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The seminiferous epithelial cycle and microanatomy of the koala (*Phascolarctos cinereus*) and southern hairy-nosed wombat (*Lasiorhinus latifrons*) testis

Motoharu Oishi,[1] Mei Takahashi,[1,2] Hajime Amasaki,[1] Tina Janssen[3] and Stephen D. Johnston[4]

[1]*Department of Veterinary Anatomy, School of Veterinary Medicine, Nippon Veterinary and Life Science University, Tokyo, Japan*
[2]*Morioka Zoological Park, Morioka, Japan*
[3]*Australian Animals Care and Education, Mt Larcom, Qld, Australia*
[4]*Wildife Biology Unit, School of Agriculture and Food Science, The University of Queensland, Gatton, Qld, Australia*

Abstract

The koala (*Phascolarctos cinereus*) and southern hairy-nosed wombat (*Lasiorhinus latifrons*) are iconic Australian fauna that share a close phylogenetic relationship but there are currently no comparative studies of the seminiferous epithelial cell or testicular microanatomy of either species. Koala and wombat spermatozoa are unusual for marsupials as they possess a curved stream-lined head and lateral neck insertion that superficially is similar to murid spermatozoa; the koala also contains Sertoli cells with crystalloid inclusions that closely resemble the Charcot–Bottcher crystalloids described in human Sertoli cells. Eighteen sexually mature koalas and four sexually mature southern hairy-nosed (SHN) wombats were examined to establish base-line data on quantitative testicular histology. Dynamics of the seminiferous epithelial cycle in the both species consisted of eight stages of cellular association similar to that described in other marsupials. Both species possessed a high proportion of the pre-meiotic (stages VIII, I – III; koala – 62.2 ± 1.7% and SHN wombat – 66.6 ± 2.4%) when compared with post-meiotic stages of the seminiferous cycle. The mean diameters of the seminiferous tubules found in the koalas and the SHN wombats were 227.8 ± 6.1 and 243.5 ± 3.9 μm, respectively. There were differences in testicular histology between the species including the koala possessing (i) a greater proportion of Leydig cells, (ii) larger Sertoli cell nuclei, (iii) crystalloids in the Sertoli cell cytoplasm, (iv) a distinctive acrosomal granule during spermiogenesis and (v) a highly eosinophilic acrosome. An understanding of the seminiferous epithelial cycle and microanatomy of testis is fundamental for documenting normal spermatogenesis and testicular architecture; recent evidence of orchitis and epididymitis associated with natural chlamydial infection in the koala suggest that this species might be useful as an experimental model for understanding *Chlamydia* induced testicular pathology in humans. Comparative spermatogenic data of closely related species can also potentially reflect evolutionary divergence and differences in reproductive strategies.

Key words: koala; seminiferous epithelium; southern hairy-nosed wombat.

Introduction

Spermatogenesis of mammals results in the timed sequence of cellular associations in the seminiferous epithelium resulting in repeated generations of germ cells surrounded by supporting Sertoli cells (Hess, 1999). This process can be separated into three phases; the mitotic or proliferative phase, the meiotic phase, and the spermiogenic phase, in which three types of germ cells (spermatogonia, spermatocytes and spermatids) are arranged in the seminiferous tubules and whereby their relative association can be characterized as different stages of the seminiferous epithelium cycle (Courot et al. 1970; Russell et al. 1990).

In marsupials, the main events of spermatogenesis are generally similar to those of eutherian mammals (Harding et al. 1979, 1982); however, the spermatids of most marsupials show unusual morphological changes during spermiogenesis and include: (i) a flattening of the nucleus at right angles to the long axis of the flagellum; (ii) nuclear rotation

Correspondence
Motoharu Oishi, Department of Veterinary Anatomy, School of Veterinary Medicine, Nippon Veterinary and Life Science University, 1-7-1 Kyonnan-cho, Musashino, Tokyo 180-8602, Japan.
E: oishi@nvlu.ac.jp

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about the point of attachment to the flagellum, possibly caused by invasions by Sertoli cell cytoplasm; and (iii) an incomplete coverage of the acrosome as cap over the spermatid nucleus, whereby it is restricted to a relatively small proportion of the dorsal nuclear surface (Harding et al. 1976, 1979; Tyndale-Biscoe & Renfree, 1987). The mode of nuclear flattening during spermiogenesis in both the koala and wombat follows the basic marsupial pattern except that the flagellar connection is laterally rather than centrally placed and as a result the neck insertion is disto-ventral rather than centro-ventral as in other marsupials (Harding & Aplin, 1990).

Although the koala and the wombat are categorized into the sub-order Vombatiformes, they are also thought to have had a long evolutionary separation (Kirsch et al. 1997; Osborne et al. 2002; Munemasa et al. 2006; Phillips & Pratt, 2008). Consequently, sperm production in the koala and wombat might be expected to exhibit convergent or divergent structural solutions with respect to spermiogenesis and characterisation of the various stages of the seminiferous epithelial cycle. Comparative studies of qualitative and quantitative evaluation of the testicular histology can potentially provide answers to important questions about testicular structure and function and may even reveal insights into differences of reproductive strategy, for example temporal changes and species differences in reproductive hormone secretion (Leydig cell) and sperm production.

Recently, Deif (2011) has reported the first description of orchitis and epididymitis associated with ascending chlamydiosis in the koala, with this organism being identified using histochemistry and PCRq. Deif (2011) has also reported the histopathology of these lesions and identified the organism in the tissues at the ultra structural level. An understanding of the histopathology of this infection requires a detailed comprehension of normal spermatogenesis and testicular cellular architecture. In addition, and given that chlamydiosis in the male koala is a natural infection and more widespread than previously thought (Deif, 2011), the koala may in fact be a useful model for studying the pathology of this organism in the human testis.

Although spermiogenesis in the koala and the wombat has been described at the electron microscopy level (Harding et al. 1987; Harding & Aplin, 1990), the kinetics of spermatogenesis has not yet been investigated. This study aimed to establish base-line data on the quantitative testicular histology of koala and southern hairy-nosed (SHN) wombat, with a focus on light microscopic characterisation of the seminiferous epithelial cycle and relative frequency of cellular associations. Our findings will be discussed in light of the close phylogenetic relationship between the koala and wombat, functional differences in testicular microanatomy between these species, and the potential use of the koala as a model for understanding *Chlamydia* mediated testicular histopathology in humans.

