

Observations of spermiogenesis and epididymal sperm maturation in the rufous hare wallaby, *Lagorchestes hirsutus* (Metatheria, Mammalia)

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Abstract

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Acrosomal development in the early spermatid of the rufous hare wallaby shows evidence of formation of an acrosomal granule, similar to that found in eutherian mammals, the Phascolarctidae and Vombatidae. Unlike the other members of the Macropodidae so far examined, the acrosome of this species appears to be fully compacted at spermiation and extends evenly over 90% of the dorsal aspect of the nucleus. During spermiogenesis, the nucleus of the rufous hare wallaby spermatid showed evidence of uneven condensation of chromatin; this may also be related to the appearance of unusual nucleoplasm evaginations from the surface of the fully condensed spermatid. This study was unable to find evidence of the presence of Sertoli cell spurs or nuclear rotation during spermiogenesis in the rufous hare wallaby. The majority of spermatozoa immediately before spermiation had a nucleus that was essentially perpendicular to the long axis of the sperm tail. Nuclei of spermatozoa found in the process of being released or isolated in the lumen of the seminiferous tubule were rotated almost parallel to the long axis of the flagellum; complete parallel alignment occurred during epididymal maturation. At spermiation spermatozoa have characteristically small cytoplasmic remnants compared to those of other macropods. Unlike the majority of macropodid spermatozoa so far described, the spermatozoa of the rufous hare wallaby showed little evidence of morphological change during epididymal transit. There was no formation of a fibre network around the midpiece or of plasma membrane specializations in this region; the only notable change was a distinctive flattening of midpiece mitochondria and scalloping of the anterior mitochondrial sheath to accommodate the sperm head. Preliminary evidence from spermiogenesis and epididymal sperm maturation supports the classification of the rufous hare wallaby as a separate genus but also indicates that its higher taxonomic position may need to be re-evaluated.

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Introduction

Marsupial spermatozoa exhibit significant morphological change during epididymal maturation and transit; the diversity of which has been used to infer phylogenetic affinities

(Harding *et al.* 1979, 1982; Harding 1987; Temple-Smith 1987, 1994; Harding and Aplin 1990; Johnston *et al.* 1995). A recent investigation of mature spermatozoa of the cauda epididymidis of the rufous hare wallaby *Lagorchestes hirsutus* (Johnston *et al.* 2003) revealed apomorphies with respect to

sperm structure, particularly the relative dimensions and shape of the nucleus and midpiece, the distribution of the acrosome and an extremely unusual form of nucleoplasm evagination from the condensed nucleus *in vivo*. To determine the origin of these features in mature *L. hirsutus* spermatozoa, we investigated spermiogenesis and examined changes in sperm morphology from spermiation through epididymal transit.

Materials and Methods

Testicular tissue and epididymal spermatozoa were recovered during August from two sexually mature *Lagorchestes hirsutus* housed at Western Plains Zoo, Dubbo, New South Wales, a 3-year-old and an 11-year-old. The younger male was euthanased after sustaining severe physical trauma but was otherwise in healthy condition. The older male was euthanased following a prolonged period of idiopathic illness. Both animals were originally part of a central Australian captive population located at the Alice Springs Desert Park, Northern Territory. Testicular and epididymal tissues (caput and cauda) were recovered while the animals were under gaseous isoflurane (Forthane, Abbott Australasia Pty Ltd, Sydney) prior to euthanasia.

Testicular and epididymal tissue of the younger male was obtained for electron microscopy after the tissue had been prepared for cryopreservation at the Animal Gene Storage Resource Centre of Australia (Monash Institute of Reproduction and Development, Clayton, Australia). The cryopreservation protocol consisted of storing the reproductive tissue at 4 °C for 2 days prior to freezing in 10% glycerol in phosphate-buffered saline at 10 °C/min. Post-thaw cryopreserved epididymal tissue from this animal was only used to confirm the major ultrastructural features observed in the other male.

All tissue was prepared for standard transmission electron microscopy as previously described by Johnston *et al.* (1995). Briefly, all tissues were finely diced into 1–2-mm³ portions on dental wax and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 6% sucrose at 4 °C for 6 h. The material was then rinsed in 0.1 M phosphate buffer three times over 15 min and post-fixed for 80 min in phosphate-buffered 1% osmium tetroxide. Following post-fixation, the tissue was further rinsed in phosphate buffer (pH 7.2) and dehydrated through an ascending ethanol series before being infiltrated and embedded in Spurr's epoxy resin.

