Ultrastructure of Spermatozoa of Australian Blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata): First Observations on the Mature Spermatozoon of Scolecophidian Snakes

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ABSTRACT

The structure of mature, epididymidal spermatozoa of three species of Australian blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata) was investigated by a combination of scanning and transmission electron microscopy. Special attention was paid to features of potential phylogenetic significance, in the hope that aspects of sperm morphology might shed light on the evolutionary affinities of this highly specialized and enigmatic group of reptiles. Spermatozoa of *Ramphotyphlops* spp. share many features in common with other squamates (lizards and snakes), but display some striking features not known in any other taxa. Special derived features shared by *Ramphotyphlops* spp. and other Squamata include: a paracrystalline subacrosomal cone; dense intermitochondrial "rings" or "plaques"; a relatively inconspicuous annulus; an anteriorly extensive fibrous sheath which penetrates the midpiece; and the absence of an endonuclear canal. Additional derived features which may be shared exclusively by *Ramphotyphlops* spp. and other snakes include: the great elongation of the midpiece; the forward prolongation of the fibrous sheath to the level of the neck; the presence of a distinct "neck cylinder"; the extremely elongate form of the mitochondria; and the presence of a multi-layered midpiece plasma membrane. These cladistic interpretations lend support to the current taxonomic placement of Typhlopidae within Squamata, and specifically within Serpentes. Several highly distinctive features of *Ramphotyphlops* spermatozoa are identified, the most striking being the extreme length and regular zig-zagging arrangement of the mitochondria.

RÉSUMÉ

Ultrastructure des spermatozoïdes des serpents aveugles australiens, *Ramphotyphlops* spp. (Typhlopidae, Squamata): premières observations sur le spermatozoïde mûr des Serpents Scolecophidiens.

La structure des spermatozoïdes mûrs épididymaires de trois espèces de serpents aveugles australiens, *Ramphotyphlops* spp. (Typhlopidae; Squamata) a été étudiée en microscopie électronique à balayage et à transmission. Une attention particulière a été portée aux détails d'importance phylogénique potentielle, dans l'espoir que des caractéristiques de la morphologie du spermatozoïde puissent éclairer les affinités évolutives de ce groupe de Reptiles, très évolués et

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énigmatiques. Les spermatozoïdes de *Ramphotyphlops* spp. possèdent de nombreux caractères en commun avec les autres Squamata (lézards et serpents), mais montrent quelques aspects originaux inconnus dans les autres taxons. Les caractères dérivés spéciaux partagés par *Ramphotyphlops* spp. et les autres Squamata comprennent: un cône subacrosomien paracristallin, des "anneaux" ou "plaques" denses intermitochondriaux, un annulus relativement peu apparent, une gaine fibreuse s'étendant vers l'avant et qui pénètre la pièce intermédiaire et l'absence de canal endonucléaire. Les caractères dérivés supplémentaires qui pourraient être communs seulement à *Ramphotyphlops* spp. et aux autres Serpents comprennent: le grand allongement de la pièce intermédiaire, le prolongement vers l'avant de la gaine fibreuse jusqu'au niveau du cou, la présence d'un "cylindre du cou" distinct, la forme extrêmement allongée de la mitochondrie et la présence d'une membrane plasmique à plusieurs couches dans la pièce intermédiaire. Ces interprétations cladistiques soutiennent le placement actuellement admis des Typhlopidae dans les Squamata, et spécifiquement dans les Serpentes. Quelques caractères très originaux du spermatozoïde de *Ramphotyphlops* sont identifiés, dont le plus frappant est la longueur extrême et la disposition régulièrement zigzaguante des mitochondries.

Despite the pioneering efforts of FURIERI [6] to promote interest in the ultrastructure of reptilian spermatozoa, many significant gaps in taxonomic coverage still mar our understanding of spermatozoal structure and evolution in this large and important group of vertebrates. This is particularly marked within the taxonomically diverse Order Squamata (collectively, the lizards and snakes), for which information is available for less than one third of the extant families.

In this paper we report the first observations on spermatozoa from members of the Family Typhlopidae, a group commonly known as blindsnakes. These highly specialized, fossorial snakes are represented on all continents, with major radiations in Australia, Africa and Central America. Together with members of at least two other families, Leptotyphlopidae and Anomelepidae, blindsnakes are generally included in the higher taxon Scolecophidia, as one of the two major groups of living snakes [4, 13]. However, some authors have suggested a possible closer relationship with certain groups of lizards, thereby challenging the monophyly of both Scolecophidia and Serpentes [9, 14]. Even more remarkably, one worker has questioned the placement of Typhlopidae within Squamata, based on peculiar features of the male reproductive tract [17].

Material examined in this study comes from three species of the Australasian blindsnake genus *Ramphotyphlops*, which includes approximately 50 species spread between Malaysia and southeastern Australia. This study forms one part of a wider comparative survey of spermatozoal morphology among Australian and Indonesian squamates, from which we hope not only to fill many of the major gaps in taxonomic coverage, but also to further knowledge of squamate phylogeny and classification.

MATERIAL AND METHODS

Spermatozoal samples were collected from three *Ramphotyphlops* species. The associated voucher specimens are lodged in the collection of the Western Australian Museum as follows: *Ramphotyphlops waitii* 114891 Waggrakine Pass, W. A. and 119239 Walyunga National Park, W. A.; *R. endoterus* 115000 38 km ENE Laverton; *R. australis* 115125 11 km ESE North Dandalup. Samples from all three species were processed for TEM and for *R. waitii* also for SEM.

