The ultrastructure of the spermatozoa of Epipedobates flavopictus (Amphibia, Anura, Dendrobatidae), with comments on its evolutionary significance

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Abstract

We describe, for the first time, the spermatozoon ultrastructure of a dendrobatid frog, Epipedobates flavopictus. Mature spermatozoa of E. flavopictus are filiform, with a moderately curved head and a proportionally short tail. The acrosomal vesicle is a conical structure that covers the nucleus for a considerable distance. A homogeneous subacrosomal cone lies between the acrosome vesicle and the nucleus. The nucleus contains a nuclear space at its anterior end, and electron-lucent spaces and inclusions. No perforatorium is present. In the midpiece, the proximal centriole is housed inside a deep nuclear fossa. Mitochondria are scattered around the posterior end of the nucleus and inside the undulating membrane in the anterior portion of the tail. In transverse section the tail is formed by an U-shaped axial fiber connected to the axoneme through an axial sheath, which supports the undulating membrane. The juxta-axonemal fiber is absent. The spermatozoon of E. flavopictus has several characteristics not observed before in any anurans, such as a curved axial fiber, absence of a juxta-axonemal fiber, and presence of mitochondria in the typical undulating membrane. Our results endorse the view that, in anurans, the conical perforatorium and subacrosomal cone are homologous and that Dendrobatidae should be grouped within Bufonoidea rather than Ranoidea.

Keywords: sperm; ultrastructure; Anura; Dendrobatidae; Epipedobates

Introduction

The phylogenetic relationships among families of Anura are still largely unresolved (Duellman & Trueb, 1986; Ford & Cannatella, 1993). Groups widely accepted as monophyletic have often been challenged with new phylogenetic reconstructions and the continuous accumulation of new data. For example, Hillis et al. (1993) using 28S fragments of rRNA found Neobatrachia to be polyphyletic. Contents of groups such as Bufonoidea and Ranoidea are in a constant state of flux because of the addition and exclusion of families, such as Dendrobatidae (Ford, 1993; Ford & Cannatella, 1993). At the family level, the resolution of most phylogenetic trees is very poor, the relations between most clades being largely unresolved, while two of the major families (Leptodactylidae and Ranidae) are generally considered polyphyletic (Ford & Cannatella, 1993).
Reasons for this lack of resolution range from the limited utility of external morphology (Inger, 1967) to the great paucity of data for tropical lineages. Analyses using alternative data sets, such as molecular markers (de Sá & Hillis, 1990; Hillis et al., 1993 and Hay et al., 1995), have slowly added new insights to the problem but also have refuted well-established clades. Filling the gaps on existing data sets and exploring new kinds of characters are important ways to improve phylogenetic hypotheses among anurans (Ford, 1993).

The ultrastructure of spermatozoon has been used as an alternative data set to investigate the phylogeny of many taxa such as fishes (Jamieson, 1991 and Tanaka et al., 1995), amphibians (Lee and Jamieson, 1992 and Lee and Jamieson, 1993; Jamieson et al., 1993 and Scheltinga et al., 2001), reptiles (Jamieson, 1995; Teixeira et al., 1999a and Teixeira et al., 1999b), and invertebrates (Jamieson, 1987). An advantage of sperm ultrastructure data is that they provide more conservative characters for groups with highly derived body plans, such as Amphisbaenia, which cannot be scored for some traditional morphological traits (Teixeira et al., 1999b). Spermatozoon ultrastructure data have also been useful in clarifying relationships among Polyplacophora, where traditionally used characters are either too conserved or too variable (Buckland-Nicks, 1995). Spermatozoon morphology, therefore, seems to be useful for groups where, for some reason, external morphology cannot be scored, either because of evolutionary conservativeness (as in some traits of Polyplacophora) or specialization (as for Amphisbaenia).

Some conjectures on anuran phylogeny have been made based upon spermatozoon ultrastructure and the cladistic significance of some characters has been investigated. For example, the conical perforatorium has been proposed as a tentative synapomorphy of Bufonoidea (Lee & Jamieson, 1993), whereas the presence of an undulating membrane or a rod-shaped perforatorium have been scored as symplesiomorphies of Anura. Yet, due to the paucity of data on spermatozoon morphology for several families, no comprehensive cladistic analysis of sperm ultrastructure data, such as those made for squamate reptiles (Jamieson, 1995 and Teixeira et al., 1999b) and fishes (Tanaka et al., 1995), has been conducted for anurans (but see Scheltinga et al., 2001).

