

The ultrastructure of the spermatozoa of bufonid and hylid frogs (Anura, Amphibia): implications for phylogeny and fertilization biology

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Comparison of the spermatozoa of *Bufo marinus*, six Australian species of the family Hylidae, and the myobatrachine *Adelotus brevis*, with those of 41 other species of frogs, in a total of 12 investigated families allows the following phylogenetic inferences: the bufonoids (myobatrachids, leptodactylids, hylids, and bufonids) form a monophyletic assemblage with a single synapomorphy: the presence of a conical subacrosomal perforatorium. This structure is analogous to, rather than homologous with, the perforatorium in archaeobatrachians, which differs notably in being an endonuclear structure. The hylid–leptodactylid–bufonid assemblage is the sister-group of the Myobatrachidae (Australian ‘leptodactylids’). Myobatrachids are distinguished by two, albeit weak, synapomorphies, the presence of well-defined pericentriolar material, and the extension of the axial rod up the centriolar fossa, the latter condition approached in the bufonid *Nectophrynoides*. The bufonid, leptodactylid (*sensu strictu*), and hylid families are united, and separated from myobatrachids, by a single synapomorphy: a thick collar-like cytoplasmic sheath that emanates from the centriolar region, is separated from the flagellum by a cytoplasmic canal, and contains the mitochondria. *Litoria fallax*, *L. gracilenta*, and *L. lesueuri* are associated by a unique synapomorphy, hypermorphosis of the minor fibre (juxta-axonemal fibre), though this is approached in *Bufo bufo*. However, there is no spermatological evidence supporting the recognition of Australian hylids (pelodyadids) as a group distinct from the remaining eubufonoids and, specifically, from the Hylidae. Evidence is presented in support of the tentative proposal that the Lissamphibia were primitively internally fertilizing.

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Introduction

The anurans are characterized by an extremely conservative bauplan (Inger 1967). Most taxa, even suprafamilial groupings, are defined according to minor morphological features. Complex (and correspondingly convincing) apomorphies are rare. As a result, phylogenetic relationships within the Anura remain largely unresolved. A recent analysis by Hillis (1991), using morphological and molecular data, has helped to determine the positions of the more plesiomorphic families. Sperm ultrastructure has been used to clarify the relationships of *Ascaphus* (Jamieson *et al.* 1992) and of myobatrachids (Jamieson & Lee 1992), but relationships within the neobatrachians remained largely intractable.

In the present study, the phylogeny of the bufonoid neobatrachians is re-examined in the light of new information derived from sperm ultrastructure. Within the bufonoids, ultrastructural descriptions of sperm exist for many bufonids, New World leptodactylids and hylids (see table of examined species in Lee & Jamieson 1992). Sperm ultrastructure in myobatrachids (Australian ‘leptodactylids’) suggests (Lee & Jamieson 1992) that myobatrachids are the sister-group of all other bufonoids

Papuan family Pelodyadidae (Australasian ‘hylids’) have not previously been described.

Sperm from six species of pelodyadids, and a bufonid introduced into Australia, are here described. The spermatozoon of the curious tusked myobatrachine, *Adelotus brevis*, is illustrated in confirmation of distinctive myobatrachid synapomorphies. The new information, with that previously obtained for myobatrachids, offers an additional perspective on the relationships of these problematic groups.

Material and methods

Species collected and localities were as follows: the myobatrachine, *Adelotus brevis* (Brisbane, Queensland, November 1991, collector David Hillis); *Bufo marinus* (Kenmore, Queensland, February 1990); *Litoria peronii* (Mt Glorious, Queensland, February 1990), *L. lesueuri*, *L. fallax*, *L. gracilenta* (Brookfield, Queensland, February 1990), *L. caerulea* (Stafford Heights, Queensland, March 1990), all collected by M. Lee, and *L. rubella* (Boorooloola, New South Wales, collector Michael Mahoney). Material of *L. rubella* was obtained from museum specimens which had been fixed and preserved in buffered formalin. Specimens of the other species were pithed and dissected under cold amphibian ringer (0.65 g NaCl, 0.03 g CaCl₂, 0.025 g KCl, and 0.02 g NaHCO₃ per 100 ml of distilled water). Testes of fresh specimens were removed by dissection, macerated, and fixed in cold (4°C) glutaraldehyde (3% solution in 0.1 M phosphate buffer, pH 7.2) for at least 2 h.

tissue was rinsed three times in cold buffer for 15 min. It was then postfixed in cold osmium tetroxide (1% solution in 0.1 M phosphate buffer, pH 7.2) for approximately 80 min, then rinsed three times in cold buffer for 15 min, and dehydrated in a series of ascending ethanols (20, 40, 60, 80, 90, 95%, and two changes of 100%), for 30 min in each. The alcohol solutions up to 70% were kept cold (4°C), thereafter at room temperature. The material was then impregnated with and embedded in Spurr's resin which was allowed to polymerise at 60°C for at least 8 h. Thin sections were cut using glass and diamond knives on a LKB III and IV ultramicrotomes and were placed on copper grids (coated with 1% collodion in amyl acetate), and stained using either of the following methods: (1) 40 min in uranyl acetate and 20 min in lead citrate, with three rinses in distilled water after each solution. (2) 1 min in lead citrate, 2 min in uranyl acetate, and 1 min in lead citrate, with three rinses in distilled water after each solution. The thin sections were then viewed, and micrographs taken, on an Hitachi 300 electron microscope operating at 75 kV.

For scanning electron microscopy portions of macerated testes in buffer were placed on polylysine coated coverslips and the buffer replaced with distilled water, an ascending series of ethanol, the amyl acetate, critical point dried and sputter-coated with gold before photography on stubs in a Philips 505 scanning electron microscope at 20–30 kV.

Abbreviations used in figures

<i>a</i>	axoneme or flagellum
<i>af</i>	axial fibre
<i>av</i>	acrosome vesicle
<i>cl</i>	proximal centriole
<i>c2</i>	distal centriole
<i>cc</i>	cytoplasmic canal
<i>f</i>	centriolar (nuclear or implantation) fossa
<i>fi</i>	fibres radiating from proximal centriole
<i>m</i>	mitochondrion
<i>mc</i>	mitochondrial collar
<i>mf</i>	minor (juxta-axonemal) fibre
<i>n</i>	nucleus
<i>p</i>	conical perforatorium
<i>pr</i>	paraxonemal fibre (union of axial and juxta-axonemal fibres)
<i>su</i>	subacrosomal space
<i>rt</i>	ring of blind tunnels
<i>u</i>	undulating membrane

Results

Bufo spp.

A diagrammatic representation of a generalized pelodyadid-bufoiid spermatozoon is shown in Fig. 1 and should be referred to throughout this account.

Bufo marinus (Linnaeus, 1758). The testicular spermatozoon of *Bufo marinus* is composed of an 18 µm long region containing the acrosomal structures, nucleus and mitochondrial sheath, and a 50 µm long tail region (Fig. 2A). The nucleus is electron-dense, cylindrical, and approximately 13 µm long and 0.85 µm in diameter in its middle region (Figs 2A, 3B, D, L). At its anteriormost 3 µm, it tapers gradually to a point. In this region it is capped by a perforatorium and an acrosome: both structures also taper gradually, continuing anteriorly for about 2 µm beyond the nucleus, eventually terminating at the blunt, rounded anterior end of the sperm head (Fig. 3A). The acrosome consists of a membrane-bound vesicle (approximately 0.04 µm thick) of moderately electron-dense material (Fig. 3F–K). The perforatorium is slightly more electron-dense than the acrosome, and is not membrane-bound. It consists of isolated elongated

ery of the extensive subacrosomal space (Fig. 3A), and therefore appears discontinuous in transverse sections (Fig. 3F–K). A considerable asymmetry is sometimes apparent (Fig. 3J, K): the perforatorium occasionally appears to continue further posteriorly along one side of the nucleus than along the other. Each bundle of perforatorial material is composed of very fine rods orientated parallel to each other, and therefore appears grainy in transverse section (Fig. 3F–K).

