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Animal Reproduction Science 91 (2006) 307–316

ANIMAL REPRODUCTION SCIENCE

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Cycle and duration of the seminiferous epithelium in puma (*Puma concolor*)

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Received 29 November 2004; received in revised form 1 April 2005; accepted 8 April 2005

Available online 31 May 2005

Abstract

Puma or sussuarana (*Puma concolor*) is the second largest feline in the American continent and has an ample latitudinal distribution in very diverse habitats. In relation to its conservation status, the puma is considered an extinction-threatened species. The study of the testis morphology and the spermatogenic process in a species is fundamental for establishing the physiologic patterns that will make possible the selection of the protocols for assisted reproduction. A number of peculiarities associated with the reproductive biology of specific species such as the duration of spermatogenic process can be used to determine the frequency of sperm collection. Nine adult male pumas maintained in captivity were used to determine the relative frequency of stages in the seminiferous epithelium cycle. Three of them received intra-testicular injections of 0.1 ml tritiated thymidine to determine the duration of the seminiferous epithelium cycle, and were subjected to biopsy 7 days later. The cycle of the seminiferous epithelium in puma was didactically described into eight stages by the tubular morphology method. The total duration of one seminiferous epithelium cycle in puma was calculated to be 9.89 days, and approximately 44.5 days are required for development of spermatozoon from spermatogonia. The duration of spermiogenesis, prophase and other events of meiosis were 14.08,

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0378-4320/$ – see front matter
doi:10.1016/j.anireprosci.2005.04.003

15.20 and 1.79 days, respectively. The relative frequency of the pre-meiotic, meiotic and post-meiotic phases were 3.98, 1.79 and 4.12 days, respectively.

Keywords: *Puma concolor*; Spermatogenesis; Cycle of seminiferous ephitelium

1. Introduction

Puma or susuarana (*Puma concolor*) is the second largest feline on the American continent. It may weigh more than 100 kg and is overcome by only the cougar (*Panthera onca*) in weight, although the equatorially distributed individuals are smaller than those in extreme latitudes (Kitchener, 1991). The latitudinal distribution of Puma is wide, embracing the most diverse habitats from Canada to Chile, including Brazil. (Redford and Eisenberg, 1992). In relation to its conservation status, puma is considered an extinction-threatened species but in some areas of the Atlantic forest, savannah and thorn forest, this species has been practically exterminated.

The presence of predators belonging to the carnivora order may be used as indicator of an ecosystem's state of health because these species occupy the top of the food chain. These animals are co-responsible for the ecological balance in the areas they occupy. In the neotropical area, the felids are the main representatives of this order. The lack of information on the reproductive morpho-physiology in puma (*P. concolor*) and its relative abundance under captive management makes this species a good experimental model for understanding their reproductive biology.

The spermatogenic process occurs in a spatial and chronological manner that is highly organized into associations predetermined among different cellular types. It may be divided into three phases based on morphologic and functional considerations: (1) proliferative reproductive phase (spermatogonia), when the cells undergo rapid and successive mitotic divisions; (2) meiotic phase (spermatocytes), when the genetic material is duplicated and undergo genetic recombination; (3) differentiation or spermiogenic phase (spermatids), when spermatids undergo significant modifications into highly specialized cells that are structurally equipped to reach the site of fertilization and fertilize oocytes, the spermatozoon (Russell et al., 1990).

The spermatogenic cycle of the seminiferous epithelium is referred to as the time period when a given stage is repeated in the same tubular segment. A total of approximately 4.5 cycles are necessary for completion of spermatogenesis, that is, at each repeating cycle the germ cell is under a more advanced phase of the process until the time when a spermatogonium develops into the spermatozoon that is released into the tubular lumen (Paula, 1999).

The duration of the seminiferous epithelium cycle is a species-specific biological constant, which is controlled by the genotype of the germ cell and is not affected by any other known factor (Clermont, 1972; Amann and Schanbacher, 1983).

