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Variability of testes morphology and the presence of testis-ova in the European blind cave salamander (*Proteus anguinus*)

Variabilnost v morfologiji testisov in prisotnost jajčnih celic v testisih pri proteusu (*Proteus anguinus*)

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Abstract: The European blind cave salamander, *Proteus anguinus,* is a neotenic, permanently aquatic amphibian with a very long life span, late sexual maturity, and the ability to reproduce for at least 30 years. *Proteus* is considered to be vulnerable species, and yet very little is known about its reproductive biology. The objective of this study is to describe the detailed morphology of the testes of adult *Proteus* and determine the maturation state of the gonads and gametogenesis with respect to body size and seasonality. This research showed that testis size increases with adult male body length, but the shape and meiotic condition of the testes are highly variable and independent of the length of the specimen. The testis of *Proteus* has a simple cystic type of organization in which cysts are enclosed in lobules, with synchronous maturation of the germ cells within each cyst. Spermatogenesis progresses in a caudo-cephalic direction within the testis, as in other salamanders, and appears to be seasonal, despite the fact that *Proteus* is a cave animal living in stable environmental conditions. Surprisingly, the testes of approximately one third of the specimens, regardless of their morphology or meiotic condition, contained testis-ova located randomly among groups of spermatogonia and spermatocytes. These testes-ova contained germinal vesicles with lampbrush chromosomes, and thus correspond to primary oocytes. The presence of testis-ova may be related to a sex-chromosome turnover involving X-Y translocation that was recently discovered in *Proteus*.

Keywords: testis, morphology, spermatogenesis, proteus, *Proteus anguinus*

Izvleček: *Proteus anguinus* je neotenična jamska dvoživka z zelo dolgo življenjsko dobo, pozno spolno zrelostjo in reproduktivno sposobnostjo najmanj 30 let. Proteus ima status ranljive vrste, vendar o njeni reproduktivni biologiji pravzaprav ne vemo veliko. Cilj raziskave je podrobno opisati morfologijo testisov odraslih živali proteusa in določiti zrelostno fazo gonad glede na velikost telesa in sezono. Raziskava je pokazala, da se velikost testisov povečuje s telesno dolžino živali, vendar sta oblika in zrelost testisov zelo variabilni in popolnoma neodvisni od velikosti živali. Testisi proteusa imajo preprost cistični tip organizacije, s številnimi lobuli, ki vključujejo ciste s sinhronim zorenjem spolnih celic v vsaki od njih. Tako kot pri ostalih repatih dvoživkah, spermatogeneza poteka v kavdalno - cefalični smeri testisa. Navkljub, da je proteus jamska žival, ki živi v okolju s stabilnimi abiotskimi dejavniki, je videti, da je spermatogeneza v korelaciji s sezono. Presenetljivo je tudi to, da so v testisih proteusa prisotne jajčne celice (testis-ova), in sicer kar pri tretjini proučevanih osebkov ter popolnoma neodvisno od morfologije in zrelosti testisov. Jajčne celice so v lobulih testisa med spermatogoniji ali pa spermatociti. Po morfologiji in krtačastih kromosomov v jedrih ustrezajo primarnim oocitom v jajčnikih proteusa. Prisotnost oocitov v testisih povezujemo z nedavno odkrito preureditvijo spolnih kromosomov proteusa, ki vključuje translokacijo kromosoma Y na kromosom X.

Ključne besede: testis, morfologija, spermatogeneza, cave salamander, *Proteus anguinus*

Introduction

The European blind cave salamander, *Proteus anguinus*, is endemic to underground waters in the Dinaric karst of the Balkan region of Europe. Because of its subterranean habitats, *Proteus* is thought particularly vulnerable to environmental degradation, and thus we need solid baseline studies especially on its reproductive biology. The species is divided into two subspecies, the strongly troglomorphic (i.e. cave-adapted) “white” proteus, *P. a. anguinus*, and the recently discovered (1986), troglobitic but non-troglomorphic, “black” proteus, *P. a. parkelj* (Sket and Arntzen 1994). *Proteus a. anguinus* is geographically the more wide-spread subspecies and is distributed from Italy in the northwest to Montenegro in the southeast. Altogether, almost 250 locations are known, most of them in Slovenia (Sket 1997). The general troglomorphic characteristics include specialization of the sensory organs (e.g. mechano-, electro-, and chemoreceptors), especially of the head, elongation of individual body parts, asymmetric growth of the head and body, degenerate eyes, and skin depigmentation (Bulog et al. 2000, Langecker 2000, Schlegel et al. 2009, Bulog and Bizjak Mali 2014). *Proteus a. parkelj* is different from *Proteus a. anguinus* in having small but otherwise well-developed larval eyes (Kos in Bulog 2001) and dark pigmented skin, and is known only from an area of less than 2 km^2 in the region of Bela Krajina, southeastern Slovenia (Sket 1997, Gorički et al. 2017). Both subspecies of *Proteus anguinus* are obligate neotenes, and their inability to metamorphose is presumably due to the lack of response of target tissues to thyroid hormones (Langecker 2000). Neotenic characteristics are retained in adults, e.g. three pairs of external gills, two pairs of gill slits, an integument with many larval characteristics, and a typical larval visceral skeleton with no maxillary bones (Noble 1931, Langecker 2000). *Proteus* is the longest-lived amphibian, with an estimated lifespan of over 100 years in captivity (Voituron et al. 2011). *Proteus* also has a very low metabolic rate and can cope with long-term starvation (Briegleb 1962, Vandel and Bouillon 1959, Vandel 1965, Hervant et al. 2001, Bizjak Mali et al. 2013). Some of these characteristics of *Proteus*, such as low metabolic rate, large cells, and low rates of growth and development, may be related to its large genome size (Gregory 2001, 2005) which, at approximately 49 billion base pairs, is about 16 times that of humans and one of the largest among salamanders.

Proteus reproduction has been studied over many years in the Cave-laboratory of Moulis, France, revealing extremely long reproductive cycles and delayed sexual maturity in comparison with other amphibians. Females become sexually mature after 15 years at 11-12ºC (or even later, after 17 years, at lower temperature) (Juberthie et al. 1996). Males mature earlier than females, at 11 years. Both mature at a total body length of 140 to 180 mm, but they do not start to reproduce until they reach 200 to 240 mm total length (Durand and Delay 1981) These studies estimate that a *Proteus* female lays eggs at intervals of 6 to 12.5 years, with a total reproductive period that lasts 30 years or even more (Jubertie et al. 1996). *Proteus* has a sex ratio of nearly 2:1 in favor of females (our own observation based on a sample over 100 individuals) but, unlike most other salamanders, *Proteus* males and females are indistinguishable by external morphological criteria, which hampers demographic studies using non-invasive techniques. As in many other salamanders, *Proteus* reproduces via internal fertilization, with the male producing spermatophores (Briegleb 1961). Females lay eggs at any time of the year with a slight preference for winter-time (Jubertie et al. 1996). The large, yolky eggs, each approximately 4-5 cm in diameter, are laid in small clutches of 35 -70 eggs, and are constantly guarded by the female. Embryonic development is slow and lasts 130 days to hatching at 11-12ºC, and the embryonic mortality rates are quite high (≥ 50%) (Juberthie et al. 1996).

We have performed studies of the gonads and gametogenesis in *Proteus* to gain better base-line knowledge of their reproductive biology, with potential applications in conservation issues as well as in the management of their reproduction in captivity. Several of our previous studies were focused on the detailed morphology of the ovaries of *Proteus*, including descriptions of the developmental stages of the oocytes (Bizjak Mali and Bulog 2010, Bizjak Mali et al. 2010, Bizjak Mali and Bulog 2011, Bizjak Mali et al. 2013). These studies showed that ovarian maturation is not seasonal, although it is positively correlated with body length and mass (Bizjak Mali et al. 2010, 2013). However, except for the research of Kezer (1962), who worked on meiotic chromosomes from testes of *Proteus* from the summer season and briefly mentioned testis condition, the detailed external and internal morphology and process of meiosis of the testes of *Proteus* have never been described. The objective of this study was therefore to perform a detailed analysis of the morphological and histological variation of the testes of adult specimens of *Proteus,* and to determine the maturation state of the gonads, including the processes of spermatogenesis and spermiogenesis. From previous studies of the ovaries of female *Proteus* (Bizjak Mali et al. 2010, 2013), I predicted that the condition of the testes, especially size, would be positively correlated with body size (a proxy for age), and that there would be no correlation between reproductive state of the testes and season of the year, as is seen in other urodelan species living in constant temperature environments (Chan 2003, Ogielska and Bartmanska 2009). Our results confirm some of these predictions but not others, and also include the unexpected discovery of a high frequency of testis-ova in male *Proteus*. The significance of these findings is discussed.

