Ultrastructure of the spermatozoon of the tuatara (*Sphenodon punctatus*) and its relevance to the relationships of the Sphenodontida

J. M. HEALY[1]† AND B. G. M. JAMIESON[2]‡

[1]*Queensland Museum, South Bank, South Brisbane, Queensland, Australia*
[2]*Department of Zoology, University of Queensland, Brisbane, Queensland 4072, Australia*

SUMMARY

Spermatozoa of the New Zealand tuatara, *Sphenodon punctatus punctatus* (Gray), are described from light and electron microscopic observations and compared with spermatozoa of other living 'reptiles' (Chelonia, Crocodilia, Squamata), birds and mammals. Mature *Sphenodon* spermatozoa consist of an acrosomal complex (length 4 μm), elongate, helical nucleus (54–56 μm), a relatively short midpiece (7–8 μm), elongate principal piece (74–78 μm) and short end piece (2–4 μm). The acrosomal vesicle and underlying subacrosomal material form a double, curved, conical sheath around the nucleus anteriorly. Two parallel, loosely helical, endonuclear canals each containing perforatorial material, extend posteriorly from the apex of the nucleus to at least 2.5 μm below the base of the acrosomal complex. Rings of several spherical mitochondria are stacked around the elongate distal centriole to form the midpiece. Each mitochondrion has concentric cristae surrounding a dense central body. Proximal and distal centrioles, although differing markedly in length, are similar in having triplets with an open C tubule. Nine peripheral fibres are intimately associated with the triplets of the distal centriole. A well developed annulus defines the posterior extremity of the midpiece. The principal piece consists of a 9 + 2 axoneme (accompanied anteriorly by nine peripheral fibres) surrounded by a highly electron-dense fibrous sheath and the plasma membrane. Absence of a penis in *Sphenodon* has not resulted in recognizable modifications of the spermatozoon. *Sphenodon* shares many spermatozoal features here interpreted as plesiomorphies with crocodiles and turtles, particularly the latter group, but exhibits none of the advanced character states (apomorphies) diagnostic of the Squamata. These data not only underscore the primitive status of living tuatara (recently questioned in the literature) but also militate against a close, sister-group relationship between the Sphenodontida and Squamata.

1. INTRODUCTION

Tuatara (*Sphenodon* spp.) are of special scientific interest because they are the only survivors of an ancient order of lizard-like amniotes, the Sphenodontida (formerly the Rhynchocephalia *sensu* Günther (1867)). Living species of *Sphenodon* (*S. punctatus*, *S. guntheri*) are confined to offshore islands of New Zealand where they are strictly protected and monitored (Cree & Daugherty 1990; Daugherty *et al.* 1990). Various factors such as the small size of many breeding populations and the long time (13 years) required to attain sexual maturity have meant that declines in adult numbers can and have led to local extinctions, including the possible recent extinction of a subspecies (Cree & Daugherty 1990; Daugherty *et al.* 1990). Bearing this in mind, there exists a need to fully understand tuatara reproductive biology. Over the past ten years many important aspects of tuatara reproduction have been examined including courtship, mating, embryology, nesting and hatchling survival (Gans *et al.* 1984; Moffat 1985; Cree & Thompson 1988; Cree & Daugherty 1990). At the cellular level, basic organization of the testis and ovary, and gametogenic cycles (particularly in relation to sex hormone levels) have also been recently studied (Saint Girons & Newman 1987; Bradshaw *et al.* 1988; Cree *et al.* 1990, 1991). By using light microscopy, Hogben (1921) described changes in chromosomal morphology occurring in spermatogonia and spermatocytes of *S. punctatus* but was prevented from tracing later events of spermatogenesis by the inadequate fixation of available tissue.

Sphenodon has not previously been examined for sperm ultrastructure, although light microscopic work on testis development (Saint Girons & Newman 1987; Cree *et al.* 1991) shows the sperm of *S. punctatus* to be filiform. Inspired by the same reasons as Hogben (1921) to document gametogenic events of this important and still threatened group, we here present an ultrastructural study of the spermatozoa and late spermatids of *S. punctatus*. Our interest in *Sphenodon* also stems from the fact that it differs from all other amniotes excepting higher birds (the Chelonia, Crocodilia, Squamata, ratites, some other birds and mam-

† Present address: Department of Zoology, University of Queensland, Brisbane, Queensland 4072, Australia.
‡ To whom correspondence should be sent.

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mals) in lacking an intromittent organ (Crook 1975; Cree & Daugherty 1990) and as such might exhibit important modifications of sperm ultrastructure. The penis is often said to be absent in squamates but they have a bifold eversible intromittent organ (Heilmann 1972). A detailed examination of spermiogenesis in *S. punctatus* by the present authors is currently in progress and will be presented elsewhere.

The taxonomic and phylogenetic value of comparative sperm ultrastructure has been firmly established for invertebrates (see, for example, Baccetti (1970, 1979); Afzelius (1979); Wirth (1984); Justine *et al.* (1985); Jamieson (1983, 1987); Healy (1988); Alberti (1990)) and vertebrates (see, for example, Harding *et al.* (1982); Rouse & Robson (1986); Temple-Smith (1987); Jamieson (1991)). As the primitive status of *Sphenodon* has been challenged by a number of writers in recent years (Whiteside 1986; Gauthier *et al.* 1988a,b), we conclude our account with a comparison of *Sphenodon* sperm structure with that of other tetrapods, especially other 'reptiles', and briefly outline the phylogenetic implications of such cytological data. This latter aspect is dealt with in greater detail by Jamieson & Healy (1992) as part of a cladistic analysis of amniote relationships based on comparative sperm morphology.

2. MATERIAL AND METHODS

The three testis samples of *Sphenodon punctatus punctatus* (Gray) used in this study were generously made available to us by Dr Alison Cree (University of Otago) after the completion of her work on the sex steroid cycles in wild females and males in this species (see Cree *et al.* 1991). As both living tuatara species (*S. punctatus* and *S. guntheri*) are fully protected animals, availability of freshly collected testis material is very limited. The samples used, all of which had been fixed and stored in neutral buffered 10% formalin, derived from males collected, under permit, from Stephens Island, New Zealand between July 1987 and February 1989. Our CITES number permitting research on *S. punctatus punctatus* is AU 005.

The following testis tissues were used: sample 1, July 1987 collected by A. Cree and L. J. Guillette (primary and secondary spermatocytogenesis only); sample 2, February 1989 collected by M. McIntyre, M. Brown, R. Ainsworth (full spermiogenesis and mature sperm); sample 3, March 1988 collected by B. Firth and M. B. Thompson (late spermiogenesis, some mature spermatozoa). All samples were examined, but most of the observations presented here were made from sample 2 which contained the greater proportion of mature spermatozoa. For transmission electron microscopy (TEM) testis samples were diced into 1–2 mm^3 portions in the original 10% formalin fixative, rinsed thoroughly in 0.1 M phosphate buffer (30 min), osmicated (2 h, 1% osmium tetroxide in phosphate buffer), dehydrated in ethanol (ascending from 20%, 40% etc. to absolute, 30 min each step) then gradually infiltrated, and embedded, in Spurr's epoxy resin. All stages from the initial buffer rinse to 70% ethanol were conducted at 4°C and thereafter at room temperature. A LYNX el Microscopy Tissue Processor was used to prepare testis samples for TEM. Semithin and ultrathin sections were cut by using glass or diamond knives and an LKB IV 2120 Ultrotome. Ultrathin sections were collected on coated or uncoated 200 mesh copper grids, then stained with 6% uranyl acetate and Reynold's lead citrate according to the double stain method of Daddow (1983). Specimen bearing grids were examined with an Hitachi 300 TEM operated at 80 kV and a JEOL 100S at 60 kV. For negative staining, pieces of tissue (sample 2) were washed and minced in distilled water; a drop of the resulting suspension was mixed with a drop of 3% (aqueous) uranyl acetate. Some of this stained suspension was placed onto a colloidin-coated wide-bar TEM grid and allowed to air dry after excess stain-suspension was blotted off. Light microscopic observations of spermatozoa, from tissue squashes, were made by using an Olympus microscope adjusted for phase-contrast microscopy.

For comparison with *Sphenodon* we include representative TEM micrographs of freshwater turtle spermatozoa (*Emydura krefftti*, *E. signata*, *Chelodina expansa*, *Elseya latisternum*). These derive from unpublished observations by Jamieson & Georges on spermatozoa (from the vas deferens) of the Australian Chelonia (including above mentioned species in addition to *Emydura macquarii* and *Elseya dentata*).

3. RESULTS

(*a*) *Light microscopy*

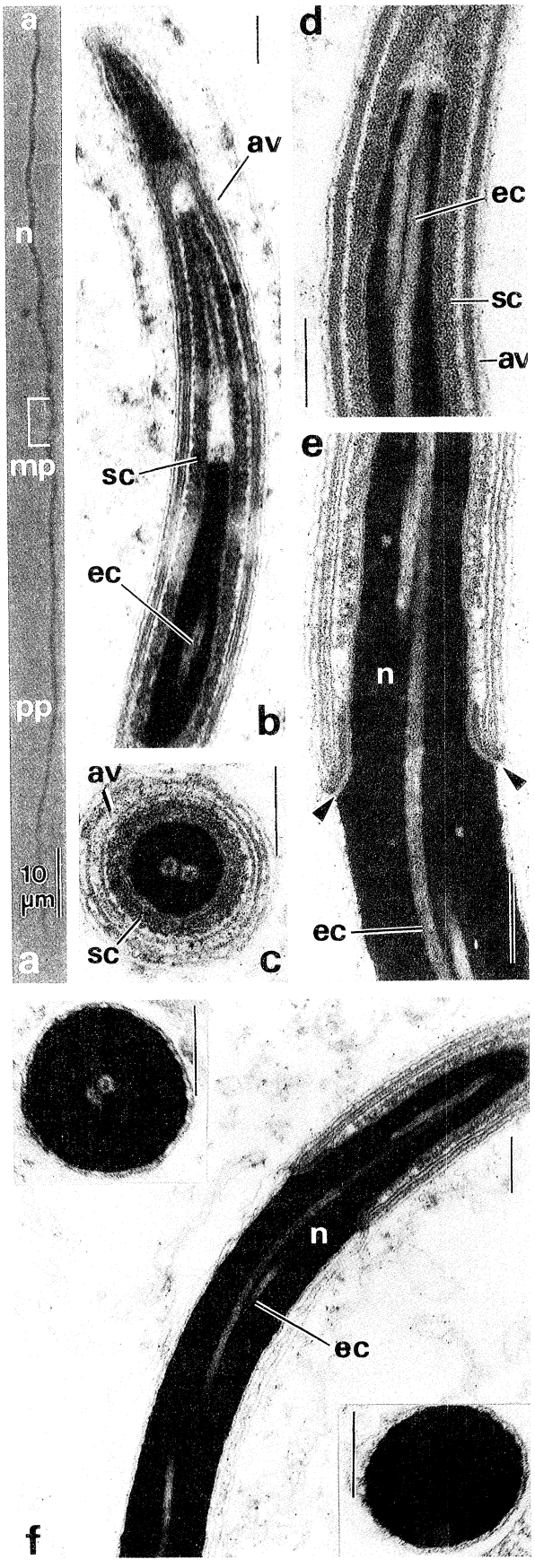
Spermatozoa of *Sphenodon punctatus* are filiform, approximately 140–144 μm long, with a head region (acrosome plus nucleus) which forms a loose helix. Examination of air-dried spermatozoa gives a length of 58–60 μm for the head, 7–8 μm for the midpiece, 74–78 μm for the principal piece and 2–4 μm for the end piece (figure 1a).

(*b*) *Transmission electron microscopy* (TEM)

The description that follows is based on mature or almost mature spermatozoa from the testis. For a few features such as the neck region we could only obtain details from the late spermatid stage. This is noted in the text. In some instances it was necessary to trace the developmental origins of sperm components to determine their precise homology. We therefore include pertinent micrographs of such developing structures.

Acrosomal complex

The acrosomal complex consists of three elements: (i) a conical and membrane-bound acrosomal vesicle (length approximately 4 μm), (ii) a hollow subacrosomal cone and (iii) perforatorial (subacrosomal) material occupying the paired endonuclear canals. The acrosomal vesicle and subacrosomal cone are curved and ensheath the tapered, anterior region of the nucleus for a distance of 1.6 μm (figure 1b–e). A 20 nm



thick, electron-lucent layer separates the acrosomal vesicle from the subacrosomal cone (figure 1b–e). The base of the acrosome rests on a shoulder-like widening of the nucleus; anterior to this the nucleus tapers narrowly within the subacrosomal cone (figure 1e,f).

Nucleus

The nucleus is very elongate, 54–56 μm long (figure 1a), coiled in a loose helix, and tapers to a point anteriorly (figure 1b). A small, cuboidal fossa containing dense material and a portion of the proximal centriole is present at the base of the nucleus of late spermatids (figure 2a), and presumably also occurs in mature sperm. The anterior, tapered portion of the nucleus is ensheathed by the posterior portion of the acrosomal complex (figure 1b). Two narrow, endonuclear canals (diameter 40–45 nm) are present anteriorly, each filled with a perforatorial deposit and opening within the subacrosomal cone (figure 1d–f). Longitudinal sections (figure 1e, f) clearly indicate that the canals, though closely parallel, are helically twisted around each other. Although length of the canals was not determined, they were followed in longitudinal sections to at least 2.5 μm beyond the base of the acrosomal complex (figure 1f) and possibly extend much deeper. Nuclear contents of mature spermatozoa are highly electron-dense, are invested by nuclear and plasma membranes and exhibit occasional electron-lucent lacunae (figure 1e). Observations on late spermatids reveal that nuclear condensation proceeds via aggregation of coarse (44 nm) granules and gradual, although not total, elimination of lacunae (figure 2b).

