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RESEARCH ARTICLE

Histological analysis of spermatogenesis and the germ cell development strategy within the testis of the male Western Cottonmouth Snake, *Agkistrodon piscivorus leucostoma*

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Summary

Cottonmouth (*Agkistrodon piscivorus leucostoma*) testes were examined histologically to determine the germ cell development strategy employed during spermatogenesis. Testicular tissues from Cottonmouths were collected monthly from swamps around Hammond, Louisiana. Pieces of testis were fixed in Trump's fixative, dehydrated in ethanol, embedded in Spurr's plastic, sectioned with an ultramicrotome, and stained with toluidine blue and basic fuchsin. Spermatogenesis within Cottonmouths occurs in two independent events within a single calendar year. The testes are active during the months of March–June and August–October with spermiation most heavily observed during April–May and October. To our knowledge, this is the first study that describes bimodal spermatogenesis occurring in the same year within the subfamily Crotalinae. During spermatogenesis, no consistent spatial relationships are observed between germ cell generations. Typically, either certain cell types were missing (spermatocytes) or the layering of 3–5 spermatids and/or spermatocytes within the same cross-section of seminiferous tubule prevented consistent spatial stages from occurring. This temporal pattern of sperm development is different from the spatial development found within birds and mammals, being more reminiscent of that seen in amphibians, and has now been documented within every major clade of reptile (Chelonia, Serpentes, Sauria, Crocodylia). This primitive-like sperm development, within a testis structurally similar to mammals

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and birds, may represent an intermediate testicular model within the basally positioned (phylogenetically) reptiles that may be evolutionarily significant.

Introduction

Two different germ cell development strategies exist within vertebrates. Anamniotic vertebrate testes are composed of tubules or lobules that are lined with cysts. Germ cells develop as a single cohort through mitosis, meiosis, spermiogenesis, and upon completion of spermatogenesis mature spermatozoa are released from these cysts into the lumina of the tubules or lobules in a single spermiation event (Lofts, 1964; Van Oordt and Brands, 1970). Amniotic testes contain seminiferous tubules that are lined with seminiferous epithelia (Leblond and Clermont, 1952). Spermatogenesis occurs in association with Sertoli cells, which make up the seminiferous epithelium of amniotic testes.

Throughout the spermatogenic cycle in most amniotes studied to date (limited mostly to temperate and continually breeding birds and mammals), germ cells within the seminiferous epithelium are organized in consistent spatial arrangements as generations of germ cells move from the periphery of the epithelium to the lumen of the seminiferous tubules. This type of synchronized germ cell development strategy results in consistent cellular associations that can be seen and predicted. These stages are typically numerically arranged within large segments of seminiferous tubules, which lead to waves of sperm release throughout the reproductively active months in mammals (Russell et al., 1990).

Current research provides evidence that most temperate pitvipers mate, have a period of testicular quiescence, and then begin spermatogenesis during the late spring/early summer (Srivastava and Thapliyal, 1965; Saint Girons, 1982; Aldridge, 2002). Although spermatogenesis generally starts during the late spring/early summer, many variations and testicular cycles exist. These cycles differ from species to species and are associated with internal and external androgenic cues (Shea, 2001; Bertona and Chiaraviglio, 2003). Recent research has also shown that temperate snakes, like the Black Swamp Snake (*Seminatrix pygaea*), may rely on a completely different germ cell development strategy than that observed in birds and mammals. Black Swamp Snakes (Gribbins et al., 2005) and other temperate reptiles (Gribbins and Gist, 2003; Gribbins et al., 2003, 2006) studied to date have testes with typical amniotic structures (seminiferous tubules, seminiferous epithelium, Sertoli cells). However, their germ cell development strategy is more reminiscent of that found in anamniotes. This germ cell development may be conserved and considered primitive compared with the derived spatial spermatogenesis seen in birds and mammals. The basal position of reptiles within the amniotic clade and the reoccurring theme of a temporal germ cell development strategy in every temperate reptile studied to date suggests that this pleisomorphic-like spermatogenic cycle within a structurally amniotic testis may be evolutionary significant.

The present study on *Agkistrodon piscivorus leucostoma* is a continuation of this long-term investigation on germ cell development in reptiles. The Cottonmouth is a viviparous snake that can be found within lowlands of the southeastern United States (Conant and Collins, 1991). Cottonmouths are semi-aquatic snakes that in many cases occupy permanent water sources in abundant numbers within their geographic range. Pairings of males and females have been seen in Cottonmouths by Wharton (1966) throughout the entire year except January with female ovulation occurring in the spring (Zaidan et al., 2003). Spermatogenesis begins in April and spermiogenesis is completed by late October (Johnson et al., 1982) for populations of Cottonmouths found in Alabama. The climax of spermatogenesis, along with increased testosterone levels, occurs in mid July in all Cottonmouth populations studied to date (Johnson et al., 1982; Zaidan et al., 2003; Graham, 2006).

The purpose of this study is to provide a detailed histological evaluation of the major events associated with spermatogenesis so that the germ cell development strategy of a southeastern Louisiana Cottonmouth population (Johnson et al. (1982) did not provide information about the germ cell development strategy within Alabama male Cottonmouths) can be compared with that of the Swamp Snake and other temperate reptiles. Cottonmouths are unique to the other temperate species studied to date in that there is evidence that spermatogenesis is seasonal, but breeding may occur any time during the year (assuming intersexual pairings are indicative of mating activity). Furthermore, within the population of Cottonmouths from this study mature sperm can be found in the distal regions of

the female reproductive tract in late spring and fall suggesting that there may be two major breeding seasons in Louisiana (Siegel and Sever, 2008). This information leads to the hypothesis that male Cottonmouths in Louisiana either store sperm that is produced from one seasonal peak of spermatogenesis as observed by Johnson et al. (1982) or there may be more than one spermatogenic event (i.e. early summer and late fall) that would provide sperm for the majority of matings, which are assumed to occur most often during the warmer months of the year. This type of bimodal spermatogenesis has not been documented within a species of snake belonging to the subfamily Crotalinae. However, a similar biannual germ cell development in males has been previously described during the summer months for species within Colubridae (Goldberg, 1995, 1998). If a bimodal type of spermatogenesis occurs in this Louisiana population of Cottonmouths, then this new annual pattern of reproduction would be quite different from the postnuptial pattern of spermatogenesis typical of temperate-zoned snakes that was previously described by Johnson et al. (1982) for Alabama Cottonmouths. A biannual pattern of spermatogenesis within Louisiana Cottonmouths would provide the opportunity to retest the hypothesis that reproductive strategy, which has already been examined in postnuptial (*Seminatrix pygaea*, Gribbins et al., 2005), prenuptial (*Alligator mississippiensis*, Gribbins et al., 2006), and mixed (*Podarcis muralis*, Gribbins and Gist, 2003) reproductive cycles, may predetermine the type of germ cell development strategy utilized by seasonally breeding reptiles.

Materials and methods

Animal collection

Twenty-two adult male Cottonmouth snakes, *A. piscivorus leucostoma*, were collected during the months of January 2005 through November 2006 (2 snakes were captured per month except: 1 specimen per month for September, November, and January and 5 specimens for June) from three localities; the Amite River Diversion Canal (North 30°22.616/West 090°68.506, Livingston Parish, LA), Turtle Cove Environmental Research Station on Pass Manchac (North 30°29.426/West 090°35.592, Tangipahoa Parish, LA), and the private residence of Dr. Clifford Fontenot, 10 km Northwest of New Albany (North 30°30.871/West 090°36.202, Livingston Parish, LA). The snakes were sacrificed with a 0.2–0.5 ml intraperitoneal injection of sodium pentobarbital (1 g sodium pentobarbital in 10% ethanol/40% propylene glycol solution) and the testes were removed and fixed in Trump's fixative (EMS, Hatfield, PA). The testes were then cut into transverse sections and stored in 70% ethanol at 4 °C.

