

Ultrastructure of Spermiogenesis in the American Alligator, *Alligator mississippiensis* (Reptilia, Crocodylia, Alligatoridae)

Kevin M. Gribbins,^{1*} Dustin S. Siegel,² Marla L. Anzalone,¹ Daniel P. Jackson,¹ Katherine J. Venable,¹ Justin L. Rheubert³ and Ruth M. Elsey⁴

¹Department of Biology, Wittenberg University, Springfield, Ohio 45501

²Department of Biology, Saint Louis University, St. Louis, Missouri 63103

³Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402

⁴Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, Grand Chenier, Louisiana 70643

ABSTRACT Testicular samples were collected to describe the ultrastructure of spermiogenesis in *Alligator mississippiensis* (American Alligator). Spermiogenesis commences with an acrosome vesicle forming from Golgi transport vesicles. An acrosome granule forms during vesicle contact with the nucleus, and remains posterior until mid to late elongation when it diffuses uniformly throughout the acrosomal lumen. The nucleus has uniform diffuse chromatin with small indices of heterochromatin, and the condensation of DNA is granular. The subacrosome space develops early, enlarges during elongation, and accumulates a thick layer of dark staining granules. Once the acrosome has completed its development, the nucleus of the early elongating spermatid becomes associated with the cell membrane flattening the acrosome vesicle on the apical surface of the nucleus, which aids in the migration of the acrosomal shoulders laterally. One endonuclear canal is present where the perforatorium resides. A prominent longitudinal manchette is associated with the nuclei of late elongating spermatids, and less numerous circular microtubules are observed close to the acrosome complex. The microtubule doublets of the midpiece axoneme are surrounded by a layer of dense staining granular material. The mitochondria of the midpiece about the proximal centriole resulting in a very short neck region, and possess tubular cristae internally and concentric layers of cristae superficially. A fibrous sheath surrounds only the axoneme of the principal piece. Characters not previously described during spermiogenesis in any other amniote are observed and include (1) an endoplasmic reticulum cap during early acrosome development, (2) a concentric ring of endoplasmic reticulum around the nucleus of early to middle elongating spermatids, (3) a band of endoplasmic reticulum around the acrosome complex of late developing elongate spermatids, and (4) midpiece mitochondria that have both tubular and concentric layers of cristae. *J. Morphol.* 000:000–000, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: spermatids; alligator; ultrastructure; spermiogenesis; acrosome

INTRODUCTION

Members of the Crocodylia are large, oviparous, and aquatic (fresh, brackish, or salt water) vertebrates distributed in subtropic and tropic zones

(Ferguson, 1985). Males copulate with females via insertion of a single penis into the female cloaca (Gadow, 1887), and sperm is stored in the female oviduct (Gist et al., 2008; Bagwill et al., 2009) until ovulation. Eggs are deposited in a hole or mound (Thorbjarnarson, 1996) and sex is determined by nest temperature in all species of Crocodylia investigated (Lang and Andrews, 1994). Based on morphological data, extant Crocodylia can be broken up into the Alligatoroidea (alligators and caiman), Crocodylinae (crocodiles), Tomistominae (false gavial), and Gavialinae (true gavials; Gatesy et al., 2004).

Mature sperm ultrastructure has been described in two Crocodylia members, *Caiman crocodylus* (spectacled caiman; Saita et al., 1987) and *Crocodylus johnsoni* (freshwater crocodile; Jamieson et al., 1997). Ultrastructural changes that occur during spermiogenesis within Crocodylia testes have been described for *C. crocodylus* (Saita et al., 1987) and *Alligator sinensis* (Chinese alligator; Wang et al., 2008). No additional ultrastructural investigations exist on the sperm structure or spermiogenic stages of other Crocodylia taxa; however, Gribbins et al. (2006) evaluated the cytology of spermatogenesis in *Alligator mississippiensis* (American alligator) in an effort to describe the germ cell developmental strategy of this species. Also, Moore et al. (2009) illustrates the development of the testes in post-hatchling juveniles.

Contract grant sponsor: Wittenberg Summer Undergraduate Research Grants.

*Correspondence to: Kevin M. Gribbins, Department of Biology, Wittenberg University, PO Box 720, Springfield, OH 45501-0720. E-mail: kgribbins@wittenberg.edu

Received 5 January 2010; Revised 4 March 2010; Accepted 6 March 2010

Published online in
Wiley Online Library (wileyonlinelibrary.com)
DOI: 10.1002/jmor.10872

Interestingly, unlike their fellow Archosaurs (birds), *A. mississippiensis* and *A. sinensis* have a germ cell development strategy more similar to other members of the paraphyletic Reptilia (Gribbins and Gist, 2003; Gribbins et al., 2003, 2005, 2008; Wang et al., 2008; Rheubert et al., 2009), and thus, *A. mississippiensis* has a pleisiomorphic-like (temporal) germ cell development strategy.

While sperm morphology is an invaluable tool for comparative and phylogenetic analyses (review: Jamieson, 1995), few studies have investigated the effects of aquatic environmental toxicants on sperm development in amniotic models. It is well established that baseline data on the ultrastructural changes during spermiogenesis are necessary to understand how potential reproductive toxins (e.g., pesticides; Russell et al., 1990) alter spermatogenesis in vertebrates. These types of studies may provide data on sperm abnormalities upon exposure to man-made toxins and may be integral in future species conservation. Considering the affinity of members of the Crocodylia to water, these reptiles may represent sentinel species for histopathological studies on how potential aquatic reproductive toxicants, such as pesticides, affect spermatogenic output in the testis of semi-aquatic reptiles.

Here we describe the ultrastructure of the spermiogenic stages in *Alligator mississippiensis*. We then compare these data with those published for *Caiman crocodylus* (Saita et al., 1987) and *Alligator sinensis* (Wang et al., 2008). We will also note any similarities or differences between alligator spermatids and the products of spermiogenesis within the testes of the Tuatara (*Sphenodon punctatus*; Healy and Jamieson, 1994), snakes (Gribbins et al., 2009), lizards (Clark, 1967; Dehlawi et al., 1992; Gribbins et al., 2007; Rheubert et al., in press), chelonians (Sprando and Russell, 1988; Healy and Jamieson, 1992; Al-Dokhi and Al-Wasel, 2001a,b, 2002; Zhang et al., 2007), and birds (Lin and Jones, 1993; Soley, 1997; Aire, 2007).

MATERIALS AND METHODS

Animal Collection

Five adult male American Alligators (*Alligator mississippiensis*) between 2.5 and 3 m were collected during May 2002 and May 2003 (peak month of spermiogenesis: Gribbins et al., 2006) from the Rockefeller Wildlife Refuge in Grand Chenier, LA. Alligators were captured by noose or by baited hooks that were placed in canals within the refuge. Upon capture, alligators were sacrificed and the testes were removed immediately by dissection. The testes were submerged in Trump's Fixative (EMS, Hatfield, PA) and minced thoroughly into small pieces. The testicular pieces were then placed in fresh Trump's fixative and stored under refrigeration for at least 48 h (4°C).

Tissue Preparation

Testicular tissues cut into 2-3 mm blocks were washed twice with cacodylate buffer (pH 7.0) for 20 min each. They were then

post-fixed in 2% osmium tetroxide for 2 h, washed with cacodylate buffer (pH 7.0) three times for 20 min each, dehydrated in a graded series of ethanol (70%, 85%, 95% X2, 100% X2), and cleared twice with 10 min treatments of propylene oxide. Each piece of alligator testis was then gradually introduced to epoxy resin (Embed 812, EMS, Hatfield, PA) (2:1 and 1:1 solutions of propylene oxide: epoxy resin). Testicular tissues were then placed in pure Embed 812 for 24 h. Fresh resin was prepared and the tissues were embedded in small beam capsules, and subsequently cured for 48 h at 70°C in a Fisher isothermperature vacuum oven (Fisher Scientific, Pittsburgh, PA). Sections (90 nm) were obtained by use of a diamond knife (DDK, Wilmington, DE) on an LKB automated ultramicrotome (LKB

Produkter AB, Bromma, Sweden). Sections were then placed on copper grids and stained with uranyl acetate (18 min) and lead citrate (5 min).