Thick and thin sections were cut with a diamond knife on an LKB 2128 UM IV ultramicrotome. Thin sections (50–80 nm thick) were collected on carbon stabilized, colloidal-coated, 200- μ m mesh copper grids, stained for 30 s in Reynold's lead citrate, rinsed in distilled water and stained for a further 2 min in lead citrate before final rinsing. Electron micrographs were taken on a Hitachi 300 electron microscope. Approximately 25 sections of each tissue type were examined by transmission electron microscopy. Thick sections (1 μ m) were stained with toluidine blue and examined

using conventional light microscopy under oil immersion; digital light micrographs of testicular spermatozoa at spermiation were taken using a Nikon 995 Coolpix camera attached to a Nikon E400 light microscope.

Results

Observations of spermiogenesis

The most conspicuous feature of the early round spermatid nucleus was the presence of a dense granule within the acrosomal vacuole (Fig. 1A); however, a proacrosomal granule and vacuole were not found in the sections examined. Further acrosomal development at the round spermatid stage prior to nuclear flattening resulted in the eventual contraction of the acrosomal vacuole to form a homogeneous, electron-dense structure over the dorsal spermatid surface (Fig. 1B,C). During dorsal–ventral nuclear flattening there was evidence of uneven stranded chromatin condensation (Fig. 1F). Approximately halfway through the nuclear flattening/condensation process, the acrosome and nucleus were still orientated symmetrically (Fig. 1E) so that the spermatid at this stage exhibited a characteristic T-shape; the flagellum was located centrally on the ventral surface of the nucleus attached at the implantation fossa and arranged perpendicularly to the long axis of the nucleus. By the time the nucleoplasm appeared fully condensed there was an even distribution of acrosomal matrix covering more than 90% of the dorsally flattened nucleus (Fig. 1G). There was also evidence in some condensed spermatids of nuclear evaginations protruding from the bulk of the nucleoplasm but within the confines of the nuclear membrane (Fig. 1D). These extensions of the nucleus have been previously described in cauda epididymal spermatozoa (Johnston *et al.* 2003). Some of these nucleoplasm extensions ultimately separate from the nucleus and can be found as distinct vesicles of nucleoplasm in both caput (Fig. 2B) and cauda spermatozoa (Fig. 2C). The acrosome of some spermatozoa in the latter stages of spermiogenesis also contained electron-lucent vesicles; the significance of these structures to the final compaction of the acrosome is yet to be determined. Surprisingly, at no stage of spermiogenesis were Sertoli cell spurs identified nor was there evidence of nuclear rotation.

The sperm nucleus immediately prior to spermiation was perpendicular or obtusely oblique to the long axis of the flagellum (Fig. 2A). However, all spermatozoa were released into the lumen of the seminiferous tubule and were subsequently found in the caput epididymis with their sperm nucleus aligned essentially parallel to the flagellum with only minimal amounts of cytoplasmic remnant (Fig. 2B).

Changes in sperm morphology during epididymal transit

Paradoxically, the most notable feature of sperm maturation during epididymal transit in this species was the almost

