Ultrastructural analysis of spermiogenesis in the Eastern Fence Lizard, *Sceloporus undulatus* (Squamata: Phrynosomatidae)

Justin L. Rheubert¹, David M. Sever², Dustin S. Siegel³, Kevin M. Gribbins⁴

¹Department of Natural Sciences, The University of Findlay, Findlay, OH 45840 USA

²Department of Biology, Southeastern Louisiana University, Hammond, LA 70402 USA

³Department of Biology, Southeast Missouri State University, Cape Girardeau MO 63701 USA

⁴Department of Biology, University of Indianapolis, Indianapolis, IN 47201 USA

Correspondence: Justin L. Rheubert

Department of Natural Sciences

The University of Findlay

1000 N. Main St, Findlay, Ohio 45840

Email: rheubert@findlay.edu

Phone: 419-434-5681

Abstract

Studies on reptilian sperm morphology have shown that variation exists at various taxonomic levels but studies on the ontogeny of variation are rare. Sperm development follows a generalized bauplan that includes acrosome development, nuclear condensation and elongation, and flagellar development. However, minute differences can be observed such as the presence/absence of manchette microtubules, structural organization during nuclear condensation, and presence/absence of a nuclear lacuna. The purpose of this investigation was to examine sperm development within the *Sceloporus* genus. The process begins with the development of an acrosomal complex from Golgi vesicles followed by nuclear condensation and elongation, which results in the presence of a nuclear lacuna. As the acrosomal complex differentiates, flagellar development commences with elongation of the distal centriole. Spermatid development culminates in a mature spermatid with a highly differentiated acrosomal complex, a condensed nucleus with a nuclear lacuna, and a differentiated flagellum. Although the overall developmental pattern is consistent with other squamate species, minute differences are observed, even within the same genus. For example there is variation in the presence/absence of an endoplasmic reticulum complex during acrosome development, presence/absence of a nuclear lacuna, and presence/absence of manchette microtubules within the three species of *Sceloporus* studied to date. Future studies concerning sperm morphology in closely related species will aid in our understanding of variation in sperm development and may prove to be useful in testing phylogenetic and evolutionary hypotheses.

Keywords: Sperm, development, reptile, reproduction, ultrastructure

Introduction

Reptiles exhibit morphological, physiological, and ecological differences from other vertebrates in terms of reproductive biology. Previous studies demonstrated that reptiles exhibit a unique variation of germ cell development that combines aspects from both the mammalian model (Russel et al., 1990), which is shared by aves (Kumar, 1995), and the anamniotic model exhibited by fishes and amphibians (Lofts, 1964). The testes of reptiles are comprised of seminiferous tubules and a permanent seminiferous epithelium where germ cells develop and then are released in a temporal pattern upon maturation into a centrally located lumen (for review see Gribbins, 2011). This model of spermatogenesis relies on consistent cell to cell communication to allow germ cells to develop as cohorts through the various stages of spermatogenesis, spermiogenesis, and spermiation (Gribbins et al., 2003; Wang et al., 2008; Rheubert et al., 2009).

Lepidosaurians (Squamata + Rhynchocephalia) have recently received much attention in terms of reproductive morphology; i.e., morphology of the testicular ducts (Akbarsha et al., 2007; Siegel et al., 2009; Rheubert et al., 2010; Sever, 2010), testes including sperm and sperm production (for review see Gribbins and Rheubert, 2011, 2014; Cree, 2014; Jamieson, 2014), female gonadoducts including the cloaca (for review see Siegel et al., 2011a,b, 2014a; Cree, 2014), oogenesis (for review see Ramírez-Pinilla et al., 2014), and kidneys (for review see Aldridge et al., 2011; Rheubert et al., 2014) that unequivocally produce copulatory material (Friesen et al., 2013). Some studies have discussed the diversity of reproductive attributes in the context of squamate phylogeny; e.g., the phylogenetic distribution

of cloacal glands in natricine snakes (Siegel et al., 2014b). However, no reproductive attributes have been examined in a diverse enough sampling of taxa to propose robust hypotheses on the evolution of reproductive structure in lepidosaurs.

Morphology of mature spermatozoa has traditionally been a major focus in lepidosaur reproductive biology with sperm from over 60 species described. Furiera (1965) provided the first ultrastructural description of a lepidosaur spermatozoon in the snake *Vipera aspis*. Healy and Jamieson's (1994) study on the sperm of *Sphenodon punctatus* was undoubtedly the most significant of early studies due to *Sphenodon*'s proposed position as the closest extant relative to the Squamata. Subsequently, numerous attempts have been made to use spermatozoa characters in a phylogenetic context with the addition of the *Sphenodon* outgroup (Oliver et al., 1996; Tavares-Bastos et al., 2008; Rheubert et al., 2010). These studies have isolated approximately 28 sperm characters (for review see Rheubert et al., 2010) as possible synapomorphies or apomorphies for clades within Squamata.

The ontogenetic stages of lepidosaur spermatid development have received less attention compared to morphological description of mature spermatozoa, especially within lizards. Ontogeny of spermatid development was previously described in the phrynosomatid family (Scheltinga et al., 2000) and a number of species within the Iguanomorpha (Clark, 1967; Rheubert et al., 2010; Rheubert et al., 2012; Gribbins et al., 2014). The purpose of this study is to give a detailed anatomical account of the steps of spermatid development in *Sceloporus undulatus* in comparison with other lepidosaurs, specifically those within the *Sceloporus* genus. Data from this study will aid in our understanding of variation in the

development of the spermatozoa at the genus level by comparing the developmental stages steps of the spermatids between species. The *Sceloporus* genus is one of the more diverse lineages of squamates (Leaché 2010), and the morphological changes during spermiogenesis will help to further understand the evolutionary trajectory of sperm evolution within *Sceloporus*. Optimistically, the ontogeny of spermatids may provide insights into the evolutionary relationships among species within this genus, which is currently controversial (Wiens and Reeder, 1997; Leaché, 2009).