Neck region, centrioles

The morphology of the nucleus–midpiece junction or 'neck' region could be established only for advanced and late spermatids (figure 2a, b) as favourable sections were not obtained for fully mature sperm. Longitudinal sections of late spermatids show the presence of a nuclear fossa; a proximal centriole consisting of nine triplets of microtubules of which the C tubule often appears open (figure 2a, b); and an extensive deposit of pericentriolar material, here termed the lateral body, which extends into the nuclear fossa and posteriorly lies in contact with the

Figure 1. *Sphenodon punctatus* (a) Whole spermatozoon (phase-contrast light microscopy). Positions of acrosomal complex (a), nucleus (n), midpiece (mp) and principal piece (pp) indicated. (b) Longitudinal section of acrosomal complex sheathing the nuclear apex. Note acrosomal vesicle (av), subacrosomal cone (sc), endonuclear canals (ec). (c) Acrosomal complex and nucleus in transverse section. Endonuclear canals (containing perforatorial material) visible. (d) Anterior extremity of nucleus. (e) Basal region of acrosomal complex with arrow heads indicating nuclear shoulder. Note twisting of endonuclear canals. (f) Penetration of endonuclear canals posterior to acrosomal complex. Upper inset: endonuclear canals posterior to acrosome. Lower inset: nucleus posterior to endonuclear canals. Scale bars = (a) 10 μm; (b–f) 0.25 μm

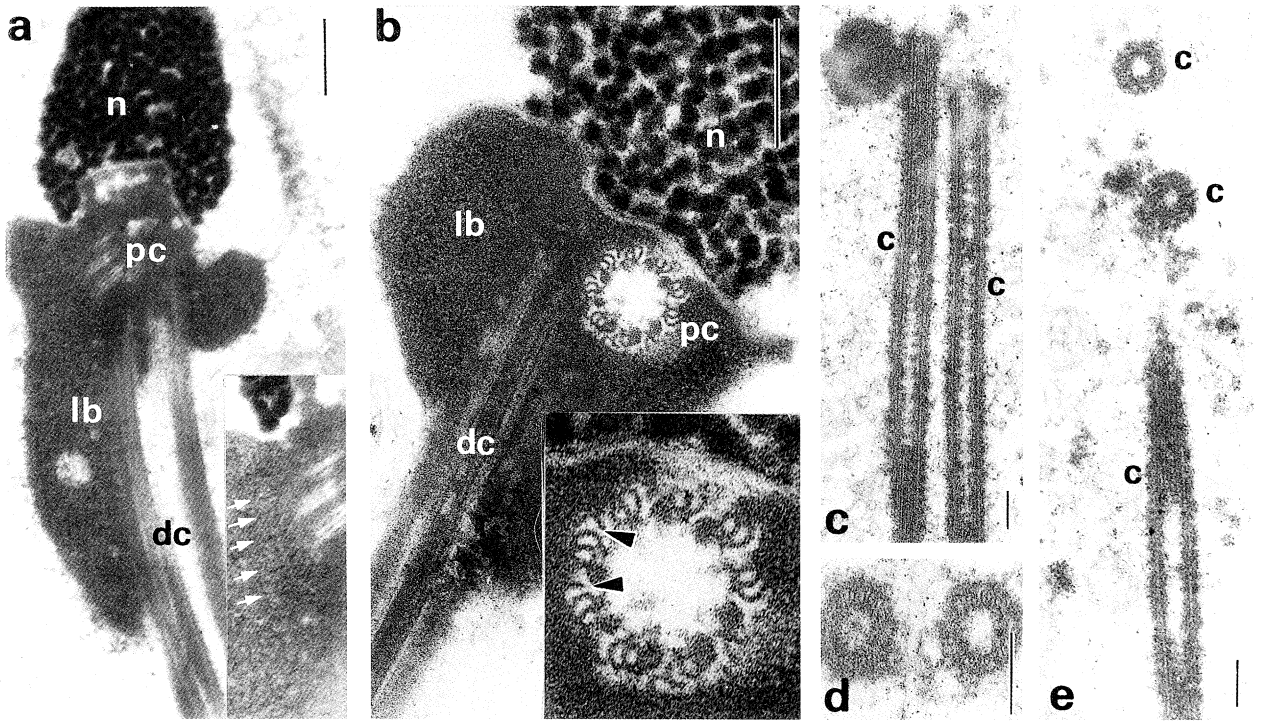


Figure 2. *Sphenodon punctatus* (a) Neck region of late spermatid showing proximal and distal centrioles (pc, dc), extent of lateral body (lb) and condensing nucleus (n). Inset: arrows indicate faint periodic striations present within lateral body. (b) Proximal centriole and elongate distal centriole embedded in matrix of lateral body at base of condensing nucleus in advanced spermatid. Inset: detail of proximal centriole showing open C tubule (arrow heads). (c) Pair of elongate centrioles of spermatocyte. (d) Transverse section of paired centrioles. (e) Three centrioles visible in spermatocyte. All scale bars = 0.25 μm

elongate distal centriole (figures 2a and 3b). The lateral body is collar-shaped anteriorly and cylindrical distally (within the midpiece) with occasional electron-lucent lacunae (figure 2a). In the immediate vicinity of the proximal centriole, faint periodic striations (repeat distance 30–40 nm) are seen within the lateral body (figure 2a inset). These suggest that the lateral body of the mature spermatozoon is also periodically striated.

Our preliminary observations on spermatogenesis of *S. punctatus* show that a pair of elongate centrioles (figure 2c, d), each centriole often seen to be associated with a short centriole (figure 2e), is present in the spermatocyte. The elongate centrioles are hollow (lacking a central pair of tubules), are at least 4 μm long and lie parallel to each other. The single, elongate distal centriole of each spermatid clearly must be a persistence of an elongate centriole of the spermatocyte. The central pair of tubules (also termed central singlets) within the distal centriole of the spermatid is presumably acquired during formation of the axoneme for which the centriole forms the basal body. The distal centriole of the mature spermatozoon is unusual both in its great elongation and in containing central singlets (see Discussion).

Midpiece

Following the nucleus and neck is the midpiece, occupying only 7–8 μm of the length of the sperm but nevertheless long in comparison with 'primitive', externally fertilizing sperm. It consists of spherical mitochondria (each with concentric cristae) which surround an elongate, distal centriole (figure 3b–e). Eight or nine mitochondria are present in cross section anteriorly (figure 3c) decreasing to five or six posteriorly (figure 3e). Each mitochondrion has 11 to 14 concentric cristae around a dense central body (figure 3b, e). Mitochondria of early and middle stage spermatids (and spermatocytes), by contrast, contain only regular (linear) cristae (figure 3a) indicating that transformation from regular to concentric cristae takes place relatively late in spermiogenesis. The structure here regarded as a true distal centriole runs the entire length of the midpiece. It consists of nine triplets of microtubules, often showing an open C tubule; nine peripheral fibres which partly ensheath the triplets and project into the centriolar lumen; and the pair of central tubules which are embedded in and partly obscured by dense material (figure 3b–e). The distal centriole is embedded in the lateral body near the nuclear fossa (figure 2a, b), while posteriorly it is continuous with the axoneme of the principal piece (figure 4a).

Annulus

A dense ring, constituting a well developed annulus, is present at the distal extremity of the midpiece. This structure is electron-dense, approximately 1.0 μm in diameter and is closely applied to the inside surface of the plasma membrane (figure 4a, b). Electron-dense

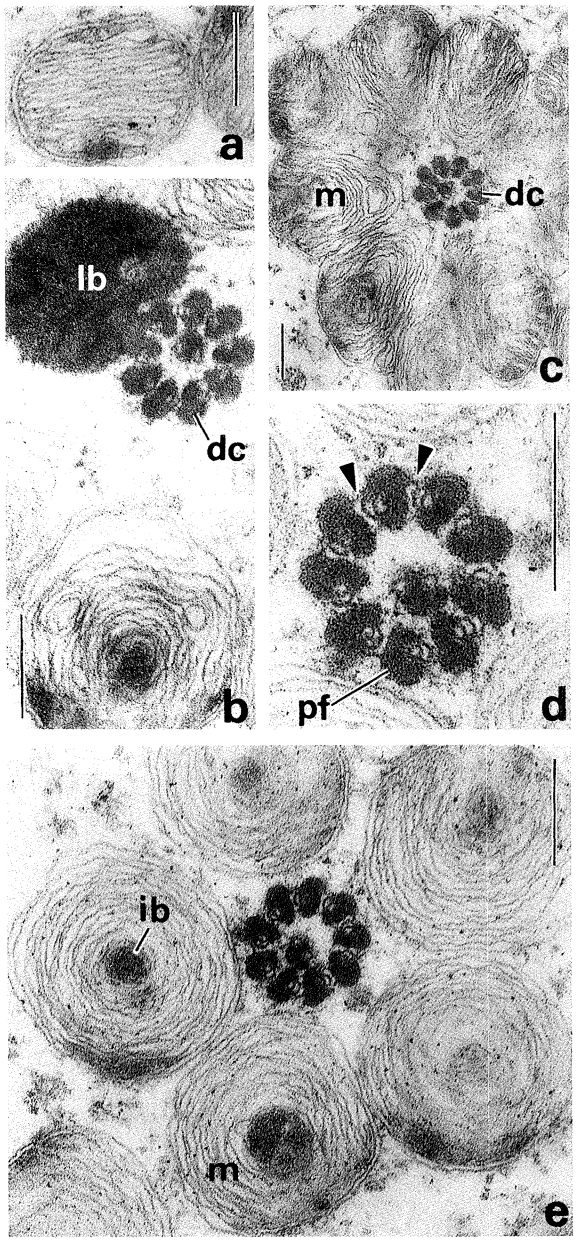


Figure 3. *Sphenodon punctatus.* (a) Mitochondrion with unmodified cristae from mid-spermatid (b) Lateral body (lb) and distal centriole (dc) of late spermatid. (c) Transverse section of mature midpiece showing mitochondria (m, with concentric cristae) packed around distal centriole. (d) Detail of distal centriole showing peripheral fibres (pf) between and partly sheathing centriolar triplets. Note open C tubules (arrow heads) of triplets. (e) Transverse section through posterior region of midpiece showing concentric cristae and dense intramitochondrial bodies (ib). All scale bars = 0.25 μm.

material is also observed at the point of reflection of the plasma membrane giving an inner periaxonemal ring in transverse sections (figure 4b). Transverse sections also show that the transition from distal centriole to axoneme occurs within the annular region and that peripheral fibres associated with doublets 3 and 8 are appreciably thicker than the remaining seven fibres (figure 4a inset, 4b). The thicker fibres at doublets 3 and 8 continue posteriorly in contact with the inner surface of the fibrous sheath of the principal piece.

Principal piece

The principal piece occurs posterior to the midpiece (figure 4a). This is the longest region (74–78 μm) of the spermatozoon and consists of the 9 + 2 axoneme surrounded by an electron-dense fibrous sheath and the plasma membrane (figure 4a, c–h). The A tubule of each doublet is filled with electron dense material, and bears two dynein arms which project towards the B tubule of the succeeding doublet (figure 4d–f). Initially the axoneme emerges from the midpiece accompanied by all nine peripheral fibres (figure 4b, c) but these rapidly decrease in diameter and, with the exception of fibres 3 and 8, terminate within the anterior region of the principal piece (figure 4d). Diameter of the principal piece decreases from 0.73 μm anteriorly (figure 4c, d) to 0.38 μm approaching the end piece (figure 4e, f). The anastomosing, and probably helical, arrangement of the fibrous sheath can best be observed in oblique longitudinal sections (figure 4a, g) and in negative-stained preparations (figure 4h).

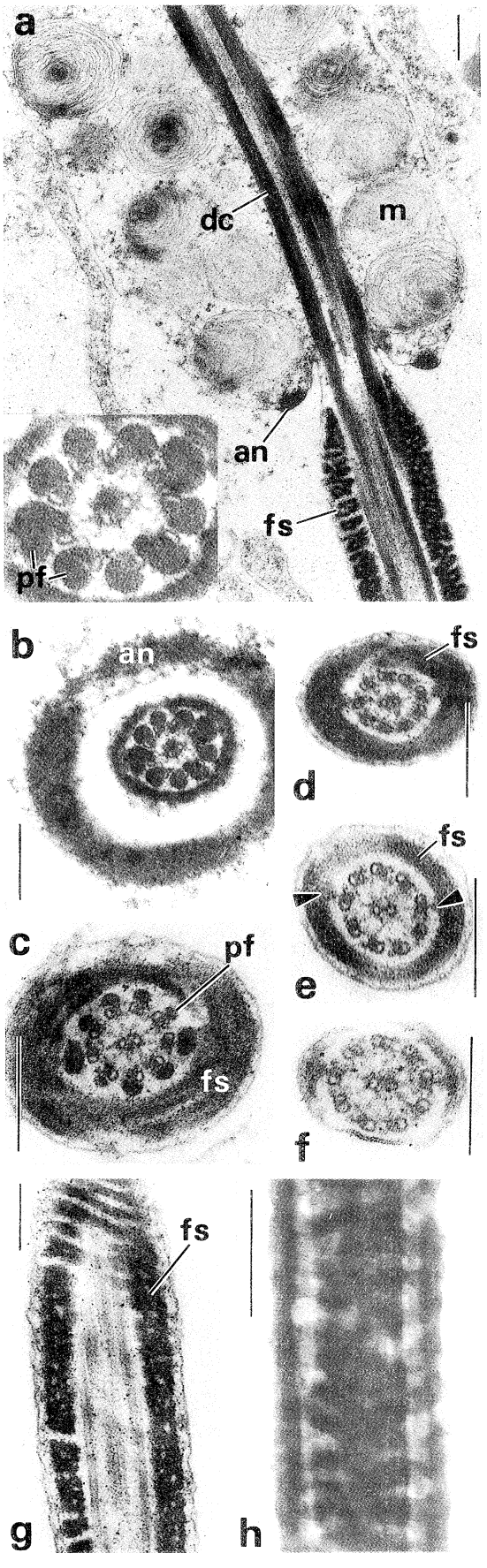
End Piece

The end piece (length 2–4 μm) of the spermatozoon was observed only by light microscopy.

Spermatozoa of the Chelonia

Spermatozoa of turtles strongly resemble those of *Sphenodon.* In order to highlight these similarities, we include representative micrographs from *Emydura krefftii*, *E. signata*, *Chelodina expansa* and *Elseya latisternum* (all Emydidae) to illustrate the basic morphology of turtle sperm (figure 5).

The acrosomal complex of turtle sperm consists of an acrosomal vesicle and subacrosomal cone collectively ensheathing the apical portion of the nucleus (figure 5a–d). Paired, helically twisted endonuclear canals containing perforatorial material extend posteriorly from the nuclear apex (figure 5a–d). A step-shaped nuclear shoulder marks the posterior limit of the acrosomal complex (figure 5a, b). Two centrioles are present, the proximal being short and embedded in dense material near the nuclear fossa (figure 5f). The distal centriole (also embedded in dense material) is elongate and surrounded by mitochondria to form the midpiece (figure 5f–h). A central pair of microtubules penetrates the distal centriole (figure 5f–h). We have recognized the open nature of the C tubule of the distal centriole by close examination of the centrioles illustrated in figure 5g, h. Midpiece mitochondria are spherical with concentric cristae surrounding a dense, intramitochondrial body (figure 5f, g). Posterior to the midpiece, the dense fibrous sheath encloses the axoneme to form the principal piece (figure 5f). An annulus, associated with the plasma membrane cups the basal mitochondria of the midpiece (figure 5f). Turtles differ from *Sphenodon* principally in having a much shorter nucleus which is curved rather than helically coiled, and also in the more copious quantity



of dense material associated with the central tubules of the distal centriole.

