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ULTRASTRUCTURE OF THE SPERMATOZOA OF *LITORIA LONGIROSTRIS* (HYLIDAE, ANURA, AMPHIBIA): MODIFICATIONS FOR PENETRATION OF A GELATINOUS LAYER SURROUNDING THE ARBOREAL EGG CLUTCH

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Spermatozoa of *Litoria longirostris* are highly modified for its unique mode of fertilisation and differ distinctively from the sperm of the 10 *Litoria* species previously examined. *L. longirostris* spermatozoa are longer in head, tail and total. The head is long and straight with a distinct acrosome vesicle compared to a short curved head in other *Litoria* spermatozoa. The acrosome vesicle is well developed and surrounds approximately the apical third of the perforatorium only. The perforatorium is a solid homogenous rod that attaches to the nucleus asymmetrically along one side. The nucleus and midpiece are similar in size and structure to those of other *Litoria* species. The axial fibre is greatly enlarged and a juxta-axonemal fibre at doublet 3, usual in anuran sperm, is absent.

Although the spermatozoa of *L. longirostris* are highly modified, having secondarily lost the bufonoid synapomorphy of a putative conical perforatorium consisting of fibres, it can still be distinguished as a eubufonoid by the mitochondrial collar. Thus, we assert that sperm morphology is correlated with phylogenetic relationships as well as mode of fertilisation and that spermatozoal morphology can provide useful information in resolving phylogenetic relationships. ❐ *Frog, spermatozoa, ultrastructure, fertilisation, Litoria longirostris.*

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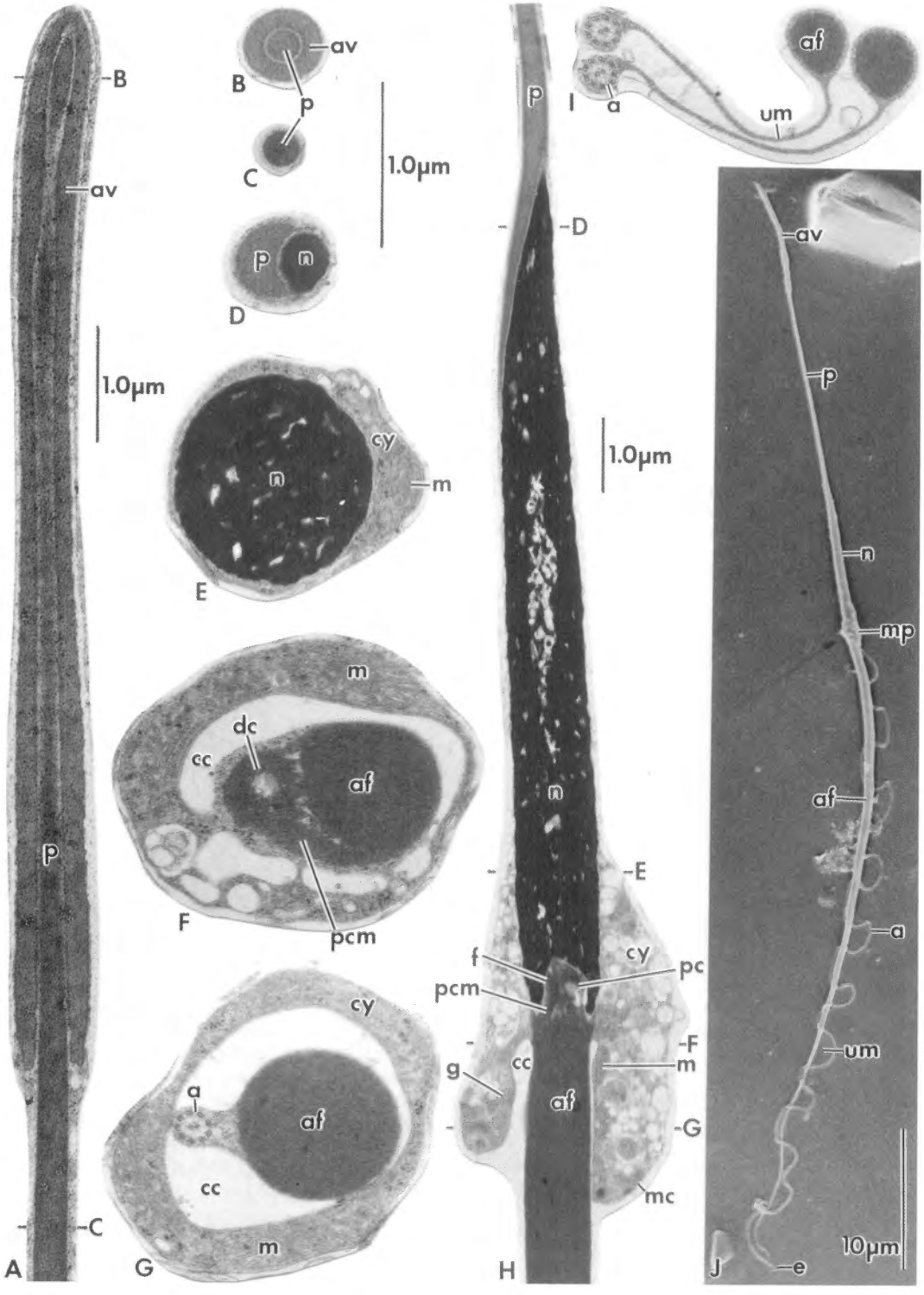
Litoria longirostris is a relatively small (ca 25mm) grey-brown frog inhabiting the granite upland, rainforest streams of the McIlwraith Range, Cape York Peninsula, Queensland. It is unique amongst Australian frogs in its reproductive mode of depositing egg clutches on vegetation and rocks overhanging water bodies in rainforest streams (McDonald & Storch, 1993). The lime green eggs are large (2.0-2.4mm diameter) and surrounded by a 6mm thick gelatinous capsule. Tadpoles in the clutch are similar in colour to the eggs during development, before turning a pale brown on the dorsal surface prior to hatching. Parental care has been observed with adult frogs straddling the clutch (McDonald & Storch, 1993). The exact method of amplexus and fertilisation remains unknown.

