

The ultrastructure of the spermatozoon of the northern water snake, *Nerodia sipedon* (Colubridae, Serpentes), with phylogenetic considerations

B.G.M. JAMIESON

Zoology Department, University of Queensland, Brisbane 4072, Australia

AND

L. KOEHLER

Department of Biology, Central Michigan University, Mount Pleasant, MI 48859, U.S.A.

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The ultrastructure of the spermatozoon of *Nerodia sipedon* conforms closely to that of other described snake sperm: it is filiform; the acrosome vesicle is in the form of a hollow, concentrically zoned cone that basally overlies a subacrosomal cone which invests the tapered anterior end of the nucleus; the putative perforatorium is a slender rod extending anteriorly from the subacrosomal cone; the midpiece contains dense bodies and mitochondria; the axonemal fibrous sheath extends anteriorly into the midpiece (squamate autapomorphy); 9 peripheral dense fibres surround the distal centriole and the axoneme in the midpiece, of which fibres adjacent to 3 and 8 are enlarged; and the endpiece lacks peripheral fibres and the fibrous sheath. The midpiece is very long (a synapomorphy of the Serpentes) and is surrounded by a multilaminar membrane (an autapomorphy). In the squamates, only snakes, including *N. sipedon*, retain microtubules external to the plasma membrane of the mature spermatozoon. Helically arranged zigzag mitochondria are shared (probably homoplasmically) with iguanid sperm. A poorly developed "stopperlike" putative perforatorial base plate in *N. sipedon*, unknown in other snakes, is questionably homologous with that of gekkonids. An electron-lucent space caps the nuclear point, as in the snakes *Boiga irregularis* and *Stegonotus cucullatus* and in some other squamate orders.

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Le spermatozoïde de *Nerodia sipedon* est conforme, par son ultrastructure, à celui d'autres serpents : il est filiforme, la vésicule de son acrosome affecte la forme d'un cône peu profond à zones concentriques qui recouvre à la base un cône subacrosomien, lequel pénètre l'extrémité antérieure amenuisée du noyau, le bourgeon de son perforatorium est un bâtonnet mince qui a son origine dans le cône subacrosomien et se prolonge vers l'avant, sa région médiane contient des corpuscules et des mitochondries opaques, le manchon fibreux de son axonème se prolonge vers l'avant en une pièce médiane (autapomorphie des squamates), 9 fibres périphériques opaques entourent son centriole distal et la partie médiane de son axonème dont les fibres adjacentes 3 et 8 sont élargies, sa partie terminale ne comporte ni fibres périphériques, ni manchon fibreux. La région médiane est très longue (synapomorphie chez les Serpentes) et est entourée d'une membrane multilaminaire (autapomorphie). Chez les squamates, seuls les serpents, y compris *N. sipedon*, ont encore des microtubules extérieurs à la membrane plasmique dans le spermatozoïde à maturité. Des mitochondries en zigzag disposées en hélices sont présentes à la fois chez les squamates et chez les iguanidés (probablement une homoplasie). Une plaque basale probablement perforatrice, en forme de « bouchon », existe chez *N. sipedon* mais chez aucun autre serpent et est peut-être l'homologue d'une structure semblable chez les gekkonidés. Un espace translucide aux électrons recouvre la pointe nucléaire, comme chez les serpents *Boiga irregularis* et *Stegonotus cucullatus* et chez d'autres ordres de squamates.