A small but well-defined fossa (0.35 µm deep) is present in the posterior region of the nucleus (Fig. 3B–E). This cavity contains the proximal centriole, which is usually orientated at an angle of approximately 70° to the long axis of the sperm nucleus (Fig. 3E, M, N). A bundle of fibrils emanates from this centriole (Fig. 3C). The distal centriole is located posterior to the proximal centriole and is orientated parallel to the nuclear axis. It is continuous with the 9+2 axoneme of the flagellum (Fig. 3D, O, P). Both centrioles consist of the usual 9 triplets of short microtubules in a circular arrangement.

An electron-dense 'paraxonemal rod', initially round in cross-section (diameter 0.22 µm), begins posterior to the distal centriole and runs beside the axoneme for a short distance. In this region, the paraxonemal rod and flagellum are surrounded by a thin layer of cytoplasm. Distally, the cytoplasm disappears and a constriction within the paraxonemal rod develops near the flagellum (Fig. 2B). Further distally, this constriction is continuous with the undulating membrane (Fig. 3Q). Hence, proceeding distally along the tail region, the paraxonemal rod gradually splits into unequal portions as a constriction, and then an undulating membrane, develops between its two portions. The small portion (the minor fibre or juxta-axonemal fibre), remains adjacent to the flagellum and is triangular in cross-section (sides 0.08 × 0.1 µm), while the larger portion (the axial or major fibre) is teardrop-shaped in cross-section (longest axis 0.24 µm). The undulating membrane attains its maximum length (approximately 2 µm), as seen in cross-section of the tail, in the middle region of the sperm tail and connects with the minor fibre in the plane containing the two central axonemal singlets (Figs 2B, 3Q). The major and minor fibres are continuous via a thin dense lamina sandwiched within the undulating membrane. Towards the tip of the sperm tail, the undulating membrane again becomes shorter and finally ends, major and minor fibres again merging (Fig. 3R). The paraxonemal rod (= coalesced major and minor fibres) tapers then finally ends, leaving a short length of the flagellum free.

Numerous small ovoid mitochondria (longest axis 0.4 µm, shortest axis 0.3 µm) with prominent parallel cristae occur in a sheath or collar of cytoplasm that surrounds, but is separated from, the proximal region of the sperm tail (Figs 2B, 3B). This 'mitochondrial collar' is approximately 2.3 µm long and 1.3 µm in diameter (Figs 2B, 3B) and is attached to the rest of the spermatozoon only in the region of the distal centriole (Fig. 3B). It has been observed to degenerate, with its mitochondria, in late testicular sperm and during storage in the seminal vesicle (Pugin-Rios 1980; Garrido *et al.* 1989; see *Litoria fallax* below). Transverse sections of the mitochondrial

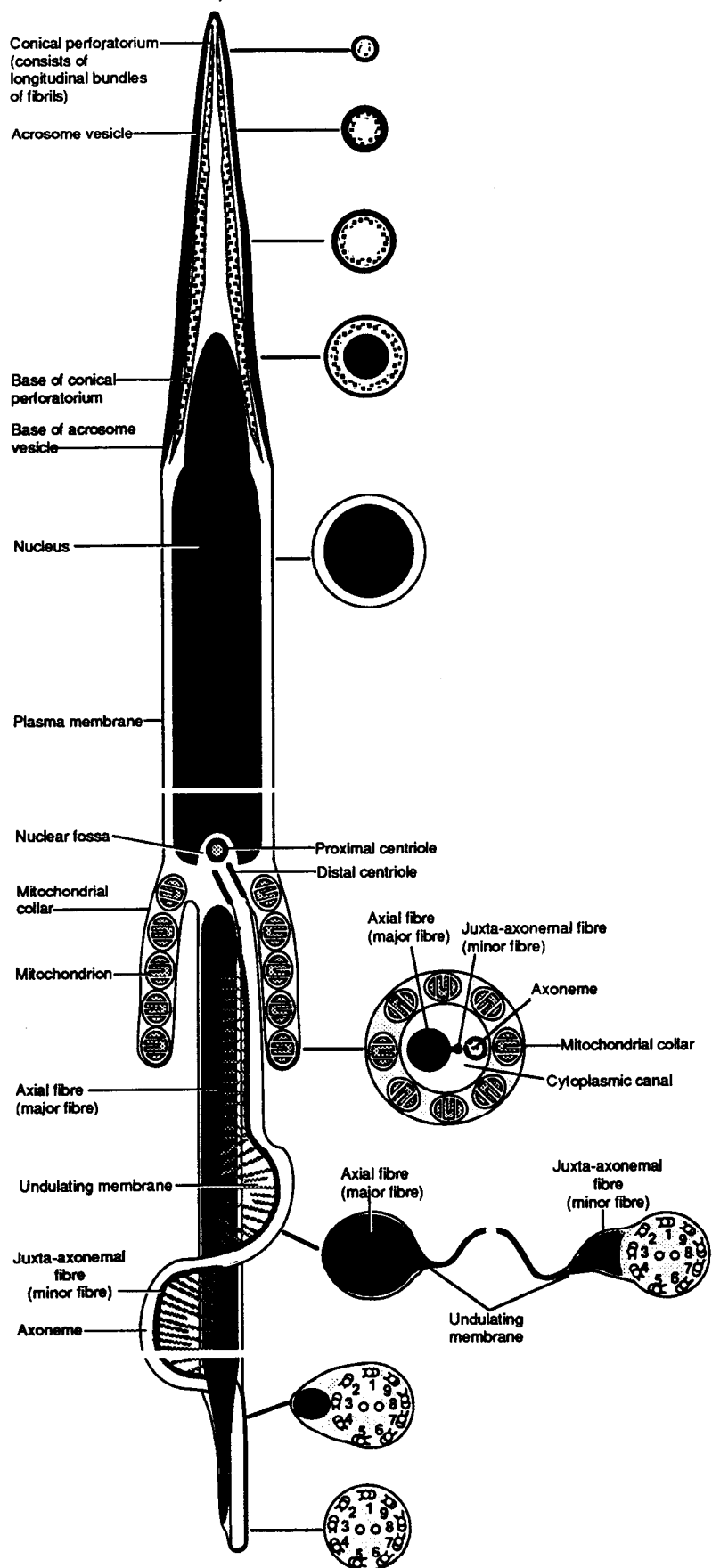


Fig. 1. Highly diagrammatic representation of a generalized pelodyrid (Australian hylid)-bufonid spermatozoon. The conical perforatorium is diagnostic of the Bufonoidea. The mitochondrial collar or sheath distinguishes eubufonoids, including, *inter alia*, bufonids and hylids, from the Myobatrachidae.

between the mitochondrial sheath and the sperm tail is known as the cytoplasmic canal (Fig. 2B; see Jamieson 1991). This canal terminates anteriorly as a series of short blind tunnels, arranged in a ring, that end in slightly expanded chambers (Fig. 3B, P).

Pelodyadidae (Hylidae part.)

Sperm of all six species of *Litoria* examined were similar to each other. All closely resembled *Bufo* sperm, though their tails were consistently shorter. The taxonomic sig-

nificance, if any, of the minor differences in sperm head length cannot be established as yet: the scarcity of mature testicular sperm in many of the animals examined precluded large sample sizes. Because *Litoria* sperm were so similar to *Bufo marinus* sperm, only differences from *Bufo* are noted in the following descriptions, and where such differences might merely represent ontogenetic and individual variability, this is also noted.

