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ORIGINAL ARTICLE

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Frequency of stages of the seminiferous cycle in the thick-tailed bush baby (*Otolemur garnetti*), a prosimian primate: possible phylogenetic implications?

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Abstract Spermatogenesis in the thick-tailed bush baby, *Otolemur garnetti*, was studied using light microscopy. The stages and stage frequencies of the cycle of the seminiferous epithelium were determined using semithin sections stained with methylene blue-azure II. These sections were obtained from the testes of six healthy adult males ($n = 6$). They revealed 11 stages of the seminiferous epithelial cycle in this species. The mean relative frequencies of the stages I–XI were 10.9, 6.0, 5.9, 7.3, 13.2, 10.7, 11.7, 9.2, 7.6, 8.9 and 8.6, respectively. Comparisons were made between the frequency data in the thick-tailed bush baby and equivalent data in the rat, hamster, macaque, baboon, chimpanzee and man. There was a significant correlation ($P < 0.05$) between the *Otolemur* data and equivalent stage frequency data of two rodent species (rat and hamster) and monkey (*Macaca arctoides*). However, there was no significant correlation between the present data and those of the baboon, chimpanzee and man. Possible phylogenetic implications of these findings are discussed.

Keywords Bush baby · Spermatogenesis · Seminiferous epithelium · Phylogeny · Testis

Introduction

In various mammalian species, the seminiferous epithelium in any given part of the seminiferous tubule undergoes continuous periodic changes of the germ cell component in form of successive cellular associations, the complete cycle of which is termed the cycle of the seminiferous epithelium (Leblond and Clermont 1952a). The seminiferous epithelial cycle has been characterised in various rodents, including rats (Leblond and Clermont 1952a, b; Russell et al. 1990; Hess 1990), mice (Oakberg 1956; Russell et al. 1990) and hamsters (Leblond and Clermont 1952b; Van Haaster and de Rooij 1993). The same aspect has also been investigated in a number of primates, including macaques (Clermont and Antar 1973), baboon (Chowdhury and Steinberger 1976), lesser bush baby, *Galago senegalensis* (Pardue 1978), man (Clermont 1963) and chimpanzee (Smithwick and Young 1996). In the species studied to date, there are substantial differences in actual duration of the seminiferous epithelial cycle (Hochereau-de Reviers et al. 1990), although comparisons of such temporal differences do not reveal significant information regarding spermatogenesis. It is our opinion that comparative data of relative frequencies of equivalent stages of the seminiferous epithelial cycle in various species may perhaps yield more useful information.

The bush babies and other prosimians occupy a unique position in the evolutionary hierarchy of mammals. There is, however, a paucity of data regarding spermatogenesis in these species. In this study, certain aspects of spermatogenesis in the thick-tailed bush baby, particularly the seminiferous epithelial cycle, was examined and the data obtained compared with those of other animals.

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Materials and methods

Fixation and tissue processing

Six adult male thick-tailed bush babies weighing between 920 and 1,200 g were used in the study. The animals were deeply anaesthetised by intramuscular injections of a combination of ketamine hydrochloride

(15 mg/kg body weight) and xylazine hydrochloride (0.5 mg/kg body weight), then laparotomised, abdominal aorta located and cannulated cranial to the branches of the testicular arteries. The bush babies were then euthanased with an intravenous overdose of 20% sodium pentobarbitone. Immediately, the caudal vena cava was opened, followed by gravitational perfusion of the lower trunk, genitalia and limbs; first with 0.85% sodium chloride solution warmed to 35°C for 3 min, then later by aldehyde fixative for 15 min. Fixation was done using a 3% glutaraldehyde–1% paraformaldehyde mixture in 0.2 M phosphate buffer (pH 7.4). The testes of each animal were dissected out and sectioned into slices, then immersed in the same fixative for 24 h. These were then diced, washed in 0.1 M veronal acetate buffer, post-fixed in osmium (IV) oxide, dehydrated in ascending concentrations of acetone and embedded in araldite. Semi-thin sections were cut with glass knives on a Sorvall MT-1 microtome and subsequently stained with methylene blue-azure II stain.

Determination of stages and stage frequencies of the cycle of seminiferous epithelium

Stages of the seminiferous epithelium were identified, based on the changes in morphology of acrosomes and nuclei of developing spermatids (Leblond and Clermont 1952b) and the germ cell associations found within a given part of the epithelium. Using these criteria, 11 major stages of the seminiferous epithelium could be distinguished in the methylene blue-azure II stained sections.

