Morphology and dynamics of testicular gametogenesis in *Sympterygia bonapartii* (Chondrichthyes, Rajidae).

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ABSTRACT. Chondrichthyans constitute a successful group with a long and intricate evolutionary history that makes them highly vulnerable. The smallnose fanskate, *Sympterygia bonapartii* (MÜLLER & HENLE, 1841) is one of the most disembarked items in commercial harbors in Argentina. In this work, the microscopic architecture of mature male gonads and the dynamics of cysts development are analyzed as an interesting tool for understanding the reproductive biology of this specie. Some biological data related to reproduction are given as well. Two seasons were sampled (fall and spring) and length classes' frequency distribution and maturity stages frequency distribution are given. LT_{50} for males was estimated as 58.01 cm of total length. Testes are symmetric, peer, lobated, with several germinal zones. Inside the gonads, there are many spermatocysts, which contain reproductive cells at the same developmental stage. On the basis of their cytological and microanatomical features, seven maturative degrees of the spermatogenic series were differentiated. Few Leydig cells were recognized at the interstitial tissue between cysts. The microscopic quantitative analysis performed in this work provides a promising tool that may contribute to a better knowledge of the reproductive cycles of this economically and ecologically important species.

KEY WORDS: Histology, testis, smallnose fanskate, spermatogenesis.

RESUMEN. Morfología y dinámica de la gametogénesis testicular en *Sympterygia bonapartii* (Chondrichthyes, Rajidae). Los Condrictios constituyen un grupo exitoso de peces, con una historia evolutiva larga y compleja que los hace altamente vulnerables a la sobrepesca. La raya marmorada, *Sympterygia bonapartii* (MÜLLER & HENLE, 1841) es una de las especies de rayas más desembarcadas en los puertos argentinos. Este trabajo analiza la dinámica y arquitectura microscópica de las gónadas de machos maduros, como una herramienta interesante para la comprensión de la biología reproductiva de esta especie. También se aportan algunos datos biológicos relacionados con la reproducción. Se tomaron muestras en dos estaciones (otoño y primavera) y se analizó la distribución de frecuencias de tallas y de estadios madurativos. Se estimó una talla de primera madurez sexual para machos de 58,01 cm de longitud total. Los testículos son pares, simétricos y lobulados, con múltiples zonas germinales. Dentro de los lóbulos hay espermatocistos, cada uno de los cuales contiene células sexuales en el mismo estadio de desarrollo. Se diferenciaron siete grados madurativos de la serie espermatogénica, en base a sus características citológicas y microanatómicas. En el tejido intersticial, entre los cistos, se encontraron células de Leydig. El análisis cuantitativo microscópico llevado a cabo en este trabajo resulta una herramienta interesante, que podría contribuir a un mejor conocimiento del ciclo reproductivo de esta importante especie, desde el punto de vista económico y ecológico.

PALABRAS CLAVE: Histología, testículo, raya marmorada, espermatogénesis.

INTRODUCTION

Chondrichthyes constitute a successful group with a long and intricate evolutionary history (NAYLOR *et al.*, 2005), based mainly on the diversity of reproductive modes that they exhibit, quite different from those of teleost (HAMLETT, 1999). The reproductive strategies vary from the exclusive lecitotrophism, to several degrees of matrotrophism (MUSICK & ELLIS, 2005; GALÍNDEZ *et al*., 2010). On the other hand, this clade is characterized by a low growth, an extensive period of gestation and a scarce number of large offspring, all this resulting in a low reproductive potential that makes this fish highly vulnerable to overfishing (DULVY *et al*., 2008). As a consequence of this, chondrichthyan fisheries have been strongly disturbed around the word (WARD PAIGE *et al.*, 2012). For instance, in Argentina the increase in the Chondrichthyes exploitation has been resulting in a decline of stocks for the last 20 years (TAMINI *et al*., 2006; MASSA & HOZBOR, 2011).

The genus *Sympterygia* is endemic of the Southwest Atlantic (COUSSEAU *et al*., 2000; MENNI & STEHMANN, 2000). The smallnose fanskate, *S. bonapartii*, extends from Río Grande do Sul in Brazil, to the Magallanes strait (FIGUEIREDO, 1977; MASSA *et al*., 2004). Is a relatively medium-size skate, present longwise the coastline of Argentina. This species is oviparous and uses mainly the estuarine waters for mating, giving birth and as a breeding ground (MABRAGAÑA *et al*., 2002; LOPEZ CAZORLA, 2007). In Argentina, this species is one of the most disembarked item in the commercial harbors (MASSA & HOZBOR, 2011) and according to the paucity in the complete knowledge of their reproductive features, the IUCN Red List for threatened species (2014) considers *Sympterygia bonapartii* as “insufficient data for evaluation”. In spite of the economic and ecological importance of this species and the

vulnerability of its actual situation, there are few reports about the morphofunctional aspects of its reproduction.

The aim of this work is to describe the morphology of mature male gonads and the dynamics of cysts development in *S. bonapartii,* as well as providing some information about the life history of this species.

MATERIALS AND METHODS

This study was performed using data collected from two different sources: for histological study, a total of 20 sexually active males of *S. bonapartii* were collected monthly during spring and autumn (March, April, May, October and November) by line fishing in the inner zone of Bahía Blanca estuary (61°30´-62°30´ O y 38°45´-39°20´ S). For morphometric and biological analysis, the specimens were collected during spring and autumn (March, April, May, September, October and November) from artisanal fishery landings at Roucomar processing plant (Ingeniero White port, Bahía Blanca).

The maturity stage of the fish was classified as immature, maturing and mature according to STEHMANN's (2002) criteria. Total length and disc width were measured at lesser millimeter, as well as the clasper length, from the insertion below the pelvic fin to its end, according the COMPAGNO (1984) criteria. Gonad weight (Wg) was recorded to the nearest 0.1g for the specimens from the artisanal fishery. A logistical ogive was fitted to the data using a maximum-likehood approach to determinate size at maturity. The symmetry in gonads weight and claspers length was analyzed using a *t* student statistical test.

Each animal collected for histological study, was sacrificed by a head blow (CICUAE-Prot.022/2014). The dissection of the reproductive system was carried out on board. Small pieces of the gonads were fixed in Bouin's solution in seawater for at least

24 hours. Afterwards, all material was dehydrated through a graded series of alcohols and embedded in Paraplast®. Sections of 5-6 μm thick were stained by Masson's trichromic stain, hematoxilyn-eosin and periodic acid Schiff reaction (PAS). Selected sections were photographed using an Olympus BX51 light microscope equipped with an Olympus C-7070 digital camera. To determine the coverage of each spermatogenic stage, one lobe of the medial region of each gonad was employed to delineate every gametogenic area and thus determine their significance. The analysis was made from dorsal to ventral sides of the testis and employing UTHSCSA ImageTool V. 3.0

RESULTS

A total of 212 males from the artisanal fishery landings, were analyzed. The length classes' frequency distribution and maturity stages frequency distribution in both seasons sampled are shown in **Figs. 1** and **2**, respectively. The largest immature male was 55cm Lt, the largest maturing male was 66,5cm Lt and the smallest mature male was 54cm Lt. LT_{50} was estimated as 58,01cm, corresponding to the 79% of the maximum total length observed in males.

