Ultrastructure of the spermatozoon of *Leiopelma hochstetteri* (Amphibia, Anura, Leiopelmatidae)

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**ABSTRACT**

The spermatozoon of the basal frog *Leiopelma hochstetteri* Fitzinger, 1861 is examined and compared with previously investigated lissamphibian spermatozoa. *L. hochstetteri* spermatozoa share with those of urodeles and *Ascaphus* the pleisiomorphic (primitive) features of an elongate conical acrosome, and an elongate nucleus, with distinct nuclear shoulders, which tapers to a point (as the nuclear rostrum) in the subacrosomal space. The spermatozoon of *L. hochstetteri*, alone among the Anura, resembles those of urodeles in having a juxta-axonemal fibre at doublet 8. *L. hochstetteri* shares with *Ascaphus truei* (homoplastically) the reduction of the undulating membrane to a short, thick paraxonemal rod. *L. hochstetteri* spermatozoa have the plesiomorphic condition, also seen in dipnoans, of a cytoplasmic collar, containing mitochondria, around the axoneme. Although the sperm of *L. hochstetteri* lacks the endonuclear perforatorium seen in *Ascaphus*, Bombinidae, urodeles and caecilians, it has what may be remnants of an endonuclear perforatorium within the nuclear rostrum. No synapomorphic spermatozoal characters are present to support the placement of *Leiopelma* and *Ascaphus* in the same family. Spermatologically, *Leiopelma* is the pleisiomorph sister taxon of all other anurans and is thus confirmed as the most basal (primitive) anuran. A further finding is that the Pelodytidae, sometimes considered basal archaeobatrachians, share the synapomorphy of a conical perforatorium divided into fibres with the Bufonoidea.

**KEY WORDS**

Amphibia, Anura, Leiopelmatidae, spermatozoa, ultrastructure, phylogeny.
RÉSUMÉ

Ultrastructure du spermatozoïde de Leiopelma hochstetteri (Amphibia, Anura, Leiopelmatidae).

Le spermatozoïde de la grenouille primitive Leiopelma hochstetteri Fitzinger, 1861 est examiné et comparé aux spermatozoïdes de lissamphibiens précédemment étudiés. Le spermatozoïde de L. hochstetteri partage avec les Urodèles et Ascaphus les caractéristiques plésiomorphes ( primitives) suivantes: un acrosome conique et allongé, un noyau allongé, avec des épaules nucléaires distinctes, et qui s’effile dans l’espace subacrosomal, formant un rostre nucléaire. Le spermatozoïde de L. hochstetteri est le seul parmi les Anoures à ressembler à ceux des Urodèles par une fibre juxta-axonémale associée au doublet 8. L. hochstetteri a en commun avec Ascaphus truei (de manière homoplasique) la réduction de la membrane ondulante en une baguette paraxonémale courte et épaisse. Les spermatozoïdes de L. hochstetteri ont l’état de caractère plésiomorphe, également rencontré chez les Dipneustes, d’un collier cytoplasmique contenant des mitochondries, autour de l’axonème. Bien que le spermatozoïde de L. hochstetteri ne possède pas le perforatorium endonucléaire trouvé chez Ascaphus, les Bombiniidae, les Urodèles et les Caeciliens, il présente ce qui peut être les restes d’un perforatorium endonucléaire dans le rostre nucléaire. Aucune synapomorphie spermatique ne soutient l’inclusion de Leiopelma et d’Ascaphus dans la même famille. Du point de vue spermatologique, Leiopelma est le taxon-frère plésiomorphe de tous les autres Anoures, ce qui confirme sa position d’Anoure le plus basal. On remarque aussi que les Pelodytidae, parfois considérés comme des Archaeobatrachiens basaux, partagent la synapomorphie d’un perforatorium conique divisé en fibres avec les Bufonoida.

MOTS CLÉS
Amphibia, Anura, Leiopelmatidae, spermatozoïdes, ultrastructure, phylogénie.

