Ann. Limnol. - Int. J. Lim. 2020, 56, 19
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https://doi.org/10.1051/limn/2020017

Available online at:
www.limnology-journal.org

RESEARCH ARTICLE

Characterization of testicular histology and spermatogenesis in the Levantine frog, *Pelophylax bedriagae* (Amphibia: Anura: Ranidae)

Esra Akat*

Faculty of Science, Biology Department, Zoology Section, Ege University, 35040 Bornova, İzmir, Turkey

Received: 14 April 2020; Accepted: 26 June 2020

Abstract – Amphibians occupy a position of great interest in terms of vertebrate evolution. Additionally, amphibians are known as a transitional group between amniotes and anamniotes. However, there are few studies on the gametogenesis of anamniotes vertebrates, especially anurans. Therefore, the purpose of this study was to analyze the histological feature of germ cells and their arrangement in the testis of Levantine frog, *Pelophylax bedriagae* (Camerano, 1882). Spermatogenic cells were organized in spermatocysts. Each spermatocyst contained cells at the same stage of the spermatogenic cycle. Identification of each cellular type in seminiferous tubule was carried out according to the size and morphology of cells and the degree of nuclear material compaction. Spermatogonia were large cells localized at the base of the seminiferous epithelium. Primary spermatocytes were examined in different phases of first meiotic division and distinguished from other cell types by their dark spherical nuclei or looser chromatin. Two types of spermatids, spherical and elongated cells, were observed. Seminiferous tubules were surrounded by peritubular myoid cells, and they contained no lumen. The lack of lumen in the seminiferous tubules and the cystic spermatogenesis probably provide synchronously production of a large number of sperms. The location of hyaluronic acid was also determined in interstitial tissue between seminiferous tubules to probably provide testicular integrity and viscoelasticity.

Keywords: Amphibian / hyaluronic acid / testis / spermatocyst / gametogenesis

1 Introduction

The Levantine frog, *Pelophylax bedriagae* is widely distributed in the eastern Mediterranean, and in Turkey this species is found throughout the Aegean coast and in the southern part of the Anatolian highlands. Additionally, this species is present on the Island of Rhodes in Greece and in much of Cyprus. *P. bedriagae* is an aquatic frog, and the dorsal side of frog is typically greenish or brownish with large dark spots over the body (Budak and Göçmen, 2008; AmphibiaWeb, 2020).

Amphibians are the first tetrapods, so they have great interest in terms of phylogeny. Additionally, amphibians are transitional group between amniotes and anamniotes, and they are an important part in the success of using key species to discover new information about all animals. Amphibians provide many advantages including a well understood basic physiology, comparative studies because of being animal model (Burggren and Warburton, 2007). However, there are few reports on gametogenesis and structures of the anuran male reproductive system. Testicular histology and gametogenesis of *P. bedriagae* are prominent to understand gametogenesis of anurans, particularly of Palearctic water frogs, for which data remain insufficient.

Glycosaminoglycans (GAGs) are linear carbohydrate polymers, which are composed of repetitive saccharide units. These groups are composed of exchangeable uronic acid and hexamine units. The change in these saccharide units within GAG causes to be formed different GAG groups, such as dermatan sulfate, keratan sulfate, heparin, heparan sulfate, chondroitin sulfate and hyaluronic acid (Kennett and Davies, 2009; Pomin and Mulloy, 2018). Hyaluronic acid (HA) has important functions in many groups from bacteria to vertebrates including human (Almond, 2007). HA has a prominent role in many biological process. It regulates normal structural integrity, development, cell migration, regeneration, cancer formation and resistance (Garantziotis and Savani, 2019). HA is distributed in different parts of body such as connective tissues, the vitreous fluid of eye and synovial fluid to lubricate movable part of body (Kogan *et al.*, 2007). The present study was carried out to describe histological,

*Corresponding author: esra.akat@ege.edu.tr

structural features of germ cells and their organization in testis of *P. bedriagae.* Furthermore, the location of HA in testis was determined to explain its role.