Macroscopic structure of the testis

The internal reproductive organs encompass: the testis, the efferent ducts, the epididymis, the deferens ducts and the seminal vesicles **(Fig. 3)**. Testes are peer, lobated and dorsoventrally flattened organs, enveloped by the epigonal organ, a yellowish lymphomyeloid tissue. There were no statistical differences in testes weight (t Student's test, p= 0.46) and clasper length (t Student's test, p= 0.95).

Microanatomy of the testis

Testes are lined by a simple squamous to cuboidal epithelium and a thin layer of dense connective tissue **(Fig. 4 insert).** This capsule emits irrigated trabeculae that define lobes. In this connective tissue, there is also evidence of the initiation of the ductal system. The lymphomyeloid tissue is represented by a small outline at the posterior surface of the testes **(Fig. 4).**

The testicular parenchyma of mature skates comprises an aggregate of spermatocysts. Each cyst includes reproductive cells at the same stage of spermatogenesis **(Fig. 5)**. New spermatocyst form in a germinal zone, located at the dorsal surface of the lobe. As new spermatocysts form, the old ones move diametrically though the lobe, like an "open fan" **(Fig. 4)**.

Histological features of the spermatogenesis

Both testes include multiple lobules, each of which shows the complete spermatogenic series. The differentiation of sperm takes place within spermatocysts. These are spherical structures defined by a basement membrane and lined by a germ epithelium that includes Sertoli and spermatogenic cells. According the maturative wave progress, germ cells depict not only different cytological features but also a distinctive arrangement within the cyst. On the basis of these characteristics, it is possible to distinguish seven kinds of spermatocysts.

The morphometric characteristics of cells and cysts are shown in **Table I** and **Fig. 6** respectively.

Table II and **Fig. 7** show the average coverage of spermatogenic stages within the testicular lobes, in each sampled season.

Spermatocysts of the Germinal Zone: The germinal zone is a restricted dorsal area where spermatogenesis initiates **(Fig. 5)**. The first step of the spermatogenic testicular process is the spermatogonium, which is distinguished as a rounded cell with large euchromatic nucleus, containing patches of heterochromatin **(Fig. 8)**. Spermatogonia associate with few Sertoli cells, which are medium large cells, of lightly eosinophilic and irregular cytoplasm and with an oval-shaped heterochromatic nucleus with 2-3 nucleoli. At this point, the deposit of the PAS (+) basement membrane that will define the cyst, initiates **(Fig. 8)**. These primary spermatogonia cysts are massive acinar-like structures.

Spermatocysts with spermatogonia: At this point, the basal membrane envelopes completely the group of cells, so that the cysts acquire identity as such. Initially they are unilaminar nests, formed by a single layer of spermatogonia and Sertoli cells interspersed, surrounding an incipient lumen **(Fig. 8)**. As the cysts progress through the lobe, all cells proliferate and the cyst wall stratifies. Spermatogonia are more abundant than Sertoli cells, so that they push the Sertoli cell nucleus toward the lumen **(Fig. 9)**. When cyst acquires several layers, the nucleus of the Sertoli cells locate at their definitive position, adjacent to the basement membrane. At this point the lumen is still present.

Spermatocysts with spermatocytes: As spermatogonia proliferate and differentiate into primary spermatocytes, the cysts increase in size **(Fig. 10a)**. Spermatocytes are rounded, large cells with the distinctive chromatin pattern of the first meiotic division. This process results in spermatocysts containing secondary spermatocytes **(Fig. 10b)**. These are smaller than primary ones, with a heterochromatic nucleus. Sertoli cells dispose abutted to the basement membrane and the lumen occludes gradually.

Spermatocysts with spermatids: The second meiotic division leads to spermatids. They are smaller than spermatocytes, with a round, heterochromatic nucleus. As differentiation progresses, these cells define three different kinds of cysts: those with immature spermatids, where the germinative epithelium completely fills the cyst; bigger cysts with intermediate spermatids which upholsters an incipient lumen **(Fig. 11),** and the cysts with mature spermatids, which group in lax bundles with their body partially embedded in the apical cytoplasm of Sertoli cells. The nucleus of these cells is elongated compared with the immature ones **(Fig. 12)**.

Spermatocysts with immature sperm: Immature sperm cells characterize by their lengthened shape, the high degree of chromatin condensation and the sketch of the spermatic features. They organize in lax bundles attached to Sertoli cells **(Fig. 13)**. These cysts are of similar size than the previous stage.

Spermatocysts with mature sperm: At this stage**,** sperm cells complete their differentiation and organize in discrete packages, embedded in the apical cytoplasm of Sertoli cells. Their spiral-shaped tails arrange toward the lumen. At this point, cysts occupy the periphery of the lobe **(Fig. 14)**.

Degenerate zone: This area is composed by spermatocysts plenty of cellular debris or with few degenerating cells **(Fig. 15).**

Interspersed in the interstitial connective tissue between cysts, there are some cells depicting a large, round nucleus and scarce acidophilic cytoplasm. The nucleus characterizes by the disposition of the chromatin within it: centrally euchromatic and peripherally heterochromatic. Due to these cytological features, these were considered Leydig cells. These are more abundant in the zone of the lobe where cysts with spermatids and sperm are present **(Fig. 16)**.

DISCUSSION

Elasmobranchs inhabit the planet from the Devonian and their survival has been associated, in part, to their reproductive strategies (MC MILLAN, 2007). Curiously, these are the same reproductive features that have leaded them at high risk levels all over the world because overfishing (DULVY & REYNOLDS., 2002; DULVY *et al.*, 2008). Argentina is not an exception to this, so that the local fisheries have shown clear evidences of overexploitation (TAMINI *et al.*, 2006; MASSA & HOZBOR, 2011).

The sizes ranges observed in this work for *S. bonapartii* agree with previous reports of other authors (MABRAGAÑA *et al.*, 2002; ODDONE & VELASCO, 2004; COUSSEAU *et al*., 2007; JAÑEZ & SUEIRO, 2007). On the other hand, the seasonal maturity stages frequency observed in this study is concordant with the reports of MABRAGAÑA *et al.* (2002).