4. DISCUSSION

(*a*) *Comparison with sperm of other tetrapods*

Figure 6 summarizes our results for sperm ultrastructure in *Sphenodon punctatus* and will serve as a pictorial basis for this discussion.

Most of the features of *Sphenodon* sperm have been reported in studies of other amniotes. They are synapomorphies for the Amniota relative to Lissamphibia but are simultaneously basic amniote symplesiomorphies (Jamieson & Healy 1992). However, similarity is closest between sperm of *Sphenodon* and those of turtles (many species examined: Furieri, 1970; this study; B. G. M. Jamieson & A. Georges unpublished results) and the only studied crocodilian, *Caiman crocodilus* (Saita *et al.* 1987). Phylogenetically this similarity, although consisting of additional plesiomorphies, is highly significant (Jamieson & Healy 1992). From the standpoint of structure and fertilization biology it is noteworthy, however, that absence of a penis in *Sphenodon* (an absence unique among 'reptiles') has not resulted in recognizable modifications of the spermatozoon.

To facilitate comparison of the spermatozoon of *Sphenodon* with spermatozoa of other tetrapods a summary, below, of its chief characters precedes the main discussion of comparative sperm morphology.

Sphenodontidae (Sphenodon punctatus), summary description

The chief features of spermatozoon (figure 6) are: (i) an acrosomal complex consisting of acrosomal vesicle and subacrosomal cone ensheathing the anterior end of the nucleus and the pair of perforatoria; (ii) a long, helically coiled nucleus with two deep endonuclear canals; (iii) a short proximal centriole embedded in a lateral body; (iv) a very elongate distal centriole (penetrated by central pair of singlet tubules) which is surrounded by mitochondria which have concentric cristae around a dense body; (v) open C tubules in both centrioles; (vi) a prominent annulus; (vii) a principal piece with a fibrous sheath; (viii) peripheral fibres within the midpiece and principal piece; (ix) peripheral fibres at doublets 3 and 8

Figure 4. *Sphenodon punctatus*. (a) Longitudinal section through annulus (an) at the junction of midpiece (note mitochondria, m) and principal piece (note fibrous sheath, fs). Inset: peripheral fibres (pf) associated with axonemal doublets. (b) Transverse section through annulus. (c) Anterior region of principal piece showing fibrous sheath surrounding axoneme and peripheral fibres. (d) Anterior region of principal piece posterior to figure 4c showing loss of seven peripheral fibres. (e) Posterior region of principal piece (peripheral fibres near doublets 3 and 8: arrow heads). (f) Distal region of principal piece. (g) Portion of principal piece in oblique longitudinal section. (h) Negative stained principal piece showing anastomosing fibrous sheath. All scale bars = 0.25 μm.

continuing far posteriorly of the other fibres, within the principal piece.

Acrosomal complex and nucleus

The double-layered sheathing of the nucleus by the acrosomal vesicle and subacrosomal cone in *Sphenodon* is observed widely among other tetrapods, being recorded for example in turtles, crocodiles, non-passerine birds (chicken, guinea fowl), squamates (all groups) and monotremes (Furieri 1970; Carrick & Hughes 1982; Butler & Gabri 1984; Saita *et al.*, 1987; Thurston & Hess 1987; Carcupino *et al.* 1989; this study; B. G. M. Jamieson & A. Georges, unpublished results). Only in squamates, however, does the subacrosomal cone exhibit paracrystalline substructure (Furieri 1970; Butler & Gabri 1984).

Nuclear length and shape are extremely variable among tetrapods ranging from very long and helical as in *Sphenodon* (e.g. monotremes, some passerine birds), through moderately long and curved (Chelonia, *Caiman*, Squamata, amphibians) to short and straight (some mammals, passerines) (Fawcett 1970; Furieri 1970; Carrick & Hughes 1982; Rouse & Robson 1986; Asa & Phillips 1987; De *et al.* 1987). Helical (or helically keeled), elongate nuclei are also recorded in a variety of invertebrates (cephalopod and gastropod molluscs (Maxwell 1974; Healy 1990); chilopods (Jamieson, 1986); leeches (Garavaglia *et al.* 1974)) reflecting a general trend towards spiralling of this and other sperm components in many internally fertilizing groups. Perhaps of more phylogenetic significance than gross nuclear morphology is the occurrence in *Sphenodon* of two parallel, helically twisted endonuclear canals, each containing perforatorial material. Precisely the same configuration occurs in turtles, although three canals may be present in some turtle sperm (Yasuzumi & Yasuda 1968; Yasuzumi *et al.* 1971; Furieri 1970; B. G. M. Jamieson & A. Georges unpublished results; this study). Elsewhere among vertebrates, multiple endonuclear canals have only been observed in certain fish such as the white sturgeon *Acipenser transmontanus* (three helical canals, Cherr & Clark 1984), the lungfish *Neoceratodus forsteri* (two superficial canals, Jespersen 1971) and the coelacanth *Latimeria chalumnae* (three or four canals

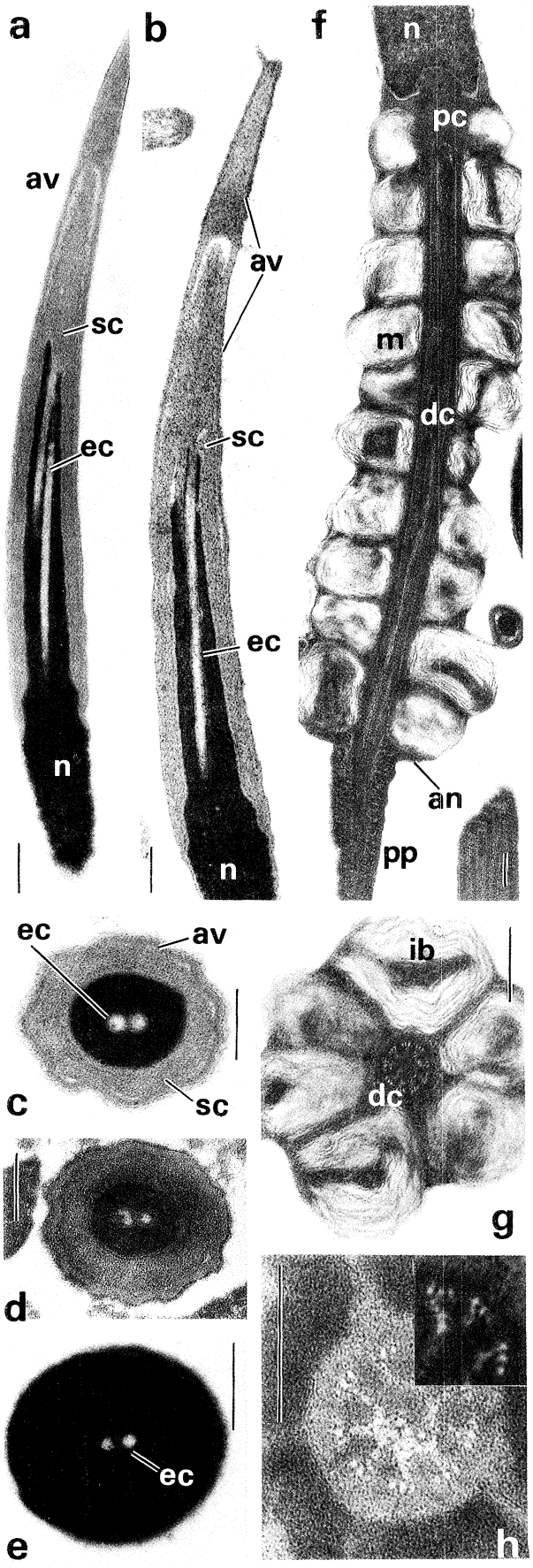


Figure 5. Spermatozoa of Chelonia. (a) Acrosomal vesicle (av), subacrosomal cone (sc) endonuclear canals (ec) and anterior region of nucleus (n) in *Emyduras signata*. (b) Acrosomal complex and anterior region of nucleus of *E. kreffti*. (c) Transverse section through acrosomal complex and nucleus in *E. kreffti*. (d) Transverse section through acrosomal complex and nucleus in *Chelodina expansa*. (e) Transverse section of nucleus below level of acrosomal complex in *E. kreffti* (canals persistent). (f) Nuclear fossa, proximal and distal centrioles (pc, dc), midpiece mitochondria (m), annulus (an) and proximal portion of principal piece (pp) in *E. kreffti*. (g) Transverse section of midpiece in *E. kreffti* showing distal centriole, concentric cristae and dense, intramitochondrial body (ib). (h) Detail of distal centriole showing triplets (*E. longisternum*). Inset: open condition of C tubule of triplets in *E. kreffti*. All scale bars = 0.25 μm.

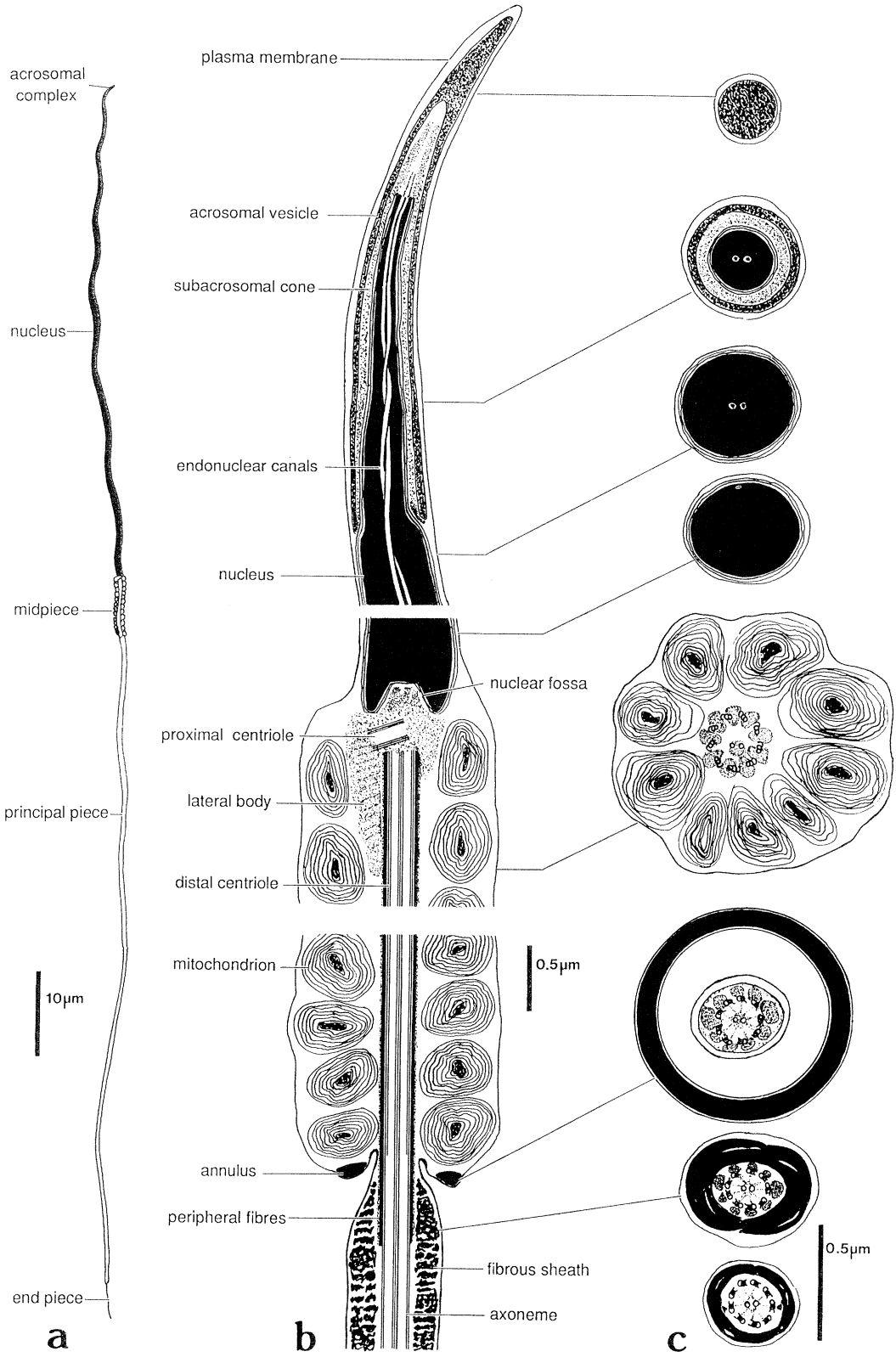


Figure 6. Spermatozoa of *Sphenodon punctatus* (semi-diagrammatic summary). (a) Whole spermatozoon showing relative positions and lengths of acrosomal complex, nucleus, midpiece, principal piece and end piece. (b) Ultrastructural features of spermatozoon as viewed in longitudinal section. Note striations of lateral body and substructure of mitochondria (concentric cristae and dense intramitochondrial body). (c) Corresponding transverse sections through the spermatozoon. Middle region of principal piece (at bottom of figure) not shown in longitudinal section. Scale bars, (a) = 10 μm; (b, c) = 0.5 μm.

occur only in 'abnormal' sperm of this species; Mattei *et al.* 1988). A single endonuclear canal occurs in *Caiman* (Saita *et al.* 1987), ratite and non-passerine birds (Asa *et al.* 1986; Asa & Phillips 1987; Thurston & Hess 1987; Phillips & Asa 1989), urodeles (Picheral 1967), lampreys (see Jamieson 1991) and the coela-

canth (Mattei *et al.* 1988). Primitive frogs (*Ascaphus truei* (B. G. M. Jamieson, M. S. Y. Lee, & K. Long, unpublished results); discoglossids (Sandoz, 1969, 1970; Furieri, 1975)), possess an endonuclear canal (Jamieson 1990) and a single canal may be plesiomorphic for tetrapods. An endonuclear canal is also absent in squamates (Furieri 1970) and mammals (Fawcett 1970; Carrick & Hughes 1982).

In the white sturgeon, contents of all three endonuclear canals (and other subacrosomal material) participate in the acrosome reaction, but only a single acrosomal process is formed (Cherr & Clark 1984). Further research will be needed to determine whether the same events, particularly extrusion of a single process, occur in other groups possessing multiple endonuclear canals such as turtles and *Sphenodon*. Further evidence for involvement of the endonuclear canals in the acrosome reaction is seen in the sperm of lampreys (*Lampetra* spp.). In these the single endonuclear canal extends throughout the length of the nucleus and into the flagellum (see references in Jamieson (1991). It contains an acrosomal filament or perforatorium capable, in *Lampetra fluviatilis*, of extrusion as a 50 μm long 'head filament' (Afzelius & Murray 1957).