[Traduit par la Rédaction]

Introduction

Several publications have dealt with the ultrastructure of components of developing or mature snake spermatozoa. Austin (1965) gave a detailed account of the fine structure of the sperm tail in *Lampropeltis getulus*, *Coluber constrictor*, *Drymarchon corais*, *Crotalus adamanteus* (Colubridae), *Micrurus fulvius* (Elapidae), and *Constrictor* sp. (Boidae). Boissin and Mattei (1965, 1966) described spermiogenesis in *Python sebae* (Boidae), Hamilton and Fawcett (1968) gave details of the neck and midpiece of *Lampropeltis getulus* and *Constrictor constrictor* sperm, Saita et al. (1988) described spermiogenesis in *Coluber viridiflavus*, and Phillips and Asa (1993) very briefly described the formation of the midpiece with reference to the behaviour of the annulus in the sperm of the colubrid *Masticophis flagellum flagellum*. The ultrastructure of the entire spermatozoon has been described in only three works. Furieri (1970) gave a general account for four species of Colubridae, *Coluber viridiflavus viridiflavus*, *Natrix tessellata tessellata*, *Natrix natrix*, and

Coronella austriaca, and one species of Viperidae, *Vipera aspis aspis*. The latter species had also been the subject of a brief paper by Furieri (1965). Oliver et al.¹ have described the ultrastructure of the sperm of four Australian species: *Boiga irregularis* (Merrem, 1802) and *Stegonotus cucullatus* (Dumeril, Bibron and Dumeril, 1854) (Colubridae); *Oxyuranus microlepidotus* (McCoy, 1879) (Elapidae); and *Aspidites melanocephalus* (Kreffft, 1864) (Boidae). The present work describes the sperm of *Nerodia sipedon* for its interest per se and with a view to establishing characters of utility for future cladistic analysis of the Squamata.

Material and methods

Males of *Nerodia sipedon* were collected on Beaver Island in northern Lake Michigan during the breeding season. They were euthanized with sodium pentobarbital and the vasa deferentia were

¹S.C. Oliver, B.G.M. Jamieson, and D.M. Scheltinga. The ultrastructure of the spermatozoa of Squamata. II. Agamidae, Varanidae, Colubridae, Elapidae, and Boidae (Reptilia). Submitted for publication.