The criteria employed usually to quickly determine the stage of sexual maturity in chondrichthyan males is the length and hardening of claspers. In the smallnose fanskate, the clasper elongation is the first morphologic event in male maturation, followed by clasper calcification and the development of alar thorns (MABRAGAÑA *et al.*, 2002). According to DÍAZ ANDRADE *et al.* (2011), claspers elongation in immature males is exponential. Otherwise, mature males show a linear pattern of clasper growth. The observations made by these authors support the idea that length growth and hardening of the claspers is a valid and simple criteria to determine maturity on board at least for this species. This reproductive pattern has also been reported for other Rajiformes as *Amblyraja radiata* (DONOVAN, 1808) (SULIKOWSKI *et al.*, 2005).

In *S. bonapartii*, as in other Elasmobranchs, epigonal organ is associated to the gonad and is the most important granulopoietic organ (PRATT, 1988; LUTTON &

CALLARD, 2007). Its produces mainly granulocytes, related to innate immunity, as well as lymphocytes associated to adapted immunity (GALÍNDEZ & AGGIO, 2002).

The simetry found regarding to claspers length and gonadal weight has also been observed in other Chondrichthyes (HAMLETT, 1999) and seems to be the rull for this group.

Spermatocyst constitute the structural and funcional unity of male gonads (WALKER, 2005). The production and maturation of spermatocysts in the "cystic testicle" of Chondrichthyes may follow different paths. In the smallnose fanskate, this progression involves the migration of maturing cysts draw away radially from the germinal zone, and through the testis diameter. This model agrees to the "testicular compound model" defined by PRATT (1988) and is also present in other skates and rays. This organization pattern could be interpreted as an adaptation to the " flattened" body scheme of these fish.

PERSONS & GRIER (1992) examined the changes in spermatocysts diameter of *Sphyrna tiburo* (LINNAEUS, 1758), and reported that there was an increase in diameter until the stage of cysts containing secondary spermatocytes. However, in *S. bonapartii* there was an increase in diameter until the cysts with intermediate and mature spermatids, which are the largest ones. Therefore, the cysts where the process of spermiogenesis occurs present a constant diameter.

Results show that, in both seasons, the more represented cystic stage, according to its coverage percentage, was that where differentiation occurs. Even though, the observations made in this work correspond only to two seasons, this preliminary results seems to agree with PERSONS & GRIER (1992) who stated that defining the testicular coverage of each stage is a useful tool to determine a seasonal cycle of activity

in this group of fish, especially when this data is correlated with mating activity observations and gonadosomatic indexes.

Sperm production is a complex, highly organized and coordinated process, involving morphologic and functional interactions between germ cells and more types of somatic cells and their secretions (SKINNER, 1991; PIERANTONI *et al.*, 2009). In cartilaginous fish, the succesives stages of spermatogenesis are arranged in a strict temporal and spatial order, constituting a cystic testicular pattern of organization. This model is cleary different from the seminiferous tubule pattern, and depicts a distinctive model of annamiontes (PIFERRER & CALLARD, 1995; BATLOUNI *et al.*, 2009). According to GRIER (1993), the continue renewing of both, germinal and Sertoli cells, occurs in each succesive spermatogenic cycle or breeding season, and cysts degenerate after spermiation, and this seems to be the case for *S.bonapartii*.

Some Rajiforms, as *Himantura signifier* (COMPAGNO & ROBERTS, 1982) (CHATCHAVALVANICH *et al.*, 2004) and *Dasyatis sabina* (LESUEUR, 1824) (MARUSKA *et al.*, 1996) and other Elasmobranchs , as *Torpedo marmorata* (RISSO, 1810) and *Scyliorhinus canicula* (LINNAEUS, 1758) (STANLEY, 1966), exhibit precursor cells, located in a “germinal papilla” on the dorsal surface of the testis. However, such distinctive papilla and cystic cells do not seem to be present at the germinal zone of *S. bonapartii* testes.

The cytological features of the spermatogenesis in the shortnose fanskate are consistent with that seen from cyclostomes to mammals (HAMLET, 1999). Some authors (GIRARD *et al*., 2000) have observed three different types of spermatids, based on the nuclear shape: immature spermatids with small round nuclei, intermediate spermatids with pyriform nuclei and mature spermatids with large oval nuclei. In *S. bonapartii* spermatids seem to show a different pattern of aggregation, with no

differences in the nuclei shape between immature and intermediate spermatids, but with different arrangement of immature nuclei within the cysts.

SIMPSON & WARDLE (1967) and DOOD & SUMPTER (1984) reported some variations on the localization of this zone according to the season in *Squalus acanthias* (LINNAEUS, 1758) and *Scyliorhinus canicula,* respectively. However, in the smallnose fanskate there were no differences at the degeneration zone localization between sampled seasons.

On the other hand, TESHIMA (1981) has not reported a degeneration zone al all in *Mustelus manazo* (BLEEKER, 1854) and *M. griseus* (PIETSCHMANN, 1908).

The main endocrine functions of the testes have been attributed to the Leydig cells (BANKS, 1992). In this work, few Leydig cells were recognized by its cytological characteristics, not different from those of mammals. Nevertheless, quantitative or endocrine analyses regarding those cells were not the aim of this work.

The unique testicular organization that elasmobranches exhibit, makes them ideal models to analyze the spermatogenesis, as this process is naturally “dissected” in different cysts that act as insolated units, housing only one maturative cellular kind. According to ENGEL & CALLARD (2005), they are also excellent models for understanding germ cell-Sertoli cell interactions, the role of hormones, and effects of environmental toxicants stage-by-stage during the spermatogenic progression.

Usually biological and morphological studies on fish reproduction have focused on female`s gonad and males have been often considered of less importance for this point of view. A deeper study on the use of the techniques referred to this work could indicate that males can offer such valuable information as females. Moreover, in the last years, the histological tools have been recognized in the last years as crucial for

reproductive studies, allowing improvements in the efficacy of management options (ALONSO FERNANDEZ *et al*., 2011).

The microscopic analysis performed in the testes of *S. bonapartii* in this work, provides not only a more accurate information on the male gonad morphology and dynamics, but also has proved to be an interesting tool that may contribute to a better knowledge and care of this important specie.

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FIGURE LEGENDS

Fig. 1. Length class frequency distribution of *Sympterygia bonapartii* males on fall and spring at Bahía Blanca estuary.

Fig. 2. Maturity stages frequency distribution of *Sympterygia bonapartii* males on fall and spring at Bahía Blanca estuary.

Fig. 3. General view of reproductive organs in a mature male of *S.bonapartii*. Only the left size is shown. T:testis; Ep: proxymal epididymus; Ed: distal epididymus;Dd: deferens duct; Sv: seminal vesicle. Scale bar: 1.5 cm.