Ultrastructural Analysis

Testicular samples were viewed using a Jeol JEM-1200EX II transmission electron microscope (Jeol). Micrographs were taken of representative spermatids and structural components associated with spermiogenesis via a Gatan 785 Erlangshen digital camera (Gatan, Warrendale, PA). The micrographs were then analyzed and composite plates were assembled using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

RESULTS

Spermatids develop within the apical portion of the seminiferous epithelium (Fig. 1) of the seminiferous tubules of *Alligator mississippiensis* as described previously by Gribbins et al. (2006). Once spermiogenesis is completed mature spermatozoa are shed into the lumina of the seminiferous tubules for transport to the excurrent duct system. The beginning of spermiogenesis is marked by the accumulation of round spermatids within the seminiferous epithelium of *A. mississippiensis* upon the completion of meiosis. During these early stages, an acrosome vesicle forms (Fig. 2A,B, Av), and a juxtapositioned Golgi apparatus (Fig. 2C, black arrowhead) dominates the spermatid cytoplasm near the apex of the nucleus. Budding from the most proximal cisternum of the Golgi are transport vesicles (Fig. 2C, white arrowhead) that merge with the developing acrosome and are responsible for its increase in size during the round spermatid stage. The acrosome vesicle is not in contact with the nuclear membrane during the vesicle's early development (Fig. 2A,) and an acrosome granule is not seen within the vesicle until contact has been made with the nucleus (Fig. 2B, white arrowhead). The cytoplasm at this time is packed with mitochondria (Fig. 2A,B, white arrows) and abundant endoplasmic reticula (Fig. 2A, SR). As the acrosome grows in size, it collapses and flattens the apical surface of the round spermatid nucleus at the climax of round spermatid development (Fig. 2D, Av) and at this point the subacrosome space can be observed (Fig. 2D, black arrowhead). The most unusual feature of the early acrosome phase of development in the *A. mississippiensis* is the long thin smooth endoplasmic reticulum that cov-

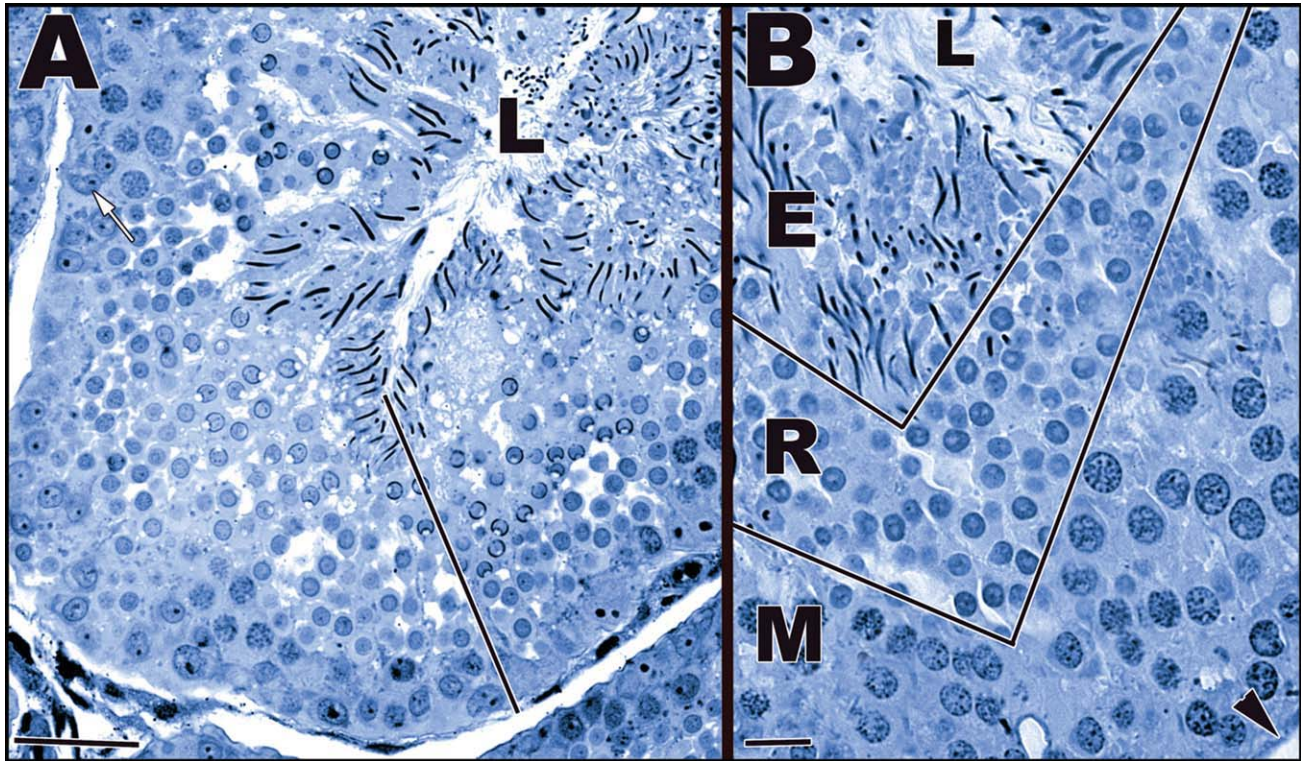


Fig. 1. Light microscope views of an April seminiferous tubule in the American Alligator testis. (A) Low power view of the seminiferous tubule in transverse section. The tubule has a lumen (L) where sperm will be released after the completion of spermiogenesis. The germinal epithelium is thick (black line) and contains developing germ cells and Sertoli cells (white arrow). Bar = 50 μ m. (B) High power view of the germinal epithelium showing generations of developing germ cells. The spermatogonia and spermatocytes (M) are located near the basement membrane (black arrowhead) at the periphery of the seminiferous tubule. The round spermatids (R), which are situated between the meiotic/mitotic cells and the elongating spermatids. The elongating spermatids (E) are located near the apex of the germinal epithelium in close proximity to the lumen (L). Bar = 20 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ers the rostral acrosome, which we term the endoplasmic reticular cap (Fig. 2A–C, black arrows).

Towards the termination of the round spermatid stage, the most distal part of the nucleus starts to elongate (Fig. 2D). As the spermatid nucleus stretches, it takes on a cylindrical appearance (Fig. 3A, Nu). The same organelles, mitochondria (Fig. 3A, black arrow) and smooth endoplasmic reticulum (Fig. 3A, white arrowheads; 3B, black arrows), are observed within the cytoplasm of elongating spermatids as are seen within the round spermatid stage. The acrosome begins to envelop and migrate laterally along the apex of the nucleus (Fig. 3A–C). The acrosome is sandwiched between the cell membrane (Fig. 3B, black arrowhead; 3C, white arrow) and the nuclear apex. The acrosome granule is large and basally located (Fig. 3B, white arrow; 3C, black arrowhead), and it appears that dense material from the granule is diffusing out into the acrosome vesicle lumen. There is a prominent and dark staining subacrosome space between the inner acrosome membrane and the apex of the nucleus (Fig. 3C, black arrow). During

the elongation phase of spermiogenesis the nucleus contains mostly diffuse euchromatin with only a few pockets of dense staining heterochromatin (Fig. 3B, white arrowhead) randomly located within the nucleoplasm. A transverse view through the early elongate spermatid nucleus reveals that the smooth endoplasmic reticulum is organized in a concentric layer located adjacent to the nuclear membrane (Fig. 3D, white arrows).

As elongation continues, the acrosome vesicle begins to envelop the nucleus by moving caudally along its lateral edges, and the acrosome granule has completely diffused throughout the acrosome lumen (Fig. 4A). This results in flocculent material within the lumen of the acrosome vesicle (Fig. 4A, *). Chromatin condensation has commenced and the chromatin begins to bead in a granular style (Fig. 4A, Nu). As the acrosome shoulders (Fig. 4A white arrow; 4B, *) migrate laterally they appear to squeeze down on the apex of the nucleus resulting in a thin papilla shaped apical head that will continue to thin into the mature rostrum of the nucleus (Fig. 4B). Within the developing rostrum

