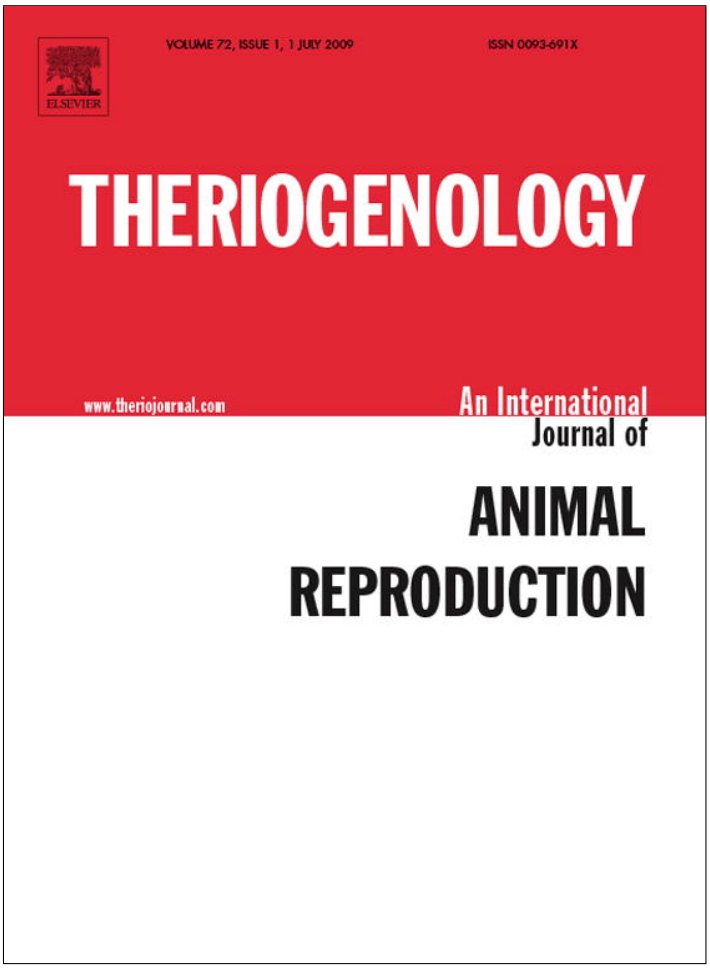

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Temporal germ cell development strategy during spermatogenesis within the testis of the Ground Skink, *Scincella lateralis* (Sauria: Scincidae)

J.L. Rheubert [a,b], H.H. McHugh [a], M.H. Collier [a], D.M. Sever [b], K.M. Gribbins [a,*]

[a] *Department of Biology, Wittenberg University, PO Box 720, Springfield, OH 45501-0720, USA*
[b] *Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402, USA*

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Abstract

Ground Skink (*Scincella lateralis*) testes were examined histologically to determine the testicular organization and germ cell development strategy employed during spermatogenesis. Testicular tissues were collected from 19 ground skinks from Aiken County, South Carolina during the months of March-June, August, and October. The testes consisted of seminiferous tubules lined with germinal epithelia in which germ cells matured in close association with Sertoli cells. As germ cells matured, they migrated away from the basal lamina of the epithelia towards the lumina of the seminiferous tubules. The testes were spermatogenically active during the months of March, April, May, June, and October (largest seminiferous tubule diameters and epithelial heights), but entered a quiescent period in August (smallest seminiferous tubule diameter and epithelial height) where only spermatogonia type A and B and early spermatocytes were present in low numbers within the seminiferous epithelium. Although the testicular organization was similar to other amniotes, a temporal germ cell development strategy was employed during spermatogenesis within Ground Skinks, similar to that of anamniotes. Thus, this skink's germ cell development strategy, which also has been recently reported in all other major reptilian clades, may represent an evolutionary intermediate in terms of testicular organization between anamniotes and birds and mammals.