Sperm ultrastructure has previously been examined in 10 species of the Australian hylid *Litoria*: *aurea*, *caerulea*, *eucnemis*, *fallax*, *gracilenta*, *lesueuri*, *moorei*, *peronii*, *rheocola* and *rubella* (Lee & Jamieson, 1993; Meyer et al., 1997; Jamieson, 1999). Although these frogs reproduce in a wide variety of habitats, their sperm are similar. With regard to correlation of reproductive mode with sperm structure, Garrido et al. (1989) noted that complex flagella occur in species in which fertilisation occurs in a non-aquatic environment and in which development may be viviparous, direct, or aquatic. In terrestrial breeders, notably some of the foam-nesting rhacophorids, the spermatozoon reaches its highest degree of modification. Male *Rhacophorus arboreus*, *R. schlegelii* and *Chiromantis xerampelina*, which shed their sperm onto a foam nest, have highly modified "corkscrew shaped" sperm (Oka, 1980; Mainoya, 1981; Mizuhira et al., 1986; Wilson et al., 1991; Jamieson, 1999). However, in some foam-nesting rhacophorids, as well as those like *Buergeria buergeri* which do not build a foam-nest but lay their eggs in streams, the spermatozoa have a long head and thin tail, neither of which is spiral (Fukuyama et al., 1993; Kuramoto, 1996; Kuramoto & Joshy, 2000, 2001).

The 'arboreal-nesting' reproductive method of *L. longirostris* is similar to that of several species from divergent anuran families (Duellman &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Trueb, 1986) and thus allows correlation between terrestrial nesting and sperm ultrastructure to be further examined. We here give the first description of the spermatozoon of *L. longirostris*.

MATERIALS AND METHODS

Three male *L. longirostris* Tyler & Davies, 1977 were collected from near egg clutches at Upper Peach Creek, McIlwraith Range, North Queensland, on 26-27 September 1995 (QMJ62099, J62100, J62102). The frogs were killed with a lethal injection of sodium pentobarbital shortly after capture. The testes were quickly removed and fixed for transmission electron microscopy (TEM) in 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) at 4°C for at least two hours before being transported at ambient temperature to Brisbane for processing and sectioning. On arrival in Brisbane, material for TEM examination was diced into $1mm^3$ pieces and rinsed in 0.1M sodium phosphate buffer; post-fixed for 80 min in similarly buffered 1% osmium tetroxide; rinsed in buffer; dehydrated through an ascending ethanol series; and infiltrated and embedded in Spurr's epoxy resin (Spurr, 1969).