Materials and methods

The testes of 16 adult male *Proteus anguinus* were examined. Specimens included both subspecies of *Proteus anguinus* (*P. a. anguinus,* N=11 and *P. a. parkelj,* N=5) collected at different seasons of the year. Seasons of the year were defined as Fall (September –November), Winter (December –February), Spring (March – May), and Summer (June – August). No animals were available for April to June. Six specimens of *P. a. anguinus* were from southwest Slovenia (Planina), and five specimens were from southeast Slovenia (Otovec, Krupa and Grčarske Ravne). Specimens of *P. a. parkelj* were from Jelševnik (southeast Slovenia). The total body lengths of the animals from all localities ranged from 200 to 360 mm, and body weight ranged from 11.4 to 76.9 g.

The testes of *Proteus* analysed for this study were from an archived collection of specimens and tissues of the Department of Biology, University of Ljubljana. The testes had been fixed in 10% buffered formalin, rinsed with water, and stored in 70% ethanol. Some of the testes were already embedded in Paraplast®. The animals were collected for other research purposes with permission of the Ministry of the Environment and Spatial Planning of the Republic of Slovenia. (35701-81/2004-9 and 35601-1/2010-6). Using archived specimens allowed me to perform this study without sacrificing additional animals.

The total length, and the diameter at the widest part of testes were measured. A gonadosomatic index (GSI = [testis weight/total body weight] x 100) was calculated for six archived specimens for which data on testis weight were available (ranging nearly 40-fold from 0.016 to 0.63 g).

Gross morphology and histological structure of the testes were analyzed using stereo and light microscopes. For histology, testes fixed in 10% buffered formalin and stored in 70% ethanol were dehydrated through graded alcohols, cleared in xylene, and embedded in Paraplast®. Serial 5µm-thick sections were stained by Weigert hematoxylin–eosin or Masson Trichrome methods, or Feulgen staining (Humason 1979, Kiernan 1990, Presnell and Schreibman 1997). Meiotic cells were staged according to the size of the nuclei and condensation of chromatin (Uribe 2003). Stages of meiosis were identified as primary or seconday spermatogonia (SgI and SgII), primary or secondary spermatocytes (ScI and ScII), spermatids (Sd), and spermatozoa (Sz). The slides were examined by light microscopy using a Zeiss OPTON-Axioskop and images were captured by a DFC290 HD digital camera (Leica) and LAS 4 program (Leica).

Results

External Anatomy of the Testes

The testes are paired, small, and non-pigmented organs lying parallel to the ventral side of the kidneys, adjacent to the mesonephric or Wolffian ducts and *paramesonephric ducts* (rudimentary Müllerian ducts, a pair of embryonic ducts parallel to the Wolffian ducts that in female develop into the oviducts), and are attached to the dorsal body wall with a dorsal mesorchium. Anteriorly, the testes are attached to the lung via mesentery (Fig. 1A). The position of the testes in *Proteus* is always asymmetrical, with the right testis positioned more anteriorly than the left one.

The testes showed extensive variability in their size and shape. At least four testis morphologies were distinguished in our study (Figs. 1A - D): **1)** simple narrow testis (SNT; n = 8 specimens) (Fig. 1A), which is mostly uniformly narrow, 14 ± 3.5 mm long and 1 ± 0.4 mm wide; in three cases the caudal part was slightly wider (1.25 mm in diameter); **2)** narrow single-lobed testis (NLT; n = 4 specimens) (Fig. 1B), is widest in the posterior half and is narrower at the cephalic and extreme caudal ends with the mean total length 13.3 ± 3.6 mm with a diameter of 3.4 ± 1.0 mm; **3)** broad single-lobed testis (BLT; n = 2 specimens) (Fig. 1C), is uniformly wide over the whole length with short narrow parts at the cranial and caudal end. The mean length of the BLT testes was 14.4 ± 0.6 mm with a diameter of 4.6 ± 1.5 mm; **4)** multilobed testis (MLT; n = 2 specimens) (Fig. 1D), has two, well-developed lobes connected by a narrow region. MLT testes also have narrow regions at the cephalic and caudal ends of the testis, and a much smaller third lobe at the caudal end. The mean length was 30 ± 7.1 mm and a diameter of 3.8 ± 0.4 mm.

The SNT and NLT testes were found in specimens of both subspecies of *Proteus anguinus*, BLT testes were found only in the white subspecies *P. a. anguinus*, and MLT testes were found only in the black subspecies *P. a. parkelj* (Table 1).

Table 1: The morphological forms and stages of spermatogenesis of the testis in both subspecies of *Proteus anguinus*. The Table also includes locality, total body length, months of the capture of animals and presence of testis-ova.

Tabela 1: Morfološke oblike testisov in faze spermatogeneze pri obeh podvrstah proteusa *Proteus anguinus*. Tabela vključuje tudi lokaliteto, dolžino telesa, mesece ulova živali in prisotnost oocitov v testisih.

Locality	**Body length (mm)**	**Month / season**	**Testis forms**	**Testis-ova**	**SgI**	**SgII**	**ScI**	**ScII**	**Sd**	**Sz**
Pap-SE-J	211	jan (W)	SNT[a]	yes	x	x				
Paa-SW-P	246	jul (Su)	SNT[a]	yes	x	x				
Paa-SE-O	210	aug (Su)	SNT[a]	/	x	x				
Paa-SE-K	241	nov (F)	SNT[a]	/	x	x				
Paa-SE-G	240	nov (F)	SNT[b]	/	x	x	x			
Paa-SW-P	248	oct (F)	SNT[b]	/	x	x	x			
Pap-SE-J	200	nov (F)	SNT[b]	/	x	x	x			
Pap-SE-J	253	dec W)	SNT[b]	/	x	x	x			
Paa-SE-O	265	mar (Sp)	NLT	/	x	x	x			
Paa-SW-P	282	mar (Sp)	NLT	/	x	x	x			
Paa-SW-P	210	dec (W)	NLT	yes	x	x	x			
Paa-SW-P	250	sept (F)	NLT	yes	x	x	x	x	x[1]	
Paa-SE-O	280	aug (Su)	BLT	/	x	x	x	x	x[2]	
Paa-SW-P	255	dec (W)	BLT	yes	x	x	x	x	x	x
Pap-SE-J	360	jan (W)	MLT	/	x	x	x			
Pap-SE-J	247	sept (F)	MLT	/	x	x	x			

Legend: Paa – *P.a.anguinus*, Pap – *P.a.parkelj*; SW – south west Slovenia, SE – south east Slovenia; G – Grčarske Ravne, J – Jelševnik, K – Krupa, O – Otovški breg, P – Planina cave; Sp – Spring, Su – Summer, F – fall, W- Winter; BLT – broad single-lobed testis, NLT – narrow single-lobed testis, MLT – multilobed testis, SNT[a] – uniformly narrow simple narrow testis, SNT[b] – simple narrow testis with slightly wider posterior part; SgI – primary spermatogonia, SgII – secondary spermatogonia, ScI – primary spermatocytes in pachyten I, ScII – secondary spermatocytes, Sd – spermatids, Sz – spermatozoa; [1] – early spermiogenesis, [2] – late spermiogenesis.

Legenda: Paa – *P.a.anguinus*, Pap – *P.a.parkelj*; SW – jugozahodna Slovenija, SE – jugovzhodna Slovenija; G – Grčarske Ravne, J – Jelševnik, K – Krupa, O – Otovški breg, P – Planinska jama; Sp – pomlad, Su – poletje, F – jesen, W- zima; BLT – širok testis, NLT – ozek testis z razširitvijo, MLT – testis z več razširitvami, SNT[a] – enakomerno širok ozek testis, SNT[b] – ozek testis z rahlo razširjenim posteriornim delom; SgI – primarni spermatogoniji, SgII – sekundarni spermatogoniji, ScI – primarni spermatociti v pahitenu I, ScII – sekundarni spermatociti, Sd – spermatide, Sz – spermatozoji; [1] – zgodnja spermiogeneza, [2] – pozna spermiogeneza.

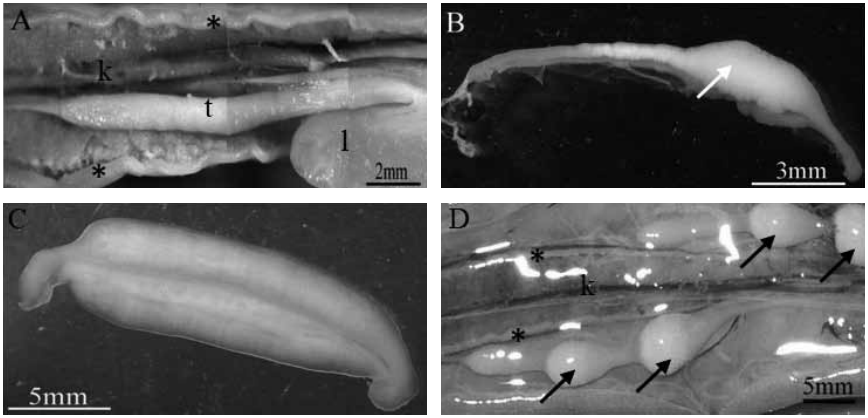

Figure 1: Diversity in the morphology of testes in *Proteus anguinus*. **A** – Simple narrow testis (SNT) of *P. a. anguinus*. **B** – Narrow single-lobed testis (NLT) of *P. a. anguinus*. **C** – Broad single-lobed testis (BLT) of *P. a. anguinus*. **D** –Multilobed testis (MLT) of *P. a. parkelj*. Cranial part of testes in all Figures is on the right. Arrow – lobe, asterisk – Müllerian duct, l – posterior region of lung, k – kidney.