INTRODUCTION

Frogs of the genus Leiopelma Fitzinger, 1861 are endemic to New Zealand and are considered to be among the most primitive (basal) of all living anurans (Cannatella 1985; Green & Cannatella 1993; Duellman & Trueb 1994). Leiopelma hochstetteri Fitzinger, 1861 is a semi-aquatic, highly cryptic and sedentary frog which lives and breeds in scattered remnant populations in moist seepages and along the edges of forest streams on the North Island and Great Barrier Island of New Zealand (Bell 1978; Tessier et al. 1991). They grow to a maximum known snout-vent length of 46 mm (Mclennan 1985; Slaven 1992) in close agreement with lengths of 50 mm in subfossil deposits (Worthy 1987). They are mostly dull brown frogs but dark green morphs also occur (Barwick 1961; Whitaker 1996). As observed in captive Leiopelma hochstetteri, amplexus is inguinal and occurs on land, in shallow water, or at the waters edge between the months of October and May (Bell 1978). Breeding sites are generally under rocks and logs in and around water seepages and smaller streams, where clutches of 10 to 22 eggs are laid and from which tailed swimming larvae hatch (Bell 1982, 1985). Males may occur in association with egg clutches but do not actively incubate the eggs, unlike other species of this genus. Furthermore, the larvae differ from their congeners in that they do not metamorphose on the back of a resident male frog, but metamorphose post-hatching and disperse soon after (Bell 1985).

Among the extant Anura only Ascaphus Stejneger, 1899 and Leiopelma retain the plesiomorphic characters of nine presacral amphicoelous vertebrae and “tail-wagging” muscles. Although these are sym-
plesiomorphies, *Ascaphus* and *Leiopelma* have been united in a single family, the Leiopelmatidae, together with the extinct Patagonian genera *Notobatrachus* Reig, 1957 and *Vieraella* Reig, 1961 (Duellman & Trueb 1994). However, Green & Cannatella (1993) concluded that, as *Ascaphus* and *Leiopelma* share no synapomorphic characters, they should be placed in separate families, Ascaphidae and Leiopelmatidae respectively. Other research into the phylogenetic relationships of these basal anuran families has also produced conflicting conclusions. Cannatella (1985) on the basis of morphological data considered *Ascaphus* *truei* Stejneger, 1899 to be the sister taxon of all other anurans (see also a reanalysis of Cannatella’s data by Hillis 1991), whereas, from an analysis of isozyme loci, Green et al. (1989) considered *Leiopelma* to be the probable sister group of all other frogs. In contrast to the above views, an extensive study of amphibian 12S and 16S rRNA data by Hay et al. (1995) indicated that the two anuran suborders Archaeobatrachia and Neobatrachia are each monophyletic. That *Ascaphus* and *Leiopelma* are sister groups within the Archaeobatrachia, with the most basal lineage of the Archaeobatrachia being the Pelobatoidea (Pelodytidae + Pelobatidae). While Hay et al. (1995) found *Ascaphus* and *Leiopelma* to be sister groups, this needs not necessitate placing them in the same family, the Leiopelmatidae, as suggested by them.

In this study we attempt to clarify the confused phylogenetic relationships of *Leiopelma hochstetteri* to other families of frogs by comparing its spermatozoal ultrastructure with that of previously examined lissamphibian spermatozoa, in particular that of *Ascaphus truei*.

**ABBREVIATIONS**

svl = snout-vent length;
TEM = transmission electron microscopy.

**MATERIALS AND METHODS**

**SPERMATOZOAL ULTRASTRUCTURE**

Four specimens of *Leiopelma hochstetteri* collected from Whareorino forest, North Island, New Zealand on June 29th 1998 were enclosed in 10 cm × 6 cm plastic containers lined with damp tissue paper. Each container was then placed within a 20 cm × 30 cm polystyrene container, maintained at 10 °C for transport to Massey University. Frogs were maintained on a diet of crickets and wax moth larvae for one week before transport to Victoria University. At Victoria University each frog was euthanased with Benzocaine. The testes from the only male frog collected, svl = 34.4 mm, (FT4071, C. Daugherty field collection number, Victoria University), were fixed for transmission electron microscopy (TEM) in 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) at 4 °C for 2 h, being agitated for the first hour. Testis material was then posted to Brisbane, Australia, at ambient temperature for TEM processing. The testicular tissue was diced into 1-2 mm³ portions and then rinsed in 0.1M 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. Sections were cut with diamond knives, on a LKB 2128 UM IV microtome. Thin sections, 50-80 nm in thickness, were collected on 200 µm mesh copper grids, rinsed in distilled water, stained for one minute in Reynold’s lead citrate, rinsed again, and stained for a further three minutes in lead citrate before final rinsing. Electron micrographs were taken on an Hitachi 300 electron microscope at 75 kV.

Light microscopic observations of spermatozoa from glutaraldehyde-fixed tissue squashes, were made under Nomarski contrast using an Olympus BH2 microscope.

**PARSIMONY ANALYSIS**

A parsimony analysis using PAUP* version 4.0b2 was used to reconstruct the phylogenetic relationships of *Leiopelma* with other Anura based on sperm morphological characters. Species used, together with their sources, were:
**Urodela**
Plethodontidae *Eurycea quadridigitata* (Holbrook, 1842) (Jamieson 1999; pers. obs.).