The study of the testis morphology and the spermatogenesis process in a species is fundamental for establishing the physiologic patterns that will make possible the determination

of the protocols for assisted reproduction. Thus, the duration of spermatogenic process can be used to determine the frequency of sperm collection. These studies also increase our understanding of testis biology in different species (Fawcett et al., 1973). The objective of the present work was to determine the cycle length and duration of spermatogenesis of the seminiferous epithelium of puma (*P. concolor*).

2. Material and methods

Nine adult male pumas from zoos in Distrito Federal, Minas Gerais and São Paulo, were used in this study. The research protocols were approved by the Centro Nacional de Conservação de Felinos Neotropicais (CNCFN), Jundiaí, SP.

The animals were chemically restrained by anesthetic darts and kept under general anesthesia with 10 mg/kg ketamin hydrochloride (Dopalen®-Vetbrand) associated to 1 mg/kg xylazin hydrochloride (Dopaser®-Calier) The procedure was monitored throughout, by measuring the body temperature, respiration, and heart rate at 10 min intervals.

After being anesthetized, the animal was weighed, while the biological materials (hair, skin, blood and feces) and biometric body data were collected according to the protocol of the 'Centro Nacional de Conservação de Felinos Neotropicais'. Before preparation for surgical procedure, testicular width, length and thickness were recorded.

For determining the duration of the seminiferous epithelium cycle, a 1% tritiated thymidine (methyl-^{3}H thymidine, Amersham, Life Science, England) solution was used at a concentration of 1.0 mCi/ml as marker of the spermatogenical-line cells. Specifically, an adult animal was given intra-testicular injections of 0.1 ml tritiated thymidine at three different time points, through a hypodermic syringe, in the caudal extremity of the right testis, which was subjected to biopsy 7 days later.

The testicular biopsy was performed following scrotal trichotomy and local antisepsis. A small skin incision was made, and using a 4 mm diameter skin punch, a fragment from the medium area of the right or left testis was removed. This fragment was carefully manipulated and immediately immersed in glutaric aldehyde (Merck®) 4% in 0.1 M phosphate buffer (pH 7.4) for 2 h; it was then stored under refrigeration at 4 °C in this buffer until processed for histological analysis. The fibrous tunica albuginea and skin were separately sutured, using an absorbable thread (Vicryl®). The animal was then given prophylactic antibiotic therapy.

For light microscopy analysis, the testis fragments were dehydrated in ethanol at increasing concentrations, infiltrated into glycol metacrylate (Historesin, Leica Instruments) and included into the same medium by addition of the hardener (dimethyl sulfoxide). A number of 4 μm-thick sections were obtained, by using a rotary microtome equipped with a glass razor. These sections were placed on glass slides and then stained with 1% toluidine blue–sodium borate solution.

The slides were analyzed using a light microscope for characterizing the eight stages of the seminiferous epithelium cycle according to the tubular morphology method (Berndtson, 1977). The relative frequencies of these stages were computed in approximately 150 transverse sections of the seminiferous tubules randomly observed in each animal.

For identification of the cells marked by tritiated thymidine, sections were subjected to autoradiography as previously described (Bundy, 1995). The histological preparations of the testis fragments subjected to biopsy near the site of tritiated thymidine injection were covered with Kodak NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, NY, USA). The entire procedure was performed in dark room. Slides were soaked in an emulsion, which was maintained as a liquid in a water-bathing at 45 °C. These slides were subsequently conditioned in a light-proof box and stored in refrigerator at 4 °C for 2 months. This duration is considered to be sufficient for sensitization of the silver grains in emulsion by the radioisotope. After this period, the slides were revealed with an aqueous solution (1:1) of the revelator Kodak D-19 for 4 min at 15 °C. Then, the slides were washed in distilled water and fixed in fixer Kodak F5 for 5 min, at environmental temperature. After washing in distilled water for the second time, the slides were dried at room temperature, covered with glass cover slips and analyzed under a light microscope. Based on the position of the most advanced cell with the label after injection, as well as on the relative frequencies of the stages in the seminiferous epithelium cycle, the total duration of the seminiferous epithelium cycle was calculated.