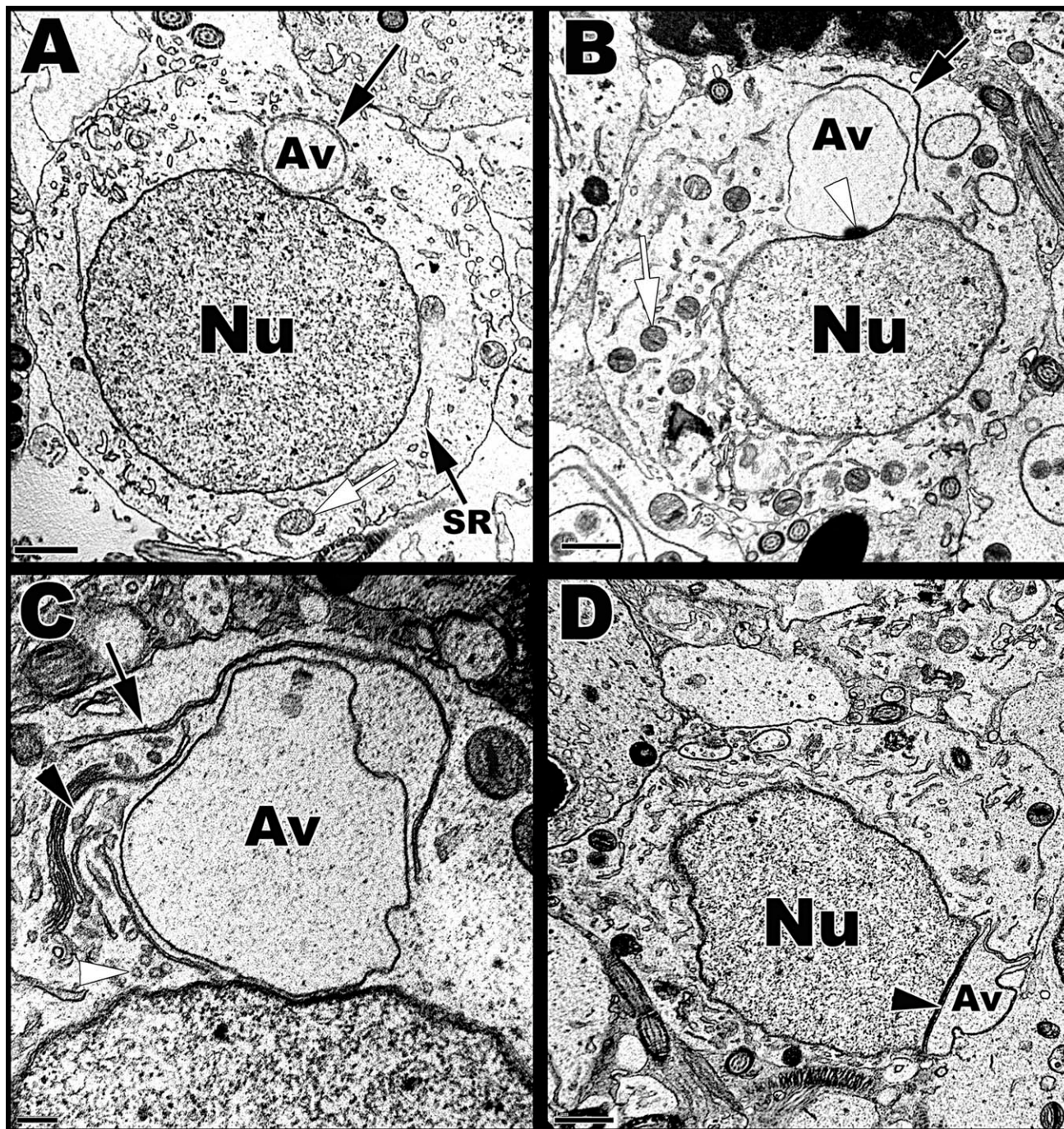


Fig. 2. Round spermatids undergoing acrosome development during the early stages of spermiogenesis within the testes of *Alligator mississippiensis*. Common features of the round spermatids include smooth endoplasmic reticulum (SR) and mitochondria (white arrow) in the cytoplasm, and a thin band of smooth endoplasmic reticulum (black arrow) lying apical to the acrosomal vesicle (Av). (A) The acrosomal vesicle lies apical to the nucleus (Nu), but is not yet in contact with the nucleus. Bar = 1 μ m. (B) The acrosomal vesicle is in contact with the nucleus and the acrosomal granule (white arrowhead) has now formed. Bar = 1 μ m. (C) Transport vesicles (black arrowhead) of the Golgi apparatus (black arrow) merging with the acrosomal vesicle. Bar = 0.5 μ m. (D) The acrosome vesicle has collapsed and flattened the apical surface of the nucleus (Nu), and the subacrosomal space (black arrowhead) can now be observed. Bar = 1 μ m.

an endonuclear canal starts to form and will house the perforatorium in more mature spermatids (Fig. 4A, white arrowhead; inset, white arrowhead; 4B,

black arrow). The subacrosome space becomes more prominent and is filled with a condensed dark staining granular layer (Fig. 4B, white

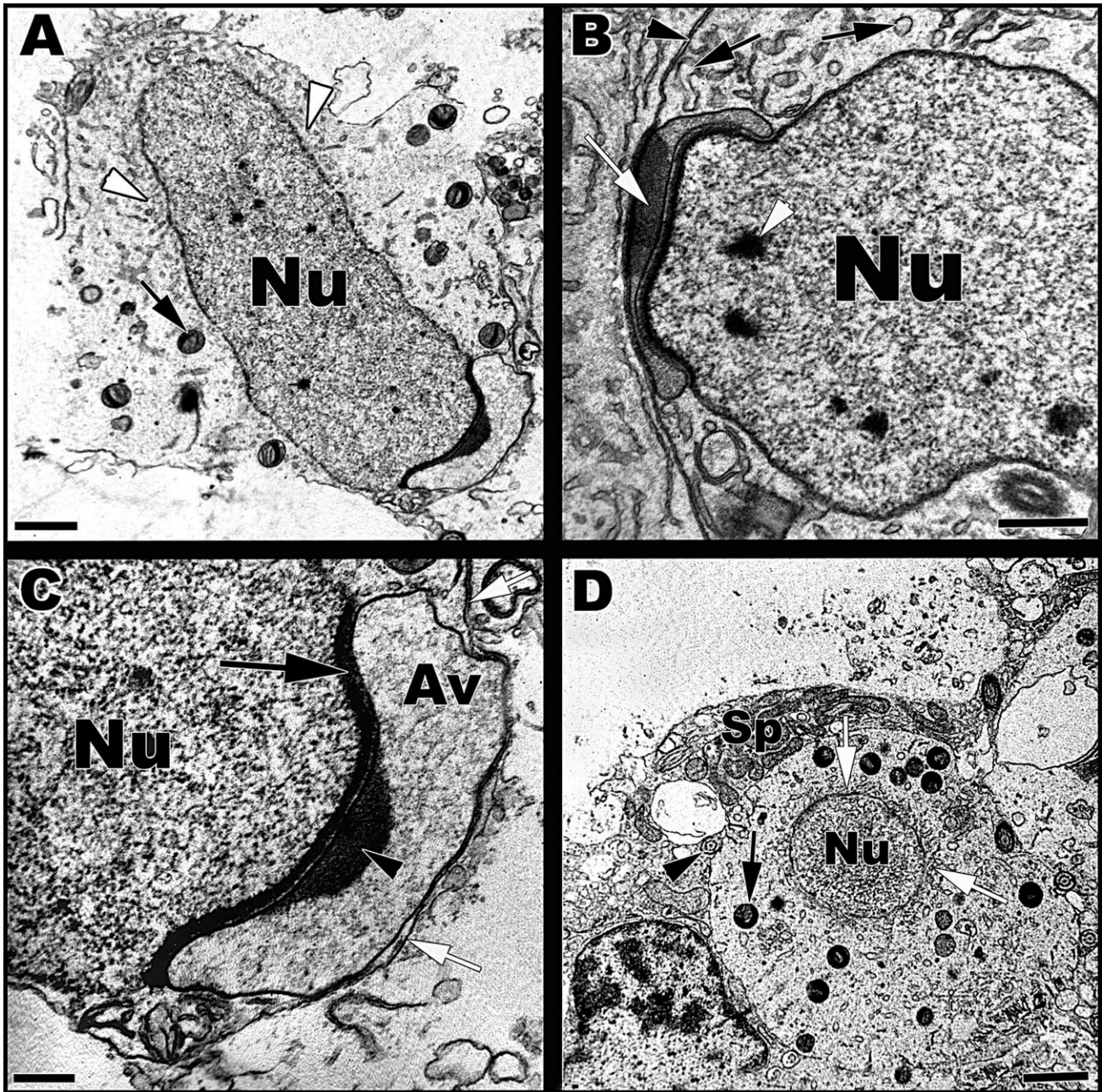


Fig. 3. Germ cells immediately after the round spermatid stage when spermatid elongation begins within the testes of *Alligator mississippiensis*. (A) Nucleus (Nu) elongating and capped by the acrosomal vesicle stretching posteriorly over the nucleus. Mitochondria (black arrow) and smooth endoplasmic reticulum (white arrowheads) are common in the cytoplasm. Bar = 1 μ m. (B) High magnification of the acrosomal region of the elongating spermatid demonstrating the dense material of the acrosomal granule (white arrow) diffusing within the lumen of the acrosomal vesicle. At this stage the acrosomae is sandwiched between the cell membrane (black arrow) and nuclear apex, and cisternae of smooth endoplasmic reticulum are present cytoplasmically (black arrows). During elongation the nucleus (Nu) is euchromatic with pockets of dense staining chromatin (white arrowhead). Bar = 1 μ m. (C) Apex of the nucleus (Nu) highlighting the orientation of the nucleus, cell membrane (white arrow), acrosomal vesicle (Av), and acrosomal granule (black arrowhead) in an early elongating spermatid. Subacrosome space (black arrow). Bar = 0.5 μ m. (D) Transverse section through an early elongating spermatid nucleus (Nu) showing the concentric layers of smooth endoplasmic reticulum (white arrow) organized adjacent to the nuclear membrane. The axoneme (black arrowhead) can also be observed abutting the cell membrane, and mitochondria (black arrow) are scattered throughout the early elongating spermatid cytoplasm. Sertoli cell process (Sp). Bar = 1 μ m.