Tissue preparation for light microscopy

Sections of each testis were cut into approximately 3 mm cubes, dehydrated in a graded series of ethanol (70%, 85%, 2 × 95%, 2 × 100%), incubated in 1:2 Spurr's plastic (EMS, Hatfield, PA): 100% ethanol for 30 min, and then once in 1:1 Spurr's plastic: 100% ethanol for 30 min. The tissues were then infiltrated in pure Spurr's plastic overnight. New plastic was prepared and the testes were embedded and cured at 60 °C for 48 h in a Fisher isotemperature vacuum oven (Fisher Scientific, Pittsburg, PA). Sections (2–3 μm) were cut from the plastic blocks using a dry glass knife and an LKB-Ultramicrotome III (LKB Produkter AB, Bromma, Sweden). The tissues were visualized using a basic fuchsin and toluidine blue composite stain as described by Hayat (1993).

Histological analysis

The testicular tissues were examined using an Olympus compound microscope (Olympus America, Center Valley, PA) to determine the cytological changes that occurred during spermatogenesis. Sagittal and cross-sectional areas of the seminiferous tubules were selected at random and germ cell morphologies and the presence or absence of spatial stages were determined. Photographs were taken with a SPOT digital camera (Diagnostic Systems Laboratories, Webster, TX) and were viewed and edited using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Data analysis, measurements, and morphometrics

Upon capture, each snake's snout-to-vent length (SVL) was determined and ranged from 36.0 to 73.1 cm. The width and length (in mm) were measured for the right and left testes shortly after dissection and testis volumes were estimated using the formula for a prolated spheroid: $V = 4/3\pi(1/2\,L)(1/2\,W)^2$ (Selby, 1965; Ramirez-Bautista and Gutierrez-Mayen, 2003). Right and left testicular volumes were pooled in order to increase data collection for each month of the year. Thirty

cross-sections of seminiferous tubules were chosen for each represented month and the tubular diameter and germinal epithelial heights were measured using an ocular micrometer.

Data analysis was performed using Minitab 15 (Minitab Inc., State College, PA) for Windows. Results were deemed significant if $P \leqslant 0.005$. Testicular volume, tubular diameter, and germinal epithelial height data were tested for normality and homogeneity of variances using the Kolmogorov–Smirnov and Bartlett's tests, respectively, before statistical analyses were performed (Sokal and Rohlf, 1995; Flemming, 1993). These data did not meet assumptions of normality; thus the Kruskal–Wallis analysis of variance was used to test for significant seasonal variation in testicular volume, seminiferous tubular diameter, and germinal epithelial height.

Results

Germ cell morphology and the cell cycle

The *A. piscivorus leucostoma* testis contains seminiferous tubules lined with seminiferous epithelia consisting of Sertoli cells and developing germ cells. Germ cells develop spermatogenically in close association with the Sertoli cells of this epithelium. Histological examination shows that testes of this southeastern Louisiana population of Cottonmouths are spermatogenically active during two periods within the same year, the months of March–June and again in August–October. Spermatogenesis commences in March and after a brief quiescence in July starts again in August with the proliferation of spermatogonia A and B near the basal lamina (basement membrane) of the seminiferous epithelia. The majority of spermatogonia undergo meiosis during the months of April and August/October and continue through spermiogenesis in May–June and then again in October. Spermatogonia are present and undergo mitosis in all months of the year; however, divisions are slowed during the months of June/July, November, and January/February. Although there is a decrease in mitotic activity, there are no periods during the year in which the testes of these Cottonmouths are inactive in terms of proliferation.

Testicular volume has been used in reproductive studies performed on reptiles as an indicator of spermatogenic activity (Srivastava and Thapliyal, 1965; Flemming, 1993; Ramirez-Bautista and Gutierrez-Mayen, 2003). However, previous studies (see Brown and Bomberger Brown, 2003) have shown that SVL can bias testicular volume data; therefore, linear regression was first used to determine whether mean testicular volumes were significantly affected by variations in male mean SVL. Linear regression analysis indicated that testicular volumes were not significantly correlated with variations in mean male SVL (right testicular volumes: $R^2 = 0.217$, $P \leqslant 0.05$; left testicular volumes: $R^2 = 0.261$, $P \leqslant 0.05$). Conversely, mean testicular volumes in these Cottonmouths do indeed show a significant annual trend (Kruskal–Wallis; $H = 27.7$, $\mathrm{df} = 10$, $P = 0.002$, Figure 1). Testis volumes were at a minimum during the months of quiescence (November, 59.00 cm^3; January, 16.6 cm^3; February, 70.6 cm^3; July, 50.15 cm^3) and during the month of September (61.77 cm^3). The specimen caught during September should have a high testis volume and also show spermiogenic activity similar to the August and September testis. However, there are only spermatogonia present in the seminiferous epithelium and the testis volume is low. It also should be noted that this Cottonmouth was caught 2 weeks after hurricane Katrina and outside of its normal habitat.

Testicular volumes (Figure 1) increase steadily as spermatogenic activity increases (April, 68.42 cm^3; May, 215.30 cm^3) and volumes peak during the climax of spermiogenesis and spermiation in June (292.60 cm^3). Testicular volume then drops significantly during July (50.15 cm^3) when the testis is quiescent and then increases again as spermatogenesis and spermiogenesis increases during August (142.60 cm^3) and October (153.60 cm^3). Testis volume is much larger during the spring spermatogenic climax (292.60 cm^3) when compared with the fall peak of sperm development (153.60 cm^3).

Pre-meiotic cells

The seminiferous epithelium contains two morphologies of pre-meiotic cells (Spermatogonia A and B) (Figure 2; 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 is ovoid in shape with one large nucleolus and B type is more round in shape and usually lacks a prominent nucleolus. Both spermatogonial types are generally found near the basement membrane of the epithelium away from the lumen and associated with the basal compartments formed by Sertoli cells. During the spermatogenic cycle both types of spermatogonia undergo mitosis to maintain the spermatogonial population and many of the B spermatogonia

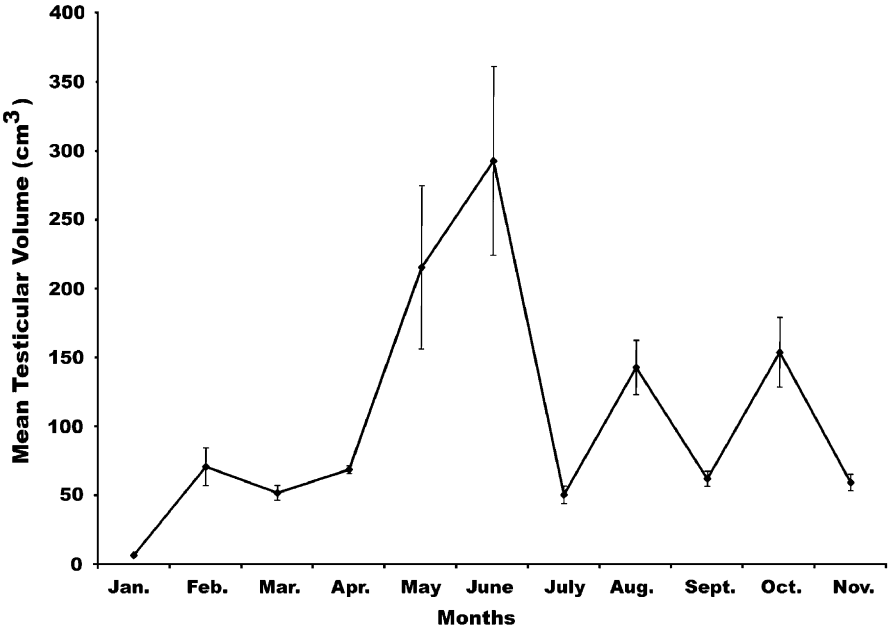

Figure 1. Variation in testicular volume (mean $\pm$ 1 SE) during the annual reproductive cycle of the male Cottonmouth, *Agkistrodon piscivorus leucostoma*.

divide to form pre-leptotene spermatocytes that then enter meiosis. Although spermatogonia A and B can be seen throughout all months of the year, they are most abundant during the months of March, April, and August.