An alternative hypothesis concerning the function of sperm endonuclear canals (in turtles) has been advanced by Yasuzumi *et al.* (1971). These authors suggest that the canals of turtle sperm are 'more likely to be responsible for the metabolism of the nucleus than to be an apparatus concerned with the sperm-egg interaction' (Yasuzumi *et al.* 1971, p. 126). They base this idea firstly on ultrastructural resemblances between the sperm canals and the 'intranuclear caniculus' of the Ehrlich ascites tumour cells and secondly on unpublished work (1971) by Yasuzumi & Sugihara, who found DNA synthesis in nuclei of tumour cells to be initiated at the caniculus. Although evidence cited above provides strong support for an acrosomal function of endonuclear canals in turtles and *Sphenodon*, the 'nuclear metabolism' theory of Yasuzumi *et al* (1971) deserves further investigation through detailed biochemical work. The two interpretations need not be incompatible: endonuclear canals could function in the acrosome reaction while having additional functions in nuclear metabolism and DNA replication.

Centrioles, lateral body and midpiece

Although the presence of a proximal centriole is well established for tetrapod sperm including *Sphenodon*, the presence and microtubular substructure of the distal centriole is less clear. A distal centriole is not seen in mature mammalian sperm (Fawcett 1970), but is present in sperm of anurans (Sandoz 1969, 1970; Furieri 1975; B. G. M. Jamieson, M. S. Y. Lee & K. Long, unpublished results), birds (Asa & Phillips 1987; Asa *et al.* 1986) and squamates (Furieri, 1970). In *Sphenodon* the distal centriole consists of true triplets partly embedded in nine peripheral fibres. The triplets, like those of the proximal centriole, show an open C tubule. The distal centriole runs the full length of the midpiece and is penetrated by a pair of central tubules partly ensheathed by dense material. An almost identical arrangement occurs in sperm of turtles and the caiman. Furieri (1970) and Saita *et al.* (1987) interpreted the axial component of the midpiece in these groups as being axonemal and not centriolar. They did not recognize the existence of triplets and interpreted the third, C, microtubule as merely a space giving the spurious impression of a microtubule. We reject this conclusion on the basis that open C microtubules have been shown in centrioles of the sperm of other tetrapods (for instance, in the primate *Macaca arctoides* (Pedersen 1974)) and, more importantly, are clearly visible in the proximal as well as the distal centriole of *Sphenodon* (figure 2b). Our conclusion that the axial region of the midpiece in *Sphenodon*, turtles, *Caiman* and non-passerine birds is a true centriole and not an anterior extension of the axoneme, despite containing two central singlets, is also supported by the presence of elongate centrioles in spermatocytes of *Sphenodon* (figure 2c–e and J. M. Healy & B. G. M. Jamieson, unpublished results) and in spermatids of non-passerine birds (Asa & Phillips 1987; Phillips & Asa 1989). In spermatids of the ratite *Rhea*, the distal centriole is elongate, and late in spermiogenesis, becomes penetrated by a central pair of tubules from the developing axoneme, giving it an axoneme-like appearance (albeit with triplet construction) in the mature sperm (Phillips & Asa 1989). Although some studies have dealt with aspects of spermiogenesis in turtles and crocodilians (Yasuzumi & Yasuda 1968; Yasuzumi *et al.* 1971; Saita *et al.* 1987) detailed information on the structure and fate of the centrioles in these groups is still required.

Our detection of cross-striations within the lateral body of late spermatids of *Sphenodon* suggests that this region of mature sperm is likewise striated, as observed in turtles (Furieri 1970; present study), *Caiman* (Saita *et al.* 1987) and most mammals (Fawcett 1970; evidently absent in monotremes (Carrick & Hughes 1982)). Figure 6 reconstructs the neck region of the mature *Sphenodon* spermatozoon as having a striated lateral body extending into the midpiece.

Sphenodon, turtles and *Caiman*, alone among tetrapods, exhibit spheroidal sperm mitochondria with concentric cristae and a dense intramitochondrial body (Yasuzumi & Yasuda 1968; Furieri 1970; Phillips 1970; Saita *et al.* 1987; B. G. M. Jamieson & A. Georges unpublished results; this study). Other amniotes, with the single reported exception of the woolly opossum, *Caluromys philander*, possess conventional cristate mitochondria (Fawcett 1970; Furieri 1970; Phillips 1970; Carrick & Hughes 1982; Rouse & Robson 1986; Temple-Smith 1987; Carcupino *et al.* 1989). Midpiece mitochondria of the woolly opossum, although exhibiting similar substructure to *Sphenodon*, turtles and *Caiman*, are elongate, flattened and helically arranged around the axoneme (Phillips 1970). Observations on early spermatids of *Sphenodon* (this study) and *Caiman* (Saita *et al* 1987) reveals only normal, cristate mitochondria, indicating that the concentric condition is attained late in spermiogenesis. It has been suggested that closely packed concentric cristae in sperm or spermatids of turtles (Yasuzumi &

Yasuda 1968) and the woolly opossum (Fawcett 1970) may reflect a demand for greater mitochondrial efficiency through maximization of available cristal surfaces. In support of this idea, Yasuzumi & Yasuda (1968) cite unpublished work of Sakamoto who found larger amounts of ATPase in mitochondria with concentric cristae compared with those with conventional cristae. The animal groups and cell types studied by Sakamoto were not specified. Certainly there are examples of increased development of cristae, albeit linear, in cell types which have high energy demands, for instance mouse cardiac muscle, while cristae are greatly reduced in the facultative anaerobe, *Fasciola hepatica* (references in Threadgold, (1976)). However, on the basis of their cladistic analysis of sperm characters, Jamieson & Healy (1992) argue in favour of the concentric cristae being a primitive rather than advanced feature of amniotes. We envisage that the presence of relatively unmodified cristae in sperm of birds (Asa & Phillips, 1987) and squamates could be brought about through suppression of a final, concentric cristal phase of mitochondrial maturation in spermiogenesis. By the same reasoning, presence of the concentric condition in the woolly opossum (Phillips 1970) may represent a failure to suppress this phase. Presence in this didelphid possibly indicates that ancestral mammals had mitochondria with concentric cristae as a persistence of the condition attributed here to basal amniotes.

If the interpretation of concentric cristae as allowing increased metabolic activity of the mitochondria and our view that they are plesiomorphic for amniotes are correct, it appears enigmatic on first consideration that concentric cristae have been lost in most amniotes. An explanation should be sought in the metabolic requirements of sperm which have reverted to 'conventional' cristae. This possibly is an evolutionary response to different conditions, or periods of storage, in the male or female tract relative to turtles, *Sphenodon*, crocodile and the woolly opossum.

The mitochondria of these four taxa with concentric cristae each contain a highly electron-dense central body but no obvious matrix between the cristae (Furieri 1970; Phillips 1970; Saita *et al.* 1987; this study). Although sperm of squamates lack a central, dense body (Furieri 1970; B. G. M. Jamieson, unpublished results), it is likely that the prominent intermitochondrial rings are mitochondrial in origin and homologous with the dense, central body (Carcupino *et al.*, 1989; Jamieson & Healy, 1992).

Annulus

An annulus occurs at the junction of the midpiece and principal piece in most tetrapod spermatozoa, and is also recorded for many invertebrate groups (Baccetti & Afzelius 1976). Among the amniotes, it is well developed in *Sphenodon*, *Caiman*, birds and most mammals (Fawcett 1970; Asa *et al.* 1986; Asa & Phillips 1987; Xia *et al.* 1986; Saita *et al.* 1987; Thurston & Hess 1987), but it is vestigial in monotremes (Carrick & Hughes 1982). Fawcett (1970) recognizes two structural categories of annulus in mammalian sperm, based on the profile of the annulus as viewed in longitudinal section : triangular (e.g. bats, dormouse, Chinese hamster, antelope) and semicircular (e.g. mouse, guinea pig, chinchilla, ram). The annulus of *Sphenodon* is of the semicircular-profile type, at least in being rounded rather than angular, as is that of *Caiman* (see Saita *et al.* 1987), while in the rhea, a triangular-profile annulus is present (Phillips & Asa 1989). The taxonomic significance of the shape of the annulus is questionable, given that in the Rodentia for example (see Fawcett 1970), both categories are encountered. Furieri (1970) neither mentions nor illustrates an annulus in turtle sperm, but our observations show this feature to be present though not always clearly visible in longitudinal sections. The apparent absence of the annulus in sperm of squamates (Furieri 1970) may be connected with the extensive overlap of the fibrous sheath between midpiece and principal piece. However, if Fawcett's (1970) hypothesized function of the annulus is accepted, that it prevents posterior displacement of midpiece mitochondria during motility, then an alternative explanation for the absence of an annulus in squamates must be sought. As the annulus in vertebrates and invertebrates develops in close association with (? and formed by) the distal centriole (Phillips 1974; Buckland-Nicks *et al.* 1983), a detailed analysis of this aspect of spermiogenesis in squamates could provide an answer to this problem.

Principal piece

A principal piece is present in spermatozoa of all amniotes, including *Sphenodon*. It consists of the axoneme (with peripheral fibres proximally) enveloped by a sheath of fibrous, highly electron-dense material (Fawcett 1970; Furieri 1970; Carrick & Hughes 1982; Asa *et al.* 1986; Asa & Philips 1987). Substructure of the fibrous sheath varies between major groups. In chelonians and squamates (Furieri 1970), mammals (Fawcett 1970) and ratite birds (Asa *et al.* 1986; Phillips & Asa 1989), the sheath consists of more or less regular rings, which, in *Sphenodon* (present paper) as in some mammals, anastomose; in non-passerine birds the sheath is amorphous. In *Sphenodon,* as in other 'reptiles', peripheral fibres at doublets 3 and 8 continue far posteriorly of the other fibres, within the principal piece. In most mammals the fibrous sheath, seen in cross section, has two inward triangular projections (longitudinal columns *sensu* Fawcett (1970)) opposite doublets 3 and 8, with or without persistence of the corresponding peripheral fibres (according to the level of section) and peripheral fibres 3 and 8 do not appear to extend posteriorly of the others (Fawcett 1970). In monotremes inward projections of the sheath are apparently absent or very weakly developed and there is, again, no evidence for a greater extent of peripheral fibres 3 and 8 (Carrick & Hughes 1982). The triangular projections are also present in the rhea and tinamou (Phillips & Asa 1989; Asa *et al.* 1986). If, as suggested by Fawcett (1970), the fibrous sheath possesses elastic properties, attachments to the axoneme would undoubtedly function in motility.

The isolated peripheral fibres 3 and 8 in amniotes may well be homologous with columns at this point in other sarcopterygians, the coelacanth, *Latimeria*, and Dipnoi. However, modifications at doublets 3 and 8, which are approximately in the plane of the two central singlets could be independent acquisitions as they presumably are in chondrichthyan sperm (see references in Jamieson (1991)).

In *Sphenodon*, turtles, crocodiles, mammals and birds the fibrous sheath is confined to the principal piece and almost certainly this represents the ancestral state of this feature. By contrast, in squamates the fibrous sheath is observed not only in the principal piece but also in the midpiece where it extends to the distal centriole (Furieri 1970; Carcupino *et al.* 1989).

(*b*) *Phylogenetic importance of Sphenodon spermatozoa*

The most significant point to emerge from this study is the extraordinarily close resemblance of *Sphenodon* sperm to those of turtles (Furieri 1970; this study; B. G. M. Jamieson & A. Georges unpublished) and crocodiles (Saita *et al.* 1987). The similarities are shared plesiomorphies but nevertheless underline the paucity of advanced characters that link these taxa with other amniotes. Recently the primitive status of *Sphenodon* has been questioned on anatomical grounds (Whiteside 1986; Evans 1988). With the notable exception of Løvtrup (1985), most authors favour a sister-group relationship between the Sphenodontidae and Squamata (these two united as Lepidosauria; Fraser 1986; Evans 1984, 1988; Gauthier *et al.* 1988*a*, *b*). Whiteside (1986) has gone so far as propose that *Sphenodon* is advanced relative to lizards. However the only spermatozoal features shared by *Sphenodon* and the Squamata also occur in turtles, *Caiman*, some birds and monotremes. Squamatan sperm apomorphies such as the paracrystalline inner core of the acrosome, penetration of the fibrous sheath to the base of the distal centriole, absence of endonuclear canals and presence of intermitochondrial dense deposits, do not occur in *Sphenodon* (Jamieson & Healy 1992). The absence of distinctive apomorphies shared between Sphenodontida and Squamata does not endorse the supposed sister-group relationship between the two taxa. The low degree of sperm apomorphy for *Sphenodon* reasserts its primitive status. Cladistic analysis of sperm ultrastructure in the Amniota using turtles as the outgroup (see Jamieson & Healy 1992), gives a single most parsimonious tree in which *Sphenodon* emerges as the sister-group to all amniotes above turtles. In the next branching sequence Crocodilia emerge as the sister-group of birds and monotremes on the one hand and a unified Squamata on the other. The bird–monotreme relationship (Haemothermia of Gardiner (1982)) is to be regarded only as heuristic but the basal position of *Sphenodon* can confidently be asserted.

To conclude, our study has shown that spermatozoa of the tuatara strongly resemble those of turtles and crocodiles. The results unequivocally indicate that the living tuatara (and by association other sphenodontids) is truly primitive and is a basal amniote with no special relationship to the Squamata.

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Note added in proof (20 December 1991): While this paper was 'in press' we received the following publication: Hess, R.A., Thurston, R.J. & Gist, D.H. 1991 Ultrastructure of turtle spermatozoon. *Anat. Rec.* **229**, 473–481. Its findings for *Chrysemys picta* sperm are in essential agreement with our own for Australian freshwater turtles, including demonstration of an annulus and presence of an elongate, distal centriole with open C tubules. However, Hess *et al.* make no comparison with crocodiles. Most, if not all, features which they consider unique to turtles, including the perforatorial rod and membranous mitochondria, are shown in our paper to occur in other amniote groups.