3. Results

The cycle of the seminiferous epithelium in puma was didactically divided into eight stages based on the form, presence and location of the nuclei in the spermatogonia, primary spermatocytes and spermatids, as well as upon stages of the meiotic division, as follows.

3.1. Stage 1

Stage 1 was characterized by the absence of elongated spermatids and presence of a spermatid generation with round and dark nuclei that generally formed three to four layers at the upper part of the seminiferous epithelium. The nuclei of the Sertoli cells exhibited a highly developed nucleolus with approximately 2.5 μm diameter and a flaccid chromatin. Some A-type spermatogonia and the primary spermatocytes under transition from preleptotene to leptotene were observed close to the basal membrane. The spermatocytes in pachytene were located between the round spermatids and spermatocytes in pre-leptotene/leptotene (Fig. 1, stage 1).

3.2. Stage 2

This stage was characterized by presence of spermatids in which the nuclei were at their elongating phase, and directed toward the nuclei of the Sertoli cells. A number of primary spermatocytes in leptotene stage were located near the basal lamina, as well as some primary spermatocytes in pachytene undergoing transition to spermatocytes in diplotene were also observed. The nuclei and nucleoli of the Sertoli cells as well as the A-type spermatogonia had a morphology that was similar to the one observed at the previous stage (Fig. 1, stage 2).

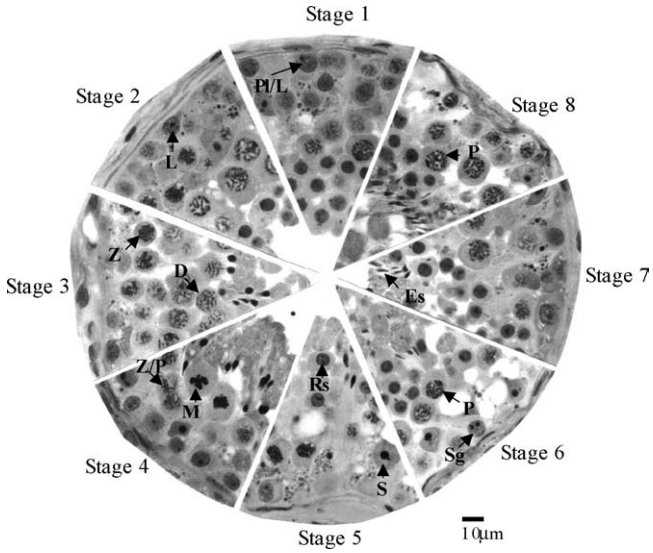


Fig. 1. Photographic mounting showing eight stages of the seminiferous epithelium cycle in puma. Sertoli cell (S); spermatogonia (Sg); primary spermatocyte undergoing transition from pre-leptotene/leptotene (Pl/L); primary spermatocyte in leptotene (L); primary spermatocyte in zygotene (Z); primary spermatocyte undergoing transition zygotene/pachytene (Z/P); primary spermatocyte in pachytene (P); primary spermatocyte in diplotene (D); metaphasic figure (M); round spermatids (Rs); elongated spermatids (El) using toluidine blue.

3.3. Stage 3

A number of elongated spermatids were grouped into bunches composed by a few nuclei. Two generations of primary spermatocytes were present at this stage: spermatocytes in zygotene and diplotene with their characteristically large nuclei. The nuclei of Sertoli cells with their prominent nucleoli and the A-type spermatogonia were observed near the basal lamina (Fig. 1, stage 3).

3.4. Stage 4

The occurrence of two meiotic divisions was the most characteristic aspect at this stage, according to observations made on metaphasic plates. The spermatocytes in diplotene stage generate the secondary spermatocytes, which divide to produce the round spermatids. Some bunches of elongated spermatids and the primary spermatocytes undergoing transition from zygotene to pachytene were also observed. A number of nuclei in the Sertoli cells were similar to those observed at the previous stage (Fig. 1, stage 4).