Animals were euthanased with sodium pentabarbitol (Nembutal). Immediately following death, samples of testis and epididymides were removed, cut into small pieces and fixed for 30-60 minutes in Karnovsky's fixative with picric acid added. They were washed and stored in 0.1 M cacodylate buffer pending further treatment.

Samples for TEM were postfixed in 1% osmium tetroxide in cacodylate buffer, stained en bloc in 4% alcoholic uranium acetate, dehydrated in alcohol and acetone, and embedded in SPURR resin. Sections were stained in 4% alcoholic uranium acetate and lead citrate, and examined using a JEOL 100 TEM.

For SEM a drop of sperm released from the tissue was placed on a cover slip coated with platinum and then with poly-L-lysine. Samples were postfixed in osmium tetroxide in cacodylate buffer, dehydrated, critical point dried in CO_2, and examined using field emission, high resolution JEOL JSM-6000, at the accelerating voltage of 3 kV.

Measurements, which are of *R. waitii*, were taken from micrographs and using the 40/0.7 and 100/1.3 objective lenses of a Leitz Diaplan microscope with a video camera attached and connected to a MD30 Automated Image Analysis System (Leading Edge, Australia). However, owing to the extreme length of the spermatozoon and the difficulty of distinguishing the midpiece and principal piece junction in whole-sperm light microscopy, the measurements given in this paper should be regarded as approximate only.

OBSERVATIONS

The mature spermatozoon in all three species is clearly very elongate, with lengths up to 179 µm recorded for *R. waitii* under the image analyser.

Head

The sperm head is elongate and essentially cylindrical in form, but with a slight flexion just behind the base of the acrosomal cap. Measurements taken from micrographs indicate a total head length of 15 µm and a basal diameter of 0.6 µm. The nucleus consists of a basal cylindrical portion measuring 10 µm in length, and a tapered apical portion which projects 2.1 µm into the acrosomal cap (Fig. 1a). A distinct "nuclear shoulder" marks the transition from basal to apical regions (Fig. 1a, h). The nuclear fossa is broad and relatively shallow except in the very centre above the basal plate where it is more deeply excavated (Fig. 2c). The nucleus is evenly electron-dense and is not penetrated by an endonuclear canal.

The acrosomal complex forms a complete cap which sits atop the tapered portion of the nucleus (Fig. 1a); it measures 3.8-4.0 µm from nuclear shoulder to acrosomal tip. In common with other reptiles, the acrosome consists of three main components: an inner subacrosomal cone; an outer acrosomal sleeve; and a central, rod-like perforatorium (Fig. 1a). The perforatorium projects from the tip of the subacrosomal cone as a slender transversely striated rod (Fig. 1a, f-g). In *R. waitii* it measures 1 µm. As shown by comparison of Figs 1f and 1g the perforatorium varies in length between the species, being a much longer structure in *R. waitii* than in *R. endoterus*. Basally it is embedded within the subacrosomal cone (Fig. 1c, f-g). A narrow electron-lucent zone surrounds much of the striated perforatorium. In *R. waitii* and *R. australis* this vacuity is irregularly subdivided by a series of vertical folds which arise from the inner surface of the acrosomal sleeve (Fig. 1a, f). In *R. endoterus* the acrosomal vacuity is more prominent and is regularly partitioned by four vertical "vanes" of the acrosomal sleeve (Fig. 1b, g). The subacrosomal cone has a paracrystalline structure (Fig. 1a, e, g). It is surrounded by the acrosomal sleeve which is complex in form. Basally, where the sleeve overlies the subacrosomal cone, it consists of a single amorphous layer (Fig. 1a, d-e). Proximally, it is composed of two distinct units separated by a narrow, electron-dense band (Fig. 1a-b, f-g). A well-defined "trigger area" [5] covers the proximal tip of the sperm head (Fig. 1a, f-g).

The plasma membrane covering the acrosomal portion of the sperm head appears as a rather heavy and relatively "loose" membrane over most of the acrosome but it is attached to the nuclear membrane proximally around the margin of the "trigger area" and basally at the "post-acrosomal ring", an annulus-like structure which rests upon the nuclear shoulder (Fig. 1a, h). In common with various other groups of snakes and lizards [1, 7, 15], the plasma membrane of the sperm head is surrounded by a pallisade of closely-applied, longitudinally aligned microtubules (Fig. 1a, d-e, g-h).

Neck region

The neck region in *Ramphotyphlops* is exceptionally complex, comprising both proximal and distal centrioles; a basal plate which lines the nuclear fossa; a series of longitudinal columns which extend anteriorly from the dense outer fibres (peripheral fibres) of the midpiece; an electron dense body (referred to here as the "capitulum" in view of its similarity to the structure of that name in mammalian spermatozoa) which conforms to the shape of the basal plate anteriorly, surrounds the proximal centriole and joins to the longitudinal columns; and a "neck cylinder" which encloses the remaining neck elements and extends distally to surround the anterior extremity of the fibrous sheath.

