J. SUBMICROSC. CYTOL. PATHOL., **29** (2), 265-274, 1997

The ultrastructure of spermatozoa of the Australian freshwater crocodile, *Crocodylus johnstoni* Krefft, 1873 (Crocodylidae, Reptilia)

B.G.M. JAMIESON, D.M. SCHELTINGA and A.D. TUCKER

Department of Zoology, University of Queensland, St. Lucia, Qld, Australia

SUMMARY - Mature spermatozoa of *Crocodylus johnstoni* are filiform with a curved head, consisting of acrosome complex (length 5.6 µm) and nucleus (length 10.7 µm); midpiece (length 4.8 µm); and elongate tail (length 49-61 µm). The acrosome vesicle and underlying subacrosomal material form a conical sheath around the anterior tapered nuclear rostrum. One to three endonuclear canals, each containing putative perforatorial material, extend posteriorly from the tip of the nuclear rostrum for approximately 5 µm, nearly half the length of the nucleus. The midpiece is composed of 11 oblique rings of varying regularity, each usually of six mitochondria, surrounding the elongate distal centriole. The mitochondria are subspheroidal to slightly elongate and possess few septate to (more externally) concentric cristae; a central dense body reported for *Caiman crocodilus* is questionably present. Nine peripheral dense fibres, closely associated with the triplets of the distal centriole, are lost within the anterior principal piece; those at doublets 3 and 8 are the longest and are separated from their doublets in the principal piece, as in other reptiles. A well defined annulus separates the midpiece from the principal piece. The principal piece consists of a 9+2 axoneme, investing fibrous sheath and plasma membrane and is followed by the endpiece which lacks the fibrous sheath. The spermatozoon of *C. johnstoni* is apomorphic relative to Chelonia and *Sphenodon* in reduction of concentric mitochondrial cristae but is less similar to that of ratites than is that of *Caiman crocodilus*, differing from ratites in the longer, multiplied perforatoria. The compact dense sheath around the central singlets of the distal centriole is a possible crocodilian autapomorphy.

KEY WORDS *Crocodylus johnstoni - Crocodylidae - Reptilia - spermatozoon - ultrastructure - phylogeny*

INTRODUCTION

The ultrastructure of spermatozoa or spermiogenesis has been studied in most major groups of reptiles (Jamieson and Scheltinga, 1993, 1994; Jamieson, 1995*a,b*; Jamieson *et al.*, 1996; Oliver *et al.*, 1996) though often limited to descriptions of only one or two species. Ultrastructural information on the male gametes of the Crocodilia is confined to four published descriptions. Phillips and Asa (1993) briefly described the formation of the midpiece of *Alligator mississippiensis*, Saita *et al.* (1987) described spermiogenesis in *Caiman crocodilus*, Kitiyanant *et al.* (1994) gave a few measurements, and scanning electron micrographs, of sperm of *Crocodylus siamensis*, and Jamieson (1995*a*) briefly compared the sperm of *Crocodylus johnstoni* with those of other amniotes. The present account more fully describes the mature spermatozoa of *C. johnstoni* and reconsiders similarities with other amniotes, particularly chelonians, *Sphenodon* and ratites.

Mailing address: Prof. B.G.M. Jamieson, Zoology Department, University of Queensland, St. Lucia, Qld. 4072, Australia.

MATERIALS AND METHODS

Sperm samples were collected from four specimens of *Crocodylus johnstoni* Krefft, 1873 from the Lynd River, in north central Queensland (near 17° 50'S, 144° 20' E). The sperm samples were obtained from the penile groove and immediately fixed for transmission electron microscopy (TEM) in cold 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The samples were then processed in a CR1000 Jouan refrigerated centrifuge at 4 °C, being centrifuged at 1,000 rpm for 5 min after each of the following stages: rinsing in 0.1 M phosphate buffer; post-fixation for 80 min in similarly buffered 1% osmium tetroxide; rinsing in buffer; dehydration through an ascending

ethanol series; and infiltration and embedding in Spurr's epoxy resin. Sections were cut with diamond knives, on an LKB 2128 UM IV microtome. Thin sections, 50-80 nm thick, were collected on carbon stabilized, colloidin-coated, 200 µm mesh copper grids, stained for 30 sec in Reynold's lead citrate, rinsed in distilled water, then placed in 6% aqueous uranyl acetate for 40 min, rinsed in distilled water, and stained for a further 20 min in lead citrate before final rinsing. Electron micrographs were taken on an Hitachi 300 electron microscope at 75 kV and a JEOL 100-s electron microscope at 60 kV. Light microscopic observations of spermatozoa, from glutaraldehyde-fixed sperm smears, were made under Nomarski contrast using an Olympus BH2 microscope.

RESULTS

Spermatozoa of *Crocodylus johnstoni* are filiform (Figs. 1 and 2A), and approximately 72.4 µm (mean of 4, SD = 3.6) long. Lengths of components for several sperm are: acrosome complex 5.6 µm (mean of 5, SD = 0.6); nuclear rostrum 2.7 µm (mean of 5, SD = 0.4); midpiece 4.8 µm (mean of 6, SD = 0.2), from transmission electron microscopy, and flagellum behind the midpiece (principal piece and endpiece) 54.3 µm (mean of 4, SD = 5.2), from light microscopy. No longitudinal sections of the entire nucleus were seen, but the sum of the length of rostrum + postrostral nucleus is 10.7 µm. The spermatozoon is circular in cross section throughout its length with a maximum diameter, at the midpiece, of approximately 0.95 µm.

Acrosome complex

The acrosome complex consists of a conical, membrane-bound acrosome vesicle surrounding the subacrosomal cone, and putative perforatorial material which extends into the one to three endonuclear canals (Fig. 2B-K). The acrosome complex is curved and envelops 2.7 µm of the tapered anterior end of the nucleus (nuclear rostrum). The subacrosomal cone is separated from the acrosome vesicle and nuclear rostrum by a thin electron lucent layer. A narrow elongate epinuclear electron lucent structure occurs within the subacrosomal cone directly above the tip of the nuclear rostrum (Fig. 2B); this is deduced to be an anterior extension of the perforatoria. The base of the acrosome complex rests on distinct nuclear shoulders (Fig. 2C).

