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# UTERO-MUSCULAR TWISTING AND SPERM STORAGE IN VIPERIDS

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***Abstract.*—Recent work on the reproduction of tropical viperids including *Crotalus durissus* and 13 species of *Bothrops* have suggested that females store sperm after mating and prior to ovulation/fertilization by "utero-muscular twisting" (UMT). Basically, the posterior region of the uterus becomes convoluted and contracted. Previous workers have indicated that this mechanism also occurs in temperate taxa, including *Agkistrodon piscivorus*, and others have suggested that UMT is an ancestral reproductive strategy of viperids. The work of these authors ignores earlier histological studies that found infundibular sperm storage tubules (SSTs) in old world viperids *Cerastes cerastes* and *Viperus aspis*. The SSTs in these two viperids were similar to those of harmless snakes, including some more basal (Leptotyphlophidae and Typhlopidae) and others more derived (Colubridae) than the Viperidae. In addition, our studies on seasonal variation in sperm storage in *Agkistrodon piscivorus* show sperm in infundibular SSTs in all reproductively active females and even those with fetuses *in utero*, with no indication of UMT. Our histological examination of the crotalines *Sisturus miliarius* and *Crotalus durissus* indicates that SSTs occur in these forms as well. The evidence for UMT as a sperm storage strategy needs anatomical and physiological confirmation.**

*Key Words*.—*Agkistrodon piscivorus*; histology; oviducts; sperm storage; Viperidae

## INTRODUCTION

Because of asynchrony in the timing of mating and ovulation, sperm storage in the oviduct is obligatory in the reproductive cycles of many reptiles (Sever and Hamlett 2002). The oviduct of squamate reptiles (snakes, lizards, and amphisbaenids) is functionally divided into an anterior infundibulum, middle uterus, and posterior vagina (Blackburn 1998). Sperm storage tubules (SSTs) have been described in the posterior infundibulum (or "tuba") or the vagina of a variety of lizards (Cuellar 1966; Sever and Hamlett 2002; Sever and Hopkins 2004). Few snakes have been examined histologically by light microscopy for the presence of SSTs, and most have been harmless snakes in the families Leptotyphlopidae: *Leptotyphlops duclis, L. humilis* (Fox and Dessauer 1962); Typhlopidae: *Typhlops bramius*, and *T. angolensis* (Fox and Dessauer 1962); or traditionally placed in the family Colubridae: *Thamnophis elegans* and *T. sirtalis* (Fox 1956; Hoffman and Wimsatt 1972), four species of *Tantilla* (Fawcett et al. 1972; Aldridge 1992), *Diadophis punctatus* (Perkins and Palmer 1996), *Nerodia sipedon* (Blackburn 1998, but see Bauman and Metter 1977), and *Seminatrix pygaea* (Sever and Ryan 1999). These snakes all store sperm in the posterior infundibulum although sperm may also be stored in the vagina or posterior uterus prior to passage to the infundibulum (Saint-Girons 1973; Halpert et al. 1982; Perkins and Palmer 1996). The only studies utilizing ultrastructural examination of sperm storage were Hoffman and Wimsatt (1972; transmission electron microscopy, TEM), Perkins and Palmer (1996; scanning electron microscopy, SEM), and Sever and Ryan (1999; both TEM and SEM).

The snake family Viperidae is phylogenetically more recently derived than the Leptotyphlopidae and Typhlopidae and sister to the family Colubridae (Lawson et al. 2005). Viperids are characterized by a relatively enlarged vagina, called the vaginal pouch (Ludwig and Rahn 1943). In some non-viperids, a sphincter has been found between the oviduct and cloaca (Uribe et al. 1998), and the existence of a sphincter in the viperid *Viperus berus* has been suggested (Nilson and Andrén 1982). Ludwig and Rahn (1943) proposed that during copulation, a hemipenis extends through the cloaca into the vaginal pouch.

