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Research Article

Spermiogenesis within the Testes of the Tokay Gecko, *Gekko gecko*

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Abstract

The Tokay Gecko, *Gekko gecko,* is a common house gecko that is native to Asia, has a widespread distribution across its native range, and is invasive in Florida. The purpose of this research is to elucidate the ultrastructural changes that occur to spermatids during the process of spermiogenesis within the Tokay Gecko using transmission electron microscopy. Spermiogenesis is the final stage of spermatogenesis and is the reproductive process that mature spermatids undergo to become mature spermatozoa. This research is vital because it will extend the knowledge of spermiogenesis within the Gekkonidae family, which previously had only one species account for spermiogenesis. This type of morphological data may also be valuable in future studies that incorporate the ultrastructural data gathered here for life history, phylogenetics, or histopathological research within squamates. Micrographs and the ultrastructural description of spermiogenesis in the Tokay Gecko are compared in this study to that of the Mediterranean Gecko, *Hemidactylus turcicus*, as well as other saurians to observe and compare similarities and difference between spermatid ultrastructure as they proceed through spermiogenesis.

Keywords: *Spermiogenesis; Ultrastructure; Spermatids; Gekko; Tokay Gecko*

Introduction

Over the last few decades, there has been an increase in studies that provide complete ultrastructural analyses on spermiogenesis within lizards [1-6]. This type of research benefits the field of herpetology and vertebrate biology because of the constant shifts within taxonomic relationships and the level of difficulty in determining evolutionary kinship between species of reptiles, including lizards; thus, spermiogenic studies provide large non-traditional data sets that can be applied to morphological phylogenetic analysis within these taxa [1,7]. Current phylogenic comparisons make the study of sperm maturation and the morphology of spermatozoa important because it has been shown that there is more variation within spermatids and spermatozoa within reptiles and lizards than that of other amniotic taxa [7-9]. By studying spermiogenesis in multiple species of lizards, a large character matrix of comparable ultrastructural features can be created that provides similarities and differences between the spermatids of different species. These ultrastructural character traits may allow for future phylogenetic analysis if larger numbers of families or several species within a genus have comparable data on spermatid development [10].

Currently, there are only 13 studies that have complete ultrastructural descriptions of spermatid development during spermiogenesis in lizards. These studies include the Common Lizard (*Zootoca vivipara*; 11), the Oscillated Skink (*Chalcides ocellatus*; 12), the Amazon Lava Lizard (*Tropidurus torquatus*; 13), the Green Iguana (*Iguana iguana*; 14), the Ground Skink (*Scincella lateralis*; 15), the Jamaican Gray Anole (*Anolis lineatopus*; 4), the Mediterranean Gecko, (*Hemidactylus turcicus*; 16), the Bunchgrass Lizard (*Sceloporus bicanthalis*; 17), the Imbricate Alligator Lizard (*Barisia Imbricata*; 9), the Rosebelly Lizard (*Sceloporus variabilis*; 18), the Eastern Fence Lizard (*Sceloporus undulatus*; 6), the Sandfish Skink (*Scincus scincus*; 19), and the

Prairie Lizard (*Sceloperus consobrinus*; 20). Of these studies listed, only one analysis has been performed in the Gekkonidae family, the Mediterranean Gecko [16]. Within the Gekkota infraorder, one of the largest clades of squamates, there are seven families and more than 1500 species [21]. Since the Tokay Gecko also resides in the Gekkonidae family, the current study will allow the direct comparison of the ultrastructural features of spermiogenesis between two species within the same family of gecko and provide sorely needed data for Gekkota overall.

In a recent survey of spermatid characters within the genus *Sceloporus*, *S. variabilis* spermatids differed in several characteristics when compared to the sibling species *S. undulatus* and *S. bicanthalis*. For example, in *S. variabilis* mitochondria separate the Golgi apparatus from the acrosome during development, which is not detected in the other two sceloporids. Myelin figures observed in acrosome development were common in *S. undulatus* and *S. bicanthalis*, but rare in *S. variabilis* [6,17,18]. These slight differences within the acrosome between species of *Sceloporus*, as well as differences within the midpiece number of mitochondria [10], provide us with specific areas of spermatid morphology that are the focus in the present study. As mentioned earlier, these differences are of importance to our future understanding of the morphological relationships of sperm development within geckos and other lizards. Furthermore, geckos are thought to be a basal taxon within Squamata [22,23] and thus spermatid architecture within geckos could be an important feature within future morphological phylogenetic analyses once enough species data on spermiogenesis is gathered for lizards.