2. Materials and Methods

Adult male *Sceloporus undulatus* were collected from Sandy Hollow Nature Reserve (Amite, LA). Specimens were euthanized using a 0.2cc intraperitoneal injection of sodium pentobarbital and dissected within one day of capture. The reproductive tracts were removed. The testis were isolated from the efferent ducts, placed in Trump's fixative (EMS, Hatfield, PA, USA), and stored at 4°C. Carcasses were catalogued into the Southeastern Louisiana Vertebrate Museum.

Predetermined spermatogenically active testicular tissues (Rheubert et al., 2014) were post-fixed with a 2% solution of osmium tetroxide and dehydrated through a graded series of ethanol solutions. Tissues were then cleared in propylene oxide and embedded in epoxy resin (Embed 812; EMS, Hatfield, PA, USA). Resin blocks were trimmed using a LKB ultramicrotome (LKB Produkter AB, Bromma, Sweden) and dry glass knife. Sections at 1 µm were placed on pre-wet slides and stained with toluidine blue. Light microscopy was used to determine whether all spermiogenic cells were available for transmission electron microscopy analysis. Using a Leica UC7 automated ultramicrotome (Leica Microsystem, Vienna, Italy),

sections were cut at 70 nm with a DDK diamond knife (DDK, Wilmington, DE, USA) and placed on copper grids. Tissues were then stained with uranyl acetate and lead citrate and then viewed with a JEOL JEM-1200EX II transmission electron microscope (JEOL Inc., Peabody, MA, USA). Each stage of germ cell development was photographed using a Gatan 785 Erlangshen digital camera (Gatan Inc., Warrendale, PA, USA). Composite plates of transmission electron microscopy micrographs were constructed using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

3. Results

The onset of spermiogenesis is marked by a swollen Golgi complex (Fig. 1A, Gb) surrounded by multiple mitochondria (Fig. 1A, Mi) at the apical portion of a round spermatid. Vesicles budding from the Golgi complex merge into an acrosomal vesicle (Fig. 1A, Av) that is approximately 0.25 μm from the membrane of the nucleus (Fig. 1A, Nu). As the acrosomal vesicle reaches its terminal size, a densification of proteinaceous material forms the acrosomal granule (Fig. 1B, Ag) in the basal position of the vesicle. The acrosomal granule rests on the basal membrane of the acrosomal vesicle after fully condensing (Fig 1C). During this time, a collection of electron dense material accumulates in the acrosomal vesicle before being shed from the cell as concentric membranous material (Fig 1D, Mf). The acrosomal vesicle (Fig. 1D, Ag) pushes against the nuclear membrane leaving a small protein dense space, the subacrosomal space (Fig 1D, Sas). The membrane of the acrosomal vesicle comes in close contact with the spermatid plasma membrane, and the apical portion of the cell is devoid of cellular machinery as the cytoplasm shifts to the posterior portion of the cell, where an accumulation of rough endoplasmic

reticulum (Fig. 1D, Rer) is observed in close association with the distal centriole (Fig. 1D, Dc) of the developing flagellum.

Figure 1. Early developmental stages during spermiogenesis in *S. undulatus*. A.) The acrosomal vesicle (Av) develops at the apical portion of the nucleus (Nu) from vesicles budding from the Golgi complex (Gb). Mitochondria (Mi). B.) The acrosomal vesicle (Av) reaches its final size and the formation of the acrosomal granule can be observed. Nucleus (Nu), mitochondria (Mi). C.) The acrosomal granule (Ag) migrates to its basal position within the acrosomal vesicle (Av) as debris (black arrow) can be observed within the acrosomal vesicle. Nucleus (Nu). D.) The indentation of the nucleus (Nu) increases in size with the acrosomal vesicle (Av) situated deep within the fossa. The acrosomal granule (Ag) is electron dense and in its basal position. The subacrosomal space (Sas) becomes evident and myelin figures (Mf) is observed in the extracellular matrix. Rough endoplasmic reticulum (Rer) is concentrated in the basal portion of the germ cell near the elongating distal centriole (Dc).

The nucleus (Fig. 2, Nu) undergoes chromatin condensation and nuclear elongation due to chromatin compacting in a spiral and granular fashion, leaving spirals and pits of open nucleoplasm (Fig. 2C). Longitudinal microtubules of the manchette (Fig. 2A, Lmm) are present and the additional manchette microtubules

(Fig. 1B, Mm) develop, accumulate (Fig. 2C, Mm), and surround the nucleus (Fig. 2C, Nu). Transport vesicles (Fig. 2C, Tv) are seen juxtapositioned to the spermatid cell membrane as the cytoplasm disintegrates and is then exocytosed. The acrosome envelops the apical nucleus (nuclear rostrum; Fig. 2D, Nr), which extends into the subacrosomal space (Fig 2D, Sas). The entire acrosomal complex pushes against the plasmalemma resulting in a protruding appearance and forcing the remaining cytoplasm and cellular organelles towards the nucleus proper. The proximal and distal centriole (Fig. 2A, Dc) rest within the indentation of the nucleus (nuclear fossa; Fig. 2A, Nf).