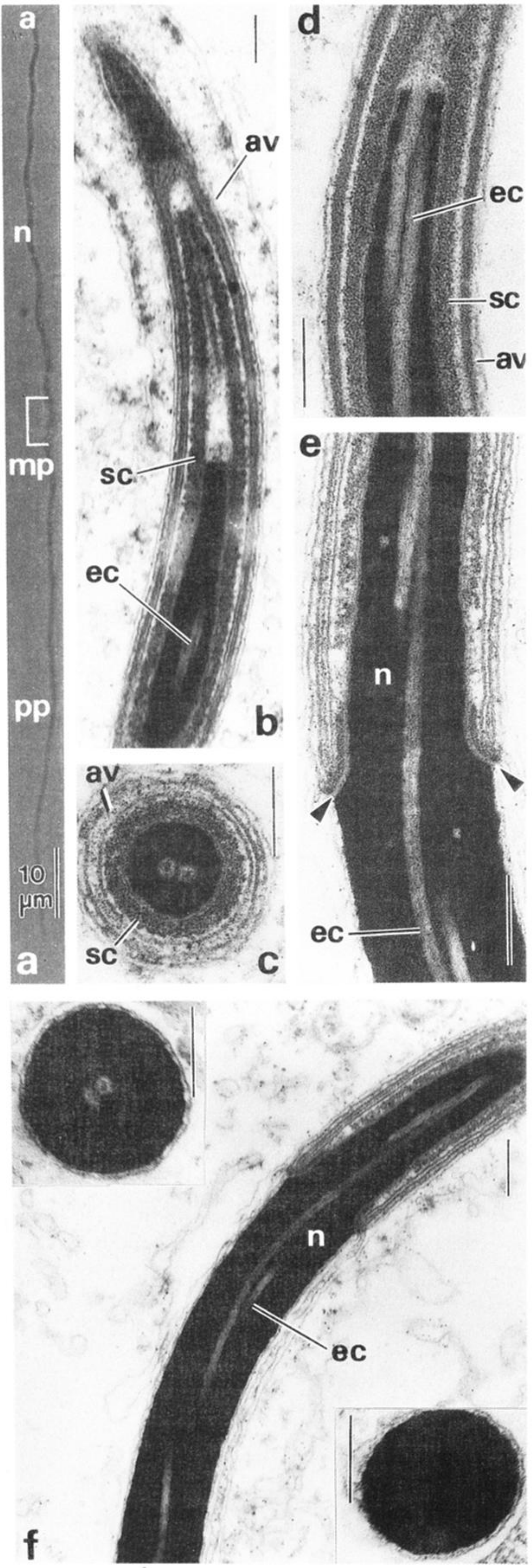


Figure 1. *Sphenodon punctatus* (a) Whole spermatozoon (phase-contrast light microscopy). Positions of acrosomal complex (a), nucleus (n), midpiece (mp) and principal piece (pp) indicated. (b) Longitudinal section of acrosomal complex sheathing the nuclear apex. Note acrosomal vesicle (av), subacrosomal cone (sc), endonuclear canals (ec). (c) Acrosomal complex and nucleus in transverse section. Endonuclear canals (containing perforatorial material) visible. (d) Anterior extremity of nucleus. (e) Basal region of acrosomal complex with arrow heads indicating nuclear shoulder. Note twisting of endonuclear canals. (f) Penetration of endonuclear canals posterior to acrosomal complex. Upper inset: endonuclear canals posterior to acrosome. Lower inset: nucleus posterior to endonuclear canals. Scale bars = (a) 10 µm; (b–f) 0.25 µm

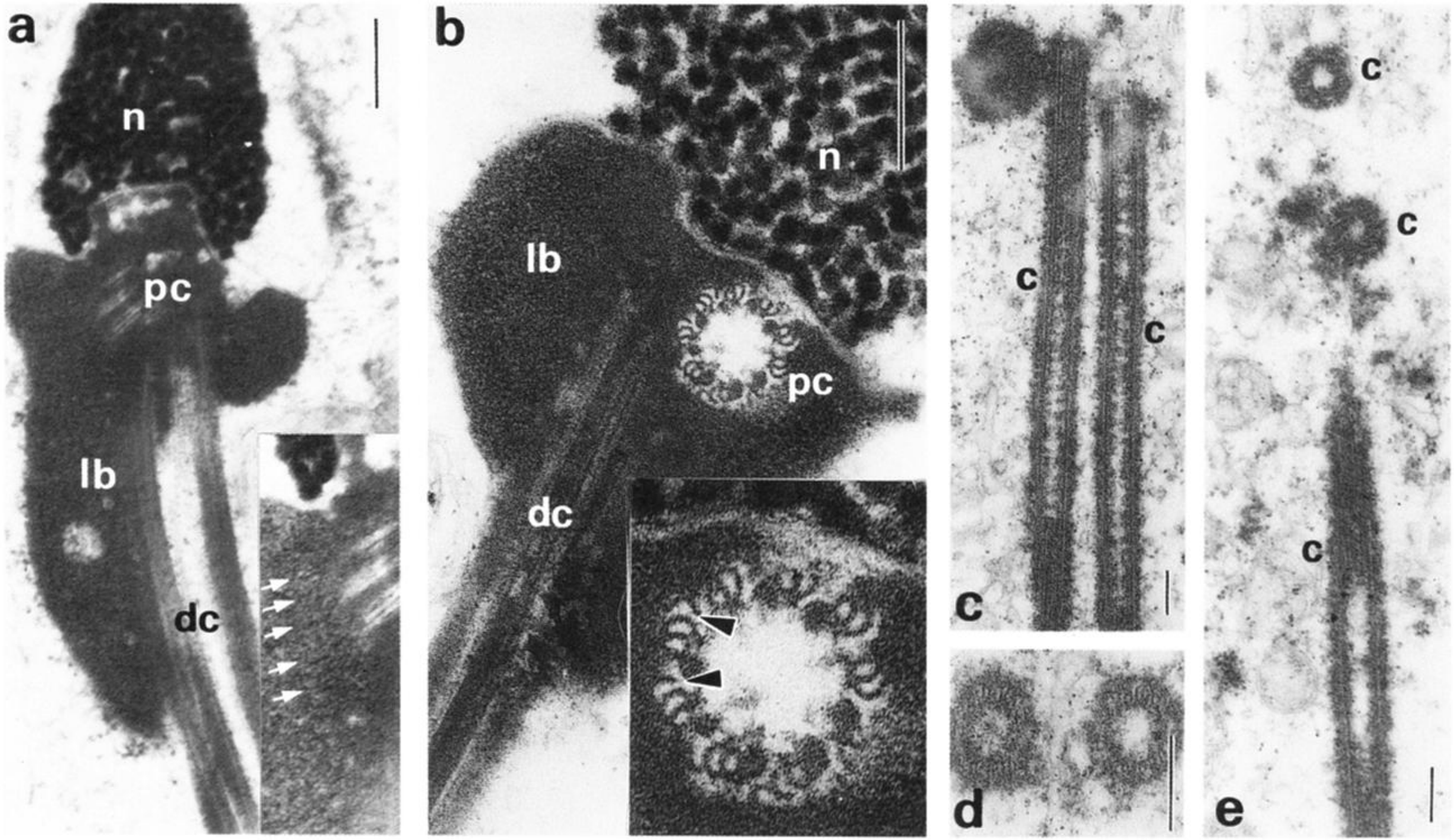
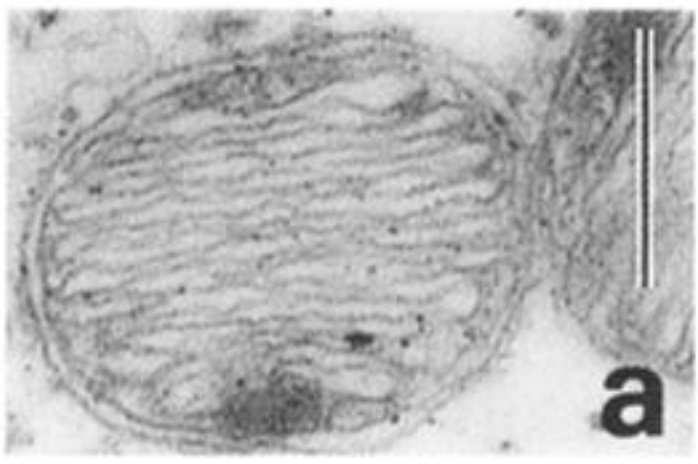
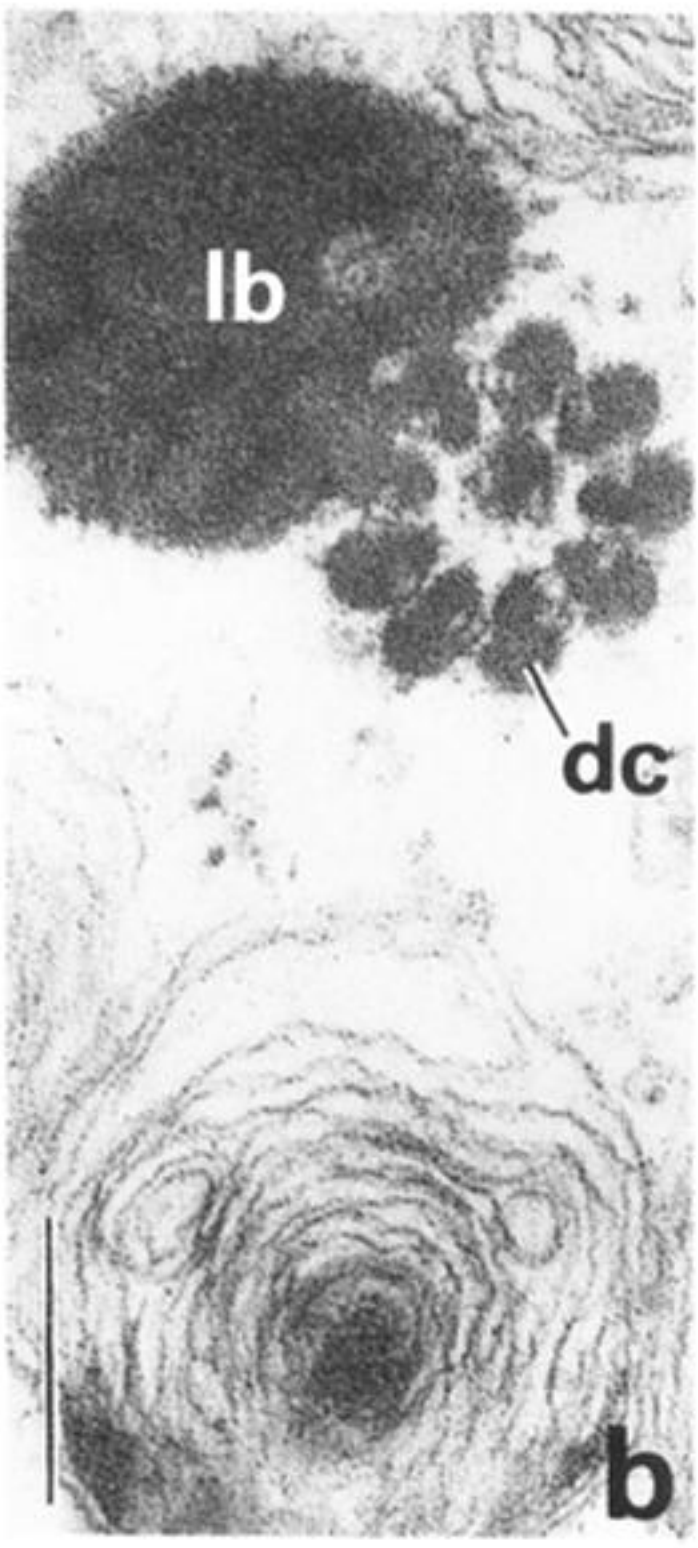
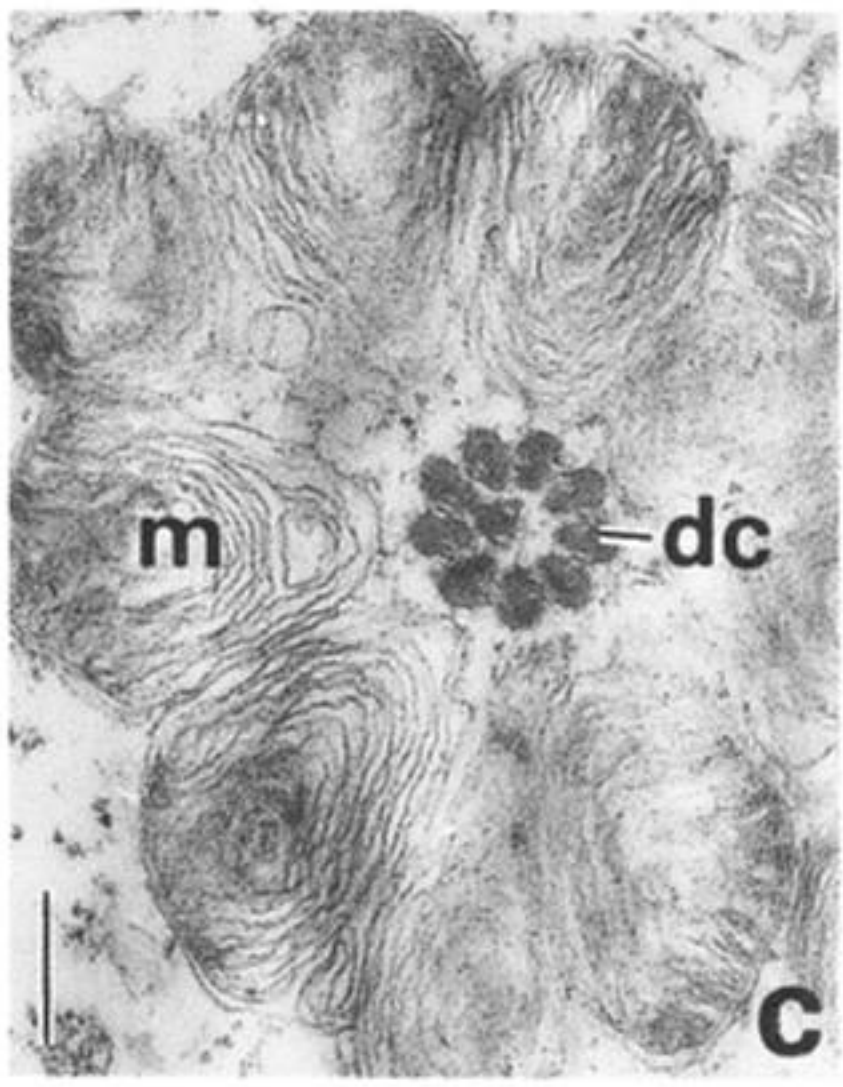
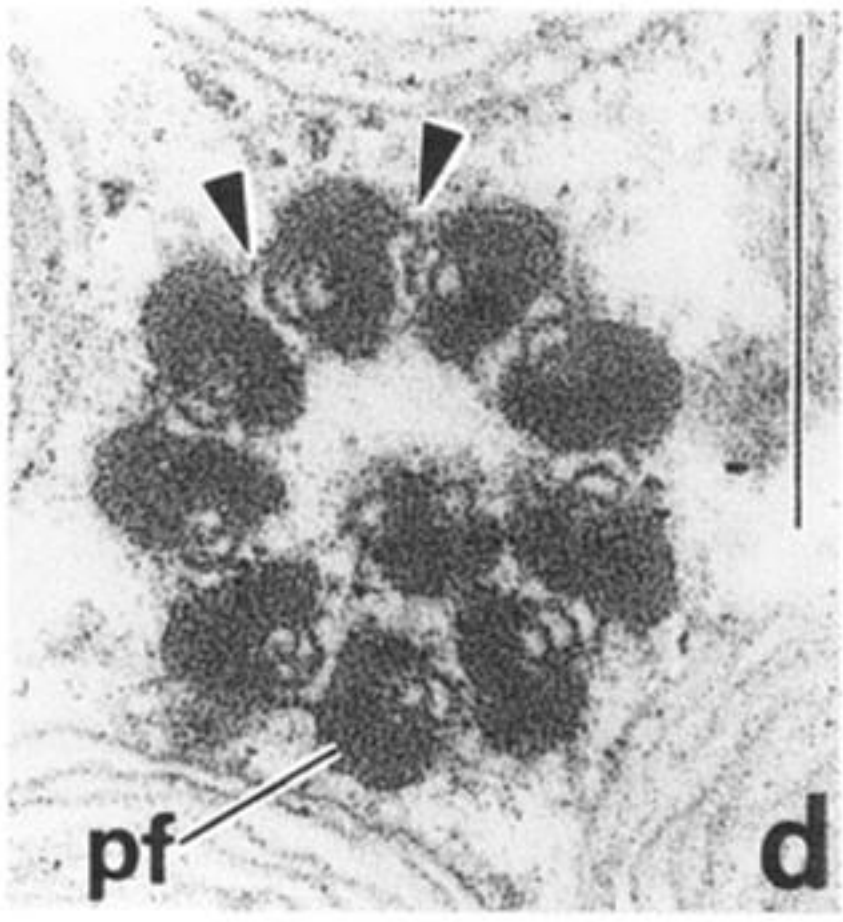