Ludwig and Rahn (1943) did histological work on at least the posterior end of the oviduct of *Crotalus viridis* and only found sperm in the portion of the oviduct immediately anterior to the vaginal pouch, which they still termed vagina but which all subsequent workers have called uterus (e.g., Blackburn 1998; Almeida-Santos and Salomão 1997). Ludwig and Rahn (1943) proposed that after storage in this area over winter, sperm migrate anteriorly to fertilize eggs that are ovulated in spring. However, the authors chose to sacrifice their specimens shortly after time of hibernation so if migration of sperm to the posterior infundibulum is slow, they could have easily missed the presence of sperm in infundibular SSTs.

Aside from Ludwig and Rahn (1943), very little histological work has been done on sperm storage in the Viperidae. The best studies are by Saint-Girons on *Vipera aspis* (Saint-Girons 1957, 1959) and *Cerastes cerastes* (Saint-Girons 1962). In both species, he reported that sperm initially present in the posterior oviduct move anteriorly to infundibular SSTs at least several weeks prior to ovulation (a mechanism Ludwig and Rahn 1943 potentially missed). The only two other histological studies on vipers are less extensive. Nilson (1981) did histological sections of the oviduct of *Viperus berus* and reported sperm was found in the uterus prior to ovulation but provided no descriptive details; it is unclear whether Nilson (1981) looked for the presence of infundibular SSTs. Schuett (1992) mentions unpublished ultrastructural observations of sperm in infundibular SSTs in *Agkistrodon contortrix*.

Up to this point, with the exception of the studies by Ludwig and Rahn (1943) and Nilson (1981), which lacked examination of the anterior oviduct, the studies on sperm storage in viperids indicated the presence of infundibular SSTs like those found in other snake families. In view of earlier literature, therefore, we find puzzling some recent papers on sperm storage in *Crotalus durissus* (Almeida-Santos and Salomão 1997; Yamanouye et al. 2004) and 13 species of *Bothrops* (Almeida-Santos and Salomão 2002; Hartman et al., 2004) in which sperm storage has been described as uterine. This conclusion was made on the basis of swabs of the cloaca and lower oviduct and a non-comprehensive histological examination of only the posterior oviduct.

In addition, these authors proposed that "utero-muscular twisting" (UMT) is the means by which sperm storage is