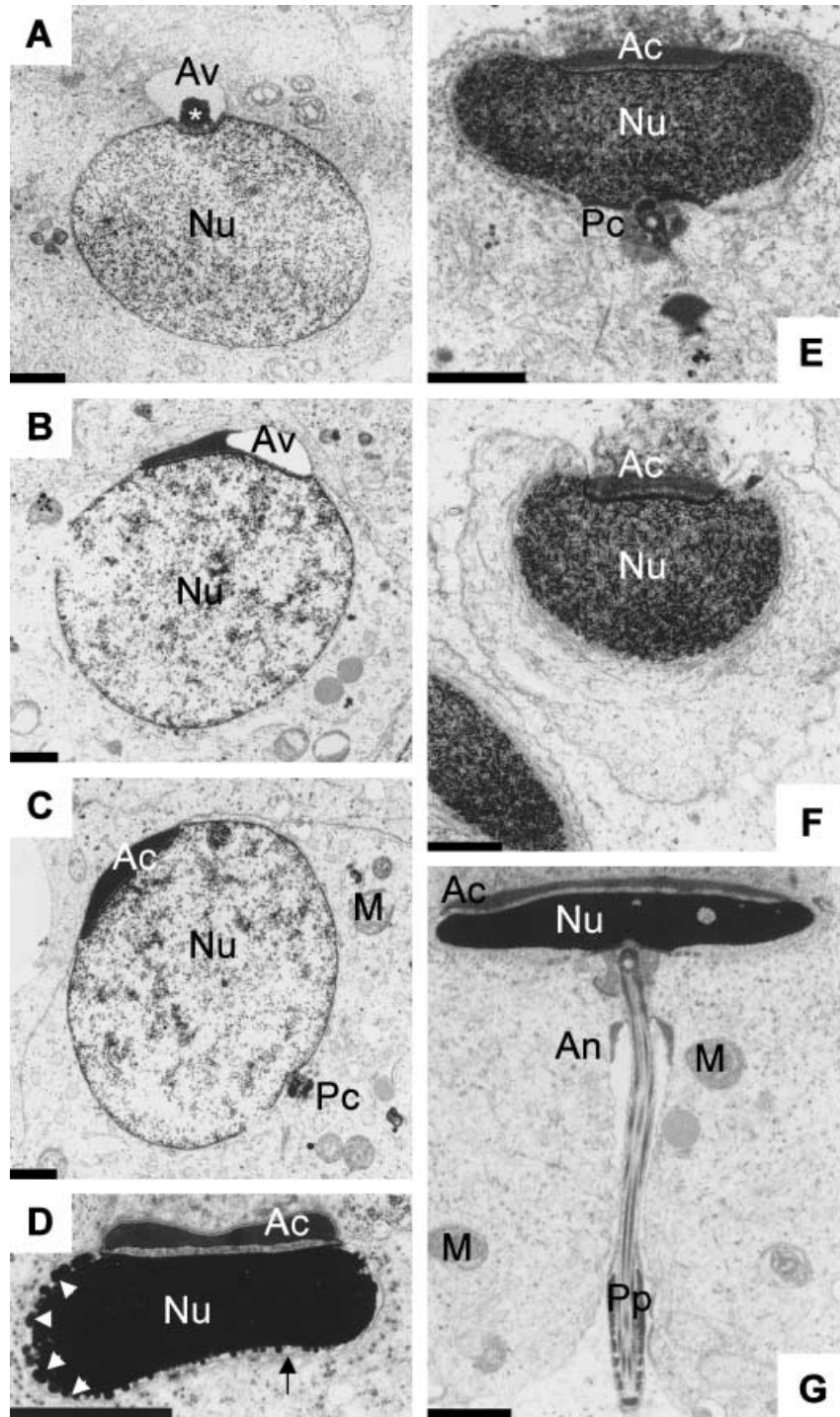


Fig. 1—Various stages of spermiogenesis in *Lagorchestes hirsutus*; —**A**. Early spermatid showing distinctive acrosomal vacuole and granule; —**B**. Early spermatid showing acrosomal vacuole in the process of collapsing and formation of the acrosomal matrix; —**C**. Early spermatid showing the acrosome over the dorsal pole of the nucleus – note the proximal centriole has shifted to the ventral pole of the spermatid; —**D**. Transverse section of fully condensed nucleus of maturing spermatid depicting evaginations (arrow heads) extending from the bulk of the nucleoplasm but within the confines of the nuclear membrane (black arrow); —**E**. Condensing spermatid showing evidence of symmetrical nuclear flattening and uneven nuclear condensation; —**F**. Condensing spermatid nucleus with distinctive chromatin strands; —**G**. Fully condensed nucleus and compacted acrosome of a late T-shape spermatid – note how the acrosome extends evenly over 90% of the dorsal surface of the nucleus. Asterisk indicates acrosomal granule; Ac, acrosome; Av, acrosomal vacuole; An, annulus; M, mitochondria; Nu, nucleus; Pc, proximal centriole; Pp, principal piece.

complete lack of obvious morphological change between spermated spermatozoa and those found in the cauda epididymides (Fig. 2B). While the plasma membrane overlying the ventral aspect of the nucleus of some caput epididymal spermatozoa appeared slightly elevated, there was little evidence of any cytoplasmic remnant (excess endoplasmic

reticulum, etc.) typical of that found in droplets of other macropodid spermatozoa. The anterior terminal portion of the axoneme of some caput spermatozoa when viewed in longitudinal section was slightly recurved so as to allow the nucleus to take on a parallel orientation to the axoneme (Fig. 2B).