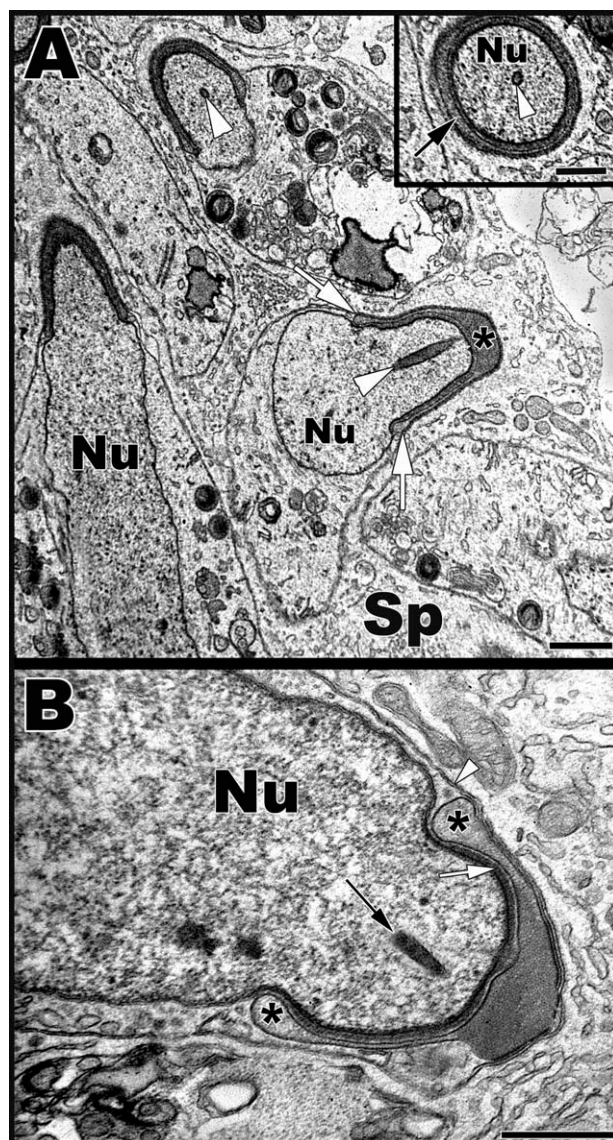


Fig. 4. Spermatids in the middle stages of elongation within the testes of *Alligator mississippiensis*. (A) The acrosomal vesicle moves caudally and envelops the nucleus (Nu) along its lateral edges, and the acrosome shoulders (white arrows) appear to squeeze the apex of the nucleus. The acrosomal granule has completely diffused throughout the acrosome lumen creating a flocculent material (*) within the acrosome vesicle. An endonuclear canal (white arrowheads) forms within the developing rostrum of the nucleus (inset is a spermatid in transverse section showing the endonuclear canal [white arrowhead] and the acrosomal space filled with flocculent material [black arrow]). Bar = 1 μ m. (B) High magnification of the nuclear apex highlighting the orientation of the nucleus (Nu), cell membrane (white arrowhead), subacrosomal space (white arrow), and acrosome shoulders (*) in sagittal section of a spermatid during middle elongation. Bar = 1 μ m.

arrow). At the peak of elongation, the acrosome vesicle envelops the entire nuclear apex and the developing rostrum is triangular in shape (Fig. 5, black arrows). The nucleus has reached its final length of over 25 μ m (Fig. 5, black brackets). The

endonuclear canal and the enclosed developing perforatorium have increased in length and span the entire immature rostrum, and they extend all the way to the tip of the nucleus (Fig. 5, white arrowheads).

During late elongation the chromatin becomes more condensed and large chromatin granules are closer together and little to no nucleoplasm is observed (Fig. 6A, NU). The microtubules of the manchette (Fig. 6B, black arrows and D, white arrows) become evident on the lateral aspects of the nucleus as the spermatids continue to thin.

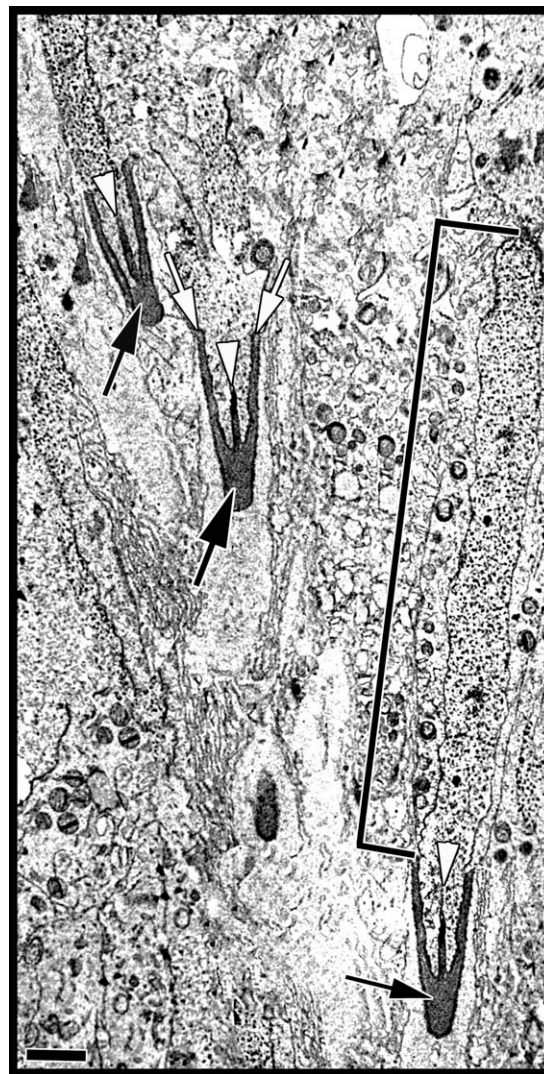


Fig. 5. Highly elongated spermatids during the climax of elongation within the testes of *Alligator mississippiensis*. The rostrum of the spermatid (black arrows) is triangular in shape and the acrosome shoulders (white arrows) have reached their most posterior position. The entire nucleus (bracket) is over 25 μ m in length, and the endonuclear canal and perforatorium (white arrowheads) span the entire immature rostrum and extend to the tip of the nucleus. Bar = 2 μ m.