Litoria peronii (Tschudi, 1838). The elongated bundles of perforatorial material are slightly narrower in cross-



Fig. 2. *Bufo marinus* spermatozoa.—A. Scanning electron micrograph (SEM) of testicular spermatozoon.—B. Various sections through the cytoplasmic sheath (mitochondrial collar), showing mitochondria, cytoplasmic canal, flagellum, axial (major) fibre and beginning of the undulating

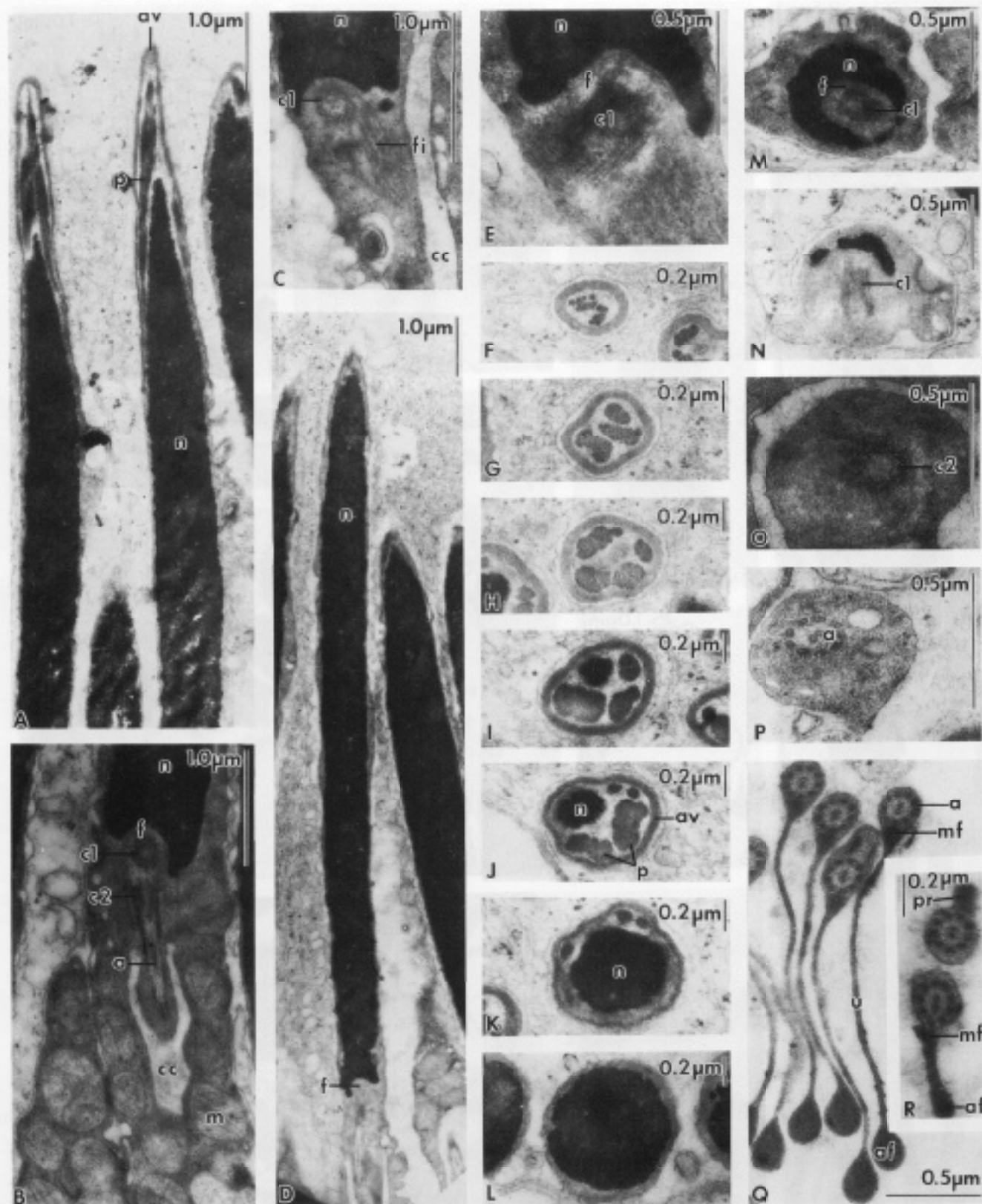


Fig. 3. *Bufo marinus*.—A. LS acrosome region.—B. LS cytoplasmic sheath (mitochondrial collar), showing proximal centriole in TS and distal centriole in LS.—C. LS nuclear fossa showing proximal centriole in TS. Note fibres that appear to emanate from the centriole.—D. LS nucleus.—E. TS nuclear fossa, showing proximal centriole in LS.—F–K. Successive transverse sections of the acrosomal region, showing acrosome, discontinuous perforatorium and nucleus.—L. TS nucleus.—M. TS nuclear fossa showing proximal centriole.—N. Same posterior to M.—O. TS regions between nucleus and cytoplasmic sheath, showing triplets of distal centriole.—P. Same, posterior to O, showing 9+2 axoneme.—Q. TS tails, showing major (axial) fibre, minor (juxta-axonemal) fibre and flagellum.—R. TS tips of tails, showing shortening of undulating membrane (lower section) and ultimate fusion of major and minor fibres as a paraxonemal fibre.

section, more numerous, more restricted to the periphery of the subacrosomal space, and have smaller intervening spaces (Fig. 4A, B) than in *Bufo marinus*. The sperm

38 µm long (Fig. 5A). In some spermatozoa, the acrosome was observed to terminate in a distinct knob: this structure might be present only during certain stages of

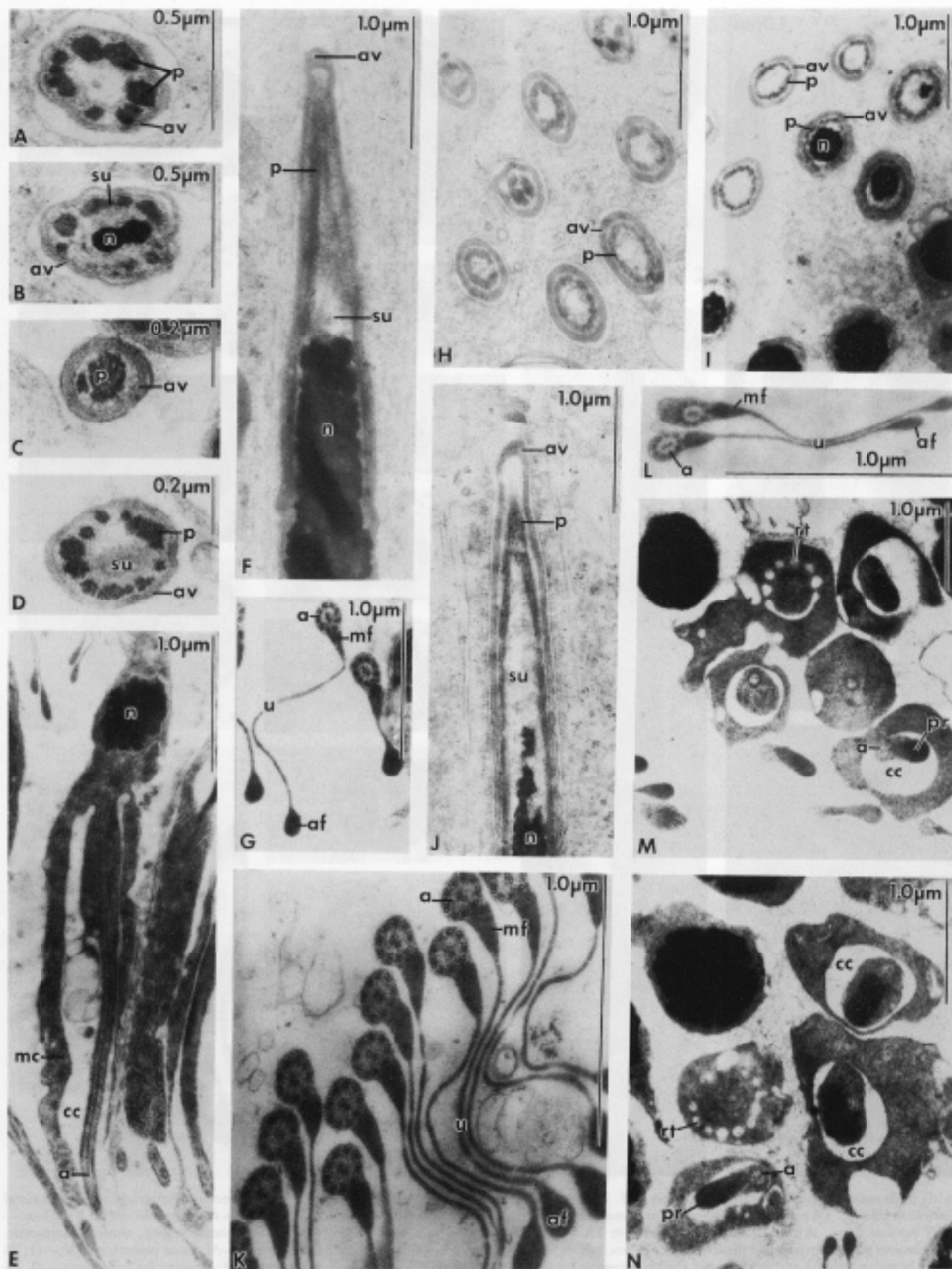


Fig. 4.—A–B. *Litoria peronii*. Successive transverse sections of the acrosome region, showing acrosome, discontinuous perforatorium, subperforatorial space, and nucleus.—C–D. Same, for *Litoria fallax*.—E. *Litoria gracilentia*. LS centriolar region. The mitochondrial collar is long in this species.—F. *Litoria rubella*. LS acrosome region, showing acrosome, conical perforatorium, subperforatorial space and nucleus.—G. *Litoria fallax*. TS tails. Note the prominent hyalid minor fibre.—H–I. *L. lesueuri*. Various slightly oblique sections of the acrosome region, showing acrosome vesicle and perforatorium without discontinuities.—J. *L. lesueuri*. LS acrosome region.—K. *Litoria gracilentia*. TS tails, showing flagellum, undulating membrane, axial fibre and enlarged minor fibre.—L. *Litoria lesueuri*. TS tails, with prominent hyalid minor fibre.—M–N. *Litoria rubella*. Various transverse sections of the centriolar region. As in all *Litoria* species and in *Rufa marinus*, the cytoplasmic canal ends as a ring of round