Relative stage frequencies were determined by scoring cross-sectional profiles of a minimum of 326 seminiferous tubules per animal. Generally, round or nearly round profiles of seminiferous tubules were preferred for this purpose. A total of 2,083 tubules were scored in the six animals used for the study (Table 1).

Moreover, tubular stages of *O. garnetti* were further compared with those documented in other species. This involved comparison of stages of acrosomal development in spermatids as well as cellular associations within the seminiferous epithelium. Where the specific stages did not correspond exactly, the equivalent stages were grouped together and the relative frequencies considered additively (Table 2). The extent of correlation between the *Otolemur* stage frequency data to equivalent stages in other species was determined by linear regression analysis and the findings tabulated (Table 2).

Results

The cycle of the seminiferous epithelium

Eleven stages of the cycle of the seminiferous epithelium were identified in the thick-tailed bush baby. These were:

Stage I

This stage was characterised by presence of two generations of spermatids; one generation being round spermatids in early to late Golgi phase, while the other was made up of older maturing spermatids (Fig. 1). Proacrosomal granules were not visible at this stage in early round spermatids. In maturing spermatids, the mitochondrial sheath was not yet distinctly outlined at this stage. Also evident was a single generation of early pachytene spermatocytes.

Stage II

During this stage there was further maturation of round spermatids, which resulted in their acrosomal vesicles making contact with the nuclei (Fig. 2). The maturing spermatids had a distinctly well-formed mitochondrial sheath. Often observed at this stage were aggregations of lipid droplets on the luminal side of the seminiferous tubules.

Stage III

The predominant feature at this stage was the increased area of contact between the round spermatid nuclei and

Table 1 Percentage frequencies of stages of the seminiferous epithelium, as determined in the testes of six adult male thick-tailed bush babies

	Stages											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Tubulescounted (*n*)
G1	11.6	6.2	3.1	2.8	11.6	12.5	11.1	10.5	7.6	14.2	8.8	353
G168	10.3	6.9	4.0	6.0	10.6	13.2	12.3	9.5	8.3	10.9	8.0	349
G167	9.8	2.4	5.6	8.9	17.2	8.6	12.8	14.5	4.5	5.9	9.8	337
G5	14.0	7.9	9.3	7.9	5.9	13.8	8.7	9.6	5.6	6.7	10.6	356
G3	11.1	6.4	7.1	11.0	19.0	9.5	12.6	3.4	6.4	4.9	8.6	326
G7	8.8	6.1	6.4	7.5	14.9	6.6	12.7	7.5	13.0	11.0	5.5	362
Means	10.9	6.0	5.9	7.3	13.2	10.7	11.7	9.2	7.6	8.9	8.6	2083[a]
SEM	0.7	0.8	0.9	1.1	2.0	1.2	0.7	1.5	1.3	1.5	0.7	

[a]Total number of tubular profiles counted

Table 2 Comparative mean frequencies (%) of equivalent stages of the seminiferous epithelium in different mammalian species. The correlation coefficient, *r*, was determined by linear regression analysis of *Otolemur* data versus that of the particular species. Sources of data: (1) rat (Sprague dawley): Hess (1990), Hess et al. (1990); (2) hamster (*Phodopus sungorus*): van Haaster and de Rooij (1993); (3) baboon (*Papio anubis*): Chowdhury and Steinberger (1976); (4) monkey (*Macaca arctoides*): Clermont and Antar, (1973); (5) man (*Homo sapiens*): Clermont (1963); (6) chimpanzee: Smithwick et al. (1996)

	Stages											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	*r*
Galago	10.9	6.0	5.9	7.3	13.2	10.7	11.7	9.2	7.6	8.9	8.6	–
Rat	19.0	2.3	4.9	42.8		3.0	3.2	3.0	8.7	6.2	6.8	0.861*
Hamster	12.7	4.2	7.9	7.4	49.9				4.5	6.7	6.8	0.992*
Monkey	15.9	5.8	5.3	7.2	7.9	13.4	13.5	8.3	7.2	7.4	8.3	0.670*
Baboon	15.5	13.3		8.8		9.3	17.7	9.4	9.1	6.1	10.8	0.055
Man	29.8				19.6	6.4		7.7	31.3		5.2	0.532
Chimpanzee	29.8				14.5	30.9		11.0	9.5		4.3	0.212

*Significantly correlated data ($P < 0.05$)

their large acrosomal vesicles (beginning of cap phase) (Fig. 3). The maturing spermatids were found closer to the lumen of the seminiferous tubule.

Stage IV

This was characterised by the spread of acrosomal cap over the apical portion of the nucleus of the round spermatids to cover almost one-fifth of its surface (Fig. 4). There are also maturing spermatids close to the lumen and pachytene spermatocytes in the basal third of the epithelium.

