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Cytological evaluation of the germ cell development strategy within the testis of the American alligator, *Alligator mississippiensis*

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Abstract

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The cytological changes to germ cells were investigated within the seminiferous epithelium of the American alligator (*Alligator mississippiensis*). Testicular tissues were collected, embedded in plastic, sectioned on an ultramicrotome, and stained with the periodic acid–Schiff+ procedure followed by a haematoxylin counterstain. Alligators have a prenuptial pattern of germ cell development, where spermatogenesis begins in early spring and sperm is mature by the time mating begins in May. Consistent spatial relationships between germ cells are absent within the seminiferous epithelium of the alligator. Their germ cells progress through the phases of spermatogenesis as a single cohort, leading to one continuous spermiation event that occurs during their mating season (May–June). This temporal germ cell development is different from the consistent spatial development seen within seasonally breeding birds and mammals but is similar to the recently described germ cell development strategies of two other temperate breeding reptiles, the slider turtle and the European wall lizard. The germ cell development strategy shared by these three temperate reptiles representing three different taxa within the class Reptilia is reminiscent of the temporal strategy seen within the anamniotic testis. Thus, alligators and at least two other temperate reptiles exhibit primitive spermatogenic cycles within derived amniotic testes and may be consider intermediates in terms of testicular organization, which may have significance phylogenetically.

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The American alligator, *Alligator mississippiensis*, is found within the south-eastern United States and is considered a living descendant of an ancient lineage of dinosaurs known as Archosaurs. The alligator's primitive reptilian characters and its important phylogenetic placement between dinosaurs and birds makes it ideal for comparative, developmental, and evolutionary studies on the vertebrate reproductive system. Alligators reside in many areas of the south-eastern United States with the densest populations occurring in the fresh-water coastal marshes of Louisiana (Joanen and McNease 1986, 1987; Lance 1989) and the lakes of central Florida (Hines 1979). The American alligator is also one of the largest North American species of reptile and little was known about its reproductive biology until recently (Lance 1989). Most of these recent data have focused on reproductive behaviour, nesting biology, clutch sizes, parental care, and reproductive/hormonal cycles (Joanen and McNease 1970, 1971, 1972, 1975, 1979, 1980; Joanen 1974; Lance 1989).

The alligator was chosen for this study as part of a series of studies examining the germ cell development strategy employed during spermatogenesis within the reptilian testis. Though extensive data exist on the reproductive cycles of both sexes, little qualitative information has been gathered on the specific cytological events of germ cell development within the alligator testis. Lance (1989) briefly described the male reproductive cycle of the alligator, focusing on the beginning, end, and major events of spermatogenesis with morphological descriptions of specific germ cell types. At present, there has been no comprehensive study performed that focuses on the sequence of germ cell development and

on whether a temporal or spatial germ cell development strategy exists within the alligator testis.

The importance of such a study is two-fold: first, reptilian ancestors were the first amniotes to evolve, and within Reptilia many derived reproductive characters allowed for their successful invasion of the terrestrial environment (Pudney 1990). These modifications or adaptations, such as a tubular testis lined with Sertoli cells and an epididymis for sperm storage, are conserved and are found in most male amniotes (Robert 1975). These adaptations allow for the protection, storage, and safe transfer of fragile sperm directly to the female reproductive tract and prevent exposure of sperm to the harsh terrestrial environment. Recent studies on *Trachymes scripta* (slider turtle) and *Podarcis muralis* (European wall lizard), two species representing different orders within the class Reptilia (Chelonia and Squamata), have provided evidence for germ cell development strategies that are quite different from those of other amniotes (Gribbins and Gist 2003; Gribbins *et al*. 2003). Germ cells develop as a single population temporally within the wall lizard and slider turtle testes, which leads to a single spermiation event at the end of spermatogenesis. This episodic germ cell development strategy is more reminiscent of that seen within the anamniotic (amphibian) testis, which is very different from the continuous spatial germ cell development that results in waves of sperm release during the breeding season in derived amniotic taxa (Lebonde and Clermont 1952; Farner and Lewis 1971; Lofts 1977; Roosen-Runge 1977; Follett and Robinson 1980; Russell *et al*. 1990; Kumar 1995). Understanding how germ cells develop within the alligator's structurally amniotic testis may provide further support for the hypothesis that temperate reptilian testes are transitional or intermediate in organization.

Second, major differences in the spermatogonia populations were also observed in these earlier studies on spermatogenesis within *T. scripta* and *P. muralis*. The slider turtle has three distinctly different spermatogonia (resting, type A, and type B) and proliferation of type A and B spermatogonia occurs over a very short period of time (30–60 days) in May and June. Once the A and B populations are exhausted in the slider turtle in early summer, they are replaced by non-dividing, darkly staining, resting-type spermatogonia for the rest of the spermatogenic cycle. In contrast, only spermatogonia A and B are observed within the wall lizard testis and they divide mitotically throughout the entire spermatogenic cycle. The importance of such differences in spermatogonial populations is not known; such information does not exist for reptilian species.

Slider turtles have long refractory periods where spermatogenesis does not occur even though resources and temperatures are adequate to support germ cell development; wall lizards lack these refractory periods. Do different spermatogonial populations affect the physiology of the testis such as the presence or absence of refractory periods or when hormonal stimulation causes recrudescence of spermatogenesis? Understanding when and what types of spermatogonia and other germ cell types are found within the seminiferous epithelium might aid in answering some of these questions.