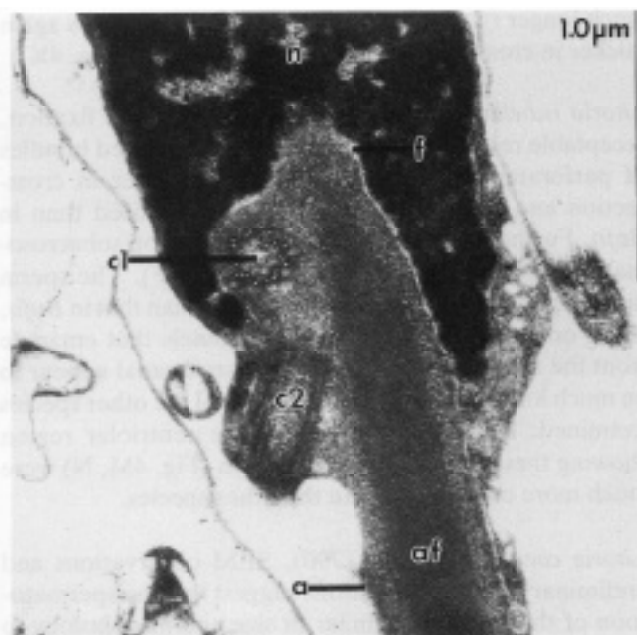
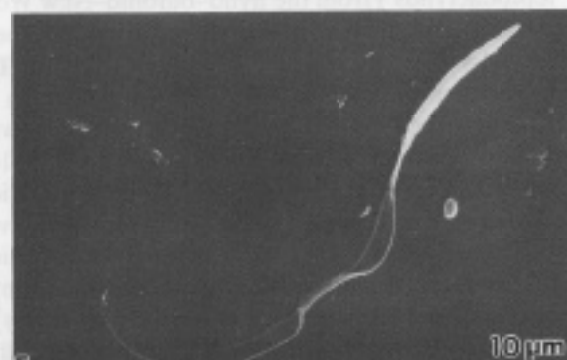
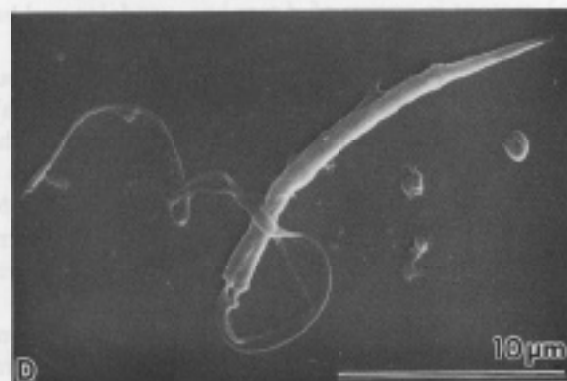
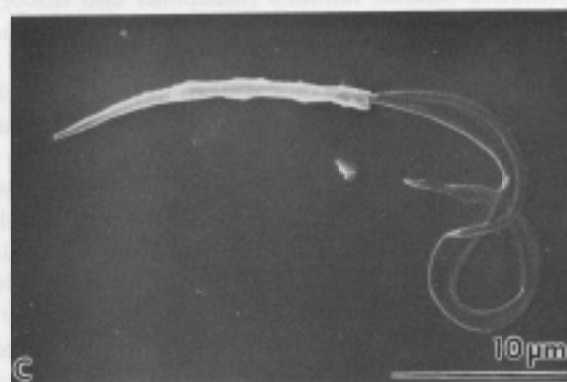
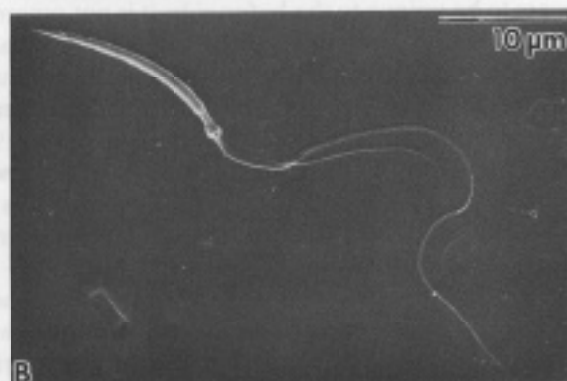
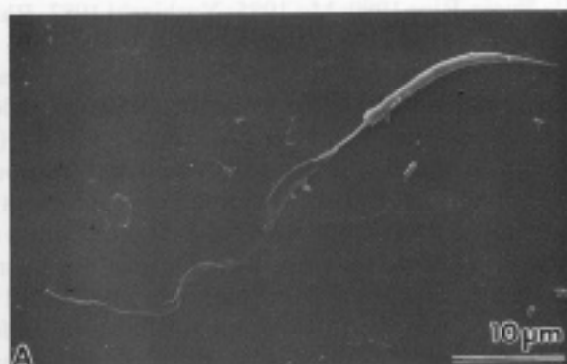


Fig. 6. *Adelotus brevis* (Myobatrachidae). Longitudinal section of spermatozoon through centriolar fossa, showing the myobatrachid synapomorphies: axial rod extending into the centriolar fossa, and mitochondria located, not in a collar around the base of the axoneme but adjacent to the axial fibre.

Litoria fallax (Peters, 1880). The perforatorium (Fig. 4C, D) is similar to that in *L. peronii*. The sperm head is slightly shorter than in *Bufo*, being 16 μm in length, and the tail is slightly shorter, being approximately 40 μm in length (Fig. 5B). The minor fibre (Fig. 4G) is thicker in cross-section (sides $0.13 \times 0.26 \mu\text{m}$). SEM micrographs were obtained for this species showing the degeneration and loss of the mitochondrial sheath, a process thought to occur late in spermiogenesis in all bufonoids (Garrido *et al.* 1989), excepting presumably the periaxonemal mitochondrial sheath in the bufonid *Nectophrynoides*.

Litoria lesueuri (Dumeril & Bibron, 1841). The perforatorium in this species is almost continuous in transverse section: the bundles of fibres are much narrower in cross-section, closely juxtaposed, and restricted to the periphery of the subacrosomal space (Fig. 4H, I). The sperm head has similar proportions to that in *Bufo*, but the sperm tail is again slightly shorter, being approximately 35 μm long (Fig. 5C). As in *L. fallax*, the minor fibre is thicker in cross-section (sides $0.13 \times 0.26 \mu\text{m}$; Fig. 4L). Again, as with *L. peronii*, in some spermatozoa the acrosome was observed to terminate in a distinct knob (Fig. 4J).

Litoria gracilentia (Peters, 1869). The perforatorium is again continuous in transverse section (see *L. lesueuri*). The sperm head has similar proportions to that in *Bufo*, but an acrosomal knob was observed in most spermatozoa (Fig. 5D; see also *L. peronii*). The mitochondrial sheath is

Fig. 5. Scanning electron micrographs of *Litoria* spermatozoa.—A. *Litoria peronii*.—B. *L. fallax*.—C. *L. lesueuri*.—D. *L. gracilentia*.

much longer (4 μm : Fig. 4E) and the minor fibre is again thicker in cross-section (sides $0.13 \times 0.26 \mu\text{m}$: Fig. 4K).