The short proximal centriole (Fig. 2b, j) is set at an angle of approximately 80 degrees to the distal centriole and axoneme. It conforms to the usual pattern of nine sets of peripheral triplets and no central tubules. The central space is penetrated by a body of diffuse, electron dense material

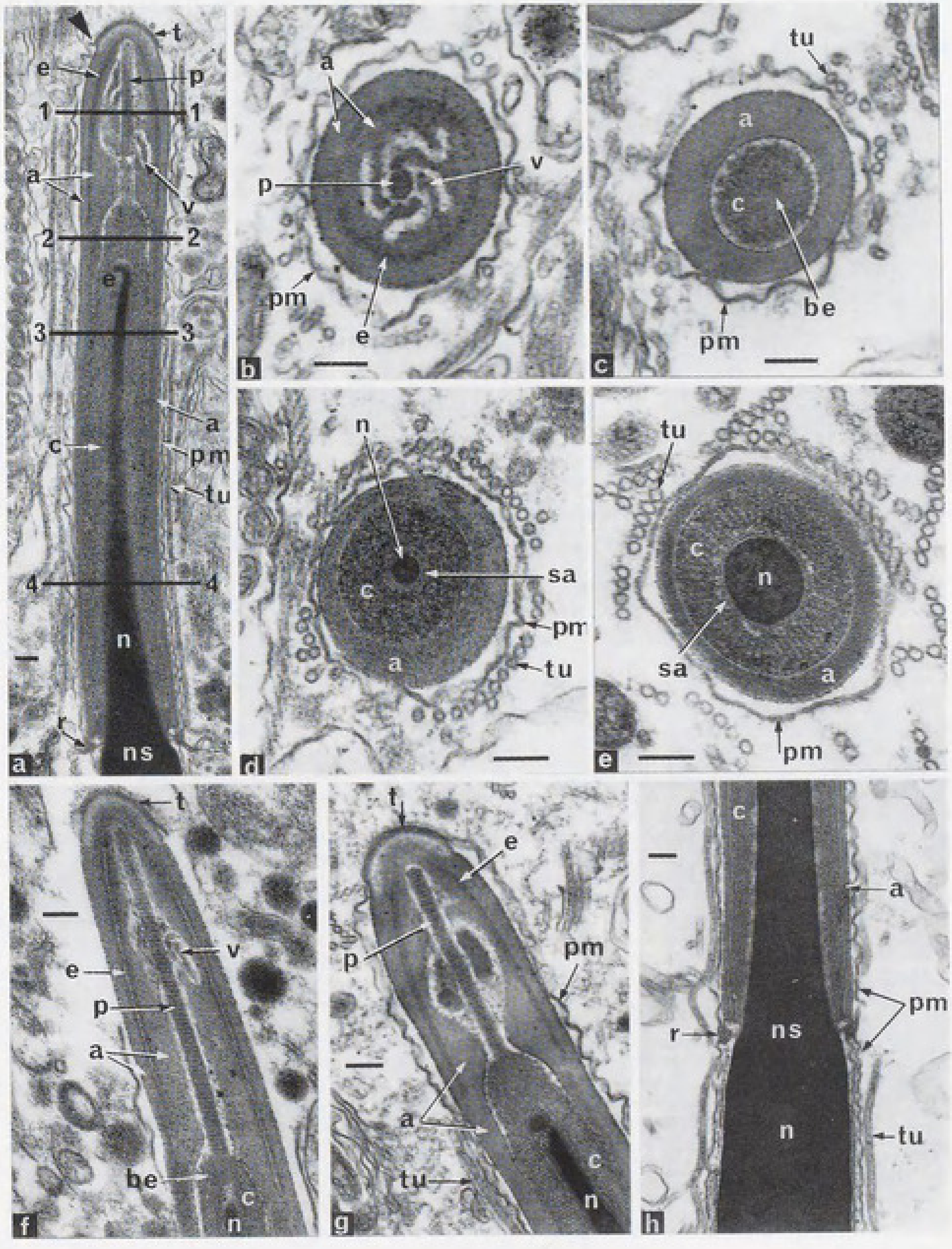
representing a "centriolar pedicle" of the neck cylinder (Fig. 2b). This unusual structure is prominent in *R. endoterus*, in which the neck cylinder and its various processes are relatively electron dense (Fig. 2c, f-g). In contrast the neck cylinder in *R. waitii* and *R. australis* appears relatively electron lucent and granular (Fig. 2a-b, d-e). The proximal centriole as a whole is embedded within an irregular-shaped, electron dense body (the "capitulum") which closely parallels the form of the nuclear fossa (Fig. 2a-c, i). This structure forms the "ball" of a mortise and tenon joint between the sperm head and flagellum, and is therefore at least analogous with the capitulum that forms the anterior region of the "connecting piece" of mammalian sperm.

The basal plate forms the functional interface between the nuclear and flagellar portions of the spermatozoon. It appears to be of composite origin, including both nuclear membranes and membranes which envelope the midpiece and neck structures (Fig. 2 a-c). The space between the basal plate and the capitulum is occupied by an unbounded body of granular material, with a suggestion of radial structure in some micrographs (Fig. 2 a-b).