**Anura, Archaeobatrachia**

**Anura, Neobatrachia**

### Data matrix

The characters and character coding used were as follows:

1. Endonuclear canal containing perforatorium: 0, present; 1, absent.
2. Juxta-axonemal fibre at doublet 8: 0, present; 1, absent.
3. Conical perforatorium divided into fibres: 0, absent; 1, present.
4. Distinct nuclear shoulders: 0, present; 1, absent.
5. Subacrosomal cone: 0, present; 1, absent.

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*Eurycea quadridigitata* was used as the outgroup. Plesiomorphic character states were scored as zero. An exhaustive search was used, employing maximum parsimony as the optimality criterion. All character states were of type unordered and equally weighted. For PAUP, the initial “Max-Trees” value was set to 100, branches were collapsed (creating polytomies) if maximum branch length was zero, the “MulTrees” option was in effect and topological constraints were not enforced. Character-state optimization was by accelerated transformation (ACCTRAN).

### RESULTS

The ultrastructure of the spermatozoon of *Leiopelma hochstetteri* is illustrated diagramatically in Figure 1. For ease of comparison the terminology of Jamieson *et al.* (1993) for *Ascaphus truei* is used throughout this account. Data are restricted to the numerous sperm from a single individual owing to the vulnerable status of *L. hochstetteri* and its full protection under New Zealand law. The use of a single individual is not of concern as Lissamphibia show very little variation in sperm morphology within a species and sperm abnormalities are too rare to be commonly sampled.

The testicular spermatozoa of *Leiopelma hochstetteri* are filiform and average 224 µm (n = 24, sd = 12) in total length (range 207 to 250 µm). The spermatozoon is composed of a curved cylindrical head region (acrosome and nucleus) 66.8 µm long (n = 32, sd = 4.6), a midpiece 2.3 µm long (n = 3, sd = 0.02), and a tail 154 µm long (n = 27, sd = 12) (Fig. 2A).

### ACROSOME COMPLEX

The acrosome complex is composed of an elongate conical acrosome and an underlying subacrosomal cone (putative perforatorium) which caps the nuclear rostrum (Fig. 2C-G, Q). The acrosome is a membrane bound vesicle and is filled with moderately electron-dense material (Fig. 2C-O, Q). The subacrosomal cone lies free within the subacrosomal space. It consists of diffuse material basally and is not bounded by a membrane. Apically the material of the subacrosomal cone compacts to form a dense rod which is square-ended anteriorly (Fig. 2D, E, I, J). Basally the subacrosomal cone is closely adpressed to the nuclear rostrum (Fig. 2G, N, O, Q).
The subacrosomal cone extends posteriorly for a short distance beyond the base of the acrosome vesicle (Fig. 2G). In transverse section the base of the acrosome vesicle is peripherally heptagonal (Fig. 2N). Further apically it decreases in polygony, becoming trilobed at the level of the apical tip of the subacrosomal cone (Fig. 2I, J) and circular at its apex (Fig. 2H).

**Nucleus**
The nucleus is elongate and cylindrical, and is composed of electron-dense material (Fig. 2G, P). Anteriorly, within the acrosomal complex, the nucleus tapers to a point (Fig. 2G, N-Q). This nuclear rostrum is 3.24 µm long (n = 3, sd = 0.06) and contains several axial lacunae which contain material which is possibly the homologue of a perforatorium (Fig. 2G, N, Q). Although no continuous endonuclear canal is present, what appear to be the vestige of a short, more continuous endonuclear canal has been observed in the very tip of the nuclear rostrum in a late spermatid (Fig. 2B). Distinct nuclear shoulders are present at the base of the nuclear rostrum (Fig. 2G). At their level the nucleus is 0.41 µm in diameter (n = 8, sd = 0.03); this increases throughout its length to a maximum diameter of 0.80 µm (n = 9, sd = 0.06) at the level of the basal nuclear fossa. The fossa is conical and 0.92 µm deep (Fig. 3A, E).

**Neck/Midpiece**
Two parallel centrioles, orientated in the long axis of the nucleus, lie at the base of the nuclear fossa (Fig. 3B). Each centriole is composed of nine, circularly arranged, triplets of short microtubules. The two centrioles lie adjacent to each other embedded in a common mass of electron-dense material, and do not extend into the nuclear fossa. One of the centrioles forms the basal body of the axoneme (Fig. 3E). The axial fibre of the flagellum extends through the neck region to the level of the base of the nucleus (Fig. 3B, E). A short cytoplasmic collar surrounding the flagellum contains a few, scattered mitochondria. A gap (termed the “cytoplasmic canal” for many vertebrate sperm) separates the cytoplasmic collar from the flagellum (Fig. 3B-E).