Materials and methods

Animals

Testes were collected from 18 sexually mature necropsied koalas (Queensland in August and September in from 1992 to 1994) and four sexually mature southern hairy-nosed wombats (South Australia in September in 2010). Koala testes were recovered from animals euthanized at the Moggill Koala Hospital (Environment Protection and Heritage, Queensland Government) and wombat testes were collected as part of population management program conducted under the authority of the South Australian Department of Environment and Heritage. Experimental procedures were approved by Animal Ethics Committee in the University of Queensland (SAS/261/10) and Nippon Veterinary and Life Science University (AEC1).

Tissue preparation

Following dissection, each testis was immediately placed in Bouin's fixative (Humason, 1977) after removing the top and bottom of the testis to facilitate penetration of the fixative. After an initial fixation of 2–3 h, testes were cut into about 5 mm thick slices and then re-fixed in Bouin's fixative overnight. Fixed tissues were transferred to 70% ethanol. For studies of testicular quantitative histology, six to eight tissue blocks were obtained from different sections of the right testis that were separated by at least 200 μm. Sections for histology were prepared using conventional histology and stained with haematoxylin-eosin staining (Humason, 1977). Histological preparations were viewed under standard light microscopy (Olympus BX51) for both quantitative measurements of microanatomy and photomicroscopy (Olympus DP2-BSW).

Quantitative histology

Random samples of 100 tubules from each testis were examined and measured using a light microscopy at 100× magnification with a calibrated micrometer eyepiece and high resolution photography using IMAGEJ 1.45 software (http://rsbweb.nih.gov/ij/). Transverse seminiferous tubule diameters were determined by taking two measurements at right angles to each other. The longitudinal and transverse lengths of the Sertoli cell nucleus were also measured in the same way using light microscopy at 200× magnification. Relative proportions of the various stages of the seminiferous epithelial cycle observed in this study were also determined by light microscopy and high resolution images (200× magnification) using photographic software (GIMP 2.6.12, http://www.gimp.org/) with a Weibel randomized grid system (Weibel, 1979); a total of 2000 points were made for each individual animal.

The epithelium of seminiferous tubules in marsupials has essentially the same cell types as those found in eutherian mammals, namely, the various germinal cells (spermatogonia, spermatocytes and spermatids) and Sertoli cells. Descriptions of the various stages of the seminiferous epithelial cycle in the present study were therefore partly based on observations of eutherian mammals (Courot et al. 1970; Russell et al. 1990) and other marsupials (Mason & Blackshaw, 1973; Setchell & Carrick, 1973; Orsi & Ferreira, 1978; Queiroz & Nogueira, 1992; Lin et al. 2004; Lloyd et al. 2008; Phillips et al. 2008). The relative frequency of each stage was calculated.

Statistical analysis

Mean seminiferous tubule diameters, mean longitudinal and transverse length of Sertoli cell nucleus, mean ratios of the seminiferous tubule and intersititial tissue, and mean stage ratios of the various stages of the seminiferous epithelium in the koala and wombat were compared with a nonparametric *U*-test ($P < 0.05$) using PASW statistics 18.0 (SPSS Japan Inc., Tokyo, Japan).

Results

Seminiferous tubule diameter and relative proportions of testicular tissue

The seminiferous tubule cross-sectional diameter and relative proportions of interstitial tissue in the koala and the SHN wombat are shown in Table 1. There were no significant differences in the seminiferous tubule diameter between the koala and the SHN wombat. The proportion of mean interstitial tissue in the koala was greater than that in the SHN wombat (Table 1, Fig. 1).

Stages of the seminiferous epithelial cell cycle

Eight stages of the seminiferous epithelial cycle in the koala and the SHN wombat as defined in terms of spermatid nuclear morphology (Fig. 2) and cellular associations were recognized and are summarized in Table 2 and Figs 3 and 4. Type-A spermatogonia possessed an ovoid nucleus, their chromatin was homogeneous and dust-like and contained a prominent nucleolus. Intermediate spermatogonia had a small ovoid nucleus equipped with a small amount of heterochromatin lying along the inner aspect of the nuclear membrane. Type-B spermatogonia possessed a rounded nucleus and contained a moderate amount of heterochromatin lying along the inner aspect of the nuclear membrane. Preleptotene spermatocytes appeared similar to Type-B spermatogonia except that they were slightly smaller. Preleptotene–leptotene transition nuclei had a speckled appearance. Leptotene spermatocytes contained a round nucleus with fine chromatin threads, whereas the zygotene spermatocytes had thicker chromosomes than those of leptotene spermatocytes. Pachytene spermatocytes had a larger nucleus and nucleolus, and their chromosomal material was more disperse. Secondary spermatocytes had a round nucleus and were larger in nuclear diameter than round spermatids. The nuclear morphology of rod-shaped spermatids was different from that of round spermatids, possessing an elongated and cylindrical nucleus. Condensed spermatids had condensed chromatin and a small spherical nucleus. Elongated spermatids were defined by a dorsal-ventral flatting of the nucleus and the long axis of the nucleus orientated perpendicular to the flagellum but characteristically possessed a neck insertion in the caudal region of the nucleus. As the spermatids developed during the

Table 1 Seminiferous tubule diameter (STD), longitudinal (SCLL) and transverse length (SCTL) of Sertoli cell nucleus (μm ± SEM), and mean proportions (% ± SEM) of the seminiferous tubule (ST) and intersititial tissue (IT) in the koala and the SHN wombat.

	STD (μm)	SCLL (μm)	SCTL (μm)	ST (%)	IT (%)
Koala	227.8 ± 6.09	23.4 ± 0.34*	15.8 ± 0.24*	67.3 ± 1.87	32.8 ± 1.87*
Wombat	243.5 ± 3.89	17.5 ± 0.18	11.4 ± 0.18	85.0 ± 0.92*	15.0 ± 0.92

Seminiferous tubule refers to all tissue within the seminiferous tubules including the lumen. Interstitial tissue includes Leydig cells, blood vessels, and connective tissue.
*Statistically significant larger value (nonparametric *U*-test; $P < 0.05$).