Litoria rubella (Gray, 1842). Despite formalin fixation, acceptable results were obtained. The elongated bundles of perforatorial material are much narrower in cross-section and are much more closely juxtaposed than in *Bufo*. Furthermore, they occupy most of the subacrosomal space, not only the periphery (Fig. 4F). The sperm head appears to be considerably shorter than that in *Bufo*, being only 14 μm long. The blind tunnels that emanate from the anterior end of the cytoplasmic canal appear to be much longer in this species than in all the other species examined: transverse sections of the centriolar region showing these tunnels in cross-section (Fig. 4M, N) were much more common than in the other species.

Litoria caerulea (White, 1790). SEM observations and preliminary TEM observations suggest that the spermatozoon of this species is similar in size and morphology to the other members of its genus, though exact details of its internal ultrastructure have yet to be obtained. The sperm head, with the mitochondrial sheath attached, is approximately 18 μm long (Fig. 5E), and the sperm tail is approximately 35 μm long. The mitochondrial sheath is long (as in *L. gracilentia*), and appears to degenerate late in spermiogenesis.

Myobatrachidae

Adelotus brevis (Günther, 1868). A longitudinal section of the posterior nuclear fossa, with contained proximal centriole, and adjacent flagellum is shown in Fig. 6. The axial fibre of the flagellum is seen to deeply penetrate the fossa. Mitochondria are not located in a collar surrounding the base of the axoneme but were seen in other micrographs to border the axial fibre.

Discussion

Implications of sperm ultrastructure for anuran phylogeny

Spermatozoal ultrastructure of the following anuran families has been examined by transmission electron microscopy, accounts which form a basis for this discussion: Ascaphidae: James 1970; Jamieson *et al.* 1992. Discoglossidae: Favard 1955; Furieri 1975; Folliot 1979; Sandoz 1969, 1970, 1973, 1974a, b; Pugin-Rios 1980. Pipidae: James 1970; Reed & Stanley 1972; van der Horst 1979; Pugin-Rios 1980; Bernardini *et al.* 1986, 1988; Yoshizaki 1987. Pelobatidae: Asa & Phillips 1988; James 1970; Morrisett 1974; Pelodytidae: Pugin-Rios 1980. Myobatrachidae: Lee & Jamieson 1992, 1993. Leptodactylidae: Pisano & Adler 1968; Pugin-Rios 1980; Pugin & Garrido 1981; Garrido *et al.* 1989; Bão *et al.* 1991; Nottola *et al.* 1991. Bufonidae: Burgos & Fawcett 1956; Furieri 1961; Nicander 1970; Morrisett 1974; Pugin-Rios 1980; Pugin & Garrido 1981; Yoshizaki & Katagiri 1982; Mo 1985; Rhinodermatidae: Pugin-Rios 1980; Pugin & Garrido 1981. Hylidae: Morrisett 1974; Pugin-Rios 1980; Rastogi

1971; Pugin-Rios 1980; Mo 1985; Yoshizaki 1987. Rhacophoridae: Mainoya 1981; Mizuhira *et al.* 1986.

We know of no somatic apomorphies corroborating the monophyly of the bufonoid neobatrachians—indeed, recent phylogenies based on general morphology (e.g. Duellman & Trueb 1986) have suggested that the group is paraphyletic. However, information from the present and previous studies on anuran spermatozoa listed above (see also table in Lee & Jamieson, 1992, for species examined) suggests that the bufonoids are monophyletic, united by a single but prominent synapomorphy, the conical perforatorium. Within the Anura, this morphological novelty appears to be unique to the bufonoids: it is not present in other anurans, or in urodeles. A conical layer of subacrosomal material between the acrosome and the nucleus in urodeles and ascaphids (Jamieson *et al.* 1992) and in many amniotes including chelonians (Healy & Jamieson 1992; Jamieson & Healy 1992) does not appear to be homologous with the conical perforatorium of bufonoids as the two structures differ ultrastructurally (Jamieson *et al.* 1992).

The conical subacrosomal perforatorium of bufonoids appears to be analogous to, rather than homologous with, the rod-shaped endonuclear perforatorium characteristic of primitive anuran families (ascaphids and discoglossids), urodeles and primitive amniotes, including chelonians. In addition to profound differences in shape and location, the bufonoid conical perforatorium develops late in spermiogenesis (well after chromatin condensation and nuclear elongation) and in association with the nuclear membrane (Burgos and Fawcett 1956; Rastogi *et al.* 1988), while the endonuclear perforatorium develops early in spermiogenesis and originates from a granule at the base of the acrosome vesicle (e.g. Sandoz 1969). Additionally, as discussed elsewhere (Jamieson *et al.* 1992), the conical bufonoid perforatorium is not homologous with a superficially similar structure (subacrosomal cone) present in sperm of *Ascaphus*, urodeles and basal amniotes.

The conical perforatorium appears to be a valid bufonoid synapomorphy: out of the 27 species (17 genera) of bufonoids so far examined for sperm ultrastructure, there are only two cases where the conical perforatorium has not been observed: *Pleurodema thaul* and *Caudiverbera caudiverbera* (see Pugin-Rios 1980; Pugin & Garrido 1981). These two taxa, which are unusual in other ways, will be discussed later.

Within the bufonoids, sperm ultrastructure strongly supports the separation of the myobatrachids (Australasian 'leptodactylids') from the hylid-bufonid-New World leptodactylid assemblage (which we may term the true or eubufonoids) (see below, Lee & Jamieson 1992). This phylogenetic and taxonomic arrangement has been repeatedly proposed in the past, though on somewhat questionable grounds. For instance, Lynch's (1973: 161) phylogeny had no characters supporting the separation, while Duellman & Trueb's (1986) cladogram tentatively separated the groups on the basis of amplexic position, though (as these authors noted) limnodynastine myobatrachids possess the same type of amplexus (axillary) as eubufonoids. There does not appear to be any compelling

batrachids from the other bufonoid families (Kluge and Farris 1969; Tyler 1985). However, on the basis of testicular sperm ultrastructure, the three eubufonoid families examined—New World leptodactylids, hylids, and bufonoids—all share a single but striking characteristic, the long mitochondrial sheath or collar separated from the centriolar region and tail by a cytoplasmic canal (see also Lee & Jamieson 1992). Out of the 23 species and 13 genera of eubufonoids for which sperm ultrastructure is known, the mitochondrial collar was absent in only two taxa. Again, these were *Pleurodema thaul* and *Caudiverbera caudiverbera* (see below). The mitochondrial collar is uniformly absent in myobatrachids and other anurans, in urodeles and in all other tetrapods, and thus its presence represents a valid synapomorphy uniting the eubufonoid families. The myobatrachids are united by their own synapomorphy, the extension of the major fibre up the centriolar fossa (Lee & Jamieson 1992), though this condition is approached in the bufonid *Nectophrynoides* and is present, presumably independently, in *Discoglossus* (Pugin-Rios 1980). This condition has been confirmed here for the myobatrachine *Adelotus brevis*, having previously been demonstrated for limnodynastine myobatrachids. The myobatrachids therefore appear to be the sister group of the eubufonoids.

There remain the two species which fail to conform to the otherwise clear criteria for separation of myobatrachids and eubufonoids: *Caudiverbera caudiverbera* and *Pleurodema thaul*, both New World leptodactylids (Pugin-Rios 1980; Pugin & Garrido 1981). Neither has a conical perforatorium, distinctive of bufonoids *sens. lat.*, nor any other recognizable perforatorium. Neither shows any trace of a mitochondrial collar and cytoplasmic canal, distinctive of eubufonoids, though mitochondria are transiently present in the spermatid in the cytoplasm of the centriolar region. *Caudiverbera* has a simple sperm tail, a free flagellum with no paraxonemal rod or undulating membrane. Taken literally, sperm ultrastructure suggests that *Caudiverbera* and *Pleurodema* diverged from the remaining bufonoids before the myobatrachids. Alternatively, *Caudiverbera* and *Pleurodema* might be part of a monophyletic eubufonoid clade, in which case absence of the conical perforatorium and mitochondrial collar would be secondary. Within the Anura there is a general trend towards simplification of sperm, and given the rather limited suite of features in sperm, parallel simplifications are inevitable. At present, it is difficult to decide which of the alternative relationships of these two genera is correct.