2 Materials and methods

The current study was carried out according to the guidelines for animal research established by Animal Ethics Committee of Ege University (Number: 2018-077). Five adult male specimens of *Pelophylax bedriagae* were captured from Izmir/Turkey (38°30′N 27°17′ E) in April. They were anaesthetized and euthanized by decapitation. Testes were removed by cutting the mesentery (mesorchium), and fixed in Bouin's fluid or 4% paraformaldehyde. They were then transferred in ethanol (70%, 96%, 100%) for dehydration and put into xylol for transparency. Thereafter, the samples were embedded in paraffin following standard histological method. 5 μm-thick deparaffinized sections were stained with Gill's hematoxylin-eosin (HE) and photographed by Zeiss Axio Scope A1 microscope attached to an Axiocam Erc 5s digital camera. When it comes to immunohistochemistry staining, 2% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline was used to block the nonspecific antibody binding sites, and then sections were incubated in biotinylated hyaluronan binding protein (B-HABP). Visualization was performed using streptavidin-fluorescein isothiocyanate (FITC) (Sigma Chemical Company). Sections were examined and photographed by Leica DM3000 microscope with Leica digital camera (DFC290). While negative control was carried out utilization hyaluronidase digested sections, positive control was performed via incubation of sections with B-HABP/HA. Additionally, morphometric analyses were evaluated by measuring the diameter of one hundred cells for each round-shaped germ cell. The measurements were directly made on the sections using micrometric ocular. Values were given as mean ± standard deviation by oneway ANOVA, using SPSS 16.0.

3 Results

The testes of *Pelophylax bedriagae* were located on the ventral region of kidney in abdominal cavity, and the kidneys were closely linked to testes by the mesentery. They were compact, oval in shape and milky-white color.

According to light microscopy examination, the testis was composed of seminiferous tubules and surrounded by tunica albuginea. Contractile cells in the tunica albuginea, peritubular myoid cells, formed a single layer around the seminiferous tubules. Each of them contained germ tissue with many spermatocysts, which were germ cell clusters in the same stage of cytodifferentiation (Fig.1A).

Interstitial tissue localizated between seminiferous tubules consisted of loose connective tissue and blood vessels. Leydig cells (interstitial cells), the testosterone producing cells, were arranged in clusters and embedded in the interstitial compartment of the testis. Efferent ducts were also observed in the interstitial areas (Fig. 1A). Identification of each cellular type in seminiferous tubules was carried out according to the size and morphology of cells and the degrees of nuclear material compaction (Fig. 1B–D). Morphometric analyses were also presented in Table 1.

Spermatogonia were large voluminous cells, which were localized at the base of the seminiferous epithelium (Fig. 1C). They did not form spermatocysts. The spermatogonia undergo mitotic divisions that give rise to a large clonal population of germ cells. After morphological alterations, these cells differentiate into two distinct types of spermatocytes; primary spermatocyte and secondary spermatocyte. Although primary spermatocytes (spermatocytes I) were large cells with different degrees of nuclear material compaction, they were smaller cells than spermatogonia (Tab. 1). Primary spermatocytes were distinguished from other cell types by their dark spherical nuclei or looser chromatin. They were examined in different phases of first meiotic division based on the degree of their chromatin compaction (Fig. 1B and D). Secondary sperma-

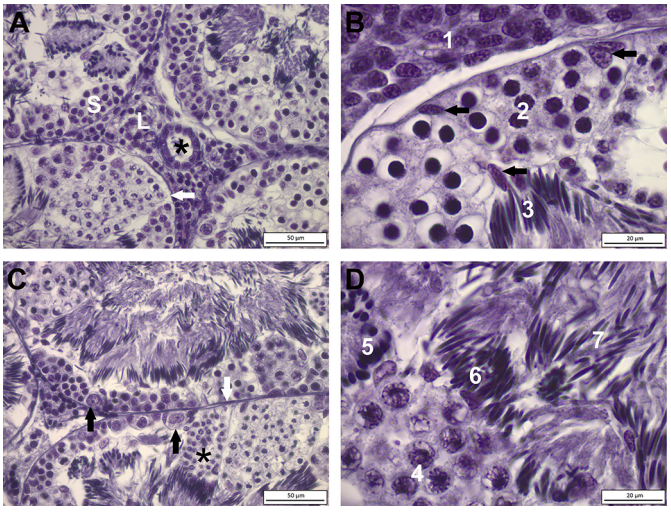

Fig. 1. Cross-sectional view of testis and differentiation stages during spermatogenesis in *P. bedriagae*. A. S: spermatogenic cysts, L: Leydig cells, white arrow: tunica albuginea, asterisk: efferent duct. B. 1: Leydig cells in interstitial area, 2: Primary spermatocytes, 3: Bundle of elongated spermatids. Black arrow: Sertoli cells C. Black arrow: Spermatogonia, white arrow: Peritubular myoid cell, asterisk (*): A spermatocyst containing secondary spermatocytes D. 4: Prophase of the first meiotic division 5: round spermatids, 6: Bundle of elongated spermatids, 7: Spermatozoa.