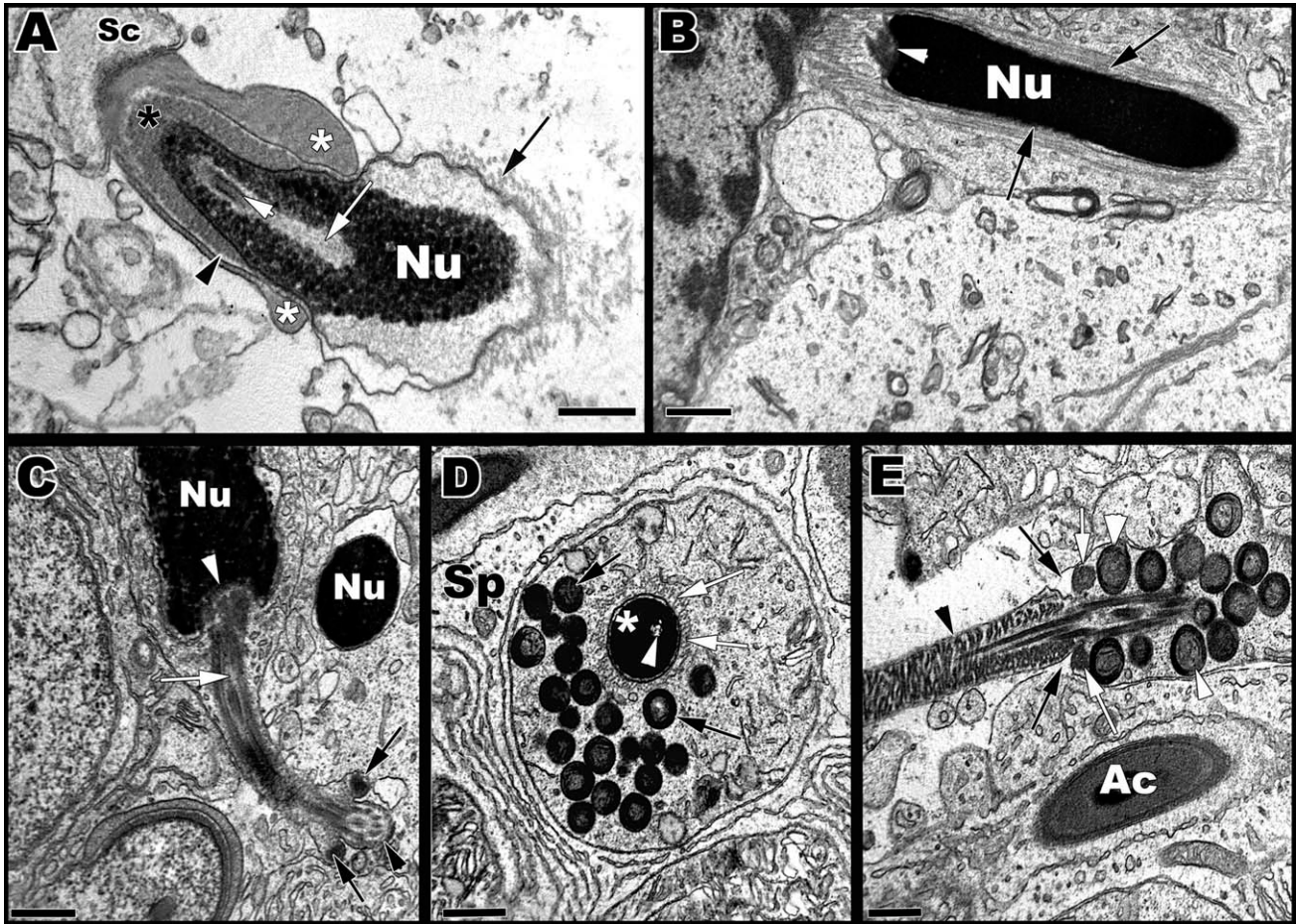


Fig. 6. Spermatids in late elongation/condensation within the testes of *Alligator mississippiensis*. (A) The chromatin becomes more condensed in the nucleus (Nu) and forms large chromatin granules. Circular microtubules (black arrow) are located around the nucleus just caudal to the acrosome shoulders (white *). The endonuclear canal (white arrow) becomes enlarged and the microtubules of the perforatorium (white arrowhead) can be seen within the canal. The subacrosome space (black *) becomes enlarged and is filled with dense granular material. There is a thin clear lucent zone between the granular layer and the inner acrosome membrane (black arrowhead). Bar = 1 μ m. (B) Parallel microtubules of the manchette (black arrows) surrounding the entire length of the nucleus (Nu), and the proximal centriole (white arrowhead) lying in the nuclear fossa. Bar = 1 μ m. (C) The distal centriole (white arrow), posterior to the proximal centriole (white arrowhead), elongating into the neck axoneme (black arrowhead), while the annular ring (black arrows) migrates away from the nucleus. Bar = 1 μ m. (D) Transverse section through a spermatid in late elongation highlighting the orientation of the nucleus (white *), endonuclear lacuna (white arrowhead), manchette (white arrows), mitochondria (black arrows), and Sertoli cell process (Sp). Bar = 1 μ m. (E) Mitochondria (white arrowheads) are arranged in a single row between the nucleus and annulus (white arrows). The midpiece of the flagellum is surrounded by Sertoli cell processes forming the flagellar tunnel (black arrows) as the flagellum continues posterior to the annulus (principal piece) and is surrounded by the fibrous sheath (black arrowhead). Bar = 1 μ m.

Most of the manchette is made up of parallel microtubules (Fig. 6B, black arrows) that can be found surrounding the length of the nucleus beginning just caudally to the shoulders (Fig. 6A, white *) of the acrosomal vesicle. There are also circular microtubules (though they are less numerous and organized) either observed earlier in elongation or more frequently along the region of the nucleus just under the acrosome shoulders (Fig. 6A, black arrow). The endonuclear canal (Fig. 6A, white arrow) has enlarged and the dark staining perforatorium (Fig. 6A, white arrowhead) can be seen within the canal. The nucleus is reduced into a

thinner rostrum apically, which extends up into the acrosome complex (Fig. 6A). The subacrosome space is also enlarged and a dense staining granular layer occupies this space (Fig. 6A, *). However, there is a thin lucent layer within the subacrosomal space just under the inner acrosome membrane that lacks dark staining granules (Fig. 6A, black arrowhead). The proximal centriole can be seen within the nuclear fossa caudally (Fig. 6B,C, white arrowheads). As the distal centriole (Fig. 6C, white arrow) elongates to form the neck axoneme (Fig. 6C, black arrowhead), the attached annular ring (Fig. 6C, black arrows) migrates away from

