Animal Reproduction Science 184 (2017) 102–109

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Full length article

Spermatogenesis in a neotropical marsupial species, *Philander frenatus* (Olfers, 1818)

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ARTICLE INFO

Keywords:
Spermatogenesis
Testis
Philander frenatus
Marsupial
Reproduction

ABSTRACT

Despite the singular morphology of the male genital system and the different reproductive strategies of marsupials, little emphasis has been given to the testis morphology and spermatogenic kinetics in this mammalian order. The present study aimed to investigate the testis function and the duration of spermatogenesis in the southeastern four-eyed opossum, *Philander frenatus*. Testes of six adult males were routinely processed for histological and stereological analyses. In order to determine the duration of spermatogenesis, intratesticular injections of tritiated thymidine were performed 1 h, 13 days and 21 days before the sacrifice. Based on the development of the acrosomic system, ten stages of the seminiferous epithelium cycle were characterized. The mean body and testis weights for the *P. frenatus* were respectively 326 ± 20 g and 0.4 ± 0.05 g, providing a gonadosomatic index of 0.3 ± 0.02%. The most advanced germ cell types labeled at 1 h, 13 days and 21 days after thymidine injections were, respectively, preleptotene spermatocytes at stage IV, pachytene spermatocytes at stage IV and diplotene spermatocytes at stage IX. Based on the stages frequencies and the most advanced labeled germ cells, each spermatogenic cycle and the entire spermatogenic process lasted respectively 13.5 ± 0.5 and 60.9 ± 2.4 days. When compared to the vast majority of eutherian mammals already investigated, these data indicate that the *Philander frenatus* presents a relatively long duration of spermatogenesis.

1. Introduction

The *Philander frenatus* is a nocturnal, semi arboreal member of one of the oldest extant mammal families – the Didelphidae family, inhabiting South American tropical rainforests (Patton et al., 1996; Jansa et al., 2014). Evidences from the literature suggest that this species has a seasonal pattern of reproduction (Cerqueira et al., 1993; Hingst et al., 1998). In fact, neotropical marsupials in general exhibit a seasonal reproduction pattern (Fleming, 1973; Streilein 1982; Cerqueira, 1984; Nogueira, 1988; Cerqueira, 1989, Cerqueira et al., 1993), which seems to be triggered by changes in the photoperiod, food and habitat availability (Cerqueira and Bergallo, 1993; Bergallo and Cerqueira, 1994). Although some studies have been thoroughly conducted in *Didelphis virginiana* describing the sperm pairing in epididymis (Temple-Smith and Bedford, 1980) as well as later events in the female tract leading up to and including fertilization (Rodger and Bedford, 1982a,b), very few studies concerning the reproductive aspects have been performed

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http://dx.doi.org/10.1016/j.anireprosci.2017.07.004
Received 9 February 2017; Received in revised form 4 July 2017; Accepted 10 July 2017
Available online 13 July 2017
0378-4320/

in this marsupial family. Actually, the importance of studying its reproduction (spermatogenesis and oogenesis) has increased since the discovery of natural inhibitors of snake venom in its sera (Moussatché et al., 1979, 1980, 1981; Bastos et al., 2016). Several ophiophagous opossums resist to toxic effects of venomous snakes (Voss and Jansa, 2012). The mechanisms of snake-venom resistance have evolved by complex trophic adaptation and have been the subject of many recent studies (Bastos et al., 2016).

Spermatogenesis is a process in which male haploid germ cells are produced from diploid spermatogonial cells over an extended time period within the seminiferous tubules (Hess and França, 2007). This process is made up of different cellular associations, called stages, which are classified based on the changes in the shape of the spermatid nucleus and its acrosome, the arrangement of spermatids within the germinal epithelium and the occurrence of meiotic divisions (França and Russel, 1998; França et al., 1998). These stages can be defined considering the development of the acrosomic system (Leblond and Clermont, 1952; Leblond and Clermont, 1952; Russel et al., 1990; Hess and França, 2007), or taking into account the tubular morphology (Amann, 1962; Courot et al., 1970; França and Russell, 1998; Almeida et al., 2006; Leal and França, 2008).