Stage V

Typically, this stage showed a large number of residual bodies close to the lumen and mature spermatids that either lined or were free in the lumen of the tubules (Fig. 5). Spermiation took place in the course of this stage. The acrosomal vesicles of round spermatids covered almost one-third of the nucleus. The predominant cells in this stage were pachytene spermatocytes and round spermatids.

Stage VI

Following spermiation, only one generation of spermatids was maintained in the tubular wall. The main primary spermatocytes found in the epithelium were pachytene spermatocytes (Fig. 6). Towards the end of this stage, type B spermatogonia divided mitotically to give rise to preleptotene spermatocytes predominantly found in stage VII.

Stage VII

Following mitotic division of type B spermatogonia in late stage VI, two generations of spermatocytes were observed in this stage namely pachytene and preleptotene spermatocytes. Compared with pachytene spermatocytes, preleptotene spermatocytes are relatively smaller and characterised by pale-staining flocculent chromatin material scattered throughout the nucleus. Round spermatids displayed abundant lipid droplets in their caudal cytoplasm that often assumed linear arrangements along the axis of their developing tails (Fig. 7). The acrosomal ends of the spermatids became oriented towards the basement membrane of the seminiferous epithelium and nuclear elongation began, signifying commencement of the acrosomal phase.

Stage VIII

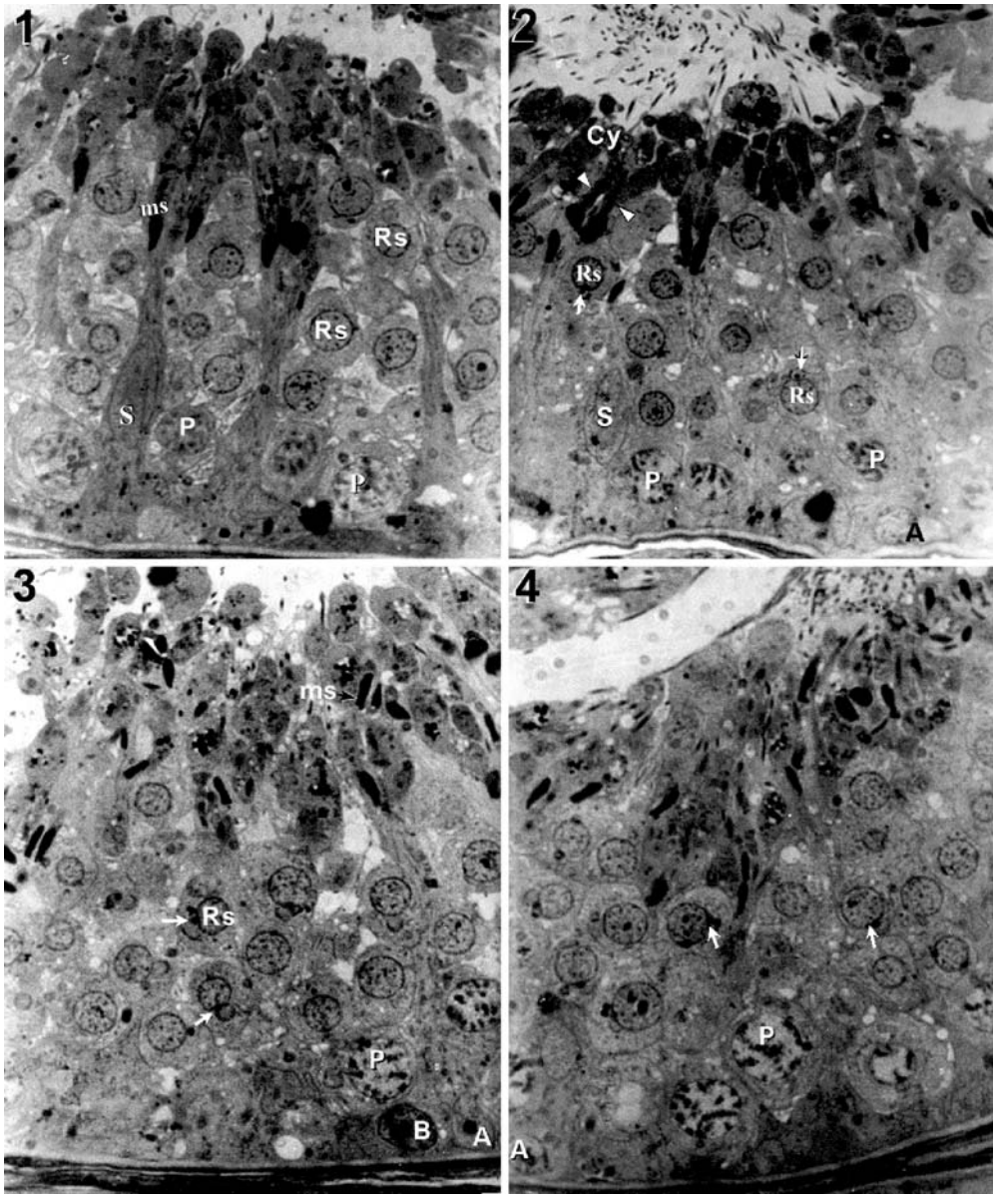
At this stage, profiles of elongating spermatids had pointed and relatively longer acrosomes (Fig. 8). Preleptotene spermatocytes matured into leptotene spermatocytes that were typified by more distinctive darker staining chromosomal aggregations in their nuclei. Pachytene spermatocytes were also present within the epithelium.