Fig. 4. General view of a cross section of the testis of *S. bonapartii*. T: testis; Eo: epigonal organ. Oval indicates one testicular lobule and the arrows point the maturative wave direction. Asteriscs point to the cysts. Gz: germinal zone; dz: degeneration zone. Scale bar: 0,5 cm. Insert: medium magnification of the testicular surface. Arrow shows the cuboidal linning epithelium. Scale bar: 15 µm.

Fig. 5. Low magnification of a testicular lobe showing the zonation of the stroma. Gz: germinal zone. Circles show different cysts. Scale bar: 400 µm.

Fig. 6. Minimum and maximum diameter of spermatocyst of each maturative stage. Note that size increases constantly during spermatogenesis and remains constant in the spermiogenesis.

Fig. 7. Average coverage (in percentage) of each lobular zone of the mature testes of *S. bonapartii* during spring and autumn.

Fig. 8. High magnification image of the germinal zone. Arrowheads indicate sparmatogonia, while arrow points to the nucleus of Sertoli cell.Oval shows an unilaminar cyst (uc). Scale bar: 12.5 µm.

Fig. 9. Multilaminar cysts with spermatogonia.Arrow depicts the cysts. Scale bar: 50 µm.

Fig. 10: Spermatocysts containing primary spermatocytes **(a)** and secondary spermatocytes **(b)**. Scale bar: 100 µm.

Fig. 11. Spermatocyst with immature spermatids.Note the incipient lumen. Scale bar: 62.5 µm.

Fig. 12. Spermatocyst with mature spermatids undergoing spermiogenesis. Scale bar: 50 µm.

Fig. 13. Immature sperm. Note the lax bundles of immature sperm with forming patches (arrows). Arrowheads indicate the nucleus of Sertoli cells. Scale bar: 70 µm. Insert shows a detail of lax bundles. Scale bar: 20 µm.

Fig. 14. Mature sperm. The well defined cysts show discrete packages of sperm (arrows). Scale bar: 75 µm. Insert shows the association between bundles and Sertoli cells (arrowhead). Scale bar: 20 µm.

Fig. 15. Degenerate zone. Arrows show spermatocysts with cellular debris. Scale bar: 200 µm.

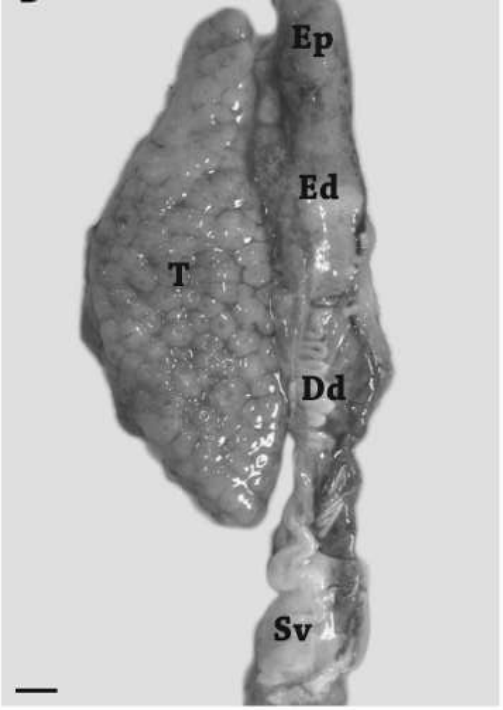
Fig. 16. High magnification picture of the interstitial tissue between cysts. Arrow points to a Leydig cell. Scale bar: 7.5 µm.

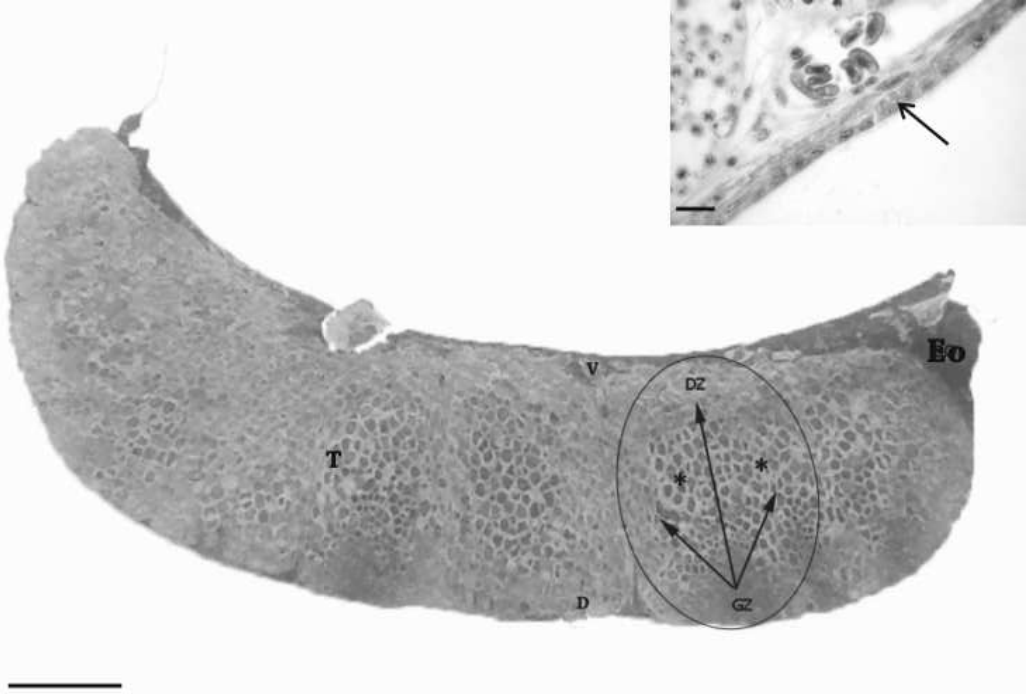
Celltype	Cellsize (µm)	Nuclear diameter(µm)
Spermatogonia	12,00 - 15,00	7,00 - 10,00
Primaryspermatocytes	5,00 - 12,50	3,75 - 7,50
Secondaryspermatocytes	4,00 - 7,50	2,50 - 3,75
Spermatids	2,50 - 3,75	1,50 - 2,50
Immaturesperm	ND	12,5 - 15,00 (head)
Maturesperm	30,00 - 40,00 (taillength)	15,00 - 20,00 (head)

Tab.I: Size and nuclear dimensions of maturativespermatogenic cells. ND: not determined

Lobulezone	Average		
	Spring	Fall	
Germinal zone	1,2	0,5	Mitosis and meiosis
Immaturespermatocytes	10,3	15	
Primary and secondaryspermatocytes	28,5	12,8	
Spermatids	16,6	30,8	
Sperm (immature and mature)	10,6	18,6	Differentiation
Degeneratezone	32,8	22,3	

Tab. II: Average coverage of each lobule zone, in percentage, during spring and autumn.