**Tail complex**
The tail complex (Fig. 3C-I) is composed of a 9 + 2 axoneme; an axial fibre, connected by a thickened undulating membrane to a juxta-axonemal fibre at doublet 3; and a further juxta-axonemal fibre at doublet 8. Anteriorly (in transverse section) the undulating membrane is shorter and with the juxta-axonemal fibre at 3...
Fig. 2. — *Leiopelma hochstetteri*: **A**, Whole testicular spermatozoon shown by light microscopy; **B-Q**, transmission electron microscopy; **B**, longitudinal section (ls) of the nuclear rostrum region of a spermatid, showing what appears to be the remnant of a short endonuclear canal; **C**, ls of the apical region of the acrosome vesicles; **D, E**, ls’s of the anterior acrosome, showing condensed "rod-like" subacrosomal material; **F**, ls of the mid region of the acrosome; **G**, ls of the base of the nuclear rostrum. Note the distinct nuclear shoulders (ns), the axially located lacunae (l) and that the subacrosomal material extend beyond the base of the acrosomal vesicle (arrow); **H-Q**, successive transverse sections (ts’s) through the acrosomal region indicated; **P**, ts through nucleus; **Q**, is through the base of the acrosome surrounding the nuclear rostrum. Note the lacunae containing putative perforatorial material (see also N). Abbreviations: av, acrosome vesicle; f, flagellum; h, head (acrosome and nucleus); l, lacunae; mp, midpiece; n, nucleus; nr, nuclear rostrum; ns, nuclear shoulders; sc, subacrosomal cone. Scale bars: **A**, 10 µm; **B-Q**, 1 µm.
Spermatozoon of *Leiopelma* (Amphibia, Anura, Leiopelmatidae)

**FIG. 3.** — *Leiopelma hochstetteri*; spermatozoon by transmission electron microscopy; **A**, transverse section (ts) through the nuclear fossa; **B**, ts through neck region, showing the two centrioles lying adjacent and parallel to each other; **C, D**, ts’s through the mid-piece, showing the "cytoplasmic" canal. Note the juxta-axonemal fibres at doublets 3 and 8, the short thickened undulating membrane and the axial fibre; **E**, longitudinal section (ls) of the midpiece. Note that the centriole and axial fibre do not enter the nuclear fossa; **F-I**, successive ts’s through the flagellum; **J**, ts through the distal end of tail (endpiece). All to the same scale as indicated.

Abbreviations: **3**, juxta-axonemal fibre at 3; **8**, juxta-axonemal fibre at 8; **af**, axial fibre; **ax**, axoneme; **c**, centriole; **cc**, cytoplasmic canal; **cy**, cytoplasm; **m**, mitochondrion; **ms**, mitochondrial sheath (cytoplasmic collar); **n**, nucleus; **nf**, nuclear fossa; **pm**, pericentriolar material; **pr**, paraxonemal rod; **um**, undulating membrane. Scale bar: 1 µm.
and the axial fibre, forms an electron-dense, hourglass shaped paraxonemal rod (Fig. 3C, D). Posteriorly, the juxta-axonemal fibre at 3 becomes less distinct from the undulating membrane (Fig. 3F-I). The juxta-axonemal fibre at doublet 8 extends along much of the length of the axoneme, decreasing in size posteriorly (Fig. 3C, D, F-I). For some of its length this fibre has two thin extensions, as seen in transverse section, attaching it to the axoneme (Fig. 3F).

A short portion of the axoneme, the endpiece, extends beyond the juxta-axonemal and axial fibres (Fig. 3J). Within the endpiece the 9 + 2 structure of the axoneme becomes increasingly disrupted posteriorly.

**DISCUSSION**

**ACROSOME**

An anterior acrosome, with or without a perforatorium, is present in all lissamphibian sperm. The acrosome of many urodeles ends subapically in a distinct barb (Retzius 1906; Selmi *et al.* 1997; Wortham *et al.* 1982). Although the acrosomal vesicle in *Hynobius* spp. lacks a barb, in transverse section it is strongly trifoliate (Picheral 1967, 1979; Lee & Kwon 1996), a condition which is compatible with the presence of a barb or at least some unilateral modification of the tip of the vesicle (see review by Jamieson 1999). The barb has no equivalent in anurans. However, the acrosome vesicle of *Leiopelma* shows some similarities to that of *Hynobius* Tschudi, 1838 in that it is trilobed anteriorly.