Nucleus

The nucleus, estimated to be 10.7 µm long, is curved and tapers to a point anteriorly, as the nuclear rostrum, within the acrosome complex (Fig. 2B,C). Usually two narrow endonuclear canals are seen to twist helically around each other in at least the anterior 5 µm (and possibly more) of the nucleus. Occasionally only one but up to three endonuclear canals are present (Fig. 2F-K). The endonuclear canals are lined by the nuclear membrane and contain a dense core of the putative perforatorial material (Fig. 2G). They open into the subacrosomal cone. The nuclear contents are highly electron-dense, and are invested by nuclear and plasma membranes. Basally a small dome-shaped nuclear fossa containing dense material houses the anterior moiety of the proximal centriole (Fig. 3A,C,L,Q).

Neck region and centrioles

The neck region is the junction between the nucleus and the midpiece, it contains the proximal centriole which, like the distal centriole, consists of nine triplets and is surrounded by pericentriolar material (Fig. 3A,D,L,Q). This pericentriolar material extends, with the proximal centriole, into the nuclear fossa. Posteriorly it is cylindrical and surrounds the anterior portion of the distal centriole in the midpiece (Fig. 3E). The proximal centriole is short and located immediately anterior to the elongate distal centriole with its long axis at slightly less than a right angle to it (Fig. 3A). The C microtubules of the distal centriole appear to be open, as in Chelonia and *Sphenodon*, though this requires confirmation.

Midpiece

The midpiece is composed of eleven oblique rings of varying regularity, each usually of six mitochondria, surrounding the elongate distal centriole. The mitochondria are subspheroidal to slightly elongate and possess few cristae which may vary from a septate to, more externally in the mitochondrion, a concentric arrangement; a central dense body is questionably visible (Fig. 3A,E,F,N,O,P). The distal centriole extends through the entire length of the midpiece. It consists of nine triplets penetrated by two central microtubules ensheathed by dense material (Fig. 3A,E,F). Nine peripheral dense fibres are associated with the triplets of the distal centriole, each fibre ensheaths the triplet and projects into the centriolar lumen (Fig. 3E,F,N). The pair of central microtubules are also embedded in dense material (dense sheath in Fig. 3E). The pericentriolar material extends for some distance around the peripheral dense fibres (Fig. 3E). Posteriorly the distal centriole is continuous with the axoneme of the principal piece. The midpiece terminates at a well defined annulus. This is a dense ring the cross section of which is triangular (Fig. 3A,R).

Principal piece

The principal piece is the longest region of the spermatozoon (49-61 µm) and consists of the 9+2 axoneme surrounded by the electron-dense fibrous sheath and plasma

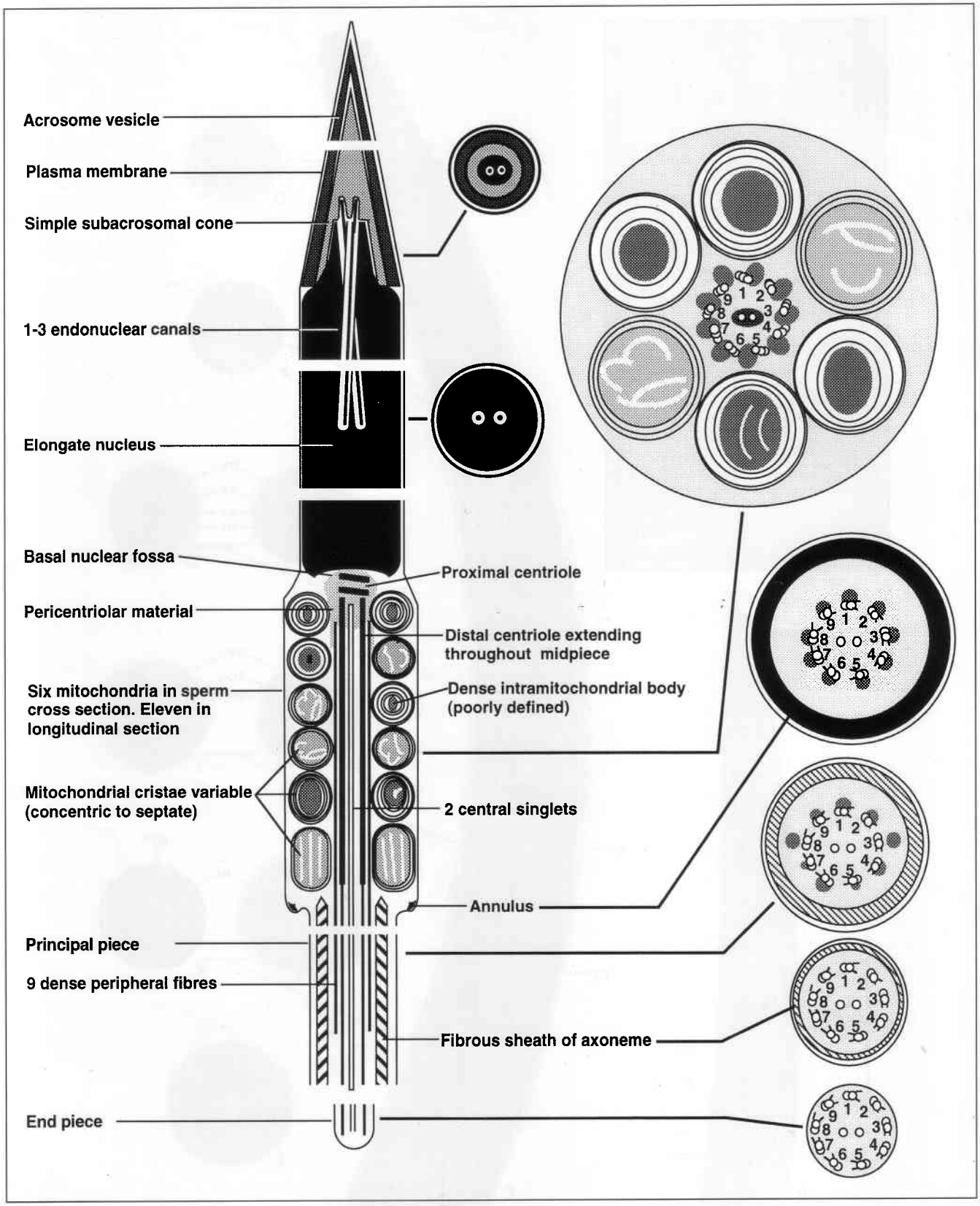

FIGURE 1 *Crocodylus johnstoni*. Diagrammatic representation of the chief components of the spermatozoon.