In this study, we provide the first complete histological analysis of spermatogenesis and the germ cell development strategy within a species of the order Crocodilia. We relate these observations to the accumulating data that suggest that temperate reptiles retain a temporal germ cell development pattern similar to anamniotes and in the context of reproductive cycles, spermatogonial population composition, and hormonal and temperature control of spermatogenesis.

Materials and Methods

Animal collection

Adult male American alligators (*A. mississippiensis*) between 2.5 and 3 m in total length, were collected from the Rockefeller Wildlife Refuge in Grand Chenier, Louisiana from February 2001 to May 2001, from March 2002 to June 2002, and from April and May 2003. Thirteen alligators in all were collected for this study (12 from the present collecting dates and one late June sample collected from the same area was provided by Valentine Lance from the San Diego Zoo).

Although 13 animals are the limit to our sample size, comparisons of overlapping months from the 3 years revealed consistent morphological data for each month represented. A complete series of specimens representing each month of the year is not presented in this study. However, the months represented do occur over at least one complete cycle of spermatogenesis and allow for a detailed analysis of the germ cell development strategy. The focus here is on the germ cell development strategy and not the histological details of the entire reproductive cycle, such as the beginning and ending of spermatogenesis. Lance (1989) provides more complete information on the male reproductive cycle of American alligators, which includes a description of annual changes to the testis.

Alligators were captured by noose or by baited hooks that were placed in canals within the refuge. Upon capture, they were killed and the testes were removed immediately by dissection. The testes were sliced into transverse pieces and were fixed and stored in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) under refrigeration until the time of embedding.

Tissue preparation for light microscopy

Transverse sections of testis were cut into 3-mm pieces and dehydrated through a graded series of alcohols. Pieces were infiltrated with a catalysed acrylic monomer before embedding in glycol methacrylate (GMA) resin (Polysciences, Inc., Warrinton, PA). Resin blocks were allowed to cure for 2–4 days. Sections (2 μm) then were cut from resin blocks using a dry glass knife and an LKB-Ultrotome III (LKB

Produkter AB. Bromma, Sweden). Finally, sections were stained with the Periodic Acid Schiff's Stain (PAS) procedure for acrosome granule visualization followed by a Mayer's haematoxylin counterstain for nuclear detail.

Histological analysis

Ten random 3-mm pieces of testis were embedded for each alligator testis. Five serial sections from each 3-mm piece were placed on glass slides, examined using an Olympus compound microscope, and digital pictures were taken with a high-resolution digital camera. Digital pictures were evaluated for cytological changes occurring to germ cells during spermatogenesis. Cytological changes accompanying spermatogenesis are common to the testes of all vertebrates (Volsoe 1944; Hess 1990; Russell *et al*. 1990). Therefore, the alligator germ cells were scored for changes to the acrosomal granule and vesicle, changes to the chromatin of the nucleus, and nuclear elongation and condensation. Once the morphologies of the germ cells were determined, cross-sections of the alligator seminiferous epithelium were evaluated for consistent cellular associations or stages (spatial relationships between germ cells) for each month represented.

Results

Adult male *A. mississippiensis* have typical amniotic testes containing highly convoluted seminiferous tubules. These tubules are lined with a seminiferous epithelium consisting of Sertoli cells. Germ cells develop through the phases of spermatogenesis in association with Sertoli cells. The testis is spermatologically active from at least February to late June. The proliferative phase of spermatogenesis has already begun in the February sample and the majority of the germ cell population consists of spermatogonia undergoing mitosis in the basal portion of the seminiferous epithelium. By early March, most of the germ cells within the epithelium have entered meiosis I, but a very small proportion of the germ cell population has progressed as far as early spermiogenesis. The April and May seminiferous epithelia are packed full of germ cells in different phases of spermiogenesis. The major events of mitosis and meiosis are completed by April and most of the germ cell population consists of developing spermatids. By early June, most of the germ cell population is either completing spermiogenesis or undergoing spermiation and in our samples spermiation was completed by late June.

Germ cell morphology and development

Spermatogenesis within alligator testes may be broken down into three phases: the proliferative (mitotic), meiotic, and spermiogenic (maturational) phases. Nineteen germ cell morphologies were recognized within the seminiferous tubules of the alligator.

Proliferative (pre-meiotic cells)

Two morphologies of spermatogonia are represented within the seminiferous epithelium of the alligator testis. Type A spermatogonia (Fig. 1A, white arrowhead) are found within the epithelium in all the slides examined in this study. They represent the majority of the germ cell population during February and are most frequently observed dividing from February to March. Spermatogonia A are ovoid in shape and have one flattened cellular surface resting directly on the basement membrane. Their nuclei contain prominent nucleoli and heterochromatin concentrated close to the nuclear membrane.

Type B spermatogonia (Fig. 1B, white arrowhead) have nuclei that are round and contain large globules of heterochromatin dispersed throughout the nucleoplasm. Type B cells typically have more than one nucleolus and are found in the basal portion of the seminiferous epithelium in all the slides examined in this study. The proliferative phase (early events) of spermatogenesis produces a large population of type B spermatogonia, which accumulate just above the basement membrane. Type B spermatogonia are most numerous in the month of March.

Meiotic cells

Meiotic cells are characterized by increasing nuclear and cytoplasmic size and the gradual condensation of nuclear chromatin into distinct chromosomes. The majority of spermatogonia B undergo mitotic divisions during March to produce preleptotene cells. Pre-leptotene cells (Fig. 1C, white arrowhead) appear from late February through March. They have nuclei with prominent nucleoli and fine granular chromatin. Pre-leptotene cells, along with step 1 spermatids (Fig. 1K), are the smallest germ cells within the alligator seminiferous epithelium. Their small size easily distinguishes them from the larger spermatogonia A and B at the base of the seminiferous epithelium.