Stage IX

The elongated spermatids at this stage had more condensed nuclei that frequently assumed a V-shaped profile in cross-section (Fig. 9). Also present in the epithelium were leptotene and late pachytene spermatocytes.

Stage X

Further condensation of spermatid nuclei continued while leptotene and pachytene spermatocytes in the tubular wall progressively differentiated into zygotene and diplotene spermatocytes, respectively (Fig. 10). Zygotene spermatocytes had definite paired condensed chromatids within their nuclei. Diplotene spermatocytes



on the other hand, were characterised by the presence of comparatively larger nuclei.

Stage XI

In early stage XI, secondary spermatocytes emerged, arising from first meiotic division of diplotene spermatocytes (Fig. 11). The nuclei of secondary spermatocytes were comparatively smaller than those of primary spermatocytes. Zygotene and diplotene spermatocytes remained the predominant primary spermatocytes of the tubular wall in this stage. Towards the end of the stage, dividing cells, most likely secondary spermatocytes undergoing second meiotic division, became the main feature of the seminiferous epithelium. Other cell types found in the epithelium at this time were maturing spermatids, secondary spermatocytes, zygotene primary spermatocytes and occasionally very early spermatids (Fig. 12). The demarcation between early and late stage XI at times was not very distinct. The cycle then proceeded to stage I.

In *O. garnetti*, therefore, stages I–V of the cycle were characterised by two generations of spermatids and one generation of spermatocytes. Stage VI had single generations of both spermatocytes and spermatids, while stages VII–XI had a single generation of spermatids and two of spermatocytes. Of interest too, was the observation that in the thick-tailed bush baby spermiation precedes mitotic division of the B spermatogonia that give rise to preleptotene spermatocytes. The scope of this study, however, did not entail determination of the mode of spermatogonial renewal in this species.

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Fig. 1 Photomicrograph corresponding to stage I of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Both round (*Rs*) and maturing spermatids (*ms*) are evident in the seminiferous epithelium together with early pachytene spermatocytes (*P*). *S* Sertoli cell

Fig. 2 Photomicrograph corresponding to stage II of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Maturing spermatids have distinctly well developed mitochondrial sheaths (*arrowheads*). Round spermatids (*Rs*) have acrosomal vesicles (*arrows*) in contact with the nuclei of the cells. Contents of cytoplasmic droplets (*Cy*) form aggregations close to the lumen of the tubules. *A* type A spermatogonia; *P* pachytene spermatocytes; *S* Sertoli cell

Fig. 3 Photomicrograph corresponding to stage III of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Large acrosomal vesicles (*arrows*) of round spermatids (*Rs*) have increased area of contact with the nuclei of the cells. Pachytene spermatocytes (*P*) have more condensed chromosomal material in their nuclei. *A* type A spermatogonia; *B* type B spermatogonia; *ms* maturing spermatids

Fig. 4 Photomicrograph corresponding to stage IV of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). There is further spread of the acrosomal cap (*arrows*) of round spermatids to cover about one-fifth of the nuclear envelope. *A* type A spermatogonia; *P* pachytene spermatocyte

Relative duration of the stages of the cycle of the seminiferous epithelium

Data on frequencies of stages of the seminiferous epithelium (Table 1) indicate that the longest stage was stage V, with a mean frequency of $13.2 \pm 2.0\%$, which was almost twice as long as the shortest stages, stages II and III, with mean frequencies of $6 \pm 0.8\%$ and $5.9 \pm 0.9\%$, respectively. Stages V–X consumed the bulk of the time for spermatogenesis in this species (Fig. 13). When mean stage frequencies in the bush baby were compared with those of equivalent stages in other species, there was a significant correlation ($P < 0.05$) between stage frequencies in the bush baby and those of two rodents, the rat and hamster, and the monkey, *Macaca arctoides* (Table 2). However, no significant correlation ($P > 0.05$) between bush baby stage frequencies and those of equivalent stages in the baboon, chimpanzee and man was observed.