3.5. Stage 5

Two spermatid generations were present at this stage: the recently-formed round and the elongated spermatids. Although the morphology of the nucleus in the round spermatids

exhibited a smaller diameter, it was similar to that observed in the secondary spermatocytes. Some bunches of elongated spermatids were located in crypts of the Sertoli cells, and the observation of deeper nuclei was not uncommon in the seminiferous epithelium. A number of primary spermatocytes undergoing transition from zygotene to pachytene were observed between the round spermatids and the basal compartment. The presence of some A-type spermatogonia was observed at the base of the tubule. Nuclei of the Sertoli cells with prominent nucleolus generally exhibited their longitudinal axis perpendicularly positioned at the basal lamina (Fig. 1, stage 5).

3.6. Stage 6

All cellular types observed at the previous stage were present, except for the spermatocytes in zygotene. In general, the spermatid bunches were closer to the tubular lumen, which is a characteristic aspect for this stage. Primary spermatocytes in pachytene were laying in the middle region of the seminiferous epithelium. Nuclei of the Sertoli cells and A-type spermatogonia were close to the basal lamina. Some intermediate spermatogonia were also observed at this stage. This cellular type exhibited a lower and darker nucleus, compared to those of the A-type spermatogonia (Fig. 1, stage 6).

3.7. Stage 7

At this stage, the bunched groups of the elongated spermatids were dissociated from each other and located close to the tubular lumen. Some nuclei of the primary spermatocytes in pachytene were observed in the mid-region of the seminiferous epithelium. The B-type spermatogonia were also observed at this stage, presenting either a round or ovoid nucleus and greater content of heterochromatin. The presence of other cellular types was observed at this stage, such as the round spermatids, A-type spermatogonia and Sertoli cells (Fig. 1, stage 7).

3.8. Stage 8

The most characteristic aspect of this stage was the location of the elongated spermatids ready to be delivered from the seminiferous epithelium. Residual bodies were laying in the luminal border of the seminiferous epithelium. Spermatocytes in pachytene, round spermatids, A-type spermatogonia, and Sertoli cells were also observed. Some spermatocytes in preleptotene were observed near the basal lamina (Fig. 1, stage 8).

3.9. Characteristics of seminiferous epithelium cycle

Data for the relative frequency for each stage of the seminiferous epithelium cycle in puma are shown in Tables 1 and 2 with a grouping of these stages at the pre-meiotic (stages 1–3), meiotic (stage 4) and post-meiotic (stages 5–8) phases being observed.

The total duration of the seminiferous epithelium cycle in puma was calculated to be 9.89 days because 1 week after applying the radioisotope the cells at the most advanced stage containing the radioactive marker was the primary spermatocytes in pachytene at

Table 1
Relative frequency and duration of the stages in the seminiferous epithelium cycle in puma

Stages	Frequency (%)[a]	Duration (days)
1	13 ± 3.3	1.29
2	14.4 ± 2.0	1.43
3	12.7 ± 2.6	1.26
4	18 ± 1.8	1.79
5	12.6 ± 4.6	1.25
6	7.9 ± 2.2	0.78
7	8.4 ± 1.7	0.83
8	12.7 ± 2.1	1.26

[a] Mean ± standard deviation, $n = 9$ males.

Table 2
Relative frequency and duration of the pre-meiotic, meiotic and post-meiotic phases of the seminiferous epithelium cycle in puma

	Pre-meiotic phase	Meiotic phase	Post-meiotic phase
Frequency (%)[a]	40.1 ± 4.5	18.0 ± 1.8	41.7 ± 3.9
Duration (days)	3.98	1.79	4.12

[a] Mean ± standard deviation, $n = 9$ males.

stage 5 (Fig. 2) because 70.7% of the whole cycle had already occurred during this period (Fig. 3). Data for the duration of the different stages over the seminiferous epithelium cycle, as well as that of the pre-meiotic, meiotic and post-meiotic phases are included in Tables 1 and 2. Because a total of approximately 4.5 cycles of the seminiferous epithelium is necessary for the spermatogenic process to be completed, approximately 44.5