Materials and Methods

Tissue collection

Testicular blocks from adult male *Gekko gecko* (n = 3) were provided by Stanley Trauth from the Department of Biological Sciences, Arkansas State University. These geckos were euthanized using an intraperitoneal injection of sodium pentobarbital following AVMA guidelines and approved by the Arkansas State University Institute of Animal Care and Use Committee where euthanasia occurred. Following euthanasia, testicular tissues were removed and fixed in Trump's glutaraldehyde fixative.

Tissue preparation

Testicular tissues were post-fixed in osmium tetroxide, washed in sodium cacodylate buffer for 30 minutes, and then dehydrated in a graded series of ethanol solutions. Following dehydration, tissues were cleared using propylene oxide and gradually introduced to epoxy resin (Embed 812, EMS diasum) following methods described in Rheubert., *et al.* [20]. Tissues were then embedded in pure epoxy resin and allowed to cure for 48hrs in a vacuum oven. Embedded tissues were sectioned at 1um using a dry glass knife, and sections were placed on slides for tissue verification. Once the tissues were deemed acceptable for active spermiogenesis, they were sectioned at 90 nm using a diamond knife; and sections were placed on to copper grids. Tissues were stained with lead citrate for 5 minutes and uranyl acetate for 30 minutes.

Micrograph acquisition

Tissues were viewed using a Zeiss EM 900 transmission electron microscope and photographed. Negatives was developed in a dark room and scanned at high resolution. Following full analysis, composite micrographs of representative images were created using Adobe Photoshop CS7.

Results

In *Gekko gecko*, spermiogenesis begins with the formation of the acrosome vesicle (Figure 1, Av) and the acrosome granule (Figure 1, Ag) near the apical surface of the nucleus (Figure 1, Nu). Ithas been suggested that the acrosome vesicle and granule form from the transport vesicles arising from the Golgi apparatus (Figure 1B, Ga) [10]. The acrosome vesicle and its granule grow and begin to move toward the nucleus where it creates a deep nuclear indentation and helps forms the mature round spermatid. The acrosome vesicle then flattens the apex of the nucleus of the late-staged round spermatid, leaving the acrosome granule intact (Figure 1E), and begins to compartmentalize into the two main subdivisions of the acrosome complex, the acrosome vesicle and the subacrosomal space.

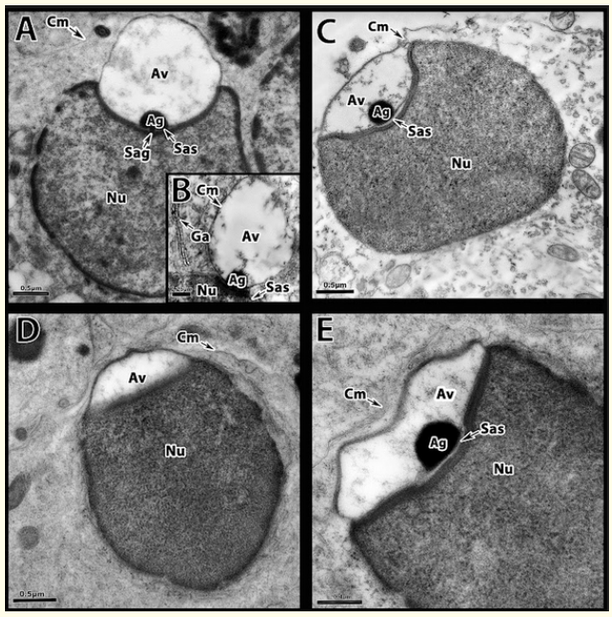

Figure 1: *Early stages of spermiogenesis with the round spermatids within G. gecko testis. Acrosome vesicle (Av); Cell membrane (Cm); Acrosome granule (Ag); Subacrosomal space (Sas); Subacrosome granule (Sag); Nucleus (Nu); Golgi Apparatus (Ga).*