The ultrastructure of sperm can therefore provide an alternative data set for the phylogenetic analysis of amphibians. Unfortunately, until now only about half of the families of Anura have had the spermatozoa of at least one species described (Kwon & Lee, 1995; Jamieson, 1999) and several characters mentioned in the literature are poorly defined. Consequently, there is a need both for the detailed description of the sperm ultrastructure in families not yet studied and for the continued data accumulation on families already studied. Herein we describe, for the first time, the ultrastructure of the spermatozoon of a member of
the family Dendrobatidae, Epipedobates flavopictus, from the central Brazilian Cerrado. Further, we discuss the evolutionary significance of the results regarding the evolution of the subacrosomal cone in anurans and the phylogenetic position of dendrobatid frogs.

Material and methods

We collected two individuals of E. flavopictus on September 1998, at Minaçu, Goiás, Brazil (13°38′ S, 48°15′ W), and one individual on November 1995 at Serra do Cipó, Minas Gerais, Brazil, during the reproductive season. We killed animals by rubbing xylocaine onto the abdomen skin and deposited them at the Coleção Herpetológica da Universidade de Brasília (CHUNB 09581, 09582) and Museu de História Natural ‘Professor Adão José Cardoso’, Universidade Estadual de Campinas (ZUEC 11434). We removed testes by dissection and placed them in a Petri dish with phosphate buffer (PBS) pH 7.2 and saline solution. We cut testes into small pieces and fixed them overnight at 4 °C in a solution of 2.5% glutaraldehyde, 5 mM CaCl2, and 5% sucrose in 0.1 M sodium cacodylate buffer pH 7.2. After rinsing in sodium cacodylate buffer, we postfixed testes for 1 h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM CaCl2 in 0.1 M sodium cacodylate buffer pH 7.2, and left them overnight in a solution of 0.5% uranyl acetate for ‘in block’ contrast. We proceeded with dehydration in a series of ascending acetone (30–100%) and embedded the material in Spurr’s epoxy resin. We made ultrathin sections with glass and diamond knifes on a Leica Reichert ultramicrotome and stained the sections with uranyl acetate and lead citrate. We observed the sections in a Jeol® 100C transmission electron microscope and took micrographs at 80 kV. We also made light microscope observations of spermatozoa under interferential contrast using a Zeiss® Axiophot microscope.

Results

Under the light microscope, the spermatozoon is filiform, approximately 59 μm long, with a short tail (ca. 33 μm) when compared to the head region (Fig. 1A). The head is curved and the midpiece is very short and not clearly visible. An undulating membrane is distinguishable in the tail, and the axoneme is seen describing a very sinuous path along the axial fiber.
Fig. 1. Spermatozoa of *E. flavopictus*. (A) Light microscopy showing whole spermatozoon with head (h) and flagellum (f). ×1250. (B–K) Transmission electron micrographs of head and midpiece. (B) Longitudinal section of the head region showing the end of the acrosome vesicle (arrowheads). ×38 000. (C–F) Transverse sections of the head region showing the reduction of the acrosome and enlargement of the nucleus. ×116 000, ×100 000, ×94 000, ×54 000, respectively. (G & H) Transverse and longitudinal sections of the nucleus showing the lacunae (l) and inclusions (i). ×40 000, ×25 000, respectively. (I) Oblique section of the midpiece. Note the scattered distribution of mitochondria. ×50 000. (J & K) Longitudinal view of the midpiece at the level of the distal centriole and paraxonemal rod, respectively. ×28 000, both. Abbreviations: ac, acrosome vesicle; c, subacrosomal cone; dc, distal centriole; m, mitochondrion; n, nucleus; ns, nuclear space; pc, proximal centriole; pm, pericentriolar material; pr, paraxonemal rod. Scale bars: (A) 20 μm; (B & I) 0.2 μm; (C) 0.05 μm; (D & E) 0.1 μm; (F & G) 0.3 μm; (H, J & K) 0.5 μm.
**Acrosome complex**

The acrosome of *E. flavopictus* sperm is located at the anterior portion of the head and is composed of a single and narrow vesicle, filled with a homogeneous material of low electron density (Fig. 1B–E). Under the acrosome vesicle, the subacrosomal cone forms a conical cap that reaches the anterior portion of the nucleus. In cross-section, the acrosome and subacrosomal cone are circular (Fig. 1C–F). The acrosome vesicle surrounds the anteriormost portion of the nucleus, below which the nucleus thickens and is enveloped only by the subacrosomal cone (Fig. 1B–F).