Keywords: Testis; Spermatogenesis; Skink; Lizard; Histology

1. Introduction

Recently, within the European Wall Lizard, *Podarcis muralis*, a new germ cell development strategy has been described for lizards and other squamates that is much different than the typical spatial arrangement of germ cells within the amniotic (bird and mammal) testes [1,2]. Germ cells within the testis of *P. muralis* developed through the stages of spermatogenesis as a single population and produced mature spermatozoa in a single spermiation event at the end of the spermatogenic cycle. This episodic germ cell development strategy, in which the entire seminiferous tubule/epithelium participated in spermiation, was more reminiscent of that seen within anamniotic (amphibian) testes and was distinct from the continuous spatial germ cell development that results in waves of sperm release at certain portions along the length of the seminiferous tubules within the amniotic testes of avian and mammalian taxa during their breeding seasons [3–7].

* Corresponding author. Tel.: +1937 327 6478.
E-mail address: kgribbins@wittenberg.edu (K.M. Gribbins).

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Furthermore, this new temporal germ cell development strategy during spermatogenesis also has been described in all major taxa within Reptilia (Sauria: [1]; Chelonia: [8]; Serpentes: [2,9]; Crocodylia: [10]).

Historically, spermatogenesis has been explained using various stages (I-VIII), which describe the presence or absence of groups of cells within the seminiferous epithelium, presence/absence of the lumen of the seminiferous tubule, and spermatozoa in the epididymis in reptiles [11]. These studies did not concentrate on the individual cell types and omitted details pertaining to germ cell morphological changes during mitosis, meiosis, and spermiogenesis. For the current study, these stages were not included; spermatogenic activity was based on the presence or absence of specific cell types, and whether consistent associations of groups of cells were present within the seminiferous epithelium.

The purpose of this study was to expand our understanding of the method of germ cell maturation and developmental strategy employed during spermatogenesis within the temperate Ground Skink, and to determine if the temporal germ cell development strategy observed in other reptiles also existed within the family Scincidae. Ground Skinks are medium-sized ''brown-backed'' lizards (ranging from 75–146 mm) that are common in the moist woodlands of the southeastern United States [12]. In Florida populations, male Ground Skinks have the greatest enlargement and activity within the testis from October through January and the testes reach minimal size in August [13]. Populations of Ground Skinks in South Carolina are