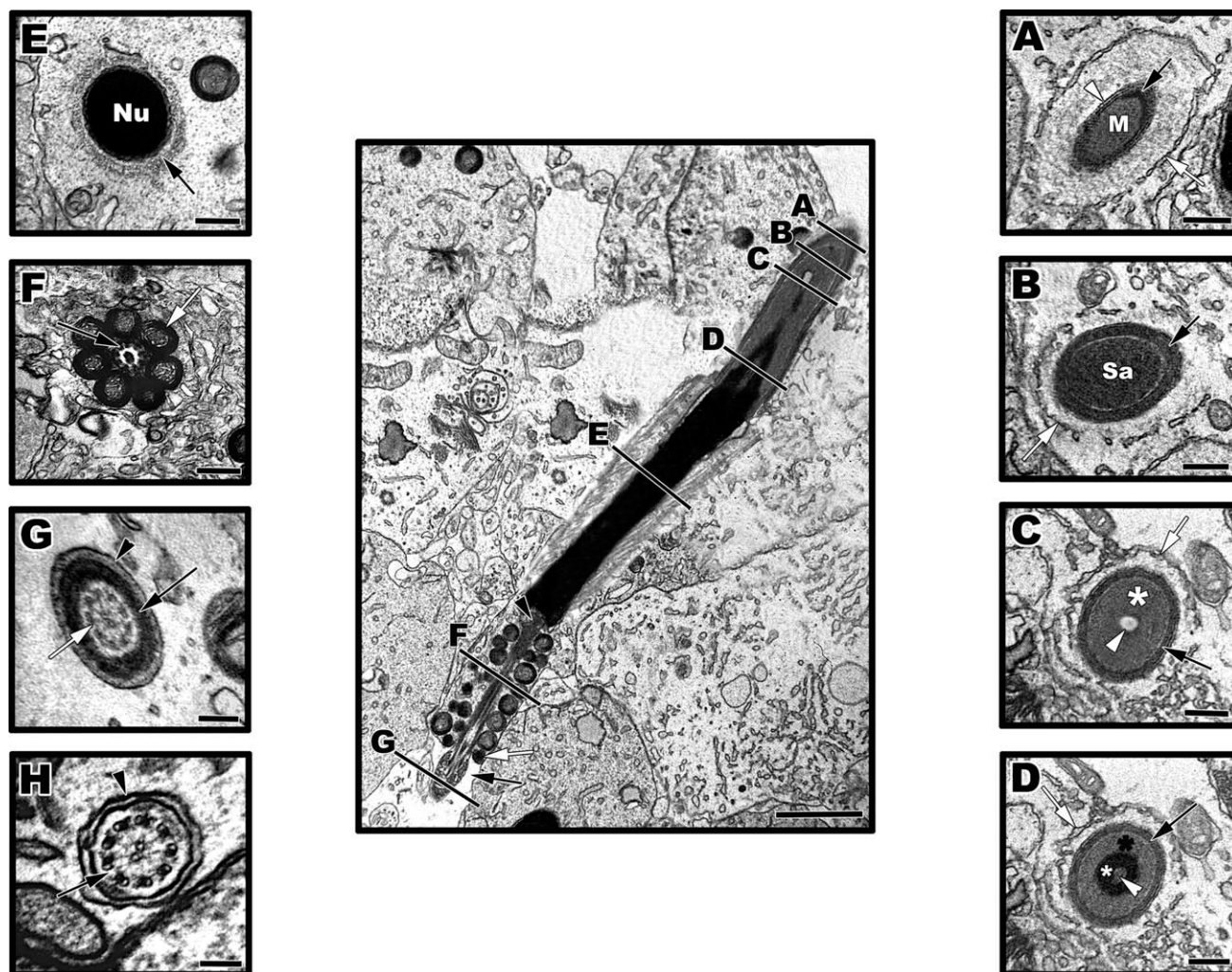


Fig. 7. Spermatids in the final stage of spermiogenesis within the testes of *Alligator mississippiensis*. (A)–(G) on the medial sagittal section represent the approximate location where the peripheral transverse sections were taken from. Sagittal section. Proximal centriole (black arrow). Bar = 1 μ m. (A) The tip of the acrosome demonstrating the dark staining cortex (black arrow) and lighter staining medulla (M) surrounded by the multilaminar layers of the Sertoli cell membrane (white arrowhead) and smooth endoplasmic reticulum [white arrow; labeled identically in (B)–(D)]. (B) The acrosome (black arrow) rests upon a dense subacrosome space (Sa). (C) An epinuclear lucent zone (white arrowhead) rests on the tip of the nuclear rostrum within the subacrosome space (white arrowhead), lying medial to the acrosome (black arrow). (D) Medial to the acrosome (black arrow) and subacrosome space (*) lays the rostrum (white *), in which microtubules of the perforatorium reside within the endonuclear canal (white arrowhead). (E) The body of the nucleus (Nu) is surrounded by parallel microtubules of the manchette (black arrow). (F) Large mitochondria (white arrow), with tubular cristae surrounded by concentric layers of cristae, surround the axoneme and dark staining columns (black arrow). (G) A fibrous sheath (black arrow) internal to the cell membrane (black arrowhead) surrounds the axoneme (white arrow) in the principal piece of the spermatid. (H) The endpiece axoneme (black arrow) lacks a fibrous sheath and is encompassed only by the cell membrane (black arrowhead). Bars = 0.2 μ m.

the nucleus. Mitochondria also migrate (Fig. 6D, black arrow) toward the developing flagellum caudally and move in the direction of the annulus (Fig. 6E, white arrows) and away from the nucleus, and arrange themselves in a single row (Fig. 6E, white arrowhead) in sagittal section. The Sertoli cell processes enveloping late elongate spermatids extend caudally to the midpiece of the flagellum forming the flagellar tunnel (Fig. 6E, black arrows). The flagellum continues to elongate cau-

dally past the annulus and becomes surrounded by a surplus of fibrous blocks (Fig. 6E, black arrowhead) creating a fibrous sheath around the principal piece of the flagellum. The axoneme of the midpiece, principal piece, and endpiece is made up of the typical 9 + 2 arrangement of microtubule doublets.

The final stage of spermiogenesis demonstrates many of the presumed mature structures that will be present within the American Alligator sperma-

tozoa. This final stage represents the climax of spermiogenesis and upon completion of development the spermatids will be transferred to the lumina of the seminiferous tubules during spermiation. The acrosomal tip/body is slightly oblong in shape (Fig. 7A) and sits on the subacrosome space (Fig. 7B, Sa), and a thin nuclear rostrum extends into the subacrosome space (Fig. 7D, white *). The main body of the acrosome has a lighter staining medulla (Fig. 7A,M) and a dark staining thin cortex (Fig. 7A, black arrow). Multilaminar layers of Sertoli cell membrane (Fig. 7A, white arrowhead) are common around the acrosome complex of these late developing spermatids. In these late developing spermatids, a ring of smooth ER is common around the acrosome complex (Fig. 7B–D, white arrow). A small epinuclear lucent zone rests on the tip of the nuclear rostrum (Fig. 7C, white arrowhead) within the subacrosome space (Fig. 7C, white asterisk). Within the nuclear rostrum (Fig. 7D, white asterisk) of a mature spermatid, a distinct endonuclear canal (Fig. 7D, white arrowhead) exists and has the enclosed perforatorium. The body of the nucleus is cylindrical and is surrounded by the parallel microtubules of the manchette (Fig. 7E, black arrow). The midpiece has large mitochondria that have tubular cristae internally and concentric layers of membranes externally (Fig. 7F, white arrow) surrounding the axoneme (Fig. 7F, black arrow). The midpiece axoneme lacks a fibrous sheath; however, the microtubule doublets of the midpiece are surrounded by dark staining granular columns (Fig. 7F, black arrow). The principal piece beyond the annular ring does have a fibrous sheath that is seen in cross section (Fig. 7G, black arrow), and the endpiece is easily distinguished from the principal piece, as it lacks a fibrous sheath around its axoneme (Fig. 7H, black arrow).

DISCUSSION

Most of the ultrastructure features of spermiogenesis within *Alligator mississippiensis* are similar to those described for other reptilian sauropsids. The early development of the acrosome complex within the *A. mississippiensis* testes is akin to what has been described for other amniote taxa. The single acrosome vesicle forms from transport vesicles delivered from the Golgi apparatus. This is comparable to what has been described for *Sphenodon* (Healy and Jamieson, 1994), squamates (Gribbins et al., 2007, 2009; Rheubert et al., in press), and other archosaurs including birds (Aire, 2007) and crocodilians (Saita et al., 1987; Wang et al., 2008). However, at least one species of agamid lizard was reported to have two acrosome vesicles and granules (Dehlawi et al., 1992), and within *Sphenodon* there are intra-acrosomal vesicles that deliver granules to the exterior of the outer acrosome mem-

brane apically (Healy and Jamieson, 1994). We identified a single unique feature in early acrosome development in *A. mississippiensis*: a thin layer of endoplasmic reticulum forms a cap on top of the developing acrosome vesicle.

A single large acrosome granule forms and remains in contact with the inner acrosome membrane throughout sperm development until mid to late elongation when it diffuses uniformly throughout the acrosome lumen. The nucleus of the American alligator has uniform diffuse chromatin with small indices of heterochromatin, which is much different from the intermediate to heavily heterochromatic nuclei of *Sphenodon* (Healy and Jamieson, 1994) and chelonians (Sprando and Russell, 1988; Healy and Jamieson, 1992; Zhang et al., 2007). Conversely, like other crocodilians (Saita et al., 1987; Wang et al., 2008), the condensation of DNA in *Alligator mississippiensis* packs into progressively larger granules until the nucleus contains only homogeneous dark staining DNA, resembling *Sphenodon* (Healy and Jamieson, 1994) and chelonians (Sprando and Russell, 1988; Healy and Jamieson, 1992; Al-Dokhi and Al-Wasel, 2001b; Zhang et al., 2007). In squamates, such as *Agkistrodon piscivorus* (Gribbins et al., 2009), *Anolis carolinensis* (Rheubert et al., in press), and *Scincella laterale* (Gribbins et al., 2007), the DNA condenses in a filamentous helical fashion. In *A. mississippiensis*, early to mid elongating spermatids have an adjacent layer of endoplasmic reticulum that surrounds the nucleus, which may be an autoapomorphic feature of spermiogenesis in *A. mississippiensis* as this has not been reported in any other taxa, including previously crocodilians (Saita et al., 1987; Wang et al., 2008). The subacrosome space develops early in the round spermatid stage in *A. mississippiensis* and continues to enlarge during elongation, and this space accumulates a thick layer of dark staining granules similar to that described in other reptilian sauropsids (Sprando and Russell, 1988; Aire, 2007; Gribbins et al., 2007, 2009). Also like other reptilian sauropsids (Clark, 1967; Sprando and Russell, 1988; Aire, 2007; Gribbins et al., 2007, 2009), once the acrosome has completed its development and growth, the nuclei of the early elongating spermatids become associated with the cell membrane. This contact with the cell membrane flattens the acrosome vesicle on the surface of the anterior nucleus, which aids in the migration of the acrosomal shoulders laterally over the apical nuclear head.

In *Sphenodon* (Healy and Jamieson, 1994), chelonians (Sprando and Russell, 1988; Healy and Jamieson, 1992; Al-Dokhi and Al-Wasel, 2001a,b, 2002; Zhang et al., 2007), non-passarine birds (Aire, 2007) and other crocodilians (Saita et al., 1987; Wang et al., 2008), at least one endonuclear canal is present that is rod shaped and houses the perforatorium. Within crocodilians and other arch-

osaurs (this study; Saita et al., 1987; Aire 2007; Wang et al., 2008), these endonuclear canals are restricted to the rostral region of the nucleus, whereas in *Sphenodon* (Healy and Jamieson, 1994) and chelonians (Sprando and Russell, 1988; Healy and Jamieson, 1992; Al-Dokhi and Al-Wasel, 2001a; Zhang et al., 2007) the rods extend past the acrosomal region deep into the nuclear body in middle elongating spermatids. There has been some controversy on whether a perforatorium exists within the endonuclear canals of some reptilian sauropsids (Saita et al., 1987), however, in *A. mississippiensis*, a clearly visible perforatorium (dark staining rods) develops within the endonuclear canal, much like that observed in the *Caiman* (Saita et al., 1987). In contrast, all squamates studied to date have an extranuclear perforatorium (with no endonuclear canals) located in the subacrosome space within their spermatids and spermatozoa (Jamieson, 1995; Gribbins et al., 2007, 2009; Rheubert et al., in press).