The acrosome vesicle of *Leiopelma* is similar to that of *Ascaphus* in being symmetrical and differentiated into regions of varying thickness and

**PARSIMONY ANALYSIS**

Analysis using PAUP evaluated a total of 10395 trees with only one shortest tree being found. This one best tree (Fig. 4) had a length of six steps. The worst tree found was 14 steps in length.
with different contents. However, the polygonal structure of the acrosome vesicle, when seen in transverse section, in Leiopehma is unique (autapomorphic) within the Lissamphibia. A subacrosomal cone covering the tip of the nucleus in Leiopehma is a synapomorphy of Lissamphibia and amniotes (Jamieson 1995, 1999) and therefore symplesiomorphic in Leiopehma. In Leiopehma the subacrosomal cone extends for a short distance beyond the posterior limit of the acrosome vesicle. A similar condition is seen in urodeles (Picheral 1979) and Ascaphus (James 1970; Jamieson et al. 1993). The acrosome complex of Leiopehma differs from that of Ascaphus in lacking a crenulated interior margin of the acrosome vesicle and in not being underlain by a perinuclear space.

At the base of the acrosomal region, the nucleus of basal amniotes (Healy & Jamieson 1992; Jamieson 1995), urodeles (Picheral 1979; Selmi et al. 1997), Ascaphus (Jamieson et al. 1993) and Leiopehma flares out abruptly to form distinct nuclear shoulders (tetrapod synapomorphy). Behind this, the nucleus forms a very elongate cylinder.

**Perforatorium**

A rod-like perforatorium and endonuclear canal are characteristic of sperm of basal tetrapods. They occur in urodeles (Picheral 1967, 1979; Fawcett 1970; Selmi et al. 1997). They are seen in caecilians (Gymnophiona), though, there lodged posteriorly in a greatly shorter endonuclear canal (Van der Horst et al. 1991). They also characterize the more basal anurans, Ascaphus (Jamieson et al. 1993) and the bombinids Bombina Oken, 1816 and Alytes Wagler, 1830 (Sandoz 1970a, b; Furieri 1975a, b; Folliot 1979; Pugin-Rios 1980). Furthermore one or more are present in sarcopterygian fish (Dipnoi and Latimeria chalumnae Smith, 1939) (Mattei et al. 1988; see reviews in Jamieson 1991). It is thus reasonable to deduce that an endonuclear canal containing a long rod-like axial perforatorium is plesiomorphic for the Lissamphibia. This is endorsed by persistence of this condition in lower amniotes, the Chelonia, Sphenodontida and Crocodilia, and in palaeognath birds (Jamieson & Healy 1992; Jamieson 1995, 1999; Jamieson et al. 1997). However, although Leiopehma lacks a definite endonuclear canal, there are what appear to be remnants of an endonuclear canal and axial perforatorium in the form of axial lacunae, containing electron-pale material, within the nuclear rostrum. In the pelobatid Scaphiopus couchi Baird, 1854 an electron-lucent vesicle containing a “dense body” is present in the centre of the nucleus (Morrisett 1974). Nuclear vacuoles of variable size and location, and with granular contents, are also frequently seen in Xenopus laevis and Bombina variegata (Linnaeus, 1758) (Pugin-Rios 1980). A perforatorium traverses the endonuclear canal in the late spermatid of Discoglossus pictus (Sandoz 1970b) but is restricted to the prenuclear subacrosomal space in the mature spermatozoon, though the endonuclear canal persists (Pugin-Rios 1980).

**Nuclear Fossa**

Within the Anura a basal nuclear fossa containing either one or both centrioles, which may constitute an implantation fossa, is poorly developed in species with a simple flagellum, well-developed in those with an undulating membrane and especially well-developed in Discoglossus pictus in which the tail is very long (1000 µm). No neck-piece of the type seen in salamandroid urodeles is seen in the Anura but the shape of the basal nuclear fossa of Ascaphus and Leiopehma appears to differ little from that of cryptobranch and hynobiid urodeles (pers. obs.). Leiopehma differs from Ascaphus and most other Anura in that no centriole is present within the nuclear fossa.