**Nucleus**

Below the nuclear envelope, a nuclear space that probably results from the condensation of chromatin process is seen (Fig. 1B, D & E). The nucleus is circular in transverse section (Fig. 1G) and conical in longitudinal section (Fig. 1B). The anterior portion of the nucleus is enveloped by the acrosome complex and gradually tapers (nuclear shoulders absent) to a point within the subacrosomal cone (Fig. 1E & F). The chromatin is highly condensed and electron-dense. Despite the high degree of condensation, several small electron-lucent nuclear lacunae and inclusions are seen (Fig. 1G & H).

**Midpiece**

The midpiece is the transitional region between the spermatozoon head and tail, containing the nuclear fossa, proximal and distal centrioles, axoneme, mitochondria, and paraxonomal rod (sensu Jamieson et al., 1993) (Fig. 1J). The proximal centriole is seen in Figure 1I inside the nuclear fossa and surrounded by pericentriolar material. The posteriormost portion of the nucleus is curved, forming a deep nuclear fossa that completely surrounds the proximal centriole and the pericentriolar material (Fig. 1E and Fig. 2). The paraxonomal rod inserts into the nuclear fossa, reaching the proximal centriole, and is embedded in the pericentriolar material (Fig. 2A). The proximal centriole lies at an approximate angle of 50° with respect to the longitudinal axis of the spermatozoon (Fig. 1K). Numerous round mitochondria are seen scattered in the midpiece (Fig. 1 and Fig. 2). They completely surround the posterior region of the nucleus and extend into the anterior portion of the tail (Fig. 1 and Fig. 2).
Tail

In transverse section, the tail of E. flavopictus sperm consists of the axoneme, undulating membrane and axial fiber (Fig. 2B). In transverse section the axial fiber is curved or U-shaped and apparently of the same composition of an electron-dense structure that supports the undulating membrane, to which it is connected. Since other structures are found inside the undulating membrane of E. flavopictus, such as cytoplasm and mitochondria, it is necessary to name this structure to which the axial fiber is connected, hereafter called ‘axial sheath’. It is formally defined as the connection between the axial fiber and the axoneme (or juxta-axonemal fiber, when it is present). In E. flavopictus the axial sheath is directly connected to the axoneme through the doublet 3, without a juxta-axonemal fiber (Fig. 2B & C).

Mitochondria are observed inside the undulating membrane in the anterior portion of the tail (Fig. 2B). At the end of the tail, the undulating membrane no longer contains mitochondria and the plasma membrane is completely adhered to the axial fiber and axial sheath. In Figure 3, the intermediate piece of a spermatid is seen. Contrary to the condition
seen in the mature spermatozoon, the mitochondria are organized in a mitochondrial collar around the flagellum.

Fig. 3. Section of spermatid showing presence of mitochondrial collar in the early development of the sperm. ×40 300. Abbreviations: af, axial fiber; ax, axoneme; m, mitochondrion; n, nucleus. Scale bar: 0.3 μm.
**Discussion**

The basic structure of the spermatozoon of *E. flavopictus* (Fig. 4) is similar to that of most neobatrachians (Ford, 1993) described to date, such as *Bufo arenarum* (Burgos & Fawcett, 1956), *B. marinus* (Swan et al., 1980; Lee & Jamieson, 1993), *Melanophryniscus cambaraensis* (Báo et al., 2001), *Odontophrynus cultripes* (Báo et al., 1991), *Lepidobatrachus laevis* (Waggener & Carroll, 1998), and *Pachymedusa dacnicolor* (Rastogi et al., 1988). However, it possesses several traits not seen in these other species and which may possibly be shared with other dendrobatids.

*Epipedobates flavopictus* has a subacrosomal cone below the acrosomal vesicle. We advance that this is homologous with the subacrosomal cone of *Ascaphus truei* and the conical perforatorium of bufonoids, contrary to the proposition of Lee and Jamieson, 1992 and Lee and Jamieson, 1993 and Jamieson et al. (1993). These authors indicated that the subacrosomal cone seen in *A. truei*, urodeles, and basal amniotes is absent in all other anurans they examined and that in bufonoids a similar structure, the conical perforatorium, lies beneath the acrosome vesicle. Further, they provide four reasons why the conical perforatorium is not homologous with the subacrosomal cone of *A. truei*: (1) a rod-shaped endonuclear perforatorium exists in *A. truei*; (2) the close proximity of the subacrosomal cone, in its posterior region, to the nucleus and acrosome vesicle in *A. truei*, whereas in bufonoids the conical perforatorium lies free in the subacrosomal space; (3) the homogeneous nature of the subacrosomal material in *A. truei*, whereas loose bundles of coarse fibers running parallel to the nucleus are seen in bufonoids; and (4) the subacrosomal cone in *A. truei* is less electron-dense than the acrosome vesicle, while in bufonoids the conical perforatorium is more electron-dense than the acrosome vesicle. Later, Jamieson (1999) suggested that the conical perforatorium may be homologous with the subacrosomal cone, but provided no rationale to his proposition.