Figure 2. Nuclear condensation and elongation during spermiogenesis in *S. undulatus*. A.) The nucleus (Nu) begins to elongate as longitudinal microtubules (Lmm) become present. The flagellar components set deep within the nuclear fossa (Nf) as the distal centriole (Dc) elongates. Endpiece (Ep). B.) The chromatin of the nucleus (Nu) condenses in a spiral and granular fashion and more manchette microtubules (Mm) develop and migrate to their final destination surrounding the nucleus. C.) The manchette microtubules (Mm) reach their final destination in an organized fashion surrounding the nucleus (Nu). Transport vesicles (Tv) are seen on the germ cell periphery. D.) The nucleus (Nu) becomes more elongated as the acrosomal complex (Acc) pushes against the germ cell membrane giving it a protruding appearance. A portion of the nucleus extends into the subacrosomal space (Sas) as the nuclear rostrum (Nr). The germ cell cytoplasm and organelles such as mitochondria (Mi) are pushed to the posterior portion of the cell.

As the nucleus (Fig. 3A, Nu) becomes homogenous in electron density, an electron lucent lacuna (Fig. 3A, La) is visible in many of the cross sections of the nucleus proper. Manchette microtubules (Fig 3A, Mm) remain present as the nucleus elongates. The acrosome becomes further compartmentalized with the nuclear rostrum extending into the subacrosomal space. An acrosomal lucent ridge

(Fig. 3B, Alr), historically termed the inner acrosomal membrane, separates the subacrosomal space (Fig. 3B, Sas) from the acrosomal proper (Fig. 3B, Acp).

During late nuclear elongation the spermatid nucleus proper becomes curved, forcing the rough endoplasmic reticulum-filled cytoplasm (Fig 3C, Cy) to the inner radius of the germ cell. The developing flagellum is situated in the posterior nuclear indentation, the nuclear fossa (Fig. 3D, Nf) and mitochondria (Fig. 3D, Mi) are associated with the flagellum, dense bodies (Fig. 3D, Db) separate the individual mitochondria, and the fibrous sheath (Fig. 3D, Fs) is evident.

Figure 3. Late stages of spermiogenesis in *S. undulatus*. A.) The nucleus (Nu) becomes homogenous in electron density and a nuclear lacuna (La) becomes evident. Manchette microtubules (Mm) continue to surround the nucleus and lipid droplets (Li) become present within the germ cell cytoplasm. Lipid inclusion (Li). B.) The nucleus (Nu) becomes highly elongate as the acrosome becomes highly compartmentalized. A protrusion of the nucleus, the nuclear rostrum (Nr), extends into the subacrosomal space (Sas). The subacrosomal space is separated from the acrosomal proper (Acp) by an acrosomal lucent ridge (Alr). C.) The nucleus (Nu) becomes more elongate and arched. The cytoplasm (Cy) and its organelles are forced under the arc of the nucleus. Acrosome (Ac). D.) The flagellum sets within the nuclear fossa (Nf) and begins to differentiate with mitochondria (Mi) and dense bodies (Db) surrounding the axoneme of the distal centriole. A fibrous sheath (Fs) also becomes present during this time.

The late stage spermatid prior to spermiation is filiform in shape. The acrosome is highly compartmentalized and externally surrounded by an acrosomal cortex (Fig 4A, Acc). Deep to the acrosomal cortex, the acrosomal medulla (Fig 4A, Acm) is separated from the subacrosomal space (Fig. 4A, Sas) by the acrosomal lucent ridge (Fig. 4A, Alr). A slender portion of the apical nucleus extends into the subacrosomal space and is capped by an epinuclear lucent zone (Fig. 4B, Elz). A

single perforatorium (Fig. 4C, Pe) extends into the acrosomal medulla but no perforatorial base plate was observed. Multiple Sertoli cells (Fig. 4D, Sc) are bound to the acrosomal complex by ectoplasmic specializations (Fig. 4F, Es). The superficial Sertoli cells, typically 2, contain multiple cellular organelles including rough endoplasmic reticulum. The nuclear lacuna (Fig. 4G, La) remains present and the manchette microtubules (Fig. 4G, Mm) persist.

Figure 4. Anterior portion of the late stage spermatid in *S. undulatus*. A.) Sagittal section of the apical nucleus and acrosomal complex detailing the compartmentalization of the acrosome. The nuclear rostrum (Nr) extends into the subacrosomal space (Sas) which is surrounded by an acrosomal lucent ridge (Alr, separating it from the acrosomal medulla (Acm). The acrosomal complex is surrounded by an acrosomal cap (Acc). Ectoplasmic specializations (Es) can be observed with the surrounding Sertoli cell. B.) Oblique section through the acrosome detailing the epinuclear lucent zone (Elz) capping the nuclear rostrum (Nr). C.) Oblique view through the acrosome detailing the perforatorium (Pe) within the acrosomal medulla (Acm). D.) Cross sectional view of the acrosome detailing multiple Sertoli cells (Sc, Sc2) surrounding the acrosome. Acrosomal cap (Acc), subacrosomal cone (Sac). E.) Transverse view showing the concentration of rough endoplasmic reticulum (Rer) within the Sertoli cell (Sc) surrounding the acrosome. Acrosomal lucent ridge (Alr), subacrosomal space (Sas). F.) Oblique section through the acrosome detailing the nuclear rostrum (Nr) within the subacrosomal space (Sas). Ectoplasmic specializations (Es) can also be observed in association with the surrounding Sertoli cells. Acrosomal lucent ridge (Alr). G.) Cross sectional view through the apical nucleus (Nu) detailing the nuclear lacuna (La) and manchette microtubules.

The posterior portion of the nucleus supports the nuclear fossa (Fig. 5A, Nf), which houses the proximal centriole (Fig. 5A, Pc) of the flagellum. The flagellum displays the 9+2 microtubule arrangement. The midpiece (Fig. 5B) of the flagellum

is surrounded by mitochondria with multiple dense bodies (Fig. 5B, Db). A fibrous sheath (Fig. 5B, Fs) surrounds the axoneme beginning at mitochondrial tier 2.