Table 1. Cell diameters in seminiferous tubules of *Pelophylax bedriagae* testes. Values are given as mean ± standard deviation.

Cell types	Spermatogonium	Primary spermatocyte	Secondary spermatocyte	Round spermatid
Diameter of cell (μm)	19.29 ± 2.34	13.42 ± 1.64	8.12 ± 1.08	3.41 ± 0.64

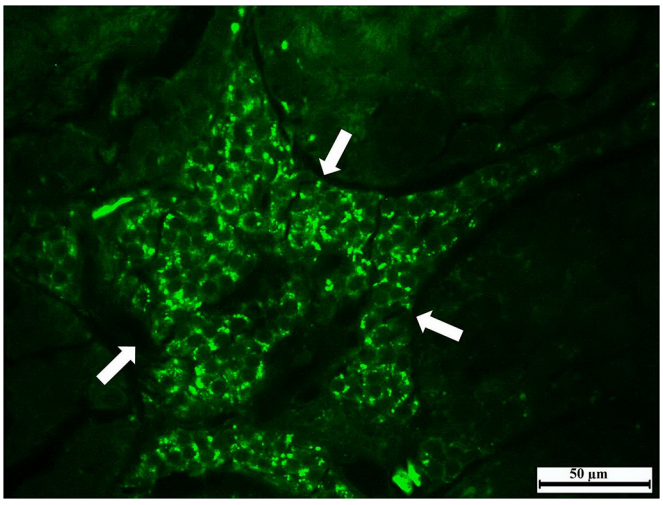

Fig. 2. Cross-sectional fluorescence microscope image of *P. bedriagae* testis. Immunofluorescence localization of hyaluronic acid (HA) in the interstitial area of testis.

tocytes (spermatocytes II) were smaller than primary spermatocytes (Fig. 1C and Tab. 1). The population of spermatids was heterogeneous in terms of their morphology. Two types of spermatids were observed. Spherical spermatids (spermatids I) were rounded cells with a slightly compressed nucleus, other spermatid types (spermatids II) were slightly elongated cells with nuclear elongation and compaction (Fig. 1D). Sertoli cells located within the seminiferous tubules were easily identified in different forms which were pyramid, column or irregular shapes (Fig. 1B).

Hyaluronic acid (HA) immunoreactivity were mainly examined in the interstitial tissue between seminiferous tubules to maintain testicular integrity and viscoelasticity (Fig. 2).

4 Discussion

Amphibians act an important role in the success of using model organism to discover new information related to animals (Burggren and Warburton, 2007). In recent years, amphibians have significant roles in researches on excretory (Droz and McLaughlin, 2017), cardiovascular (Hoppler and Conlon, 2019) and reproductive systems (Calatayud *et al.*, 2019), neurobiology and respiratory physiology (Janes, 2019). The current study describes histological and structural features of spermatogenic cells and their organization in the testis of *Pelophylax bedriagae*. Additionally, the localization of hyaluronic acid (HA) in testis was determined to explain its role.

The testes of *P. bedriagae* were situated on the ventral region of kidney and they were oval-shaped genital organs with milky-white color. However, in some species, such as *Eupemphix nattereri* (Franco-Belussi *et al.*, 2009), *Physalaemus cuvieri* (Oliveira *et al.*, 2002), the testes possess dark coloration due to the abundance of melanocytes in both tunica albuginea and interstitial tissue.

The testes of *P. bedriagae* consisted of seminiferous tubules including a germ tissue with many spermatocysts, inside which spermatogenic cells at the same stage of cycle were observed. Çakıcı (2013) reported the diazinon effects on testes of *P. bedriagae*. Germ cells in cystic organization were also noted in the report. In several other amphibian species, *Rana catesbeiana* (Go and Lee, 2001), *Scinax fuscovarius* (De Oliveira *et al.*, 2003), *Dendropsophus minutus* (De Souza Santos and De Oliveira, 2008), *Bombina orientalis* (Yi and Lee, 2015), *Bufotes variabilis* (Çakıcı, 2015) the cystic spermatogenesis were reported. Additionally, in some fish species, *Acroteriobatus annulatus* (former *Rhinobatos annulatus*), *Sebastiscus marmoratus* the cystic spermatogenesis were examined by Rossouw (2014) and Li *et al.* (2017), respectively. The spermatocysts have been known as the formation of blood-testis barrier, which provides a microenvironment for the maturity of haploid male germ cells (Cavicchia and Moviglia, 1983; Bergmann *et al.*, 1984). However, De Oliveira *et al.* (2003) reported that cystic spermatogenesis immediately provided the beginning of the process after the first mitotic division.