Leptotene cells (Fig. 1D) are larger in diameter and stain more intensely than preleptotene cells and fine filamentous chromatin pack their nuclei. They appear from early March through April and constitute a large portion of the primary spermatocyte population within the seminiferous epithelium from mid-March to early April. Zygotene cells (Fig. 1E) appear in their largest numbers within the seminiferous epithelium of the alligator in April. They are marked by a slight increase in cellular size and by a thickening of the filamentous chromatin fibres within the nucleus.

Pachytene cells (Fig. 1F) first appear in late March and remain in the seminiferous epithelium until the end of spermatogenesis. They make up a large percentage of the germ cell population in April. These germ cells undergo a substantial size increase and their nuclei contain very thick chromatin fibres that are interspersed with areas of open nucleoplasm. Pachytene cells are the most common of the

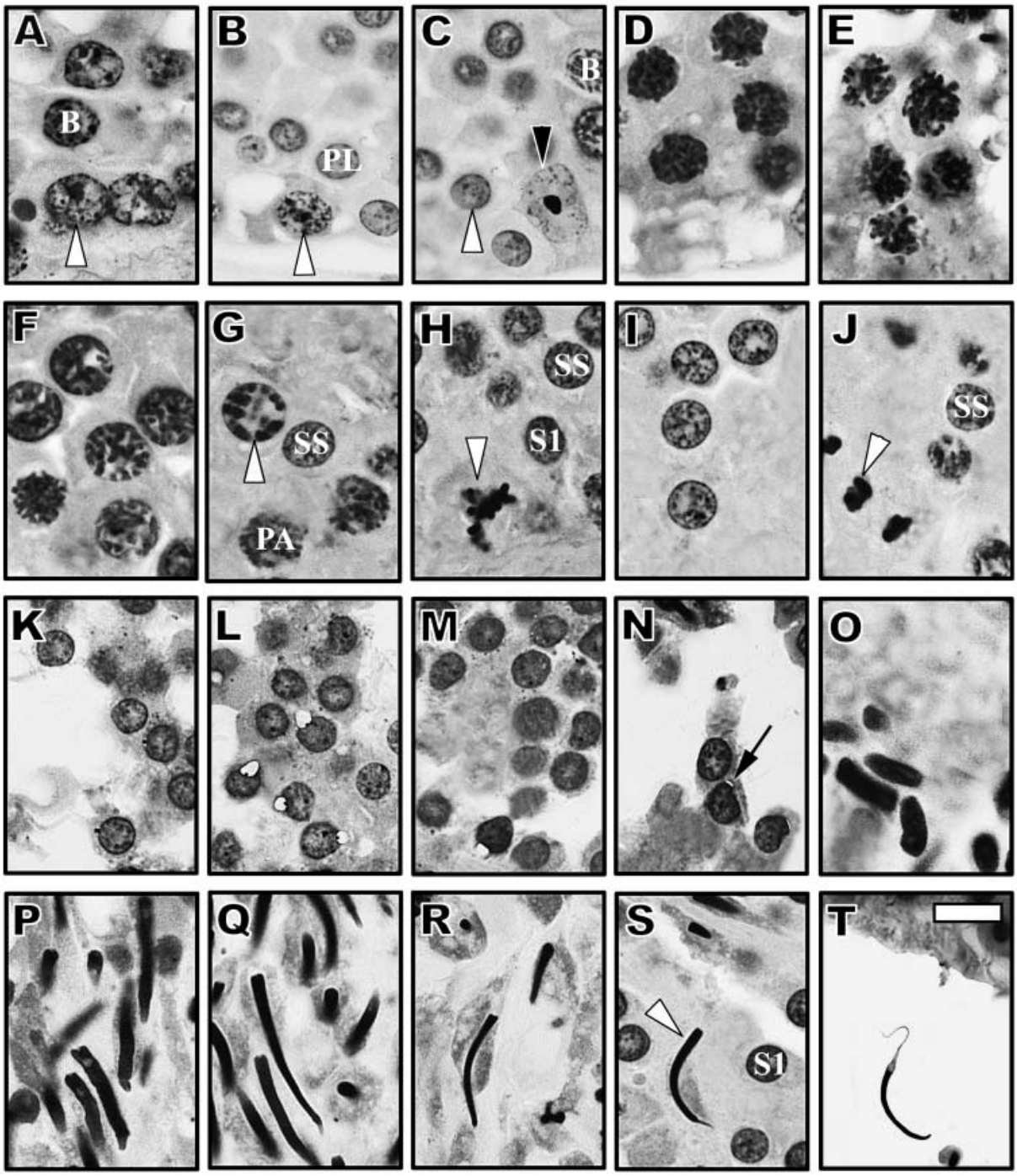

Fig. 1—Cell types found within the seminiferous epithelium of *Alligator mississippiensis*. Bar = 15 μm. —**A**. Spermatogonia A (white arrowhead). B, spermatogonia B. —**B**. Spermatogonia B (white arrowhead). PL, preleptotene spermatocyte. —**C**. Pre-leptotene spermatocyte (white arrowhead). Sertoli cell nucleus (black arrowhead). B, spermatogonia B. —**D**. Leptotene spermatocytes. —**E**. Zygotene spermatocytes. —**F**. Pachytene spermatocytes. —**G**. Diakinesis (white arrowhead). SS, secondary spermatocyte. PA, pachytene spermatocytes. —**H**. Meiosis I (white arrowhead). S1, step 1 spermatid. SS, secondary spermatocytes. —**I**. Secondary spermatocytes. —**J**. Meiosis II (white arrowhead). SS, secondary spermatocytes. —**K**. Step 1 spermatids. —**L**. and —**M**. Step 2 spermatids. —**N**. Step 3 spermatids. Black arrow point to developing acrosome. —**O**. Step 4 spermatids. —**P**. Step 5 spermatids. —**Q**. Step 6 spermatids. —**R**. Step 7 spermatids. —**S**. Step 8 spermatid (white arrowhead). S1, step 1 spermatid. —**T**. Mature spermatozoon. Note: If white or black arrows are not present within a section then all the cells within a box represent the listed germ cell type.

meiotic germ cells observed in the seminiferous epithelium during spermatogenesis.