The distal centriole extends posteriorly from the proximal centriole (but parallel to the long axis of the flagellum) to approximately the region of the flagellum marked by the anterior extremity of the fibrous sheath (Fig. 2a, c, d-f), where it meets the axoneme. It possesses the usual peripheral triplets as well as a pair of central singlets (Fig. 2d). The dense outer fibres associated with each of the nine axonemal doublets continue forward into the neck where they meet the longitudinal columns. These nine columns diverge progressively from the centriolar triplets as they extend in the direction of the sperm head and at their anterior extremity they join the capitulum, forming a support for the proximal centriole and capitulum (Fig. 2a, c, h-j). Transverse sections through the distal centriole show a supernumerary dense column lying between one of the pair of central singlets and one of the centriolar triplets which, on the basis of presently incomplete information, is judged to be triplet 3 (Fig. 2 d-f). One of the longitudinal columns is very poorly developed relative to the others and generally is found close to its adjacent

FIG. 1. — **a:** Longitudinal section through the acrosomal region of the nucleus in a *Ramphotyphlops waitii* spermatozoon. The arrow head marks the attachment of the plasma membrane and nuclear membranes at the trigger area and the post-acrosomal ring marks the basal attachment. The numbered lines indicate the approximate position of the transverse sections in Fig. 1b-e. **b:** Transverse section through the head of a *R. endoterus* spermatozoon in the position marked 1–1 in Fig. 1a. Note the regular arrangement of the vertical folds in the acrosomal vacuity. **c:** Transverse section through the head of a *R. endoterus* spermatozoon in the position marked 2–2 in Fig. 1a. A very slight variation in the appearance of the centre of the subacrosomal cone marks the basal portion of the perforatorium. **d:** Transverse section through the head of a *R. waitii* spermatozoon in the position marked 3–3 in Fig. 1a. Note the thin layer of subacrosomal material surrounding the nucleus. **e:** Transverse section through the head of a *R. waitii* spermatozoon in the position marked 4–4 in Fig. 1a. The paracrystalline structure of the subacrosomal cone is very evident. **f:** Longitudinal section through the anterior region of the acrosome in a *R. waitii* spermatozoon. Compare the length of the perforatorium with that of *R. endoterus* in Fig. 1g printed at approximately the same magnification. **g:** Transverse section through the anterior region of the acrosome in a *R. endoterus* spermatozoon. Note that the vertical folds enclosed within the acrosomal vacuity are more prominent and regular than in *R. waitii*. **h:** Longitudinal section through the head of a *R. waitii* spermatozoon in the region of the post-acrosomal ring. a-h, scale bar = 0.1 µm.

Abbreviations for all figures: a, acrosomal sleeve; an, annulus; b, basal plate; be, basal portion of perforatorium embedded in the subacrosomal cone; c, subacrosomal cone; ca, capitulum; cy, neck cylinder; d, distal centriole; df, dense outer fibres; e, electron dense band separating inner and outer layers of acrosomal sleeve; f, fibrous sheath; g, granular material surrounding fibrous sheath; i, intermitochondrial plaque; lc, longitudinal columns of the neck; m, mitochondria; n, nucleus; ns, nuclear shoulder; p, striated rod of the perforatorium; pd, centriolar pedicle; pm, plasma membrane; px, proximal centriole; r, post-acrosomal ring; s, supernumerary longitudinal column; t, trigger area; tr, centriolar triplets; tu, palisade of longitudinally orientated microtubules surrounding the spermatozoon; v, vertical folds within acrosomal vacuity; x, axoneme.



triplet (Fig. 2 d-e). The spatial relationship between this column and the supernumerary column is constant in the various transverse sections (Fig. 2 d-e) and, again on the basis of as yet incomplete information, this poorly developed column appears to be adjacent to triplet number 9.

The fibrous sheath, normally confined to the principal piece in tetrapod sperm, extends in *Ramphotyphlops* all the way forward to the level of the axoneme–distal centriole junction. In longitudinal sections the columns are seen to diverge radially from this point moving anteriorly (Fig. 2a, c, h-i).