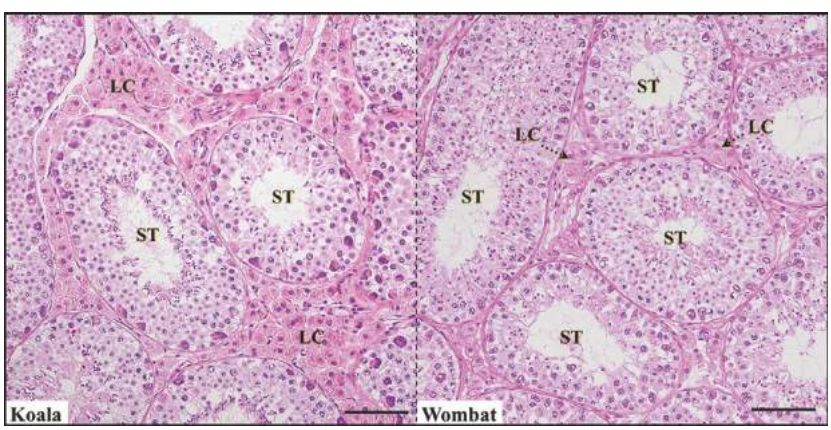

Fig. 1 The seminiferous tubule and interstitial tissue of the koala and the SHN wombat. LC, Leydig cell; ST, seminiferous tubule. Scale bar: 100 μm.

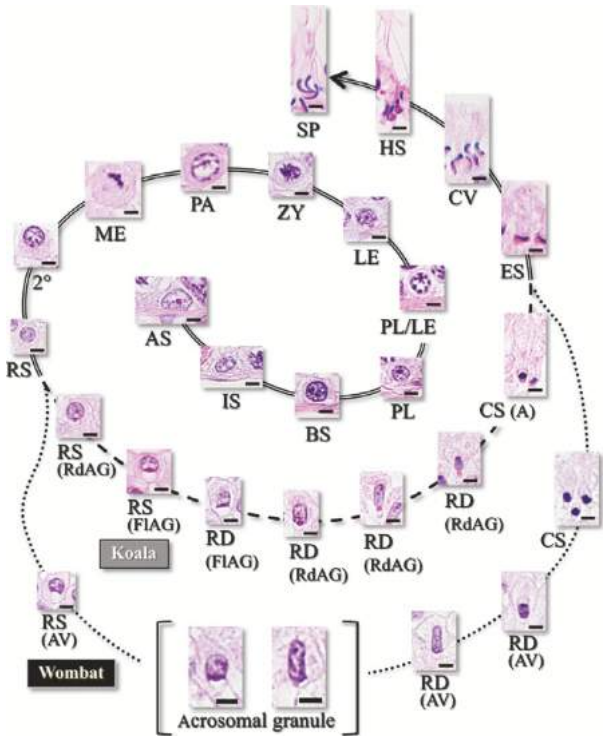

Fig. 2 Germ cells found in the koala and the SHN wombat seminiferous epithelium. AS, type-A spermatogonia; IS, intermediate spermatogonia; BS, type-B spermatogonia; PL, preleptotene spermatocyte; PL/LE, preleptotene-leptotene transition cell; LE, leptotene spermatocyte; ZY, zygotene spermatocyte; PA, pachytene spermatocyte; ME, meiosis; 2°, secondary spermatocyte; RS; round spermatid; RD, rod-shaped spermatid; CS, condensed spermatid; ES, elongated spermatid; CV, curved spermatid; HS, hook-shaped spermatid; SP, spermatozoon; RdAG, round acrosomal granule; FlAG, flattened acrosomal granule; A, well-developing acrosome. Each germ cell type is defined according to previous studies (Courot et al. 1970; Russell et al. 1990). Cellular morphological changes during spermatogenesis in the koala and the SHN wombat were similar (double line); however, round and rod-shaped spermatids in the koala possessed acrosomal granules (dashed line), whereas the SHN wombat did not (dotted line). Scale bar: 5 μm.

Table 2 Cell types and cellular associations of the eight stages of the seminiferous epithelial cycle in the koala and the SHN wombat.

	Stage of the seminiferous epithelial cycle								
Cell type	Ia	Ib	II	III	IV	V	VI	VII	VIII
Spermatogonia									
Type A	■	■	■	■	■	■	■	■	■
Intermediate					?	■	?		
Type B	■	■					■	■	■
Primary Spermatocyte									
Preleptotene	■	■							
PL/LE			■						
Leptotene				■	■	■			
Zygotene							■	■	■
Pachytene	■	■	■	■	■	■	■	■	■
Diplotene					?				
Meiosis					■				
Secondary Spermatocyte					■				
Spermatid									
Round	■				■	■	■	■	■
Rod-Shaped		■							
Condensed			■	■					
Elongated				■	■	■			
Curved						■	■		
Hooked							■	■	
Spermatozoon								■	■
Acrosomal vacuole	■	■					■	■	■
Acrosomal granule									
Round		K					K	K	
Flattened	K							K	K
Residual body								■	■

? = Intermediate spermatogonia and diplotene spermatocytes not yet positively identified in these stages. K = In the koala, the round and rod shaped spermatids possessed a distinctive eosinophilic acrosomal granule within its vacuole, whereas this granule was rarely found in the wombat spermatids.

later stage of spermiogenesis, the nucleus became progressively curved proximally, ultimately resulting in a hook or sickle-shaped mature sperm nucleus.

Stage I

The lumen of the early stage I tubule was lined with round spermatids in both species that elongated into rod-shaped nuclei prior to nuclear condensation. Round spermatids initially possessed a large round acrosomal vacuole that progressively collapsed during nuclear protrusion. The acrosomal granule in the koala spermatid during stage I was substantially more prominent and eosinophilic than that found in the SHN wombats. Outside the spermatids, there was a layer of pachytene spermatocytes and then a layer of Sertoli cells and ovoid type-A spermatogonia, which were present in all stages. Preleptotene spermatocytes lay adjacent to the basement membrane, as were preleptotene –leptotene transition cells.

Stage II

The spermatids lining the lumen of the tubule had condensed round nuclei that had not yet elongated. The nucleus of the koala spermatid possessed a highly eosinophilic acrosomal matrix that was not discernible in the wombat nucleus. Pachytene spermatocytes were present outside the spermatid nuclei and the preleptotene spermatocytes had now developed to leptotene spermatocytes adjacent to the basement membrane.