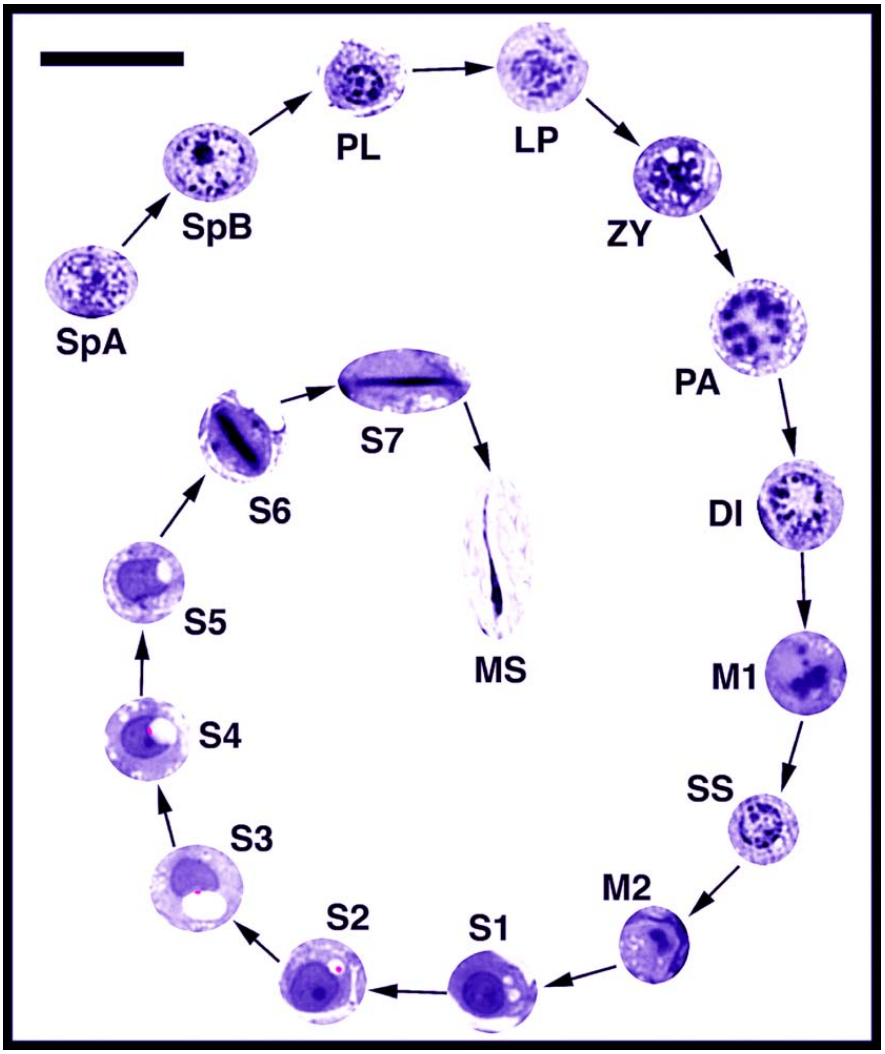


Fig. 1. Germ cell types found within the seminiferous epithelium of the Ground Skink. Bar = 20 μm. SpA, spermatogonia A; SpB, spermatogonia B; PL, pre-leptotene spermatocyte; LP, leptotene spermatocyte; ZY, zygotene spermatocyte; PA, pachytene spermatocyte; DI, diplotene spermatocyte; M1, meiosis I; 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.

spermatogenically active March through July, with quiescence in August [14]. The Ground Skink is found in the largest and most diverse family of lizards, Scincidae, which contains more than 1200 species, is a cosmopolitan taxon, with representative species on every continent except Antarctica [15]. Furthermore, skinks have recently become popular in the pet trade.

To date, *Podarcis muralis* of the family Lacertidae, *Seminatrix pygaea* of the family Colubridae, and *Agkistrodon piscivorous* of the family Viperidae are the only families within Squamata (~40 families) with a complete description of the germ cell development strategy exhibited during active spermatogenesis. The histological data obtained on spermatogenesis within *Scincella lateralis* will be compared to the temporal germ cell development of *P. muralis*, to the known histology of the testes in other skinks, and to what is known of the life history and reproductive characters within the Ground Skink. Also, data from this study may eventually help to elucidate or strengthen the phylogenetic relationships (by adding nontraditional characters such as germ cell development morphologies to molecular phylogenetic information) of skinks and other squamates, which is highly controversial at present [18,19].

2. Materials and methods

Adult male Ground Skinks were collected within the United States Department of Energy's Savannah River Site in Aiken County, SC, during the months of April (n = 3), May (n = 3), and October (n = 3) 2001 and March (n = 3), June (n = 3), August (n = 4) 2002. Although a complete series of testicular tissues from every month of the year were not collected, individuals were collected during what is believed to be the reproductively active and inactive seasons. Skinks were killed by exposure to ether (technique approved by Animal Care and Use Committee of Saint Mary's College, Notre Dame, IN, USA where the animals were killed), and the testes were excised and fixed in Trump's fixative (Electron Microscopy Sciences, Hatfield, PA, USA).

Testicular tissues were dehydrated in a graded series of ethanol, infiltrated with a 1:2 solution of Spurr's plastic (Electron Microscopy Sciences)/100% ethanol, and again in 1:1 Spurr's plastic/100% ethanol, before being infiltrated in 100% Spurr's plastic overnight. Fresh plastic then was used to embed tissues and polymerized blocks were allowed to cure for 2 d in a Fisher Isotemperature Vacuum Oven (Fisher Scientific, Pittsburg, PA, USA). Thin sections (2-3 μm) were cut from blocks using an LKB-Ultramicrotome III (LKB Produkter AB, Bromma, Sweden) and a dry glass knife. The sections were stained using a basic fuchsin/toluidine blue composite stain, as described by Hayat [20].