Peripheral fibers associated with microtubule doublets 3 and 8 are grossly enlarged (Figure 5B, Ef). The midpiece ceases and continues as the principal piece (Fig. 5C) with the termination of the mitochondria but a continuation of the fibrous sheath (Fig. 5C, Fs). The fibrous sheath terminates at the beginning of the endpiece (Fig. 5D), which is only surrounded by minimal cytoplasm and the germ cell membrane.

Figure 5. Posterior portion of the mature spermatid in *S. undulatus*. B-D represent cross-sectional views of the main regions of the flagellum. The proximal centriole (Pc) sets deep within the nuclear fossa (Nf). Aligned at approximately 90° the distal centriole (Dc) is elongated. The midpiece is characterized by the surrounding mitochondria (Mi) and dense bodies (Db). The fibrous sheath (Fs) begins in the midpiece at mitochondrial tier 2 and extends into the principal piece. The axoneme continues into the endpiece but the fibrous sheath is absent and only the cytoplasmic membrane (Cm) persists.

4. Discussion

The events of spermiogenesis in *Sceloporus undulatus* parallel that of other amniotes (see Gribbins and Rheubert, 2014 for review). The initial stages are marked by the development of the acrosome, followed by nuclear condensation and elongation, and culminate with the differentiation of the flagellum. This process remains highly conserved in amniotes and only as new studies are added do we start to see the minor differences that occur during the steps of spermiogenesis.

Acrosome formation begins with Golgi vesicles merging at the apex of the nucleus. Contrary to that seen in *Iguana iguana* (Ferreira and Dolder, 2002), the rough endoplasmic reticulum is in close association with the Golgi complex. A single acrosomal granule is seen condensing in the middle of the acrosomal vesicle similar to *Anolis carolinensis* (Clark, 1967). This differs from that seen in *Chalcides ocellatus* (Carcupino et al., 1989), *Norops lineatopus* (Rheubert et al., 2010), and *Tropidurus torquatus* (Vieira et al., 2001) where the granule is initially observed in a basal position and also from *Mabuya quinquetaeniata* (Ismail, 1997) where two acrosomal granules are observed within the vesicle.

The final result of acrosome development is a highly compartmentalized acrosome, which is consistent among squamates. In *Sceloporus undulatus* these compartments consist of an acrosomal cortex, acrosomal medulla, acrosomal lucent ridge, subacrosomal space, perforatorium, nuclear rostrum, and epinuclear lucent zone. The division of the exterior acrosome into the acrosomal cortex and medulla is consistent among reptilian models (Gribbins and Rheubert, 2014). The acrosomal lucent ridge, historically termed the inner acrosomal membrane (Huang Jr and

Yanagimachi, 1985), seems to be present in all amniotic models but has been ignored in reptiles. It is believed that this structure is responsible for binding to the zona pellucida during fertilization (Huang Jr and Yanagimachi, 1985). The acrosomal lucent ridge surrounds the subacrosomal space, a space sometimes referred to as the subacrosomal cone (Cunha et al., 2008).

A highly compartmentalized acrosome in reptiles, specifically squamates, may represent unique physiological degradation of the cumulus granulosa cells, zona pellucida, and oocyte membrane through sperm-egg interaction. Enzymes responsible for degrading the cumulus granulosa cells may be located inside the acrosomal cortex and medulla and are released upon membrane fusion during fertilization. Following calcium influx, enzymatic proteins may then be released from the subacrosomal space to degrade the zona pellucida; however, this hypothesis needs to be tested.

During nuclear condensation and elongation, the chromatin condenses in a spiral and granular fashion, which is common (but not ubiquitous) among squamates studied to date (Ferreira and Dolder, 2002; Gribbins et al., 2007; Rheubert et al., 2011) but differs from that of archosaurs and mammals in which it is solely granular (Lin and Jones, 2000; Gribbins et al., 2010; Gribbins, 2011). The manchette microtubules are believed to aid in the elongation of the nucleus (Soley, 1997) and are present during nuclear elongation in *Sceloporus undulatus*. However, these structures were not observed in *Norops lineatopus*, which suggests that other cellular contents/interactions may play a more pivotal role in elongation. As the nucleus elongates and condenses it becomes arc shaped and the cytoplasm is

pushed towards the inside of the arc, which may indicate formation of the cytoplasmic residuum (Newton and Trauth, 1992).

The flagellum begins to develop early on with the appearance of the proximal and distal centrioles, which are derived from the parental centrioles during meiotic divisions (Mizukami and Gall, 1966). The proximal centriole migrates deep to the nuclear fossa. Unlike some reptilian species, no electron lucent shoulders surround the nuclear fossa of *Sceloporus* (Rheubert et al., 2010; Rheubert et al., 2014). The distal centriole elongates and a fibrous sheath becomes evident. As the cytoplasmic shift occurs, mitochondria are forced to the distal portion of the developing germ cell where they will surround a portion of the flagellum termed the midpiece. The mitochondria end at their final destination and individual mitochondria are separated by dense bodies. The fibrous sheath is pulled distally and begins at mitochondria tier 2, similar to all other iguanian species (Teixeira et al., 1999; Vieira et al., 2005; Vieira et al., 2007) except *Uta stansburiana* (Scheltinga et al., 2000) in which the fibrous sheath begins at mitochondrial tier 1.