Stage III

The nuclei of the spermatids in stage III were dorso-ventrally flattened and elongated parallel to the basement membrane. Pachytene spermatocytes remained essentially

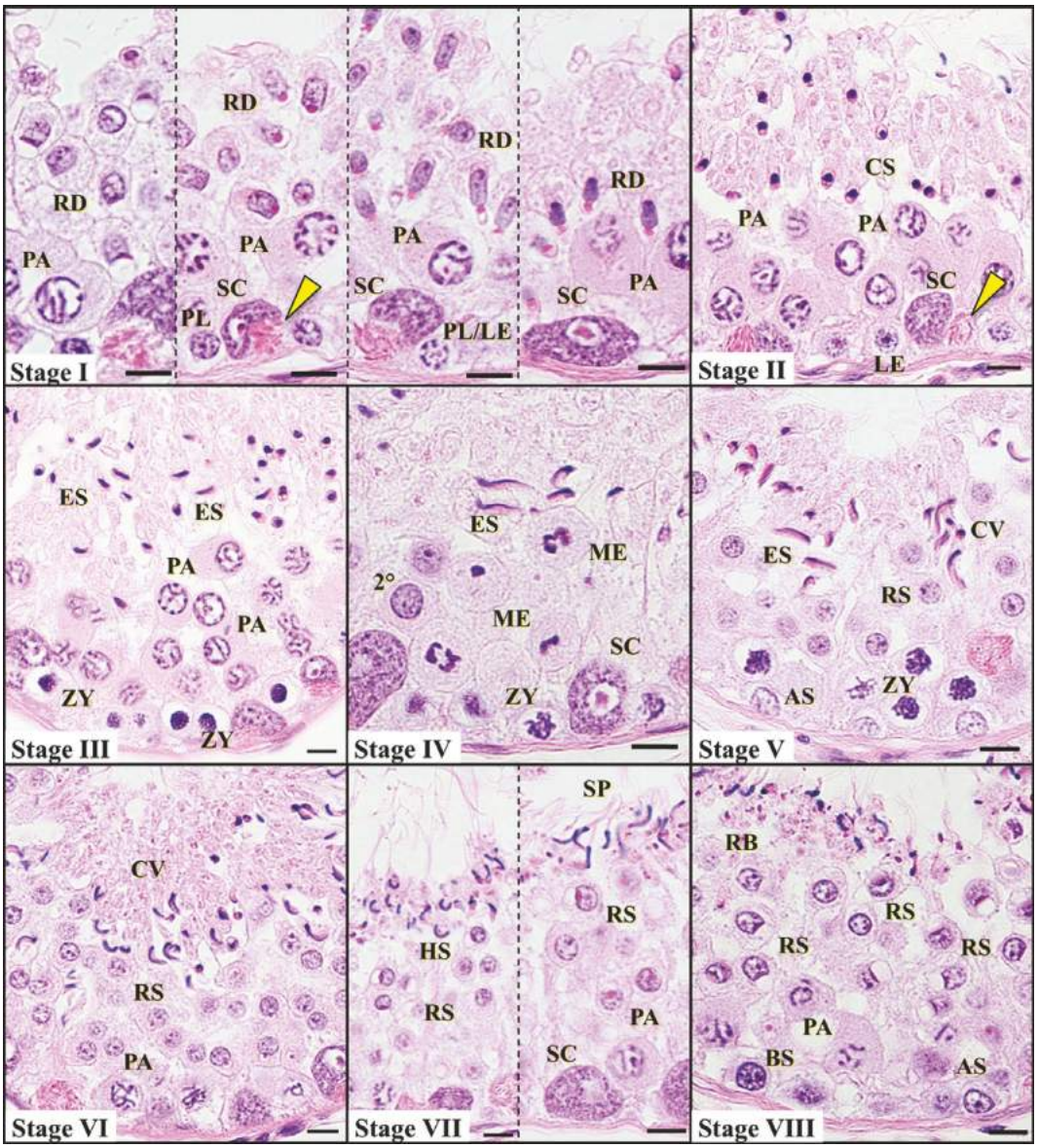

Fig. 3 Eight stages of the seminiferous epithelial cycle in the koala. RB, residual body; SC, Sertoli cell; arrowhead, crystalloid inclusions in Sertoli cell. Other abbreviations are as detailed in Fig. 2. Scale bar: 10 μm.

unchanged but the nuclei of the outer leptotene spermatocytes had now formed homologous chromosomal pairs and entered the characteristic 'bouquet configuration' (Clermont, 1972) of the zygotene spermatocytes.

Stage IV

While the spermatid nuclei of stage IV remained essentially unchanged from stage III, stage IV was clearly differentiated by the presence of meiotic figures from the previous pachytene spermatocytes or, in some sections, the presence of the newly produced secondary spermatocytes and round spermatids; zygotene spermatocytes were also present along the adjacent basement membrane. It was not possible to distinguish the inter chromosomal clear areas of the diplotene spermatocytes.

Stage V

The elongated spermatid nucleus on the luminal surface of the tubule had taken on a slightly curved morphology approaching the appearance to the nuclei of mature spermatozoa, with their long axes orientated perpendicular to the basement membrane of the seminiferous tubule. These curved spermatids surrounded a layer of early stage round spermatids that showed no light microscope evidence of early acrosomal formation. There were no pachytene spermatocytes in this stage, as zygotene spermatocytes were still