Meiotic cells

Meiotic cells are characterized by an increase in nuclear size and a condensation of chromatin into chromosomes. Spermatogonia B undergo mitotic divisions and enter prophase of meiosis I in March and then again in August. These pre-leptotene spermatocytes (Figure 2, PL) contain a nucleus with a well-defined dark staining nucleolus. Pre-leptotene cells along with step 1 spermatids (Figure 2, S1) are the smallest of the developing germ cells. The pre-leptotene spermatocyte small size (1/2 the size of spermatogonia B) allows them to be easily distinguished from spermatogonia B within the basal compartment of the seminiferous epithelium.

Leptotene spermatocytes (Figure 2, LP) are close in size to pre-leptotene cells and are more easily distinguished based on their darker staining filamentous chromatin. Leptotene cells are present in March–June and then again in August/October. Their largest numbers are seen in April and August. Zygotene spermatocytes (Figure 2, ZY) are larger and stain less intensely than leptotene cells (more open nucleoplasm). Their nuclei are filled with increasingly thick chromatin fibers. Zygotene cells are found within the germinal epithelium of the March and August testis and are the most infrequently seen spermatocytes in the germinal epithelium of the Cottonmouth. Pachytene spermatocytes (Figure 2, PA) are the largest and most commonly observed meiocyte within the Cottonmouth testis. They are similar in morphology to zygotene cells; however, their nuclei have almost double the volume compared with zygotene cells and there are much more open nucleoplasm and thicker chromatin fibers. Pachytene cells are present in March–June and August–October.

Diplotene spermatocytes (Figure 2, DI), metaphase I (Figure 2, M1), secondary spermatocytes (Figure 2, SS), and metaphase II (Figure 2, M2) cells can be found within the seminiferous epithelium throughout all active months of spermatogenesis (March–June and August/October). These germ cells are typically found together in tight clusters within the germinal epithelium. In diplotene spermatocytes, the nuclear membrane begins to degenerate and the almost fully condensed chromosomal 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. The chromatin fibers of secondary spermatocytes are dispersed randomly throughout the nucleoplasm. These cells are about twice the size of Step 1 spermatids, which are typically clumped with secondary

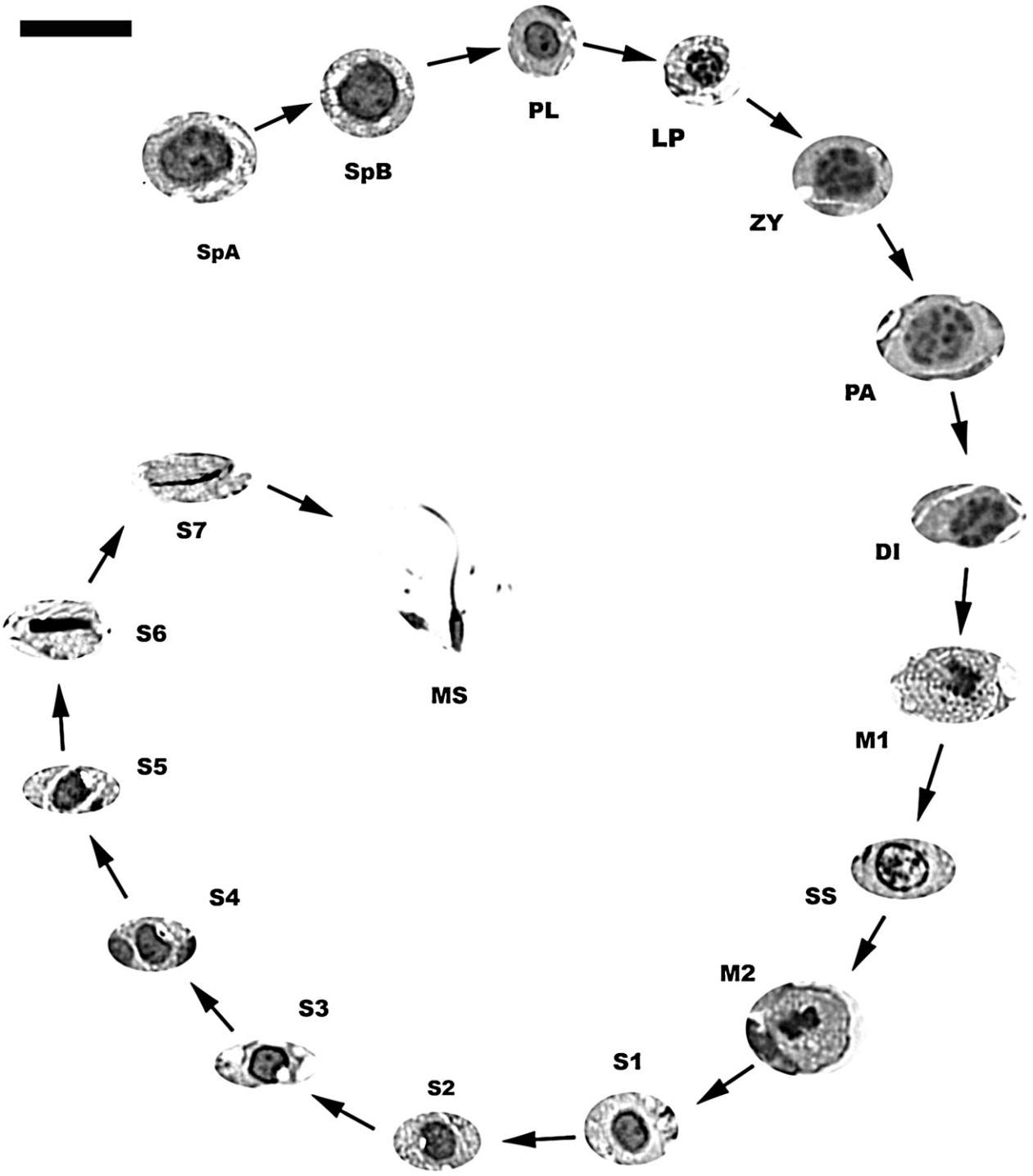

Figure 2. Germ cell types found within the seminiferous epithelium *Agkistrodon piscivorus leucostoma*. Bar = 15 μm. SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene spermatocyte; LP, leptotene spermatocyte; ZY, zygotene spermatocyte; PA, pachytene spermatocyte; DI, diplotene spermatocyte; 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 spermatozoa.

spermatocytes within the seminiferous epithelium. During metaphase 2, chromosomes aggregate around the metaphase plate again. The only differential factor between metaphase 1 and metaphase 2 is the germ cell size and the amount of chromatin present. The metaphase 2 cells are slightly smaller and contain about half the amount of chromatin found in metaphase 1 cells.