Sections were cut with a diamond knife on a LKB 2128 UM IV microtome. Thin sections, 50-80nm thick, were collected on carbon stabilised, colloidin-coated, 200μm mesh copper grids, stained for 30 s in Reynold's lead citrate (Reynolds, 1963), rinsed in distilled water, then placed in 6% aqueous uranyl acetate for 4 min, rinsed in distilled water, and stained for a further 2 min in lead citrate before final rinsing. Electron micrographs were taken on an Hitachi 300 electron microscope at 75kV and a JEOL 100-s electron microscope at 60kV.

A drop of glutaraldehyde-fixed spermatozoa, for examination by scanning electron microscopy (SEM), was placed on a clean small round cover slip. Dehydration of the spermatozoa through an ascending ethanol series and final amyl acetate was achieved by adding a drop of each reagent in turn, after removal of the previous fluid with filter paper. The spermatozoa were subjected to critical point drying, gold sputter coated, and photographed on a JEOL 6400 SEM.

Photographs of spermatozoa, from glutaraldehyde-fixed tissue squashes, were made using an Olympus BH2 microscope with Nomarski interference contrast and attached OM-2 camera.

RESULTS

The testicular spermatozoa of *L. longirostris* are filiform and average 82.7μm (n=20, SD=3.3) in length (range 74.1-87.2μm). The spermatozoon is composed of a straight head region (acrosome complex and nucleus) 34.2μm long (n =9, SD=1.9), a midpiece 2.49μm long (n=5, SD=0.32), and a tail 46.3μm long (n=11, SD= 1.7) (Fig. 1J). Under light microscopy a distinct acrosome vesicle, perforatorium, nucleus, midpiece, axial fibre, undulating membrane and axoneme can be clearly seen.

ACROSOME COMPLEX. The acrosome complex is 24.6μm long (n=6, SD=1.1) and composed of an elongate cylindrical acrosome vesicles surrounding the apical portion of the putative perforatorium (Fig. 1A,J). The acrosome vesicle is 10.4μm long (n=5, SD=0.08), membrane bound and filled with moderately electron-dense material (Fig. 1A,B). The perforatorium continues posteriorly without any associated structures for some distance before attaching asymmetrically to one side of the nucleus (Fig. 1C,D,H). The perforatorium is a single unit, not divided into separate sheaves/ fibres, with a constant diameter throughout its

FIG. 1. *Litoria longirostris*. A-I, TEM. A, Longitudinal section (L.S.) of the apical region of a spermatozoon, showing the acrosome vesicle surrounding only the anterior region of the perforatorium; B-G, Successive transverse sections (T.Ss) through the spermatozoon as indicated; B, through the apical region of the acrosome complex; C, through the perforatorium; D, through the junction of the perforatorium and nucleus; E, through the posterior region of the nucleus; F, through the distal centriole; G, through the midpiece; H, L.S. of the nucleus (showing the perforatorium lying along one side of the nucleus, see also D) and midpiece (showing the mitochondrial collar); I, T.S. of the tail. Note the absence of a juxta-axonemal fibre at doublet 3 and the relatively large axial fibre (see also F and G); J, Whole testicular spermatozoon shown by SEM, showing the head (acrosome vesicle, perforatorium and nucleus), midpiece and tail (axial fibre, undulating membrane and axoneme). B-G and I to the same scale as indicated. A, H and J scales as indicated. Abbreviations: a = axoneme; af = axial fibre, av = acrosome vesicle; cc = cytoplasmic canal; cy = cytoplasm; dc = distal centriole; e = endpiece; f = nuclear fossa; g = putative glycogen granules; m = mitochondrion; mc = mitochondrial collar; mp = midpiece; n = nucleus; p = perforatorium; pc = proximal centriole; pcm = pericentriolar material; um = undulating membrane.