**Centrioles**

The neck region contains the centrioles, the elements of the so-called centriolar annexe and the anterior part of the axial fibre if this is present. Within the Lissamphibia both centrioles are always present and have the classic structure of nine triplets of short microtubules; the proximal (anterior) centriole lies either parallel (Leiopehma, this study; Scaphiopus Holbrook, 1836, Morrisett 1974), oblique (Ascaphus, Jamieson et al. 1993; Xenopus Wagler, 1827, Pugin-Rios 1980; urode-
les, Picheral 1979) or perpendicular (Bombina, Pelodytes Wagler, 1830, Pugin-Rios 1980; most Neobatrachans, Lee & Jamieson 1992, 1993; Meyer et al. 1997) to the long axis of the spermatozoon. In sperm with a simple flagellum the distal (posterior) centriole is situated at the base of the axoneme, for which it forms the basal body, and is orientated in the long axis, but in sperm with an undulating membrane the distal centriole is often oblique. Where there are two flagella, as in Telmatobufo australis Formas, 1972, there is no differentiation into proximal and distal centrioles but each centriole forms the basal body of a flagellum (Pugin-Rios 1980). In Leiopelma both centrioles lie adjacent and parallel, and in the long axis of the spermatozoon, conditions which are unique to Leiopelma among lissamphibians with only one flagellum.

AXIAL FIBRE
In Leiopelma, as in most Anura, the base of the axial fibre is situated further posteriorly than, or adjacent to the distal centriole. However, in Discoglossus pictus the base of the axial fibre protrudes deeply into the implantation fossa and the centrioles occupy a lateral position at the caudal end of the fossa (Pugin-Rios 1980). This condition is also seen in the Myobatrachidae where it is considered a distinctive synapomorphy (Lee & Jamieson 1992) presumably homoplastic relative to Discoglossus Otth, 1837. In urodeles the axial fibre reaches the base of the fossa or extends only just into the fossa (Werner 1970; Picheral 1979; Selmi et al. 1997).

MIDPIECE
The number and disposition of the mitochondria in lissamphibian sperm are variable and they are often absent at maturity. In the primitive cryptobranch urodeles Cryptobranchus alleganiensis bishopi Grobman, 1943 (Baker 1963) and Hynobius spp. (Picheral 1979; Kuramoto 1995, 1997) the mitochondria are located in a protoplasmic bead around the nucleus and not around the tail, even in the mature sperm. In salamandrids, ambystomatids and plethodontids small, ovoid mitochondria are present in cytoplasm around a long anterior region of the axial fibre where, in transverse section of the sperm, they form an arc (Baker 1966; Picheral 1967, 1979; Fawcett 1970; Wortham et al. 1977; Jamieson et al. 1993). The urodele type of midpiece has been reported to be absent in anurans (Pugin-Rios 1980) but in the myobatrachids Limnodynastes peronii and Neobatrachus pelobatoides (Werner, 1914) an incomplete ring of mitochondria surrounding the axial fibre occurs, much as in salamandrids (Lee & Jamieson 1992). A somewhat similar arrangement is also seen in Bombina variegata (Furieri 1975b; Folliot 1979; Pugin-Rios 1980). The mitochondria lie in a cytoplasmic mass in Xenopus laevis, Mixophyes fasciolatus Günther, 1864, Caudiverbera caudiverbera (Linnaeus, 1758), Pleurodema thal (Lesson, 1826), Nectophrynoides occidentalis Angel, 1943, Rana esculenta (Linnaeus, 1758) and Rana temporaria (Linnaeus, 1758) (Pugin-Rios 1980; Lee & Jamieson 1992) but this location may be secondary in at least Rana and possibly in all of these species.

In Xenopus laevis a very short cytoplasmic canal is occasionally evident. In contrast, in bufonids, and most other eubufonoids including leptodactylids, the mitochondria are distributed around the base of the sperm tail in a collar, which may be lost at maturity in many of these species (Pugin-Rios 1980; Garrido et al. 1989; Lee & Jamieson 1992; Meyer et al. 1997). Mitochondria occur in one or more longitudinal series in a groove in the paranemal fibre in Ascaphus truei, a condition which does not appear to be plesiomorphic (arguments having been presented for a paedomorphic origin) despite the primitive (basal) status of this species (Jamieson et al. 1993). Large, elongate mitochondria are known only from the rhacophorids, Rhacophorus arboreus (Okada & Kawano, 1924), Rhacophorus schlegelii (Günther, 1859) and Chiromantis xerampelina Peters, 1854, which are highly aberrant, being “corkscrew-shaped” and biflagellate (Mizuhira et al. 1986; Wilson et al. 1991). In the caecilian Typhlonectes natans (Fischer, 1879), the collar forms a cylinder around the base of the axoneme and contains many mitochondria clustered in a spiral near its inner wall (Van der Horst et al. 1991).
The conditions seen in *Leiopelma* of slight development of a cytoplasmic collar and rudimentary cytoplasmic canal is similar to that seen in the lungfish *Neoceratodus forsteri* (Krefft, 1870) (Jamieson 1999; Jespersen 1971). Until now no extant species were known which presented an arrangement of mitochondria which could confidently be regarded as plesiomorphic for the Anura. It seems reasonable to deduce that the *Neoceratodus*-like condition seen in *Leiopelma* is plesiomorphic for anurans. The well-developed, but transient, mitochondrial collar in eubufoonoids (Lee & Jamieson 1993), resemble a condition seen in many acanthopterygian fish (Jamieson 1991) and therefore on first analysis appear plesiomorphic, but may well be a reversal to a pre-lissamphibian condition.