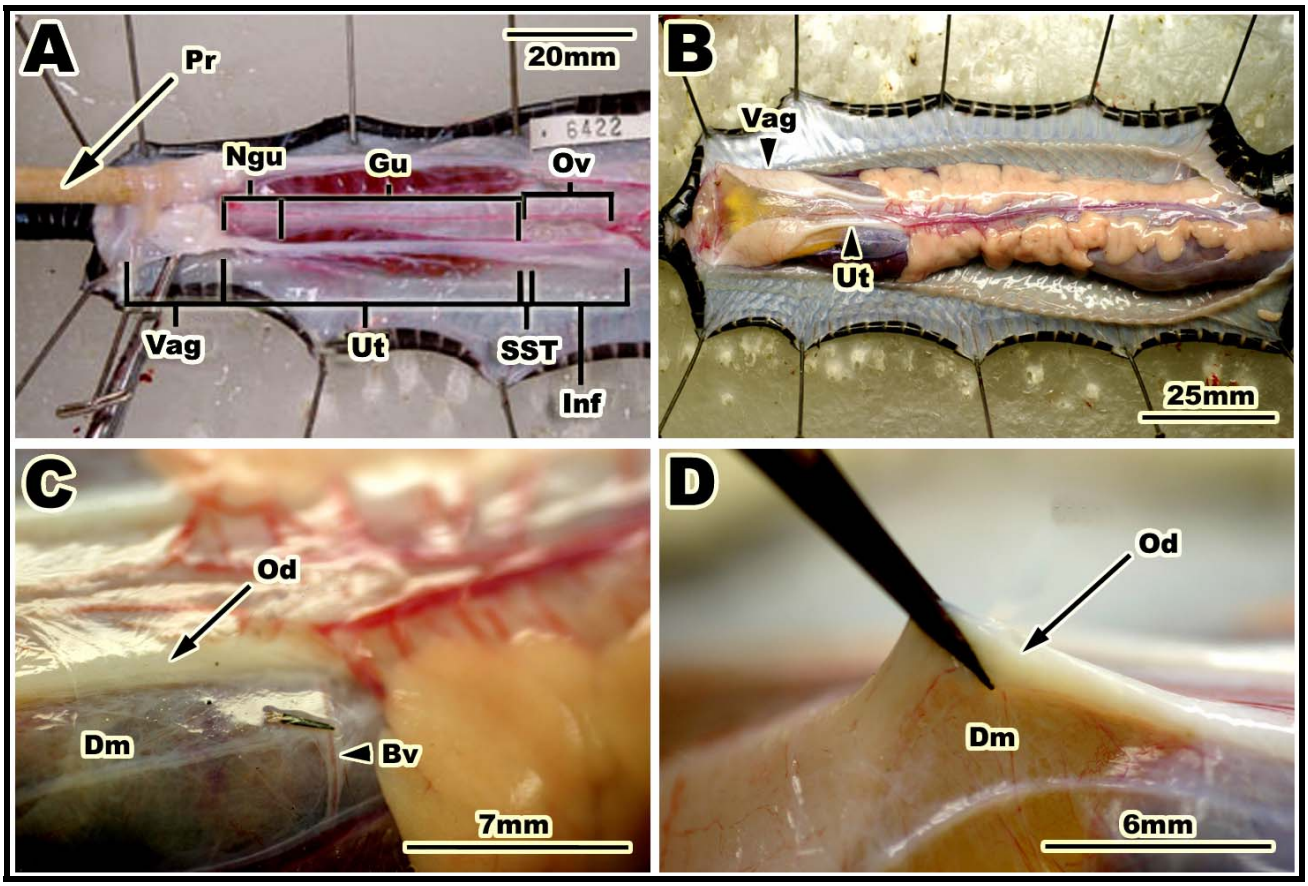


**FIGURE 1.** Gross reproductive anatomy of *Agkistrodon piscivorus* females. **A**. Reproductive anatomy of a June collected cottonmouth with intestinal tract and mesenteries removed to show divisions in the oviduct and area of sperm storage. Note wooden probe in right vaginal orifice. **B**. Reproductive anatomy of a May collected *Agkistrodon piscivorus* with no other viscera removed to show natural orientation of reproductive anatomy. **C**. Enlargement of **B** illustrating relationship of serous membranes to the oviduct. **D**. Enlargement of **B** demonstrating the tightness of the connection between the dorsal mesentery and oviduct. Bv, blood vessel; Dm, dorsal mesentery; Gu, glandular uterus; Inf, infundibulum; Ov, ovary; Od, oviduct; Pr, probe; Ngu, non-glandular uterus; Ut, uterus; Vag, vaginal pouch.

maintained in the viperid snakes they examined. Basically, the posterior region of the uterus becomes convoluted and contracted, which acts as a copulatory plug and sequesters sperm in the posterior oviduct until ovulation. A similar contraction of the posterior oviduct was reported by Nilson and Andrén (1982) in *Viperus berus*. Those authors believed that such muscular contractions function solely as a copulatory plug and not in sperm storage (Andrén and Nilson 1987), although the efficiency of the role as copulatory plug has been questioned (Stille et al. 1986; Stille and Niklasson 1987).

In their Table 1, Almeida-Santos and Salomão (2002) also indicated that UMT occurs in the temperate pitvipers *Agkistrodon contortrix*, *A. piscivorus*, and *Crotalus viridis*, although no supporting data are presented in the text. Yamanouye et al. (2004) suggested that UMT is an ancestral reproductive strategy in viperid snakes, and the idea that UMT is a feature of viperid reproduction has been uncritically accepted (Monteiro et al. 2006).