Figure 2. *Sphenodon punctatus* (a) Neck region of late spermatid showing proximal and distal centrioles (pc, dc), extent of lateral body (lb) and condensing nucleus (n). Inset: arrows indicate faint periodic striations present within lateral body. (b) Proximal centriole and elongate distal centriole embedded in matrix of lateral body at base of condensing nucleus in advanced spermatid. Inset: detail of proximal centriole showing open C tubule (arrow heads). (c) Pair of elongate centrioles of spermatocyte. (d) Transverse section of paired centrioles. (e) Three centrioles visible in spermatocyte. All scale bars = 0.25 μm









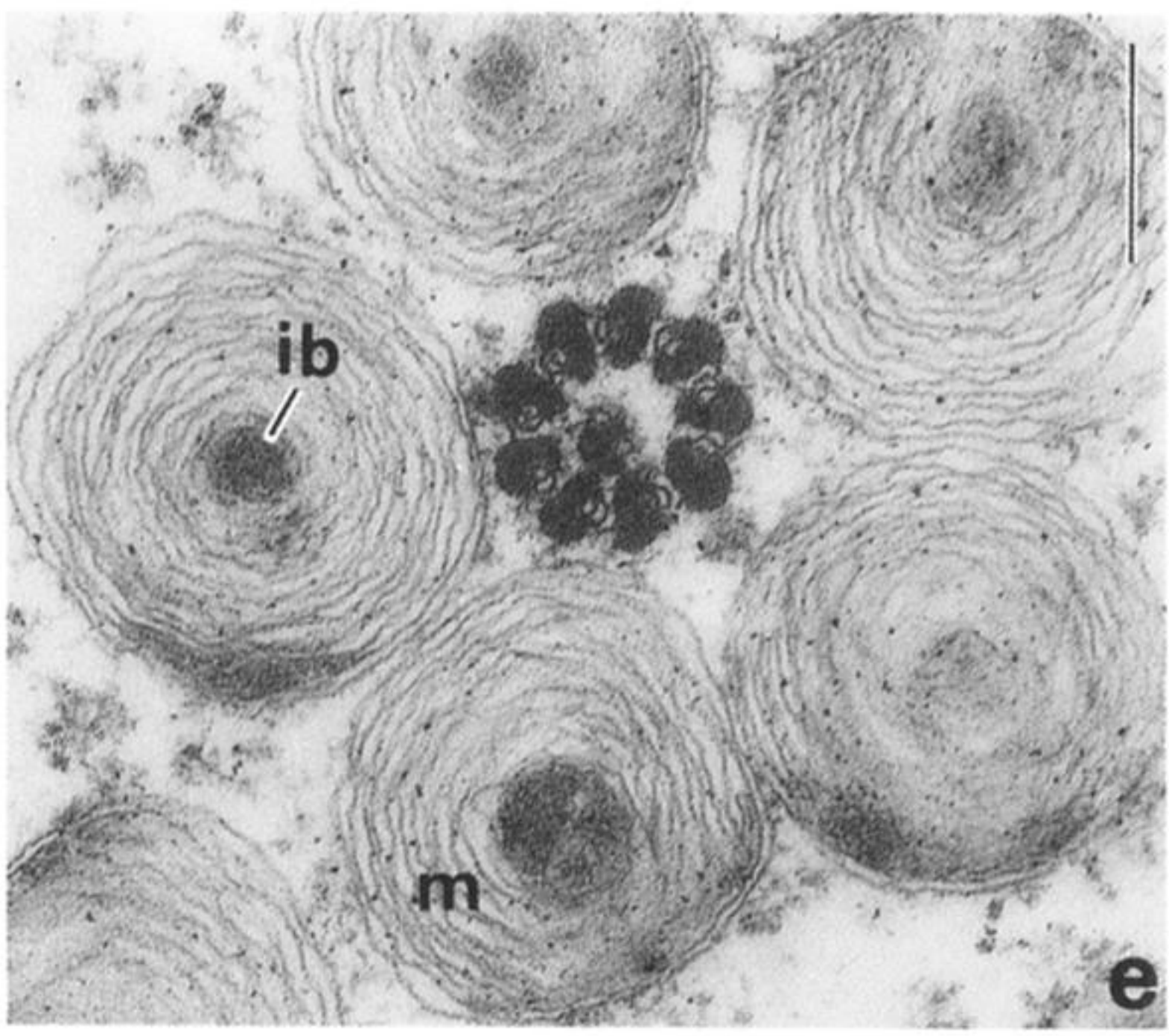
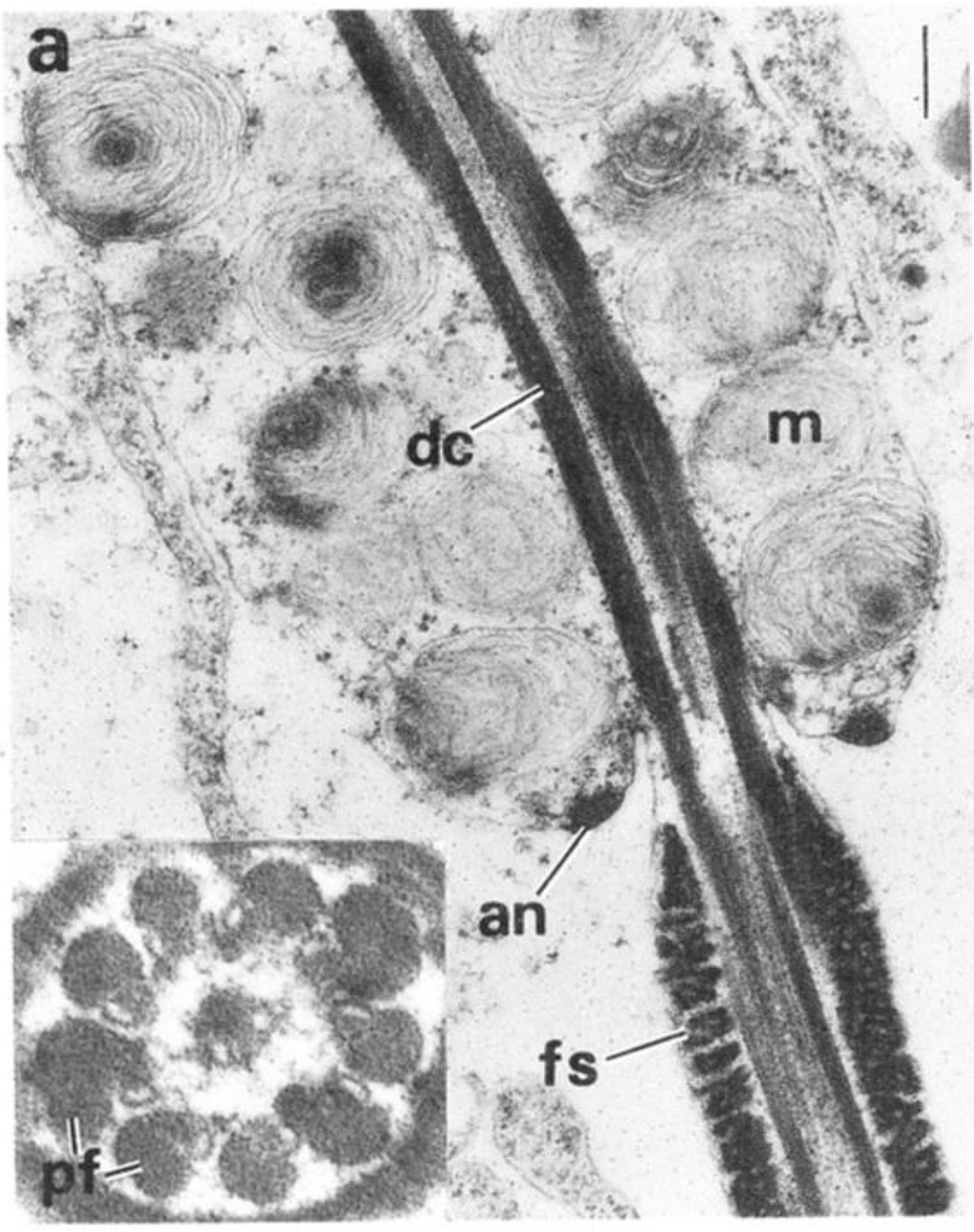
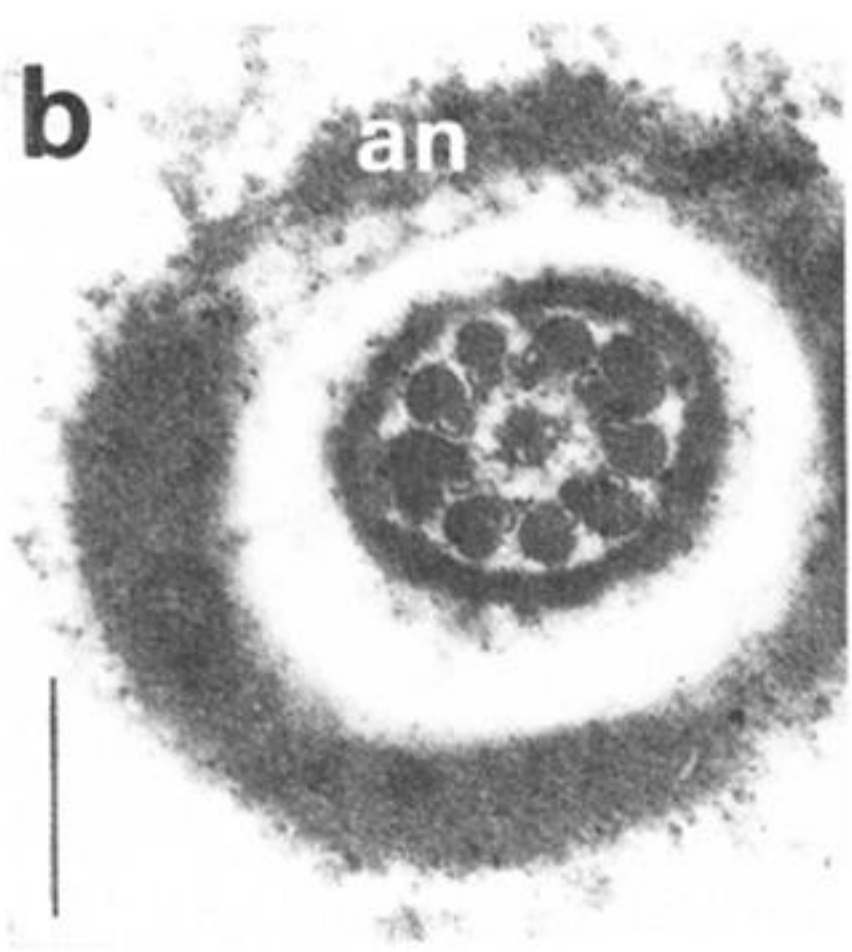
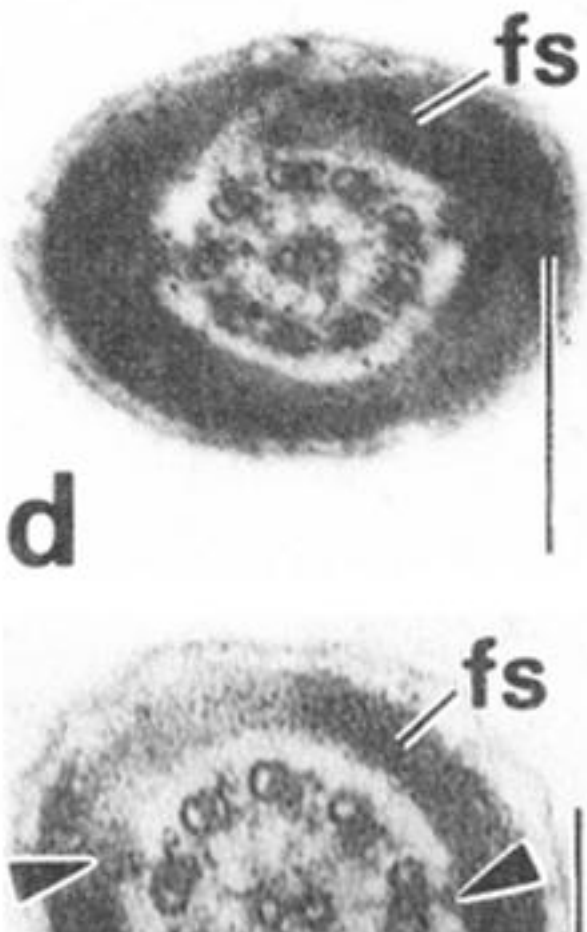
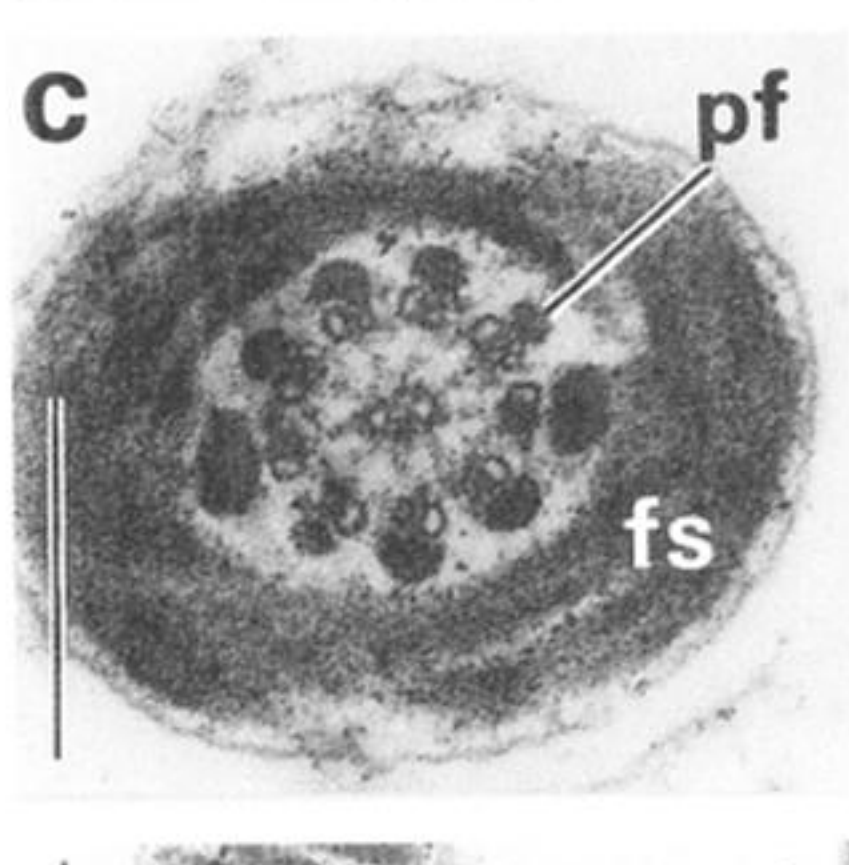