The manchette (Figure 2A and B, Ma), consisting of a series of microtubules, surrounds the circumference of the nucleus (Figure 2, Nu) and is thought to help aid the elongation of the nucleus. The nucleus begins to elongate into a rod-like cylinder and becomes more electron dense (filamentous condensation of chromatin) as it continues through elongation (Figures 2, 3), with the acrosome complex on its apical surface. As the nucleus elongates (Figure 2B, Nu), the flagellum (Figure 2B, Fl) begins to attach itself to the nuclear fossa (Figure 2B, Nf) on the base of the nucleus with the aid of the pericentriolar material (Figure 2B, Pm). While the nucleus is elongating, the acrosome complex continues to compartmentalize and mature into several different structures. The most apical structure is the acrosome cortex (Figure 2C, Acc), followed by the electron dense acrosome medulla (Figure 2C, Acm). At this point in elongation, the perforatorial base plate (Figure 2C, Pbp) is the deeply stained small circular structure at the apical edge of the acrosome lucent ridge (Figure 2C, Alr) and just above the subacrosome cone (Figure 2C, Sac). As seen in figure 3B, closer to the end of elongation, the acrosome perforatorium (Figure 2A and 3B, Pe), will also become visible.

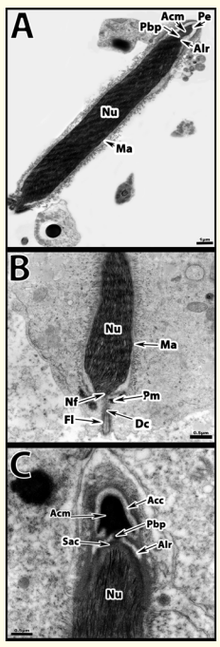

Figure 2: *Middle stages for elongation of the spermatids within G. gecko testis. Acrosome medulla (Acm); Acrosome perforatorium (Pe); Perforatorial base plate (Pbp); Acrosome lucent ridge (Alr); Nucleus (Nu); Manchette (Ma); Flagellum (Fl), Nuclear fossa (Nf); Pericentriolar material (Pm); Distal centriole (Dc); Subacrosome cone (Sac); Acrosome cortex (Acc).*

Near the end of elongation, the nucleus becomes more electron dense (Figure 3, Nu), which can be noted by its intense dark color. In the later stages of elongation, more structures within the acrosome complex will mature until all the structures of completed spermatozoa are present. As shown in an earlier stage of elongation, the acrosome cortex (Figure 3B, Acc) rests at the most apical and lateral points or shoulders of the acrosome. Just beneath the acrosome cortex, is the acrosome medulla (Figure 2B, Acm), which is deep to the cortex. Within the medulla is the acrosome perforatorium and opaque area containing rods of actin (Figure 2B, Pe). The perforatorial base plate (Figure 2B, Pbp) is just below the perforatorium, and above the acrosome lucent ridge (Figure 2B, Alr), which continues down past the nuclear rostrum (Figure 2B, Nr). The nuclear rostrum is the most superior part of the nucleus (Figure 2B, Nu) and is just below the epinuclear lucent zone (Figure 2B, Elz) and is surrounded by the subacrosome cone (Figure 2B, Sac) resting below the acrosome lucent ridge.

During the process of elongation of the nucleus, the flagellum also begins to extend and differentiate. The nuclear fossa (Figure 3C, Nf) resides at the base of the nucleus (Figure 3C, N). The midpiece is the portion of the flagella that has mitochondria (Figure 3C, Mi) surrounding the axoneme. Each mitochondrion is separated by a round electron dense body (Figure 3C, Db). The midpiece is terminated by a dense ring called the annulus (Figure 3C, An). Proximal to the midpiece, is the proximal centriole (Figure 3C, Pc), located near to the nuclear fossa and the distal centriole (Figure 3C, Dc), which is located closer to the mitochondria of the midpiece. The principal piece (Figure 3C, Pp) is the portion of the flagellum below the midpiece and can be identified by its conspicuous fibrous sheath (Figure 3C, Fs).