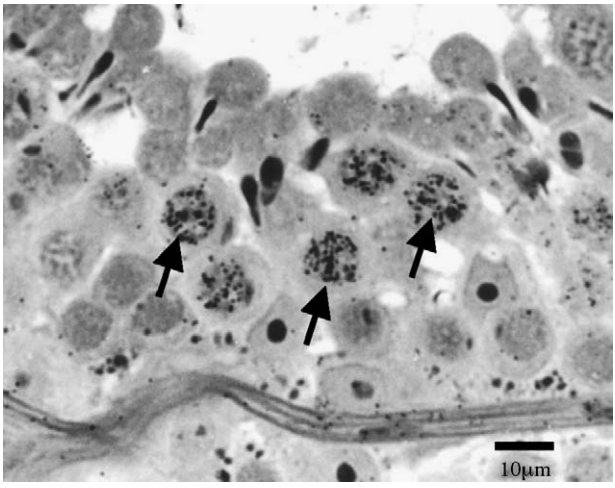
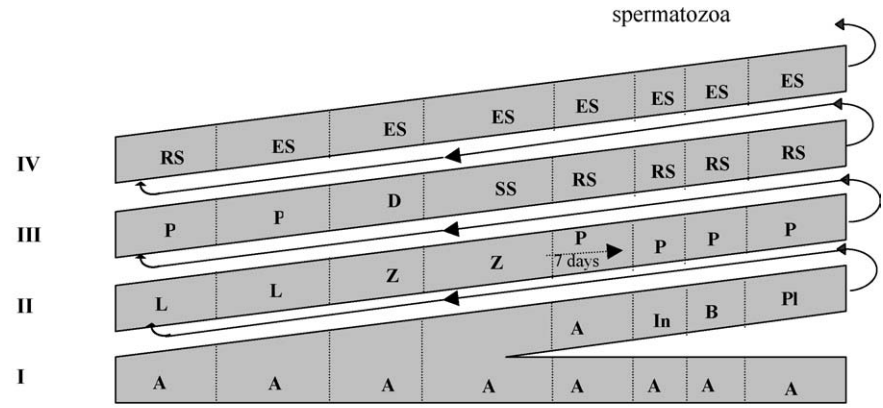


Fig. 2. Primary spermatocytes in pachytene showing the autoradiographic marcation (arrows), in the seminiferous epithelium cycle of the adult puma using toluidine blue.



Stages	1	2	3	4	5	6	7	8
Frequency (%)	13.0	14.4	12.7	18.0	12.6	7.9	8.4	12.7
Duration (days)	1.29	1.43	1.26	1.79	1.25	0.78	0.83	1.26

Fig. 3. Diagram showing the most advanced germ cell (arrow) at the eight stages of the seminiferous epithelium cycle, one week after injection with tritiated thymidine. Roman numbers indicate the spermatogenic cycle. The space given to each column is proportional to the relative frequencies of the cycle stage. Letters in each column indicate the germinative cells characteristic for each stage of the seminiferous epithelium cycle. A-type spermatogonia (A); intermediate spermatogonia (In); B-type spermatogonia (B); primary spermatocyte: in pre-leptotene (Pl); in leptotene (L); in zygotene (Z); in pachytene (P); in diplotene (D); secondary spermatocyte (SS); round spermatid (RS); elongated spermatid (ES).

days are needed for development of the spermatozoon from one spermatogonia. Based on these data, it was possible to estimate that the duration of the events spermiogenesis, prophase and the other meiotic events in the adult puma were 14.08, 15.2 and 1.79 days, respectively.

