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Duration of spermatogenesis in the bullfrog (*Lithobates catesbeianus*)

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Abstract

The bullfrog (*Lithobates catesbeianus*) has substantial economic importance and has also been used as an experimental model for biological studies in the fields of pharmacology, medicine, and reproductive biology, especially studies addressing gametogenesis. However, there is a lack of comprehensive information in the literature regarding testis structure and function in this amphibian. The main objective of the current study was to estimate the duration of the various phases of spermatogenesis in this vertebrate. Sixteen sexually mature bullfrogs received an intracoelomic administration of tritiated thymidine. Testes were analyzed at various times between 1 h and 33 d after administration to detect the most advanced germ cell types labeled at each interval, as well as labeled preleptotene spermatocytes, which presumably originated from spermatogonial stem cells. The duration of the spermatogonial, spermatocytic, and spermiogenic phases of spermatogenesis in the bullfrog were approximately 18, 14, and 8 d, respectively. Thus, the total duration of the spermatogenesis process from early spermatogonia through to spermatozoa was 40 d in this species, similar to that of most previously investigated mammalian species. To our knowledge, this is the first reliable report on the duration of the full spermatogenic process in any amphibian species. These findings will be very useful for tracking the pace of germ cells in studies involving spermatogonial transplantation in lower vertebrates.

Keywords: Bullfrog; Duration of spermatogenesis; Germ cells; *Lithobates catesbeianus*; Testis

1. Introduction

The bullfrog *Lithobates catesbeianus* (Anura, Ranidae) is an amphibian species native to North America that was introduced in South America [1–3] and other regions due to its commercial importance, particularly in the food trade [4]. Moreover, this species is easily kept under laboratory conditions and is used as an experimental model in biomedical research [5] and studies on reproductive biology [6–8].

Similar to other vertebrates, the testicular parenchyma in amphibians consists of two well-defined compartments. The tubular compartment is composed of the seminiferous epithelium, where Sertoli and germ cells are located, and the tubular wall or tunica propria, made up of a basal membrane and peritubular myoid cells. A visible lumen resulting from the fluid secretion of Sertoli cells is also found in this compartment. The intertubular or interstitial compartment contains steroidogenic Leydig cells, connective tissue, blood, and lymphatic vessels [9–11]. As a primitive characteristic of amphibians, the seminiferous tubules are connected to the rete testis by a duct system where sperm are stored [12,13].

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Spermatogenesis is an organized, synchronized process that involves the transformation of diploid spermatogonial cells into haploid spermatozoa [14]. This process is fairly conserved among vertebrates [15,16]. Based on morphofunctional characteristics, spermatogenesis can be divided into three distinct phases: the *spermatogonial* or *proliferation* phase, in which successive mitotic divisions occur; the *meiotic* or *spermatocytic* phase, which involves DNA duplication and genetic recombination; and the *spermiogenic* or *differentiation* phase, in which newly formed round spermatids undergo a dramatic morphologic transformation to form spermatozoa [16–18]. Similar to fish, spermatogenesis in amphibians occurs in cysts within the seminiferous tubules. These cysts are formed when a single spermatogonium is surrounded by Sertoli cells [11,15,19–21].

The time interval necessary for the formation of spermatozoa from a single spermatogonial stem cell is called the duration of spermatogenesis, and knowledge about this parameter is very important in determining reproductive and spermatogenic efficiency [14]. Furthermore, the duration of spermatogenesis is considered a constant for a given species, is not phylogenetically determined, and is under the control of the germ cell genotype [14,22].

There is little information regarding testis structure and function in amphibians, particularly data related to the duration of spermatogenesis. In fact, only for a few amphibian species the duration of premeiotic synthesis of DNA (S phase) as well as the meiotic phase have already been determined [23–26], and the large variation in results were attributed to the great range of temperatures in which the animals were maintained [26]. However, to our knowledge, there are no previous studies concerning the full length of spermatogenesis (including the spermatogonial phase) in any amphibian species. Therefore, the main objective of the current study was to estimate the length of the phases of spermatogenesis in sexually mature bullfrogs. Besides providing baseline information related to reproductive biology for this species, these data are necessary for future investigations addressing spermatogonial germ cell transplantation using bullfrogs as an experimental model.

2. Materials and methods

2.1. Animals

Sixteen sexually mature male bullfrogs (*Lithobates catesbeianus*), approximately 7 mo of age, were obtained from a commercial farm (Ranamig, MG, Brazil) for use in the current study. The animals were maintained under controlled temperature (26 °C) and photoperiod (12:12 h light/dark). All procedures were carried out in compliance with the guidelines for the ethical treatment of animals, and the experimental protocol received approval from the Ethics Committee for Animal Experimentation and Care of the Federal University of Minas Gerais (MG, Belo Horizonte, Brazil).

2.2. Thymidine administrations and tissue preparation

To estimate the duration of spermatogenesis, animals received an intracoelomic administration of tritiated thymidine (Thymidine [methyl–^{3}H], specific activity 82.0 Ci/mmol; Amersham, Life Science, England). Approximately 1 μCi of [^{3}H]thymidine per gram of body weight was used. One animal was used for each time interval considered (1 h to 33 d) after the thymidine administration. Two animals received two thymidine administrations and were killed at different time intervals (Table 1). Prior to death, the animals were anesthetized with thiopental (Thiopentax, 1.0 mL/100 g; Cristália, Itapira, SP, Brazil) and their testes were removed, weighed to obtain the gonadosomatic index (GSI; testis mass divided by body weight × 100), and immersed in 4% buffered glutaraldehyde for approximately 24 h. Testis fragments (2 to 3 mm in thickness) were routinely prepared and embedded in plastic (glycol methacrylate). For the autoradiographic analysis, unstained histologic sections (4 μm) were dipped in autoradiography emulsion (Kodak NTB-2; Eastman Kodak Company, Rochester, NY, USA) at 45 °C. After drying for approximately 1 h at 25 °C, the sections were placed in sealed black boxes containing silica gel as a drying agent and stored in a refrigerator at 4 °C for approximately 3 wk. The testis sections were then developed in Kodak D-19 solution at 15 °C and stained with toluidine blue. Analysis was performed using light microscopy to detect the most advanced germ cell type labeled at the various evaluation times after thymidine administration. Cells were considered labeled when four or more grains were present over the nucleus in a low-to-moderate background. Labeling in the testicular somatic cells was also qualitatively evaluated. The duration of the spermatocytic and spermiogenic phases of spermatogenesis was estimated considering preleptotene spermatocytes as the most advanced germ cell type labeled at 1 h after thymidine administration. Thus, these cells were the reference point until the meiotic divisions (spermatocytic phase)