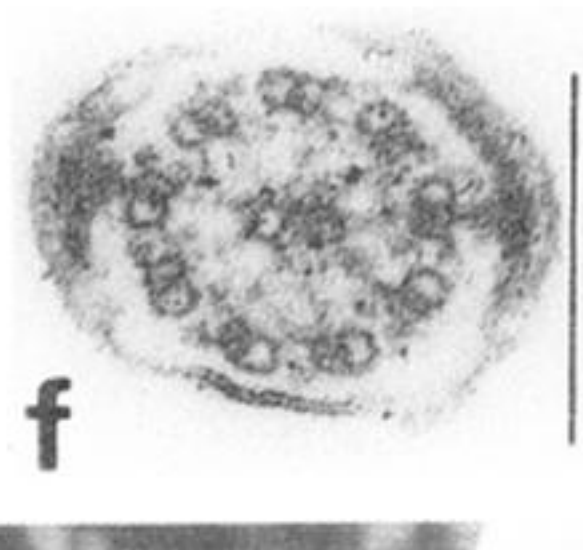
Figure 3. *Sphenodon punctatus*. (a) Mitochondrion with unmodified cristae from mid-spermatid (b) Lateral body (lb) and distal centriole (dc) of late spermatid. (c) Transverse section of mature midpiece showing mitochondria (m, with concentric cristae) packed around distal centriole. (d) Detail of distal centriole showing peripheral fibres (pf) between and partly sheathing centriolar triplets. Note open C tubules (arrow heads) of triplets. (e) Transverse section through posterior region of midpiece showing concentric cristae and dense intramitochondrial bodies (ib). All scale bars = 0.25 μm.

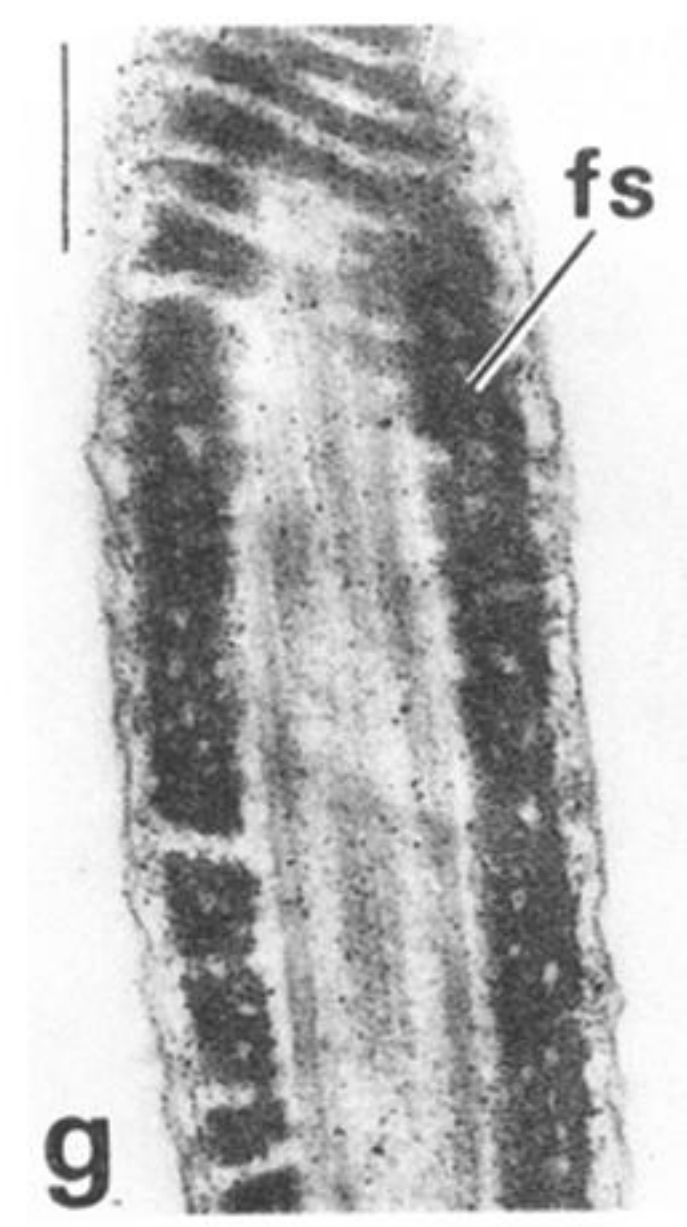












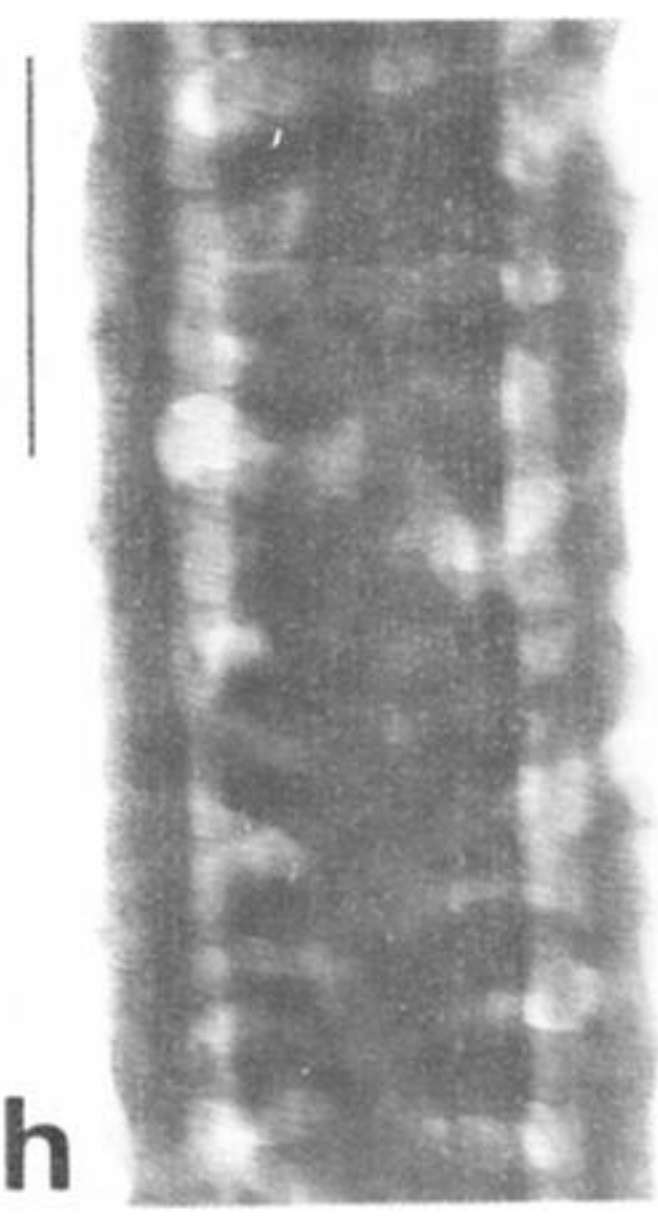
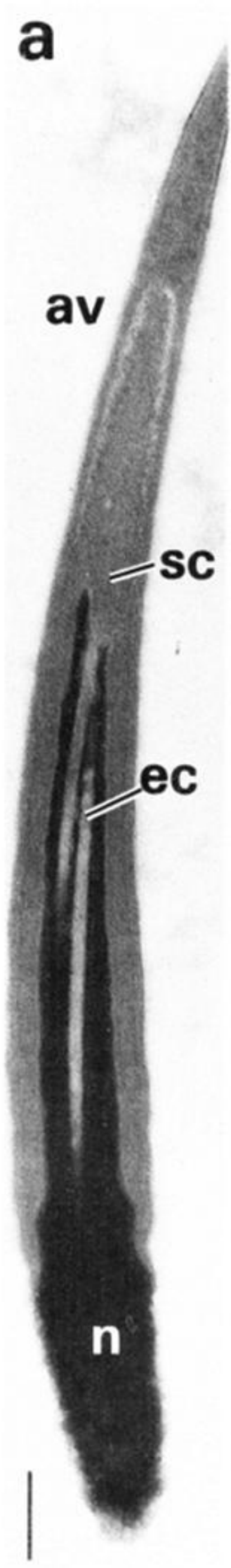
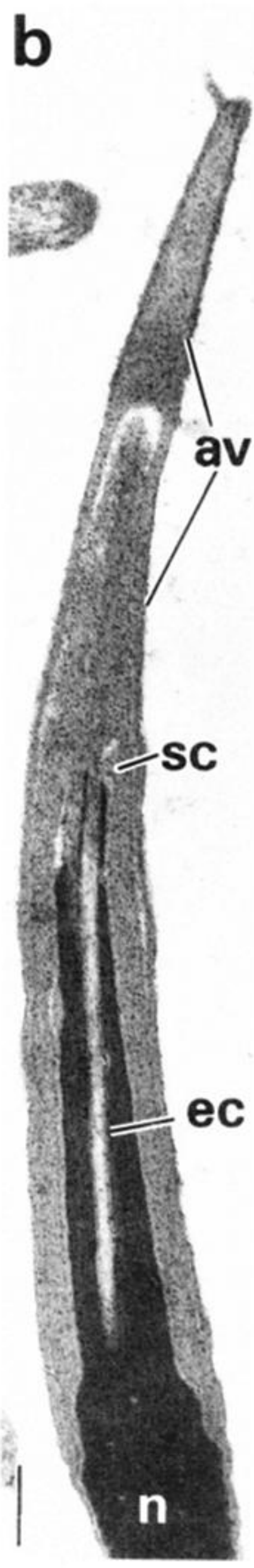
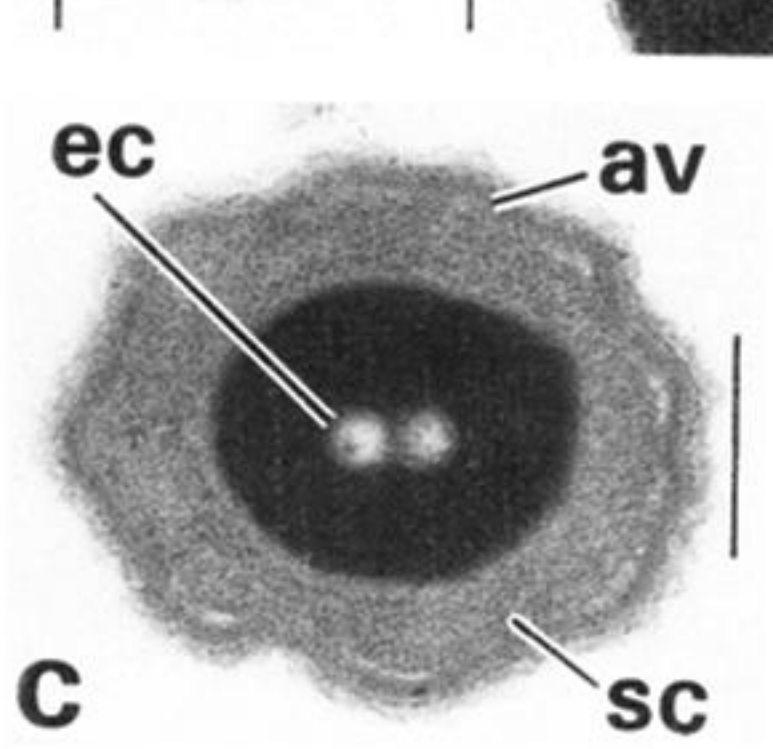
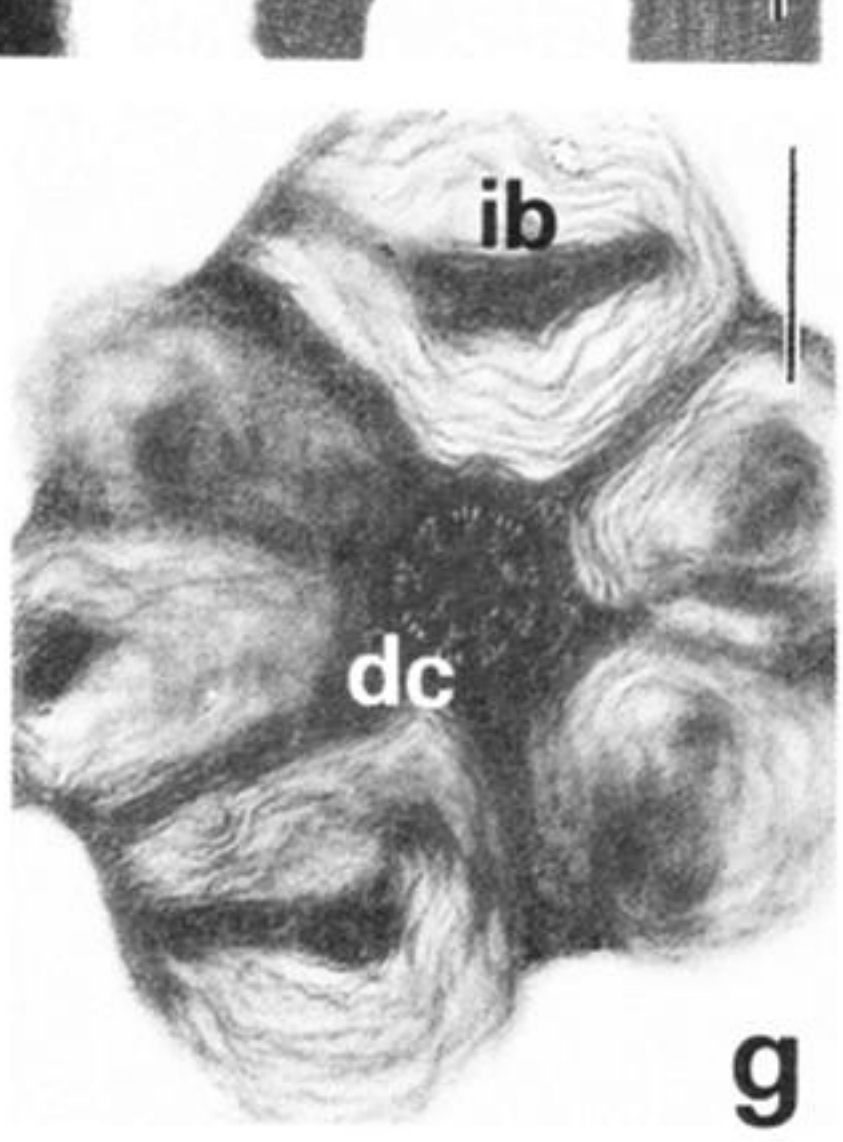