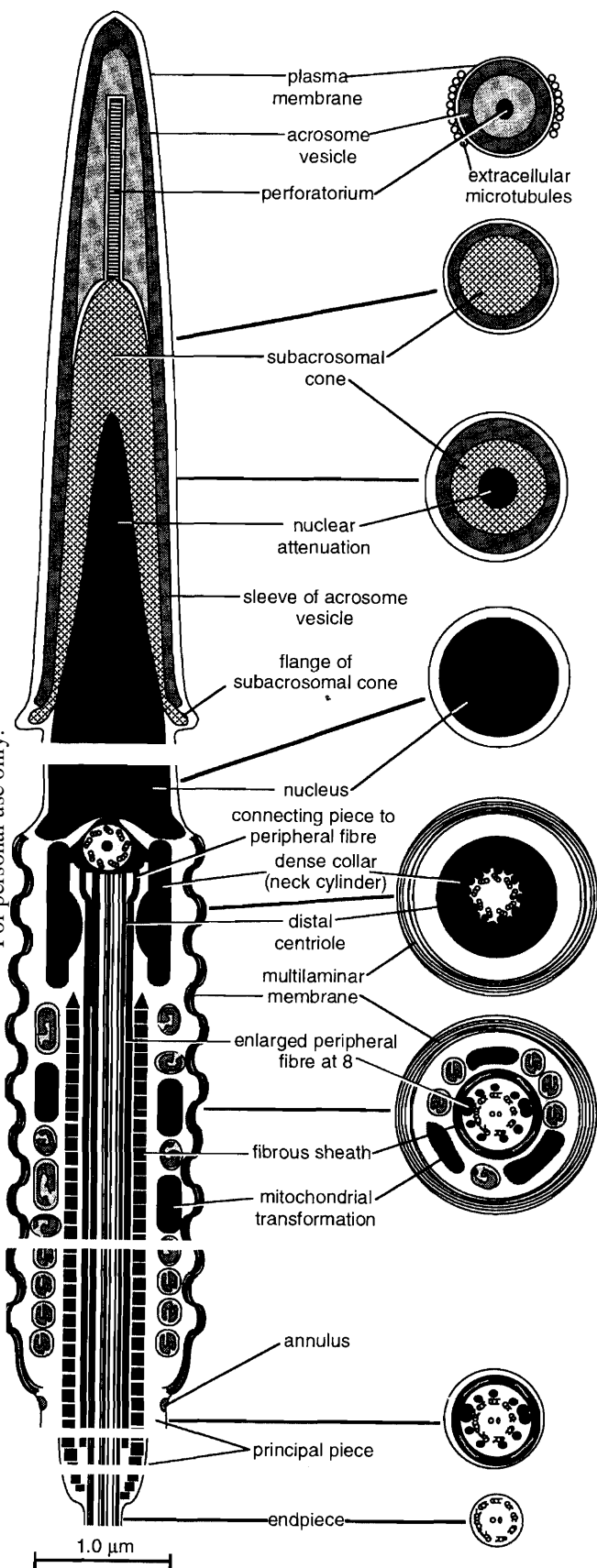


FIG. 1. Diagram of the sperm of a snake, representing all investigated snakes in the families Colubridae, Elapidae, and Boidae. The central element in the proximal centriole has not been seen in *N. sipedon*, but is known in another colubrid and a boid. Note the multilaminar membrane around the midpiece, an autapomorphy of the Serpentes.

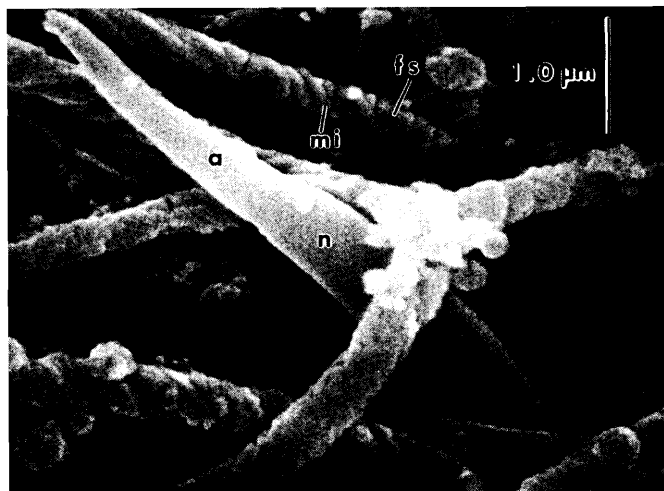


FIG. 2. Scanning electron micrograph of spermatozoa of *Nerodia sipedon*, showing the acrosome and nucleus and the spiral course of the mitochondria around the fibrous sheath. *a*, acrosome; *fs*, fibrous sheath; *mi*, mitochondrion; *n*, nucleus.

removed. Fixation was in 2% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2. Tissues were postfixed in 1% buffered osmium tetroxide, dehydrated through ethanol and propylene oxide, and embedded in Epon. Sections were cut with diamond knives on an LKB 2128 UM IV microtome. Thin sections (50–80 nm) were collected on carbon-stabilized, collodion-coated, 200 μm mesh copper grids, rinsed in distilled water, and stained for 30 s in Reynold's lead citrate and then in 6% aqueous uranyl acetate for 1–4 min and for a further 1–2 min in lead citrate before final rinsing. Electron micrographs were taken on a Hitachi 300 electron microscope at 75 kV and a JEOL 100-s electron microscope at 60 kV.

Results

A generalized serpent sperm is illustrated diagrammatically in Fig. 1. It is based on the description of the sperm of *Boiga irregularis* and *Stegonotus cucullatus* (Colubridae), *Oxyuranus microlepidotus* (Elapidae), and *Aspidites melanocephalus* (Boidae) by Oliver et al. (see footnote 1) and *Nerodia sipedon* (present account) and is applicable to all investigated snakes. The structure conforms closely to that typical of squamates (see Jamieson et al. 1995), therefore the description below is brief but stresses departures from general squamate spermatozoal ultrastructure.