Although previous reports have shown that proteins secreted by the epididymis are necessary for final sperm maturation (Depeiges and Dacheux, 1985), only a handful of studies exist looking at post-spermiation modification to morphology (Esponda and Bedford, 1987). No studies in reptiles to date have examined the amount of modification the developing germ cell undergoes post-spermiation. Future studies are needed to determine if the morphological details observed in mature spermatids prior to spermiation (like those described in this

study) are equivalent to those found in mature spermatozoa from the caudal epididymis.

The data from this study and data from previous studies suggest that the morphology of sperm development is variable among closely related species within the same genus (Rheubert et al., 2012; Gribbins et al., 2013). These variations are highlighted through observations of three species within the *Sceloporus* genus: *S. bicanthalis* (Rheubert et al., 2012), *S. variabilis* (Gribbins et al., 2014), and *S. undulatus* (this study). In *S. variabilis* a mitochondria/ER complex is found separating the Golgi body from the developing acrosome, a character that is not observed in *S. undulatus* or *S. bicanthalis*. Myelin figures in the developing acrosome are rare in *S. variabilis* but present in *S. bicanthalis* and *S. undulatus*. During development and enlargement of the acrosomal vesicle, a deep nuclear depression is formed in *S. undulatus* and *S. bicanthalis* but the depression is shallow in *S. variabilis*. Longitudinal microtubules of the manchette are present in *S. undulatus* and *S. bicanthalis* but are not present in *S. variabilis*. A nuclear lacuna is also present in *S. undulatus* and *S. bicanthalis* but absent in *S. variabilis*. Finally, nuclear condensation occurs in a spiral fashion in both *S. undulatus* and *S. bicanthalis* but not in *S. variabilis*, where it occurs in a granular fashion. Interestingly, *S. undulatus* and *S. bicanthalis* share a more recent common ancestor within *Sceloporus* and their sperm share a more similar ontogeny when compared to a more distantly related *Sceloporus* species (*S. variabilis*; Pyron et al., 2013). However, with only three species of *Sceloporus* studied, caution must be taken when interpreting these results in any phylogenetic context.

The observed development variations can be extremely valuable in future phylogenetic studies and potentially serve to test hypotheses concerning the status of species. Furthermore, these data will add to the growing database of sperm morphology characters for use in understanding the evolution of spermatozoa within various lineages. The data presented here and in previous studies suggest closely related species vary in spermatid morphology. However, additional studies concerning variation of spermatid and spermatozoal morphology in closely related (including sister taxa and populations) need to be conducted. Furthermore, the observed variation in the development of the spermatids can provide insights into variations in mature sperm morphology. For example, in *S. undulatus* and *S. bicanthalis* the nuclear chromatin condenses in a spiral fashion and both have a nuclear lacuna. In *S. variabilis* the nuclear contents condense in a non-spiral fashion and a nuclear lacuna is absent. It is possible that the spiral condensation of the nuclear contents results in the presence of a nuclear lacuna. These developmental differences in closely related species might aid our understanding of the ontogenic mechanism responsible for the variations observed in mature sperm morphology.

5. Acknowledgements

The authors would like to thank Christopher M. Murray, Jennifer Lee, and Johnathon Babin for their help in the collection of specimens. We extend our thanks to Christopher Murray for his help in proofreading this document along with students from the Rheubert lab at The University of Findlay. Funding was provided by University of Indianapolis and the National Science Foundation Grant DEB-0890381 to DMS.

6. References

- Akbarsha, M.A., Kadalmani, B., Tamilarasan, V. 2007. Efferent ductules of the fan-throated lizard *Sitana ponticeriana* Cuvier. *Journal of Morphology* 267, 713-729.
- Aldridge, R.D., Jellen, B.C., Siegel, D.S., Wisniewski S.S. 2011. The sexual segment of the kidney. In: Aldridge, R.D. and Sever, D.M. (Eds.), *Reproductive Biology and Phylogeny of Snakes*. CRC Press, Boca Raton, FL, pp. 477-510.
- Carcupino, M., Corso G., Pala M. 1989. Spermiogenesis in *Chalcides ocellatus tiligugu* (Gmelin) (Squamata, Scincidae): An electron microscope study. *Boll Zool* 56, 119-124.
- Clark, A.W. 1967. Some aspects of serpmiogenesis in a lizard. *American Journal of Anatomy* 121, 369-400.
- Cree, A. 2014. Reproductive anatomy and cycles of tuatara (*Sphenodon punctatus*), and intriguing non-squamate lepidosaur. In: Rheubert J.L., Siegel D.S., Trauth S.E. (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara*. Eds. CRC Press, Boca Raton Fl. pp 620-646.
- Cunha, L.D, Tavares-Bastos, L., Báo, S.N. 2008. Ultrastructural description and cytochemical study of the spermatozoon of *Crotallus durissus* (Squamata, Serpentes). *Micron* 39, 915-925.
- Esponda, P., Bedford, J.M. 1987. Post-testicular change in the reptile sperm surface with particular reference to the snake, *Natrix fasciata*. *Journal of Experimental Zoology* 241, 123-132.