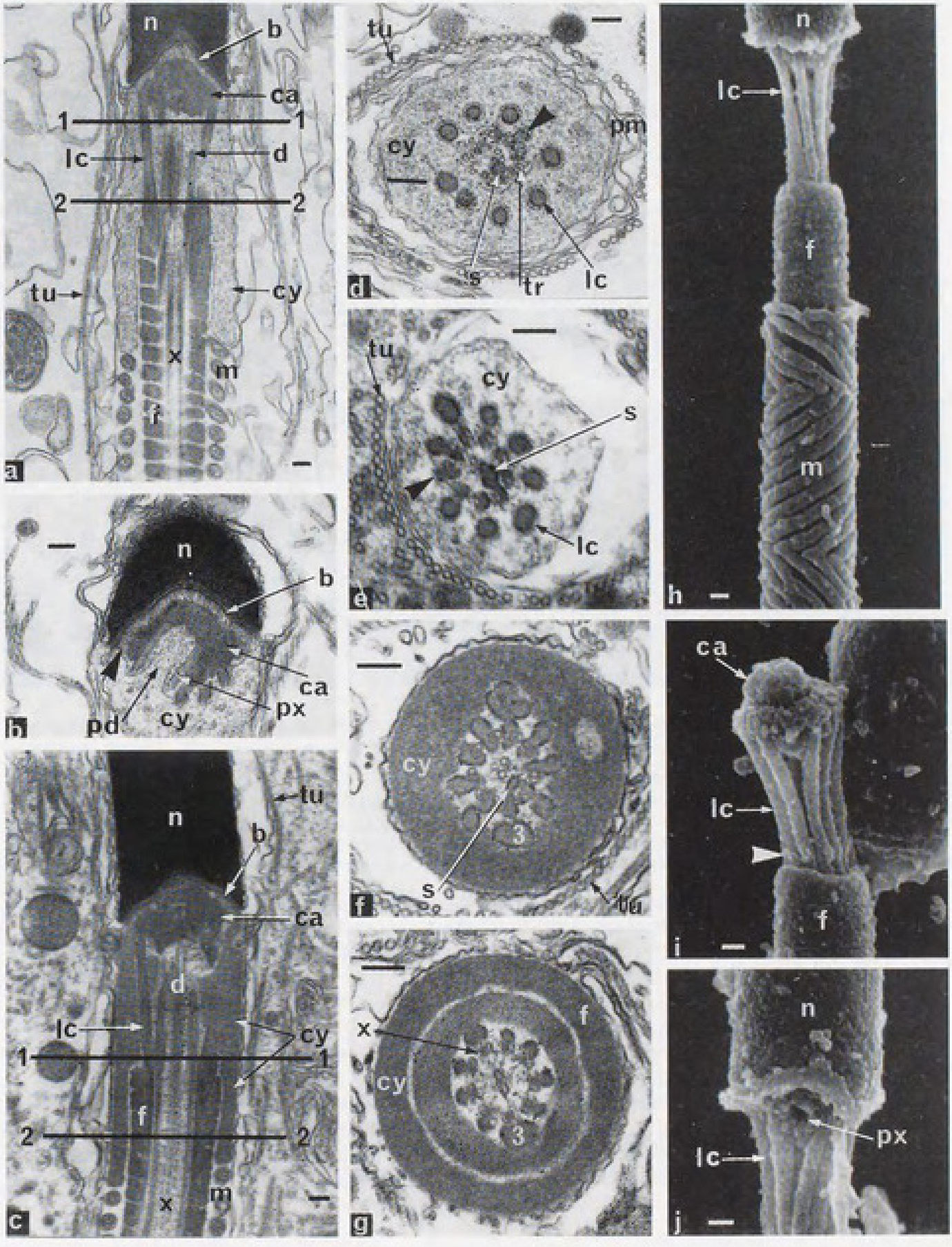
It is not clear precisely where the columns join the dense outer fibres. In a region which must be just anterior to the commencement of the fibrous sheath (since the sheath is absent but the columns/fibres are not radially displaced from the axoneme doublets) the supernumerary fibre is still seen between the central pair and outer doublet 3 (?). Dense outer fibres 3 and 8 are particularly prominent and the fibres associated with all the doublets appear not only adjacent to the outer periphery of the circle of doublets, but also extend inwards, between and partially enclosing each doublet (Fig. 2f).

The neck cylinder in *R. waitii* and *R. australis* consists of a diffuse and somewhat uneven aggregation of granular material, bounded by a narrow membrane. Anteriorly, it surrounds the capitulum and the centriolar complex, contacting the nucleus around the periphery of the nuclear fossa, where it appears to be continuous with the basal plate (Fig. 2 a-b). Posteriorly, it extends well beyond the point of the distal centriole–axoneme union to enclose the anterior portion of the fibrous sheath (Fig. 2 a). In *R. endoterus* the neck cylinder is a more solid structure, being equivalent in electron density to the "capitulum" and the dense outer fibres (Fig. 2 c). However, its basic form and relations are identical to those described for *R. waitii*.

Midpiece

The midpiece is extremely elongate, with one complete section measured from a micrograph reaching 108 µm. Typical cross-sections are circular in shape, have a diameter of 0.5 µm and show nine mitochondrial sections; eight of which are round and one very elongate (Fig. 3 c).

FIG. 2. — **a:** Longitudinal section through the neck and anterior midpiece of a *Ramphotyphlops waitii* spermatozoon. The longitudinal columns diverge as they extend from the anteriormost portion of the fibrous sheath to the capitulum, which surrounds the proximal centriole. The numbered lines indicate the approximate position of the transverse sections in Fig. 2 d-e. **b:** Oblique longitudinal section through the nuclear fossa of a *R. waitii* spermatozoon. Note the radial striations in the granular material (arrowed) separating the basal plate and the capitulum and the centriolar pedicle of the neck cylinder. **c:** Longitudinal section through the neck and anterior midpiece of a *R. endoterus* spermatozoon. The numbered lines indicate the approximate positions of the transverse sections in Fig. 2 f-g. **d:** Transverse section through the distal centriole of a *R. waitii* spermatozoon in the approximate region marked 1–1 in Fig. 2a. Note the multiple plasma membranes, the poorly developed longitudinal column (arrowed), the supernumerary column, and the central pair surrounded by the centriolar triplets. **e:** Transverse section through the distal centriole of a *R. waitii* spermatozoon in the approximate region marked 2–2 in Fig. 2a. Note that the longitudinal columns are not as radially divergent as in Fig. 2d. The poorly developed column is arrowed. **f:** Transverse section through the neck region of a *R. endoterus* spermatozoon in the approximate region marked 1–1 in Fig. 2c. Note the difference in electron density of the neck cylinder compared with that in *R. waitii* (Fig. 2e). Note that the fibres extend between the doublets. Fibres 3 (labelled) and 8 are considerably larger than the rest but all 9 fibres are well developed. **g:** Transverse section through the neck region of a *R. endoterus* spermatozoon in the approximate region marked 2–2 in Fig. 2c. Note the supernumerary column is no longer visible and dense outer fibre (3?) (marked) is particulalry large. **h:** SEM view of the neck and anterior midpiece of a *R. waitii* spermatozoon. The plasma membrane and neck cylinder have been lost to reveal the underlying structures. **i:** SEM view of the neck of a *R. waitii* spermatozoon. The head and flagellum are separated to reveal the capitulum supported by the longitudinal columns. There appears to be a junction (arrowed) just anterior to the start of the fibrous sheath, which may be the junction between the longitudinal columns and the dense outer fibres. **j:** SEM view of the nucleus-neck junction in a *R. waitii* spermatozoon. The plasma membrane and neck cylinder have been lost to reveal the proximal centriole. a-j, scale bar = 0.1 µm.