The spermatozoa of *N. sipedon* are filiform. The head is long and attenuated and is slightly curved, as is shown by scanning electron microscopy in Fig. 2 and by transmission electron microscopy in Fig. 3A. A major departure from squamates other than snakes is the great elongation of the midpiece.

Acrosome complex

The acrosome vesicle is divisible into cortex and medulla (Fig. 3A). The acrosome complex is an elongate cone, as evidenced by longitudinal and transverse sections (Figs. 3A, 3C, 3D, 3E), with a length of 3.4 μm. The subacrosomal space within the acrosomal medulla contains the putative perforatorium (Figs. 3A, 3C). The putative perforatorium is cross-striated (Figs. 3A, 3L). A poorly defined, stopperlike dense area at the base of the putative perforatorium, occupying the thickness of the apical wall of the subacrosomal cone, may be the equivalent of the perforatorial base plate

seen in some other squamates, though not previously described for snakes, but it is poorly developed (see Discussion). The subacrosomal cone does not show the paracrystalline structure characteristic of squamate sperm, but the absence of this structure requires confirmation. The subacrosomal cone projects as a posterolateral flange behind the base of the acrosome vesicle (Fig. 3A) as in other snakes. Extracellular microtubules are present in the vicinity of the acrosome and nucleus and are seen in longitudinal section in Fig. 3A.

Nucleus

The nucleus, 5.5 μm in total length, has the form of an elongate cylinder (Figs. 2, 3A), wider at its base (0.55 μm) than at the nuclear shoulders (0.50 μm). It is circular in section throughout its length (Figs. 3E–3G). The chromatin is strongly electron dense and condensed and lacks lacunae. Anteriorly the nucleus extends as a narrow cone, the nuclear attenuation (nuclear point), 1.5 μm long, being within the acrosome complex (Fig. 3A). The nuclear shoulders are well developed but smoothly sloping. The nuclear fossa is shallow and accommodates only the anterior fraction of the proximal centriole. An elongate electron-lucent space caps the nuclear point (Figs. 3A, 3L).

Neck region

The neck (Figs. 3B, 3O, 3M) contains a proximal centriole titled relative to the distal centriole (Fig. 3O). The proximal centriole is surrounded by a narrow zone of electron-dense material which is continuous (Figs. 3M, 3O) with outwardly arched thick fibres that pass caudally, contacting the distal centriole and continuing (Figs. 3B, 3O) as the dense peripheral fibres (coarse fibres) which are seen in transverse section in Figs. 3H–3J. The existence of a laminar structure projecting laterally from the pericentriolar dense area surrounding the proximal centriole, and seen in sphenomorph skinks, is questionable, but there is a suggestion of it in cross sections of the proximal centriole in the anterolateral aspects of the latter (Fig. 3B). No central dense structure or microtubules have been observed in the axis of the proximal centriole.

A dense collar (the neck cylinder of Austin 1965; Hamilton and Fawcett 1968) surrounds the distal centriole (Figs. 3B, 3H). The inner surface of the ring is scalloped, owing to the 9 dense peripheral fibres, which are associated with the triplets of the centriole and the doublets of the axoneme (Fig. 3H). No mitochondria indent the outer face of the collar, in contrast with *A. melanocephalus*. The material of the collar resembles that of dense bodies (mitochondrial transformations), with which it may be homologous. The 9 dense peripheral fibres (coarse fibres) are associated with the A tubules of the 9 triplets of the distal centriole and are continuous radially with dense areas on the inner side of and displaced slightly clockwise relative to the triplets (Fig. 3H). In addition, there is a dense area, presumably a fibre, associated with one of the central elements. The central elements in the axoneme are two singlet microtubules, but in the distal centriole these are represented by a triplet (Fig. 3H);

see Discussion). The distal centriole does not project into the fibrous sheath.