Sections of each testis were examined using a Zeiss compound light microscope (Carl Zeiss Microimaging, Inc. Thornwood, NY, USA) at various magnifications to determine the morphology of germ cells and germ cell development strategy. Photographs were taken using a SPOT digital camera (Diagnostic System Laboratories, Webster, TX, USA) and composite plates were constructed using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA). Thirty cross sections of seminiferous tubules for each represented month were chosen at random and the tubular diameter and germinal epithelial heights were measured using an ocular micrometer. Data analysis was performed using Minitab 15.0 (Minitab Inc., State College, PA, USA) for Windows. Results were deemed significant if $\alpha \leq 0.05$.

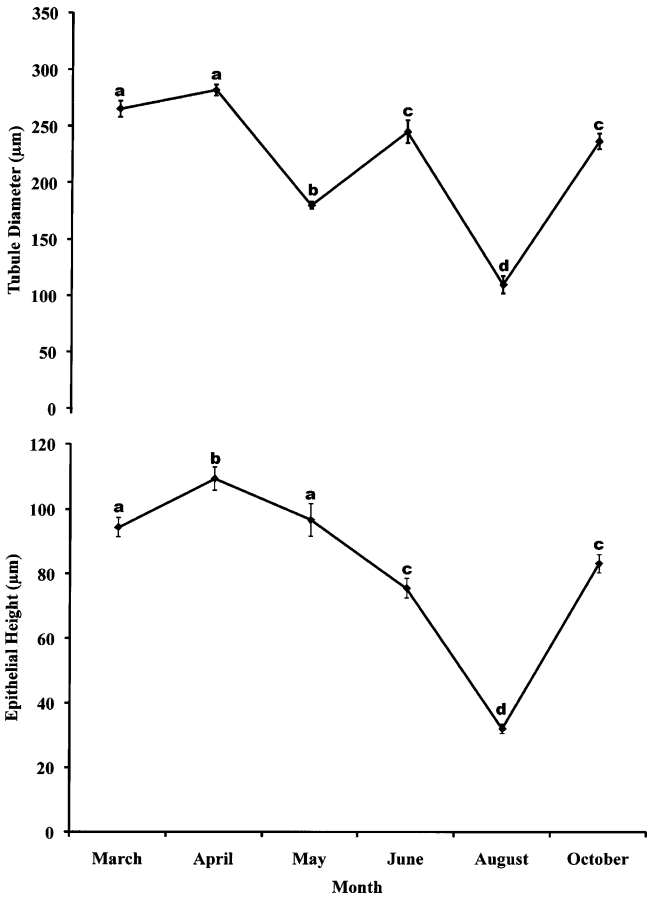


Fig. 2. **Top**: Variation in seminiferous tubule diameter and **Bottom**: Variation in germinal epithelial height during the months of March-June, August, and October within the testis of the Ground Skink. Values are means ± 1 SEM. Different superscripts indicate differences between monthly means ($P \leq 0.05$; Dunn-Sidak multiple range test).

Tubule 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 [21,22]. These data did not meet assumptions of normality; thus, nonparametric Kruskal-Wallis analysis was used to assess seasonal variation in seminiferous tubule diameter and germinal epithelial height. Post-hoc nonparametric multiple comparison tests using Dunn-Sidak procedures were then used to detect significant differences among pairs of means [2,23].