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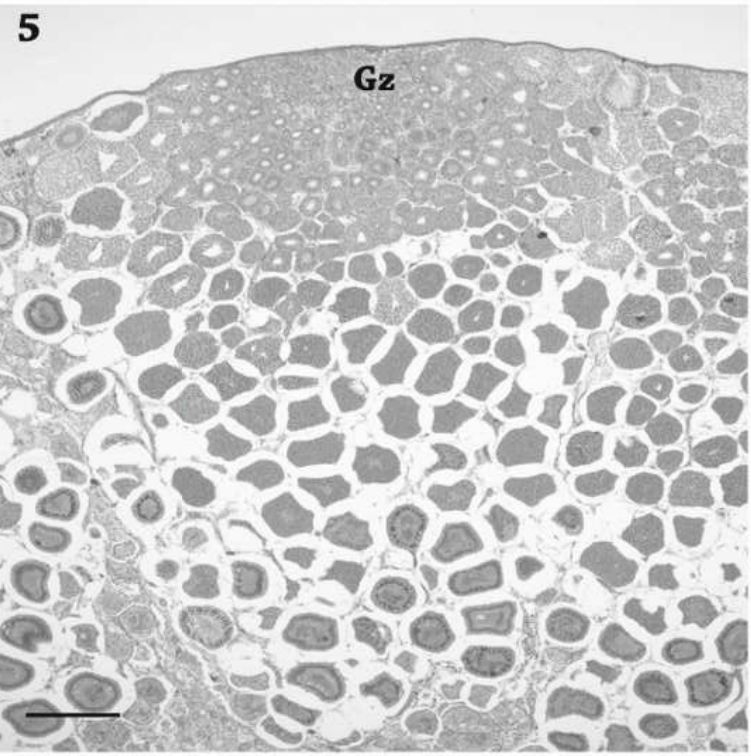

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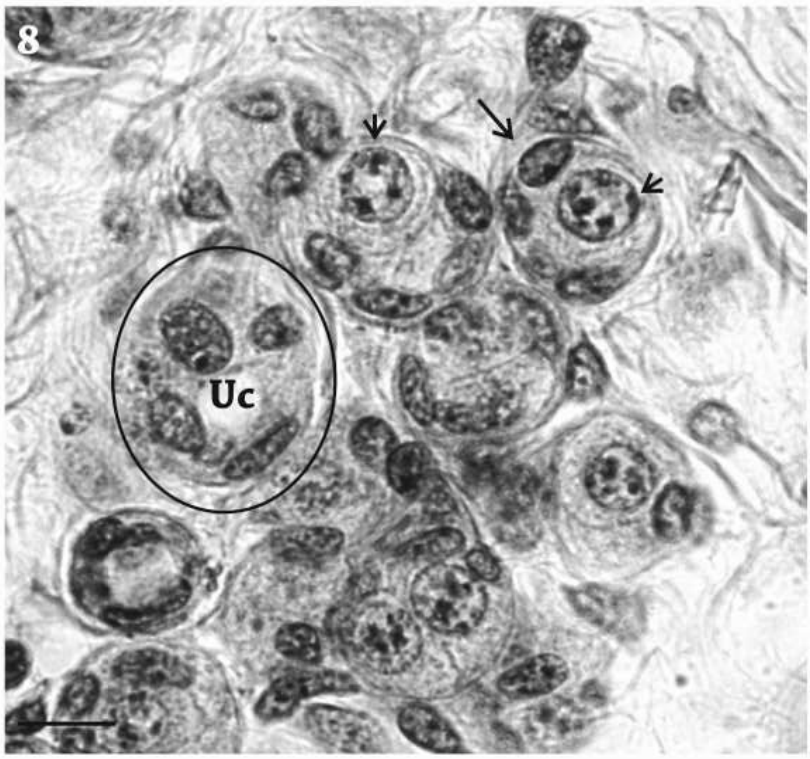

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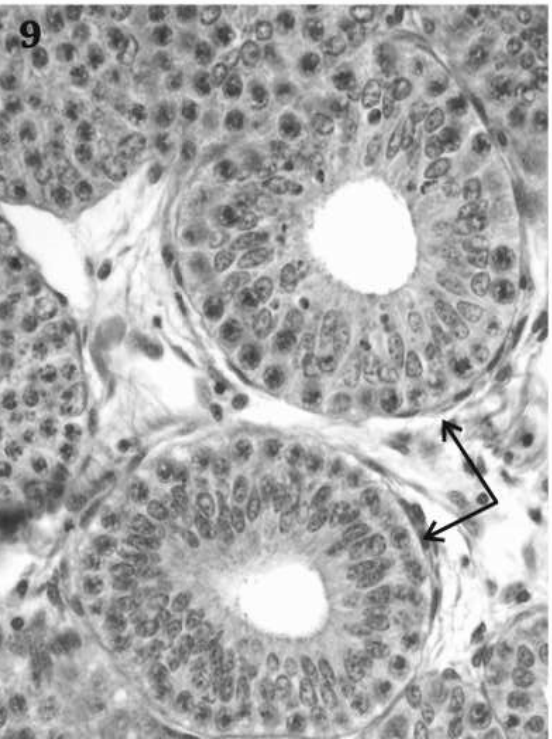

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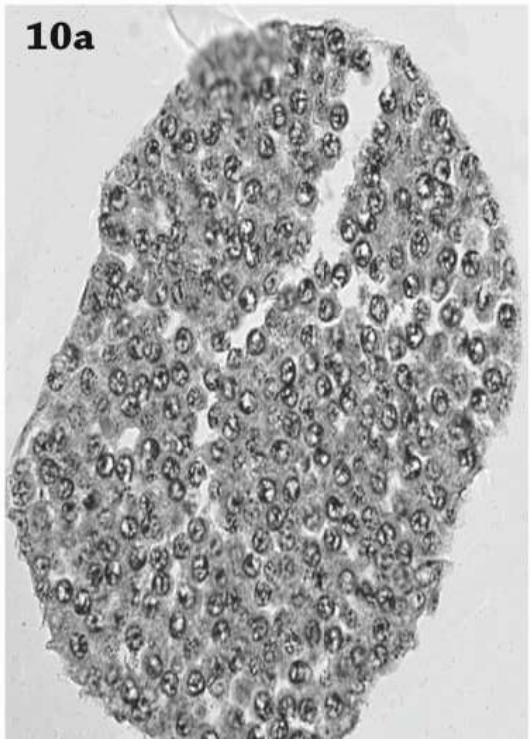