- Ferreira, A., Dolder, H. 2002. Ultrastructural analysis of spermiogenesis in *Iguana iguana* (Reptilia: Sauria: Iguanidae). *European Journal of Morphology* 40, 89-99.
- Friesen, C.R., Shine, R., Krohmer, R., Mason R.T. 2013. Not just a chastity belt: the functional significance of mating plugs in garter snakes, revisited. *Biological Journal of the Linnean Society* 109, 893-907.
- Furieri, P. 1965. Prime osservaioni al microscopio eletrronico sulla ultrastruttura dello spermatozoo di *Vipera aspis aspis*. L. *Boll Soc Ital Biol Sper* 41, 478-480.
- Gribbins, K.M. 2011. Reptilian spermatogenesis. *Spermatogenesis* 1, 250-269.
- Gribbins, K.M., Gist, D.H. 2003. Cytological evaluation of speratogenesis within the germinal epithelium of the male European wall lizard, *Podarcis muralis*. *Journal of Morphology* 258, 296-306.
- Gribbins, K.M., Mills, E.M., Sever, D.M. 2007. Ultrastructural examination of spermiogenesis within the testis of the ground skink, *Scincella laterale* (Squamata, Sauria, Scincidae). *Journal of Morphology* 268, 181-192.
- Gribbins, K.M., Siegel, D.S., Anzalone, M.L., Jackson, D.P., Venable, K.J., Rheubert, J.L., Elsey, R.M. 2010. Ultrastructure of spermiogenesis in the American alligator, *Alligator mississippiensis* (Reptilia, Crocodylia, Alligatoridae). *Journal of Morphology* 271, 1260-1271.
- Gribbins, K.M., Rheubert, J.L. 2011. The ophidian testis, spermatogenesis, and mature spermatozoa. In: Aldridge, R.D. and Sever, D.M. (Eds.), *Reproductive Biology and Phylogeny of Snakes*. CRC Press, Boca Raton, FL., pp. 183-264.

- Gribbins, K.M., Matchett, C.L., DeVello, K.A., Rheubert, J.L., Villagrán-Santa Cruz, M., Granados-González, G., Hernández-Gallegos, O. 2014. The ultrastructure of spermatid development during spermiogenesis within the rosebelly lizard, *Sceloporus variabilis* (Reptilia, squamata, phrynosomatidae). *Journal of Morphology* 275, 258-268.
- Gribbins, K.M., Rheubert, J.L. 2014. The architecture of the testis, spermatogenesis, and mature spermatozoa. In: Rheubert, J.L., Siegel, D.S., Trauth, S.E. (Eds.) *Reproductive Biology and Phylogeny of Lizards and Tuatara*, CRC Press, Boca Raton, FL. pp. 340-424
- Healy, J.M., Jamieson, B.G.M. 1994. The ultrastructure of spermatogenesis and epididymal spermatozoa of the tuatara *Sphenodon punctatus* (Sphenodontida, Amniota). *Philos Trans R Soc Lond B Biol Sci* 344, 187-199.
- Huang Jr., T.T.F., Yanagimachi, R. 1985. Inner acrosomal membrane of mammalian spermatozoa: its properties and possible functions in fertilization. *American Journal of Anatomy* 174, 249-268.
- Ismail, M.F. 1997. Unusual features of the sperm head differentiation in *Mabuya quinquetaeniata*. *Archives of Andrology* 39,19-23.
- Jamieson, B.G.M. 2014. The ultrastructure of spermatogenesis and spermatozoa of the tuatara *Sphenodon punctatus* (Sphenodontida, Amniota). CRC Press. Boca Raton, Fl. pp. 647-676.
- Kumar, M. 1995. Spermatogenesis in the house sparrow, *Passar domesticus*: histological observations. *Pavo* 33,1-4.

- Leaché, A.D. 2009. Species tree discordance traces to phylogeographic clade boundaries in North American fence lizards (*Sceloporus*). *Systematic Biology* 58, 547-559.
- Leaché, A.D. 2010. Species trees for spiny lizards (Genus *Sceloporus*): Identifying points of concordance and conflict between nuclear and mitochondrial data. *Molecular Phylogenetics and Evolution*. 54, 162-171.
- Lin, M., Jones R.C. 2000. Spermiogenesis and spermiation in a monotreme mammal, the platypus, *Ornithorhynchus anatinus*. *Journal of Anatomy* 196, 217-232.
- Lofts, B. 1964. Seasonal changes in the functional activity of the interstitial and spermatogenic tissues of the green frog, *Rana esulenta*. *General and Comparative Endocrinology* 4, 550-562.
- Mizukami, I., Gall, J. 1966. Centriole Replication II. Sperm formation in the fern, *Marsilea*, and the Cycad, *Zamia*. *Journal of Cell Biology* 29, 97-111.
- Newton, W.D, Trauth, S.E. 1992. Ultrastructure of spermatogonium of the lizard *Cnemidophorus sexlineatus* (Sauria: Teiidae). *Herpetologica* 48, 330-343.
- Oliver, S.C., Jamieson, B.G.M., Scheltinga, D.M. 1996. The ultrastructure of spermatozoa of Squamata. II. Agamidae, Varanidae, Colubridae, Elapidae, and Boidae (Reptilia). *Herpetologica* 52, 216-241.
- Pyron, R.A., Burbrink, F.T., Wiens, J.J. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evolutionary Biology* 13, 93.