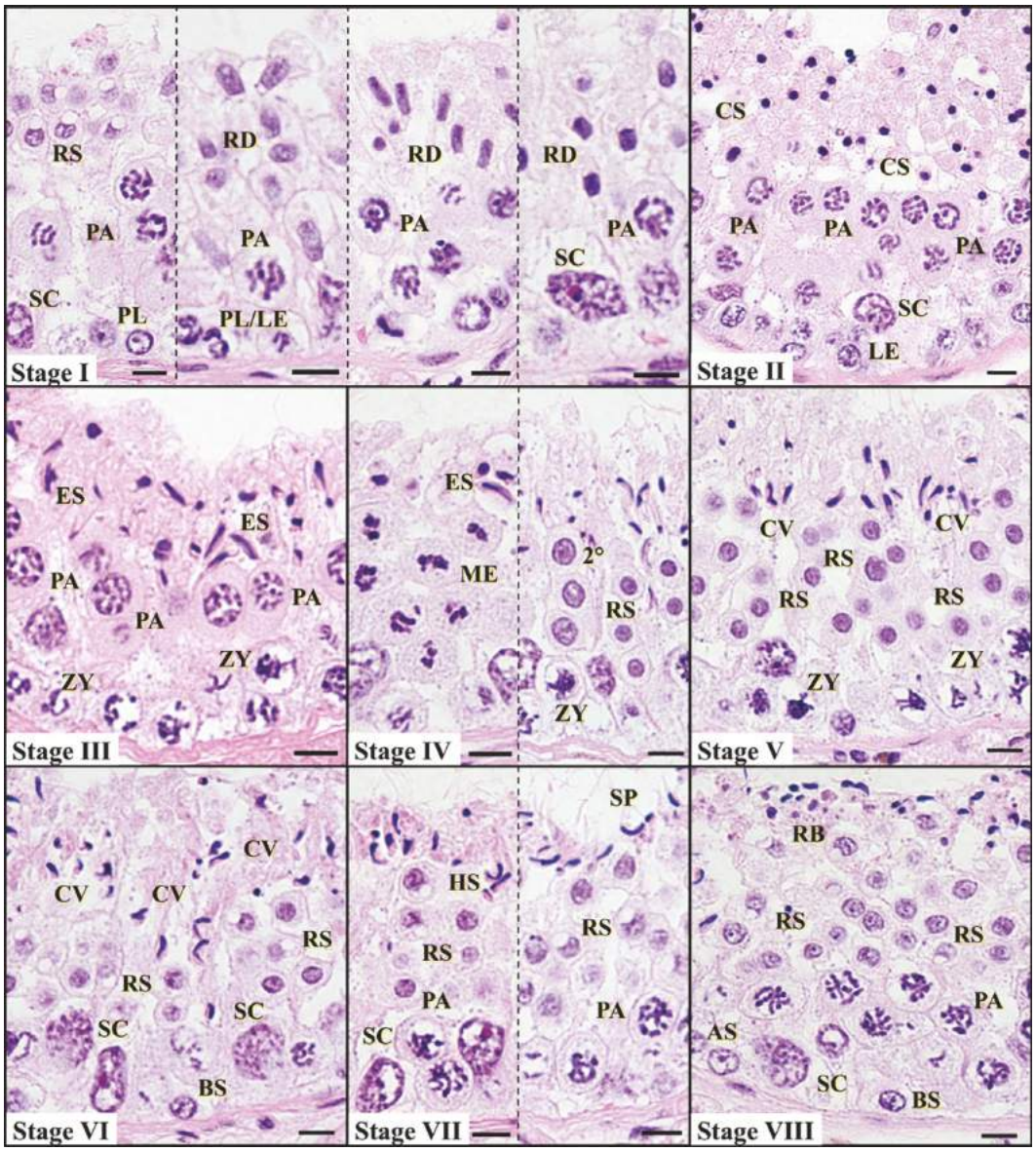

Fig. 4 : Eight stages of the seminiferous epithelial cycle in the SHN wombat. RB, residual body; SC, Sertoli cell. Other abbreviations are as detailed in Fig. 2. Scale bar: 10 μm.

present. Lining the basement membrane of stage 5 tubules were type-A and intermediate spermatogonia, although the later cells were difficult to differentiate.

Stage VI

The spermatids lining the lumen of stage VI were similar in morphology to those of stage V, although the curvature of the nucleus was slightly more pronounced. Outside the layer of curved spermatids were round spermatids with distinct acrosomal vacuoles. In the koala, the round spermatids possessed a distinctive acrosomal granule within its vacuole, whereas this granule was rarely found in the wombat spermatids. Stage VI was differentiated from stage V by the appearance of pachytene spermatocytes and type-B spermatogonia.

Stage VII

The characteristically hooked, re-curved spermatid nuclei of the koalas and the SHN wombats were clearly visible by stage VII on the luminal extremity of the tubule epithelium. The flagella of the spermatids were arranged radially and extended into the lumen. Outside this layer, round spermatids were found that possessed larger acrosomal vacuoles than those in stage VI. The round acrosomal granules found in the koala acrosomal vacuole in stage VI had flattened against the nuclear surface; smaller sized, less distinctive

granules were only rarely observed in the wombat round spermatids in stages VII to I. The acrosomal vacuole of both species appeared to have flattened and there was a corresponding a nuclear concavity. Pachytene spermatocytes and type-A and type-B spermatogonia were also present in stage VII.

Stage VIII

This was considered the stage of spermiation and was characterized by the presence of spermatozoa free within the lumen and the presence of residual bodies of cytoplasm on the luminal epithelium. The remainder of the stage VIII tubule was essentially similar to stage VII. Round spermatids with large acrosomal vacuoles were present, as were pachytene spermatocytes and type-A and type-B spermatogonia. Prominent flattened granules were found in the acrosomal vacuoles of round spermatids of the koala but were usually not detectable in the SHN wombat.

Relative frequencies of stages of the seminiferous epithelial cycle

The mean proportions (% ± SE) of each stage of the seminiferous epithelium cycle are shown in Table 3. The relative proportion of stages IV, VII and VIII was higher in the koala than in the SHN wombat, whereas stages III, VI were respectively lower. However, both the koala and the SHN wombat had a higher frequency of stages in the pre-meiotic phase (VIII, I–III) relative to the post-meiotic phase (V–VII) (Table 4).

Microanatomy of the Sertoli cell

The Sertoli cell nuclei of the koalas were significantly larger than those of the SHN wombats and possessed strongly eosinophilic crystalloid inclusions (Table 1, Fig. 5); no crystalloid inclusions were found associated with the wombat Sertoli cells.

Discussion

The dynamics of the seminiferous epithelial cycle in the koala and the SHN wombat were fundamentally similar to the cellular associations previously described in eutherian mammals (Courot et al. 1970; Russell et al. 1990) and other marsupials (Mason & Blackshaw, 1973; Setchell & Carrick, 1973; Orsi & Ferreira, 1978; Queiroz & Nogueira, 1992; Lin et al. 2004; Lloyd et al. 2008; Phillips et al. 2008). Marsupial seminiferous epithelial cycles have been broadly characterized based upon the cumulative sum of frequency of stages between pre- and post-meiotic stages (Setchell & Carrick, 1973; Phillips et al. 2008). The koala and the SHN wombat have a greater proportion of pre-meiotic (62 and 67%, respectively) than post-meiotic stages and are therefore similar to the brush-tailed possum (*Trichosurus vulpecula*; 53%), the bandicoot (*Permeles nausta*; 56%), the red-necked wallaby (*Macropus rufogriseus*; 53%) and the greater bilby (*Macrotis lagotis*; 61%), but differ with respect to the tammar wallaby (*Macropus eugenii*; 39%) and the common ring-tail possum (*Pseudocheirus peregrinus*; 29%) (Setchell & Carrick, 1973; Phillips et al. 2008; Johnston et al. 2010).

The mean diameters of the seminiferous tubules found in the koalas and the SHN wombats (227.8 and 243.5 μm, respectively) were generally similar to those found in marsupials, e.g. the Virginia opossum (*Didelphis virginiana*; 230–250 μm), the common brush-tailed possum (250 μm), and the tammar wallaby (254 μm) (Setchell, 1977). These diameters are greater than the maximum diameter in the greater glider (*Petauroides volans*; 205 μm; Setchell, 1977), but generally smaller than the mean diameters of the Dasyuridae, which ranged from 360 μm in the Tasmanian devil (*Sarcophilus harrisii*) to 510 μm in the fat-tailed dunnart (*Sminthopsis crassicaudata*) (Woolley, 1975) and the ring-tail possum (*Pseudocheirus peregrinus*; 274 μm) (Phillips et al. 2008). Although the biological significance of such differences in tubular diameter requires further investigation, Setchell & Breed (2006) have suggested that the large cross-sectional diameter of some dasyurid seminiferous tubules may be related to the large size of their spermatozoa.