Diakinesis (Fig. 1G, white arrowhead), metaphase I (Fig. 1H, white arrowhead), secondary spermatocytes (Fig. 1I), and metaphase II cells (Fig. 1J, white arrowhead) predominate from April to early May. These four transitional germ cells are usually found together in the seminiferous epithelium, but their frequency of observation is very low. Diakinesis spermatocytes are characterized by thick fully condensed spoke-like chromosomal fibres that are interspersed with large translucent areas of nucleoplasm. Metaphase I cells contain a condensed clump of chromosomes located on the equatorial plate with no apparent nuclear boundaries. Secondary spermatocytes (SS) (Fig. 1G,H,J) are usually dispersed randomly between diakinesis, metaphase I and metaphase II cells. Secondary spermatocytes have lightly stained centrally located nuclei that are roughly 10–20% larger than subsequent step 1 spermatids (S1) (Fig. 1H). Metaphase II cells are smaller in size than metaphase I cells and visually appear to have roughly half the amount of chromatin when compared to metaphase I cells.

Spermiogenic cells

Spermiogenesis can be divided into eight recognizable steps in the alligator based on the terminology of Russell *et al.* (1990) for mammalian species, and includes the development of the acrosomic system, elongation of the nucleus, and condensation of chromatin material. The appearance of step 1 spermatids in the alligator testis (Fig. 1K) marks the beginning of spermiogenesis. Their small size and lightly stained Golgi zone (early development of the acrosomic system) that lies in juxtaposition to the nuclear surface characterizes these spermatids. Their spherical nuclei are centrally located and contain one or more chromatin bodies.

A distinct acrosomic vesicle in direct contact with the nuclear envelope characterizes step 2 spermatids (Fig. 1L [early] and 1M [late]). A proper section through the acrosomic vesicle reveals a prominent PAS + acrosomic granule (Fig. 1L) that is basally located within the vesicle. A large oval acrosome, which extends off the apex of each nucleus distinguishes these spermatids early in their development. Step 1 and 2 spermatids are the most common of the round spermatids (Fig. 1K–M). They are observed in the seminiferous epithelium as early as late March but are most abundant in May.

Step 3 spermatids (Fig. 1N) appear at the same time as step 2 spermatids. It is not uncommon to observe steps 1, 2 and 3 spermatids grouped together within the seminiferous epithelium. Step 3 spermatids are a transitional step and mark the onset of sperm cell elongation. Their nuclei are irregular in shape compared to the uniform diameters of the previous round steps. Elongation begins in the apical region of the nucleus and therefore the apex is much thinner than the base of the nucleus. The acrosomic vesicle has widened further across the apex of each nucleus and flattens it completely (Fig. 1N, black arrow). As development progresses the acrosomic vesicle will continue to migrate across the apex of the nucleus until it envelops the entire apical surface.

Extreme elongation and condensation of the nucleus characterize spermatids of steps 4 to 8 spermatids (Fig. 1O–S). Step 4 spermatids (Fig. 1O) are longer and more tubular in shape compared to step 3 spermatids (Fig. 1N) because of continued elongation of the nuclear head. Step 5 spermatids (Fig. 1P) undergo further elongation and condensation and are much longer than step 4 spermatids; they often have a pointed conical apex that distinguishes them from step 4 spermatids. Step 6 (Fig. 1Q) spermatids represent the termination of nuclear elongation and the nuclear head has reached its maximum length of 30 μm or more. Step 4, 5 and 6 spermatids are commonly observed in bundles within columns of seminiferous epithelium that project into the lumen of May and June seminiferous tubules.

Steps 7 and 8 (Fig. 1R,S, white arrowhead) spermatids exhibit considerable nuclear condensation and cytoplasmic elimination and are much thinner in diameter than previous elongating steps (Fig. 1N–Q). Flagella are often seen extending from steps 6, 7, and 8 spermatids out into the luminal space. Steps 7 and 8 spermatids stain more intensely than any of the previous elongating spermatids and their cytoplasm is eliminated until distinguishable cell membrane boundaries are absent. At the same time, the apical portion of each nucleus becomes slightly curved from the middle point to the apex of the nuclear head, producing concave and convex surfaces. Once step 8 spermatids complete spermiogenesis, they are released as mature spermatozoa (Fig. 1T). The mature spermatozoa are slightly curved with very long nuclear heads (up to 30 μm). The majority of steps 6 through 8 are found within the seminiferous epithelium in May and June. Small pockets of spermatozoa release begin as early as May and the major events of spermiation are completed by June. The only other cell type found within the seminiferous tubules are Sertoli cells (Fig. 1C black arrowhead), which are recognized by their triangular nuclei containing one or more large prominent nucleoli.

Seasonal development of the seminiferous epithelium

The earliest sample of testicular tissue collected in this study was in mid-February and spermatogenesis is well underway by this time (Fig. 2A). Mitotic divisions of spermatogonia A and B are prominent along with the early events of meiosis I. The most common germ cell types are spermatogonia A and B, preleptotene, and leptotene spermatocytes. It should also be noted that it was not uncommon to see remnant germ cells (from last year's cycle) undergoing hypertrophy (HE) within the February testis.