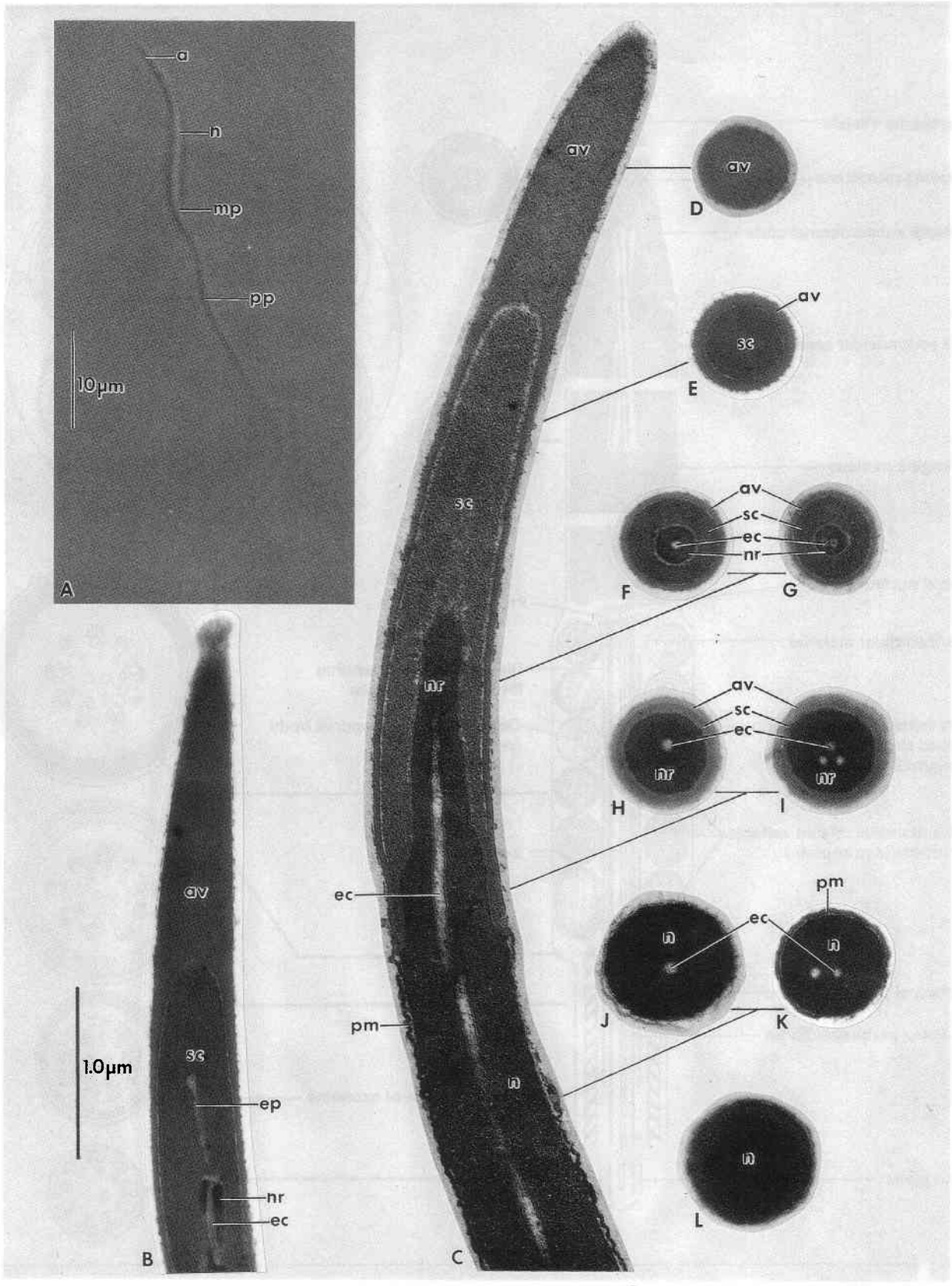

FIGURE 2 *Crocodylus johnstoni*. A: Whole spermatozoon (Nomarski contrast light microscopy). B: Longitudinal section (LS) through the nuclear rostrum showing the putative anterior extension of the perforatorium. C: LS through the acrosome. D-I: A series of transverse sections (TS) through the acrosome and nuclear rostrum. J-L: TS's through the nucleus. B-L to the same scale, as indicated. a: acrosome; an: annulus; av: acrosome vesicle; ax: axoneme; dc: distal centriole; ds: dense sheath around both central singlets; ec: endonuclear canal; ep: putative anterior extension of perforatorium; fs: fibrous sheath; m: mitochondria; mp: midpiece; n: nucleus; nf: nuclear fossa; nr: nuclear rostrum; pc: proximal centriole; pe: putative extension of pericentriolar material; pf: peripheral dense fibres; pm: plasma membrane; pp: principal piece; sc: subacrosomal cone.