In *Alligator mississippiensis*, we observed a prominent longitudinal manchette associated with the nuclei of late elongating spermatids. Also present, but less numerous, were circular microtubules associated with the nucleus close to the acrosome complex. The manchette has been implicated in the elongation process of the nucleus during spermiogenesis (Russell et al., 1990) and is considered a common structure observed during spermiogenesis in reptilian sauropsids (Ferreira and Dolder 2002). Many archosaurs have both circular and longitudinal components to their manchettes (Saita et al., 1987; Lin and Jones, 1993; Soley, 1997; Aire, 2007) as do many squamates (Da Cruz-Landim and Da Cruz-Hofling, 1977; Butler and Gabri, 1984; Dehlawi et al., 1992). However, whether both components are needed for elongation is not known and is complicated by the fact that some squamates have only the longitudinal microtubules (Gribbins et al. 2007, 2009), and at least one species of anole (Rheubert et al., in press) lacks a manchette altogether and elongation still occurs normally. There is only a single type of microtubule that makes up the circular and longitudinal manchette in *A. mississippiensis*, which is different from the *Caiman* (Saita et al., 1987) where the microtubules of the longitudinal manchette are thicker walled than those found in the circular manchette.

During the late stages of elongation in some chelonians and some squamates, there are lucent zones within the distal bodies of the elongate spermatid nuclei. These clear zones have been called nuclear lacunae or intranuclear tubules (Jamieson et al., 1996; Ibarguengoytia and Cussac, 1999). It is not unusual to see these same types of lacunae within the nucleus of *Alligator mississippiensis*, and in many non-passerine birds (Jamieson and Tripepi, 2005). The functions of these spaces are still unknown within these developing spermatids.

There is also an epinuclear lucent zone present within the *A. mississippiensis* acrosome complex as seen in many other reptilian taxa (Jamieson, 1995; Ferreria and Dolder, 2002; Gribbins et al., 2007, 2009). The *A. mississippiensis* flagellum develops similarly to that described in other reptiles to date. The proximal centriole rests within the nuclear fossa of *A. mississippiensis* spermatids. The axoneme develops in continuity with the distal centriole. There is no prominent neck region and no peritubular dense material around this region in *A. mississippiensis*. The mitochondria of the midpiece about the proximal centriole resulting in a very short neck region, which is congruent to what is observed within the *Caiman* (Saita et al., 1987) and chelonians (Sprando and Russell, 1988; Healy and Jamieson, 1992; Al-Dokhi and Al-Wasel, 2002). The microtubular doublets of the midpiece axoneme are surrounded by a layer of dense staining granular material that is similar to that observed within the midpiece axoneme of *Caiman* spermatids (Saita et al., 1987). These dense staining materials around the microtubules of the midpiece are equivalent to the striated columns found within mammalian spermatozoa (Lindemann, 1996) and most likely aid to reinforce the midpiece axoneme. The mitochondria of the midpiece, at least in the late elongating spermatids of *A. mississippiensis*, have tubular cristae internally and concentric layers of cristae superficially. This is slightly different from what is seen in the *Caiman*, which has concentric rings of cristae superficially and an electron-dense center that lacks tubular cristae. There are typically 7-9 concentric rows of mitochondria in a sagittal section of the *A. mississippiensis* midpiece and 7-8 concentric mitochondria within each row in cross section. These numbers seem to be consistent with what has been observed in other crocodilians (Saita et al., 1987). The concentric layers of cristae are also observed within chelonians (Hess et al., 1991; Healy and Jamieson, 1992) and *Sphenodon* (Healy and Jamieson, 1994). Since sperm storage is common in the female tract of turtles (Gist and Jones, 1989; Gist and Fischer, 1993; Gist and Congdon, 1998), and has recently been described in *A. mississippiensis* (Gist et al., 2008; Bagwill et al., 2009), it is possible that these concentric rings of cell membrane may provide energy reserves that aid the sperm's ability to survive long and short periods of time within the female reproductive tract before fertilization. The midpiece terminates at the annulus within *A. mississippiensis* spermatids as that described for most reptilian sauropsids. Also, like *Sphenodon*, chelonians, and other archosaurs the fibrous sheath of the principal piece does not penetrate into the midpiece, penetration being a synapomorphy for the squamates (Jamieson, 1995). The endpiece in American alligators is easily distinguished from the principal

piece, as the axoneme is not surrounded by the fibrous sheath.

Although the characteristics of spermiogenesis within *Alligator mississippiensis* are similar to what has been described in other amniotes, there are four ultrastructural features of the *A. mississippiensis* spermatids that seem to be unique and possible autoapomorphies for this species: (1) the endoplasmic reticulum cap of early acrosome development; (2) the concentric ring of endoplasmic reticulum around the nucleus of early to middle elongating spermatids; (3) the band of endoplasmic reticulum around the acrosome complex of late developing elongate spermatids; (4) composite mitochondria of the midpiece that have a tubular cristae core and concentric layers of cortical cristae superficially. *Alligator mississippiensis* spermatids also share two major features with *Sphenodon*, chelonians, and other archosaurs: (1) endonuclear canals; (2) the lack of the fibrous sheath within the midpiece. These characteristics have not been reported for Squamata. The importance of such differences and similarities in structure of the reptilian spermatids between closely and distantly related species is unknown as there are not sufficient representative data from squamates, archosaurs, and turtles to perform robust phylogenetic analyses using morphological characters from spermiogenesis. It is noteworthy to mention as do Saita et al. (1987) that the crocodilian spermatids, like that of the *Caiman*, seem to share far more ultrastructural features with those of chelonians and *Sphenodon* than they do with Squamata. Furthermore, Jamieson (2007) has shown that the sperm of paleognath birds are so similar to those of crocodilians as to be termed "crocodiloid." However, most of the similarities between the sperm of chelonians and those of *Sphenodon* and crocodilians have been shown in a cladistic analysis to be symplesiomorphies of these basal amniote groups (Jamieson and Healy, 1992). Until further morphological data sets are produced from spermiogenesis and spermatozoal ultrastructure within all the major groups of reptiles and birds answers to these questions will remain tentative. The ultrastructural study on *A. mississippiensis* spermiogenesis presented here provides baseline data that should be utilized in future evolutionary and histopathological investigations.

ACKNOWLEDGMENTS

The authors thank the hard working employees (Phillip "Scooter" L. Trosclair, III and Dwayne LeJeune) at the Rockefeller Wildlife Refuge who helped to collect and dissect the alligators used in this study. They also acknowledge Wittenberg, Saint Louis, and Southeastern Louisiana Universities for continual support of this research.