We regard, like James (1970) and Jamieson (1999), that the conical perforatorium and the subacrosomal cone are homologous based on what follows. First, argument (1) above is a syllogism: bufonoids have a conical extranuclear perforatorium whereas *A. truei* has a rod-like, endonuclear perforatorium; hence, since the function of supporting the spermatozoon head is performed by the perforatorium, the subacrosomal cone of *A. truei* cannot be homologous with the conical perforatorium of bufonoids. Similarity of function, however, is not a requirement for homology (Lauder, 1994). Lee and Jamieson, 1992 and Lee and Jamieson, 1993 and Jamieson et al. (1993) were probably influenced by the earlier work of Burgos and Fawcett (1956), the first to suggest that the coarse strands of dense material, observed around
the tapering end of the nucleus in the spermatozoon of B. arenarum, form a perforatorium. Had Burgos and Fawcett (1956) chosen a different name (without implying a function) for the same structure, say ‘subacrosomal cone’, argument (1) above would vanish.

Fig. 4. Diagrammatic representation of the spermatozoon of the dendrobatid frog E. flavopictus.
Second, arguments (2)–(4) above are not independent. The more detached aspect of the presumed conical perforatorium relative to the nucleus and acrosome vesicle, and its higher electron density in bufonoids are a direct consequence of its fibrous arrangement. Moreover, in the bufonoids Myxophyes fasciolatus (Lee & Jamieson, 1992, Fig. 3E), O. cultripes [Báo et al., 1991, Figs 12 & 13, mislabeled as acrosome (A)], and M. cambaraensis (Báo et al., 2001, Fig. 4) the presumed conical perforatorium is much more homogeneous, forming an almost continuous layer in transverse section around the nucleus, with no coarse fibers being observed. We regard this condition as intermediate between that found in A. truei and the state typical of most bufonoids.

Finally, if the view of Lee and Jamieson, 1992 and Lee and Jamieson, 1993 and Jamieson et al. (1993) is correct, six steps would be required during the evolution of anurans, when mapping the characters conical perforatorium and subacrosomal cone onto a current phylogeny of the group (Hay et al., 1995) (Fig. 5). According to this view, the conical perforatorium was absent in the common ancestor of anurans and salamanders and evolved once in the ‘bufonoid’ lineage (Fig. 5A). Furthermore, the subacrosomal cone was originally absent in anurans and evolved independently twice in the group (Fig. 5B). Conversely, if James (1970) was correct in regarding the conical perforatorium of bufonoids as homologous with the subacrosomal cone of A. truei, then only four evolutionary steps are required and the presence of the subacrosomal cone would be plesiomorphic for bufonoids (Fig. 5C). If inferring genealogy rests on the principle of parsimony, i.e. choosing genealogical hypothesis that minimize requirements for homoplasies (Farris, 1982), then James’ view is to be preferred.

The acceptance of the hypothesis proposed by James (1970) implies that the condition seen in the acrosome of Bombina (Furieri, 1975), Discoglossus (Sandoz, 1975), and Xenopus (Bernardini et al., 1986), where the subacrosomal cone is absent, is not intermediate between A. truei and bufonoids as suggested by Lee and Jamieson (1993, Fig. 7). In addition, the proposition made by Lee and Jamieson (1993) and Jamieson (1999) that the conical perforatorium is a synapomorphy of bufonoids is not supported. Instead, the replacement of a perforatorial rod (as in Ascaphus and basal amniotes) with a modified fibrillar subacrosomal cone is a bufonoid synapomorphy.