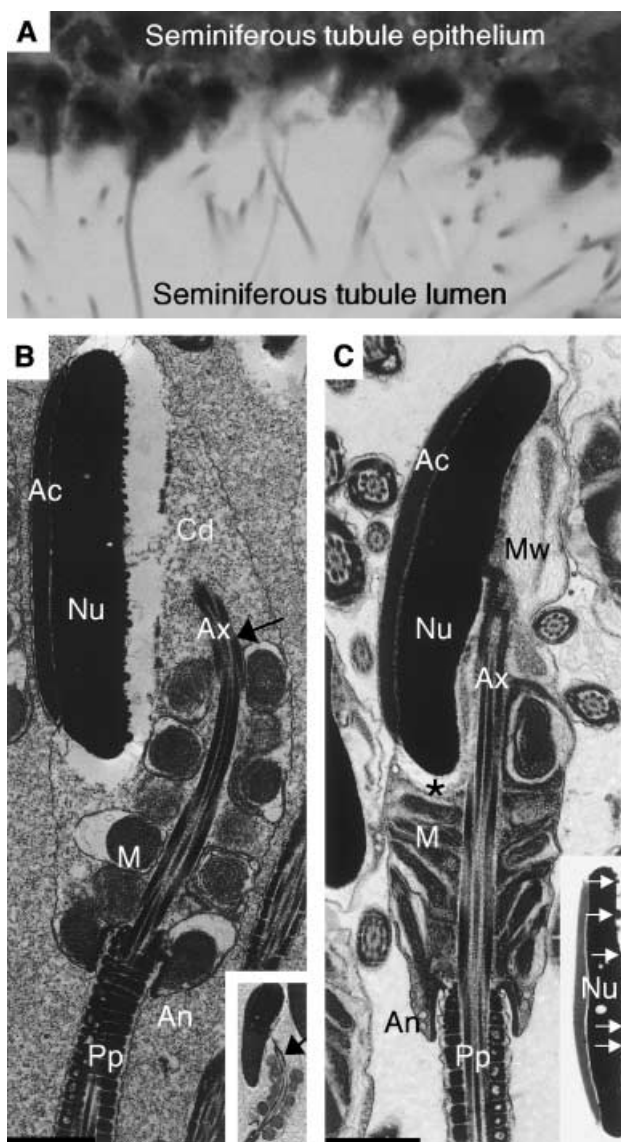


Fig. 2—*Lagorchestes hirsutus* testicular, caput and cauda epididymal spermatozoa; —A. ‘Thick Spurr’s resin section’ showing T-shaped orientation of the sperm head immediately prior to spermiation – as sperm are released into the lumen the head rotates to a parallel orientation with respect to the flagellum; —B. LS of caput epididymal spermatozoon [Insert – caput epididymal spermatozoa showing recurved portion of the distal axoneme (black arrows) to accommodate the parallel orientated sperm head – as the sperm matures during epididymal transit, the anterior mitochondrial sheath is transformed or ‘scalloped’ to further accommodate the nucleus; consequently, the axoneme straightens]; —C. LS of cauda epididymal spermatozoon [Insert – nucleus of cauda epididymal sperm showing evaginations of nucleoplasm (white arrows)]; Ac, acrosome; An, annulus; Ax, axoneme; Cd, cytoplasmic droplet; M, mitochondria; Mw, membranous whorls; Nu, nucleus; Pc, proximal centriole; Pp, principal piece; asterisk indicates scalloping of the anterior midpiece to accommodate the nucleus.

The most distinctive maturational event in the ultrastructural appearance of *L. hirsutus* spermatozoa was a change in the shape and structure of the mitochondria of the sperm midpiece. Mitochondria of caput and corpus epididymal spermatozoa appeared as large structures with circular profiles and distinctive concentric cristae (Fig. 2A), whereas the mitochondria of cauda epididymal spermatozoa were typically flattened, vacuolated, showed poor definition of cristal arrangement and appeared morphologically degenerative (Fig. 2B).

Cauda epididymal spermatozoa also showed no evidence of the presence of a helically arranged fibre network around the midpiece region; nor was there any suggestion of any plasma membrane specialization in this region. However, the mature cauda spermatozoa did possess what appeared to be membranous whorls (Fig. 2C) consisting of a series of relatively electron-lucent laminated membranes below the anterior portion of the ventral nucleus.