- Ramírez-Pinilla, M.P., de Pérez, G.R., Alvarado-Ramírez, C. 2014. In: Rheubert, J.L., Siegel, D.S., Trauth, S.E. (Eds.), Reproductive Biology and Phylogeny of Lizards and Tuatara. CRC Press. Boca Raton, Fl. pp. 213-252.
- Rheubert, J.L., Poldemann, E.H., Eckstut, M.E., Collier, M.H., Sever, D.M., Gribbins, K.M. 2009. Temporal germ cell development strategy during mixed spermatogenesis within the male Mediterranean gecko, *Hemidactylus turcicus* (Reptilia: Gekkonidae). Copeia 4, 793-800.
- Rheubert, J.L., McMahan, C.D., Sever, D.M., Bundy, M.R., Siegel, D.S., Gribbins, K.M. 2010. Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). VUU, spermatozoon morphology and evolutionary trends of sperm characters in snakes. Journal of Zoological Systematics and Evolutionary Research. 48, 366-375.
- Rheubert, J.L., Wilson, B.S., Wolf, K.W., Gribbins, K.M. 2010. Ultrastructural study of spermiogenesis in the Jamaican gray anole, *Anolis lineatopus* (Reptilia: Polychrotidae). Acta Zoologica (Stockholm) 91, 484-494.
- Rheubert, J.L., Sever, D.M., Geheber, A.D., Siegel, D.S. 2010. Proximal testicular ducts of the Mediterranean gecko (*Hemidactylus turcicus*). Anatomical Record 293, 2176-2192.
- Rheubert, J.L., Siegel, D.S., Venable, K.J., Sever, D.M., Gribbins, K.M. 2011. Ultrastructural description of spermiogenesis within the Mediterranean gecko, *Hemidactylus turcicus* (Squamata: Gekkonidae). Micron 42, 680-690.
- Rheubert, J.L., Touzinsky, K., Hernández-Gallegos, O., Granados-González, G., Gribbins, K.M. 2012. Ontogenic development of spermatids during

- spermiogenesis in the high altitude bunchgrass lizard (*Sceloporus bicanthalis*). *Spermatogenesis* 2, 94-103.
- Rheubert, J.L., Touzinsky, K., Sever, D.M., Aldridge, R.D., Wilmes, A.J., Siegel, D.S., Gribbins, K.M. 2014. Reproductive biology of *Sceloporus consobrinus* (Phrynosomatidae): male germ cell development and reproductive cycle comparisons within spiny lizards. *Journal of Herpetology* 48, 162-171.
- Russell, LD., Hikim, S.A.P., Ettlin, R.A., Legg, E.D. 1990. Histological and Histopathological Evaluation of the Testis. Cache River Press, Clearwater, Florida.
- Scheltinga, D.M., Jamieson, B.G.M., Trauth, S.E., Mcallister, C.T. 2000. Morphology of the spermatozoa of the iguanian lizards *Uta stansburiana* and *Urosaurus ornatus* (Squamata, Phrynosomatidae). *Journal of Submicroscopy Cytology and Pathology*. 32, 261-271.
- Sever, D.M. 2010. Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). VI. Anterior testicular ducts and their nomenclature. *Journal of Morphology* 271, 104-115.
- Trauth, S.E., Sever, D.M. 2011. Male urogenital ducts and cloacal anatomy. In: Aldridge, R.D., Sever, D.M. (Eds.), *Reproductive Biology and Phylogeny of Snakes*. CRC Press. Boca Raton, Fl. pp. 411-476.
- Siegel, D.S., Sever, D.M., Rheubert, J.L., Gribbins, K.M. 2009. Reproductive biology of *Agkistrodon piscivorus* Lacépède (squamata, serpents, viperidae, crotalinae). *Herpetological Monographs* 23, 74-107.

- Siegel, D.S., Miralles, A., Chabarria, R.E., Aldridge, R.D. 2011a. Female reproductive anatomy: cloaca, oviduct, and sperm storage. In: Aldridge, R.D., Sever, D.M. (Eds.), Reproductive Biology and Phylogeny of Snakes. CRC Press. Boca Raton, Fl. Pp. 347-410.
- Siegel, D.S., Trauth, S.E., Sever, D.M., Aldridge, R.D. 2011b. The phylogenetic distribution of the ampulla ureter and ampulla urogenital/uriniferous papilla in the Serpentes. Journal of Zoological Systematics and Evolutionary Research. 49, 160-168.
- Siegel, D.S., Miralles, A., Rheubert, J.L., Sever, D.M. 2014a. Female reproductive anatomy: cloaca, oviduct, and sperm storage. In: Rheubert, J.L., Siegel, D.S., Trauth, S.E. (Eds.), Reproductive Biology and Phylogeny of Lizards and Tuatara. CRC Press. Boca Raton, Fl. pp. 253-301.
- Siegel, D.S., Trauth, S.E., Rheubert, J.L., Rabe, B., Ruopp, B., Miralles, A., Murray, C.M., Aldridge, R.D. 2014b. Novel cloacal glands in snakes: the phylogenetic distribution of ventral urodaeal glands in *Thamnophiini*. Herpetologica 70, 279-289.
- Soley, J.T. 1997. Nuclear morphogenesis and the role of the manchette during spermiogenesis in the ostrich (*Struthio camelus*). Journal of Anatomy 190, 563-576.
- Tavares-Bastos, L., Colli, G.R., Bo, S.N. 2008. The evolution of sperm ultrastructure among Boidae (Serpentes). Zoomorphology 8, 62-68.

- Teixeira, R.D., Colli, G.R., Bo, S.N. 1999. The ultrastructure of the spermatozoa of the lizard *Polychrus acutirostris* (Squamata, Polychrotidae). Journal of Submicroscopic Cytology and Pathology 31, 387-395.
- Vieira, G.H.C., Wiederhecker, H.C., Colli, G.R., Bo, S.N. 2001. Spermiogenesis and testicular cycle of the lizard *Tropidurus torquatus* (Squamata, Tropiduridae) in the Cerrado of central Brazil. Amphibia-Reptilia 22, 217-233.
- Vieira, G.H.C., Colli, G.R., Bo, S.N. 2005. Phylogenetic relationships of corytophanid lizards (Iguania, Squamata, Reptilia) based on partitioned and total evidence analyses of sperm morphology, gross morphology, and DNA data. Zoologica Scripta 34, 605-625.
- Vieira, G.H.C., Cunha, L.D., Scheltinga, D.M., Glaw, F., Colli, G.R., Bo, S.N. 2007. Sperm ultrastructure of holocercid and oplurid lizards (Sauropsida, Squamata, Iguania) and the phylogeny of Iguania. Journal of Zoological Systematics and Evolutionary Research. 45, 230-241.
- Wang, L., Xiaobing, W., Xu, D., Wang, R., Wang, C. 2008. Development of testis and spermatogenesis in *Alligator sinensis*. Journal of Applied Animal Research 34, 23-28.
- Wiens, J.J., Reeder, T.W. 1997. Phylogeny of the spiny lizards (*Sceloporus*) based on molecular and morphological evidence. Herpetological Monographs 11, 1-101.