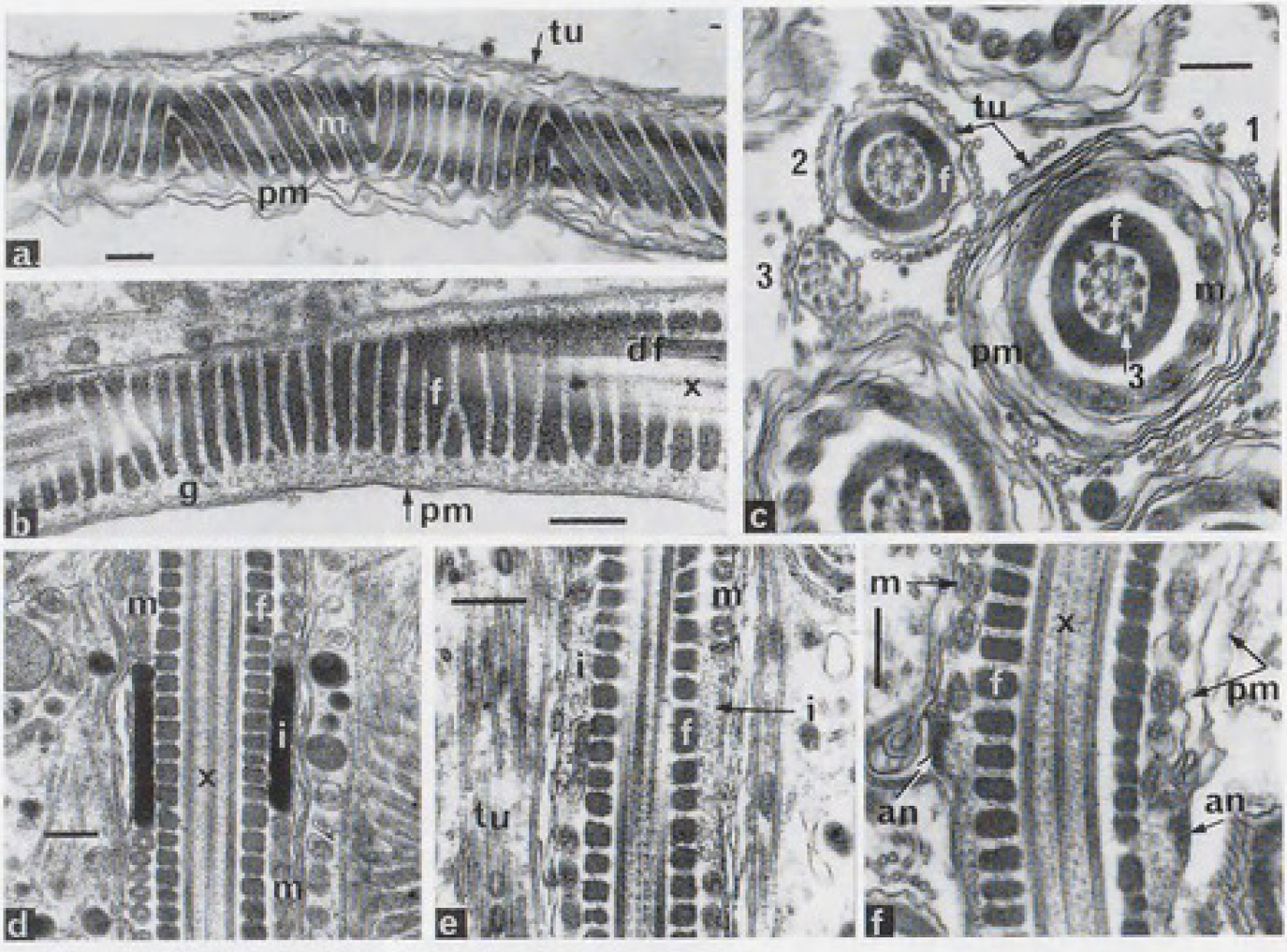


FIG. 3. — **a:** Grazing longitudinal section of the midpiece of a *Ramphotyphlops waitii* spermatozoon to show the ordered and unusual arrangement of the mitochondria. Note the multiple plasma membranes. **b:** Grazing longitudinal section of the fibrous sheath in the principal piece of a *R. waitii* spermatozoon. Note the single plasma membrane and the granular material surrounding the fibrous sheath. **c:** Three transverse sections of *R. waitii* spermatozoa to show the midpiece (labelled 1), the principal piece (2) and the endpiece (3). Note that in the midpiece the dense outer fibres 3 (numbered) and 8 are considerable larger than the others and are associated with the inner layer of the fibrous sheath, and that the fibres are absent in the principal piece section. Note also the multiple plasma membrane layers in the midpiece and the single layer in the principal and end pieces. **d:** Longitudinal section of part of the midpiece of a *R. endoterus* spermatozoon to show the intermitochondrial plaque. **e:** Longitudinal section of part of the midpiece of a *R. australis* spermatozoon to show the poorly developed intermitochondrial plaque. **f:** Longitudinal section of the annulus region of a *R. endoterus* spermatozoon. Note the multiple plasma membranes in the midpiece and single in the principal piece and the granular material in the principal piece. a-f, scale bar = 0.2 µm