We have conducted a study on the seasonal pattern of sperm storage in female *Agkistrodon piscivorus* from southern Louisiana. The full results of this extensive histological and cytological investigation will be published elsewhere. In this paper we wish to address the location of sperm storage and whether UMT occurs. We supplement these observations with those on single specimens of *Crotalus durissus* and *Sistrurus miliarius.*

## MATERIALS AND METHODS

***Specimens examined.—****Agkistrodon piscivorus* females were collected from the site of a railroad track levee along the Amite River Diversion Canal, North 30.22616/West 090.68506, Livingston Parish, Louisiana and the Turtle Cove Environmental Research Station on Pass Manchac, North 30.29426/West 090.35592, Tangipahoa Parish, Louisiana. Specimens were housed in glass aquariums (approximately 0.3 m *x* 0.6 m *x* 0.3 m) with locking screen lids for no more than three days before they were sacrificed. Water was available to these snakes *ad libitum*.

Individuals were sacrificed by a 0.2-0.5 cc I.P. injection of a solution of sodium pentobarbital at a concentration of 1 g sodium pentobarbital in 10% alcohol and 40% propylene glycol. The left side of the reproductive tracts were removed from the specimens and fixed in 10% neutral buffered solution (NBF) for light microscopy. The right side of the oviduct was prepared for examination by TEM and SEM. The number of sexually mature specimens prepared per month was: February – 1; March – 2; April – 2; May – 2; June – 2; July – 1; August – 1; September – 1; October – 1; November – 2. Snout-vent lengths (SVL) ranged from 48.8-67.8 cm. Specimens of *Sistrurus miliarius* (LSMNH 7740, collected 3 May, 1956 in St. Bernard Parish, SVL approximately 30