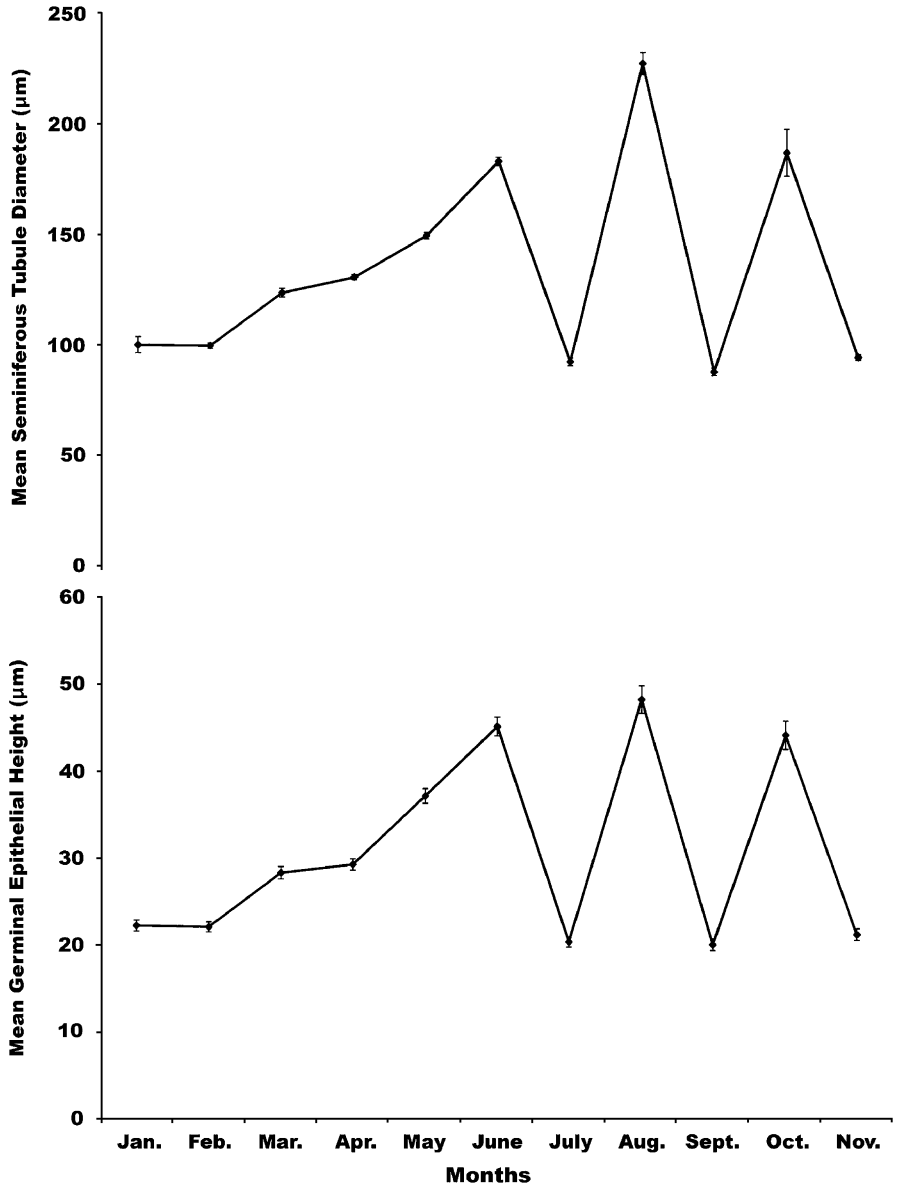

Figure 3. Top: variation in seminiferous tubule diameter (mean $\pm$ 1 SE) and bottom: variation in germinal epithelial height (mean $\pm$ 1 SE) during the annual reproductive cycle of the male Cottonmouth, *Agkistrodon piscivorus leucostoma*.

Spermiogenic cells

Spermiogenesis can be divided into seven steps in the Cottonmouth 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. The presence of step 1 spermatids (Figure 2, S1) in April and August marks the beginning of spermiogenesis. Step 1 spermatids are small in size, have a well-defined nuclear membrane, and no definable acrosomal vesicle. A well-defined acrosomal vesicle in contact with the nuclear membrane characterizes step 2 spermatids (Figure 2, S2). These germ cells have chromatin that is interspersed throughout the nucleoplasm. Step 2 spermatids are present in large numbers in May and October.

Step 3 spermatids (Figure 2, S3) and Step 4 spermatids (Figure 2, S4) are often present at the same time as step 2 spermatids. As step 3 and 4 spermatids continue to develop, the acrosome begins to widen and envelope the nuclear head, which flattens the apex of the nucleus. A centrally located acrosome granule is often present during this stage of spermatid development. Step 5 spermatids (Figure 2, S5) mark the transition between round and elongating

spermatids. Elongation begins at the opposite end of the nucleus away from the acrosome creating a nucleus that is stretched in 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.

The nuclei of step 6 spermatids (Figure 2, S6) are longer than they are wide. The acrosomal vesicle continues to extend over the head of the nucleus when visible. Step 7 spermatids (Figure 2, S7) represent the climax of elongation. They have undergone nuclear condensation and cytoplasmic elimination, resulting in nuclei more intensely stained and thinner in diameter than any of the other elongating steps. Once spermiogenesis is complete, the mature spermatozoa (Figure 2, MS) are released into the lumen of the seminiferous tubules where they will be transported to the excurrent ducts of the male reproductive system. The late steps of spermiogenesis are seen in May–June and also in October.

Seasonal development and germ cell development strategy of the seminiferous epithelium

From the 22 samples of *A. piscivorus leucostoma* testes taken over an entire year (excluding December), two distinct waves of spermatogenesis

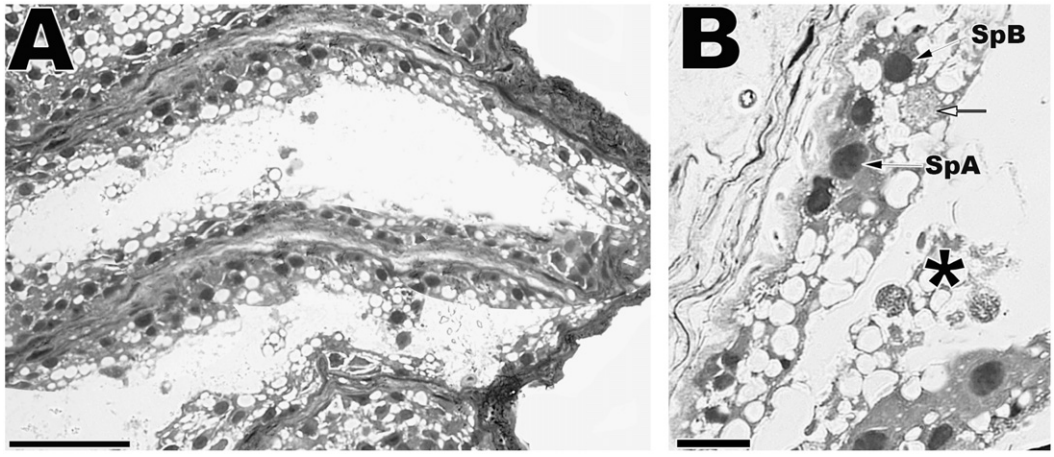

Figure 4. (A) Sagittal section (40 ×) of a January seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the January germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia. *Note*: Germ cells from the previous cycle are being shed into the lumen of the seminiferous tubule, * and many of the spermatocytes appear hypertrophic (white arrow).