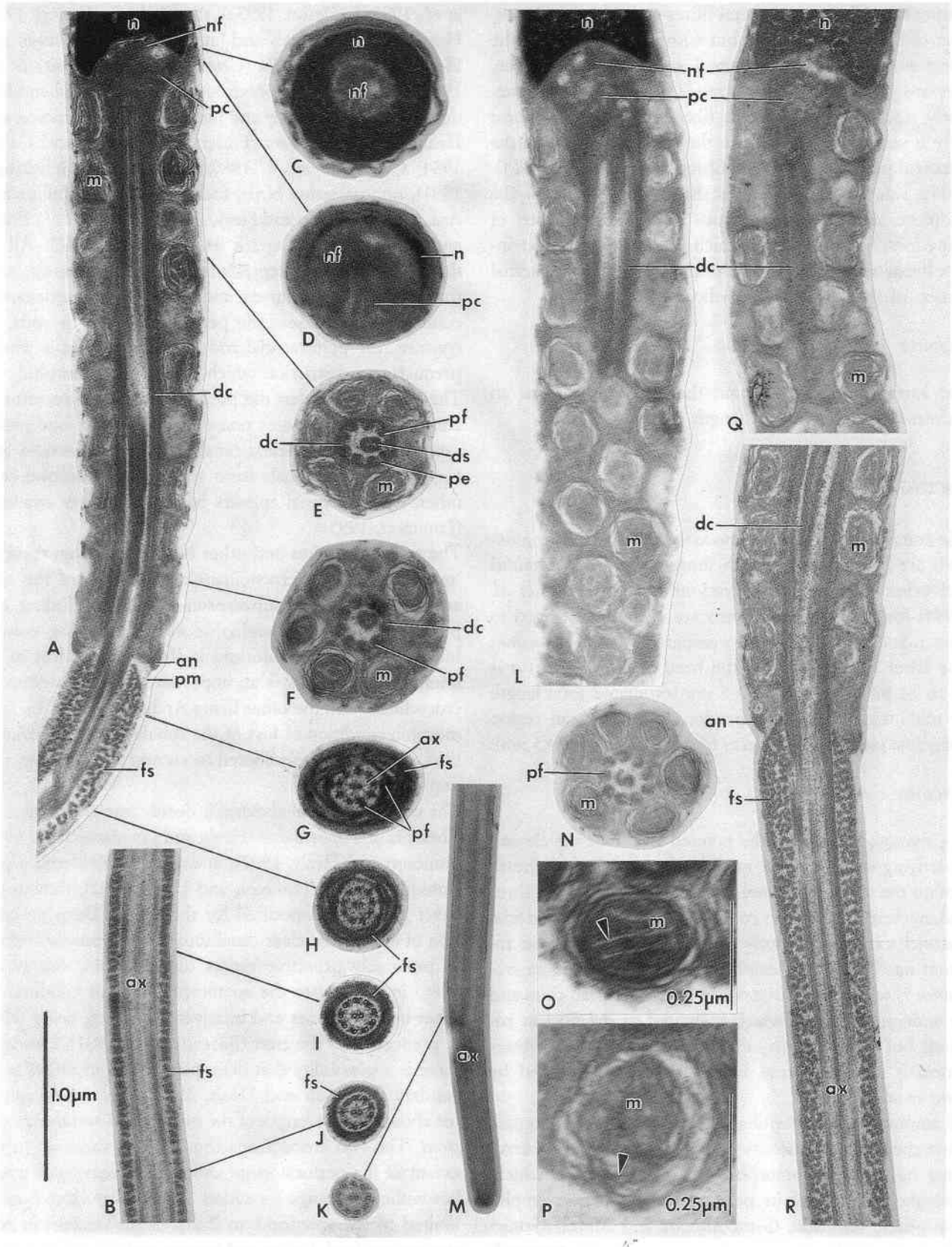

FIGURE 3 *Crocodylus johnstoni*. A: Longitudinal section (LS) through the midpiece showing the elongate distal centriole surrounded by 11 'rings' of mitochondria. B: LS through the 'mid' principal piece. C-K: A series of transverse sections (TS) through the neck (C,D), midpiece (E,F), principal piece (G-J) and end piece (K). L: Oblique LS through the midpiece showing the arrangement of mitochondria. M: LS through the endpiece. N: TS through the midpiece. O-P: TS of mitochondria (showing (O) linear cristae and (P) concentric cristae). Q: LS through the neck region showing the triplets of the proximal centriole. R: LS through the midpiece-principal piece junction showing the annulus and loosely anastomosing fibrous sheath. A-N, Q, R to the same scale, as indicated. Abbreviations as in Fig. 2.

membrane. All nine peripheral fibres enter the anterior region of the principal piece, but with the exception of the fibres associated with doublets 3 and 8 of the axoneme, they are greatly reduced in size (Fig. 3G). Further posteriorly, apart from these two fibres, only an occasional fibre is seen (Fig. 3H), and in the posterior region of the principal piece no peripheral fibres are present (Fig. 3I,J). Unlike squamates, the fibrous sheath does not enter the midpiece. Anteriorly the fibrous sheath is composed of many loosely joined fibres which posteriorly compact into a linear series of oblong blocks as seen in longitudinal section of the sheath (Fig. 3A,B,G-J,R).

Endpiece

The axoneme extends behind the fibrous sheath as an endpiece of undetermined length (Fig. 3K,M).

DISCUSSION

The components of the spermatozoon of *Crocodylus johnstoni* are here compared with those of *Caiman crocodilus* and other amniotes. Observations by Kitiyanant *et al.* (1994) for *Crocodylus siamensis* are virtually restricted to measurements and are here compared with *C. johnstoni* (the latter in parentheses): the head in *C. siamensis* was 20 to 24 µm long by 1 to 1.5 µm (estimated total length 16 µm); midpiece 5 to 6 µm long (4.6 µm); tail region (principal piece and endpiece) 65 to 80 µm long (54.3 µm).

Acrosome complex

In *Crocodylus johnstoni*, the pointed acrosome vesicle and underlying subacrosomal material form a conical sheath around the anterior tapered nuclear rostrum. One to three endonuclear canals, each containing putative perforatorial material, extend posteriorly from the tip of the nuclear rostrum nearly half the length of the nucleus. *Caiman crocodilus* is reported to have only a single perforatorium and an endonuclear canal which is limited to the nuclear rostrum but its extent requires confirmation as the observation is not supported by micrographs presented by Saita *et al.* (1987).

All amniote classes (Reptilia, Birds and Mammals) contain some species, or a majority of species, in which the acrosome has this plesiomorphic tripartite structure which, from the evidence of its presence in all three lissamphibian orders (Urodela, Gymnophiona and Anura) (Jamieson *et al.*, 1993), was already present in early tetrapods ancestral to Lissamphibia and Amniota. The pointed form of the acrosome, presence of the subacrosomal cone, and tapering of the tip (rostrum) of a cylindroid nucleus within this, are seen in *Ascaphus*, in the Lissamphibia (Jamieson *et al.*, 1993; Jamieson, 1995*a*), the Chelonia (Furieri, 1970; Hess *et al.*, 1991; Healy and Jamieson, 1992; Jamieson and Healy, 1992), Crocodilia (*Caiman crocodilus*) (Saita *et al.*, 1987), and *Crocodylus johnstoni* (this study), Sphenodontida (*Sphenodon*) (Healy and Jamieson, 1992; Jamieson and Healy, 1992), Squamata (Furieri, 1970; Butler and Gabri, 1984; Carcupino *et al.*, 1989; Jamieson and Scheltinga, 1993), non-passerine birds, including ratites (Phillips and Asa, 1986; Thurston and Hess, 1987; Jamieson *et al.*, 1996), and monotremes (Carrick and Hughes, 1982). All of these plesiomorphic representatives of their classes, with the exception of mammals, and, in the non-passerines, the columbiforms, possess the perforatorial rod or rods. In contrast, the perforatorial rod in the Squamata is wholly prenuclear, a restriction which is clearly apomorphic.

There is evidence that the presumed common ancestor of Amphibia and amniotes possessed more than one perforatorium and endonuclear canal. In turtles, *Sphenodon* and *C. johnstoni*, the canals form a loose spiral around each other. A single canal appears basic to all other amniotes (Jamieson, 1995*a*).

The sperm of ratites and other birds differ from those of 'reptiles' including crocodilians in reduction of the subacrosomal material (subacrosomal cone, excluding any perforatorium) to a negligible amount. In the columbiforms even a perforatorium is absent (references in Jamieson, 1995*a*). This is an important distinction between crocodilians and the other living Archosauria but the apomorphic condition of loss of the subacrosomal material in birds need not be considered to contest archosaurian monophyly.