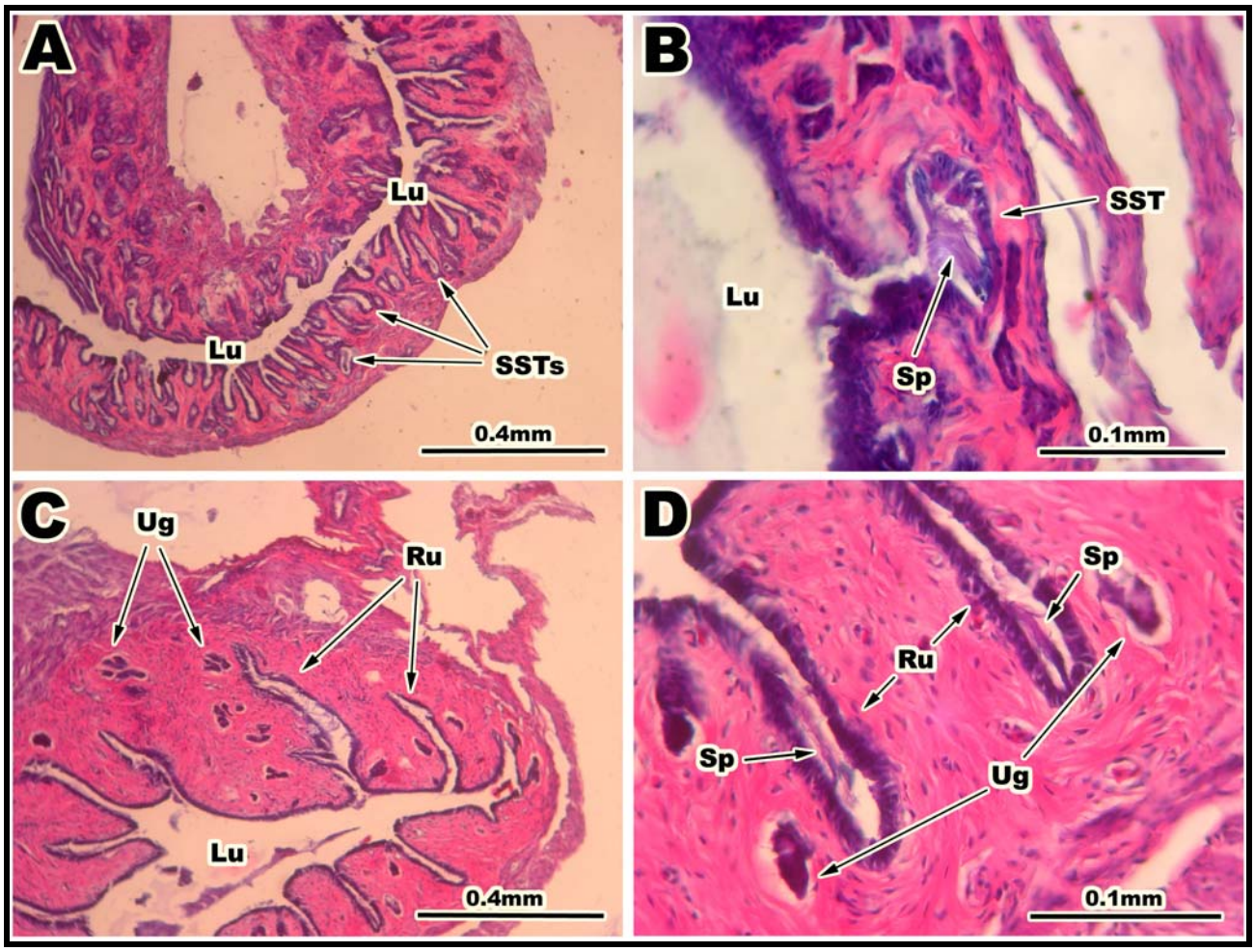


**FIGURE 2.** Sagittal oviductal sections (10 μm) of a female *Agkistrodon piscivorus*, captured and sacrificed in November of 2005, stained with hematoxylin and eosin. **A**. Simple tubular glands used for storing sperm (SSTs) in the posterior infundibulum. **B**. Higher magnification of **A** showing sperm completely filling one SST. **C**. Rugae and uterine glands that characterize the glandular section of the uterus of *Agkistrodon piscivorus*. **D**. Higher magnification of **C** with emphasis on sperm contained in the larger invaginations (rugae). Lu, lumen; Ru, rugae; Sp, sperm; SST, sperm storage tubule; Ug, uterine gland.

cm) and *Crotalus durissus* (LSMNH 22071, collected 7 June 1970 in Venezuela [no specific locality], SVL approximately 80 cm) were obtained from the Louisiana State University Museum of Natural History. These specimens were formalin fixed and stored in 55% isopropanol. The left oviduct was removed from each specimen for light microscopy preparation.

***Tissue preparation for light microscopy.***—Tissues fixed in NBF or removed from museum specimens stored in 55% isopropanol were rinsed for one hour in tap water and then dehydrated by placing the tissue in a graded series of ethanol (70%, 95%, and 100% for an hour each). After dehydration, the tissues were embedded in paraffin blocks for sectioning with a MR3 microtome (Research and Manufacturing Co., Tucson, Arizona, USA). Sections 10μm thick were cut and affixed to albuminized slides. Slides were then stained with hematoxylin and counterstained with eosin for histological examination. Histological techniques followed Kiernan (1990).

## RESULTS

The oviduct of *Agkistrodon piscivorus* was grossly and histologically divided into a posterior vaginal pouch, a middle uterus with a posterior non-glandular section and anterior glandular section, and an anterior infundibulum with SSTs located between the infundibulum and uterus (Fig. 1A).

Some authors refer to the SST area as the "tuba", but we follow Blackburn (1998) in designating the region as posterior infundibulum. We found no evidence of a vaginal/cloacal sphincter, and the vaginal orifice is quite large and easily distended in females in all reproductive conditions (Fig. 1A). The entire uterus (posterior and anterior) in females storing sperm is straight with no major folds and absolutely no twisting/coiling (Fig. 1A, B). Indeed, coiling of the uterus would be anatomically implausible because of the continuous dorsal mesentery that encloses blood vessels and nerves and attaches the oviduct to the dorsal body wall and other organs (Fig. 1C, D, for a review of mesenteries see Blackburn, 1998). No evidence of UMT was observed in any female *A. piscivorus* or in the other pit-vipers examined.