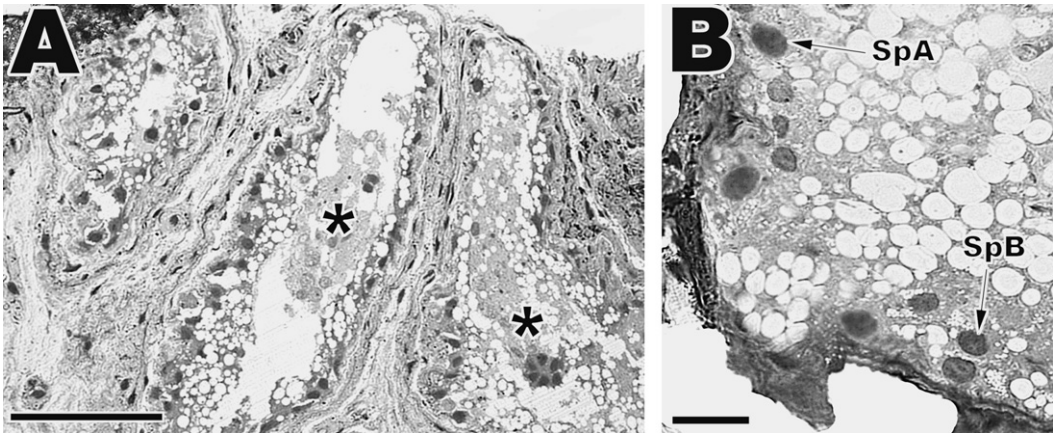

Figure 5. (A) Sagittal section (40 ×) of a February seminiferous tubules. Bar = 100 μm. Germ cells from the previous cycle are being shed into the lumen of the seminiferous tubule, *. (B) The cell types represented within the February germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia. *Note*: High vacuolation of the germinal epithelium.

are observed histologically within the seminiferous epithelium. This biannual type of spermatogenesis is supported by seminiferous tubule diameter and germinal epithelial height data. Seasonal variation in seminiferous tubule diameter (Kruskal–Wallis; $H = 287.22$, df $= 10$, $P = 0.000$, Figure 3) and germinal epithelial height (Kruskal–Wallis; $H = 270.93$, df $= 10$, $P = 0.000$, Figure 3) parallel each other and show a significant monthly trend.

Prior to March the seminiferous tubules are in a quiescent phase of development (January and February, Figures 4 and 5) and spermatogonia A and B are the only major germ cell types present within the seminiferous epithelium. Seminiferous tubule diameters (January, 101.25 μm; February, 99.80 μm) and germinal epithelial heights (January, 21.55 μm; February, 21.65 μm) are also close to their smallest values during these two months. It is not uncommon to observe large portions of the seminiferous epithelium sloughed off into the lumen (Figures 4B and 5A, *) at this time of the year.

The first wave of spermatogenesis begins in March and April (Figures 6 and 7) with an increase in spermatogonial proliferation and the early events of meiosis I dominating the seminiferous epithelia. The increase in mitosis and meiosis leads to larger seminiferous tubule diameters (March, 126.05 μm; April, 130.75 μm) and germinal epithelial heights (March, 28.20 μm; April, 29.15 μm). No consistent cellular associations are observed between early and late developing generations of germ cells because the later events of spermiogenesis are missing from the seminiferous tubules of January–April Cottonmouth testes.

Spermatogenesis continues to advance in the May testis (Figure 8). The majority of the population of germ cells has completed meiosis II and has entered

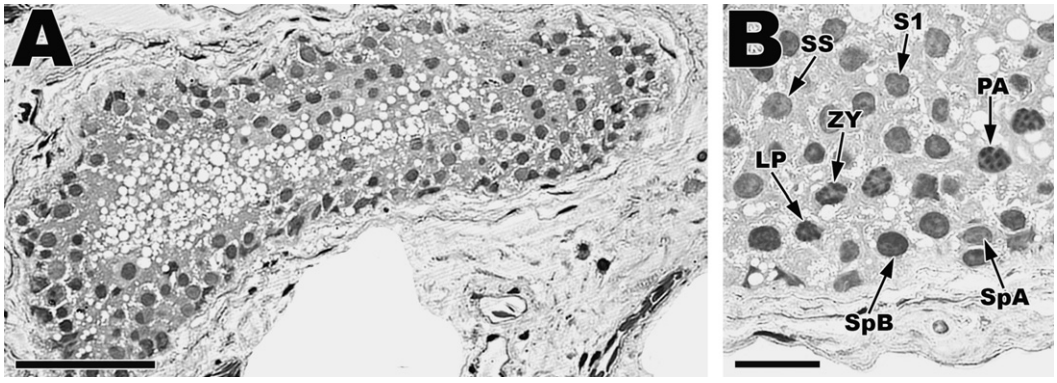

Figure 6. (A) Sagittal section (40 ×) of a March seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the March germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia; LP, leptotene spermatocytes; ZY, zygotene spermatocytes; PA, Pachytene spermatocytes; SS, secondary spermatocytes; S1, step 1 spermatid.

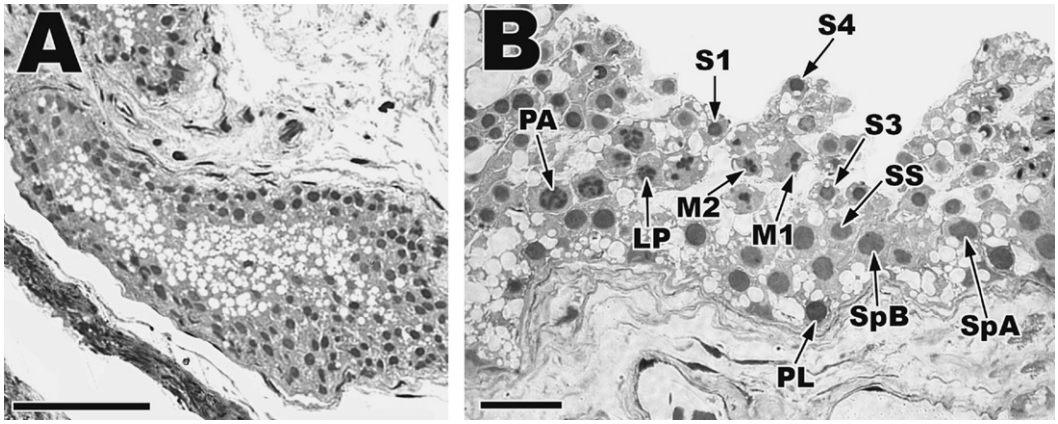

Figure 7. (A) Sagittal section (40 ×) of an April seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the April germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene spermatocytes; LP, leptotene spermatocytes; PA, Pachytene spermatocytes; M1, meiosis 1; M2, meiosis 2; SS, secondary spermatocytes; S1, step 1 spermatid; S3, step 3 spermatid; S4, step 4 spermatid.