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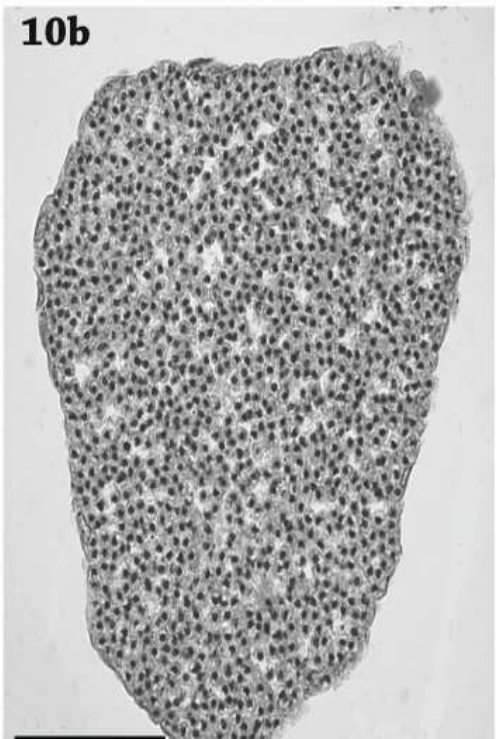

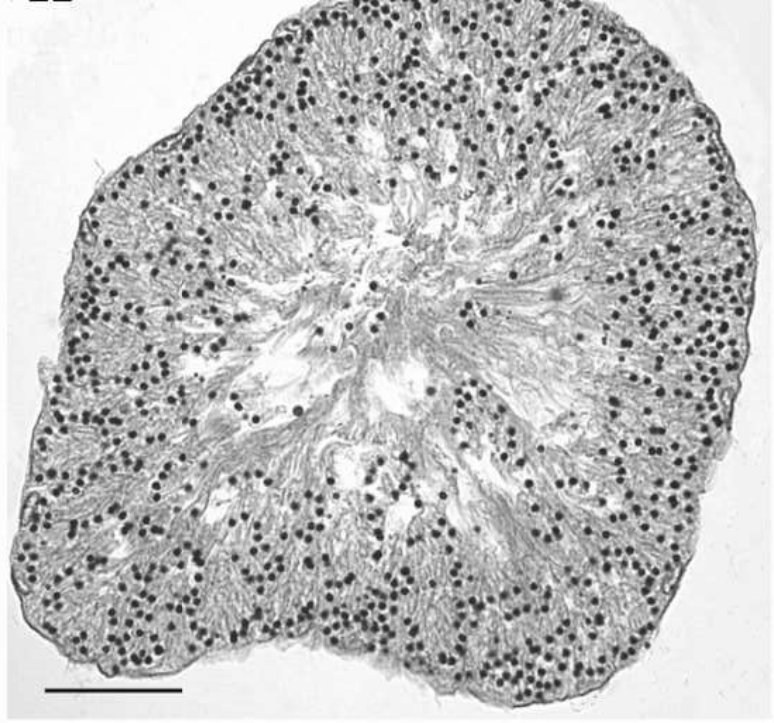

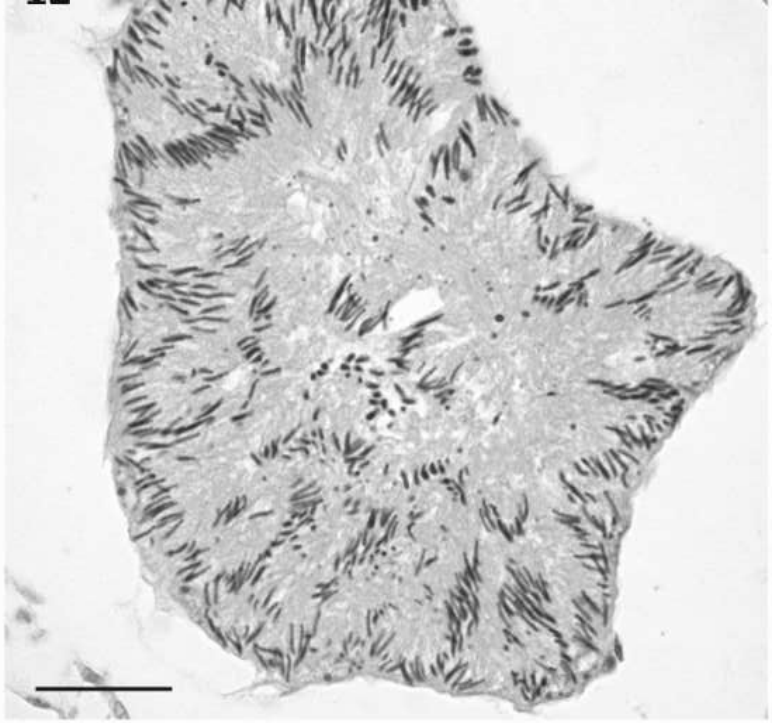

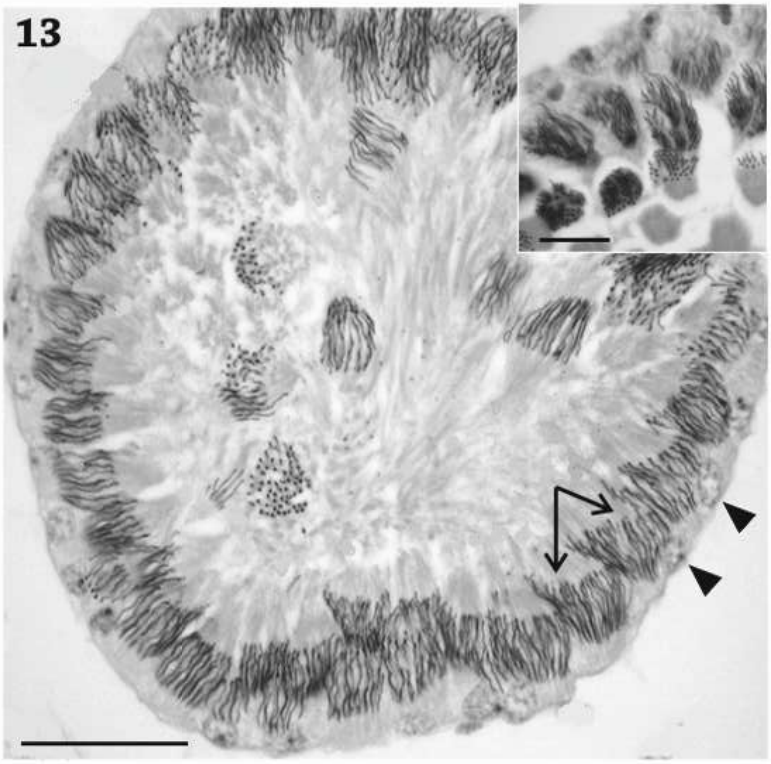