Midpiece

The midpiece includes the neck, described above. It consists of mitochondria, the dense collar and dense bodies (mitochondrial transformations; Oliver et al., see footnote 1), and the greater part of the axoneme, with its fibrous sheath, and ends posteriorly with the annulus. A distinctive feature of the midpiece of *N. sipedon*, like that of other Serpentes, in addition to its great length, is the peculiar system of enclosing membranes. In addition to the plasma membrane there is a multilaminar membrane, which in longitudinal section is tortuous. It extends from the base of the nucleus to the posterior end of the midpiece immediately anterior to the annulus (Figs. 3B, 3I, 3O, 3P). The annulus is weakly developed (Fig. 3P).

In the transverse section of the midpiece (Fig. 3I), the mitochondria present mostly circular, sometimes conjoined, profiles, numbering 10 or fewer, in single file interspersed with stout dense bodies in the form of short crescents or oblong ellipsoids. The appearance in sagittal longitudinal section is similar, with usually round mitochondrial profiles interspersed in single file with stout rodlike dense bodies (Figs. 3O, 3P). However, by scanning electron microscopy (Fig. 2) and in parasagittal longitudinal sections taken near the plasma membrane (Fig. 3N), the mitochondria are seen to be zigzag or more or less straight tubular structures that have a strong tendency to run spirally around the fibrous sheath.

As in all squamates, the fibrous sheath extends anteriorly into the midpiece (Figs. 3B, 3I, 3O, 3P). It encloses the axoneme and associated peripheral fibres. As usual, the fibres at doublets 3 and 8 are thicker than the other peripheral fibres and are apposed to the fibrous sheath at some distance from the doublets with which they are associated (Fig. 3I).

Principal piece

The principal piece consists of the continuation, behind the midpiece, of the axoneme with its surrounding fibrous sheath and plasma membrane. The plasma membrane becomes closely associated with the axoneme immediately behind the annulus, there being none of the wide separation that characterizes the commencement of the principal piece in at least skinks, teiids, agamids, and varanids (Jamieson and Scheltinga 1993; Oliver et al., see footnote 1). Only the enlarged peripheral fibres 3 and 8 persist conspicuously into the principal piece (Fig. 3J), but vestiges of the other fibres are present. Further posteriorly the fibrous sheath is not accompanied by peripheral fibres.