3. Results

Testes of Ground Skinks were composed of seminiferous tubules that are lined with a germinal epithelium consisting of Sertoli cells and developing germ cells. Spermatogonia A and B (Fig. 1, SpA and SpB) were found in the basal compartments of the epithelia during all months analyzed. Both spermatogonia types were located near the basement membrane of the seminiferous epithelium, with SpA being ovoid in shape and SpB being slightly larger and more round. The number of spermatogonia and the mitotic divisions of these gonial cells decreased during August (one layer of gonial cells) compared to all other months sampled (two or three layers of gonial cells). Little difference occurred between the abundance of spermatogonia and mitotic divisions in testes of specimens collected from March-June. There was a slight increase ($\sim$5%) in the number of layers of spermatogonia ($\leq$3) and mitotic activity in testes of animals collected in October, compared to all other months sampled.

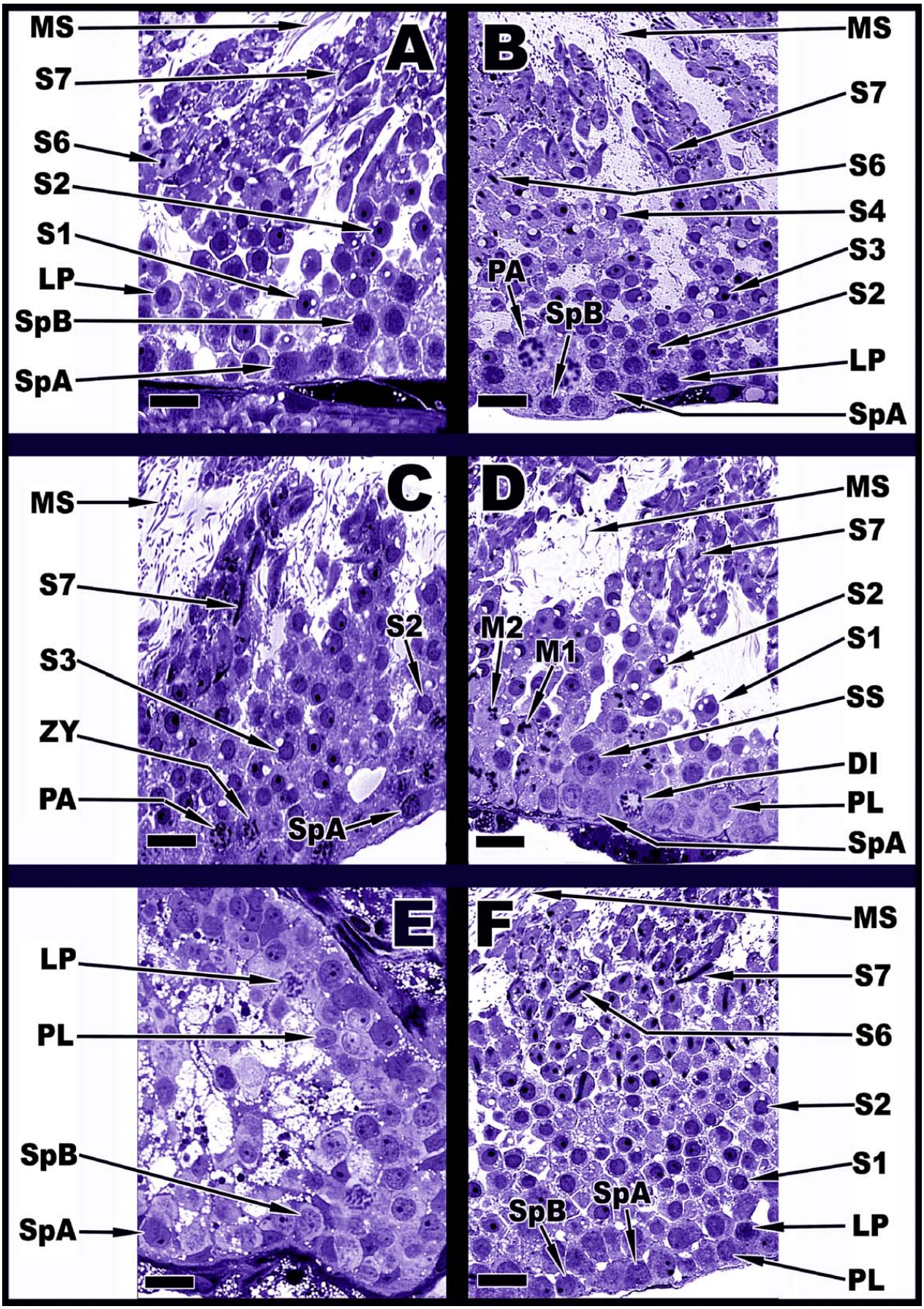
Meiotic cells were characterized by a sequential increasing in size of the nucleus and a condensation of chromatin into chromosomes. The cell types representing prophase I of meiosis were found during all months of the year, including August (usually only pre-leptotene cells) when the testis was reduced in size. Pre-leptotene cells (Fig. 1, PL) were the immediate result of mitotic divisions of Spermatogonia B. Pre-leptotene cells contained well-defined nuclear envelopes with dark staining nucleoli and were the smallest of all meiocytes.