Discussion

Spermiogenesis in the Macropodidae has recently been thoroughly described in the Tamar Wallaby (*Macropus eugenii*) (Lin *et al.* 1997). These authors observed the formation of testicular spermatozoa from round spermatids, describing the process in 14 morphologically distinctive steps. Their reconstruction of spermiogenesis in *M. eugenii* will be used here as a framework on which *L. hirsutus* spermiogenesis shall be compared and contrasted.

Observations from the current study indicated that acrosome morphogenesis involved the formation of a distinctive acrosomal granule within an acrosomal vacuole; a characteristic which *L. hirsutus* also appears to share with the Phascolarctidae, Vombatidae and the majority of eutherian spermatozoa so far examined (Harding and Aplin 1990). Acrosome formation in *M. eugenii*, and in the majority of marsupials so far investigated, does not involve formation of an acrosomal granule; thus, within the Macropodidae, this character is apomorphic for *L. hirsutus*. Recent unpublished observations (Lloyd, Carrick and Hall) have indicated that acrosomal formation in *Hypsiprymmond moschatus* may also involve the formation of acrosomal granules, so that it is possible that both species represent an early divergence from the main phylogenetic branch of kangaroo evolution. Given the presence of an acrosomal granule, it is likely that a proacrosomal granule and vacuole would also be present, although they were not identified in our preparations.

The acrosome of *M. eugenii* undergoes a complex series of morphological changes during spermiogenesis, resulting in the formation of a ‘scoop’-shaped appendage on the dorsal nucleus at spermiation (Lin *et al.* 1997); this structure is then compacted during epididymal transit to form the characteristic button-shaped acrosome of the mature spermatozoa. For a detailed description of acrosomal compaction in *M. eugenii* see Setiadi *et al.* (1997). By contrast, acrosomal

formation in *L. hirsutus* is simple and the acrosome is fully compacted at the time of spermiation; this is a radical departure from the situation found in other Macropodidae, Phalangeridae and some Petauridae and clearly represents another apomorphic trait for *L. hirsutus*.

Another characteristic feature of *L. hirsutus* spermiogenesis noted in the present study was the relatively uneven condensation of the nucleoplasm. Some sections revealed the presence of stranded or fibred chromatin in the condensing spermatid nucleus. Within the Metatheria, uneven chromatin formation has been observed in the Phascolarctidae, Vombatidae and Pseudocheiridae (Harding and Aplin 1990). Interestingly, Harding and Aplin (1990) also refer to chromatin in the spermatids of reptiles forming longitudinally or helically arranged electron-dense fibres.

Yet another feature of macropodid spermiogenesis is the asymmetrical flattening of the nucleus to give the typical 'kangaroo sperm' head appearance with a large anterior portion and a narrow, tapered posterior. In contrast to other macropodids, nuclear flattening in *L. hirsutus* was essentially symmetrical, resulting in the formation of the distinctive head morphology for this species (Cleland 1969 cited in Rodger 1978; Johnston *et al.* 2003) and a T-shaped orientation of the nucleus to the flagellum. This type of nuclear flattening process also resulted in the formation of an evenly thickened acrosome over 90–95% of the dorsal nuclear surface. While the acrosome of the mature *H. moschatus* spermatozoa also covers most of the dorsal surface of the nucleus, the bulk of the acrosomal content is located at the anterior region in a typical macropodid arrangement (Lloyd *et al.* 2002). Hence, *L. hirsutus* nuclear shape, acrosome formation and the presence of stranded nuclear chromatin are all likely spermatozoal apomorphies.

A unique feature of marsupial spermiogenesis is the formation of Sertoli cell spurs (Sapsford *et al.* 1969). These, when fully developed in *M. eugenii*, form a sheet of electron-dense material that engulfs part of the Sertoli cell cytoplasm in the region of the acrosome (Lin *et al.* 1997). Thorough observations of thin and thick transmission electron microscopy sections of *L. hirsutus* testis examined in this study were unable to identify Sertoli cell spurs during spermiogenesis; perhaps lack of Sertoli cell spurs in *L. hirsutus* is related to the unique formation of the acrosome in this species.