In March (Fig. 2B), the majority of the germ cell population is progressing through meiosis I. Spermatogonial divisions are still common, but begin to slow in March. A small

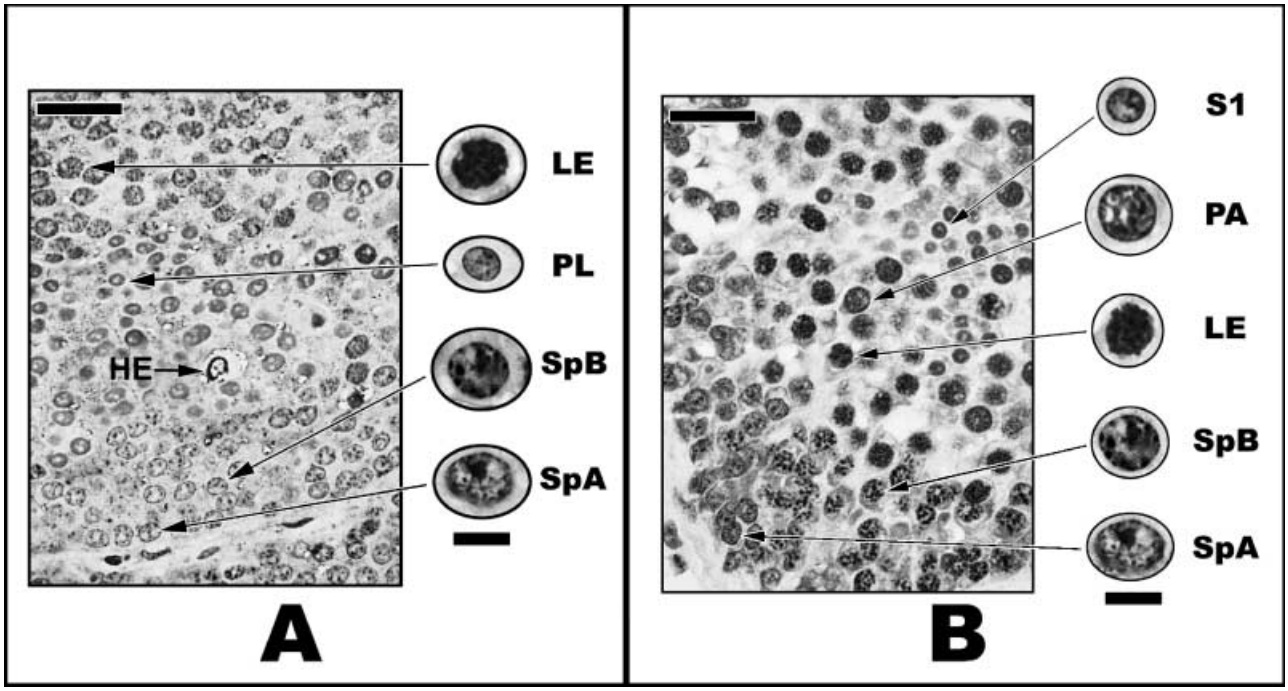

Fig. 2—Sections of seminiferous epithelia with higher magnifications of represented cell types. Bar = 100 μm for each seminiferous epithelium section and 15 μm for magnified germ cell types. —**A**. Section of the February seminiferous epithelium and represented cell types from top to bottom: leptotene (LE), preleptotene (PL), spermatogonia B (SpB) and A (SpA). Note: hypertrophic cell (HE) represented within the section of seminiferous epithelium. —**B**. Section of March seminiferous epithelium and represented cell types from top to bottom: step 1 spermatid (S1), pachytene (PA), leptotene (LE), spermatogonia B (SpB) and A (SpA). Bar = 15 μm

population of spermatogonia A will remain within the seminiferous epithelium through June (Fig. 4B) and it is not uncommon to see these spermatogonia undergoing isolated mitotic events throughout the months collected in this study. However, the major events of proliferation are over by March. The majority of the germ cell population in March are spermatocytes with a few germ cells progressing as far as the early events of spermiogenesis. Inconsistent layerings of spermatogonia and spermatocytes and the absence of late-developing spermatids prevent the identification of a consistent cellular association among germ cells within the February and March seminiferous epithelia.

Most of the germ cell population has progressed into spermiogenesis by April and May. Spermatids begin to increase in number by mid-April (Fig. 3A) and are the dominant germ cell within the seminiferous epithelium. It is not uncommon to see 5–6 spermatids layered in columns of seminiferous epithelium that project out into the lumen of the seminiferous tubules. Most of the spermatids represented within the April seminiferous epithelium are early or round spermatids that have well-developed acrosomes. By May (Fig. 3B), the majority of spermatids are progressing into the late-elongating phases of spermiogenesis. Small pockets of sperm release begin to occur within the seminiferous tubules of alligators in May marking the beginning of spermiation.

Late elongating spermatids finishing spermiogenesis and the release of spermatozoa from the seminiferous epithelium dominate the early June testis (Fig. 4A) Spermatozoa release is almost complete by late June which marks the end of spermiation within the alligator testis (Fig. 4B). Thus, these data suggest that the major events of spermiation begin in early May and continue through early June. At this time, spermatogonia remain near the basement membrane along with a few lingering spermatocytes. Most of the germ cells represented in late June (Fig. 4B) are mature spermatozoa that have been released to the lumen of each seminiferous tubule and a single layer of spermatogonia (mostly A and a few B) remaining within the seminiferous epithelium near the basement membrane.