Anteriorly, the mitochondrial sheath commences at the posterior extremity of the neck cylinder. As shown in Figs 2 a, 2 c and 2 h, it surrounds the fibrous sheath, which extends further anteriorly than the mitochondria.

Sections derived from anterior portions of the midpiece show 9 dense outer fibres, each closely applied to a peripheral axonemal doublet. As is common among non-mammalian amniote spermatozoa, fibres 3 and 8 are much larger than the others and make contact with the encircling fibrous sheath (Fig. 3 c). More distal sections show a progressive reduction, eventually leaving only fibres 3 and 8 as longitudinal "ribs" of the fibrous sheath.

The fibrous sheath as revealed in grazing sections has a predominantly transverse orientation but with irregular bifurcations and anastomoses (Fig. 3 b).

The mitochondria show a uniquely regular and complex arrangement which immediately distinguishes *Ramphotyphlops* spermatozoa from those of all other reptiles. As shown clearly in both grazing TEM sections (Fig. 3 a) and in SEM images (Fig. 2 h), the overall pattern consists of a series of stacked and alternating chevrons. SEM images of damaged sperm suggest that individual mitochondria are extremely elongate and narrow, with a diameter of around 0.1 µm and that they spiral helically around the midpiece for one complete revolution before turning sharply to repeat the pattern but with the opposite helical polarity (i.e. if clockwise, then anticlockwise). The exact length attained by any individual mitochondrion, as well as the number of flexion points per mitochondrion are presently unknown. Order is apparently maintained in this potential chaos by a very precise alignment of the points of flexion around a common circumferential point, thereby maintaining the pattern of regular, alternating chevrons. In some grazing sections of the midpiece the mitochondria show a more chaotic, intertwining and overlapping arrangement. It is not clear at this stage whether these sections are artefactual, resulting from cut spermatozoa, or whether they are a natural variant, possibly associated with posterior sections of the midpiece.

Transverse and longitudinal setions consistently show a broad translucent zone between the mitochondria and the underlying fibrous sheath (Fig. 3c, f). This apparent lack of contact between the two flagellar sheaths is surprising in view of the regular alignment of the mitochondria. Internally, the mitochondria usually show a single, circular crista enclosing granular material of variable density (Figs 2 a, 3 d).

Most transverse and longitudinal sections through the midpiece show that it is surrounded by multiple plasma membranes (generally between five and seven). The longitudinally orientated microtubules which so conspicuously surround the spermatozoal head are also evident in most sections of the midpiece (Fig. 3 a, c).

Intermitochondrial "rings" or "plaques" are clearly present in *R. endoterus*, where blocks of electron dense material are commonly observed in both tranverse and longitudinal sections, interspersed among otherwise typical mitochondria (Fig. 3 d). Available material suggests that both complete rings and discrete blocks are represented. Less compelling evidence for the presence of intermitochondrial rings is available for *R. australis* and *R. waitii*. In each species just one longitudinal section has shown apparently discrete, membrane-bound plaques of granular material interposed between typical mitochondria (Fig. 3 e). Notably, although the plaques are very definite structures in *R. endoterus*, they are not numerous in comparison to the pattern seen in sperm of many other reptile species [6].

Principal piece

The boundary between mid-piece and principal piece is marked by a small but distinct annulus. There is only a minor decrease in flagellar diameter between the midpiece and principal piece (Fig. 3f). The lack of abrupt narrowing of the flagellum posterior to the mitochondrial sheath is due to the presence of a diffuse granular material both external to, and intercalated between, the elements of the fibrous sheath of the principal piece (Fig. 3 f). Further posteriorly, this granular layer is absent, and the principal piece is correspondingly smaller in diameter.

The longitudinal fibres associated with the fibrous sheath, adjacent to axoneme doublets 3 and 8, extend an unknown distance into the principal piece. The absence of these fibres from most transverse sections of the principal piece (Fig. 3 c) suggests that they must terminate fairly close to the annulus.

A single plasma membrane is present throughout the length of the principal piece, as are the associated microtubules (Fig. 3 c). In sections of *R. endoterus* the multilayered membranes of the

midpiece are clearly seen to terminate at the annulus, posterior to which a single, heavier membrane is present (Fig. 3f).

The length of the principal piece could not be measured from available material. However, it must be at least 55 µm in *R. waitii*, based on lengths of incomplete spermatozoa and of the other major components.

Endpiece

Fig. 3 c indicates that the axoneme extends posteriorly beyond the termination of the fibrous sheath where, surrounded simply by the plasma membrane, it forms the endpiece. It is notable that the palisade of microtubules extends to the endpiece.