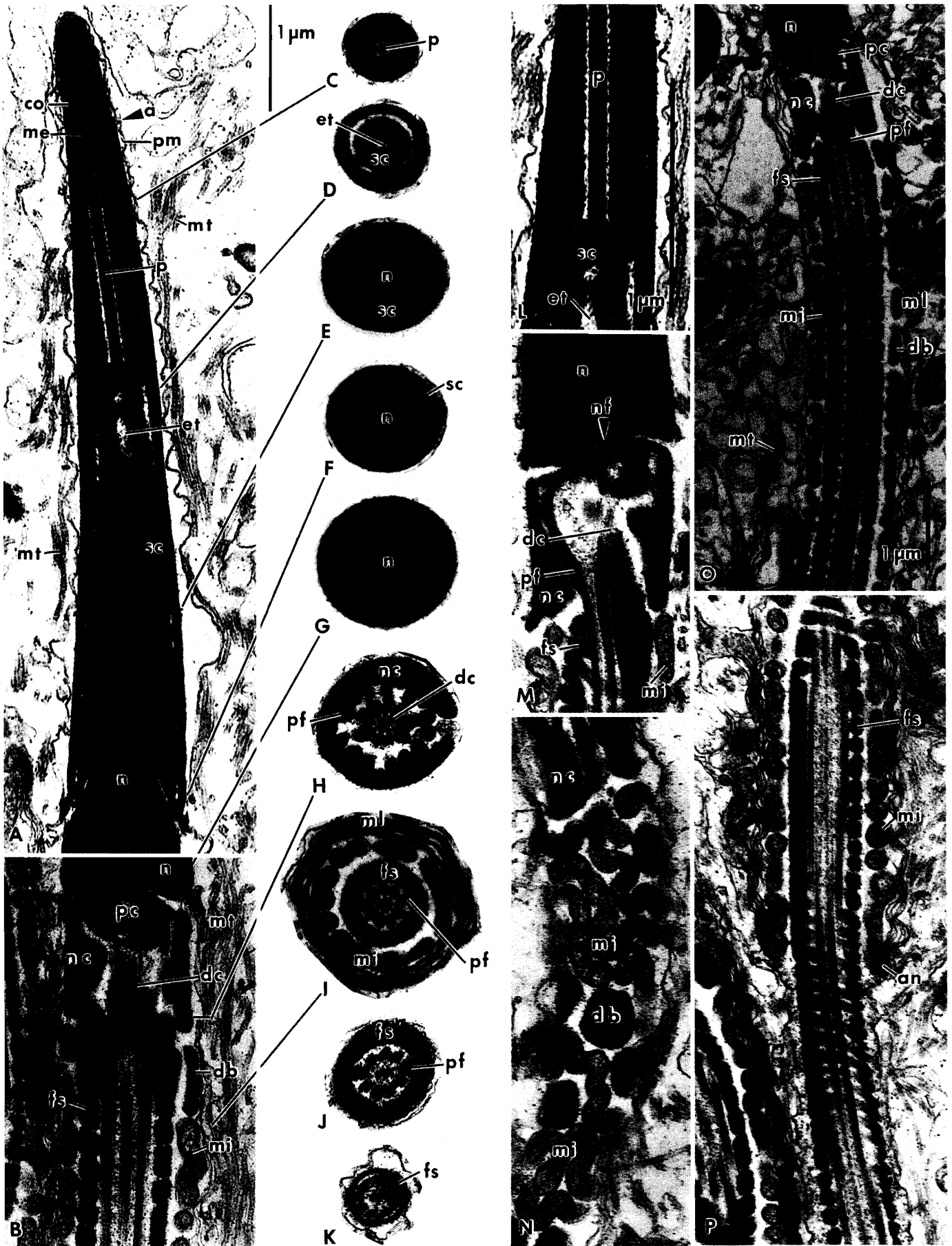
Endpiece

The endpiece (Fig. 3K) consists of the axoneme and plasma membrane but lacks peripheral fibres and the fibrous sheath.

Discussion

The ultrastructure of the spermatozoon of *Nerodia sipedon* conforms closely to that of other described snake sperm

FIG. 3. *Nerodia sipedon*, longitudinal sections (A, B) and corresponding transverse sections (C–I) of spermatozoa from the anterior end to the anterior region of the midpiece. (J) Transverse section of the axoneme and fibrous sheath posterior to the midpiece. (K) Transverse section of the endpiece (L). Detail of A, showing the transverse striation of the putative perforatorium. (M) Detail of the neck region in longitudinal section. (N) Tangential section of midpiece, showing the zigzag and helical arrangement of the tubular mitochondria. (O) Longitudinal section of the neck region, showing the oblique proximal centriole. (P) Posterior end of the midpiece and anterior principal piece, showing the annulus. *a*, acrosome; *an*, annulus; *co*, cortex of acrosome; *db*, dense body (mitochondrial transformation); *dc*, distal centriole; *et*, electron-lucent space; *fs*, fibrous sheath; *me*, medulla of acrosome; *mi*, mitochondria; *ml*, multilaminar membrane; *mt*, microtubules; *n*, nucleus; *nc*, neck cylinder; *nf*, nuclear fossa; *pc*, proximal centriole; *pf*, peripheral dense fibre (coarse fibre); *pm*, plasma membrane; *sc*, subacrosomal cone.