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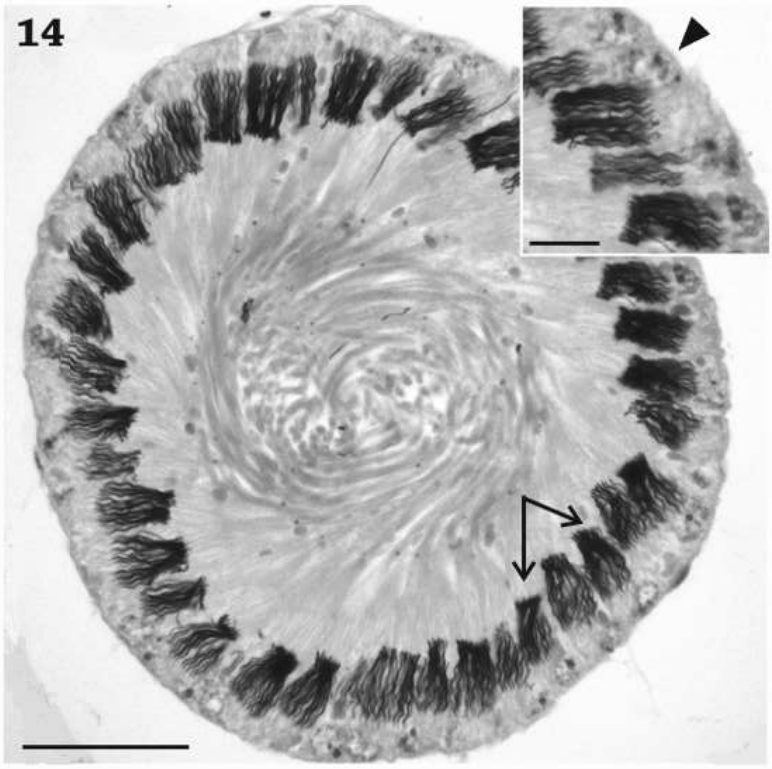
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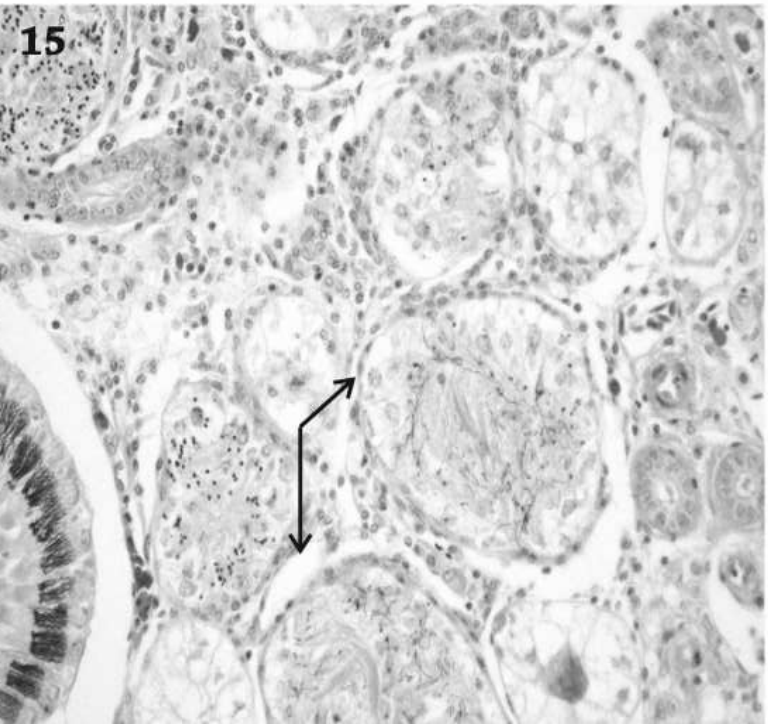
