louisiana *A. piscivorus* by qualifying the activity of major organs involved in reproduction. Because of sperm storage, the only reproductive event that must be coordinated between males and females is mating activity, however, as Fig. 17 demonstrates, mating activity is also associated with synthesis of abundant secretory material in the male excurrent ducts (Ed) and Rss. These results add to the increasing data that the Rss is highly important in reproduction in snakes, and also implicate other potential areas (e.g., distal ductuli epididymids) for synthesis of seminal fluids in snakes.

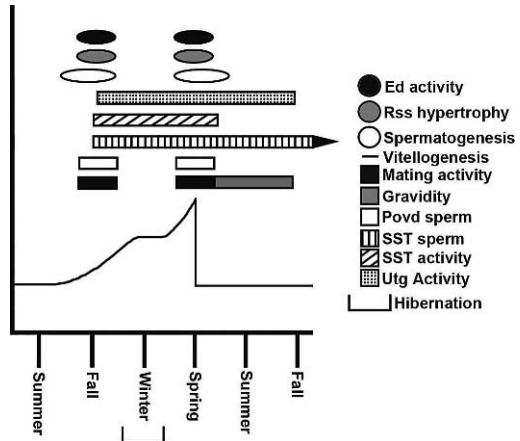


FIG. 17.—Combined male and female reproductive cycles derived from Louisiana snakes (note: spermatogenic and renal sexual segment cycles would vary in different geographic locations). Ed activity, secretory activity of the excurrent ducts; Rss hypertrophy, secretory activity of the renal sexual segment; SST activity, secretory activity of sperm storage tubules; Utg, secretory activity of uterine glands.

The fall wave of spermatogenesis also overlaps the mating season in *Agkistrodon piscivorus* from Louisiana. This is evidenced by sperm presence in the posterior oviduct of females in August while spermatogenesis is still occurring in males (Fig. 17). Additionally, the spring wave of spermatogenesis observed in Louisiana *A. piscivorus* is coordinated with mating activity. However, the ductus deferentia of male *A. piscivorus* males were never void of sperm before, during, or after spermatogenesis. Therefore, an interesting question arises from these data: When are sperm from spermatogenic events actually utilized by males in a mating? It would seem that since sperm are never absent in the ductus deferentia, new sperm produced would be aggregated at the most posterior portion of the ductus deferentia and would not be used in the first subsequent mating event to spermatogenesis. However, there are no data available that determine how many mating events must occur to utilize sperm from a specific spermatogenic event.

#### FUTURE DIRECTIONS

Obviously gaps exist in the present analyses of the reproductive cycles in *Agkistrodon*

*piscivorus*. At this time, the only information on the female oviducal cycle and seasonal variation of spermatogenesis and secondary sexual characteristics in males is from a small portion of the entire *A. piscivorus* range. Male hormonal data from southeastern Louisiana to compare with the work by Johnson et al. (1982) and Graham et al. (2008) are desired. Any data on the hormonal control of reproduction in females of this species are needed, and are lacking in North American crotalines in general. While gathering these data, attention must be paid to the extremities of the *A. piscivorus* range (e.g., Texas, North Carolina, Kentucky, etc.), and Florida where a divergent mtDNA lineage resides (*A. p. conanti*; Guiher and Burbrink, 2008). Gross morphology and histological analysis of museum specimens from different geographic regions could help supply some of the desired data, and this seems feasible with the substantial number of *A. piscivorus* present in museum collections. Other than an extensive museum survey, the only way to accomplish these goals is to further collect wild specimens throughout the reproductive and non-reproductive seasons.

The lack of hormonal data suggests more field studies are necessary to gather blood samples from *Agkistrodon piscivorus* in their natural environment. More time spent towards field studies in different geographic areas may also add to the missing data of when *A. piscivorus* are actually mating throughout their large geographic range. However, more time spent in the field does not always result in witnessing of reproductive behaviors, especially in a rather shy snake in terms of sexual activity like *A. piscivorus* (S. Graham, personal communication). Radio-telemetry is without a doubt an avenue that could help with these missing data and many projects utilizing this technique have recently been undertaken (E. Menzel, personal communication). Schuett and many collaborators have been successful in the past in utilizing captive colonies to collect hormonal data, and these types of studies are desirable, especially in females where precise reproductive condition is important in evaluating the hormonal control of reproduction.

We feel one of the more interesting phenomenon observed in *Agkistrodon pisciv-*

*orus* (and other squamates) is the ability to store sperm for very long times. However, currently no data are available that track the lifespan of one cohort of sperm from one spermatogenic event, to its utilization in mating, and finally to its utilization in fertilization. Specific chemicals like tritiated thymidine that can label sperm from one cohort in a spermatogenic event may be very useful in determining the entire lifespan of sperm, and the wealth of reproductive data on *A. piscivorus* may indicate its use in flagship investigations that employ these types of labeling techniques.

Whatever the direction of research on *Agkistrodon piscivorus* takes, we feel that the recent explosion of baseline reproductive data on *A. piscivorus* makes this snake a prime candidate for future investigation. Utilization of this species will not only benefit our understanding of reproduction in viperids, but overall, also add to our knowledge of the evolution of reproductive biology in snakes.

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