The proportion of interstitial tissue was different between the koalas (33%) and the SHN wombats (15%). This large proportion of interstitial tissue has previously been noted by Temple-Smith & Taggart (1990) but these authors gave no quantitative data. Similar high relative proportions have also been noted in the swamp antechinus (*Antechinus minimus*; 23–37%; Wilson & Bourne, 1984) and the greater bilby (37%; Johnston et al. 2010). The lower proportion of interstitial tissue reported for the SHN wombat in this study is similar to that found in the common ringtail possum (15%; Phillips et al. 2008). Although the function of such a high proportion of interstitial tissue of

Table 3 Mean (% ± SEM) ratios of the stage of the seminiferous epithelial cycle in the koalas and the SHN wombats.

	I	II	III	IV	V	VI	VII	VIII
Koala	16.1 ± 1.46	22.1 ± 1.43	14.1 ± 1.14	5.0 ± 0.60*	11.5 ± 1.35	8.0 ± 0.71	13.3 ± 0.85*	10.0 ± 0.93*
Wombat	15.9 ± 1.28	28.4 ± 2.29	19.7 ± 1.97*	2.6 ± 0.44	10.5 ± 0.46	12.6 ± 1.29*	7.7 ± 1.15	2.6 ± 0.74

* Statistically significant larger value (nonparametric *U*-test; $P < 0.05$).

Table 4 Mean (% ± SEM) proportions of the pre-meiotic and post-meiotic phases of the seminiferous epithelial cycle in the koalas and the SHN wombats.

	Pre-meiotic	Meiotic	Post-meiotic
Koala	62.2 ± 1.65	5.0 ± 0.60*	32.8 ± 1.67
Wombat	66.6 ± 2.40	2.6 ± 0.44	30.8 ± 1.99

*Statistically significant larger value (nonparametric *U*-test; $P < 0.05$).

these marsupials is equivocal, the high proportion of Leydig cells in the testis may be associated with higher androgen secretion (Akingbemi et al. 1999) or perhaps even the production of pheromones. The Leydig cells of the domestic boar, for example, are also thought to be involved with the synthesis of odoriferous steroids (Fawcett et al. 1973). It is therefore interesting that koala also possesses a large sternal gland, the size and secretion of which appears to be under androgen control (Allen et al. 2010). In the domestic boar and the stallion, large amounts of oestrogen are also apparently secreted by the Leydig cells (Fawcett et al. 1973; Raeside & Reneud, 1983); perhaps a similar function occurs in the koala, as Lee & Carrick (1989) have noted that more oestradiol is usually found in males than is typical in females.

The round spermatid nuclei in the koalas and the SHN wombats both passed through an elongation phase in late stage I to produce what we have described as 'rod'-shaped uncondensed spermatid nuclei; the formation of a 'rod'-shaped spermatid nuclei has not been previously reported for any other marsupial. Another unique feature of spermiogenesis in the koalas and the SHN wombats was the progressive curvature of the proximal sperm head, which appeared to commence in stage V and continued through to stage VII to spermiation. The curved nature of the mature sperm head was first noted by Hughes (1965) and then Harding & Aplin (1990). Temple-Smith & Taggart (1990) also noted heterogeneity in the shape of koala sperm heads during spermiogenesis and a progressive increase in the proportion of sperm with recurved nuclear morphology after epididymal transit; these authors have suggested that the variable nature of nuclear shaping during spermiogenesis and epididymal transit in the koala was likely to result in a greater level of heterogeneity in the shape of the sperm head, a phenomenon which has also subsequently been noted by Wildt et al. (1991) and Johnston et al. (1994) in ejaculated semen. It is interesting that the same degree of sperm nuclear pleiomorphy has not been described in the ejaculated spermatozoa of the common wombat (MacCallum & Johnston, 2005).

In the early stages of spermiogenesis, the round spermatids of the koala possessed a acrosomal vacuole with a distinctive eosinophilic acrosomal granule, but this structure was not as prominent and certainly not as eosinophilic in the early wombat round spermatids. Our inability to detect a prominent acrosomal granule in the wombat spermiogenesis is perplexing, as Harding & Aplin (1990) clearly noted this structure when using the electron microscope but were not able to detect a proacrosomal granule characteristic of early acrosome formation in the koala. The strongly eosinophilic nature of the acrosome granule and its persistence with the development of the mature acrosome in the koala spermatid, compared with that in the wombat, is also perplexing and may be related to the presence of the highly eosinophilic Sertoli cell crystalloid inclusions only found in the koala.

The crystalloid inclusions previously reported in the Sertoli cell of the koala (Harding et al. 1982, 1987; Kerr et al. 1987; Harding & Aplin, 1990) were not present in the SHN wombats. Our results support the incidental observations of Harding & Aplin (1990), who noted the lack of crystalloids in the testis of a single SHN wombat. Similar crystalloids have also been reported to occur in humans (Lubarsch,

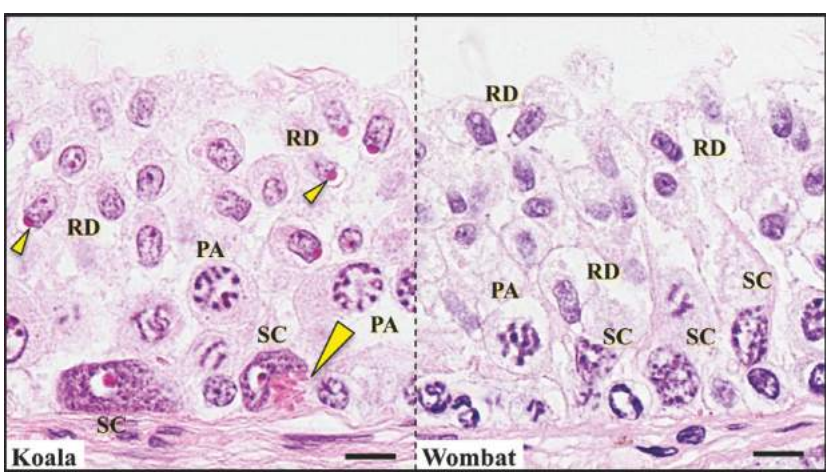

Fig. 5 Late stage I of the seminiferous epithelial cycle in the koalas and the SHN wombats. Large arrowhead refers to crystalloid inclusions in Sertoli cell; small arrowhead denotes acrosomal granule in rod-shaped spermatid. Other abbreviations are as detailed in Fig. 2. Scale bar: 10 μm.