Discussion

The thick-tailed bush baby was previously classified under the genus *Galago*, together with its lesser counterpart (Eaglen and Simons 1980). However, recent classifications have placed it in a separate genus, *Otolemur* (Nowak 1999). In this study we have adopted the latter classification, placing the thick-tailed bush baby in the genus *Otolemur*. The cycle of seminiferous epithelium in the thick-tailed bush baby was divided into 11 stages on the basis of evident morphological changes of spermatids and germ cell associations in any given part of the seminiferous tubules. Pardue (1978), recorded 12 stages in the lesser species, *G. senegalensis*. While there appears to be a strong similarity in the process of spermiogenesis in this species, including the progression of acrosomal development and spermatid maturation, to that described for the lesser bush baby (Pardue 1978), there is in fact a slight difference in the actual number of stages in the two species. However, it is not possible to make actual comparisons of the morphological changes at each stage as the earlier study by Pardue (1978) on the lesser bush baby did not provide figures for such comparisons. Two types of cellular associations have been recognised in the seminiferous epithelial cycle (Hochereau-de Reviers et al. 1990): type I, where there are two generations of primary spermatocytes and a single generation of spermatids, and type II, where only one generation of primary spermatocytes and two of spermatids occur. In the thick-tailed bush baby, as in man and the rabbit (Hochereau-de Reviers et al. 1990), the duration of types I and II cellular associations is relatively similar. This is unlike bulls and rams where type I associations are predominant. In monkeys, rats and mice, type II associations are more frequently encountered (Hochereau-de Reviers et al. 1990). Of interest too is the observation that in this bush baby, as in the bull and ram (Hochereau-de Reviers et al. 1990; Wrobel et al. 1995), buck (Onyango et al. 2000), monkey (Clermont and Antar 1973), baboon (Chowdhury and Steinberger 1976), chimpanzee (Smithwick and Young 1996) and man (Clermont 1963) spermiation occurs prior to the appearance of preleptotene spermatocytes in the seminiferous epithelium. In the rat (Hochereau-de Reviers et al. 1990) and hamster (van Haaster and de Rooij 1993); however, appearance of the latter spermatocytes precedes spermiation.

Observed differences in frequencies of the stages of the spermatogenic cycle in *Otolemur*, as in other species, is a reflection of differences in the time required for various morphological and physiological events occurring at different stages in the course of spermiogenesis. There was significant correlation between durations of different stages in the cycle of the seminiferous epithelium in the thick-tailed bush baby with equivalent stages in the rat, hamster and monkey. This could imply that the steps involved in the differentiation process of spermatids in these species require almost equivalent time intervals, suggestive of close similarity in the physiological and biochemical processes that occur during spermiogenesis in the four species.

Although evidence linking reproductive features to phylogenetic events is rather tenuous, these features have nevertheless provided useful insights into phylogenetic trends of mammalian species. In males, for instance, accessory sex glands (Voss and Linzey 1981; Bedford et al. 1998), sperm morphology (Jamieson 1995a, b; Robson et al. 1997), maturation and fertilising ability, scrotal position and relations (Luckett 1980; Bedford et al. 1998) have in the past provided important clues in tracing geneological relationships among mammalian species. More recently, it has been suggested that retention of spermatid nucleus in the middle of the