DISCUSSION

As noted in the Introduction, the phylogenetic affinities of Typhlopidae are poorly understood, with some uncertainty over both their current association with other snakes in the taxon Serpentes [9, 14], and even their current inclusion within Squamata [17]. Recent cladistic analyses of spermatozoal evolution among higher vertebrates [8, 10, 11] provide a useful framework against which to assess the broad scale phylogenetic affinities of the Typhlopidae, based on a previously unexamined aspect of their morphology.

Compared with the various major groups of living amniotes (turtles, crocodiles, birds, tuatara, mammals, squamates) spermatozoa of *Ramphotyphlops* species are, overall, most similar to those of other squamates as described by FURIERI [6] and more recent workers [11, 12, 15].

Some of the features shared by *Ramphotyphlops* spp. (e.g. the presence of an elongate and relatively straight sperm head) are undoubtedly plesiomorphic for amniotes as a whole. However, other features appear to represent derived or apomorphic characters of possible phylogenetic significance. These apomorphic, squamatan characteristics include: the paracrystalline nature of the subacrosomal cone; the absence of an endonuclear canal; the presence of electron dense intermitochondrial "rings" or "plaques"; the extension of the fibrous sheath into the midpiece ([8, 10], see also JAMIESON, Squamates, this volume); and reduction or loss of the annulus. The occurrence of these features in the spermatozoon of *Ramphotyphlops* species provides strong independent confirmation that Typhlopidae are correctly placed within Squamata.

Analysis of phylogenetic relationships within Squamata based on spermatozoal characters is hindered by the many gaps in current taxonomic coverage. Among the snakes for example, comparable observations are available only for representatives of three other families, Colubridae [1, 6, 7, 16, 18, 19], Viperidae [6] and Boidae [2,7], and no data are available for more than a dozen other families. Similarly, little or no information is available for many important groups of lizards, including some which have been putatively implicated in the evolution of snakes (e.g. varanoids [14], dibamids [4]).

Among the various squamate groups for which spermatozoal data are available, *Ramphotyphlops* spermatozoa appear particularly close to those of snakes. In all, five features appear to be uniquely shared among the various families of snakes, namely: (1) the extreme forward prolongation of the fibrous sheath to the level of the neck; (2) the presence of a distinct neck cylinder; (3) the multi-layered nature of the midpiece plasma membrane; (4) the extremely elongate nature of the individual mitochondria; and (5) the extreme elongation of the midpiece. Each of these features appears to represent a derived or apomorphic character relative to a basal squamate condition [8, 10] and they thus collectively support the current placement of Typhlopidae within Serpentes.

Ramphotyphlops spermatozoa differ from those of other snakes in the arrangement of mitochondria in the midpiece. For other species of snakes, the mitochondria are variably described as "very slender and rather convoluted" [6] and as "highly contorted and variable in their orientation ... (but with) .. a predominant direction .. in a loose spiral around the flagellum"

[7]. These observations are confirmed by our own unpublished findings for various species of boids, elapids and a viperid. Clearly they are in striking contrast with the highly regular arrangement described for *Ramphotyphlops*. Nevertheless, JAMIESON [Squamates, this volume] has identified zigzagged mitochondria as a characteristics of snakes, also seen in pygopodids.

The mitochondria of lizards and other groups of reptiles (turtles, crocodiles, tuatara etc.) are even less similar to those of *Ramphotyphlops*. In these groups, typically the mitochondria are small, rounded or brick-shaped bodies, arranged either in regular rows intercalated between complete intermitochondrial rings, or, in irregular fashion with scattered intermitochondrial bodies [6, 11, 12, 15]. Exactly how the mitochondria in *Ramphotyphlops* come to be so elongate and furthermore, how they come to be deployed in such a precise and unusual manner, are clearly issues of general interest which should be further investigated through examination of the spermiogenetic process.

Another difference between spermatozoa of *Ramphotyphlops* and those of other snakes concerns the granular layer external to the principal piece fibrous sheath. This material appears to be absent from the spermatozoa of both boid and colubroid snakes, among which the flagellum shows a more pronounced narrowing at the annulus. Among other squamates, a granular layer is present in the principal piece of at least some skinks [11, 12] and varanids [unpublished data]. Hence this may represent a plesiomorphic feature within Squamata.

Significant differences in spermatozoal structure were observed between each of the three species of *Ramphotyphlops* examined in this study. Of these, the most striking differences concern the presence and electron density of the intermitochondrial bodies of the midpieces: conspicuous, but relatively few, electron dense bodies in the midpiece in *R. endoterus* and granular structures of low electron density in *R. waitii* and *R. australis*. These appear to be even less common in the midpiece than they are in *R. endoterus*. Interestingly, these differences in the relative electron density correlate with that observed in the neck cylinder; dense in *R. endoterus* and of low electron density in the other species. The possibility that these characteristics are related is further strengthened by HAMILTON & FAWCETT's [7] finding that the neck cylinder and the intermitochondrial plaques both arise from a common body of granular material which lies in close topographical relationship to the ends of mitochondria as they assemble around the distal centriole and base of the axoneme during late spermiogenesis. However, because this observation is at odds with recent claims that derive the intermitochondrial bodies directly from mitochondria [3, 8], we defer any further speculation pending analysis of spermiogenesis in *Ramphotyphlops*.

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