Slika 1: Raznolikost v morfologiji testisov pri proteusu *Proteus anguinus*. **A** – Preprost ozek testis (SNT) pri *P. a. anguinus*. **B** – Ozek testis z razširitvijo (NLT) pri *P. a. anguinus*. **C** – Širok testis (BLT) pri *P. a. anguinus*. **D** –Testis z več razširitvami (MLT) pri *P. a. parkelj*. Na vseh slikah je kranialni del testisov na desni strani. Puščica – razširitev, zvezdica – Müllerjev vod, l – posteriorni del pljuč, k – ledvica.

Testis surface area appears to be positively correlated with total body length (Fig. 2A), although sample sizes are quite small. It is possible that the data points fit a logistic growth curve. The correlation between testis weight with body length is not as pronounced (Fig. 2B), and could also represent a growth curve. Animals less than about 250 mm in length all had very low testis mass and testes were 30-40 times heavier in larger animals.

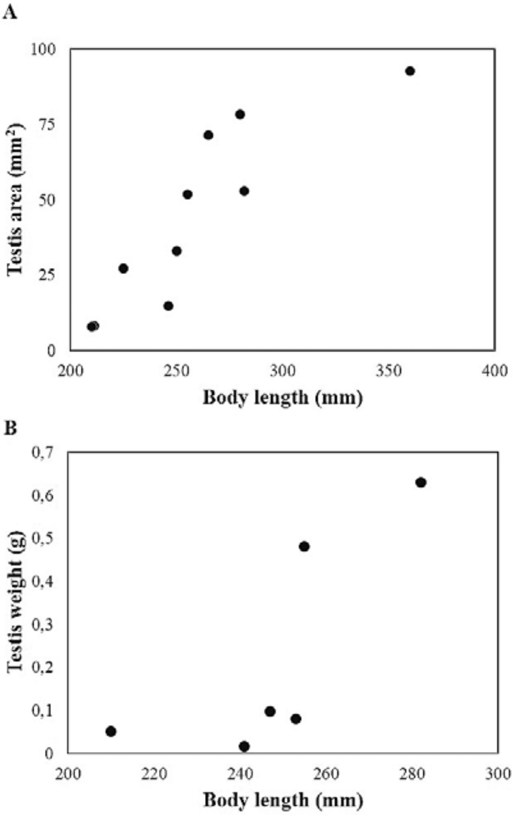

Figure 2: Testis area vs total body length (**A**), and testis weight vs total body length (**B**) in specimens of *Proteus anguinus*.

Slika 2: Površina testisa proti dolžini telesa (**A**), in teža testisa proti dolžini telesa (**B**) pri proteusu *Proteus anguinus*.

Histology of testis and germ cells

The testis is surrounded by a thin epithelium (mesothelium) and connective tissue (tunica albuginea). The internal testis arrangement consists of numerous lobules which communicate with the system of intratesticular ducts. The lobules are delimited by thin walls of loose connective tissue and contain groups of germinal cells within cysts in which spermatogenesis occurs. All of the germ cells within a particular cyst are at the same stage of development.

Spermatogonia

Primary spermatogonia (SgI) are the largest germ cells visible in the testes, around 35 -40 µm in diameter, with very large nuclei. They are round cells with light cytoplasm and round or irregular shaped nuclei with a diameter of approximately 25 µm and containing diffuse chromatin (Fig. 3A). SgI cells are found in groups or individually in the connective tissue at the base of the lobules, located near the midline in the testis. Secondary spermatogonia (SgII) form small clusters of cells, are smaller than SgI, and have heterochromatic (i.e. darkly stained) nuclei (20 µm) (Fig. 3B).

Spermatocytes

Primary (ScI) and secondary spermatocytes (ScII) are recognizable on the basis of nucleus size and condensation of the chromatin. The ScI are spherical and similar in size to SgII. Their nuclei are smaller with a diameter of about 17 µm. ScI can usually be identified by different stages of prophase I (Figs. 3C-D) and very rarely in the other stages of meiosis I (Fig. 3 E). The ScI at pachytene stage are the most abundant, reflecting the fact that this is a very long-lasting stage, whereas the other prophase I stages (leptotene, zygotene, diplotene) are rarely observed. ScII are smaller than ScI (Fig. 3F), with nuclear diameters of 13 to 15 µm, and they are scarce in sections, reflecting the fact that the stages of the second meiotic division, culminating in spermatids, are of relatively short duration.

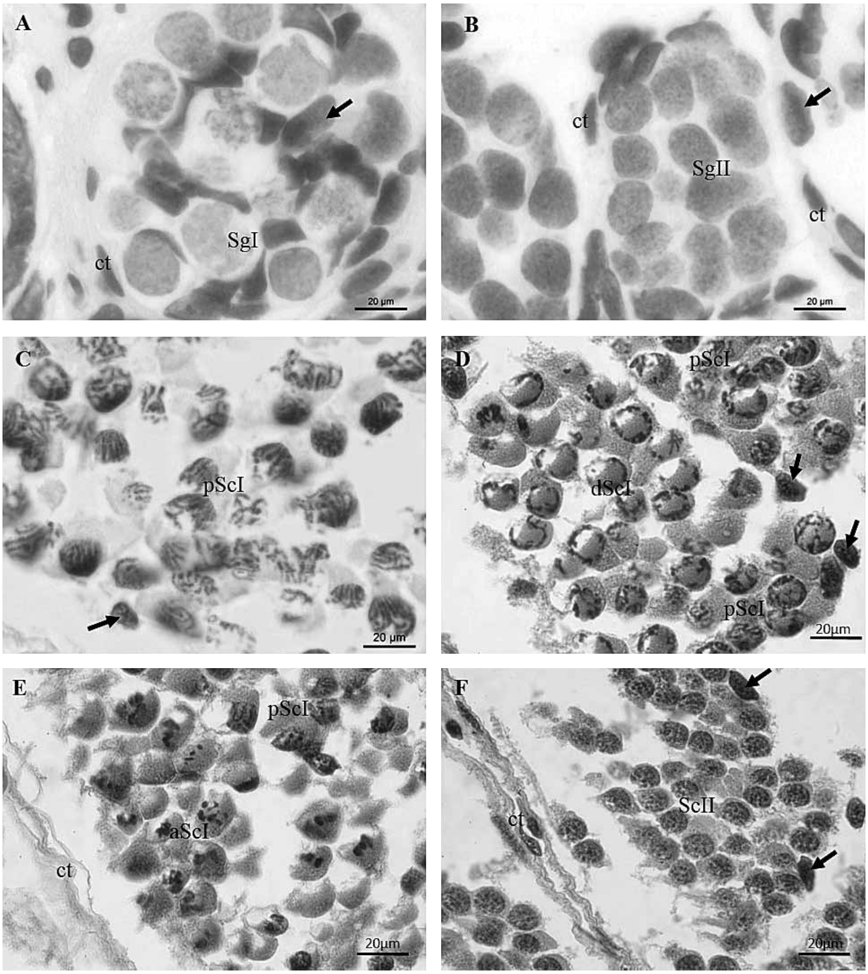

Figure 3: Spermatogonia and spermatocytes in the testis of *Proteus anguinus*. **A** – Group of primary spermatogonia (SgI) in connective tissue of the testis. **B** – Cluster of secondary spermatogonia (SgII) in cyst. **C** – **D** - Primary spermatocytes in pachytene (pScI, **Fig. C**) and diplotene stage of prophase I (dSc, **Fig. D**). **E** - Primary spermatocytes in anaphase I (aScI). **D** – Secondary spermatocytes (ScII). Arrow – nucleus of Sertoli cell, ct – the nuclei of the connective tissue between lobules. Feulgen staining (**A–C**) and H&E staining (**D-F**).

Slika 3: Spermatogoniji in spermatociti v testisu proteusa *Proteus anguinus*. **A** – Skupek primarnih sprermatogonijev (SgI) v vezivnem tkivu testisa. **B** – Gruča sekundarnih spermatogonijev (SgII) znotraj ciste. **C** – **D** – Primarni spermatociti v pahitenu profaze I (pScI, **Fig. C**) in v diplotenu profaze I (dSc, **Fig. D**). **E** – Primarni spermatociti v anafazi I (aScI). **D** – Sekundarni spermatociti (ScII). Puščica – jedro Sertolijeve celice, ct – vezivno tkivo med lobuli. Barvanje Feulgen (**A–C**) in barvanje H&E (**D-F**).