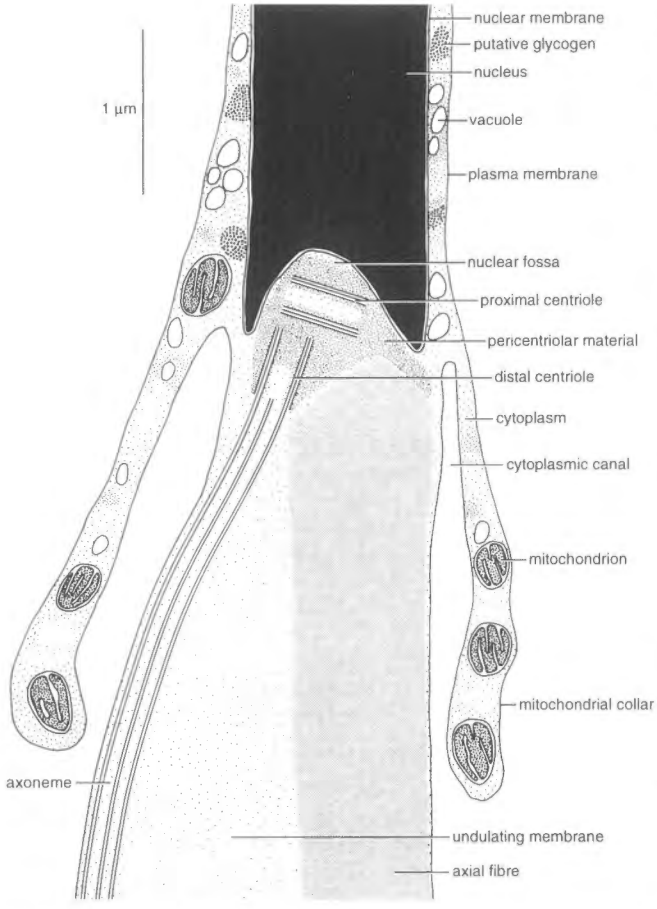

FIG. 2. Drawing of a longitudinal section through the neck region of a spermatozoon of *L. longirostris*. Drawn from a TEM micrograph.

length, is moderately electron-dense and is not bounded by a membrane.

NUCLEUS. The nucleus is 11.07µm long (n=2, SD=0.09), cylindrical, and electron-dense (condensed chromatin) (Fig. 1E,H). Anteriorly, the nucleus tapers to a distinct point alongside the perforatorium (Fig. 1H). Distinct nuclear shoulders are absent. At the base of the nucleus a well-developed nuclear fossa is present (Figs 1H; 2). At this level the nucleus is 1.07µm in diameter (n = 8, SD = 0.05).

NECK/MIDPIECE. Within the basal nuclear fossa lies the proximal centriole which is surrounded by pericentriolar material that connects it to the nuclear fossa, distal centriole and the axial fibre (Figs 1F,H, 2). The proximal centriole lies at ~75° to the long axis of the nucleus and at right angle to the distal centriole which forms the basal body of the axoneme (Fig. 2). Each centriole is composed of 9, circularly arranged, triplets of short microtubules. The axial fibre extends through the neck region to the level of the base of the nucleus (Figs 1H; 2). A short mitochondrial collar, containing scattered mitochondria, vacuoles and putative glycogen granules, surrounds the anterior portion of the tail but is separated from it by a gap, the cytoplasmic canal (Figs 1F-H, 2).

TAIL COMPLEX. The tail complex (Figs 1I,J, 2) is composed of a 9+2 axoneme and an axial fibre, the fibre being connected by a thin undulating membrane to doublet 3. A juxta-axonemal fibre at 3 is absent. The axial fibre is well-developed and circular in transverse section throughout its length. Anteriorly, it is enlarged, being ~ 0.95µm in diameter (Fig. 1F-I). It continues posteriorly, decreasing in diameter, for almost the entire length of the tail resulting in only a very short portion of the axoneme extending as an endpiece (Fig. 1J).

DISCUSSION

Spermatozoa of all 10 species of *Litoria* which have previously been examined (Lee & Jamieson, 1993, Meyer et al., 1997; Jamieson, 1999) are similar to each other. All closely resemble *Bufo* sperm and exhibit the bufonoid synapomorphy of a conical perforatorium of separate sheaves and the eubufonoid synapomorphy of a long mitochondrial collar separated from the tail by a cytoplasmic canal (Pugin-Rios, 1980; Lee & Kwon, 1992; Lee & Jamieson, 1993; Kwon & Lee, 1995). Spermatozoa of *L. longirostris* differ from these *Litoria* species. However, despite these differences the spermatozoa show characters which are synapomorphic for the eubufonoids.

Spermatozoa of *L. longirostris* are easily distinguished from those of the 10 *Litoria* species previously examined. *Litoria longirostris* spermatozoa are longer, 83µm, compared with 47-56µm in the 6 *Litoria* species examined by

Lee & Jamieson (1993), and the length of the sperm head is approximately twice the length of 14-18μm recorded in the latter species. The head is long and straight with a distinct well-developed acrosome vesicle, contrasting with a short curved head in the other investigated *Litoria* spermatozoa. The acrosome complex is highly modified relative to the other species. In the latter, a thin walled conical acrosome vesicle completely caps a putative conical perforatorium of separate sheaves of perforatorial material and the acrosome complex symmetrically caps the tapered point of the nucleus. In *L. longirostris*, the acrosome vesicle differs in surrounding only the apical third, approximately, of the perforatorium. The perforatorium differs in being a solid homogenous cylinder that attaches to the nucleus asymmetrically along one side. Most, if not all, of the increase in length of the head in *L. longirostris* is due to the great length of its acrosome complex. It is deduced that *L. longirostris* spermatozoa have secondarily lost the bufonoid synapomorphy of a conical perforatorium consisting of fibres.