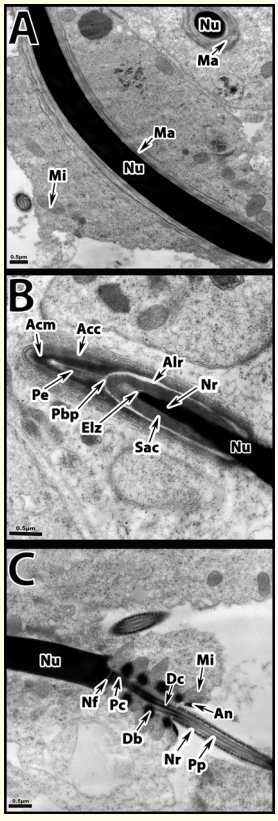

Figure 3: *Late stages of elongation of spermiogenesis within G. gecko testis. Manchette (Ma); Mitochondria (Mi); Nucleus (Nu); Acrosome medulla (Acm); Acrosome perforatorium (Pe); Acrosome cortex (Acc); Perforatorial base plate (Pbp); Acrosome lucent ridge (Alr); Epinuclear lucent zone (Elz); Subacrosome cone (Sac); Nuclear rostrum (Nr); Nuclear fossa (Nf); Proximal centriole (Pc); Distal centriole (Dc); Dense bodies (Db); Annulus (An); Fibrous sheath (Fs); Principal piece (Pp).*

Once the process of elongation is complete, the mature spermatid is ready for spermiation from the seminiferous epithelium (Figure 4). The mature spermatid consists of three main parts, the acrosome complex, the nucleus, and the flagellum. The outermost portion of the acrosome complex, the acrosome cortex (Figure 4A, B, and C, Acc), spans most of the acrosome vesicle. Deep to the acrosome cortex, there are two visibly distinct regions, which are separated by the acrosome lucent ridge (Figure 4). In longitudinal section, the portion of the acrosome above the acrosome lucent ridge is the acrosome medulla (Figure 4A, Acm) and the closely associated acrosome perforatorium (Figure 4A, Pe). The perforatorial base plate (Figure 4, Pbp) is just deep to the acrosome perforatorium and rests just above the epinuclear lucent zone (Figure 4, Elz), which sits on the apical, central, portion of the nuclear rostrum (Fig 4, Nr). Lateral to the epinuclear lucent zone is the subacrosome cone (Figure 4B, Sac), the acrosome lucent ridge (Figure 4B, Alr), and then the acrosome vesicle (Figure 4B, Acc). Distal in the acrosome complex, the nuclear rostrum (Figure 4C, Nf) is the central portion of the transverse section. Outside the rostrum is the subacrosome cone (Figure 4C, Sac), the acrosome lucent ridge (Figure 4C, Alr), and then the acrosome cortex/shoulders (Figure 4C, Acc).