Spermatids

Early spermatids (Sd), immediately following telophase II, have small round, haploid nuclei (10 - 11 µm) and densely packed chromatin (Fig. 4A). The amount of cytoplasm is minute. They gradually transform during spermiogenesis and become progressively elongated with long, thin nuclei (Figs. 4B-D). The centrosome are clearly visible at the posterior end of the nuclei, at the site of emerging flagella (Fig. 4C). The mature spermatids are arranged in bundles and are attached to the Sertoli cells by their apical parts (Figs. 4E-F). During early stages the Sd are enclosed within a cyst, while in later stages the cyst wall disintegrates and spermatids are distributed throughout the lobules.

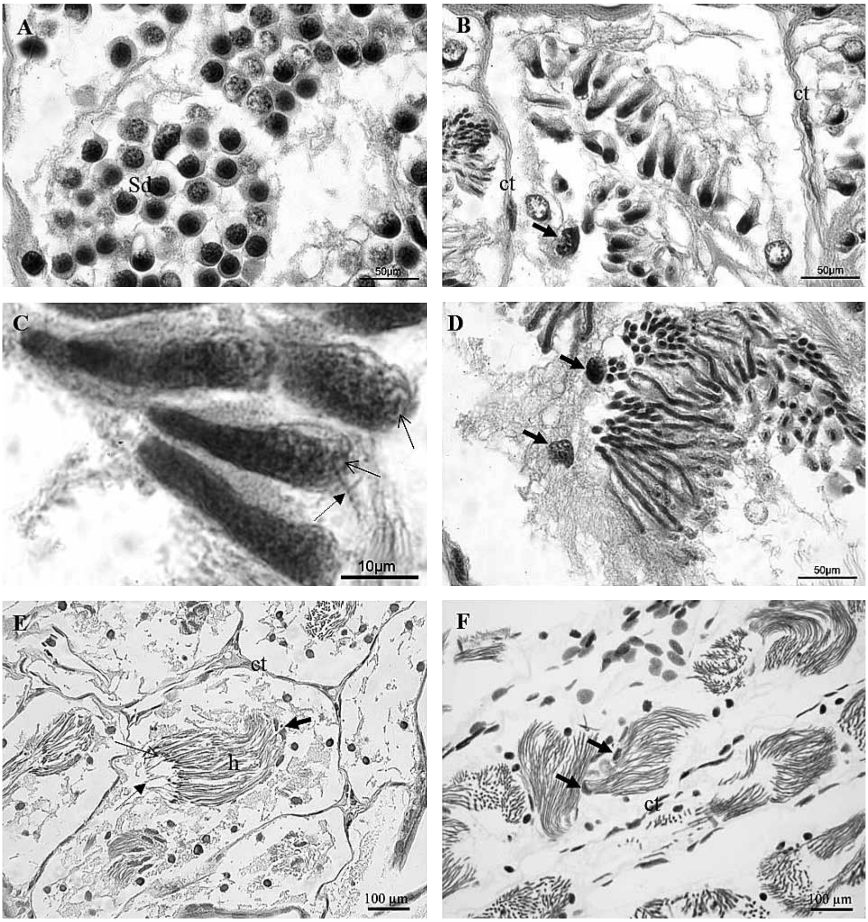

Figure 4: Spermatides in the testis of *Proteus anguinus.* **A –** Early spermatids (Sd). **B –** Stage of elongation of spermatids. **C**- Sd in the elongation process at higher magnification with lighter colored centrioles (open arrow) and protruding flagella at the proximal end of nucleus (closed arrow). **D** – Elongated Sd. **E** – A bundle of mature spermatids. **F** – Feulgen stained nuclei of mature spermatids. Thick arrows – nucleus of Sertoli cell, open arrows – centrosome, closed arrow – flagella, ct – connective tissue between lobules, h – heads of mature spermatids. H&E staining (**A-E**).

Slika 4: Spermatide v testisu proteusa *Proteus anguinus.* **A –** Zgodnje spermatide (Sd). **B –** Faza podaljševanja spermatid. **C**- Spermatida v fazi podaljševanja pod večjo povečavo s svetleje obarvanim centriolom (odprta puščica) in bičkom na proksimalnem koncu jedra (zaprta puščica). **D** – Podaljšane spermatide. **E** – Zrele spermatide. **F** – Jedra zrelih spermatid barvana po Feulgenu. Debele pu**ščice** – jedro Sertolijeve celice, odprte puščice – centrosom, zaprta puščica – biček, ct – vezivno tkivo med lobuli, h – glave zrelih spermatid. Barvanje H&E (**A-E**).

Spermatozoa

Early spermatozoa (Sz) have a distinct, elongated head, midpiece and tail region (Fig. 5A). The darkly stained nuclei are extremely thin and elongated. Spermatozoa belonging to one former cyst are oriented with the heads in the same direction towards the Sertoli cells (Fig. 5A) and gradually elongate and form tighter bundles (Fig. 5B).

The heads of mature sperm are very long (~ 350 µm) and extremely thin. Staining with Feulgen stain showed increased concentration of chromatin at the base of the nuclei (Fig. 5C), and Trichrome staining indicated the presence of a long acrosome at the apical end of the spermatozoan head (Fig. 5D).

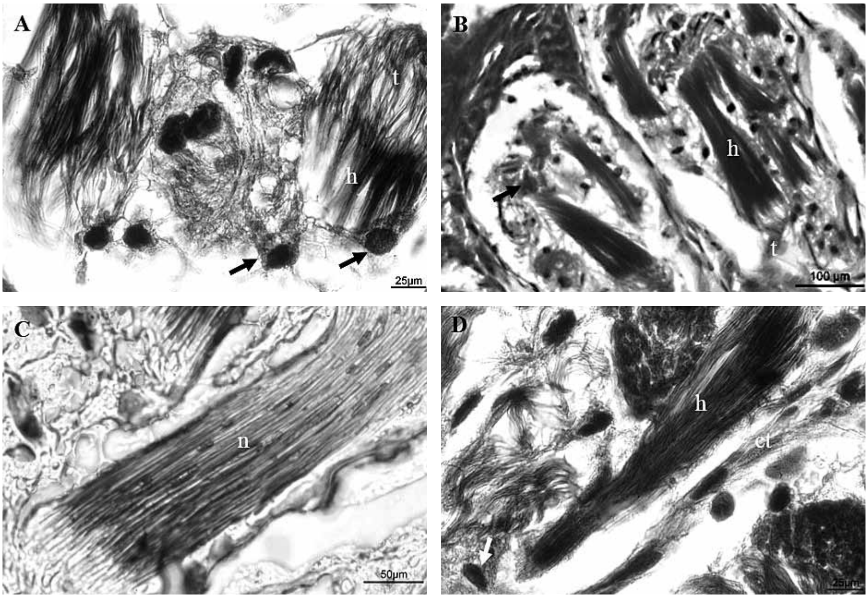

Figure 5: Spermatozoa in the testis of *Proteus anguinus.* **A** – Early spermatozoa. **B** – Mid-stage elongated spermatozoa. **C** – Feulgen stained nuclei of mature spermatozoa. **D** – Apical parts of mature spermatozoa heads with acrosomal vesicles darkly stained with Trichrome staining. Arrows – nucles of Sertoli cell, ct – connective tissue between lobules, h – heads of spermatozoa, n – nuclei, t - tails of spermatozoa. Trichrome staining (**A, B**).

Slika 5: Spermatoziji v testisu proteusa *Proteus anguinus.* **A** – Zgodnji spermatozoji. **B** – Podaljšani spermatozoji. **C** – Jedra zrelih spermatozojev barvana po Feulgenu. **D** – Apikalni deli zrelih spermatozojev s temneje obarvanimi akrosomskimi vezikli (rdeče). puščice - jedro Sertolijeve celice, ct – vezivno tkivo med lobuli, h – glave spermatozojev, n – jedra, t – repi spermatozojev. Trikromno barvanje (**A, B**).

Meiotic Condition of Different Morphological Forms of Testis

The different morphological forms of testes (SNT, NLT, MLT and BLT) have different meiotic conditions (Table 1), which is reflected in their external and internal morphology. Otherwise, all of the testes show the same general histological organization.