Leptotene spermatocytes (Fig. 1, LP) were bigger in size compared to pre-leptotene cells and were distinguished by their dense filamentous chromatin. Zygotene spermatocytes (Fig. 1, ZY) were roughly the same size as leptotene cells, but had large clumps of condensed filamentous chromatin that stained more intensely than previous germ cells. Pachytene spermatocytes (Fig. 1, PA) were the largest of all developing spermatocytes, had more open nucleoplasm, and contained thicker chromatin fibers. Pachytene spermatocytes were found during March, May, June, and October.

Diplotene spermatocytes (Fig. 1, DI), metaphase 1 (Fig. 1, M1), secondary spermatocytes (Fig. 1, SS), and metaphase 2 (Fig. 1, M2) cells were found during spermatogenically active months (March, April, May, June, and October). The nuclear membranes of diplotene cells began to degenerate and condensed chromatin fibers formed a tight circle just under these degenerating membranes. Metaphase 1 cells had fully condensed chromosomes that aligned at the metaphase plate. The results of meiosis 1 were the secondary spermatocytes. The chromatin fibers of the secondary spermatocytes were randomly dispersed throughout their nucleoplasms. During metaphase 2, chromosomes aligned again at the metaphase plate. The germ cell size and amount of chromatin present in metaphase 2 was approximately half that seen in metaphase 1. The results of metaphase 2 were step 1 spermatids.

Spermiogenesis within the Ground Skink could be divided into seven steps, based on the terminology of Russell et al. [6] for mammals. Step 1 spermatids (Fig. 1, S1) were found during every month analyzed, except August. These spermatids were small in size, had well-defined nuclear membranes, and two conspicuous acrosome vesicles in contact with the apex of each nuclear membrane. These two acrosome vesicles of each spermatid fused to produce a single acrosome in step 2 spermatids (Fig. 1, S2). Acrosome granules were most commonly seen in the acrosome of Step 2 spermatids. Step 3 spermatids (Fig. 1, S3) had defined acrosome vesicles that began to increase in size and envelop the nuclear heads. As the development of the acrosomes continued, a deep depression within the apex of the nuclear head formed, a feature that characterized step 4 spermatids (Fig. 1, S4). Nuclear elongation characterized step 5 spermatids, which began opposite of the acrosome (Fig. 1, S5) and initiated the stretching of the spermatids' dorsoventral planes. As elongating spermatids developed, they migrated towards the apical portions of Sertoli cells, with the heads of elongates facing the basement membrane and the flagella facing the lumen.