Discussion

Spermatogenesis within our samples of *A. mississippiensis* testis follows a prenuptial pattern of development, similar to the findings of Lance (1989). In this study, the spermatogenic cycle has just begun by February and progresses through late June where spermiation is close to completion. It should be noted that the sample size within this study is small (13 alligators). Collecting alligators before February and after June is difficult because the alligator population, particularly large

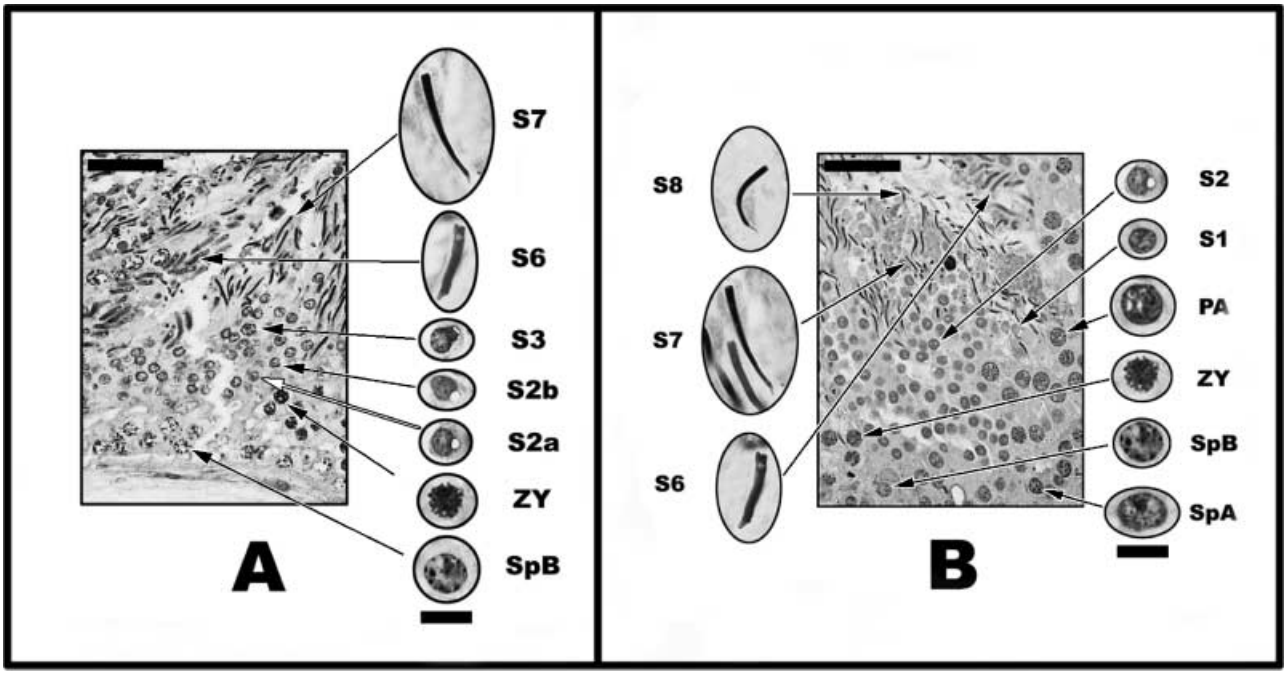

Fig. 3—Sections of seminiferous epithelia with higher magnifications of represented cell types. Bar = 100 μm for each seminiferous epithelium section and 15 μm for magnified germ cell types. —**A**. Section of the April seminiferous epithelium and represented cell types from top to bottom: step 6 spermatid (S7), step 5 (S6), step 3 (S3), late step 2 (S2b), early step 2 (S2a), zygotene (ZY), spermatogonia B (SpB). —**B**. Section of the May seminiferous epithelium and represented cell types from top to bottom: left side: step 8 spermatid (S8), step 6 (S7), step 5 (S6); right side: step 2 spermatid (S2), step 1 (S1), pachytene (PA), zygotene (ZY), spermatogonia B (SpB) and A (SpA). Bar = 15 μm

males, disperses into open water and away from the canals of the refuge where capturing animals is more efficient.

New evidence also suggests that the alligator testis is active spermatogenically in the early/late spring right before mating and then inactive for the rest of the year. A recent study by Lance (2003) states that blood testosterone levels in alligators are at their highest levels from February to May and drop off substantially in June and remain low for the rest of the summer, autumn, and winter. Similar findings were observed for blood testosterone levels collected monthly from adult male alligators in Louisiana in 2004 (Ruth Elsey, unpublished data). In these large breeding males (> 2.28 m), a seasonal increase in testis mass was correlated with the rise in testosterone levels (Lance 1989, 2003). Increased testicular mass is typically the result of increases in diameter of the seminiferous epithelium (as a result of spermatogenesis) and/or the lumen (Licht 1984). Thus, the collection of tissues in the study not only represents one complete cycle of spermatogenesis, but also is probably the only major cycle of spermatogenesis occurring in this most northern population of alligators. Collecting samples from adult male alligators past the month of June would yield little useful data histologically (especially with the focus here on the germ cell development strategy) in light of these most recent findings. Furthermore, killing large adult males (3 m) after the June sample translates into removing at least 12 additional 15- to 25-year-old alligators at or near their breeding prime from the current population, which the authors are strongly opposed to in light of the new testosterone data.