In the simple narrow testes (SNT) only the early stages of spermatogenesis, primary and secondary spermatogonia (SgI and SgII), are present, and occasional mitosis is observed among the SgI (Fig. 6A). Lobules are uniform throughout the whole SNT testis. The lobules are small, similar in size, and closely apposed one to the other. The number of cells in the cysts is larger in the caudal part of testis as compared to the cephalic part. In the SNT testes with a wider posterior part, the lobules in this region are larger and a lumen is already formed (Fig. 6B). The walls of the lobules contain very young cysts including

SgII, and the lobules are larger with an increased number of SgII in the cysts at the most caudal part of the enlargement (Fig. 6C). In a few cases the lobules also include spermatocytes (Sc). SgI always lie in the midline of the testis around the intratesticular ducts of the SNT testes.

The narrow single-lobed testes (NLT) and multilobed testes (MLT) contain pachytene stage ScI at the posterior region of the lobe, the lobules are wider, and cysts are not well defined (Fig. 7A). The slightly narrower anterior region of the lobe contains SgII and scarce Sc, and the lobules are elongated with SgI at the base of each lobule (Fig. 7B). The narrow parts at either end in NLT and MLT testes, as well as the regions between the lobes in MLT testes, have the same appearance of lobules as SNT testes (Fig. 6A), and contain only spermatogonia (SgI and SgII).

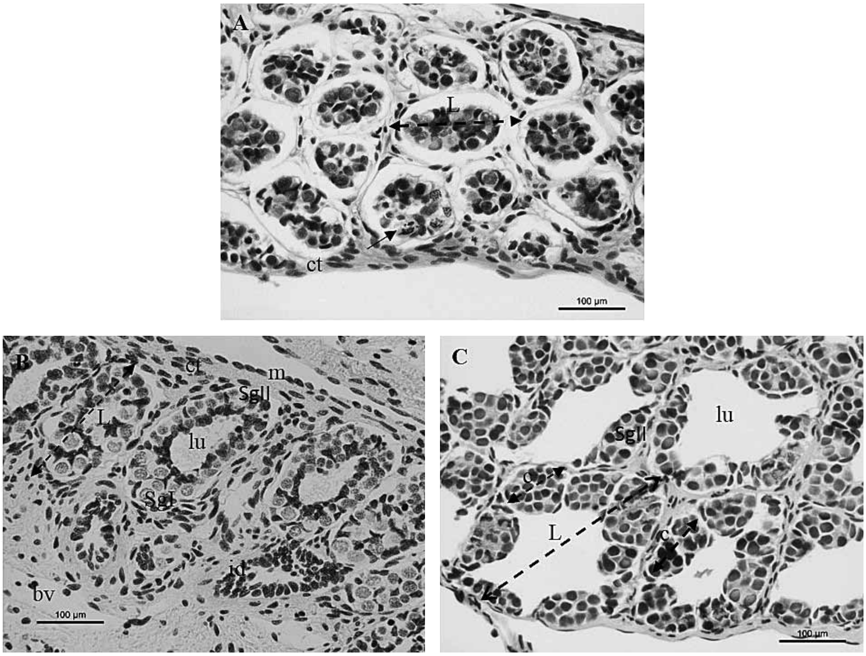

Figure 6: Histology of the simple narrow (SNT) testis of *Proteus anguinus.* **A** – Lobules of an evenly wide SNT testis with early stages of spermatogenesis. **B** - Lobules with cavity in an SNT testis with wider posterior part. **C** – Lobules of the caudal region of an SNT testis with wider posterior part. arrow – mitotic figures in Sg, bv – blood vessel, c – cyst, ct – conective tissue of tunica albuginea, it - intratesticular ducts, L – lobule, lu – lumen of lobule, m - mesothelium. H&E staining.

Slika 6: Histologija preprostega ozkega testisa (SNT) pri proteusu *Proteus anguinus.* **A** – Lobuli z zgodnjimi fazami spermatogeneze v enakomerno širokem testisu (SNT). **B** – Lobuli z lumnom v SNT testisu s širšim posteriornim delom. **C** – Lobuli kavdalne regije SNT testisa s širšim posteriornim delom. „ – mitotične figure v Sg, bv – krvna žila, c – cista, ct –tunica albuginea, it – intratestikularni vodi, L – lobul, lu – lumen lobula, m – mezotelij. Barvanje H&E.

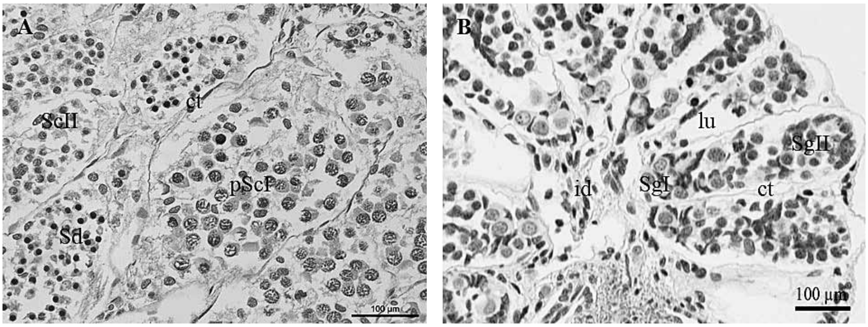

Figure 7: Histology of the lobed testes (NLT and MLT) of *Proteus anguinus.* **A** – Posterior region of a lobe of the testis with pachytene spermatocytes (pScI), secondary spermatocytes (ScII) and early spermatids (Sd). **B** – Anterior region of a lobe of the testis with spermatogonia (SgI and SgII). ct – connective tissue of the lobule, id - intratesticular ducts, lu – lumen of the lobule. Feulgen staining (**A**) and H&E staining (**B**).

Slika 7: Histologija testisov z razširitvami (NLT in MLT) pri proteusu *Proteus anguinus.* **A** – Posteriorna regija razširitve testisa s spermatociti v pahitenu I (pScI), sekundarnimi spermatociti (ScII) in zgodnjimi spermatidami (Sd). **B** – Anteriorna regija razširitve testisa s spermatogoniji (SgI and SgII). ct – vezivno tkivo med lobuli, id - intratestikularni vodi, lu – lumen lobula. Barvanje Feulgen (**A**) in barvanje H&E (**B**).

Broad single-lobed testes (BLT) contain the full range of meiotic stages including spermatids (Sd) and spermatozoa (Sz) (Figs. 8A-C). Spermatogenesis evidently progresses in a caudal-cephalic direction. The cephalic region of the testis contains mostly pachytene ScI (Fig. 8A), the middle region of the testis contains Sc at different stages of meiosis (Fig. 8B), and the caudal region of the testis is packed with cells undergoing spermiogenesis with progressive elongation of spermatids (Fig. 8C). The BLT testes of one animal also contained mature spermatozoa.

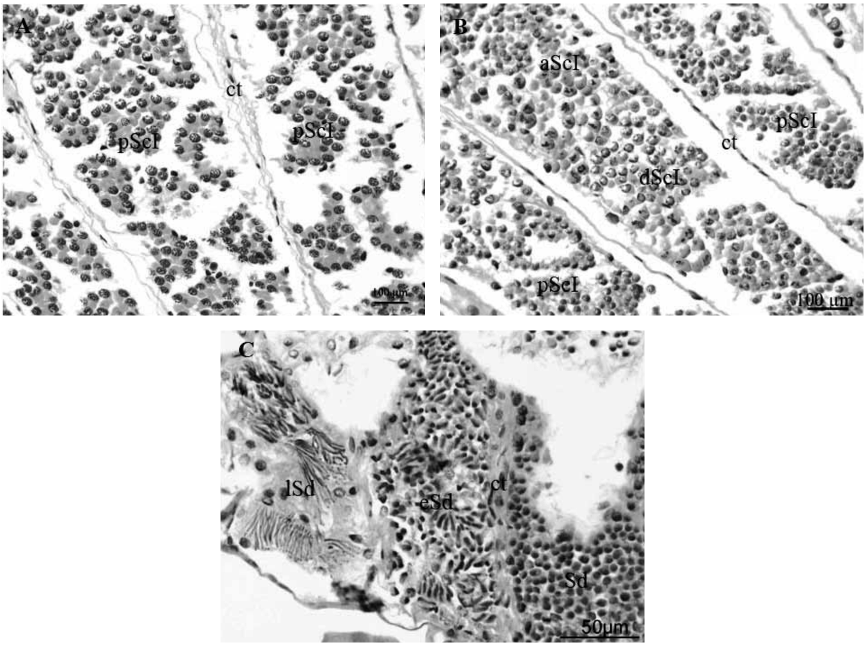

Figure 8: Histology of a broad single-lobed (BLT) testes of *Proteus anguinus.* **A -** The cephalic region of the testis with cysts of pachytene spermatocytes (pScI). **B –** Middle region of the testis with diplotene (dScI) and anaphase spermatocytes (aScI), as well as with first spermatids (Sd). **C**- The caudal region of the testis with evident spermiogenesis. Individual lobules include early spermatids (Sd), elongated spermatids (eSt), late spermatids (lSt). ct – connective tissue between lobules. H&E staining.