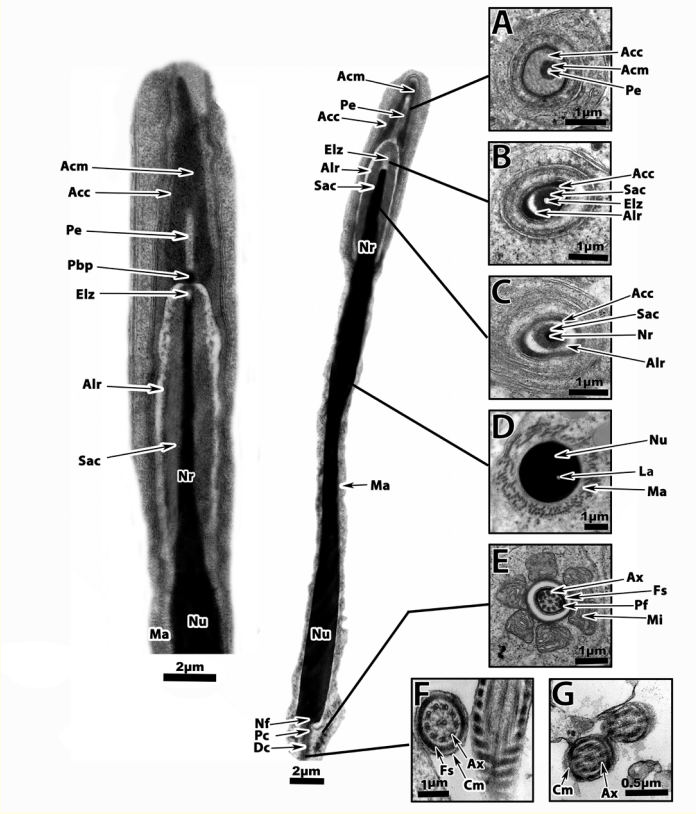

Figure 4: *Ultrastructure of the mature spermatid just before spermiation within Gekko gecko. Acrosome medulla (Acm); Acrosome cortex (Acc); Perforatorium (Pe); Perforatorial base plate (Pbp); Epinuclear lucent zone (Elz); Acrosome lucent ridge (Alr); Subacrosome cone (Sac); Nuclear rostrum (Nr); Nucleus (Nu); Manchette (Ma); Nuclear fossa (Nf); Distal centriole (Dc); Proximal centriole (Pc); Lacuna (La); Axoneme (Ax); Fibrous sheath (Fs); Peripheral fibers (Pf); Mitochondria (Mi), Cell membrane (Cm)*

Unlike the acrosome complex, the nucleus of the spermatozoa does not have much specialization and is homogeneous throughout. Apart from the occasional intranuclear lacunae (Figure 4D, La), which does not span the entirety of the nucleus, the nucleus (Figure 4D, Nu) is the central portion of

the nuclear region, with only the manchette (Figure 4D, Ma) surrounding it. The flagellum starts where the nucleus ends, at the nuclear fossa (Figure 4, Nf), and like the acrosome complex, the flagellum has several differences distally. The connecting piece is closest to the nuclear fossa and includes the proximal and distal centrioles. The proximal centriole rests in the upper portion of the connecting piece and the distal centriole (Figure 4, Dc) resides centrally in thelower portion of the connecting piece. Just below the connecting piece is the midpiece (Figure 4E). The midpiece is lined by irregular, more trapezoidal shaped mitochondria (Figure 4E, Mi). Dense bodies, which could be seen during early elongation are mostly absent in the mature spermatid's midpiece.

Within the midpiece, there is a fibrous sheath (Figure 4E and F, Fs) which contains a central axoneme (Figure 4E, Ax), as well as the continuation of the peripheral fibers three and eight (Figure 4E, Pf). The fibrous sheath begins at tier two of the mitochondria of the midpiece. However, peripheral fibers three and eight terminate within the midpiece and are not present in cross sections of the principal or the end pieces (Figure 4F, G). The fibrous sheath does continue past the terminal end of the midpiece, which marked by the annulus (Figure 3C, An), down into the principal piece (Figure 4F). Just outside the fibrous sheath is the cell membrane (Figure 4F, Cm). The terminal piece of the flagellum, the endpiece (Figure 4G), lacks a fibrous sheath and only contains the axoneme (Figure 4G, Ax) and the cell membrane (Figure 4G, Cm).

Discussion and Conclusion

As mentioned by many researchers, portions of the process of spermiogenesis in squamates is highly conserved [5,7]. The Tokay Gecko, *Gekko gecko*, is no exception, which allows for a comparative analysis of spermatid development between the Tokay Gecko and the Mediterranean Gecko, *Hemidactylus turcicus* [16], a lizard within the same family, Gekkonidae, as well as those in other saurian families. Gekkonidae is typically considered one of the basal or stem taxon within squamates and thus should have many characters that are like that of other lizards and thus homologous and symplesiomorphic (ancestral) [23]. The ultrastructure from this study will aid our understanding of phylogenetic relationships among lizards using spermiogenic characteristics as the present data aids in our comprehension of which characteristics in sperm development are possibly ancestral within these types of morphological data sets.