In some foam-nesting rhacophorids the sperm head shows modifications different from those of *L. longirostris* and forms a spiral (Oka, 1980; Mainoya, 1981). It is unknown if there are any ultrastructural modifications to the sperm of those foam-nesting rhacophorids that do not show a spiral head.

In view of the fact that the spermatozoa of the 10 previously examined *Litoria* species have such uniform structure despite differing reproductive modes (lotic vs lentic nesting). It seems likely that the acrosomal modifications observed in *L. longirostris* are an adaptation to penetration of the thick gelatinous mass surrounding the large eggs.

The nucleus and midpiece of *L. longirostris* are similar in size and structure to those *Litoria* species previously examined and show the eubufonoid synapomorphy of a mitochondrial collar separated from the tail by a cytoplasmic canal. The tail of *L. longirostris* spermatozoa differs in its length (46μm compared to 35-40μm (Lee & Jamieson, 1993)), having a greatly enlarged axial fibre and in the absence of a juxta-axonemal fibre at doublet 3. All previously examined *Litoria* spermatozoa have a juxta-axonemal fibre at doublet 3. Lee & Jamieson (1993) proposed that the enlargement of the juxta-axonemal fibre observed in *L. fallax*, *L. gracilenta* and *L. lesueuri* was a weak synapomorphy uniting them. It appears that the presence and size of the juxta-axonemal fibre may provide a useful character in the examination of phylogenetic relationships between species of *Litoria*. Conversely, loss of the juxta-axonemal fibre may not be correlated with phylogenetic relationships and may be an adaptation to the unique fertilisation biology of *L. longirostris*.

The spermatozoa of *L. longirostris* differ greatly from those of foam-nesting rhacophorids that have been examined ultrastructurally in tail, in addition to acrosomal, characters. In these rhacophorids the sperm possess two axonemes which are surrounded by microtubules in a pseudocrystalline matrix. Furthermore, juxta-axonemal fibres, axial fibre and undulating membrane are all absent (Mainoya, 1981; Mizuhira et al., 1986; Wilson et al., 1991; Jamieson, 1999). The loss of a juxta-axonemal fibre, axial fibre and undulating membrane appear each to be a synapomorphy of the Ranoidea, though these states are questionably independent of each other. The only notable similarities, albeit superficial, between the spermatozoa of *L. longirostris* and foam-nesting rhacophorids are the asymmetrical attachment of the acrosome complex to the nucleus and the absence of a juxta-axonemal fibre associated with doublet 3. On present evidence it appears that these similarities are homoplasies.

The Mexican hylid *Pachymedusa dacnicolor* has a similar reproductive mode (Bagnara et al., 1986) to *L. longirostris* and is the only other arboreal-nesting frog to have had its spermatozoa examined ultrastructurally (Rastogi et al., 1988). *P. dacnicolor* spermatozoa are very similar to those of the 10 aquatic-nesting *Litoria* species previously examined and thus differ from *L. longirostris* in its acrosome vesicle which completely caps a conical perforatorium composed of fibres and a juxta-axonemal fibre at doublet 3. There is one similarity between the spermatozoa of these 2 arboreal-nesting hylids that may reflect their shared fertilisation biology and that is the thick axial fibre. In both *P. dacnicolor* and *L. longirostris* the axial fibre is approximately 1μm in diameter compared to a diameter of approximately 0.2μm in the other *Litoria* examined. However, within the wider Anura, differences in the sperm tail appear more reflective of phylogeny than fertilisation biology (van der Horst et al., 1995; Meyer et al., 1997).

Although the spermatozoon of *L. longirostris*

is highly modified for its unique fertilisation biology it can still be distinguished as that of a eubufonoid by the mitochondrial collar. Thus, we agree with Garrido et al. (1989) that amphibian sperm morphology is correlated with broad phylogenetic relationships (here at the suprafamilial, eubufonoid level) as well as the mode of fertilisation. Therefore, spermatozoon morphology can provide useful information in resolving phylogenetic relationships at various taxonomic levels despite unique fertilisation biology resulting in highly modified spermatozoa.

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