The endonuclear canal extends deeply into the nucleus in Chelonia and *Sphenodon* (Healy and Jamieson, 1992, 1994; Jamieson and Healy, 1992), and this is considered a plesiomorphic state (Jamieson and Healy, 1992), though the exact length is unspecified for these taxa. Deep penetration of the endonuclear canal into the nucleus also occurs in putatively primitive ratites (i.e. tinamou, Asa *et al.*, 1986) in contrast to the apomorphic shorter condition in other non-passerines and in advanced ratites, being wholly prenuclear in the emu (Baccetti *et al.*, 1991). However, there is a possibility that deep penetration in ratites is secondary (Jamieson and Healy, 1992). The canals extend for about half the length of the nucleus in *Crocodylus johnstoni*. The two crocodilians thus show a variation in the extent of the perforatorium and endonuclear canal which lies within the range for ratites. The endonuclear canal is limited to the anterior 1 to 2 µm of the nucleus in rooster, guinea fowl, turkey and parrots and the anterior third of the nucleus in the ostrich (Baccetti *et al.*, 1991). The canal is lost in mammals and, homoplasically, in squamates. In mammals, rod-like perforatoria do not occur (Jamieson, 1995*a*).

The nucleus and nuclear fossa

The nucleus is plesiomorphically elongate in amniotes from Chelonia through *Sphenodon*, crocodilians, squamates, birds, monotremes and, in therian mammals, the pangolin alone (Leung and Cummins, 1988), as in lissamphibians.

The basal nuclear fossa appears poorly developed in the sperm of *Caiman crocodilus* (Saita *et al.*, 1987) but has a low dome-shaped form in *Crocodylus johnstoni*. Representation of the fossa, loosely termed the implantation fossa, is very variable in amniotes (Jamieson, 1995*a*; Jamieson *et al.*, 1996). In the ratites it has a distinctive triple profile (references in Jamieson and Healy, 1992; Soley, 1993). A compact rounded form appears to be plesiomorphic for amniotes (Jamieson and Healy, 1992; Jamieson, 1995*a*).

Midpiece

The midpiece in *Crocodylus johnstoni* is composed of eleven oblique rings of varying regularity, each usually of six mitochondria, surrounding the elongate distal centriole. The mitochondria are subspheroidal to slightly elongate and possess few cristae which may vary from a septate to, more externally in the mitochondrion, a concentric arrangement; a central dense body is questionably visible.

The number of mitochondria seen in transverse section of the midpiece is very variable in amniotes but some of the apparent variations require confirmation, particularly as there is variation along the midpiece. It appears that a maximum number, in transverse section, in the order of 6 to 9 may have been plesiomorphic; a maximum of 6 has been recorded for the Chelonia, 9 in *Sphenodon*, 8 in *Caiman crocodilus* (Saita *et al.*, 1987; Jamieson and Healy, 1992) and 6 in *Crocodylus johnstoni*. In the remaining amniotes, a trend towards reduction in numbers, in transverse section, to 4 in birds and monotremes has been suggested (Jamieson and Healy, 1992). It is 4 in ratites (Soley, 1993) and in the turkey (Thurston *et al.*, 1982) and in the order of 5 in the dove *Geopelia striata*. However, in squamates the number remains plesiomorphically high in lizards or shows apomorphic increase to as many as 14, in snakes, while a reduction to 2 in geckos is correlated with intrusion of intermitochondrial material of supposed mitochondrial origin into the transverse section of the midpiece. Further variability in numbers is now known though it cannot be fully documented here. For instance, large numbers of small mitochondria occur, it seems apomorphically, in eugongyloid skinks (Jamieson and Scheltinga, 1994) and approximately 9 have been observed in transverse section of the budgerigar sperm (Jamieson *et al.*, 1996). The mitochondria are subspheroidal in Chelonia, *Sphenodon* and, with a tendency to some elongation, crocodilians. The subspheroidal form may reasonably be inferred as the plesiomorphic condition. The number of tiers of mitochondria in longitudinal sequence is in the order of 10 in turtles (Healy and Jamieson, 1992), which is also presumed to be plesiomorphic, and is of the same order in crocodilians. In the spiral midpiece of mammals, the number of gyres varies from 55 to 300 (Fawcett, 1970) but is not specified for monotremes (Carrick and Hughes, 1982).

Structure of the mitochondria

In turtles (Furieri, 1970; Healy and Jamieson, 1992) and tuatara (Healy and Jamieson, 1992, 1994; Jamieson and Healy, 1992), the mitochondria have a form (also approached in the sperm of the Woolly opossum, *Caluromys philander*, see Phillips, 1970) in which concentric cristae surround a central dense body. This condition has been illustrated in a line drawing for *Caiman crocodilus* by Saita *et al.* (1987), though doubtfully substantiated by micrographs. It is clearly seen in the mitochondria of an immature spermatozoon of that species (their Fig. 22) but in a mature spermatozoon (Fig. 26) cristae of the septate type predominate and the central dense body is doubtfully present. In *Crocodylus johnstoni* concentric membranes (cristae?) are usually present peripherally in the mitochondria but are accompanied by septate, sometimes linear, cristae; a central body is rarely, if at all, recognizable. In all other amniotes studied, the cristae normally have a 'conventional' appearance, being linear or curved septate, as in Lissamphibia, but are not concentric, and do not surround a dense body. However, in ostrich sperm some mitochondria may occasionally have concentric cristae (Soley, personal communication).

The concentric arrangement of cristae with central dense body appears to be an apomorphy acquired early or initially in amniote evolution and retained paraphyletically in the tuatara, and turtle clades and, though less clearly developed in Crocodilia (Healy and Jamieson, 1992; Jamieson and Healy, 1992). It is here suggested that the Crocodilia are in the process of losing or reducing the plesiomorphic concentric arrangement with central body, in favour of the septate condition. In *Sphenodon* (Healy and Jamieson, 1992; Jamieson and Healy, 1992), the cristae have the linear appearance usual for metazoan sperm and the concentric arrangement is a late development. Phylogenetic 'reversion' of mitochondrial of concentric cristae to the linear condition seen in other amniotes would need only suppression of this final transformation (Jamieson and Healy, 1992). Presence of concentric cristae and the intramitochondrial body in the woolly opossum is construed as homoplasic although the possibility that ancestral mammals retained this condition from basal amniotes cannot be ruled out (Healy and Jamieson, 1992).