Slika 8: Histologija širokega testisa (BLT) pri proteusu *Proteus anguinus.* **A –** Cefalična regija testisa s spermatociti v pahitenu I (pScI). **B –** Osrednja regija testisa z diplotenimi (dScI) in anafaznimi spermatociti (aScI), ter maloštevilnimi spermatidami (Sd). **C**- Kavdalna regija testisa z lobuli v spermatogenezi. Posamezni lobuli vključujejo zgodnje spermatide (Sd), podaljšane spermatide (eSt) in zrele spermatide (lSt). ct – vezivno tkivo med lobuli. Barvanje H&E.

The relative mass of the testes (n = 6), expressed as gonadosomatic index (GSI = [testis weight/total body weight] x 100), appears to be positively correlated with meiotic stages (Fig. 9A); low GSI corresponds to early meiotic stage testes and high GSI to late meiotic stages. Although sample size is very small, this relationship suggests that testis mass increases as a proportion of total body mass as meiosis progresses.

A comparison of meiotic stages in the testes from specimens collected from different seasons of the year (Fig. 9B, Table 1) shows that individuals with immature (SNT) testes containing only Sg stages, were found in all seasons of the year except for early Spring (March). However, no animals were available for late Spring (April and May) and early Summer (June). Individuals with testes containing later stages of spermatogenesis (NLT, MLT, BLT) were found from sequentially later times of the year: spermatocytes were found only in individuals collected from Fall to Spring, and spermatids were found only in individuals collected from Summer to Fall. In our sample, only one individual, collected in Winter, had fully mature (BLT) testes containing spermatozoa (Table 1, Fig. 9B).

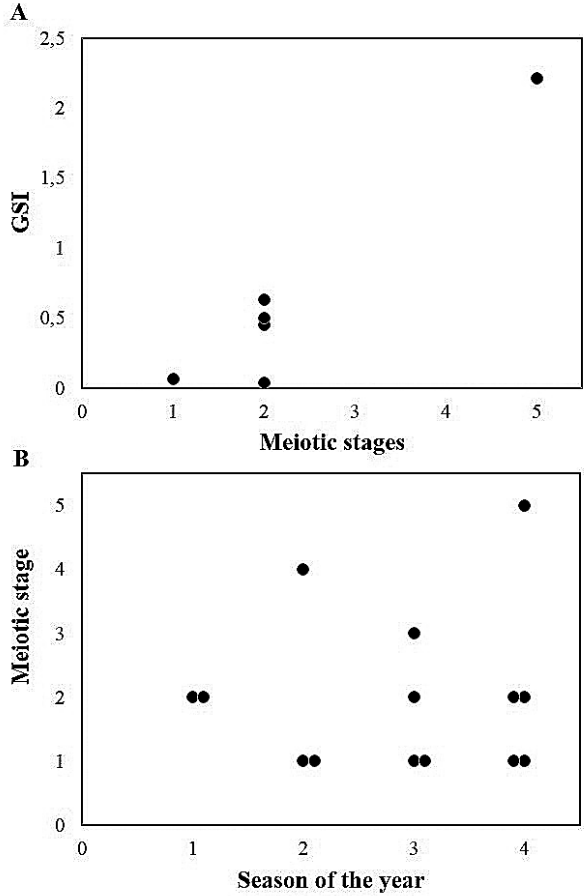

Figure 9: **A -** Correlation of gonadosomatic index (GSI) with meiotic stage in testes of *Proteus anguinus*. **B** – Comparison of meiotic stages of testes of *Proteus* with different seasons of the year. Meiotic stages: 1 – primary spermatogonia, 2 – spermatocytes, 3 – early spermatids, 4 – late spermatids, 5 – spermatozoa. Seasons of the year: 1 - Spring, 2 - Summer, 3 - Fall, 4 – Winter.

Slika 9: **A –** Korelacija med gonadosomatskim indeksom (GSI) in fazami mejoze v testisih proteusa *Proteus anguinus*. **B** – Primerjava faz mejoze v testisih proteusa z sezonami leta. Faze mejoze: 1 – primarni spermatogoniji, 2 – spermatociti, 3 – zgodnje spermatide, 4 – zrele spermatide, 5 – spermatozoji. Letni časi: 1 - pomlad, 2 - poletje, 3 - jesen, 4 – zima.

Testis-ova

Individual oocytes were observed in the testes of *Proteus* regardless of the morphology or meiotic condition of the testes. These testis-ova were found in approximately 30% of the sampled testes (Table 1). They were located randomly among spermatogonia or spermatocytes inside the lobules (Fig. 10A). The testis-ova were at diplotene stage with distinctly visible lampbrush chromosomes and numerous nucleoli (Fig. 10B), and with total cellular diameter betwen 58 and 120 µm, which is consistent with Stage III (early vitellogenic) oocytes in the normal ovary of *Proteus* (Bizjak Mali et al. 2013, 2015).

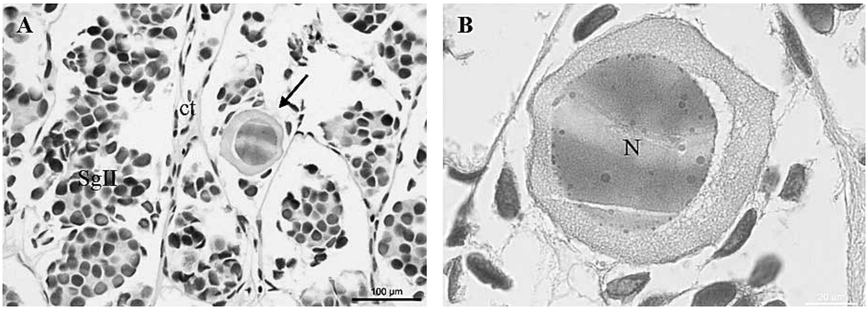

Figure 10: Oocytes in the testis of *Proteus anguinus*. **A** – A testis-ovum (arrow) in diplotene stage of meiosis among the groups of secondary spermatogonia (SgII). **B** – A testis ovum at higher magnification. N – nucleus with lampbrush chromosomes and nucleoli. H&E staining.

Slika 10: Oociti v testisih proteusa (*Proteus anguinus*). **A** – Oocit (puščica) v diplotenu mejoze I med sekundarnimi spermatogoniji (SgII). **B** – Oocit pri večji povečavi. N – jedro s krtačastimi kromosomi in jedrci. Barvanje H&E.

Discussion

The goals of this study of *Proteus* testes were three-fold, first to perform a detailed description of the morphology of the testis and meiotic stages, secondly to determine whether there is any correlation between testes morphology and body size in this long lived salamander, and third, to determine whether spermatogenesis in this cave animal shows any seasonal patterns. Since body length may reflect sexual maturity (age) in salamanders, as we found in female *Proteus* (Bizjak Mali et al. 2010, 2013), I expected to find a positive correlation between testis size and maturation state with body length in *Proteus*. Furthermore, since *Proteus* lives in stable cave environments with no extreme fluctuation in temperature, I also expected that the mature testes would show a non-seasonal pattern of spermatogenesis, as seen in other amphibian species living in constant temperature environments (Chan 2003, Ogielska and Bartmanska 2009), including female proteus (Bizjak Mali et al. 2010, 2013).

Testis morphology and meiotic condition are obviously highly variable in *Proteus*. Even though the sample size is small, the data show that testis size (length and width) is positively correlated with total body length of the animal. However, the meiotic state of the testis (Table 1) is not correlated with body length, and immature testes were found in even one of the larger animals (246 mm in total length). Nor is testes morphology or meiotic state correlated with locality (Table 1). The positive correlation seen between the meiotic state of the testis and testis mass, expressed as proportion of total body mass (GSI), indicates simply that testes increase in mass relative to overall body mass as meiosis progresses.

The basic internal structure of the testes of *Proteus* is similar to that described for other urodele amphibians (Humphrey 1922, De Sa and Berois 1986, Callard 1992, Pierantoni et al. 2002, Uribe 2003, 2009, Flament et al. 2009, Uribe and Mejía-Roa 2014). The interior of the testis of *Proteus* is divided into numerous lobules which includes cysts, with synchronous maturation of the germ cells within each cyst. A single cyst is the primary germ unit of the amphibian testis and is established when the Sertoli cell engulfs two daughter cells derived from a single primary spermatogonium SgI (Callard 1992, Pierantoni et al. 2002). SgI multiply mitotically to give rise to secondary Sg which eventually differentiate more-or-less synchronously to produce meiotic spermatocytes. The ScII then form mature haploid spermatids which elongate and transform into spermatozoa through the process of spermiogenesis. As in other urodeles, spermatogenesis in *Proteus* testes progresses in a caudo-cephalic »wave« along the length of the testis, i.e. the earlier stages of meiosis are found in the more cranial lobules while the later stages are present in more caudal lobules. The reason for this universal pattern is not currently understood. Spermatogenesis in *Proteus* testes also shows a medial-lateral pattern of differentiation in relation to internal collecting ducts in the midline of testes, such that cells form a differentiation series from Sg in the center of the testes to more mature stages found in the peripheral parts. This explains expansion of the testis in diameter over time. Finally, when the spermatogenic process in other urodeles nears completion, most of the testis becomes full of the mature stages of spermatogenesis (spermatozoa). Unfortunately, we do not know if this happens in *Proteus* since the most mature testes we have examined contain these mature stages, including spermatozoa, only at the most posterior part while the middle part is full of Sc in prophase I.