1896; Sohval et al. 1971), the pig (Toyama, 1975, 1976; Toyama et al. 1979) and sloth (Toyama et al. 1990), although there were differences in the ultrastructure and arrangement of filaments constituting these crystalloids. Although the function of these crystalloids remains unknown, the cytoplasmic processes of the Sertoli cell containing the crystalloids are cross-linked to the plasma membrane of germ cells, suggesting that they may stabilize the relationship between the Sertoli cell cytoplasm and the surrounding germ cells (Kerr et al. 1987; Temple-Smith & Taggart, 1990).

Although there is a growing literature on the effect of *Chlamydia trachomatis* on human males (Villegas et al. 1991; Satta et al. 2006; Fernandez et al. 2007; Cunningham & Beagley, 2008; Gallegos et al. 2008), studies of the effect of Chlamydiae on male non-human mammalian fertility are, by comparison, extremely limited (Deif, 2011). To date, there has been no systematic review of existing information on the pathology associated with chlamydial infection in the genital tract or semen in male non-human mammals. Inflammation of the testis usually occurs within the seminiferous tubule and can disrupt spermatogenesis or even result in complete degeneration of the seminiferous tubule (e.g. humans – Allam et al. 2008). In severe chronic orchitis, seminiferous tubules may be obliterated by connective tissue (fibrosis) or granulomas (granulomatous orchitis) so that it is likely in these situations that this pathology could result in irreversible damage to the testis and sterility (Deif, 2011). Recent observations of orchitis in the koala related to natural chlamydial infection (Deif, 2011) suggest that the koala might potentially be an important animal model for studying this disease, and therefore descriptions of normal testicular histology will help in assessing the severity of pathology.

Conclusion

This study has revealed that the microanatomy and seminiferous epithelial cycle in the koala and wombat are essentially the same and confirms the close phylogenetic relationship between these species. There were, however, some interesting differences between the species, including the koala possessing (i) a greater proportion of Leydig cells, (ii) larger Sertoli cell nuclei, (iii) crystalloids in the Sertoli cell cytoplasm, (iv) a distinctive acrosomal granule during spermiogenesis and (v) a highly eosinophilic acrosome. Our observations will provide important insights for understanding the evolution of testis structure and function in future comparative studies with other mammals and become the basis for the assessment of the impact of testis histo-pathology associated with reproductive diseases such as chlamydiosis.

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References

Akingbemi BT, Ge RS, Hardy MP (1999) Leydig Cells. In: *Encyclopedia of Reproduction*, **Vol. 2** (eds Knobil E, Neill JD), pp. 1021–1033, San Diego: Academic Press.

Allam JP, Fronhoffs F, Fathy A, et al. (2008) High percentage of apoptotic spermatozoa in ejaculates from men with chronic genital tract inflammation. *Andrologia* **40**, 329–334.

Allen CD, de Villiers DL, Manning BD, et al. (2010) Seasonal reproduction in wild and captive male koala (*Phascolarctos cinereus*) populations in south-east Queensland. *Reprod Fertil Dev* **22**, 695–709.

Clermont Y (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* **52**, 198–236.

Courot M, Hochereau-de Reviers MT, Ortavant R (1970). Spermatogenesis. In: *The Testis*, **Vol. 1** (eds Johnson AD, Gomes WR, Van Demark NL), pp. 339–452. New York: Academic Press.

Cunningham KA, Beagley KW (2008) Male genital tract chlamydial infection: implications for pathology and infertility. *Biol Reprod* **79**, 180–189.

Deif H (2011) Chlamydial disease of the male koala (Phascolarctos cinereus) reproductive tract. MPhil Thesis. The University of Queensland.

Fawcett DW, Neaves WB, Flores MN (1973) Comparative observations on intertubular lymphatics and the organisation of the interstitial tissue of the mammalian testis. *Biol Reprod* **9**, 500–532.

Fernandez JL, Ramos B, Santiso R, et al. (2007) Frequency of sperm cells with fragmented DNA in males infected with *Chlamydia trachomatis* and *Mycoplasma sp*, determined with the sperm chromatin dispersion (SCD) test. *Fertil Steril* **88**, S5–S.

Gallegos G, Ramos B, Santiso R, et al. (2008) Sperm DNA fragmentation in infertile men with genitourinary infection by *Chlamydia trachomatis* and *Mycoplasma*. *Fertil Steril* **90**, 328–334.

Harding HR, Aplin KP (1990) Phylogenetic affinities of the koala (Phascolarctidae: Marsupialia): a reassessment of the spermatozoal evidence. In: *Biology of the Koala* (eds AK Lee, KA Handasyde, GD Sanson), pp. 1–33. Sydney: Surrey Beatty & Sons.

Harding HR, Carrick FN, Shorey CD (1976) Spermiogenesis in the brush-tailed possum, *Trichosurus vulpecula* (Marsupialia). The development of the acrosome. *Cell Tissue Res* **171**, 75–90.

Harding HR, Carrick FN, Shorey CD (1979) Special features of sperm structure and function in marsupials. In: *The Spermatozoon* (eds DW Fawcett, JM Bedford), pp. 289–303. Baltimore: Urban and Schwarzenburg.

Harding HR, Carrick FN, Shorey CD (1982) Crystalloid inclusions in the Sertoli cell of the koala *Phascolarctos cinereus* (Marsupialia). *Cell Tissue Res* **221**, 663–642.

Harding HR, Carrick FN, Shorey CD (1987) The affinities of the koala *Phascolarctos cinereus* on the basis of sperm ultrastructure and development. In: *Possums and Opossums; Studies in Evolution* (ed. M Archer), pp. 353–364. Sydney: Beatty and Sons Pty Ltd and Royal Zoological Society of New South Wales.

Hess RA (1999) Spermatogenesis, Overview. In: *Encyclopedia of Reproduction, Volume 4* (eds E Knobil, JD Neill), pp. 539–545. London: Academic Press.

Hughes RL (1965) Comparative morphology of spermatozoa from five marsupial families. *Aust J Zool* **13**, 533–543.

Humason GL (1977) *Humason's Animal Tissue Techniques*. Baltimore, MD: Johns Hopkins University Press.

Johnston SD, McGowan MR, Carrick FN, et al. (1994) Seminal characteristics and spermatozoa morphology of captive Queensland koalas, (*Phascolarctos cinereus adustus*). *Theriogenology* **42**, 501–511.