Summarizing the spermatological evidence in the present study, with the exception of *Pleurodema* and *Caudiverbera*, the conical perforatorium is unique to, and ubiquitous in, the bufonoid neobatrachians (*sens. lat.*, including myobatrachids), and the mitochondrial sheath is unique to, and ubiquitous in, the eubufonoids. Thus spermatological evidence presents the only known synapomorphy uniting the bufonoids.

The striking uniformity of sperm morphology in eubufonoids, which allows such suprafamilial inferences to be drawn, conversely prevents resolution of lower-level relationships within the eubufonoids. While sperm of bufo-

species), and New World hylids are almost identical (see above description for *B. marinus*), their shared traits appear to be primitive for the bufonoid–leptodactylid–hylid clade (eubufonoids) as a whole. The tapering acrosome occurs in all other anurans (including other eubufonoids) except ranoids. The conical perforatorium occurs in other eubufonoids and in myobatrachids. The paraxonemal rod (or its equivalents the major and minor fibres) and undulating membrane occur in myobatrachids, archaeobatrachians, some other eubufonoids, and appear to be ubiquitous in urodeles (Picheral 1979) and caecilians (Seshachar 1945); the nuclear (centriolar) fossa occurs in all anurans so far examined (except for pipids and ranoids), and in urodeles (e.g. Picheral 1979), caecilians (Seshachar 1945) and amniotes (e.g. Bedford *et al.* 1984); and as emphasized previously, the mitochondrial collar occurs in almost all eubufonoids for which testicular sperm ultrastructure is known. Hence, the similarities between bufonoid, Australian hylid (pelodyradid) and New World hylid spermatozoa are symplesiomorphic and are not indicative of affinities. Therefore our results tend to corroborate the predominant view in anuran phylogeny (e.g. Duellman & Trueb 1986) which recognizes the Australian leptodactylids (myobatrachids), but not the Australian hylids, as a group distinct from the eubufonoids. *Hyla meridionalis* is apomorphic in having lost the undulating membrane while retaining the paraxonemal rod (Pugin-Rios 1980).

Within the Australian hylids, half of the species investigated, *Litoria fallax*, *L. gracilentia* and *L. lesueuri*, are united by a relatively simple (and therefore weak) synapomorphy, the enlarged minor filament. Even this condition is approached in *Bufo bufo* (see Pugin-Rios 1980). The leptodactylid genera *Batrachyla* and *Telmatobufo* appear to be united by the possession of a hypermorphosed perforatorium that occupies almost all of the subacrosomal space (Pugin & Garrido 1981; Garrido *et al.* 1989), but the condition of this character in other leptodactylids is very imperfectly known. In general, however, eubufonoid sperm are so conservative (e.g. sperm of *Bufo*, *Litoria* and New World hylids are almost indistinguishable) that they provide little information regarding the interrelationships of the various families, and resolution of these must continue to rely largely upon other features.

The plesiomorphic state of fertilization in lissamphibians

Recent studies on lissamphibians have suggested that external fertilization is primitive, and that internal fertilization has been acquired independently in the three orders, caecilians, urodeles and anurans (e.g. Boisseau and Joly 1975; Hecht & Edwards 1976; Duellman & Trueb 1986). We contend, however, that sperm ultrastructure and some morphological data together provide support for the hypothesis that the proto-lissamphibian was internally fertilizing, and that external fertilization in 'primitive' urodeles (hynobiids and the related cryptobranchids) and most anurans is a secondary development. The evidence from sperm ultrastructure is circumstantial, though compelling. A complex sperm 'ground plan' is indicated by the lissamphibians, assuming in all three orders. It

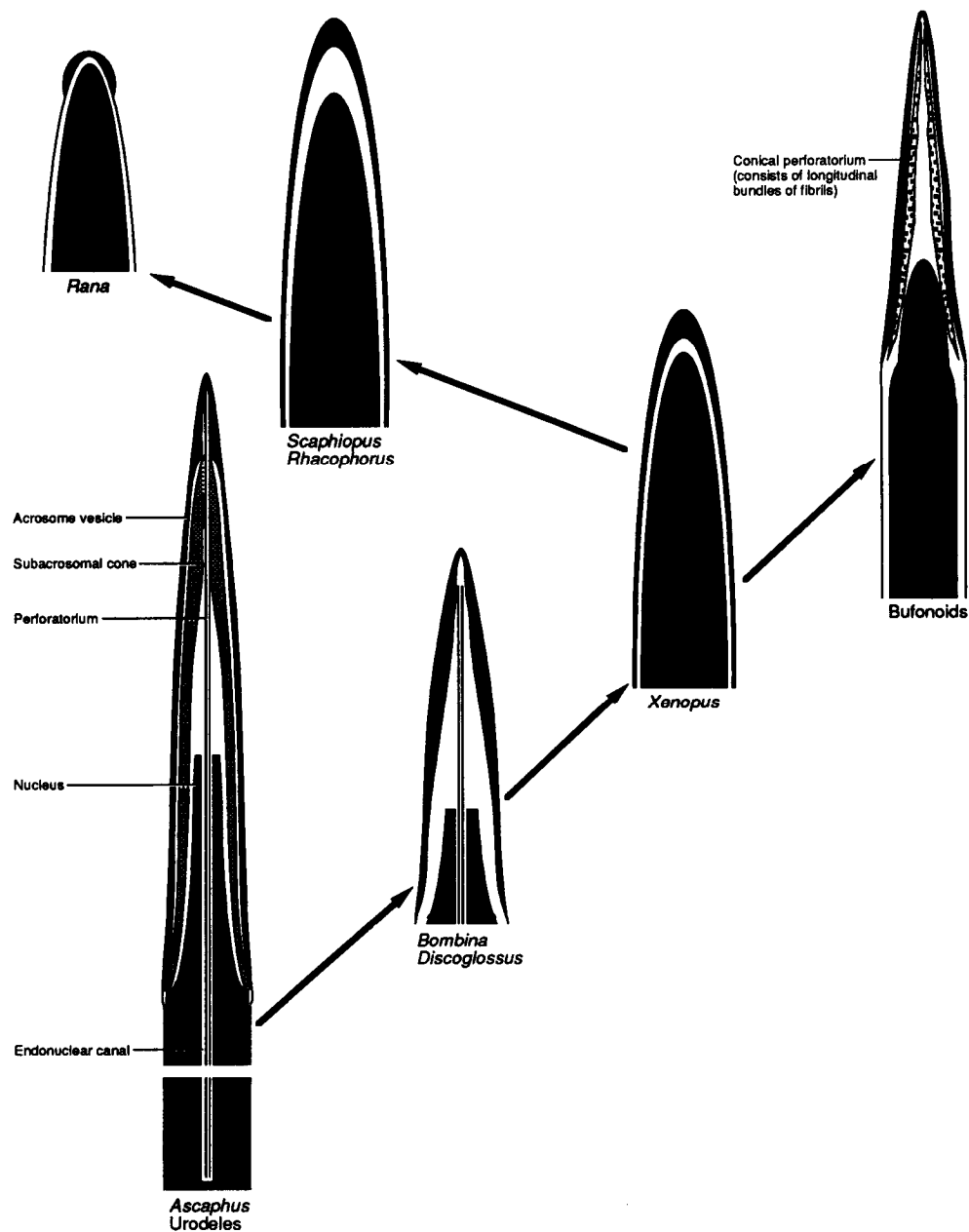


Fig. 7. Highly diagrammatic representation of some evolutionary trends in the acrosome region of anuran sperm. This is not intended as a phylogeny, though the trends are towards more highly apomorphic taxa. The rod-shaped perforatorium of urodeles and archaeobatrachians is not here regarded as homologous with the perforatorial cone (periferfatorial sheath) of *Ascaphus* and urodeles homologous with the perforatorial cone. Note a trend to simplification of the acrosome complex which culminates in the condition seen in *Rana*.