4. Discussion

The spermatogenic process consists of a series of events occurring in the seminiferous epithelium from the division of the A_1-type spermatogonia until release of spermatozoon into the tubular lumen (Ortavant et al., 1977; Russell et al., 1990). Conceptually, this process is didactically described as a cyclic sequence of ordered successions in cellular associations, or stages occurring over time in a given area of the seminiferous epithelium (Russell et al., 1990; Sharpe, 1994). The cycle of the seminiferous epithelium is referred to as the period of time when a certain stage is repeated in the same tubular segment (Leblond and Clermont, 1952; Clermont, 1972; Berndtson, 1977; Amann and Schanbacher, 1983; Russell et al., 1990; Castro et al., 1997). Approximately 4.5 cycles are needed for the whole spermatogenic process to occur (Fig. 3), that is, at each repeated cycle the germ cell appears at a more advanced phase in this process until the spermatozoon is produced and released into tubular lumen (Paula, 1999).

As mentioned before, the stages of the seminiferous epithelium cycle were classified according to the tubular morphology method (Berndtson, 1977), which is based on the form and position of the spermatid nuclei as well as on the occurrence of the meiotic divisions. All animals used in this study were maintained in an adequate nutritional state, with animals having a typical weight for this species and having a qualitative assessment of the seminiferous epithelium representative of adult animals in a healthy condition. In puma, the spermatogenic process is generally very similar to that described for cat and other species (Godinho, 1999; Russell et al., 1990; De Rooij, 1998).

Usually, just one stage of the seminiferous epithelium cycle was observed in each transverse section of the seminiferous tubule in puma. This observation is similar to the reports available in literature, where except for some primates the arrangement of the seminiferous epithelium cycle stages is a segmented one in all mammals (Sharpe, 1994).

Although the relative frequency of the stages of the seminiferous epithelium cycle is classically considered as a constant for a given species, an individual variation of up to 50% above or below the average value was observed in the present study. In spite of this variation, our observations were in accordance with previous reports by Hess et al. (1990) for rats, França and Cardoso (1998) for swine, França et al. (1999) for caprine and observations made by Godinho (1999) for the cat.

The different stages may be grouped into three phases, using meiosis as a reference point, as follows: the pre-meiotic phase includes spermatid generation and stages of the seminiferous epithelium cycle between the end of spermiation and before meiotic divisions; the meiotic phase includes the stage where two meiotic divisions occur and the secondary spermatocytes are present; and the post-meiotic phase includes the two spermatid generations, and extends from the formation of new round spermatids to spermiation of the elongated spermatids. The values observed in puma for the relative frequency of the pre-meiotic, meiotic and post-meiotic phases approach those found for the domestic cat of 45.5, 17.6, 36.9, respectively (Godinho, 1999), and are similar to other species (Guerra, 1983).

The tritiated thymidine is specifically incorporated into the nucleus of the germ cells synthesizing DNA after injection, specifically the spermatogonia and the primary spermatocytes in pre-leptotene/leptotene at stage 1 of the seminiferous epithelium cycle. Thus, by collecting the testis fragments at specific time intervals after this injection, it is possible to estimate the percentage of the cycle completed, as well as to infer its duration (days) through identification of the marked cells and their stage at the time of collection (Fig. 2). In mammals, the shortest duration of the cycle (6.7 days) generally occurs in the rodent ‘bank vole’ (*Cletheriomys glareolus*) (Grocock and Clark, 1976), whereas the longest ones were for the opossum (17.3 days, *Didelphis albiventris*, Queiroz and Nogueira, 1992) and chinese hamster (17.0 days, *Cricetulus griseus*, Oud and De Rooij, 1977). Among about 30 different mammal species that have been investigated, the duration of the spermatogenic cycle ranged from 10 to 14 days for 60% of them, while in 30% of them this cycle lasted from 7 to 9 days. Thus, the duration of spermatogenesis in the puma is within the range observed for most mammals. In puma, the duration of the seminiferous epithelium cycle as well as that of spermiogenesis, meiotic prophase, and other meiotic phases were similar to those observed in the domestic cat (Godinho, 1999), a fact that appears to corroborate with the phylogenetic proximity theory between the domestic cat and puma (Hast, 1989).

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