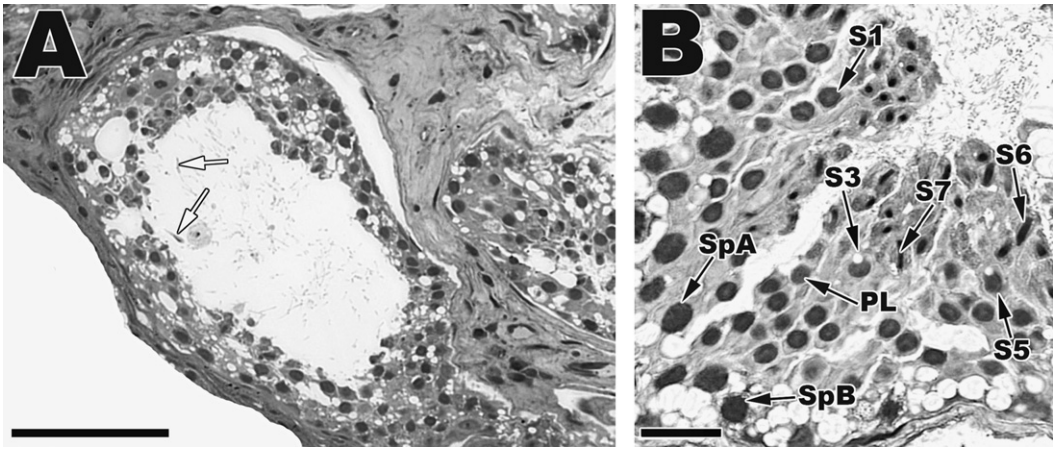

Figure 8. (A) Cross-section (40 ×) of a May seminiferous tubule. Bar = 100 μm. Note, mature spermatozoa (white arrows) in the lumen suggest spermiation has started. (B) The cell types represented within the May germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia; PL, preleptotene spermatocytes; S1, step 1 spermatid; S3, step 3 spermatid; S5, step 5 spermatid; S6, step 6 spermatid; S7, step 7 spermatid.

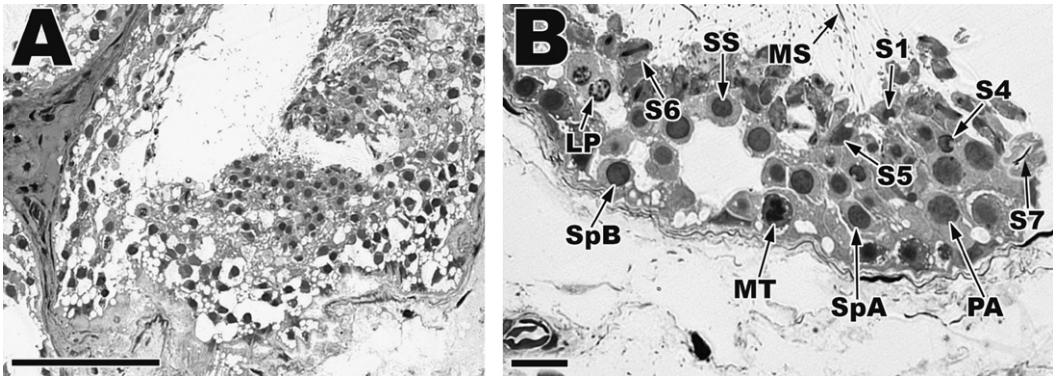

Figure 9. (A) Cross-section (40 ×) of a June seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the June germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia; MT, mitosis; LP, leptotene spermatocytes; PA, pachytene spermatocytes; SS, secondary spermatocytes; S1, step 1 spermatid; S4, step 4 spermatid; S5, step 5 spermatid; S6, step 6 spermatid; S7, step 7 spermatid; MS, mature spermatozoon.

the early phases of spermiogenesis. It is common to see 4–5 different spermatids layered together within the adluminal compartment of the seminiferous epithelium. The multiplying spermatid population causes the formation of large columns of seminiferous epithelium, which leads to a substantial size increase in seminiferous tubule diameter (150.05 μm) and germinal epithelium height (38.10 μm). The abundance of spermatids and the absence of early meiotic I cells again preclude consistent cellular associations from forming between late and early generations of germ cells. June samples of testis (Figure 9) represent the climax of spermiogenesis and an increase in spermiation. Most of the population of germ cells is completing spermiogenesis and entering the lumen as mature spermatozoa. The luminal increase in size as mature sperm are dumped into the seminiferous tubules leads to a climax in spring/early summer seminiferous tubule diameter (180.85 μm) and the accumulation of generations of elongating spermatids causes an enlargement in the germinal epithelial height (43.80 μm). The lack of early meiotic cells and accruing number of elongating spermatids prevents consistent cellular association between germ cell types. By July (Figure 10), spermiation is complete and the seminiferous tubules have entered their second phase of quiescence, which leads to a dramatic decrease in seminiferous tubule diameter (92.75 μm) and germinal epithelial height (20.05 μm). The only cell types found within the highly vacuolated germinal epithelium are a single row of spermatogonia A and B located against the

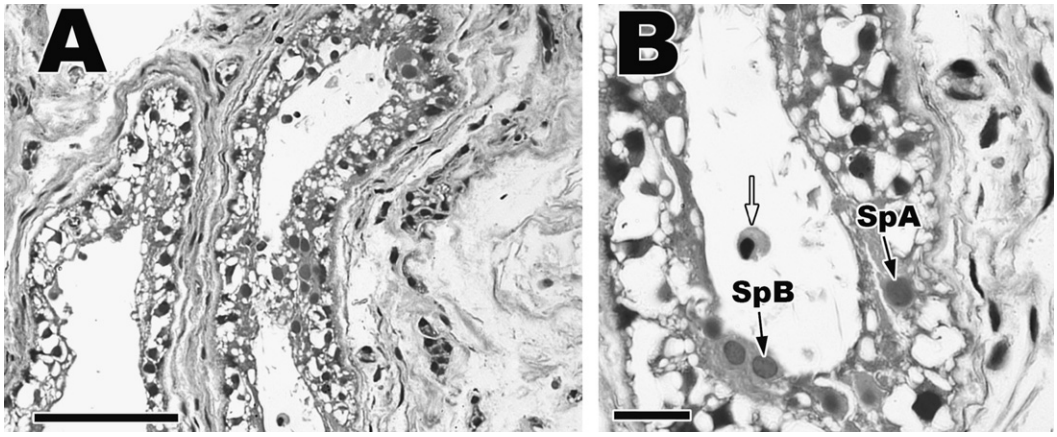

Figure 10. (A) Sagittal section (40 ×) of a July seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the July germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia. *Note*: Germ cells from the previous cycle are being shed into the lumen of the seminiferous tubule, white arrow.

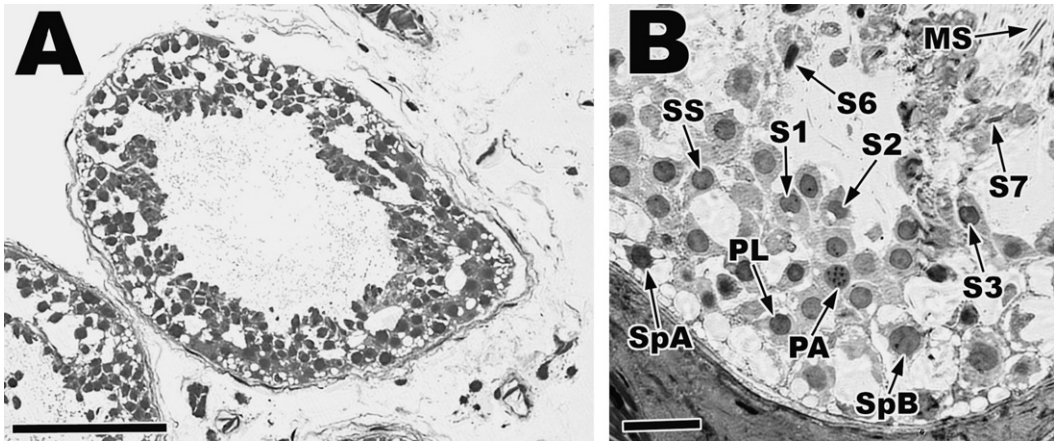

Figure 11. (A) Cross-section (40 ×) of an August seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the August germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene; PA, pachytene spermatocytes; SS, secondary spermatocytes; S1, step 1 spermatid; S2, step 2 spermatid; S3, step 3 spermatid; S6, step 6 spermatid; S7, step 7 spermatid; MS, mature spermatozoon.

basement membrane. The lumina of these tubules are void of most spermatozoa and have pieces of the seminiferous epithelium with left over germ cells (Figure 10B, white arrow) from the spring cycle of spermatogenesis.