We found SSTs in the posterior infundibulum similar to those reported in colubrids (Figs. 2A-B, 3). In *A. piscivorus*, we also found sperm in the non-glandular and most posterior glandular sections of the uterus in females collected from July (start of the breeding season) through hibernation in December (Fig. 2C-D) and in the spring before ovulation, when a second mating season occurs.

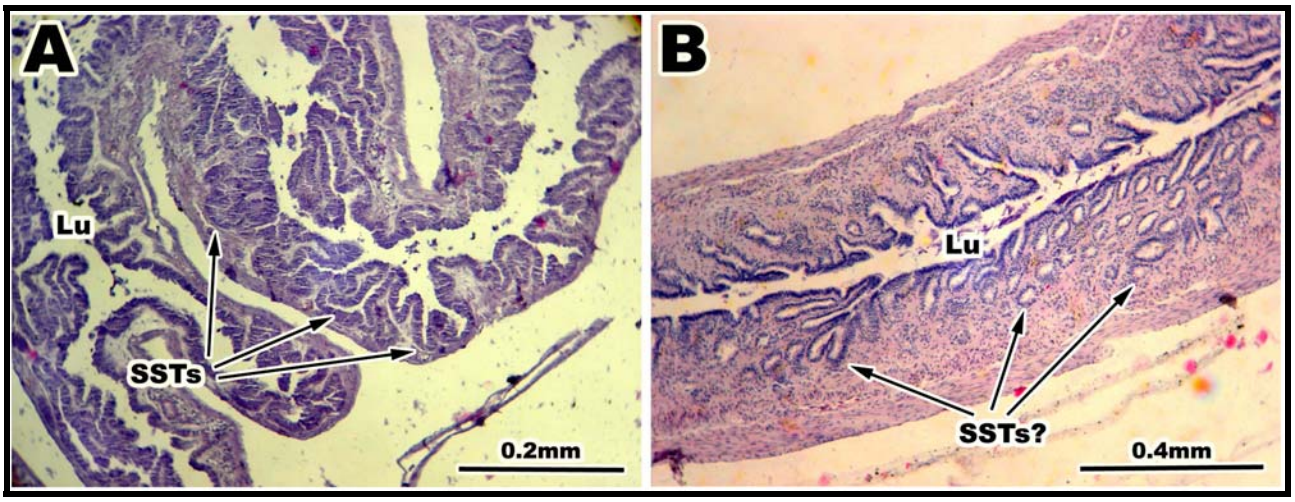


**FIGURE 3.** Sagittal oviductal section (10 μm) of two representative viperid species stained with hematoxylin and eosin. **A**. SSTs located in the posterior infundibulum of *Sistrurus miliarius*. **B**. Putative SSTs located in the posterior infundibulum of *Crotalus durissus*. Lu, lumen; SST, sperm storage tubule.

This presence of sperm in the posterior uterus could be an artifact of recent mating activity, for one would expect sperm in the posterior oviduct after copulation during mating seasons. In the specimen of *Sistrurus miliarius*, which was a preovulatory female, the SSTs were packed with sperm, and sperm were also found in SSTs of cottonmouths collected post-copulation/pre-hibernation and post-hibernation/pre-ovulation. As a matter of fact, specimens of *Agkistrodon piscivorus* that had recently ovulated and had fetuses *in utero*, or were post-partum females also contained sperm in the SSTs. This phenomenon was not consistent with the posterior uteri sections of *A. piscivorus*, which contained no sperm at this time. Specimens that were not breeding in a particular year contained no sperm in the posterior uteri sections or posterior infundibulum (some females of this species have a biennial reproductive cycle, Wharton 1966).