The annulus

A dense ring, the annulus, at the posterior end of the midpiece is a feature of many metazoan sperm. It is clearly plesiomorphic for amniotes, occurring in all classes (Jamieson and Healy, 1992) but absence in Dipnoi possibly indicates apomorphic re-acquisition (a reversal) in tetrapods. It is present in Chelonia, *Sphenodon* (Healy and Jamieson, 1992, 1994; Jamieson and Healy, 1992), *Caiman crocodilus* (Saita *et al.*, 1987), the American Alligator (Phillips and Asa, 1993), in *Crocodylus johnstoni*, in squamates (Courtens and Depeiges, 1985; Newton and Trauth, 1992; Jamieson and Scheltinga, 1993, 1994; Oliver *et al.*, 1996) and, though reduced in some species, in snakes (Jamieson and Koehler, 1995; Oliver *et al.*, 1996). The annulus is basic to mammals and birds, including ratites but is apomorphically absent in parrots (references in Jamieson, 1995*a*). It is weakly developed in monotremes (Carrick and Hughes, 1982).

The centrioles

Presence of the proximal centriole is plesiomorphic for tetrapods and is seen in all amniote classes. It persists, well developed, in monotremes (Carrick and Hughes, 1982), but is absent from mature therian mammals (Jamieson, 1995*a*).

A distal centriole is at most a vestige in mature mammalian sperm (Fawcett, 1975), but is well developed in sperm of anurans, Chelonia, *Sphenodon*, crocodilians (Healy and Jamieson, 1992; Jamieson and Healy, 1992, this study), squamates (Furieri, 1970), and birds (Asa *et al.*, 1986; Asa and Phillips, 1987). The distal centriole, forming the basal body of the axoneme, is plesiomorphically short in vertebrates, including the Lissamphibia and (as a reversal?) squamates (Jamieson and Scheltinga, 1993). In contrast, it extends the entire length of the long midpiece in turtles, the tuatara, crocodilians, and ratites, an apparent basal synapomorphy of amniotes. These elongate centrioles differ from most metazoan basal bodies in being penetrated by two central singlets from the axoneme. Thus in spermatids of the ratite *Rhea*, the distal centriole elongates and, late in spermiogenesis, becomes penetrated by a central pair of tubules from the developing axoneme (Phillips and Asa, 1989). The shorter, though still elongate distal centriole in the rooster and the somewhat shorter centriole in guinea fowl (0.6 μm) and *Geopelia striata* (0.5 μm), the short centriole in squamates, and the vestigial centriole in monotremes appear to represent secondary reduction in length of the centriole (Healy and Jamieson, 1992), culminating in almost total reduction in therian mammals (Jamieson, 1995*a*).

The distal centriole is embedded in a ring of dense material in all of the amniotes for which it has been investigated. A dense body, often cross striated, lateral to the proximal centriole appears to be a basal synapomorphy of amniotes but its homology across the various groups requires confirmation. It is seen in late spermatids of the tuatara (Healy and Jamieson, 1992), and the caiman spermatozoon (Saita *et al.*, 1987) where homology with the nine striated columns of eutherian sperm has been suggested (Healy and Jamieson, 1992) but imperfect fixation of the pericentriolar material in *Crocodylus johnstoni* does not allow detailed characterization. It shows various manifestations in squamates and does not appear to have been reported in birds.

The axonemal complex

An annulated, helical, dense fibrous sheath must have developed in the earliest amniotes as it is present in all amniote classes. In most of the amniotes investigated the fibrous sheath (or in some birds its amorphous equivalent) commences immediately behind the midpiece. This condition is seen in turtles, *Caiman crocodilus* and *Crocodylus johnstoni*, ratites, non-passerines (absent in parrots and doves) and mammals. However, in squamates the fibrous sheath extends anteriorly well into the midpiece, a clear squamate autapomorphy (Healy and Jamieson, 1992; Jamieson and Healy, 1992; Jamieson and Scheltinga, 1993; Jamieson, 1995*a,b*).

In *Crocodylus johnstoni* nine peripheral dense fibres are closely associated with the triplets of the distal centriole. In the principal piece a fibre persists for some length at doublets 3 and 8 but the other fibres disappear in its anterior region. The principal piece consists of a 9+2 axoneme surrounded by a fibrous sheath and the plasma membrane and is followed by the endpiece, lacking the sheath. The presence of nine fibres is an autapomorphy and simultaneous symplesiomorphy of the amniotes, though nine appear homoplasically in other groups, such as some lampreys and an osteoglossomorph fish (references in Jamieson, 1991, 1995*a*). The peripheral dense fibres are small in turtles, *Caiman crocodilus* and *Crocodylus johnstoni*, the tuatara, squamates, birds and monotremes. They have been observed in the anteriormost region of the principal piece of ratite spermatozoa (Asa *et al.*, 1986; Baccetti *et al.*, 1991; Soley, 1993, 1994) but are described as 'tiny' for the rhea, are absent from the tinamou (Asa *et al.*, 1986), and are greatly reduced in columbiforms. There appear to be trends to enlargement of the peripheral fibres in passerines and non-monotreme mammals, with diversification in the latter, and to reduction in ratites and doves (Jamieson, 1995*a*). In Chelonia, *Sphenodon*, *Caiman crocodilus* (but not notably *Crocodylus johnstoni*) and in squamates, the fibres at doublets 3 and 8 are enlarged

(Healy and Jamieson, 1992; Jamieson and Healy, 1992). They are possibly homologous with the axial fibre, at 3, and juxta-axonemal fibre at 8, in lissamphibian sperm (Jamieson, 1995*a*).

Synapomorphies have been suggested for amniote groups on the basis of the configuration of the peripheral axonemal fibres (Jamieson, 1995*a*). In reptiles the peripheral fibres at 3 and 8 are detached from their corresponding doublets while the other seven fibres are attached to their doublets. This reptilian feature is retained in crocodilians. In ratites, although fibres 3 and 8 are contiguous with their doublets, they appear less closely attached than the other fibres in at least the ostrich (see Fig. 10, Soley, 1993). In non-ratite birds and monotremes all of the peripheral fibres are attached to the corresponding doublets (Fawcett and Phillips, 1970). In contrast to both of these assemblages, in metatherian and eutherian mammal sperm the peripheral fibres 3 and 8 may be close to or attached to their doublets whereas the other seven fibres show a tendency to separate from them (Fawcett and Phillips, 1970). The peripheral fibres are usually situated in the midpiece with some extension into the principal piece as in turtles (Jamieson and Healy, 1992), the caiman (Saita *et al.*, 1987), non-passerines (Asa and Phillips, 1987), tuatara (Jamieson and Healy, 1992), and monotremes (Carrick and Hughes, 1982). In the rhea and tinamou these dense fibres are present only in the proximal principal piece. Very small dense fibres are present only in the distal region of the midpiece in the rooster and mallard; dense fibres in turtle dove sperm disappear before maturation is complete (see review by Asa and Phillips, 1987), though they persist through a short region of the midpiece in *Geopelia striata*. In eutherians and marsupials they extend far into the principal piece. However, in squamates, the only well developed, though small, peripheral fibres at the level of the annulus are the double fibres at doublets 3 and 8 and by the beginning of the principal piece all nine dense fibres are already vestigial or absent (Jamieson and Scheltinga, 1993, 1994). The fibres extend through most of the length of the sperm cell in oscine passerines (Phillips and Asa, 1989).