**Tail complex**

The structure of the sperm tail is highly distinctive of the Lissamphibia. The generalized lissamphibian sperm has three longitudinal fibres, one on each side of the axoneme adjacent to doublets 3 and 8 (juxta-axonemal fibres at 3 and 8), and a second fibre, the axial fibre, which is associated with doublet 3 but separated from it by an undulating membrane. The juxta-axonemal fibre at 8, characteristic of urodeles, is absent in caecilians and was previously thought to be absent in anurans. In urodele sperm, typically, there is no juxta-axonemal fibre at 3 (Baker 1963; Picheral 1979). However, *Plethodon albagula* Grobman, 1944 is exceptional, among urodeles studied, in having a dense structure on the adaxonemal end of the undulating membrane, although it is questionable if this is homologous with the anuran juxta-axonemal fibres at 3 (Jamieson et al. 1993; Lee & Jamieson 1993; Jamieson 1999).

The phylogenetic origins and, therefore, homologies of the lissamphibian flagellar complex have been discussed by Jamieson (1995, 1999) who suggested dipnoan affinities. Thus, in *Neoceratodus forsteri* where the anterior region of the sperm axoneme has a large fibre on each side, at doublets 3 and 8, and each fibre is continuous with a “fin” which terminates with a further, smaller lateral fibre (Jamieson 1999; Jespersen 1971). Jamieson (1995, 1999) has tentatively recognized homology between each fin and an amphibian sperm undulating membrane, between the fibres at doublets 3 and 8 and the amphibian juxta-axonemal fibres, and between the terminal lateral fibres and the axial fibre. He considered that this, if valid, would suggest that lissamphibians have retained, from an ancestor shared with dipnoans (and with other sarcopterygian fish?), only one of (two former, bilateral, undulating membranes, and only one of a former pair of axial fibres. He further argued that the two juxta-axonemal fibres of urodeles (and *Leiopelma*, this study) are a persistence of the paired ancestral condition, the fibre at doublet 8 normally being lost in the Anura. Thus the unilateral location of the undulating membrane and its axial fibre, rather than presence of undulating membranes per se, constitutes the synapomorphic condition for the Lissamphibia (Jamieson et al. 1993; Jamieson 1995, 1999). Sarcopterygian fish (for definition see Jamieson 1991), and particularly dipnoans, appear to be the nearest extant non-tetrapod relatives of amphibians. The plesiomorphic condition seen in *Leiopelma* of the presence of juxta-axonemal fibres at 3 and 8 is unique among the Anura. However, a temporary fibre is present in the spermatids of *Discoglossus pictus* (Sandø 1975) and also in occasional *Bufo marinus* (Linnaeus, 1758) sperm (Swan et al. 1980).

With the discovery in *Leiopelma* sperm of a longitudinal fibre adjacent to axonemal doublet 8, the Anura cannot be defined spermatologically on the negative apomorphy of loss of this fibre. This loss was formerly the sole spermatozoal synapomorphy of the Anura. A juxta-axonemal fibre at doublet 8 (as at 3) is a feature of the anuran ground plan, as it is present in the basal frog *Leiopelma* and is very rarely retained in other anurans. A juxta-axonemal fibre at 3 is absent in urodeles (questionably present in *Plethodon*) and caecilians but cannot be considered an autapomorphy of the Anura as it appears to have a dipnoan precursor.