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(Austin 1965; Hamilton and Fawcett 1968; Phillips and Asa 1993; Furieri 1970; 1965; Oliver et al., see footnote 1). It presents features common to squamate sperm. It is filiform. The acrosome vesicle is the form of a hollow, concentrically zoned cone which basally overlies a subacrosomal cone that invests the tapered anterior end of the nucleus. The putative perforatorium is a slender rod extending anteriorly from the subacrosomal material. The midpiece contains dense bodies (mitochondrial transformations) in addition to the mitochondria. The fibrous sheath surrounding the axoneme extends anteriorly into the midpiece (squamate autapomorphy). Nine dense peripheral fibres accompany the triplets of the distal centriole and the doublets of the axoneme in the midpiece. The fibres adjacent to doublets 3 and 8 are enlarged, each as a double structure associated with the fibrous sheath. The endpiece lacks peripheral fibres and the fibrous sheath. The presence of a multilaminar membrane investing the midpiece is confirmed as a unique synapomorphy (autapomorphy) of the Serpentes. The membranes appear to be less numerous in *N. sipedon* than in *Aspidites melanocephalus* and *Stegonotus cucullatus* (Oliver et al., see footnote 1). The great length of the midpiece in snake sperm was noted by Furieri (1970), who proposed that it would more appropriately be termed the mitochondrial sheath, and it constitutes a further synapomorphy. In the squamates, only snakes, as here shown for *N. sipedon*, retain microtubules external to the plasma membrane of the mature spermatozoon, but this represents retention of a spermiogenic condition widespread in squamates. Of the sperm of other squamates, the helically arranged zigzag mitochondria are known only in those of iguanids, briefly described by Furieri (1974), and this condition is probably homoplastic between the two groups.

It is uncertain whether the poorly discernible structure that penetrates the apical wall of the subacrosomal cone in *N. sipedon* is homologous with the similarly stopperlike base plate described for the sperm of the gekkonids *Lygodactylus picturatus* (see Furieri 1970) and *Heteronotia binoei* (see Jamieson et al. 1995), or the ellipsoidal perforatorial base plate that surmounts the apex of the cone in scincids of the *Eugongylus* group (Jamieson and Scheltinga 1994; Jamieson et al. 1995), the agamid *Pogona barbata*, and the varanid *Varanus gouldii* (Oliver et al., see footnote 1). No perforatorial base plate has been observed in other snakes. An electron-lucent space caps the nuclear point in *N. sipedon*, as in *Boiga irregularis* and *S. cucullatus*. It has not been seen in the other investigated snake species but is well developed in the teiid *Cnemidophorus sexlineatus* (see Newton and Trauth 1992), scincids of the *Eugongylus* group, the pygopod *Lialis burtonis* (see Jamieson et al. 1995), and the gekko *Lygodactylus picturatus* (see Furieri 1970). The presence of a laminar structure anterolateral to the proximal centriole has been tentatively demonstrated in *N. sipedon*, as in *S. cucullatus* and *B. irregularis*, but if present it does not show the clear lamination described for the sphenomorph skinks (Jamieson and Scheltinga 1993; Jamieson et al. 1995). A single dense element observed in the central axis of the proximal centriole in *Lampropeltis getulus* by Hamilton and Fawcett (1968) and *Aspidites melanocephalus* by Oliver et al. (see footnote 1) has not been seen in *N. sipedon*.

The account of the structure of the neck and midpiece above agrees closely with the detailed description of *L. getulus*

given by Hamilton and Fawcett (1968). They note the occurrence of multiple layers of membranes in *Lampropeltis* sp. and *Constrictor* sp. and suggest that these provide endogenous phospholipid that could be utilized as a source of energy for motility. They also observe the presence, unknown in other vertebrates, of extracellular microtubules, observed here in *N. sipedon*. The dense area associated with the central microtubules of the distal centriole in *N. sipedon* and other snakes (Austin 1965; Hamilton and Fawcett 1968; Oliver et al., see footnote 1) has been demonstrated for the skinks *Nangura spinosa* (see Jamieson and Scheltinga 1993), *Tiliqua scincoides*, and less certainly *Carlia pectoralis* (see Jamieson and Scheltinga 1994), and the gekko *Heteronotia binoei* (see Jamieson et al. 1995) and may well be more widely demonstrated in squamates when suitable sections of the centriole are obtained.

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