The female *Crotalus durissus* that we examined contained no sperm in the posterior infundibulum or posterior uterus. However, simple tubular glands, similar to those of *Agkistrodon piscivorus* (Fig. 2A) and *Sistrurus miliarius* (Fig. 3A), were found in the posterior infundibulum of *Crotalus durissus* (Fig. 3B). These glands are distinctly differentiated from those of uterine glands and more anterior infundibular glands. We believe that shortly after a mating, sperm would be found in these glands until the time of ovulation and thus these simple tubular glands are SSTs.

## DISCUSSION

In their original description of UMT, Almeida-Santos and Salomão (1997) illustrate a drawing of an oviduct showing a definite cork-screw twisting of the posterior uterine region. The authors do not explain how this twisting could occur when that portion of the oviduct is connected to the body wall and other viscera by a continuous dorsal mesentery that encloses blood vessels and nerves passing to the uterus (Fig. 1C-D). However, photos in Almeida-Santos and Salomão (2002) illustrating UMT seem to show coiling of the oviduct within the serous sheath of the visceral pleuroperitoneum. This anatomy appears similar to that of the ductus deferens of snakes, which is folded tightly upon itself (not circularly coiled) within its serous coverings (Sever 2005).

We found no evidence of UMT in the vipers we examined, and we question the hypothesis that this mechanism could be an ancestral mode for sperm storage in the group (Yamanouye et al. 2004). Even if some kind of utero-muscular contraction occurred in *Agkistrodon piscivorus*, this mechanism had no affect on holding sperm in the posterior uterus until ovulation because sperm were found simultaneously in the posterior uterus and anterior SSTs. The results presented here on *Agkistrodon piscivorus* mirror results from St. Girons (1973), Halpert et al. (1982), and Palmer and Perkins (1996) in which sperm were found in the posterior oviduct immediately following mating and subsequently migrated anteriorly to infundibular SSTs.

Fox and Dessauer (1962) found infundibular SSTs in the Leptotyphlopidae and Typhlopidae, considered the most basal families of snakes (Cadle 1987; Pough et al. 1998). Old world viperids like *Cerastes cerastes* and *Viperus aspis* are sister taxa to new world pitvipers like *Agkistrodon piscivorus*, which is a basal taxon in the western hemisphere Crotalinae clade of the Viperidae (Gutberlet and Harvey 2002; Castoe and Parkinson 2006), and viperids make up the sister clade to colubrids/elapids (Lawson et al. 2005). A more compelling hypothesis than UMT as the ancestral mechanism for viperid snakes is that sperm storage in infundibular SSTs is the ancestral state for all squamates, including the ancestor to the Viperidae (Sever and Hamlett 2002).

Obviously, more research is needed on the mechanisms of sperm storage in the family Viperidae. The possibility and significance of utero contraction is not being completely ignored in this study; however, more histological and physiological investigation is needed to show where, when, and why this phenomenon is taking place in the oviduct of viperids.

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**DAVID SEVER** (pictured with an Anaconda [*Eunectes sp.*]) is well-known for over thirty years of work on the secondary sexual characters of salamanders, including the anatomy, function, and evolution of their skin and cloacal glands. Other studies done by Dr. Sever have involved ecology and behavior. He also discovered and named the species *Eurycea junaluska*, a salamander restricted to the southern Appalachian Mountains. His most recent work has involved the morphology and phylogeny of sperm storage and other aspects of the urogenital systems of Chondrichthyes, amphibians, and reptiles.

**DUSTIN SIEGEL** (pictured with a Yellow-bellied water snake [*Nerodia erythrogaster flavigaster*) is a second year graduate student in Dr. Sever's lab at Southeastern Louisiana University earning his M.S. in biology. His Master's thesis includes describing seasonal ultrastructural variation in the oviduct of *Agkistrodon piscivorus*. Dustin plans on attending a Ph.D. university when he is finished at SELU and is currently interviewing at several institutions. His current interests include sperm storage, multiple paternity, sperm competition, cryptic female choice, and general reproductive biology of reptiles and amphibians.