In turtles, *Sphenodon* (Healy and Jamieson, 1992), *Crocodylus johnstoni*, and in skinks (Furieri, 1970; Jamieson and Scheltinga, 1993, 1994), the nine peripheral dense fibres are partly displaced from the radii of the triplets of the distal centriole into the gaps between adjacent triplets; the fibres are coradial with the doublets in the axoneme. These locations have been regarded as plesiomorphic for amniotes (Jamieson, 1995*a*). Dense material surrounds the central singlets in Chelonia, *Sphenodon*, crocodilians (Jamieson, 1995*a*; this study) and ostrich (Soley, 1993). In crocodilians the compact nature of the dense sheath appears to be a distinctive synapomorphy (Jamieson, 1995*a*).

In conclusion, crocodilian sperm more closely resemble those of Chelonia and *Sphenodon* on the one hand (the latter two being almost identical) and ratites on the other than they do the sperm of any other amniotes. Chelonia, *Sphenodon*, crocodilians and ratites are unified by the unique elongation of the distal centriole throughout the length of the midpiece. Penetration of the centriole by the two central axonemal singlets characterizes the four taxa but is also seen in the short centriole of mammals. A notable difference of ratites relative to Chelonia, *Sphenodon* and crocodilians is loss of the subacrosomal cone so that the perforatorium closely approaches the acrosome vesicle at the apex of the spermatozoon. A conspicuous but presumably symplesiomorphic resemblance in crocodilians (*C. johnstoni*), Chelonia and *Sphenodon*, is the presence of more than one perforatorium and endonuclear canal and the fact these are wound around each other in a loose spiral. Ratites never have more than one perforatorium. Supposed restriction of the perforatorium to the anterior region of the nucleus in the caiman is a similarity to certain non-passerines (rooster and guinea fowl) whereas its extent for half the length of the nucleus in *Crocodylus johnstoni* approaches the probably plesiomorphic deep penetration in Chelonia, *Sphenodon* and ratites. The tendency in crocodilians to substitution of concentric mitochondrial cristae, seen in Chelonia and *Sphenodon*, with 'conventional' cristae is extreme in ratites, in which concentric cristae occur only as a rare inter-individual variation. Separation of peripheral fibres 3 and 8 from their corresponding doublets is a 'reptilian' feature of crocodilians not seen in other amniotes though the connection is weakened in at least some ratites. Cladistically, *Caiman* is deduced to constitute the plesiomorphic sister-group of birds, on the basis of these and other spermatozoal characters reported by Saita *et al.* (1987), whereas from the present study *Crocodylus* appears to be only a little more apomorphic (reduction of concentric cristae) than *Chelonia* and *Sphenodon*. Pending investigation of other crocodilians and confirmation of the spermatozoal anatomy of *Caiman*, paraphyly of the Crocodilia should not, however, be presumed. The well defined electron-dense sheath around the central axonemal singlets is possibly a crocodilian autapomorphy. *Crocodylus* lies within the sister-group of the taxon which includes *Caiman* and *Alligator* in the molecular analysis of Densmore and Owen (1989) but no non-crocodilian outgroup was used and therefore apparent monophyly was inevitable.

ACKNOWLEDGEMENTS

Mrs. Lina Daddow, Mr. Tom Gorringe and Dr. Chris Tudge are thanked for excellent technical assistance. This work was made possible by an ARC grant to BJ. Crocodiles were collected during a field study undertaken by the Crocodile Research Project of the Queensland Department of Environment.

REFERENCES

ASA C.S. and PHILLIPS D.M., 1987. Ultrastructure of avian spermatozoa: a short review. In: 'New Horizons in Sperm Cell Research'. Mohri H. ed., Japan Science Society Press, Gordon and Breach, Tokyo/New York, pp. 365-373.

ASA C.S., PHILLIPS D.M. and STOVER J., 1986. Ultrastructure of spermatozoa of the crested tinamou. *J. Ultrastruct. Mol. Struct. Res.*, **94**, 170-175.

BACCETTI B., BURRINI A.G. and FALCHETTI E., 1991. Spermatozoa and relationships in Palaeognath birds. *Biol. Cell*, **71**, 209-216.

BUTLER R.D. and GABRI M.S., 1984. Structure and development of the sperm head in the lizard *Podarcis* (= *Lacerta*) *taurica*. *J. Ultrastruct. Res.*, **88**, 261-274.

CARCUPINO M., CORSO G. and PALA M., 1989. Spermiogenesis in *Chalcides ocellatus tiligugu* (Gmelin) (Squamata, Scincidae): an electron microscope study. *Boll. Zool.*, **56**, 119-124.

CARRICK F.N. and HUGHES R.L., 1982. Aspects of the structure and development of monotreme spermatozoa and their relevance to the evolution of mammalian sperm morphology. *Cell Tissue Res.*, **222**, 127-141.

COURTENS J.L. and DEPEIGES A., 1985. Spermiogenesis of *Lacerta vivipara*. *J. Ultrastruct. Res.*, **90**, 203-220.

DENSMORE L.D. and OWEN R.D., 1989. Molecular systematics of the Order Crocodilia. *Am. Zool.*, **29**, 831-841.

FAWCETT D.W., 1970. A comparative view of sperm ultrastructure. *Biol. Reprod.* (suppl.), **2**, 90-127.

FAWCETT D.W., 1975. The mammalian spermatozoon. *Dev. Biol.*, **44**, 394-436.

FAWCETT D.W. and PHILLIPS D.M., 1970. Recent observations on the ultrastructure and development of mammalian spermatozoa. In: 'Comparative Spermatology'. Baccetti B. ed., Accademia Nazionale dei Lincei, Rome, pp. 13-28.

FURIERI P., 1970. Sperm morphology of some reptiles: Squamata and Chelonia. In: 'Comparative Spermatology'. Baccetti B. ed., Accademia Nazionale dei Lincei, Rome, pp. 115-131.