consists of a long nucleus, a multilayered acrosome region and a unique tripartite tail (Fig. 8). Complex sperm are considered to be characteristic of internally fertilizing organisms (e.g. Franzén 1956; Afzelius 1972), though, admittedly, externally fertilizing fish may have complex sperm (Jamieson 1991). The caecilians (which invariably exhibit internal fertilization) and urodeles (which mostly do), have retained the primitive, yet complex lissamphibian sperm morphology. Significantly, both of the externally fertilizing urodeles that have been examined for sperm morphology (*Pseudobranchius*, a hynobiid, and *Cryptobranchius*, a cryptobranchid) possess sperm that deviate markedly from the basic lissamphibian (and urodele) pattern. *Pseudobranchius* sperm possess two flagella and two undulating membranes, all lying beside rather than behind the nucleus (Austin and Baker 1964), while

piece, and mitochondria that are located in an anterior cytoplasmic bead (Baker 1963). Furthermore, hynobiids and cryptobranchids shed the eggs in paired sacs (termed oophores by Jamieson, unpublished), one from each ovary, a condition which Estes (1981) recognizes as a synapomorphy. In the (admittedly very incomplete) fossil record, cryptobranchoids appear in the Tertiary and are greatly pre-dated by Jurassic salamandroids (Duellman & Treub 1986; Evans *et al.* 1988). Likewise, in anurans (which are mostly externally fertilizing), although many species have retained the primitive lissamphibian spermatozoon, there is unquestionably a trend towards divergence from this form. For instance, *Rhacophorus* and *Bombina* sperm are highly aberrant, with unique and complex modifications (Mainoya 1981; Mizuhira *et al.* 1986; Furieri 1975; Folliot 1979; Pugin-Rois 1980). The

fication (Figs 7, 8). Ranid sperm lack the following organelles which are all inferred to have been present in the plesiomorphic lissamphibian sperm (see below and Jamieson *et al.* 1992): subacrosomal cone, endonuclear canal, rodlike endonuclear perforatorium, subacrosomal space, centriolar fossa, cytoplasmic vesicles, neckpiece, ring, axial rod (with or without differentiation into major

and minor fibres) and undulating membrane. In addition, the ranid nucleus is shorter, the acrosome is usually less extensive, and mitochondria are fewer. At least two of the lost structures, the neckpiece and the endonuclear canal, have been postulated to be adaptations for internal fertilization (Barker and Bieseke 1967; James 1970), though the endonuclear canal is present in some externally ferti-

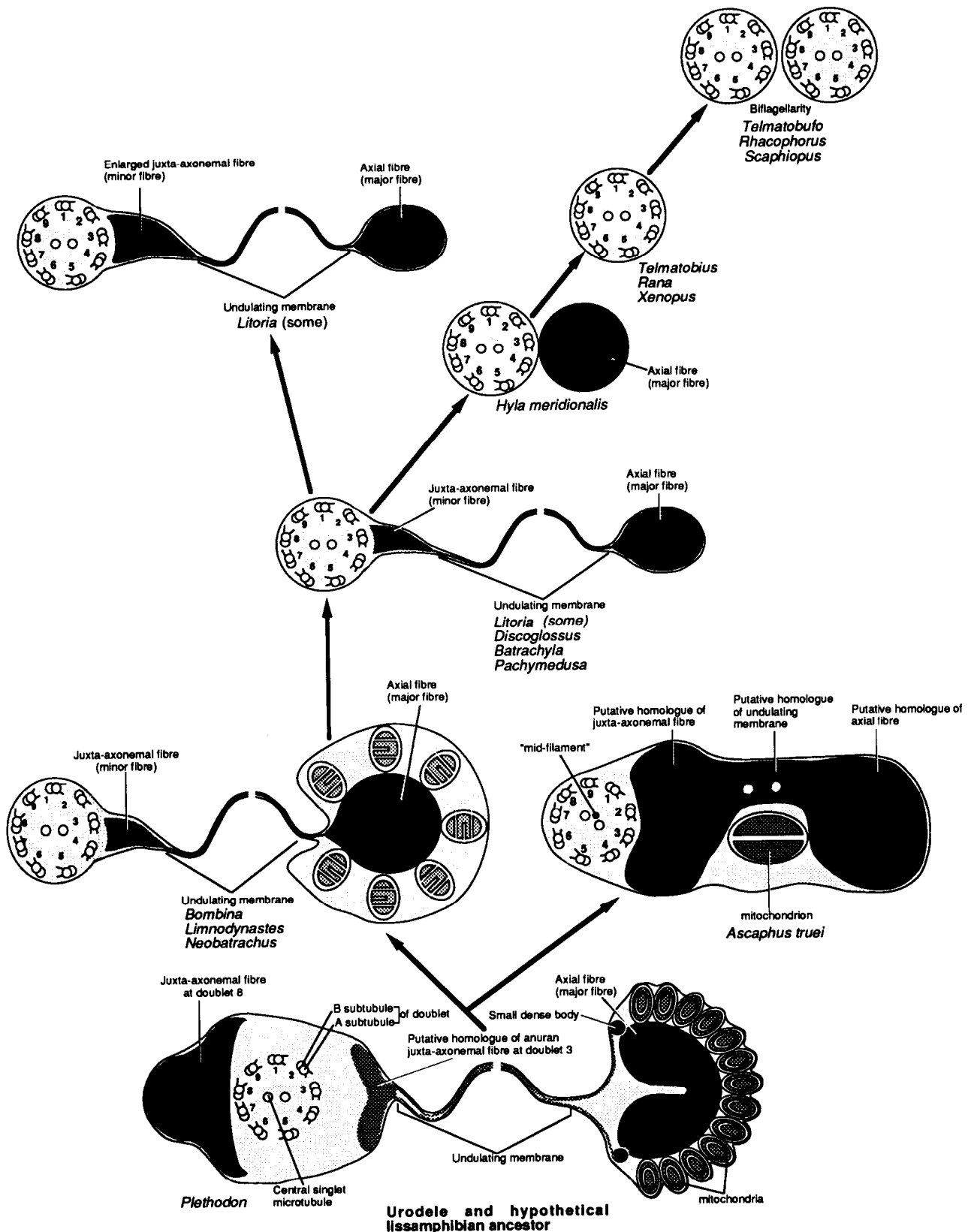


Fig. 8. Schematic illustration of evolutionary trends in the anuran sperm tail region as seen in cross-sections of the flagellum. This is not intended as a phylogeny, though the trends are towards more highly apomorphic taxa. For instance, biflagellarity in *Telmatobufo* and *Rhacophorus* has been

lizing fish (Jamieson 1991). Similar modifications of the basic lissamphibian sperm type occur elsewhere in the Anura: for instance, the axial rod and the undulating membrane have been lost in some leptodactylids. Mitochondrial numbers have also been secondarily reduced in the fully mature sperm of bufonoids: in testicular sperm, many mitochondria occur in a cytoplasmic sheath which later degenerates during storage in the seminal vesicle. The ejaculated spermatozoa possess few or no mitochondria (e.g. Garrido *et al.* 1989; Pugin-Rios 1980). This loss during ontogeny strongly suggests that many mitochondria were primitively present. In pulmonate molluscs, which are mostly internally fertilizing, loss of mitochondria is correlated with reversion to external fertilization (Favard & André 1970).

However, it is outgroup comparison that provides the strongest evidence that simple anuran sperm have been derived from more complex structures. Thus absence of a rodlike perforatorium in, for instance, *Xenopus*, bufonoids, *Scaphiopus*, *Rhacophorus* and ranids (references in Lee & Jamieson 1992) (Fig. 7) is clearly a loss as this is present in archaeobatrachians, urodeles, basal amniotes, actinistians and dipnoans. Again, absence of an undulating membrane from the sperm flagellum in, *inter alia*, *Rana* and *Xenopus* can reasonably be considered a loss in view of its presence in all three lissamphibian orders (Fig. 8). The inevitable conclusion is that simple anuran sperm are the result of secondary simplification from the complex type seen in urodeles, caecilians and some archaeobatrachians but the question is whether this simplification necessarily indicates a reversion from internal to external fertilization. All the above observations, especially that of a common complex sperm with tripartite flagellum in all three lissamphibian orders, and its modification in many lines, are explicable if the complex and unique spermatozoon basic to lissamphibians is postulated to be an adaptation for internal fertilization basic to the Lissamphibia. Hence, in taxa that have retained this mode of fertilization (caecilians and most urodeles), sperm ultrastructure has remained essentially unchanged, being modified, as far as is known, only in those urodeles (cryptobranchioids) which have external fertilization. Sperm morphology in the internally fertilizing frog *Ascapus*, which also stores sperm (Metter 1964), again differs only slightly from that of urodeles and caecilians, notably in abbreviation of the undulating membrane (Fig. 8). Parsimony suggests that elongation of the membrane must have occurred before divergence of the lissamphibian orders, and that the short bridge-like homologue of a membrane in *Ascapus* (Fig. 8) is the result of secondary abbreviation (see also Jamieson *et al.* 1992).