The second wave of spermatogenesis has begun in the August Cottonmouth testis (Figure 11). The early stages of proliferation and meiosis are similar to the March and April samples; however, spermiogenic cells are in more advanced stages than May testes. August is the only month in which proliferative, meiotic, and spermiogenic cells are found together regularly in the seminiferous epithelium, leading to the largest increase in seminiferous tubule diameter (227.70 μm) and germinal epithelial height (46.10 μm). Although all three stages of spermatogenesis are observed in August, no consistent cellular association are formed because of the 4–5 different spermatids occupying the apical portion of the seminiferous epithelium. The September testis (Figure 12) represents an aberration in the sequence of events that are occurring during spermatogenesis. The seminiferous tubules within this testis are shriveled and only A and B type spermatogonia are present in the seminiferous epithelium that is very thin in comparison to the other represented months (seminiferous tubule diameter, 89.35 μm; germinal epithelial height, 19.30 μm). Large numbers of immature spermatids have been sloughed into the seminiferous tubules in the September samples (Figure 12B, Sp). Spermatogenesis in the October seminiferous epithelium

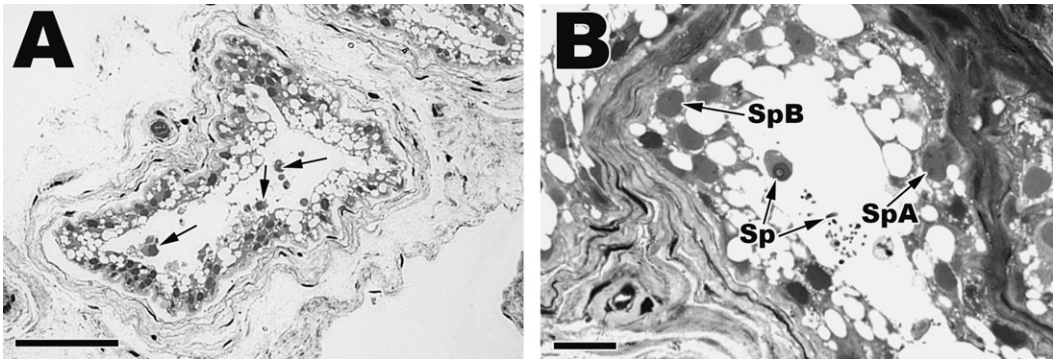

Figure 12. (A) Cross-section (40 ×) of a September seminiferous tubule. Bar = 100 μm. Note: Many shed generations of germ cells are found in the lumen of the seminiferous tubules (black arrows). (B) The cell types represented within the September germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia. *Note*: Many shed generations of spermatids are found in the lumen of the seminiferous tubules (Sp).

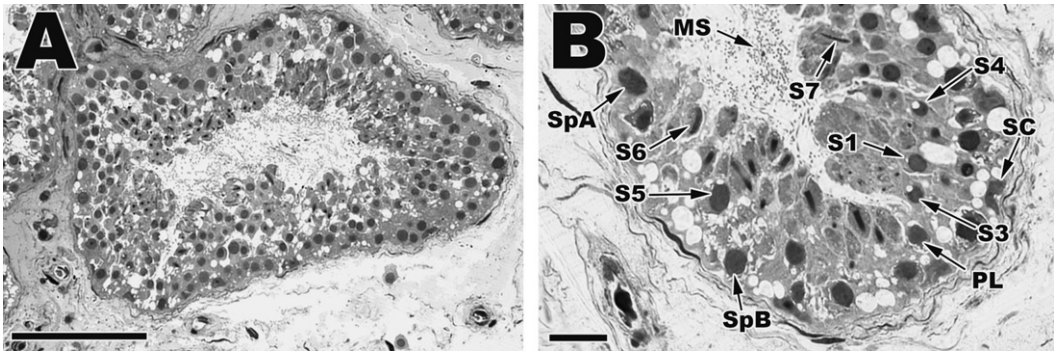

Figure 13. (A) Cross-section (40 ×) of an October seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the October germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SC, Sertoli cell nucleus; SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene; S1, step 1 spermatid; S3, step 3 spermatid; S4, step 4 spermatid; S5, step 5 spermatid; S6, step 6 spermatid; S7, step 7 spermatid; MS, mature spermatozoon.

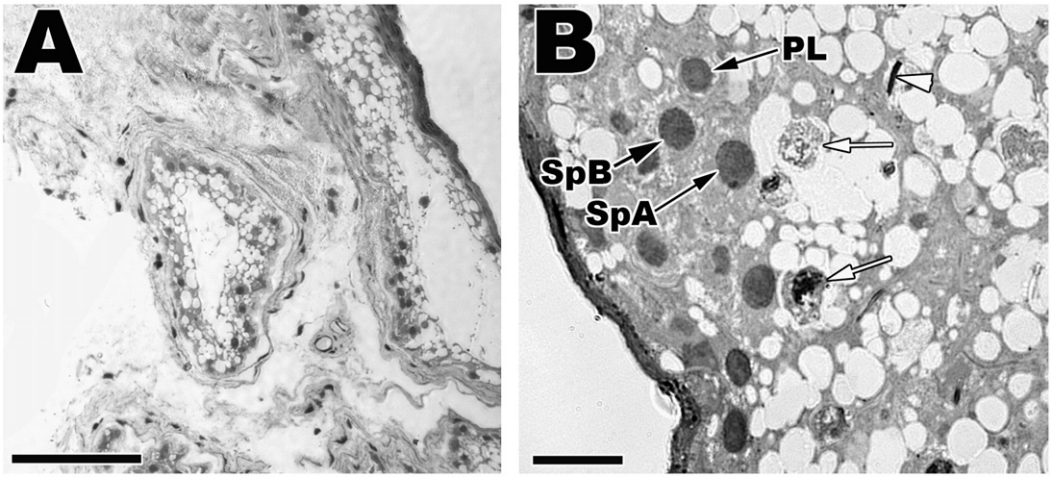

Figure 14. (A) Sagittal section (40 ×) of a November seminiferous tubule. Bar = 100 μm. (B) The cell types represented within the November germinal epithelium (100 ×). Bar = 20 μm. Labeled cell types: SpA, type A spermatogonia; SpB, type B spermatogonia; PL, pre-leptotene spermatocyte. *Note*: Remnant deteriorating germ cells are being shed into the lumen of the seminiferous tubule (white arrows: spermatocytes; white arrowhead: elongating spermatid).

(Figure 13) has advanced into spermiogenesis with round and elongating spermatids represented. Spermatocytes have been exhausted and spermatogonia A and B are found near the basement membrane of the seminiferous tubules. The loss of meiotic cells has slightly decreased the

seminiferous tubule diameter (215.40 μm) and germinal epithelial (41.55 μm) height when compared with August seminiferous tubules. Many of the developing spermatids have completed spermiogenesis and are being shed to the lumina of the seminiferous tubules as mature spermatozoa. Like the July sample, the November seminiferous tubules (Figure 14) are in a state of quiescence with spermatogonia A and B making up the majority of germ cells and lipid-rich vacuoles dominating the seminiferous epithelium. The seminiferous tubule diameter (94.25 μm) and germinal epithelial height (20.15 μm) in November also mirror that of July testes.