Generally, the spermatozoa of urodeles are longer, including proportionately longer heads, than those of other vertebrates, including other amphibians (Scheltinga and Jamieson 2003, Uribe and Mejía-Roa 2014). The size of the head of *Proteus* spermatozoa is in the range of the sizes of spermatozoa heads in *Necturus maculosus* (338-366 μm) which, with the total length of 1mm, are the longest spermatozoa among the urodeles (Scheltinga and Jamieson 2003). The very large size of the sperm heads in proteid salamanders is probably directly related to chromosome and genome size, which are large in both even compared to other salamanders (Macgregor and Walker 1973, Sessions 2008, Sessions et al. 2016).

In most urodeles, spermatogenesis is initiated cyclically, closely correlated with the seasons of the year (Uribe 2003), and changes in testis size reflect spermatogonial activity with spermatozoa present only before breeding. On the contrary, amphibians living in constant temperature environments show continuous spermatogenesis that does not correlate with seasons of the year (Chan 2003, Ogielska and Bartmanska 2009). In these species, breeding activity is potentially continuous and spermatozoa are present in the testes all year round. Results of this study suggest that, contrary to my prediction, meiotic activity in male *Proteus* shows a seasonal pattern (Table 2), but this pattern is partly obscured by the variability of the meiotic stages seen in the testes between individuals in the sample. Adult males with immature testes full of spermatogonia were found from Summer through Winter. Individuals with spermatocytes in their testes were only found from Fall through Spring, and individuals going through spermiogenesis were found from Summer through Fall. Only one individual, collected in December, had spermatozoa in its testes. Thus, despite this seasonal trend, at any given time of the year it is possible to find adult males with testes representing a wide range of maturation (as noted also by Kezer, 1962). It seems likely that part of the reason for this overlap in meiotic condition is that the rate of spermatogenesis could be very slow in *Proteus*, with at least a two-year cycle in which early stages are always present and later stages only occur from later summer to

Table 2: Interpretation of the sequential meiotic condition of the sampled testes of *Proteus anguinus* correlated with seasons over two years. One meiotic cycle extends from summer of the first year to winter of the second year.

Tabela 2: Interpretacija zaporedja faz mejoze v dvoletnem ciklu pri vzorčenih živalih proteusa *Proteus anguinus*. En cikel mejoze poteka od poletja prvega leta do zime naslednjega leta.

Meiotic stage	Summer	Fall	Winter		Spring	Summer	Fall	Winter
Sg	▨	▨	▨					
Sc		▨	▨	▨	▨			
Sd						▨	▨	
Sz								▨

Legend: Sg – spermatogonia, Sc – spermatocytes, Sd – spermatids, Sz – spermatozoa.
Legenda: Sg – spermatogoniji, Sc – spermatociti, Sd – spermatide, Sz – spermatozoji.

mid-winter (Table 2). Possible explanations for this pattern include low temperature and limited nutrient availability in cave environments, but also its large genome size, which is known to slow down cell cycles including gametogenesis (Gregory 2001). To my knowledge, meiotic rate has never been examined in *Proteus*, but it might help explain why reproductive cycles are so slow in this species.

Detection of seasonality in *Proteus* testes is surprising given the fact that *Proteus* lives in a relatively stable habitat with environmental conditions that are conducive to non-seasonal reproductive cycles. Also, we have already reported that oogenesis in female *Proteus* appears to be non-seasonal (Bizjak Mali et al. 2010, 2013), suggesting that gametogenesis in male and female *Proteus* is only loosely synchronous. This may indicate that courtship and insemination occur weeks or months before oviposition. Fertilization in most species of salamanders, including *Proteus* (Briegleb 1961), is internal via a spermatophore (a package of spermatozoa, produced by the male's cloaca, which is picked up by the female during courtship). In many species, including its closest living relative the North American mudpuppy, *Necturus maculosus*, the sperm may be stored by the female for long periods of time before actual egg laying (Shoop 1965, Sever 2002, Bruce 2003). These issues underline how incompletely we still understand reproduction in this enigmatic animal.

The testes of most urodeles are either simple with a single lobe (NLT), or multilobed (MLT) with a series of enlargements separated by narrow bridges containing only early germ cells (SgI and SgII) (Humphrey 1922, Sever 1974, Pudney 1995, Pierantoni et al. 2002, Uribe 2003, Exbrayat 2009, Flament et al. 2009, Uribe 2009, Uribe and Mejía-Roa 2014). Multilobed testes in urodeles are known in two other salamander familes, the Salamandridae and Plethodontidae. A similar testis structure has been reported for some species of Gymnophiona (De Sa and Berois 1986, Wake 1986, Smita et al. 2004). In urodeles, the lobes develop successively during adult life and each lobe actually has the structural organization of a miniature testis, and the lobes are morphologically and functionally similar (Humphrey 1922, Uribe 2003, 2009). The formation of the lobes of multilobed testes has been studied in detail in the salamandrid, *Salamandra salamandra* (Humphrey 1922), where the number of lobes is positively correlated with age of the salamander. Old males of *Salamandra* can have up to six testis lobes on each side. Likewise, adult specimens of *Triturus vulgaris* may have up to three to four lobes on each side, and a new lobe is developed every second year.

The results of this study show that *Proteus* has both types of testes described for other urodeles, single-lobed testes (NLT) as well as multilobed testes (MLT) (seen only in *P.a.parkelj* in this study, and in *P.a.anguinus* by Kezer, 1962). Such intraspecific variability in the morphological types of testes is also common for other urodeles (Sever 1974), but their interpretation is not always clear. In *Proteus*, the simple narrow testis (SNT) is clearly an immature testis where spermatogenesis has just started (Sg1 and Sg2 and in few cases also early Sc) while the other morphological forms of testis (NLT, BLT, and MLT) represent different maturation states with different stages of spermatogenesis and/or spermiogenesis.

It is interesting that these different testis morphologies, presenting different meiotic states, are not correlated with body length (age) in male *Proteus*. Working at the Moulis cave lab with *Proteus*, Durand and Delay (1981) reported that males become sexually mature at a total body length of 140 to 180 mm, but that they do not start to reproduce until they reach 200 to 240 mm total length. I examined the testes of one of their smaller specimens of known age that was born in that cave lab (151 mm total length, 8 years old) and it had a well-developed NLT testes full of pachytene ScI. The males with immature, SNT testes in my research were substantially larger, in the range of body length between 210 do 253 mm, but their testes were obviously just at the beginning of the spermatogenetic process. These results demonstrate that body size is a poor predictor of reproductive state in *Proteus* males. Instead, we conclude that the testes of *Proteus* males contain the earliest stages of spermatogenesis regardless of body size.

NLT testes with a single lobe were also reported by Kezer (1962) for white proteus and we found them in both subspecies of *Proteus*. The BLT testes, found only in white proteus from two different populations sampled (SW and SE

Slovenia), are similar in overall appearance to the fully formed testes of the other member of the family Proteidae, the mudpuppy *Necturus maculosus* (Pudney and Callard 1984), but much smaller. The simplest explanation for these two sub-types of single-lobed testis (NLT and BLT) in *Proteus* is that they represent early and late stages, respectively, in the normal progression of testis development. In other words, the NLT testes simply enlarge to become BLT testes as the germ cells proliferate and differentiate so that, at least in the white proteus, the testes form a maturation series of SNT-NLT-BLT. The two-lobed MLT testis, which we found only in *P. a. parkelj*, suggests that the series in the black subspecies is ST-MLT. But MLT testes were also reported in white proteus by Kezer (1962), which makes interpretation more difficult. Do the lobes represent individual differences that vary over time, or do the lobes eventually fuse somehow to generate a single-lobed testis? It does not seem plausible that they fuse since each lobe contains an independent, caudal-cephalic series of meiotic stages. On the other hand, fusion of lobes was described for some species of Gymnophiona (Exbrayat 2009, Wake 1986). A better understanding of these differences in morphological forms of testes will require examination of additional specimens of both subspecies of *Proteus*.