Elongation continued and condensation dominated the nuclei of steps 6 and 7 spermatids (Fig. 1, S6 and S7). As condensation of the DNA progressed, the thickness of the nuclear heads decreased, resulting in very thin and aerodynamic nuclear heads on the mature spermatozoa (Fig. 1, MS). Mature spermatozoa were shed to the lumina of the seminiferous tubules.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Spermatogenesis occurred in every month sampled; however mitosis and meiosis slowed, with no evidence of spermiogenesis observed in August testes. The number of spermatogonia and mitotic divisions in August decreased ~20% compared to the other months sampled; morphometric measurements (Fig. 2) supported this histological observation (Fig. 3). Seasonal variation in seminiferous tubule diameter (Kruskal-Wallis: H = 86.50, df = 5, P = 0.000; Fig. 2, Top) and germinal epithelial height (Kruskal-Wallis: H = 75.92, df = 5, P = 0.000; Fig. 2, Bottom) had an apparent trend over all months sampled. The absence of large numbers of spermatocytes and spermatids in August lead to the smallest values for tubule diameter and germinal epithelial height. The largest difference between means (Dunn-Sidak multiple range test) occurred when August (Fig. 2; superscript subsets a: seminiferous tubular diameter means: -71 μm minimum (May)/-172 μm maximum (April) and epithelial height means: -43 μm minimum (June)/(April) -77 μm maximum) was compared to any other month in this study. August was the only month where spermiogenic cells were not seen and meiotic cells were scarce in number.

The seminiferous tubules of March, April, May, June, and October were histologically very similar (Fig. 3, A,B,C,D,F). The greatest recorded measurements of tubule diameter and epithelial height means occurred in March (265.1 and 96.5 μm) and April (281.5 and 109.2 μm), and coincided with the largest number of spermiogenic cell types found within the seminiferous epithelium. Seminiferous tubules of May specimens had the fewest mature spermatozoa within their lumina and the fewest spermiogenic cells overall within the epithelia lining the seminiferous tubules, which lead to the smallest tubule diameter mean (183 μm; Fig. 2, Top) of the spermatogenically active months. June (76 μm) and October (82.5 μm) had the shortest epithelial heights of the spermatogenically active months and the lowest number of elongating spermatids (June) or meiocytes (October) within their seminiferous epithelia.

4. Discussion

Testicular organization of the Ground Skink was similar to other reptiles [24]. As germ cells developed spermatogenically, they migrated away from the basal lamina of the seminiferous epithelium and towards the lumina of the seminiferous tubules, which was similar to that described for other lizards such as *Podarcis muralis* [1]. Although a full set of testicular samples representing every month of the year were not obtained in this study, most months sampled occurred over the warmer months of the year, when spermatogenesis is active in other lizards including many North American skinks [25–27,29]. The largest diameters of the seminiferous tubules and the highest measurements in germinal epithelial height occurred in the spring in these Ground Skink samples, similar to other skinks, which are known to breed or show courtship behavior during this time [17,25–29]. This germinal epithelial activity was marked by elevated numbers of meiotic and spermiogenic cells along with spermiation events, which provided mature spermatozoa for breeding.

The testes, however, were spermatogenically inactive during the month of August and were in a quiescent period. Although there are multiple variations in seasonal cyclicity [5,30], most temperate reptiles have at least one period of inactivity throughout a calendar year. In North American skinks, quiescence often occurs in late summer and fall [16,25,26]. This inactivity was characterized by the presence of only spermatogonia A and B within the germinal epithelium, as well as low rates of mitotic activity. In October, spermatogenesis again had a marked increase in activity and mitotic divisions of spermatogonia were very common near the basement membrane of the seminiferous epithelium, consistent with recrudescence of spermatogenesis in the Ground Skink. This increase in spermatogenic activity, seminiferous tubule diameter and epithelial height right before hibernation and a continuation of spermatogenesis in the following spring is commonly referred to as a Mixed-Type of reproductive/spermatogenic cycle [39].

Testicular histology of North American skinks has only been explored within two other studies involving: *Eumeces anthracinus pluvialis, E. fasciatus*, and *E. skiltonianus* [16,17]. Histology of the Ground Skink testis was similar to that described in these two studies. These three skinks had similar trends in sperm production as observed in *Scincella lateralis*. Spermatogenesis peaked in the late spring/early summer, quiescence occurred in late summer/early fall, and recrudescence began in late fall. It was noteworthy that

Fig. 3. Transverse views of seminiferous tubules under high magnification from Ground Skink specimens collected in: March (A), April (B), May (C), June (D), August (E), and October (F). Bars = 20 μm. Present cell types: SpA, type A Spermatogonia; SpB, type B Spermatogonia; PL, preleptotene 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.

there was a significant decrease in tubule diameter in May in the Ground Skink. The amount of mature spermatozoa in the lumen was decreased in May, due to a slight reduction in spermiation for our samples. However, the May testis had a robust number of spermatids within the seminiferous epithelium contributing to a relatively steady epithelial height across our samples.