Discussion

The testes of the *A. piscivorus leucostoma* consist of seminiferous tubules lined by seminiferous epithelia. Sertoli cells are present at all times of the year and are associated with a continuous supply of spermatogonia. This overall testicular structure is consistent with nonmammalian amniotic testes (Pudney, 1995) and the testes of Black Swamp Snakes (Gribbins et al., 2005).

Spermatogenesis takes place during two independent events between the months of March–June and August–October in this Louisiana population of *A. piscivorus leucostoma*. Monthly morphometric data, testicular volume measurements, and histological analysis of the Cottonmouth testis all provide strong support for a biannual/bimodal type of spermatogenesis. However, it should be noted that the September testis does not fall into sequence as far as spermatogenesis with that of the other fall samples. In September, only spermatogonia are seen within the seminiferous epithelium and this snake has the smallest seminiferous tubule diameter (89.35 μm) and germinal epithelial height (19.30 μm) measured during this entire study. August testes show the early events of spermatogenesis and October seminiferous tubules show the later events of spermiogenesis and spermiation. September seminiferous epithelia should be dominated by spermatocytes and early round spermatids, similar to the September testis described for populations of Cottonmouths in Alabama (Johnson et al., 1982). This particular snake was caught outside of its normal habitat and 2 weeks after hurricane Katrina. Thus, this snake was exposed to extreme stress and the spermatogenic cycle may have been shut down in response to this environmental catastrophe due to conflict between the hypothalamic–pituitary–gonadal axis and the hypothalamic–pituitary–adrenal axis as demonstrated in stress-induced Cottonmouths (Graham, 2006) and supported by the large number of detached spermatids found in the September seminiferous tubular lumen (Figure 12B, Sp). The August, October, and November samples all show a similar histological sequence to the fall spermatogenic cycle described by Johnson et al. (1982) for *A. piscivorus* in Alabama.

The two spermatogenic events within this Cottonmouth population are separated by a quiescent period (July) in which the testes are inactive and only spermatogonia type A and B are present and the seminiferous tubule diameter, germinal epithelial height and testicular volume are smaller than all other measurements except for the months of September and January (testicular volume only). To our knowledge, this is the first evidence of bimodal spermatogenesis described for a temperate species within Crotalinae. Like other crotalids, most temperate snakes have a single annual spermatogenic cycle that typically follows a postnuptial pattern where spermatogenesis commences after spring mating (Licht, 1984; Saint Girons, 1982). Other studies (Johnson et al., 1982) on *A. piscivorus* have shown that spermatogenesis starts in the spring, peaks in late summer, and terminates in the fall. The populations of Cottonmouths studied by Johnson et al. (1982) were found in Alabama (at a more northern latitude versus southern Louisiana). The further north a population of snakes resides, the shorter the number of warm months, which may limit energy sources and the metabolism needed to maintain sperm development. This may be the reason why the early (spring) spermatogenic cycle is absent in the Alabama Cottonmouth population.

Interestingly, Johnson et al. (1982) and Sever et al. (2008) both provide data for hypertrophic renal sexual segments (RSSs) during spring and late summer/early fall even though there is only one late summer testosterone peak (Johnson et al., 1982; Graham, 2006). Though testosterone levels were not taken during the present study, it is worth noting that the literature to date suggests that hypertrophic RSSs in the Cottomouth do not parallel the timing of peak testosterone as precisely as those found in other squamates (Bishop, 1959; Misra and Prasad, 1965; Prasad and Sanyal, 1969; Krohmer, 1986). Nevertheless, RSSs do correlate strongly with the timing of spermatogenesis and when breeding may occur. Hypertrophic RSSs are often good indicators of breeding because sexual segments in reptiles are presumed to produce the seminal fluid added to spermatozoa during ejaculation (Prasad and Reddy, 1972). Furthermore, sperm aggregates found in the

posterior uterus, alleged artifacts of recent breeding (Saint Girons, 1957, 1962a, b), of female Cottonmouths, also occur during the spring and fall in Louisiana (Siegel and Sever, 2008).

In light of the breeding/endocrine/RSSs data, we suggest that the two spermatogenic cycles observed in *A. piscivorus leucostoma* from southeastern Louisiana provide mature spermatozoa for the two major breeding periods suggested by Siegel and Sever (2008) and supported by previous studies (Beyer, 1898; Martin, 1984). The majority of female *A. piscivorus* practice biennial breeding, where copulation initially begins in late summer and continues through early fall (Burkett, 1966; Wharton, 1966; Ford, 2002; Ford et al., 2004). Also, during this late summer period, vitellogenesis (yolk accumulation in the developing oocyte) presumably begins and is subsequently halted during hibernation (Aldridge and Duvall, 2002). The first spermatogenic cycle ending in June would provide spermatozoa for these late summer/fall breeding events. Vitellogenesis in females resumes again in the following early spring, when the second mating season starts, and is completed by late spring/early summer when mature ovarian follicles are ready for ovulation (Burkett, 1966). The second (fall) spermatogenic cycle is completed before hibernation and sperm is stored in the excurrent duct system until emergence from hibernation in the following spring, which is when the second mating season begins (Johnson et al., 1982).

Recent studies on temperate squamates (Gribbins and Gist, 2003; Gribbins et al., 2005) have shown a similar temporal germ cell development strategy described here for the Cottonmouth, which is different from the spatial germ cell development seen in seasonally and continually breeding birds and mammals (Yamamoto et al., 1967; Rossen-Runge, 1977; Tait and Johnson, 1982; Tsubota and Kanagawa, 1989; Tiba and Kita, 1990; Foreman, 1997). This episodic germ cell development has also been observed within the other reptilian orders (Gribbins et al., 2003: *Trachemys scripta*, Chelonia; Gribbins et al., 2006: *Alligator mississippiensis*, Crocodylia) and is very similar to the temporal germ cell development strategy of derived amphibians such as anurans (Lofts, 1964; Van Oordt and Brands, 1970). Furthermore, anuran amphibians have been described as a transitional taxon between the anamniotes and amniotes in terms of testicular organization (Van Oordt, 1955). Yet, the seminiferous tubules in anurans are lined with seasonal cysts and not a continuous epithelium like that of other amniotes. Extant reptiles and presumably their ancestors are considered the most primitive amniotes phylogenetically and have testes that are structurally similar to that of derived amniotic lineages (birds and mammals). Thus, reptiles might represent a better transitional intermediary in terms of testicular organization between anurans and the derived avian and mammalian taxa.

Temperate reproductive strategies seem to have no effect on the type of germ cell development strategy employed by the reptiles studied to date. Previously studied prenuptial (Gribbins et al., 2006), postnuptial (Gribbins et al., 2003, 2005), mixed (Gribbins and Gist, 2003), and the present results on bimodal spermatogenesis all express the same temporal type of germ cell development. It would be interesting to test whether continually reproducing populations of reptiles, such as those in the tropics, would show this same temporal germ cell development or if they have a more spatial germ cell development strategy as observed in continually breeding mammals and birds. Unfortunately, data on the details of spermatogenesis for tropical and temperate reptilian species are lacking and need to be addressed in order for such comparative models to be tested. These types of comparative, phylogenetic, and anatomical questions on spermatogenesis and the morphology of the testis cannot be answered conclusively until further information is collected from a variety of tropical and temperate species representing the major taxa within Reptilia.

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