Spermiogenesis begins with the formation of the acrosome vesicle aided by transport vesicles from the Golgi apparatus, which is very common among squamates [8,9,16,24-28]. Not surprisingly, acrosome development starts near the Golgi in *G. gecko*. The acrosome granule also forms during this time before the vesicle meets the nuclear surface. In many cases the acrosome vesicle arises close to the apical surface of the nucleus. The acrosome vesicle then indents the apical nucleus as it grows and contacts the nuclear surface, as described in *Zootoca vivipara* [11], *S. lateralis* [15], and *C. ocellatus* [12]. The acrosome vesicle forms close to the nuclear membrane and has a deep indention into their nuclei, while in some lizards, like *S. variabilis* [18], the acrosome vesicle forms further away from the membrane and has a very shallow nuclear indentation. *Gekko gecko* also has a deep fossa but appears slightly shallower than that of *H. turcicus* [16]. This could potentially mean that the acrosome vesicle formed further away from the nuclear membrane in *G. gecko.* Also, like *H. turcicus*, the formation of the subacrosomal granule within the subascrosomal space occurs during the round spermatid stage in *G. gecko*. From this analysis, it appears, as stated by Gribbins and Rheubert (5), that this granule may differentiate into the basal plate of the perforatorium based on its location and staining intensity.

Once the process of elongation starts, a manchette surrounds the nucleus. Some differences have been reported within the two types of microtubules, longitudinal microtubules and circum-cylindrical tubules, which, apart from a few species, are moderately equal in numbers within the manchette [10]. Only one reptile has been reported to lack a manchette entirely, *A. lineatopus* [4]. As for *G. gecko,* the manchette appears to be less developed than the manchette of *H. turcicus* [16]. As expected, within *G. gecko* there appears to be equal numbers of both types of microtubules; however, the density and thus number of tubules within each type appears to be less in *G. gecko*. The microtubules are known to aid in elongation [10]. *Sceloporus variabilis* has well developed circum-cylinder microtubules and under-developed longitudinal microtubules, while *S. bicanthalis* has the opposite. In both cases, nuclear elongation appears normal, but with *S.variabilis,* the mature spermatozoa seems to have more curved while in *S. bicanthalis* the spermatozoa seems to be straighter and more wide [15,18]. With respect to these noted differences, it is possible that both types of microtubules aid in elongation, the circum-cylindrical microtubules allowing for a more curved and thinner shape, while the longitudinal microtubules allow for a thicker, straighter shape. These suggestions must be made with caution as there are not many lizards with one type of microtubule dominant over the other

and obviously *A. lineatopus* spermatids still elongate even with the absence of the manchette. In the Tokay Gecko, the nucleus is long and straight but very thin in diameter, supporting that the circum-cylindrical tubules may aid in squeezing the nucleus and decreasing diameter during the elongation process.

During early elongation, it is unusual for the spermatid nucleus to have lacunae, but it becomes more common as it reaches maturity. *G. gecko* has visible lacunae, but *H. turcicus* [16] does not, a character difference that is worth noting between the two species. These intranuclear lacunae are much smaller in *G. gecko* than that described in other species of saurians. Lacunae are common after elongation in many lizards studied to date, as shown in *Scincellis lateralis* [15] and *Sceloporus consobrinus* [10]. Although not shown in all species of lizards and other reptiles, it is possible that the lacunae are there and were not sectioned or missed all together by researchers. This is possible because these lacunae do not span the entirety of the nucleus [10].

Further into the process of elongation, the acrosome complex continues to compartmentalize and become several parts, while the flagellum also develops into several regions. The acrosome complex is very similar in most previous studies of spermiogenesis within reptiles, but the developing and mature flagellum tends to vary more greatly. The proximal centriole, part of the connecting piece, is where the flagellum connects to the nuclear fossa [10]. This is common in all species of lizard including *H. turcicus* [16], *S. undulates* [6], and *S. scincus* [19]. Although this is common in lizards and other reptiles, the attachment of the flagellum has also been noted to occur in the early stages of elongation in at least three species: *S. variabilis* [18], *S. bicanthalis* [17], and *S. lateralis* [15]. Other studies suggest the proximal centriole appears during the round spermatid stage of development [10]. Since the proximal and distal centrioles are responsible for flagellar development and the flagellum shows up in all amniotes studied to date, then it is safe to hypothesize that the centrioles align themselves with the nuclear fossa during the cytoplasmic shift [10] that occurs during the late round spermatid stage, which of course is the case in *G. gecko*.