Figure Legends

Figure 1- Early developmental stages during spermiogenesis in *S. undulatus*. A.) The acrosomal vesicle (Av) develops at the apical portion of the nucleus (Nu) from

vesicles budding from the Golgi complex (Gb). Mitochondria (Mi). B.) The acrosomal vesicle (Av) reaches its final size and the formation of the acrosomal granule can be observed. Nucleus (Nu), mitochondria (Mi). C.) The acrosomal granule (Ag) migrates to its basal position within the acrosomal vesicle (Av) as debris (black arrow) can be observed within the acrosomal vesicle. Nucleus (Nu). D.) The indentation of the nucleus (Nu) increases in size with the acrosomal vesicle (Av) situated deep within the fossa. The acrosomal granule (Ag) is electron dense and in its basal position. The subacrosomal space (Sas) becomes evident and myelin figures (Mf) is observed in the extracellular matrix. Rough endoplasmic reticulum (Rer) is concentrated in the basal portion of the germ cell near the elongating distal centriole (Dc).

Figure 2- Nuclear condensation and elongation during spermiogenesis in *S.*

undulatus. A.) The nucleus (Nu) begins to elongate as longitudinal microtubules (Lmm) become present. The flagellar components set deep within the nuclear fossa (Nf) as the distal centriole (Dc) elongates. Endpiece (Ep). B.) The chromatin of the nucleus (Nu) condenses in a spiral and granular fashion and more manchette microtubules (Mm) develop and migrate to their final destination surrounding the nucleus. C.) The manchette microtubules (Mm) reach their final destination in an organized fashion surrounding the nucleus (Nu). Transport vesicles (Tv) are seen on the germ cell periphery. D.) The nucleus (Nu) becomes more elongated as the acrosomal complex (Acc) pushes against the germ cell membrane giving it a protruding appearance. A portion of the nucleus extends into the subacrosomal space (Sas) as the nuclear rostrum (Nr). The germ cell cytoplasm and organelles such as mitochondria (Mi) are pushed to the posterior portion of the cell.

Figure 3- Late stages of spermiogenesis in *S. undulatus*. A.) The nucleus (Nu) becomes homogenous in electron density and a nuclear lacuna (La) becomes evident. Manchette microtubules (Mm) continue to surround the nucleus and lipid droplets (Li) become present within the germ cell cytoplasm. Lipid inclusion (Li). B.) The nucleus (Nu) becomes highly elongate as the acrosome becomes highly compartmentalized. A protrusion of the nucleus, the nuclear rostrum (Nr), extends into the subacrosomal space (Sas). The subacrosomal space is separated from the acrosomal proper (Acp) by an acrosomal lucent ridge (Alr). C.) The nucleus (Nu) becomes more elongate and arched. The cytoplasm (Cy) and its organelles are forced under the arc of the nucleus. Acrosome (Ac). D.) The flagellum sets within the nuclear fossa (Nf) and begins to differentiate with mitochondria (Mi) and dense bodies (Db) surrounding the axoneme of the distal centriole. A fibrous sheath (Fs) also becomes present during this time.

Figure 4- Anterior portion of the late stage spermatid in *S. undulatus*. A.) Sagittal section of the apical nucleus and acrosomal complex detailing the compartmentalization of the acrosome. The nuclear rostrum (Nr) extends into the subacrosomal space (Sas) which is surrounded by an acrosomal lucent ridge (Alr, separating it from the acrosomal medulla (Acm). The acrosomal complex is surrounded by an acrosomal cap (Acc). Ectoplasmic specializations (Es) can be observed with the surrounding Sertoli cell. B.) Oblique section through the acrosome detailing the epinuclear lucent zone (Elz) capping the nuclear rostrum (Nr). C.) Oblique view through the acrosome detailing the perforatorium (Pe) within the acrosomal medulla (Acm). D.) Cross sectional view of the acrosome detailing

multiple Sertoli cells (Sc, Sc2) surrounding the acrosome. Acrosomal cap (Acc), subacrosomal cone (Sac). E.) Transverse view showing the concentration of rough endoplasmic reticulum (Rer) within the Sertoli cell (Sc) surrounding the acrosome. Acrosomal lucent ridge (Alr), subacrosomal space (Sas). F.) Oblique section through the acrosome detailing the nuclear rostrum (Nr) within the subacrosomal space (Sas). Ectoplasmic specializations (Es) can also be observed in association with the surrounding Sertoli cells. Acrosomal lucent ridge (Alr). G.) Cross sectional view through the apical nucleus (Nu) detailing the nuclear lacuna (La) and manchette microtubules.

Figure 5- Posterior portion of the mature spermatid in *S. undulatus*. B-D represent cross-sectional views of the main regions of the flagellum. The proximal centriole (Pc) sets deep within the nuclear fossa (Nf). Aligned at approximately 90° the distal centriole (Dc) is elongated. The midpiece is characterized by the surrounding mitochondria (Mi) and dense bodies (Db). The fibrous sheath (Fs) begins in the midpiece at mitochondrial tier 2 and extends into the principal piece. The axoneme continues into the endpiece but the fibrous sheath is absent and only the cytoplasmic membrane (Cm) persists.