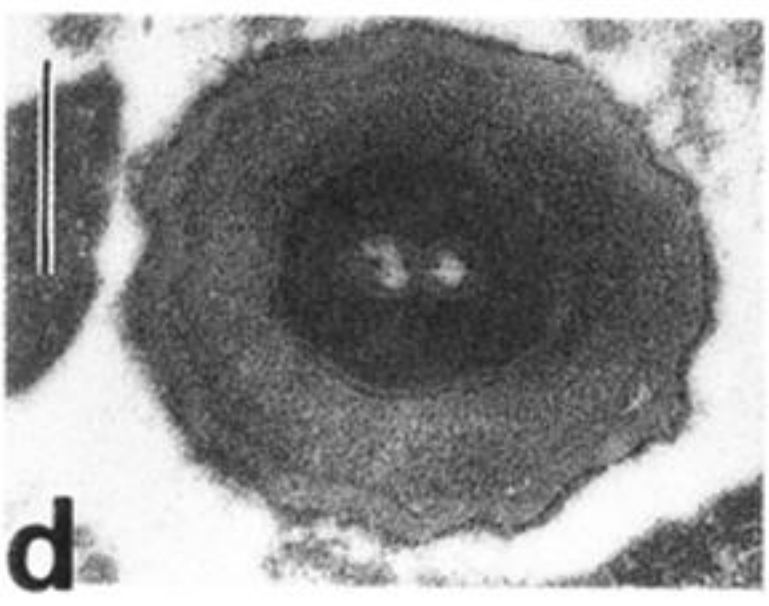
Figure 4. *Sphenodon punctatus*. (a) Longitudinal section through annulus (an) at the junction of midpiece (note mitochondria, m) and principal piece (note fibrous sheath, fs). Inset: peripheral fibres (pf) associated with axonemal doublets. (b) Transverse section through annulus. (c) Anterior region of principal piece showing fibrous sheath surrounding axoneme and peripheral fibres. (d) Anterior region of principal piece posterior to figure 4c showing loss of seven peripheral fibres. (e) Posterior region of principal piece (peripheral fibres near doublets 3 and 8: arrow heads). (f) Distal region of principal piece. (g) Portion of principal piece in oblique longitudinal section. (h) Negative stained principal piece showing anastomosing fibrous sheath. All scale bars = 0.25 μm.

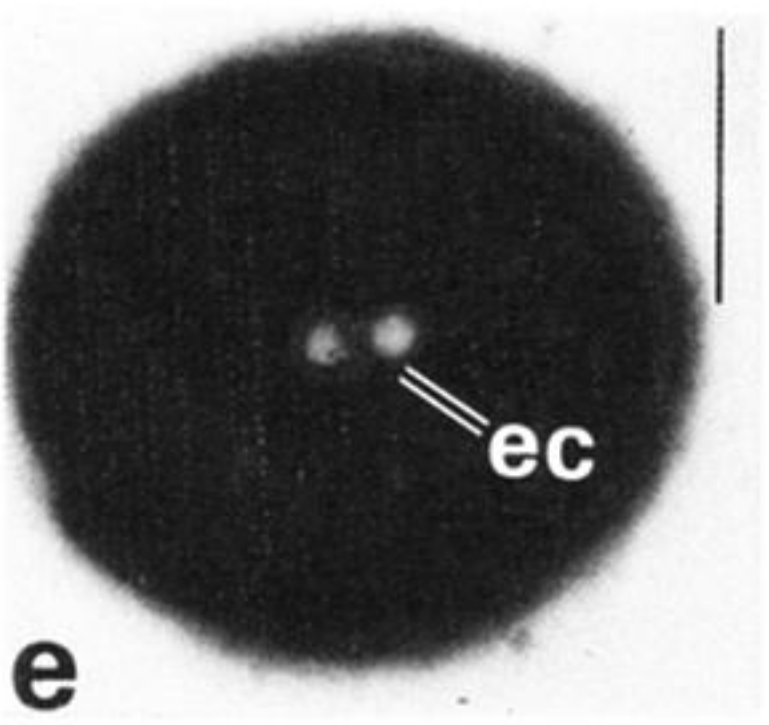












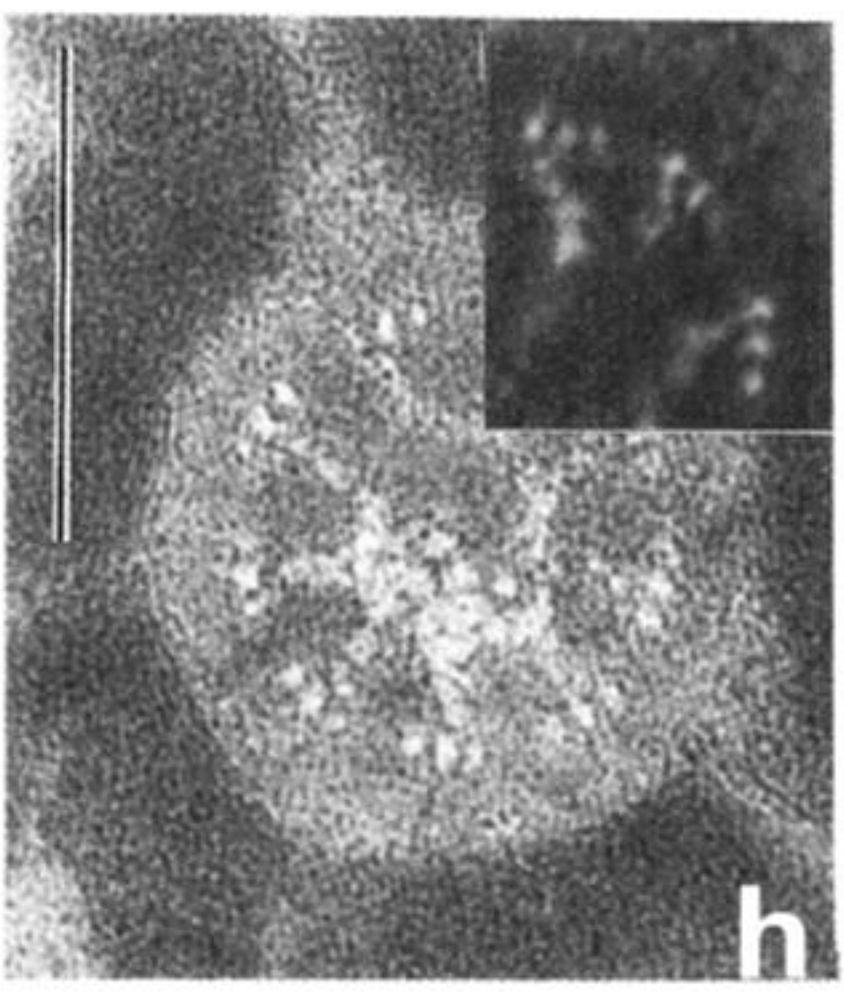


Figure 5. Spermatozoa of Chelonia. (a) Acrosomal vesicle (av), subacrosomal cone (sc) endonuclear canals (ec) and anterior region of nucleus (n) in *Emyduras signata*. (b) Acrosomal complex and anterior region of nucleus of *E. kreffti*. (c) Transverse section through acrosomal complex and nucleus in *E. kreffti*. (d) Transverse section through acrosomal complex and nucleus in *Chelodina expansa*. (e) Transverse section of nucleus below level of acrosomal complex in *E. kreffti* (canals persistent). (f) Nuclear fossa, proximal and distal centrioles (pc, dc), midpiece mitochondria (m), annulus (an) and proximal portion of principal piece (pp) in *E. kreffti*. (g) Transverse section of midpiece in *E. kreffti* showing distal centriole, concentric cristae and dense, intramitochondrial body (ib). (h) Detail of distal centriole showing triplets (*E. longisternum*). Inset: open condition of C tubule of triplets in *E. kreffti*. All scale bars = 0.25 μm.

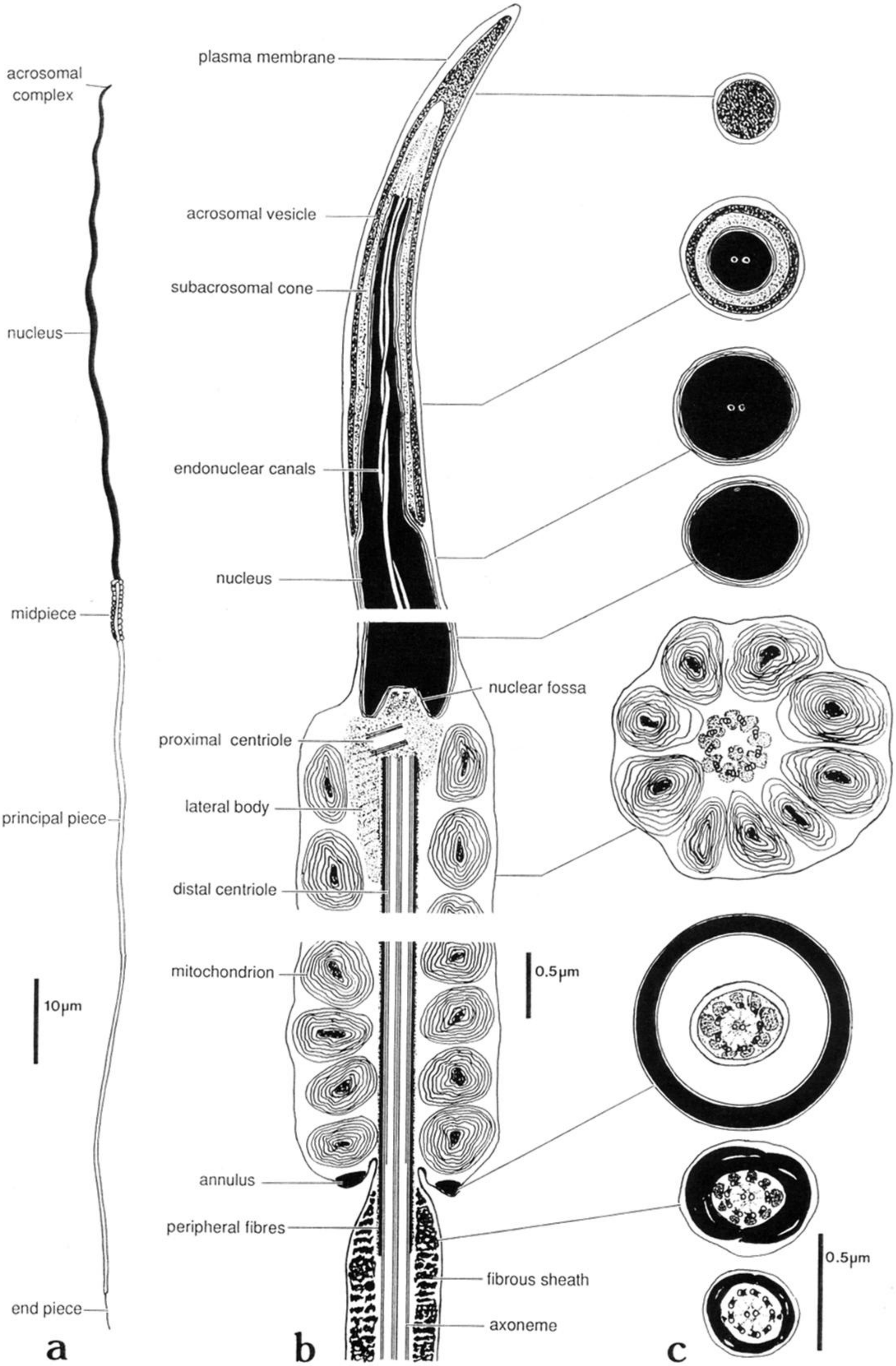


Figure 6. Spermatozoa of *Sphenodon punctatus* (semi-diagrammatic summary). (a) Whole spermatozoon showing relative positions and lengths of acrosomal complex, nucleus, midpiece, principal piece and end piece. (b) Ultrastructural features of spermatozoon as viewed in longitudinal section. Note striations of lateral body and substructure of mitochondria (concentric cristae and dense intramitochondrial body). (c) Corresponding transverse sections through the spermatozoon. Middle region of principal piece (at bottom of figure) not shown in longitudinal section. Scale bars, (a) = 10 μm; (b, c) = 0.5 μm.