In lizards, the midpiece is often shown to have only four to six tiers of mitochondria [10]. In *G. gecko*, there are typically five to six tiers of mitochondria. Although the number of mitochondria in *G. gecko* is a common trait that coincides with most lizards, it is significantly different from what was seen in *H. turcicus.* In *H. turcicus,* Rheubert., *et al.* [16] discovered that the midpiece was longer than most lizards, with twelve mitochondrial tiers, which is more commonly observed in snakes. This is uncommon in lizards, and since it is not the case for *G. gecko,* it adds to the possibility that the elongated midpiece is a possible autapomorphy for *H. turcicus* [10,16]. However, caution must be maintained as only two species of geckos to this point have complete data on spermiogenesis. Further studies might show that this elongated midpiece could be a synapomorphy of closely related sibling species of *Hemidactylus.* There are dense bodies associated with these mitochondria during the mid-elongation stage, but these bodies tend to disappear from the midpiece at the maturation point of the elongating spermatid in *G. gecko*. This is an interesting characteristic that has not been described before in lizards and requires further investigation in saurians and appears to be another significant difference between midpiece development in the Tokay versus the Mediterranean Gecko. Apart from the dissimilarities in length of the midpiece, other differences within the midpiece occur within the surrounding mitochondria. In *G. gecko,* the mitochondria in cross section are trapezoidal in shape. This is less common but has also been seen in *Iguana iguana* [3]. The more common shape of the mitochondria is more round or ovular. These can be seen in *H. turcicus* [17], *Crotaphytus bicintores* [29], and *S. bicanthalis* [17]. Lastly, the peripheral fibers 3 and 8 commonly extend past the centrioles within lizards and other reptiles [7]. However, they extent of penetration of these fibers past the midpiece depends on the species [24]. Interestingly, *G. gecko* peripheral fibers 3 and 8 terminate at the end of the midpiece and do not extend into the principal piece. This feature of the peripheral fibers has not been described in any other reptilian species to date as far as the authors know. Though this is an observable difference between *G. gecko* and *H. turcicus,* as well as other lizards, the importance of this character is hard to determine at this time as not enough geckos or other taxa of lizards have been studied to date.

The principal piece and the end piece are very similar within squamates. All squamates studied to date, including *G. gecko,* have a fibrous sheath around flagellar axonemes, starting just within the midpiece and this is a synapomorphy of squamates [24]. The fibrous sheath then ends where the end piece starts at the flagellar terminus in all lizards and end piece contains just a cell membrane surrounding the axoneme. The fibrous sheath begins at

mitochondria tier two in *G. gecko*. This is common, as almost all lizards have their fibrous sheath beginning at either tier two or three of the mitochondria, except for *S. lateralis* [15], which began at tier one. In contrast to *G. gecko,* the fibrous sheath of *H. turcicus* begins at tier three [16].

This is the first study recorded regarding spermiogenesis in the genus *Gekko*, and the second in the Gekkonidae family. As hypothesized, there are many similarities between the Tokay gecko, *G. gecko*, and the Mediterranean Gecko, *H. turcicus,* as well as within other reptiles and lizards. Some of these shared characters include the formation of the acrosome vesicle with the Golgi apparatus, the deep indent of the acrosome vesicle into the nucleus to form a round spermatid, and the presence of the fibrous sheath in the midpiece and the principal piece. Geckos also are thought to be a stem taxon within Squamata [30] and thus these shared characteristics for sperm development could be synapomorphies specifically between *G. gecko* and *H. turcicus,* or as suggested by Gribbins and Rheubert [10], symplesiomorphies for squamates as whole. Also, as expected, there were some spermiogenic differences observed between the geckos studied to date. These differences include the length and number of mitochondria of the midpiece, the shape of the mitochondria in the midpiece, the mitochondrial tier in which the fibrous sheath began, and the lacunae in the nucleus. The most interesting difference found between *G. gecko* and other lizards is the disappearance of dense bodies in the mature midpiece. This has not been recorded in any other lizard to date and is a possible autapomorphy of *G. gecko,* or a possible synapomorphy of the genus *Gekko*, differentiating between these possibilities is impossible presently as there are too few spermiogenic studies to date within this genus.

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Conflict of Interest

We declare there are no conflicts of interest that the authors know of.

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