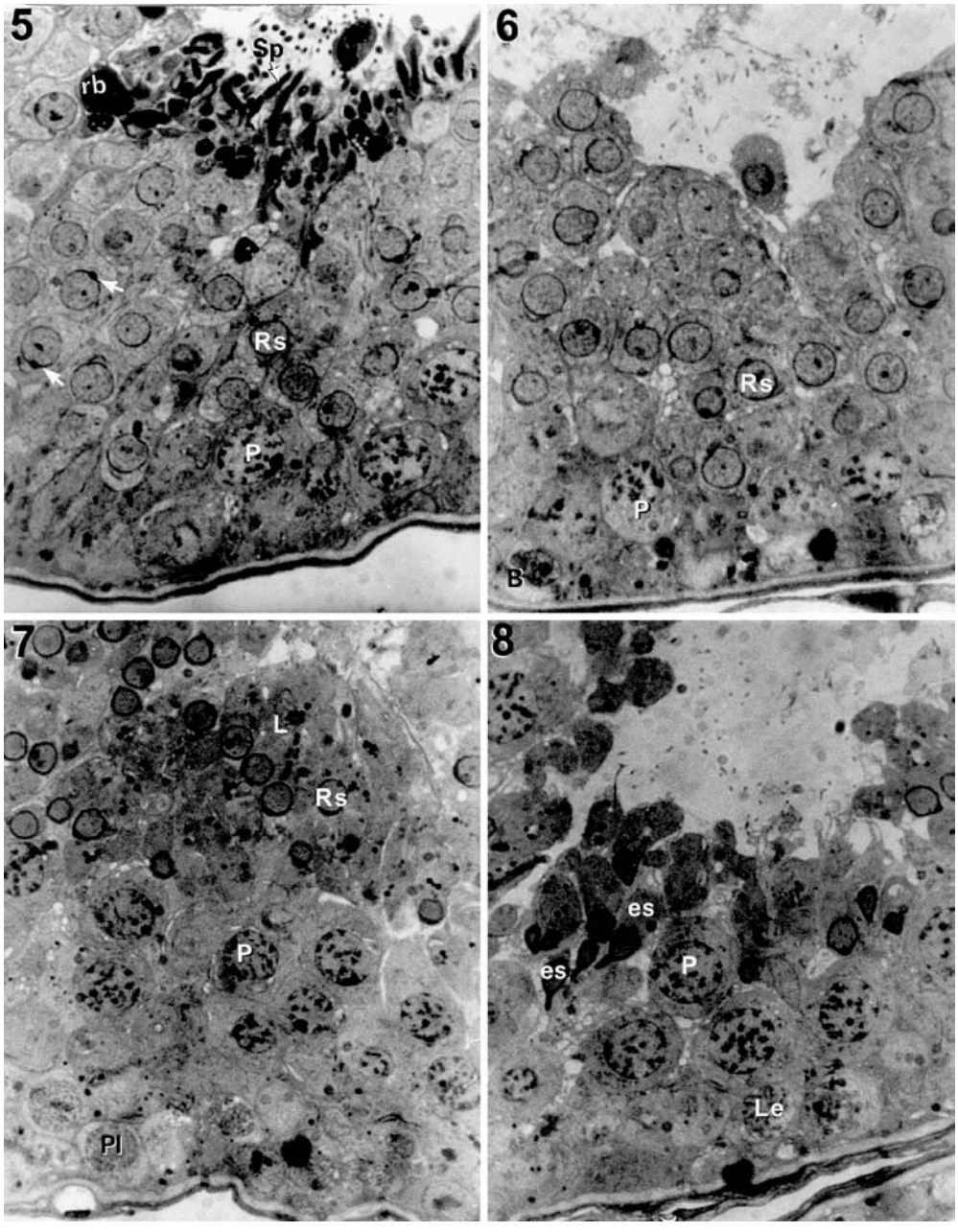

Fig. 5 Photomicrograph corresponding to stage V of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). The acrosomal cap of round spermatids covers almost one-third of the nucleus (*arrow*). Spermatozoa (*Sp*) are found close to the lumen undergoing spermiation. Some residual bodies (*rb*) occur close to the lumen of the seminiferous tubules. *P* pachytene spermatocyte

Fig. 6 Photomicrograph corresponding to stage VI of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Round spermatids (*Rs*) are the only spermatid type in the epithelium following the completion of the spermiation process in stage V. At this stage they do not have a preferred orientation. Also found in the epithelium are mid pachytene spermatocytes (*P*)

Fig. 7 Photomicrograph corresponding to stage VII of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Remodelling of round spermatids (*Rs*) begins at this stage and they then become oriented towards the basement membrane. Lipid droplets (*L*) are first observed in the caudal cytoplasm of round spermatids at this stage. *P* late pachytene spermatocytes; *Pl* preleptotene spermatocytes

Fig. 8 Photomicrograph corresponding to stage VIII of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Elongating spermatids (*es*) with pointed acrosomes, late pachytene spermatocytes (*P*) and leptotene spermatocytes (*Le*) are evident in the epithelium