Previous studies have shown that sperm are found in the penile groove from May to June (Joanen and McNease 1980) and eggs are fertilized during the month of May and are in nests by early to mid-June in Louisiana (Joanen and McNease 1979, 1989). The present data support these findings by providing histological evidence of spermatozoa release within the alligator testis starting in early May when mating begins and also shows that spermiation is winding down by late June when fertilized eggs are already in the nest. Some of the males in this study are small (2.5 m) compared to dominant males (3 m or greater). It has been observed that smaller males still show breeding behaviour in early and mid-June after larger dominant males have already bred (Ruth Elsey, personal communication). Thus, smaller alligators may produce sperm later into the summer to increase their probability of breeding after large males have finished reproducing.

A prenuptial pattern of sperm development occurs in the European wall lizard (*Podarcis muralis*) (Gribbins and Gist 2003) similar to the alligator. Sperm development in *P. muralis* however, requires almost the entire year (11 months) because spermatogenesis starts late in the summer (July) and spermiogenesis is subsequently retarded during the winter

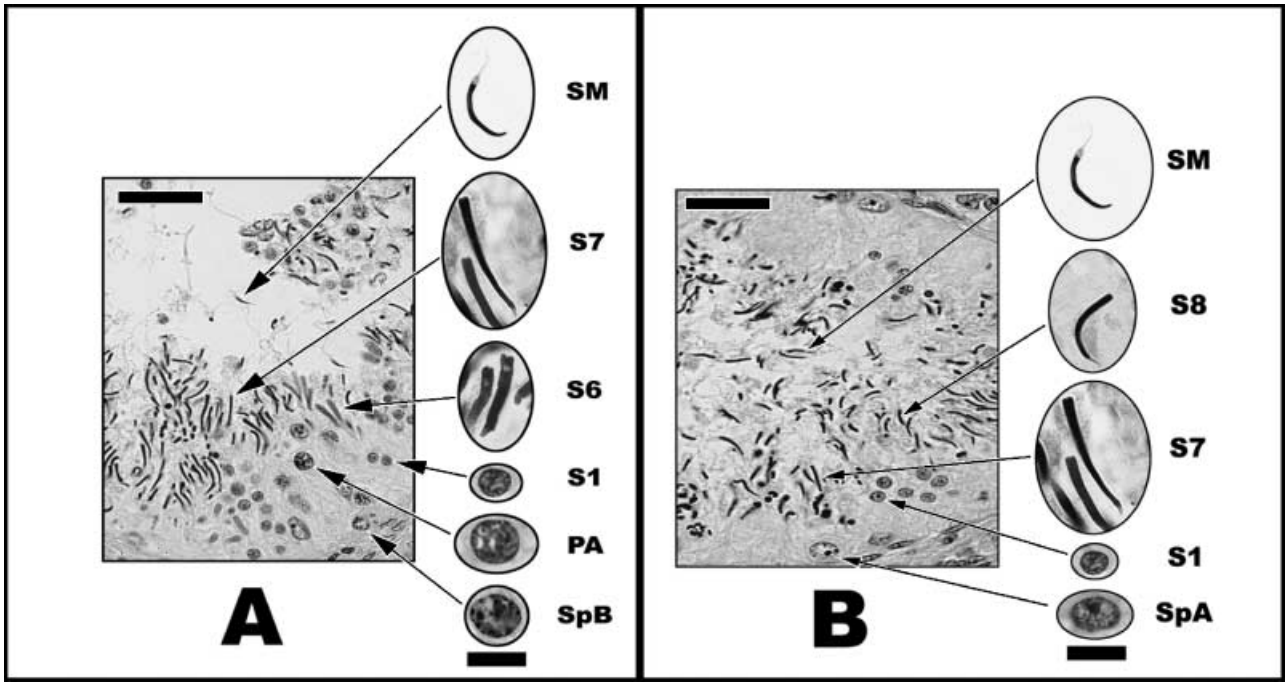

Fig. 4—Sections of seminiferous epithelia with higher magnifications of represented cell types. Bar = 100 μm for each seminiferous epithelium section and 15 μm for magnified germ cell types. —**A**. Section of the early June seminiferous epithelium and represented cell types from top to bottom: mature sperm (SM), step 6 spermatid (S7), step 5 (S6), step 1 (S1), pachytene (PA), spermatogonia B (SpB). —**B**. Section of the late June seminiferous epithelium and represented cell types from top to bottom: mature sperm (SM), step 8 spermatid (S8), step 6 (S7), step 1 (S1), spermatogonia A (SpA).

months, whereas alligators theoretically produce one complete round of spermatogenesis in 5 months (February to June). This arrest in spermiogenesis during the winter was seen in other lacertid lizards experimentally exposed to cooler temperatures at the start of spermatid accumulation (Fischer 1974; Joly and Saint Girons 1975). Thus, an adequate temperature in temperate breeding prenuptial reptiles may be crucial for completion of spermatogenesis (Licht 1984). The moderate temperatures in the southern United States may contribute to the speed of spermatogenesis in the alligator, and it would be interesting to examine other, more tropical, crocodilians to determine the importance of temperature in relation to the speed of spermatogenesis.

Temperature versus pre- and postnuptial germ cell development

Does temperature affect other aspects of spermatogenesis? The dependence of testicular function and cycles on hormones, such as gonadotropins, is well established for many reptiles (Licht 1973, 1984). However, issues and controversies exist on how these hormones interact with the testis in response to external stimuli such as temperature to generate the various types of reproductive cycles and refractory periods seen in temperate reptiles. One major problem is the lack of histological information on spermatogenesis and when germ cell types are observed within the testis during seasonal changes to the gonads (Licht 1984). The type of germ cell development strategy and the presence or absence of specific germ cell types, such as spermatogonia, may help to unravel some of the controversy that exists with hormonal control of reptilian spermatogenesis.