The nucleus is highly compact in mature spermatozoa of E. flavopictus, with nuclear lacunae and inclusions being frequently observed. The nuclear lacunae are probably formed during the condensation of chromatin. They are typically electron-lucent, with no material inside, and are usually of small diameter, as seen in A. truei (Jamieson et al., 1993). Nuclear inclusions contain a clear but not completely electron-lucent substance and are usually bigger than lacunae, as observed in Rana clamitans (Poirier & Spink, 1971).
Fig. 5. Reconstruction of the evolution of sperm ultrastructure characters of E. flavopictus. Phylogeny of anurans from Hay et al. (1995). (A) Evolution of conical perforatorium, according to Jamieson et al. (1993) and Lee and Jamieson (1993), number of steps=4. (B) Evolution of subacrosomal cone, according to Jamieson et al. (1993) and Lee and Jamieson (1993), number of steps=2. (C) Preferred hypothesis for the evolution of the subacrosomal cone, when considering the conical perforatorium homologous to the subacrosomal cone in anurans, number of steps=4. Data for families used in the analysis was obtain from the present work (Dendrobatidae) and from the following literature: Caudata (Selmi et al., 1997), Pipidae (Reed & Stanley, 1972; Bernardini et al., 1986), Bombina (Furieri, 1975 and Pugin-Rios, 1980), Discoglossidae (Pugin-Rios, 1980; Lee & Kwon, 1996), Ascaphus (James, 1970 and Jamieson et al., 1993), Leioptelmatidae (Scheltinga et al., 2001), Pelobatidae (James, 1970; Asa & Phillips, 1988), Pelodytidae (Pugin-Rios, 1980), Ranidae (Poirier & Spink, 1971; Pugin-Rios, 1980), Microhylidae (Chiasmocleis albopunctata, personal observation), Myobatrachidae (Lee & Jamieson, 1992), Leptodactylidae (Pugin-Rios, 1980 and Bao et al., 1991; Waggener & Carroll, 1998; Amara et al., 1999), Rhinodermatidae (Pugin-Rios, 1980), Bufonidae (Burgos & Fawcett, 1956; Lee & Jamieson, 1993; Bao et al., 2001), and Hylidae (Rastogi et al., 1988; Lee & Kwon, 1992; Lee & Jamieson, 1993).
The numerous and randomly distributed mitochondria distinguish E. flavopictus from several other Neobatrachia so far examined, which usually have a mitochondrial collar, as in Bufonidae (Burgos & Fawcett, 1956; Swan et al., 1980; Lee & Jamieson, 1993) and Leptodactylidae (Pugin-Rios, 1980 and Bao et al., 1991). The presence of mitochondria creates a large separation between the axial sheath and the plasma membrane in the anterior tail region of E. flavopictus. In all other amphibians with an undulating membrane, the plasma membrane is closely adhered to the axial sheath. Interestingly, early spermatids of E. flavopictus have a mitochondrial collar detached from the midpiece (Fig. 3), as in Bufonidae (Burgos & Fawcett, 1956; Swan et al., 1980; Lee & Jamieson, 1993).

The reduction of the mitochondrial collar during the spermiogenesis, the absence of a juxta-axonemal fiber, and the somewhat degenerated structure of the axial fiber in E. flavopictus (Fig. 2B) agrees with the proposition made by Lee and Jamieson (1993) that a reduction in complexity is a major trend in the evolution of anuran spermatozoa.

Our results suggest that the family Dendrobatidae should be placed within the ‘bufonoid’ lineage, as proposed by several authors (Hedges & Maxson, 1993; Hillis et al., 1993 and Hay et al., 1995). The acrosome structure resembles that seen in Leptodactylidae (Pugin-Rios, 1980, Amaral et al., 1999 and Bao et al., 2001), Bufonidae (Burgos & Fawcett, 1956; Rastogi et al., 1988; Lee & Jamieson, 1993; Meyer et al., 1997), and Myobatrachidae (Lee & Jamieson, 1992). All of these families share a plesiomorphic condition of the acrosome, where a subacrosomal cone lies below the conical acrosomal vesicle, as in the archaeobatrachian A. truei (Jamieson et al., 1993) and some Urodeles (Picheral, 1967). This feature differs markedly from the condition seen in ranoids such as Ranidae (Poirier & Spink, 1971; Pugin-Rios, 1980 and Yoshizaki, 1987) and Rhacophoridae (Mainoya, 1981, Mizuhira et al., 1986 and Jamieson, 1999), where the acrosome vesicle sits on top of the nucleus and the subacrosomal cone is absent. Similarly, the subacrosomal cone is also absent in some archaeobatrachians, such as Pelobatidae (James, 1970) and Pipidae (Reed & Stanley, 1972; Bernardini et al., 1986). Furthermore, despite some peculiarities, such as the shape of the axial fiber and the absence of a juxta-axonemal fiber, the tail of E. flavopictus is similar to that generally observed in bufonoids, where an axial fiber is connected to the axoneme through an axial sheath inside an undulating membrane. All ranoids so far studied (Ranidae, Rhacophoridae, and Microhylidae) possess a tail with only the axoneme.

The significance of anuran sperm ultrastructure needs to be evaluated under the scope of sound phylogenetic techniques. To do so, characters must be continuously evaluated and families yet to be studied (e.g. Sooglossidae, Centrolenidae, Rhinophrynidae, Mantellidae,
Hyperoliidae, and Brachycephalidae) must be investigated in order to build a consistent data set that enables parsimony analysis.

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