We consider that stabilizing selection may explain the relative uniformity of the sperm of most urodeles, caecilians and *Ascapus*. The lengthy period of sperm storage in many urodeles results in especially strong gametic selection, and any aberrant sperm are unlikely to survive for long enough to achieve fertilization (Halliday & Verrell 1984). In taxa that have secondarily acquired external fertilization (*Pseudobranchius*, *Cryptobranchius*, in the urodeles, and most anurans), this stabilizing selection has evidently been reduced and sperm morphology has both

by *Rana* sperm, which as noted above, approach the very simple 'ect-aquasperm' morphology (*sensu* Rouse & Jamieson 1987) typical of most externally fertilizing taxa: a short nucleus with a button-like acrosome and a few small mitochondria, propelled by a single flagellum. Trends to simplification of complex sperm are well exemplified by fishes (Jamieson 1991) and sabellid polychaetes (Jamieson & Rouse 1989). In both of these groups evidence for loss of earlier internal fertilization and re-acquisition of external fertilization is equivocal, tending to favour a primitive external fertilization in fish but, less certainly, internal fertilization in Sabellida.

There is also morphological evidence that internal fertilization is primitive in urodeles. Cryptobranchioids possess rudimentary spermatophore glands (Webb *et al.* 1981; Hecht & Edwards 1976). Because these glands appear to be non-functional in all cases except *Ranodon* (Hecht and Edwards 1977), they are likely to be vestigial rather than incipient. Spermatophores are produced in the externally fertilizing cryptobranchoid *Ranodon sibiricus* (Bannikov 1958). As spermatophores are linked with internal fertilization in many taxa (Jamieson 1987), the above observations suggest that cryptobranchioids formerly exhibited internal fertilization which is therefore taken to be the primitive condition in urodeles. Noble (1925) also invoked former internal fertilization in explanation of the presence of small spermatophores in *Ranodon*.

The evidence of loss of spermatophores within cryptobranchioids, coupled with the simplification of sperm in anurans, does not favour the hypothesis that lissamphibians were primitively externally fertilizing. Instead, it strongly suggests that internal fertilization is primitive in urodeles, anurans, and thus lissamphibians in general. Most recent works have suggested the opposite. Duellman & Trueb (1986: 50) asserted that 'external fertilization unquestionably is primitive in salamanders'. Boisseau and Joly (1975) and Hecht & Edwards (1976) expressed similar opinions.

Hecht & Edwards concluded that salamanders were primitively externally fertilizing for three reasons.

1. External fertilization is associated with primitive osteology, and is therefore also primitive. This assumes that all characters of 'primitive' organisms should be plesiomorphic. On the contrary, mosaic evolution appears to predominate.

2. 'Since the primitive state in anurans and caecilians is undoubtedly externally fertilizing, this mode of reproduction must be primitive for Amphibia as a whole, and postulating the gain and subsequent loss of internal fertilization in cryptobranchioids adds two extra evolutionary steps.'

The problem here is that the primitive state in caecilians (which invariably have enormous copulatory organs) (e.g. Webb *et al.* 1981; Duellman & Trueb 1986) is at least as likely to have been internal fertilization, seen also in the most primitive anuran, *Ascapus*. Hence, if current views on lissamphibian phylogeny are broadly correct, postulating internal fertilization as primitive to the Lissamphibia is the more parsimonious arrangement. Furthermore, when we extend the analysis to all tetra-

ancestor of Lissamphibia and the exclusively internally fertilizing Amniota as internally fertilizing. If *Latimeria* is taken to be the nearest living relative of the tetrapods (e.g. Schultz 1987), presence of internal fertilization in this actinistian would constitute some evidence for a basic internal fertilization in the precursors of tetrapods, with retention in amphibians and amniotes. However, Rosen *et al.* (1981) and Jamieson (1991) have argued for a closer relationship of tetrapods with the Dipnoi, all of which are externally fertilizing. In the latter case acquisition of internal fertilization is envisaged as a synapomorphy of tetrapods rather than of their finned precursors.

3. Loss of the spermatophore [as in most externally fertilizing urodeles] is unlikely because other methods of fertilization are more 'hazardous'.

However, the highly intricate manoeuvres necessary to ensure that the female urodele collects the deposited spermatophore (e.g. Duellman & Trueb 1986) suggests the opposite conclusion: that this method of fertilization is no less, and is probably more, hazardous than either copulation or external fertilization.

A difficulty in arguing for a change from internal to external fertilization is that the pathway by which previously internally fertilized eggs could be shed unfertilized may seem difficult to envisage. In most urodeles the cloaca of the female is placed over a free spermatophore and if the sperm were not accepted from the spermatophore until the egg were extruded rather than before extrusion, the change to external fertilization would be accomplished. No change in the behaviour of the male, whether or not it is of a species which facilitates contact of the female cloaca with the spermatophore, would be required. The condition in *Ranodon* closely resembles the hypothetical transitional condition. Most urodele eggs when fertilized internally are immediately shed to the exterior and basically in urodeles modification of the egg for sojourn in the oviducts is negligible. This is clearly even the case where eggs are retained in the oviducts, as exemplified by those salamanders which (Duellman & Trueb 1986: 22) retain the eggs in drought conditions but expel them when water is sufficient. Specialized dependence of the egg on the oviduct (which would result in obligatory retention of eggs and internal fertilization) does not appear to exist in urodeles. Finally, there is no ecological reason why the trend in amphibian reproduction should be from aquatic eggs and external fertilization to terrestrial eggs and ovoviviparity, necessitating internal fertilization, but never the reverse. Aquatic eggs and larvae are energetically less expensive to produce and hence allow greater fecundity (references in Duellman and Trueb 1986; Duellman 1989). While direct developing terrestrial embryos must rely solely on maternal provisions, aquatic larvae actively acquire their own food during the corresponding phase of their life cycle. Not surprisingly, amphibians with direct development and terrestrial eggs are restricted to very small adult sizes (Carroll 1970). There are definite ecological advantages in reverting to large clutches of aquatic eggs which do not require internal fertilization. It is notable that many urodeles exhibit internal fertilization yet lay aquatic eggs. Only if pressures towards terrestriality are great would

advantageous—significantly, the presumed ancestors or the lissamphibians, the dissorophid temnospondyls (Bolt 1977) were mostly highly terrestrial organisms. This is again consistent with the idea that the lissamphibians were primitively internally fertilizing.

The arguments which we have advanced for primitive internal fertilization in lissamphibians are intended to challenge too facile an acceptance that fertilization was originally external. Both views require further investigation in the light of existing and new evidence. Even if our alternative view is true, however, anurans undoubtedly exhibited external fertilization very early in their history, soon after their divergence from the other Lissamphibia. With the probable exception of *Ascaphus*, where it appears to be plesiomorphic, internal fertilization in a few distantly related extant anurans appears to be a recent reacquisition. Excepting *Ascaphus*, internally fertilizing frogs have no copulatory organs, spermathecae, or other obvious morphological specializations: cloacae are merely juxtaposed (e.g. Townsend *et al.* 1981). Hence, these non-ascaphid internally fertilizing anurans have undoubtedly been recently derived from externally fertilizing ancestors.

The hypothesis advanced above of basic internal fertilization in lissamphibians is necessarily tentative. Jamieson (1991) has discussed similar trends, in fish, from complex sperm in more basal groups to ectaquasperm in more advanced fish, the Neopterygii. Though the possibility that the complex sperm indicated a previous internal fertilization (now seen only in chondrichthyans, of the more basally derived groups) was considered, it was concluded that the weight of evidence including consideration of parsimony, supported the view that external fertilization was basic (plesiomorphic) to fish and that internal fertilization in the various groups displaying it was derived (apomorphic). For the Lissamphibia, the weight of evidence discussed above appears to favour, or at least warrant consideration of, the hypothesis that fertilization was originally internal, being retained in all caecilians, most urodeles, and the most plesiomorphic living frog, *Ascaphus*.

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