In a study of mature cauda epididymal *L. hirsutus* spermatozoa, Johnston *et al.* (2003) noted the presence of nuclear evaginations; similar nucleoplasmic extensions were also found in maturing spermatids and caput epididymal spermatozoa. The significance of this phenomenon is uncertain but there may be a possible association with uneven nuclear condensation found in *L. hirsutus* spermatids. While it is possible that these nuclear evaginations may be an artefact of tissue processing, this seems unlikely, as they have been observed associated with appropriately fixed and intact nuclear membranes. Nuclear evaginations observed in *L. hirsutus* are structurally different to indentations of the peripheral ventral

extremities of the Dasyurid sperm nucleus described by Harding *et al.* (1979); there is a need to obtain further testis and epididymal tissue from other congeneric species to confirm this observation. Perhaps, as Harding *et al.* (1979) suggests, nuclear evagination is actually associated with the shedding of excess nucleoplasm after condensation. These extensions of nucleoplasm presumably separate from the nucleus as the spermatozoa matures.

By the time the *M. eugenii* spermatozoa are ready to leave the testis, the sperm nucleus has rotated parallel to the long axis of the flagellum; in the process of the spermatozoa being released into the lumen of the seminiferous tubule they then rotate back to a T-shaped position. Hence *M. eugenii* spermatozoa undergo two separate rotations of the nucleus during spermiogenesis (Lin *et al.* 1997). The spermatozoa of *L. hirsutus* show no evidence of nuclear rotation while in the spermatid and immediately before spermiation are in a T-shaped or slightly oblique orientation with respect to their sperm tail. As spermatozoa are released from their Sertoli cell attachments into the lumen of the seminiferous tubule, all sperm heads are rotated parallel to their long axis. Therefore, the relative orientation of the *L. hirsutus* sperm head at spermiation is diametrically opposite to that found in *M. eugenii*. Testicular spermatozoa of *L. hirsutus* also differ from those of *M. eugenii* in possessing only a small remnant of cytoplasm; by the time the spermatozoa enters the caput epididymis there is no obvious cytoplasmic droplet.

Sperm maturational changes in the Macropodoidea have been summarized by Harding *et al.* (1979), Harding (1987), Jones (1989) and Setiadi *et al.* (1997); apart from biochemical changes, macropodid sperm maturation includes a range of structural modifications, such as rotation of the sperm head from a T-shape orientation to a more hydrodynamic position parallel along the axis of the flagellum, acrosomal compaction, the development of a midpiece fibre network and the formation of membranous whorls around the neck of the spermatozoon. Despite *L. hirsutus* being placed within the Macropodoidea, spermatozoa of this species undergo only one of the maturational changes typical of this taxon. In this respect, they are also dissimilar to the spermatozoa of all other Australian marsupials so far examined except the Phascolarctidae (Harding *et al.* 1979).

The most distinctive maturational change in the spermatozoa of *L. hirsutus* occurs in the morphology of the midpiece, as the concentric circular mitochondria become flattened and condensed in appearance with an associated increase in electron density of the cristae. While *Trichosurus vulpecula* (Brush-tail Possum) and *Pseudocheirus peregrinus* (Common Ringtail Possum) spermatozoa also show increased electron density of their mitochondria during epididymal maturation (Harding *et al.* 1979), there has been no documented evidence of mitochondrial flattening during epididymal transit. The severe degree of mitochondrial flattening in this species is likely to be associated with a characteristic scalloping of the anterior mitochondrial sheath; modification of

the sheath allows the sperm head to take a more streamlined hydrodynamic position relative to the flagellum and is likely to be a direct consequence of the *L. hirsutus* spermatozoa not possessing a nuclear groove along its ventral surface.

Observations of mature cauda *L. hirsutus* presented in this study revealed a series of fine electron-dense membranes against the anterior ventral nucleus; similar structures in other mature macropodid spermatozoa have been noted as membranous whorls (Harding 1987; Taggart *et al.* 1995). This structure was not reported in recent observations of mature *L. hirsutus* spermatozoa (Johnston *et al.* 2003).

Although further cladistical analysis and validation are necessary, the observations of spermiogenesis and epididymal sperm maturation described in this paper, appear to support the classification of *L. hirsutus*, not only as a separate genus but possibly as a distinct evolutionary lineage within the Macropodidae (Rodger 1978). However, these observations are not consistent with current conventional understanding of Macropodid phylogeny, which has primarily been determined using a combination of dental, skeletal and molecular evidence (Flannery 1989; Burk and Springer 2000).

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