For example, direct effects of temperature on aspects of spermatogenesis have been observed in the male alligator. Lance (1989) observed that in rare instances spermatogonial divisions can start in unusually warm weather as early as September in the alligator testis. However, advanced development past the proliferation stage and major changes in testis size were not observed in these samples. Much evidence exists for temperature-induced spermatogenesis independent of photoperiod in alligators in zoos and farms at a whole range of latitudes and photoperiods (Green 1981; Wright 1981; Ben-Moshe 1987; Eriksen 1987). In the present study, it was observed that spermatogonia A and B continued to divide throughout the entire spermatogenic cycle. The same mitotic ability of spermatogonia not only occurs during spermatogenesis but also for almost the entire year within the prenuptial European wall lizard. It has been shown that many temperate lizards that are prenuptial show this initial rapid mitotic growth of germ cells in response to elevated temperature (Licht 1973). Evidence also links the presence of spermatogonia A and B with the recrudescence of spermatogenesis upon stimulation of the proper hormone such as

gonadotropins or testosterone (Saint Girons 1976; Licht 1984). Furthermore, spermatogonia A and B have been found to have the proper hormone (testosterone) receptors (Licht 1984). Many have suggested that elevated body temperature alone probably is the major exogenous cue that either directly (Licht 1984) or by stimulating the release of the proper hormone (Licht and Pearson 1969; Licht 1973; Jalali *et al.* 1976; Saint Girons 1976; Licht 1984) causes recrudescence of spermatogenesis in these prenuptial reptiles.

In contrast, the postnuptial slider turtle lacks this population of dividing spermatogonia A and B year around and may not be able to respond to favourable temperatures in late summer or autumn because their spermatogonial population is in the resting phase and lacks the ability to divide. Slider turtle's dividing spermatogonia A and B populations are exhausted in mid-summer (July) and are replaced with dormant resting spermatogonia (Gribbins and Gist 2003). For a window running from July to December, A and B spermatogonia are rare or not found at all in the slider turtle seminiferous epithelium, which corresponds to their long refractory period during late summer and autumn.

Does the presence or absence of spermatogonia affect if and when spermatogenesis will undergo recrudescence? Having the right type of stem spermatogonia (A and B) present when hormonal or environmental stimulation occurs to the testis might influence spermatogenic recrudescence. Thus, the consistent presence of A and B spermatogonia within the testis of the alligator may be one reason why alligators have the ability to jump start spermatogonial divisions out of season in response to elevated temperatures or within artificial settings such as zoos or alligator farms. While lacking, this population of A and B cells might relate to factors such as the long refractory period seen in slider turtles (possess resting spermatogonia) versus the European wall lizard (possess only A and B spermatogonia), which virtually lacks a refractory period.

Phylogenetic implications of temporal germ cell development

Interestingly, the alligator's temporal germ cell development is similar to that of the wall lizard and slider turtle (Gribbins and Gist 2003; Gribbins *et al.* 2003). These three temperate reptiles represent three distinct orders (Chelonia, Squamata, Crocodilia) within the class Reptilia and are often considered polyphyletic in many respects (Jefferies 1986). However, reptilian ancestors represent the most primitive of amniotes (reptiles, birds, and mammals) and within their lineage arose many derived reproductive characters associated with their successful invasion of the terrestrial environment. These adaptations are conserved in most male amniotes (Robert 1975). The temporal germ cell development strategy shared by these reptiles is reminiscent of the developmental strategy seen in the anuran (anamniotic) testis. The germ cells of anurans progress as a single cohort temporally through proliferation, meiosis and spermiogenesis within cysts that line the tubules of their testes (Lofts and Boswell 1960; Lofts 1964; Van Oordt and Brands 1970), similar to the pattern seen in the slider turtle, wall lizard and alligator.

An argument can be made that the temporal pattern of spermatogenesis seen within the amphibian and reptilian testes is a strategy shared by all temperate (seasonally) breeding vertebrates and is an example of convergent evolution. Though studies on the reproductive cycle of tropical species are extensive (Fitch 1970; Howland *et al.* 1990; Vitt and Morato de Carvalho 1992), no information on the specifics of the male germ cell development strategy exist for reptilian species occupying homeothermic habitats. However, germ cell development in both seasonally breeding mammals and birds is spatial in nature, with waves of sperm release during the interval of reproductive activity, not one massive sperm release at the onset of breeding (Lebonde and Clermont 1952; Farner and Lewis 1971; Roosen-Runge 1977; Kumar 1986, 1995; Kadhim *et al.* 1987; Dasgupta and Bhattacharyya 1988; Hess 1990; Russell *et al.* 1990). These three reptiles, representing distinctly different reptilian taxa, all possess a common episodic mode of germ cell development. Thus, the temperate reptilian testis may be viewed as transitional, structurally similar to the tubular testis of amniotes, yet retaining the temporal pattern of germ cell development found within the cystic testis of anamniotes.

Perspective

The phylogenetic implications of this conserved germ cell development strategy within the most ancestral amniotes, reptiles, could be significant. However, few comparative germ cell development data exist for specific species of reptiles outside the series of studies performed for the slider turtle, European wall lizard and alligator. Do other prenuptial reptiles have spermatogonia A and B present consistently within the testis? Do tropical species of reptiles have the same temporal germ cell development strategy? Does a prenuptial or postnuptial cycle determine the type of spermatogonial population found within the seminiferous epithelium? These questions and other comparative, phylogenetic, and physiological questions cannot be answered unless more data are collected on germ cell development strategies and sperm development in tropical and temperate reptilian species.

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