The plesiomorphic narrow unilateral undulating membrane with a fibre at 3 and an axial fibre (though in the absence of a fibre at 8) occurs in
the mature sperm of *Bufo*, *Litoria* and most leptodactylids (Pugin-Rios 1980; Lee & Jamieson 1992, 1993; Meyer *et al*. 1997). The flagellum may secondarily lack an undulating membrane and fibres and be single (*Xenopus laevis, Caudiverbera caudiverbera, Rana* spp., Pugin-Rios 1980) or double (*Telmatobufo australis*, Pugin-Rios 1980). Alternatively, the axoneme may be associated with a solitary parallel paraxonemal rod (*Ascaphus*, Jamieson *et al*. 1993; *Leiopelma*, this study; some *Cyclorana* spp., Meyer *et al*. 1997; and *Hyla meridionalis* Boettger, 1874; Pugin-Rios 1980); in *Ascaphus*, *Leiopelma* and *Cyclorana* Steindachner, 1867, at least, the rod represents axial fibre, lamina (equivalent to the undulating membrane), and juxta-axonemal fibre. The paraxonemal rod of *Ascaphus* differs from that of *Leiopelma* in that it carries a longitudinal groove which houses the mitochondria. Therefore, the paraxonemal rod is not considered here to be a shared apomorphy and is probably homoplastically derived in *Ascaphus* and *Leiopelma*. Presumed reduction of the undulating membrane has been considered a major advanced feature (apomorphy) of the *Ascaphus* spermatozoon (Jamieson *et al*. 1993).

**LENGTHS OF SPERMATOZOA**

The sperm of the Anura are elongate, their length varying from 40 to 110 µm, though those of *Telmatobufo australis* and *Leiopelma hochstetteri* reach 240 µm and 250 µm, respectively, and *Discoglossus pictus*, attains the remarkable length of 2300 µm (Favard 1955; Sandoz 1975; Pugin-Rios 1980) to 2500 µm (Furieri 1975a). The length of *Ascaphus* sperm is unknown. The spermatozoa of urodeles are generally longer than those of anurans, with the exception of *Discoglossus pictus*. In urodeles, the sperm of the hynobiid *Hynobius takedai* Matsui & Miyazaki, 1984 is the shortest known, with an average length of 169 µm (Kuramoto 1997), while the longest known is that of the proteid *Necturus maculosus* (Rafinesque, 1818) which reaches a length of 920 µm (Baker 1963). The sperm of the lungfish *Neoceratodus forsteri* reaches an approximate length of 272 µm, with head, midpiece and tail lengths of 70 µm, 2 µm and 200 µm respectively (Jespersen 1971), measurements very similar to those of *Leiopelma*.

**CONCLUSION**

Plesiomorphic features which the sperm of *Leiopelma hochstetteri* share with basal amniotes, urodeles and *Ascaphus truei* include, an elongate conical acrosome vesicle, a subacrosomal cone, distinct nuclear shoulders and an elongate nucleus which tapers to a point within the acrosome. However, *L. hochstetteri* does not exhibit the plesiomorphic feature present in *Ascaphus*, Bombinidae and urodeles of a well-defined axial perforatorium extending into an endonuclear canal (and albeit reduced, in caecilians), although axial lacunae present in the nuclear rostrum and containing putative perforatorial remnants are present in *L. hochstetteri*.

In contrast to all previously examined Anura, *Leiopelma hochstetteri* sperm has a well-developed juxta-axonemal fibre at doublet 8, a condition elsewhere seen only in urodeles within the Lissamphibia. Juxta-axonemal fibres at doublets 3 and 8 are deduced to be the plesiomorphic condition as they not only occur in one or both positions in anurans and urodeles but are also present, at both sites in the dipnoan *Neoceratodus forsteri*.

Our phylogram based only on sperm characters (Fig. 4) indicates that, spermatologically, *Ascaphus* and *Leiopelma* are paraphyletic. They share no synapomorphic sperm characters and there is accordingly no support here for their placement in the same family. Spermatologically, *Leiopelma* is the plesiomorphic sister-taxon of all other anurans. Our analysis also shows the spermatozoa of the Pelodytidae to be highly derived, sharing the apomorphic condition of a conical perforatorium divided into fibres with the Bufonoidea.

**Acknowledgements**

We thank Mrs L. Daddow and Mr T. Gorringe for excellent technical assistance, Dr Brian Gill of the Auckland Museum for sending the fixed tis-
sues to Brisbane and Dr Charles Daugherty for preliminary fixation of the testes samples. Animals were collected under a New Zealand Department of Conservation permit and thanks go to D. G. Newman and C. Smuts-Kennedy (New Zealand DoC) for assistance in obtaining the permit. This work was partly supported by an Australian Research Council grant to BGMJ, University Research Grant to DMS and NSERC Canadian Research Grant to DMG.

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Submitted on 8 February 2000; accepted on 3 October 2000.