The size and morphology of germ cells and the degrees of nuclear material compaction were used to determination of each cellular type. Spermatogonia were large cells and localized at the base of germinal epithelium, and they did not form spermatocysts. Secondary spermatocytes were smaller cells than primary spermatocytes. While the localization of spermatogonia was near the basal lamina, primary and secondary spermatocytes were examined in the spermatocyst. Similar observations were reported in *Bombina orientalis* and *Bombina bombina* by Yi and Lee (2015) and Rozenblut-Koscisty *et al.* (2017), respectively. However, Li *et al.* (2017) reported that the testes of *S. marmoratus* had numerous spermatocysts including spermatogonia and spermatocytes. Similarly, Rossouw (2014) observed spermatogonia in the spermatocyst. Based on literature data, the cystic spermatogenesis has not been reported in reptile, aves and mammalian until now. Therefore, the cystic spermatogenesis should be considered a special property of anamniotes that cannot be observed in amniotes.

Two different types of spermatids were examined in *P. bedriagae* according to their morphology. Spherical spermatids were round-shaped cells with a slightly compressed nucleus, other spermatid types were elongated spermatids with nuclear condensation and elongation. The round spermatids have round proacrosomal granule close to the nucleus, and a single and simple ciliary structure. During spermiogenesis, these cells gradually become elongated, the proacrosomal granule becomes the acrosome located at the anterior of elongated cell, and ciliary structure converts into flagellum composed of the axoneme (Sáez *et al.*, 1990; 2004). Different types of Sertoli cells were observed in the seminiferous tubules of *P. bedriagae.* They were pyramid, column or irregular shaped. These cells provide essential factors necessary for the spermatogenesis. These primary factors are in the form of physical support, barriers, growth factors or nutrients (Griswold, 1998).

HA is present in some parts of body such as synovial fluid, vitreous humor, movable part of body, brain, skin, muscle, kidney, lung and submucosa of gastrointestinal system (Bernstein *et al.*, 2001; Necas *et al.*, 2008; Akat *et al.*, 2014; Temple-Wong *et al.*, 2016; Ventorp *et al.*, 2016). The HA has prominent biological functions including control

of tissue hydration, water transport and maintenance of viscoelasticity. The HA increases viscoelastic nature of the pericellular environment, forming an extremely malleable extracellular matrix that contributes the cell shape modify necessary for the migration and proliferation of cell (Lee *et al.*, 1993; Toole *et al.*, 2002; Necas *et al.*, 2008). HA immunoreactivity were mainly observed in the interstitial tissue between seminiferous tubules in the testes of *P. bedriagae*. Seminiferous tubules were surrounded by a single layer of peritubular myoid cells in *P. bedriagae*. The contraction of seminiferous tubules helps moving the spermatozoa. Considering the location of HA in testis, it probably helps the contractility of seminiferous tubules via maintaining viscoelasticity of the organ. Based on literature data about the biological functions of HA, it provides testicular integrity and viscoelasticity of testis of *P. bedriagae*.

In conclusion, microanatomical and histomorphological characteristics of the testis in *P. bedriagae* were studied. The histological organization of seminiferous tubules, the main characteristics of different cell types and spermatogenesis were described. This is the first report that demonstrates the presence of HA in amphibian testis and detailed description of the spermatogenesis of *P. bedriagae*. Compared with the studies published to date, results of this study added information for characterization of spermatogenesis of *P. bedriagae.* The lack of lumen in seminiferous tubules and the cystic spermatogenesis probably provide synchronously production of a large number of sperms. HA provides testicular integrity and viscoelasticity of testis.

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Cite this article as: Akat E. 2020. Characterization of testicular histology and spermatogenesis in the Levantine frog, *Pelophylax bedriagae* (Amphibia: Anura: Ranidae). *Ann. Limnol. - Int. J. Lim.* 56: 19