The stages progress through precisely timed and highly organized cycles, which are essential for continuous sperm production. It is known that the spermatogenic cycle is dependent upon intrinsic (Sertoli and germ cells) and extrinsic (e.g., androgens and retinoic acids) factors (Hess and França, 2007; Sugimoto et al., 2012; Kent et al., 2016). Furthermore, the length of each spermatogenic cycle and its stages are considered a biological constant for the same species (Clermont, 1972), although variations in the expected composition of cell associations and in the stage frequencies might occur among breeds/strains (Swierstra, 1968; Okwun et al., 1996; França and Russell, 1998). It is also known that in mammals already investigated the total duration of spermatogenesis usually lasts from 30 to 75 days (França and Russell, 1998; Hess and França, 2007) and is under the control of the germ cell genotype (França et al., 1998).

There are very few reports in the literature concerning the reproductive biology in the four-eyed opossum (Nogueira et al., 1984, 1985; Ribeiro and Nogueira, 1990; Hingst et al., 1998; D'Andrea et al., 2007). In this regard, the main objectives of the present study were to characterize the stages of spermatogenesis and to determine the spermatogenic cycle length in the sexually mature *P. frenatus*.

2. Materials and methods

2.1. Animals

Six adult animals, weighing 326 ± 20 g, were captured in a fragment of the Atlantic Forest [Serra do Caraça Private Reserve (20°05′S, 43°29′W), Brazil] during their active sexual time period (June to February). Following orchiectomy, testes were separated from epididymis, weighed and cut in small fragments. These samples were fixed by immersion with 4% buffered glutaraldehyde for 24 h, and then routinely processed and embedded in glycol-methacrylate for histological and autoradiographic analysis. All procedures were in compliance with approved guidelines for the ethical treatment of animals established by our institution (CEUA-UFMG, #94/2008).

2.2. Thymidine injections and autoradiographic analysis

In order to estimate the duration of spermatogenesis, intratesticular injections (75 μCi per testis) of tritiated thymidine [thymidine (methyl-3H), specific activity 82.0Ci $mmol^{-1}$; Amershan Life Science, UK] were administered near the cauda epididymis prior to orchiectomy. Three time intervals were considered after the thymidine injections: 1 h, 13 and 21 days.

For the autoradiographic analysis, unstained testis sections (4 μm) were dipped in the autoradiographic emulsion (Kodak NTB-2, Eastman Kodak Company; Rochester, NY) at 43–45 °C. After drying for approximately 1 h at 25 °C, the testis sections were placed in sealed black boxes and stored in a refrigerator at 4 °C for approximately 4 weeks. Sections were then developed in Kodak D-19 (Eastman Kodak Company; Rochester, NY) solution at 15 °C (Bundy, 1995) and stained with toluidine blue. In order to detect the most advanced germ cell type labelled at the different time periods following thymidine injections, analyses of these sections were performed using an Olympus microscope (BX60). Cells were considered labelled when four or more thymidine grains were present over the nucleus on a low to moderate background.

2.3. Stages of the seminiferous epithelium cycle and duration of spermatogenesis

Seminiferous epithelium cycle stages were characterized based on the development of the acrosomic system and the morphology of the developing spermatid nucleus (Hess and França, 2007). The relative stage frequencies were determined by evaluating at least 250 seminiferous tubule cross-sections per animal at the magnification of 400x. The seminiferous tubules analyzed were chosen at random, and both testes were examined for each animal.

The spermatogenic cycle length was estimated based on the stage frequencies and the most advanced germ cell type labelled at different time periods following the thymidine injections. The total duration of spermatogenesis took into account that approximately 4.5 cycles are necessary for this process to be completed from type A spermatogonia to spermiation (Amann and Schanbacher, 1983; Hess and França, 2007). All data are represented as mean ± SEM.

2.4. Epididymis histological analysis

The entire epididymides (n = 10) of five animals were dissected out and routinely immersed in Bouin's fixative during 24 h. The