Johnston SD, Rumph C, Lucht M, et al. (2010) Studies of male reproduction in the greater bilby (*Macrotis lagotis*). *Aust J Zool* **35**, 315–330.

Kerr JB, Knell CM, Irby DC (1987) Ultrastructure and possible function of giant crystalloids in the Sertoli cell of the juvenile and adult koala (*Phascolarctos cinereus*). *Anat Embryol* **176**, 213–224.

Kirsch JAW, Lapointe FJ, Springer MS (1997) DNA-hybridization studies of marsupials and their implications for metatherian classification. *Aust J Zool* **45**, 211–280.

Lee AK, Carrick FN (1989) Phascolarctidae. In: *Fauna of Australia, Volume 1B* (eds DW Walton, BJ Richardson), pp. 740–754. Canberra: Australian Government Publishing Service.

Lin M, Harman A, Fletcher TP (2004) Cycle of the seminiferous epithelium in a marsupial species, the brushtail possum (*Trichosurus vulpecula*) and estimation of its duration. *Reprod Fertil Dev* **16**, 307–313.

Lloyd S, Carrik F, Hall L (2008) Unique features of spermiogenesis in the Musky Rat-Kangaroo: reflection of a basal lineage or a distinct fertilization process. *J Anat* **212**, 257–274.

Lubarsch O (1896) Über das Vorkommen krystallinischer und krystalloider Bildungenin den Zellendes menschlichen Hodens. *Virchows Arch [A]* **145**, 316–338.

MacCallum CA, Johnston SD (2005) Studies on the cryopreservation of common wombat (*Vombatus ursinus*) spermatozoa. *Reprod Fertil Dev* **17**, 727–732.

Mason K, Blackshaw AW (1973) The spermatogenic cycle of the Bandicoot, *Isodon macrourus*. Proceedings of the Australian Society for Reproductive Biology. *J Reprod Fertil* **32**, 307–308.

Munemasa M, Nikaido M, Donnellan S, et al. (2006) Phylogenetic analysis of diprotodontian marsupials based on complete mitochondrial genomes. *Genes Genet Syst* **81**, 181–191.

Orsi AM, Ferreira AL (1978) Definition of the stages of the cycle of the seminiferous epithelium of the opossum (*Didelphis azarae*, Temminck, 1825). *Acta Anat* **100**, 153–160.

Osborne MJ, Christidis L, Norman JA (2002) Molecular phylogenetics of the Diprotodontia (kangaroos, wombats, koala, possums, and allies). *Mol Phylogenet Evol* **25**, 219–228.

Phillips MJ, Pratt RC (2008) Family-level relationships among the Australasian marsupial 'herbivores' (Diprotodontia: koala, wombats, kangaroos and possums). *Mol Phylogenet Evol* **46**, 594–605.

Phillips DJ, McKinnon A, Keeley T, et al. (2008) Testosterone secretion, testicular histology and the cryopreservation of cauda epididymidal spermatozoa in the common ringtail possum (*Pseudocheirus peregrinus*). *Reprod Fertil Dev* **20**, 391–401.

Queiroz GF, Nogueira JC (1992) Duration of the cycle of the seminiferous epithelium and quantitative histology of the testis of the South American white-bellied opossum (*Didelphis albiventris*), Marsupialia. *Reprod Fertil Dev* **4**, 213–222.

Raeside JI, Reneud RL (1983) Estrogen and androgen production by purified Leydig cells of mature boars. *Biol Reprod* **28**, 727–733.

Russell LD, Ettlin RA, Shinha Hikim AP, et al. (1990) *Histological and Histopathological Evaluation of the Testis*. Clearwater, IL: Cache River Press.

Satta A, Stivala A, Garozzo A, et al. (2006) Experimental *Chlamydia trachomatis* infection causes apoptosis in human sperm. *Hum Reprod* **21**, 134–137.

Setchell BP (1977) Reproduction in male marsupials. In: *The Biology of Marsupials* (eds B Stonehous, D Gilmore), pp. 411–457. London: Macmillan.

Setchell BP, Breed WG (2006) Anatomy, vasculature and innervation of the male reproductive tract. In: *The Physiology of Reproduction*. 3rd edn (ed JD Neill), pp. 794–847. New York: Raven Press.

Setchell BP, Carrick FN (1973) Spermatogenesis in some Australian marsupials. *Aust J Zool* **21**, 491–499.

Sohval AR, Suzuki Y, Gabrilove JL (1971) Ultrastructure of crystalloids in spermatogonia and Sertoli cells of normal human testis. *J Ultrastruct Res* **34**, 83–102.

Temple-Smith PD, Taggart DA (1990) On the male generative organs of the koala (*Phascolarctos cinereus*): an update. In: *Biology of the Koala* (eds AK Lee, KA Handasyde, GD Sanson), pp. 34–54. Sydney: Surrey Beatty & Sons.

Toyama Y (1975) Ultrastructural study of crystalloids in Sertoli cells of the normal, intersex and experimental cryptorchid swine. *Cell Tissue Res* **158**, 205–213.

Toyama Y (1976) Actin-like filaments in the Sertoli cell junctional specializations in the swine and mouse testis. *Anat Rec* **186**, 477–492.

Toyama Y, Obinata T, Holtzer H (1979) Crystalloids of actin-like filaments in the Sertoli cell of the swine testis. *Anat Rec* **195**, 47–62.

Toyama Y, Calderon FU, Quesada R (1990) Ultrastructural study of crystalloids in Sertoli cells of the three-toed sloth (*Bradypus tridactylus*). *Cell Tissue Res* **259**, 599–602.

Tyndale-Biscoe CH, Renfree MB (1987) *Reproductive Physiology of Marsupials*. Cambridge: Cambridge University Press.

Villegas H, Pinon M, Shor V, et al. (1991) Electron-microscopy of *Chlamydia trachomatis* infection of the male genital tract. *Arch Androl* **27**, 117–126.

Weibel ER (1979) *Stereological Methods. Practical Methods for Biological Morphometry*, vol. **1**. London: Academic Press.

Wildt DE, Bush M, O'Brien SJ, et al. (1991) Semen characteristics in free-living koalas (*Phascolarctos cinereus*). *J Reprod Fertil* **92**, 99–107.

Wilson BA, Bourne AR (1984) Reproduction in the male dasyurid *Antechinus minimus maritimus* (Marsupialia: Dasyuridae). *Aust J Zool* **32**, 311–318.

Woolley P (1975) The seminiferous tubules in dasyurid marsupials. *J Reprod Fertil* **45**, 255–261.