Hypertrophic renal sexual segments (Rss) were reported by Sever and Hopkins [13,14] for the same population of Ground Skinks during the same months of testicular activity presented here. During spermatogenic inactivity in August, the Rss become indistinguishable from other nephridian tubules. The hypertrophic Rss are often indicators of breeding because they are implicated in seminal fluid production [31] and sperm sustenance [32]. Furthermore, oviductal sperm storage studies performed by Sever and Hopkins [33] on the Ground Skink revealed that sperm were not present in the oviducts of August and October female Ground Skinks, but were in the oviducts in the spring. Thus, female sperm storage data supports our histological findings on spermatogenesis and the spring-early summer mating seasons that most likely take place in the Ground Skinks of South Carolina.

In recent studies on testicular organization and germ cell development strategies in temperate squamates (*Podarcis muralis:* [1]; *Seminatrix pygaea*: [9]; *Agkistrodon piscivorus*: [2]), there were similar testicular structure, germ cell morphologies, and seasonal variations in germ cell development as reported here in the Ground Skink. This organization of germ cells differs greatly from that of birds and mammals [3,5,7,34–37] in which a spatial germ cell development strategy is employed during spermatogenesis. Squamate reptiles including Ground Skinks have a more temporal germ cell development, where germ cell generations move and complete the stages of spermatogenesis as a single cohort during the reproductively active months. In the case of the Ground Skink, spermiogenesis seemed to be much slower in its completion to mitosis or meiosis, resulting in 3 to 5 spermatids layered in the apexes of germinal epithelia in every month except August. The result of this temporal germ cell development strategy was a lack of synchrony between the phases (mitosis, meiosis, spermiogenesis) of spermatogenesis and the lack of consistent spatial cellular associations, as present in mammals and birds. This type of temporal germ cell development was more similar to the germ cell development strategy of anuran amphibians [4,38] and has been described in other reptilian orders (*Trachemys scripta*, Chelonia: [8]; *Alligator mississippiensis*, Crocodylia: [10]). However, the seminiferous tubules of the anurans are lined with seasonal cysts rather than a continuous epithelium like that of all amniotes. Thus, spermatogenesis in Ground Skinks provides more evidence supporting an ancestral/basal mode of germ cell development within a structurally derived amniotic testis compared to anamniotes.

Evidence is building within many species of reptiles that suggested this temporal germ cell development strategy may be a pleisomorphic character within the basal reptilian clade. Thus, the occurrence of a spatial germ cell development should be considered apomorphic in birds. This would require that we consider birds derived reptiles. The spatial germ cell development strategy found in birds and mammals independently arose in both groups and is an example of convergence. This hypothesis was supported by our data and it agrees with current views that birds and mammals are not linked by a single common ancestor [40,41]. In this argument, reptiles are considered paraphyletic and mammals are a sister taxon to reptiles and birds. We argue that the anamniotic-like germ cell development within a structurally derived amniotic testis in basal reptiles could be an intermediate step between the temporal strategy observed in anamniotes and the convergent spatial strategies observed in birds and mammals. This would require that the common ancestor to extant mammals and reptiles/birds also exhibited a similar strategy to that observed in existing reptiles. Our understanding of the evolution of germ cell development strategy and the process of spermatogenesis within vertebrates may become clearer by investigating the testes in other reptilian species and in the more basal Monotremes (platypus, echidnas), which are a little-studied mammalian taxon as far as development strategy during sperm development.

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