seminiferous epithelium with a concomitant acrosomal elongation towards the base of the tubule in shrew moles is an important phylogenetic characteristic of members of the Order Insectivora (Muzukami et al. 2001). Consequently, the similarities in spermatogenesis between the thick-tailed bush baby and the two rodents (rat and hamster) and monkey appears to suggest that the bush baby shares a close phylogenetic relationship with the latter three mammalian species. This finding seems to support the classification of the bush baby as a prosimian interposed between the simians (monkeys) and the lower mammals. On the other hand, lack of correlation between our bush baby stage frequency data and equivalent stages in baboon *(Papio anubis)*, chimpanzee and man, could also have phylogenetic ramifications. In the latter three species, unique cellular associations in the seminiferous epithelium have been noted with frequent occurrence of more than one stage of the seminiferous epithelial cycle per single cross section of the seminiferous tubule (Clermont 1963; Heller and Clermont 1964; Chowdhury and Steinberger 1976; Smithwick and Young 1996; Smithwick et al. 1996). This is unlike the situation in other species studied, where single stages of the spermatogenic cycle occupy the entire cross section of individual seminiferous tubules. This "apparent anomaly" of spermatogenesis in baboon, chimpanzee and man is attributed to the occupation of patches of seminiferous tubules by various stages of the cycle, resulting into lack of a definite wave of seminiferous epithelium along the tubules (Clermont 1963;

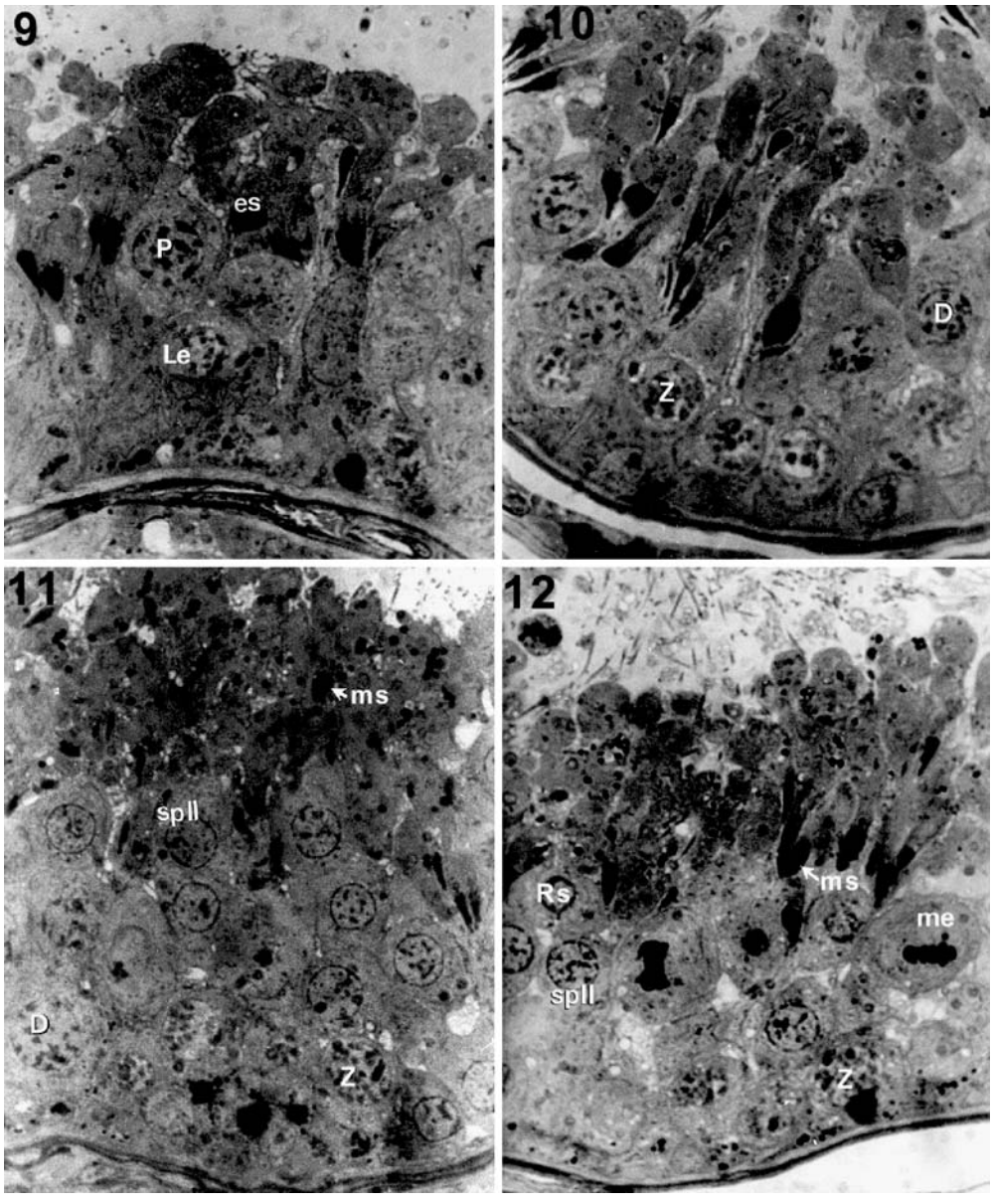

Fig. 9 Photomicrograph corresponding to stage IX of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). Elongating spermatids (*es*) have more condensed (*darker staining*) nuclei. Both late pachytene (*P*) and leptotene spermatocytes (*Le*) are also found in the epithelium