Perhaps the most remarkable discovery in this study is the high frequency of testis-ova in the testes of *Proteus*. The morphology of the testis-ova and the presence of lampbrush chromosomes and nucleoli in their nuclei confirm that these are viable, developing oocytes at the diplotene stage of maturation. The testis-ova were observed in 30% of the testes, as well as in all morphological forms of testes described (SNT, NLT, MT, BLT), and were usually located among groups of spermatogonia and spermatocytes. Testis-ova have been described for developing testes in the juvenile stages of some anuran species (Kobayashi and Iwasawa 1976, Ogielska and Bartmanska 1998, 2009, Kobayashi et al. 2014, Lambret et al 2015, Griffing et al. 2017). The presence of testis-ova in testes are usually interpreted as the result of dysfunction of hormonal control (Ogielska and Bartmanska 2009) and has even been linked to environmental endocrine disruptors (Hayes et al 2002, 2011, Hecker et al. 2006, Kosai et al. 2011, Kobayashi et al 2014, Lambret et al 2015). We consider that in *Proteus* these abnormalities might be related to current evidence that *Proteus* has undergone a sex-chromosome turnover involving an X-Y translocation (Sessions et al. 2016). Such a translocation could disrupt the function of genes involved in sex determination through “position effects” (Dimitri and Pisano 1989). In other words, an X-Y translocation can disrupt sex determination and cause gender ambiguity. In clinical cases in humans (Ferguson-Smith 1966), for example, it leads to the same kinds of abnormalities in the ovaries and especially the testes that we are seeing in *Proteus*, especially testis-ova and hermaphrodites (Bizjak Mali, unpublished observation).

In conclusion, considering the vulnerable status of *Proteus*, we need solid baseline studies, especially on its reproductive biology, in order to detect abnormalities that could be induced by environmental degradation. Further work should be done on archived collections to generate additional information about the significance of morphological variation in the testes in *Proteus*, especially in regard to the multi-lobed testes and whether gametogenesis in *Proteus* is really seasonal. It will also be important to determine if there are differences between *P. a. anguinus* and *P. a. parkelj*, since the phylogenetic relationship between these subspecies is not fully understood (Gorički and Trontelj 2006). Finally, in anticipation of the eventual need for a captive breeding program for this endangered species, these data could lead to the development of protocols to induce spermatogenesis and reproduction in *Proteus*.

Povzetek

V raziskavi smo se osredotočili na morfologijo gonad odraslih samcev proteusa *Proteus anguinus*, in zrelost gonad glede na velikost živali in sezono. Raziskava je vključevala testise osebkov obeh podvrst proteusa, troglomorfne bele podvrste *P. a. anguinus* in ne-troglomorfne podvrste *P. a. parkelj*, z razponom dolžine telesa od 210 do 360 mm. Vzorec je vključeval tudi različne populacije bele podvrste *P.a.anguinus,* in sicer iz jugozahodnega dela Slovenije, kot tudi različnih lokalitet jugovzhodnega dela (Tabela 1).

Raziskava je razkrila, raznoliko morfologijo testisov pri proteusu, ki je popolnoma neodvisna od velikosti živali. Zastopane so vsaj štiri morfološke oblike testisov (SNL, NLT, BLT in MLT) (Slika 1A-D, Tabela 1), ki predstavljajo različne zrelostne faze gonad. Preprosti ozki testisi (SNL) so nezrele gonade z zgodnjimi fazami spermatogeneze (primarni in sekundarni spermatogoniji). Imeli so jih tako manjši kot tudi večji samci. Ostale morfološke oblike (NLT, BLT in MLT) so odrasla oblika gonad na različnih stopnjah spermatogeneze. Ozki testisi z enim režnjem oziroma razširitvijo (NLT) in testisi z več razširitvami (MLT) so vključevali večinoma spermatocite in tudi spermatide, široki testisi (BLT) pa so vključevali spermatide in tudi spermatozoje. Vse tri oblike testisov (NLT, BLT in MLT) so bile zastopane pri različnih dolžinah telesa.

Osnovna notranja zgradba testisov proteusa je podobna ostalim repatim dvoživkam. Notranjost testisa je predeljena v številne lobule, ki vključujejo ciste s sinhrono zoritvijo spolnih celic v vsaki od cist. Spermatogeneza poteka v kavdalno – cefalični smeri testisa, z zrelejšimi fazami v kavdalnem delu testisa in zgodnjimi fazami mejoze v cefaličnem delu. Različne morfološke oblike testisov (SNL, NLT, BLT in MLT) se razlikujejo glede na zastopanost faz mejoze, kar se odraža tudi v organizaciji in velikosti lobulov v testisih, in nenazadnje v velikosti in zunanji morfologiji testisov.

Morfološki obliki testisov SNT in NLT smo našli pri obeh podvrstah *P. a. anguinus* in *P. a. parkelj*, in BLT testis samo pri podvrsti *P. a. anguinus.* Testis MLT pa so imeli le osebki črne podvrste *P.a.parkelj*, vendar slednjo omenja tudi Kezer (1962) pri beli podvrsti *P. a. anguinus*. Različne morfološke oblike testisov pri proteusu so lahko odraz progresivnega procesa zoritve spolnih celic. Predvidevamo, da se NLT testis v procesu proliferacije celic in njihove diferenciacije postopamo poveča, kar vodi v oblikovanje BLT testisa. Zaporedje razvoja testisa od SNT preko NLT do BLT je zastopano vsaj pri beli podvrsti *P. a. anguinus*. Pri črni podvrsti *P. a. parkelj* pa je videti, da je zaporedje razvoja in zoritve gonad od SNT do MLT testisa. Zelo verjetno, sta testisa BLT in MLT različna morfološka tipa testisov pri proteusu. Variabilnost v morfologiji testisov v okviru iste vrste je poznana tudi za nekatere druge urodele.

Nasprotno od našega predvidevanja, da spermatogeneza ni v korelaciji s sezono, smo pri samcih našli sezonski vzorec mejotske aktivnosti oziroma spermatogeneze. Nezrele gonade z zgodnjimi fazami spermatogeneze (SgI in SgII) so imeli osebki poletnega obdobja pa vse do zime, spermatociti (Sc) so bili v testisih osebkov jesenskega obdobja pa vse do pomladi, spermiogenezo (proces zoritve spermatid v zrele spermatozoje) so imeli osebki poznega poletnega in jesenskega obdobja, pri osebku iz zimskega obdobja pa so testisi vključevali tudi spermatozoje (Slika 9B). Mejotična aktivnost v testisih preučevanih samcev je zelo raznolika, vendar tudi izrazito prekrivajoča, kar je lahko nenazadnje odraz upočasnjene spermatogeneze z najmanj dve letnim ciklom zoritve spolnih celic (Tabela 2). Slednje bi lahko razložili z nizkimi temperaturami v jamskem okolju, omejeno razpoložljivostjo hrane, vlogo pri tem pa ima najverjetneje tudi velikosti genoma proteusa (slednji je med večjimi v primerjavi z ostalimi urodeli), ki dodatno upočasnjuje celične cikle (Gregory 2001), vključno z gametogenezo. Sezonska aktivnost spermatogeneze pri proteusu je sicer presenetljiva, glede na to, da živijo v okolju z dokaj stabilnimi abiotskimi dejavniki, prav tako pa se zastavlja vprašanje sinhronosti gametogeneze samcev in samic. Možno je, da se paritev in oploditev pri proteusu dogodi tedne ali pa celo mesece pred odlaganjem jajčec, kot je to značilno za njegovega najbližnjega sorodnika severno ameriškega nektura *Necturus maculosus*, in mnoge druge repate dvoživke.

Presenetljivo je tudi odkritje oocitov v testisih proteusa, ki so zastopani pri tretjini preučevanih živali, neodvisno od morfologije in zrelosti testisa. Oociti ali testis-ova so v lobulih testisa bodisi med spermatogoniji ali pa spermatociti. Po morfologiji in krtačastih kromosomih v jedrih ustrezajo diplotenim oocitom v jajčnikih samic in so videti popolnoma viabilne celice. Testis-ova navajajo predvsem za razvijajoče testise juvenilnih osebkov pri nekaterih brezrepcih in jih povezujejo s hormonskim neravnovesjem in endokrinimi motilci v okolju. Pri proteusu predvidevamo, da je za oocite v testisih odgovorna translokacija kromosoma Y na kromosom X, ki je bila nedavno odkrita (Sessions et al. 2016). Translokacija lahko moti delovanje genov za determinacijo spola preko t.i. »pozicijskega učinka«. Npr. pri kliničnih primerih pri človeku povzroča najasnost spola in razvoj različnih abnormalnosti gonad, predvsem prisotnost oocitov v testisih in hermafroditizem, ki je bil nenazadnje najden tudi pri proteusu (Bizjak Mali, neobjavljeno).

Dejstvo je, da je poznavanje reproduktivne biologije pri tej enigmatični dvoživki še vedno nepopolno, in da so nadaljne raziskave neobhodno potrebne, tudi zaradi degradacije njegovega življenskega okolja in prepoznavanja anomalij, saj je proteus zaradi svojih specifičnih prilagoditev na jamsko okolje izredno občutljiva in ranljiva vrsta živali.

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