HEALY J.M. and JAMIESON B.G.M., 1992. Ultrastructure of the spermatozoon of the tuatara (*Sphenodon punctatus*) and its relevance to the relationships of the Sphenodontida. *Philos. Trans. R. Soc. London B*, **335**, 193-205.

HEALY J.M. and JAMIESON B.G.M., 1994. The ultrastructure of spermatogenesis and epididymal spermatozoa of the tuatara *Sphenodon punctatus* (Sphenodontida, Amniota). *Philos. Trans. R. Soc. London B*, **344**, 187-199.

HESS R.A., THURSTON R.J. and GIST D.H., 1991. Ultrastructure of the turtle spermatozoon. *Anat. Rec.*, **229**, 473-481.

JAMIESON B.G.M., 1991. 'Fish Evolution and Systematics: Evidence from Spermatozoa'. Cambridge University Press, Cambridge (U.K.).

JAMIESON B.G.M., 1995*a*. Evolution of tetrapod spermatozoa with particular reference to amniotes. In: 'Advances in Spermatozoal Phylogeny and Taxonomy'. Jamieson B.G.M., Ausio J. and Justine J.-L. eds., Mémoires du Muséum National d'Histoire Naturelle, Paris, vol. 166, pp. 343-358.

JAMIESON B.G.M., 1995*b*. The ultrastructure of spermatozoa of the Squamata (Reptilia) with phylogenetic considerations. In: 'Advances in Spermatozoal Phylogeny and Taxonomy'. Jamieson B.G.M., Ausio J. and Justine J.-L. eds., Mémoires du Muséum National d'Histoire Naturelle, Paris, vol. 166, pp. 359-383.

JAMIESON B.G.M. and HEALY J.M., 1992. The phylogenetic position of the tuatara *Sphenodon* (Sphenodontida, Amniota), as indicated by cladistic analysis of the ultrastructure of spermatozoa. *Philos. Trans. R. Soc. London B*, **335**, 207-219.

JAMIESON B.G.M. and SCHELTINGA D.M., 1993. The ultrastructure of spermatozoa of *Nangura spinosa* (Scincidae, Reptilia). *Mem. Queensl. Mus.*, **34**, 169-179.

JAMIESON B.G.M. and SCHELTINGA D.M., 1994. The ultrastructure of spermatozoa of the Australian skinks, *Ctenotus taeniolatus*, *Carlia pectoralis* and *Tiliqua scincoides scincoides* (Scincidae, Reptilia). *Mem. Queensl. Mus.*, **37**, 181-193.

JAMIESON B.G.M. and KOEHLER L., 1995. The ultrastructure of the spermatozoon of the Northern Water Snake, *Nerodia sipedon* (Colubridae, Serpentes). *Can. J. Zool.*, **72**, 1648-1652.

JAMIESON B.G.M., LEE M.S.Y. and LONG K., 1993. Ultrastructure of the spermatozoon of the internally fertilizing frog *Ascaphus truei* (Ascaphidae: Anura: Amphibia) with phylogenetic considerations. *Herpetol.*, **49**, 52-65.

JAMIESON B.G.M., OLIVER S.C. and SCHELTINGA D.M., 1996. The ultrastructure of the spermatozoa of Squamata. I. Scincidae, Gekkonidae and Pygopodidae (Reptilia). *Acta Zool. (Stockholm)*, **77**, 85-100.

KITIYANANT Y., YOUNGPRAPAKORN P., SONGTHAVEESIN C., TOCHARUS C., JARUANSUWAN M., JUNPRASERT S. and PAVASUTHIPAISIT K., 1994. Seasonal changes of sperm morphology and reproductive tracts of *Crocodylus siamensis*. In: 'Crocodiles. Proceedings of the 12th Working Meeting of the Crocodile Specialist Group of the Species Survival Commission of IUCN - the World Conservation Union. Pattaya, Thailand, 2-6 May 1994'. IUCN - The World Conservation Union, Gland, Switzerland, vol. 2, pp. 268-278.

LEUNG L.K.P. and CUMMINS J.M., 1988. Morphology of immature spermatozoa of the Chinese Pangolin (*Manis pentadactyla*: Pholidota). In: 'Proceedings of the Australian Society of Reproductive Biology 1988'. Newcastle, Australia, pp. 94.

NEWTON W.D. and TRAUTH S.E., 1992. Ultrastructure of the spermatozoon of the lizard *Cnemidophorus sexlineatus* (Sauria: Teiidae). *Herpetol.*, **48**, 330-343.

OLIVER S.C., JAMIESON B.G.M. and SCHELTINGA D.M., 1996. The ultrastructure of spermatozoa of Squamata. II. Agamidae, Varanidae, Colubridae, Elapidae, and Boidae (Reptilia). *Herpetol.*, **52**, 216-241.

PHILLIPS D.M., 1970. Ultrastructure of spermatozoa of the wooly opossum *Caluromys philander*. *J. Ultrastruct. Res.*, **33**, 381-397.

PHILLIPS D.M. and ASA C.S., 1986. Ultrastructure of avian spermatozoa. *J. Cell Biol.*, **103**, 239a.

PHILLIPS D.M. and ASA C.S., 1989. Development of spermatozoa in the *Rhea*. *Anat. Rec.*, **223**, 276-282.

PHILLIPS D.M. and ASA C.S., 1993. Strategies for formation of the midpiece. In: 'Comparative Spermatology 20 Years After'. Baccetti B. ed., Raven Press, New York, pp. 997-1000.

SAITA A., COMAZZI M. and PERROTTA E., 1987. Electron microscope study of spermiogenesis in *Caiman crocodilus* L. *Boll. Zool.*, **4**, 307-318.

SOLEY J.T., 1993. Ultrastructure of ostrich (*Struthio camelus*) spermatozoa: 1. Transmission electron microscopy. *Onderstepoort J. Vet. Res.*, **60**, 119-130.

SOLEY J.T., 1994. Centriole development and formation of the flagellum during spermiogenesis in the ostrich (*Struthio camelus*). *J. Anat.*, **185**, 301-313.

THURSTON R.J. and HESS R.A., 1987. Ultrastructure of spermatozoa from domesticated birds: Comparative study of turkey, chicken and guinea fowl. *Scanning Microsc.*, **1**, 1829-1838.

THURSTON R.J., HESS R.A., HUGHES B.L. and FROMAN D.P., 1982. Ultrastructure of the guinea fowl (*Numidia meleagris*) spermatozoon. *Poultry Sci.*, **61**, 1738-1743.