Fig. 10 Photomicrograph corresponding to stage X of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). By this stage there are elongating spermatids, diplotene (*D*) and zygotene (*Z*) spermatocytes in the seminiferous epithelium

Fig. 11 Photomicrograph corresponding to stage XI of the cycle of the seminiferous epithelium in *O. garnetti* (×1,200). The early part of this stage is characterised by maturing spermatids (*ms*), diplotene (*D*), secondary (*spll*) and zygotene (*Z*) spermatocytes in the tubular epithelium

Fig. 12 Photomicrograph of the later part of stage XI, showing secondary spermatocytes (*spll*) undergoing meiotic division (*me*), most likely second meiotic division, in the seminiferous epithelium. Maturing spermatids (*ms*) are found closer to the lumen. *Z* zygotene spermatocyte (1,200×)

Heller and Clermont 1964; Schulze and Salzbrunn 1992; Smithwick and Young 1996). Lack of correlation between the bush baby stage frequency data with that of baboon, chimpanzee and man could be a further manifestation of the functional similarity of testicular function in the latter three species. Previous studies have shown differences in spermatogenesis between *M. arctoides and P. anubis* (Clermont and Antar 1973; Chowdhury and Steinberger 1976), although both species belong to the same family, *Cercopithecidae*. The results on comparisons of spermatogenetic data in these three species with that of the thick-tailed bush baby are in agreement with the earlier findings implying closer phylogeny of the baboon and chimpanzee to man and, conversely, the bush baby to the monkey.

The data in this study reveals 11 stages of the seminiferous epithelial cycle in the thick-tailed bush baby. Furthermore, the findings support the classification of the O. *garnetti* as prosimians, intermediate between primates and lower mammals. Finally, it can be inferred from the present findings that with regards to spermatogenesis, the thick-tailed bush baby may not be a suitable model for the study of the dynamics of human male reproduction.

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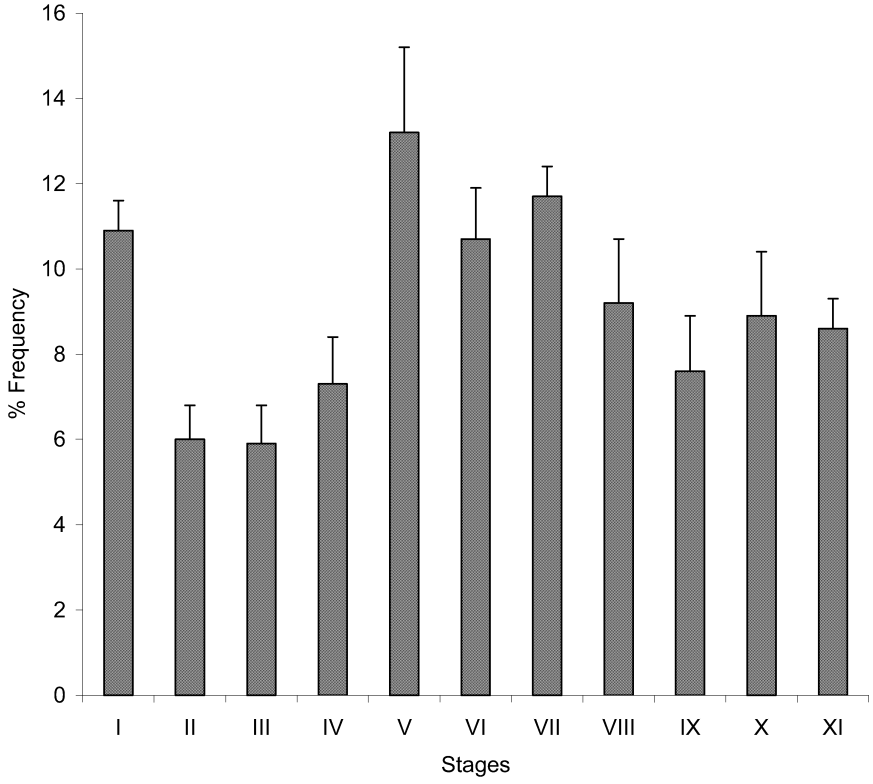

Fig. 13 Relative frequency of stages in the seminiferous epithelial cycle of the *Otolemur garnetti*

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