LITERATURE CITED

- Al-Dokhi O, Al-Wasel S. 2001a. Ultrastructure of spermiogenesis in the freshwater turtle *Maurymes caspica* (Chelonia, Reptilia). I. The acrosomal vesicle and the endonuclear canals formation. *J Egypt Ger Soc Zool* 36(B):93–106.
- Al-Dokhi O, Al-Wasel S. 2001b. Ultrastructure of spermiogenesis in the freshwater turtle *Maurymes caspica* (Chelonia, Reptilia). II. The nucleus elongation and chromatin condensation. *J Union Arab Biol Zool* 15(A):355–366.
- Al-Dokhi O, Al-Wasel S. 2002. Ultrastructure of spermiogenesis in the freshwater turtle *Maurymes caspica* (Chelonia, Reptilia). III. Sperm tail formation. *J Union Arab Biol Zool* 18(A):327–341.
- Aire TA. 2007. Spermatogenesis and testicular cycles. In: Jamieson BGM, editor. *Reproductive Biology and Phylogeny of Birds*. New Hampshire: Science Publishers. pp 279–347.
- Bagwill A, Sever DM, Else RM. 2009. Seasonal variation of the oviduct of the American alligator. *Alligator mississippiensis* (Reptilia: Crocodylia). *J Morphol* 270:702–713.
- Butler RD, Gabri MS. 1984. Structure and development of the sperm head in the lizard *Podarcis* (*Lacerta*) *taurica*. *J Ultrastruct Res* 88:261–274.
- Clark AQ. 1967. Some aspects of spermiogenesis in a lizard. *Amer J Anat* 121:369–400.
- Da Cruz-Landim C, Da Cruz-Hofling MA. 1977. Electron microscope study of lizard spermiogenesis in *Tropidurus torquatus* (*Lacertilia*). *Caryologia* 30:151–162.
- Dehlawi GY, Ismail MF, Hamdi SA, Jamjoom MB. 1992. Ultrastructure of spermiogenesis of a Saudian reptile. The sperm head differentiation in *Agama adramitana*. *Arch Androl* 28:223–234.
- Ferguson MWJ. 1985. The reproductive biology and embryology of the crocodilians. In: Gans C, Billet FS, Maderson PFS, editors. *Biology of the Reptilia*, Vol. 14: Development A. New York: John Wiley. pp 329–491.
- Ferreira A, Dolder H. 2002. Ultrastructural analysis of spermiogenesis of *Iguana iguana* (Reptilia: Sauria: Iguanidae). *Euro J Morphol* 40:89–99.
- Gadow H. 1887. Remarks on the cloaca and on the copulatory organs of the Amniota. *Phil Trans R Soc Lond B* 178:5–37.
- Gatesy J, Baker RH, Hayashi C. 2004. Inconsistencies in arguments for the supertree approach: Supermatrices versus supertrees of Crocodylia. *Syst Biol* 53:342–355.
- Gist DH, Bagwill AL, Lance VA, Sever DM, Else RM. 2008. Sperm storage in the oviduct of the American alligator. *J Exp Zool* 309A:581–587.
- Gist DH, Congdon JD. 1998. Oviductal sperm storage as a reproductive tactic of turtles. *J Exp Zool* 282:526–534.
- Gist DH, Fischer EN. 1993. Fine structure of the sperm storage tubules in the box turtle oviduct. *J Reprod Fert* 97:463–468.
- Gist DH, Jones JM. 1989. Storage of sperm in the oviducts of turtles. *J Morphol* 199:379–384.
- Gribbins K, Gist D. 2003. Cytological evaluation of the germinal epithelium and the germ cell cycle in an introduced population of European Wall Lizards, *Podarcis muralis*. *J Morphol* 256:296–306.
- Gribbins K, Gist D, Congdon J. 2003. Cytological evaluation of spermatogenesis in the Red-Eared Slider, *Trachemys scripta*. *J Morphol* 255:337–346.
- Gribbins K, Happ CS, Sever DM. 2005. Ultrastructure of the reproductive system of the Black Swamp Snake (*Seminatrix pygaea*). V. The temporal germ cell development strategy of the testis. *Acta Zoologica* 86:223–230.
- Gribbins K, Rheubert J, Collier M, Siegel D, Sever D. 2008. Histological analysis of spermatogenesis and the germ cell development strategy within the testis of the male Western Cottonmouth Snake, *Agkistrodon piscivorus*. *Annals of Anatomy* 190:461–476.
- Gribbins KM, Else RM, Gist DH. 2006. Cytological evaluation of the germ cell development strategy within the testis of the American alligator, *Alligator mississippiensis*. *Acta Zool* 87:59–69.

- Gribbins KM, Mills EM, Sever DM. 2007. Ultrastructural examination of spermiogenesis within the testis of the ground skink. *Scincella laterale* (Squamata, Sauria, Scincidae). *J Morphol* 268:181–192.
- Gribbins KM, Rheubert JL, Anzalone ML, Siegel DS, Sever DM. 2009. Ultrastructure of spermiogenesis in the cottonmouth, *Agkistrodon piscivorus* (Squamata: Viperidae: Crotalinae). *J Morphol* 271:293–304.
- Healy JM, Jamieson BGM. 1992. Ultrastructure of the spermatozoa of the Tuatara (*Sphenodon punctatus*) and its relevance to the relationships of the Sphenodontida. *Phil Trans Roy Soc London B* 335:193–205.
- Healy JM, Jamieson BGM. 1994. The ultrastructure of spermatogenesis and epididymal spermatozoa of the Tuatara *Sphenodon punctatus* (Sphenodontida, Amniota). *Phil Trans Bio Sci* 344:187–199.
- Hess RS, Thurston RJ, Gist DH. 1991. Ultrastructure of the turtle spermatozoon. *Anat Rec* 229:473–481.
- Ibarguengoytia NR, Cussac VE. 1999. Male response to low frequency of female reproduction in the viviparous lizard *Liolaemus* (Tropiduridae). *Herpetol J* 9:111–117.
- Jamieson BGM. 1995. Evolution of tetrapod spermatozoa with particular reference to amniotes. In: Jamieson BGM, Ausio J, Justine JL, editors. *Advances in Spermatozoal Phylogeny and Taxonomy*, Vol. 166. Paris: Mémoires du Muséum National d'Histoire Naturelle. pp 343–358.
- Jamieson BGM. 2007. Avian spermatozoa: Structure and phylogeny. In: Jamieson BGM, editor. *Reproductive Biology and Phylogeny of Birds*. Part A. NH: Science Publishers, Enfield. pp 349–511.
- Jamieson BGM, Healy JM. 1992. The phylogenetic position of the tuatara, *Sphenodon* (Sphenodontida, Amniota), as indicated by cladistic analysis of the ultrastructure of the spermatozoa. *Phil Trans Roy Soc London B* 335:207–219.
- Jamieson BGM, Tripepi R. 2005. Ultrastructure of the spermatozoon of *Apus apus* (Linnaeus 1758), the common swift (Aves: Apodiformes; Apodidae), with phylogenetic implications. *Acta Zool* 88:239–244.
- Jamieson BGM, Oliver SC, Scheltinga DM. 1996. The ultrastructure of spermatozoa of Squamata. I. Scincidae, Gekkonidae, and Pygionidae (Reptilia). *Acta Zool* 77:85–100.
- Jamieson BGM, Scheltinga DM, Tucker AD. 1997. The ultrastructure of spermatozoa of the Australian fresh water crocodile. *Crocodylus johnstoni* Krefft, 1873 (Crocodylidae, Reptilia). *J Submicrosc Cytol Pathol* 29:265–274.
- Lang JW, Andrews HV. 1994. Temperature-dependent sex determination in crocodilians. *J Exp Zool* 270:28–44.
- Lin M, Jones RC. 1993. Spermiogenesis and spermiation in the Japanese quail (*Coturnix coturnix japonica*). *J Anat* 183:525–535.
- Lindemann CB. 1996. Functional significance of the outer dense fibers of mammalian sperm examined by computer simulations with the geometric clutch model. *Cell Motil Cytok* 34:258–270.
- Moore BC, Hamlin HJ, Botteri NL, Lawler AN, Mathavan KK, Guille L. 2009. Posthatching development of *Alligator mississippiensis* ovary and testis. *J Morphol* 271:580–595.
- Rheubert JL, McHugh HH, Collier MH, Sever DM, Gribbins KM. 2009. Temporal germ cell development strategy during spermatogenesis within the testis of the ground skin. *Scincella lateralis* (Sauria: Scincidae). *Therio* 72:54–61.
- Rheubert JL, Wolf K, Wilson B, Gribbins KM. In press. Ultrastructure of spermiogenesis in the Jamaican Anole, *Anolis lineatopus*. *Acta Zoologica* (in press).
- Russell LD, Ettlin RA, Hikim AMP, Cleff ED. 1990. *Histological and Histopathological Evaluation of the Testis*. Clearwater: Cache River Press. pp 286.
- Saita A, Comazzi M, Perrotta E. 1987. Electron microscope study of spermiogenesis in *Caiman crocodylus* L. *Boll Zool* 4:307–318.
- Soley JT. 1997. Nuclear morphogenesis and the role of the manchette during spermiogenesis in the ostrich (*Struthio camelus*). *J Anat* 190:563–576.
- Sprando RL, Russell LD. 1988. Spermiogenesis in the red-ear turtle (*Pseudemys scripta*) and the domestic fowl (*Gallus domesticus*): A study of cytoplasmic events including cell volume changes and cytoplasmic elimination. *J Morphol* 198:95–118.
- Thorbjarnarson JB. 1996. Reproductive characteristics of the order Crocodylia. *Herpetologica* 52:8–24.
- Wang L, Wu X, Xu D, Wang R, Wang C. 2008. Development of testis and spermatogenesis in *Alligator sinensis*. *J Appl Anim Res* 34:23–28.
- Zhang L, Han X, Li M, Bao H, Chen Q. 2007. Spermiogenesis in soft-shelled turtle, *Pelodiscus sinensis*. *Anat Rec* 290:1213–1222.