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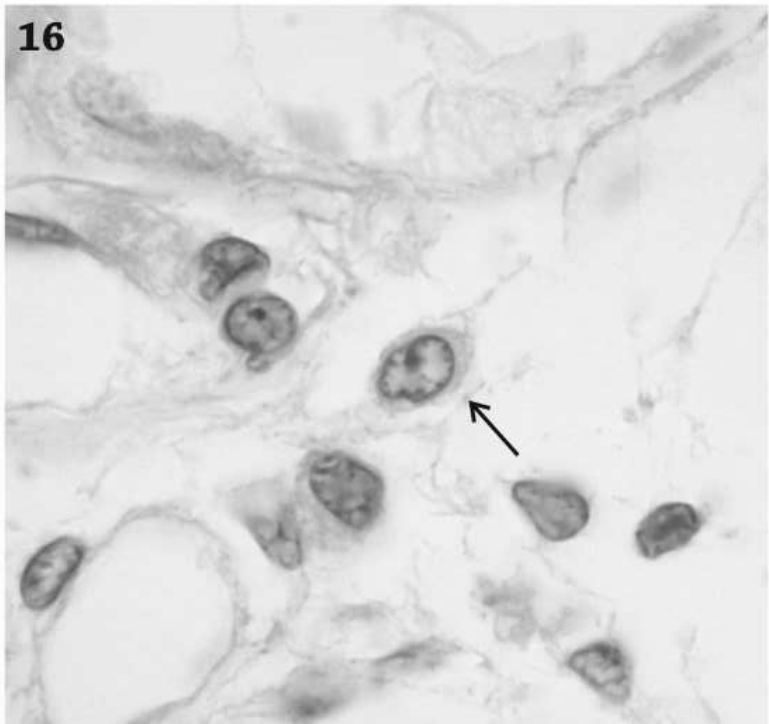
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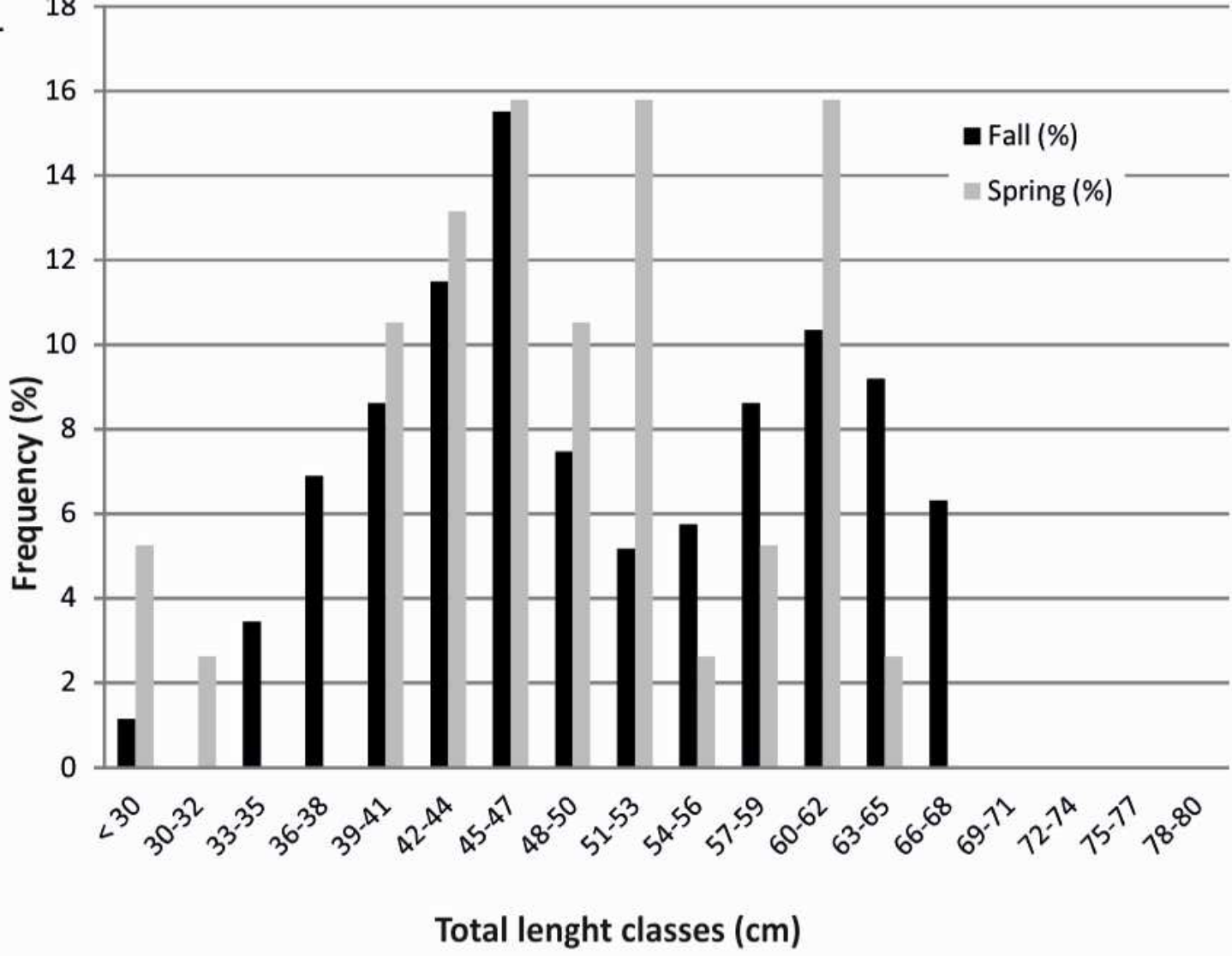
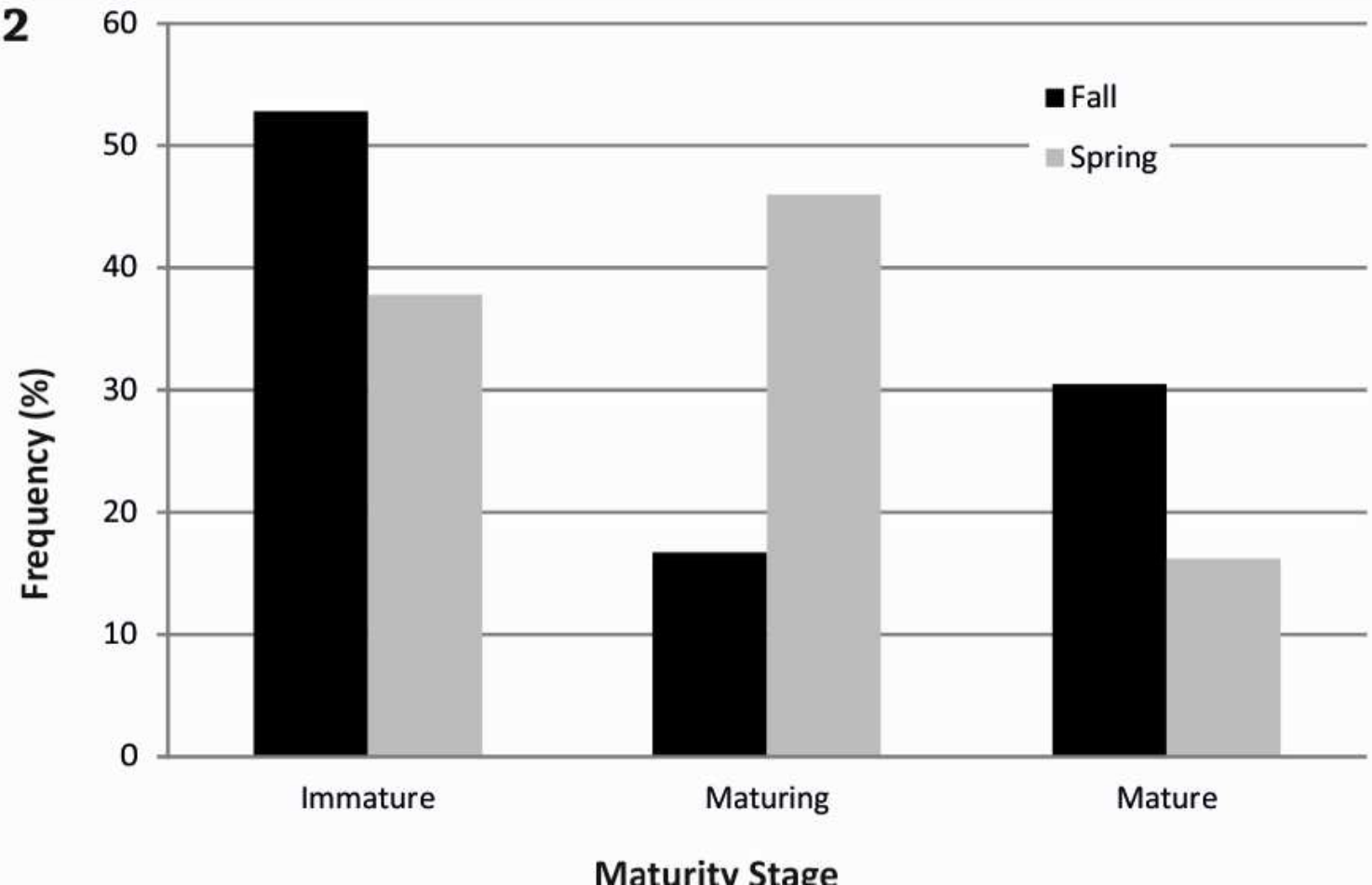
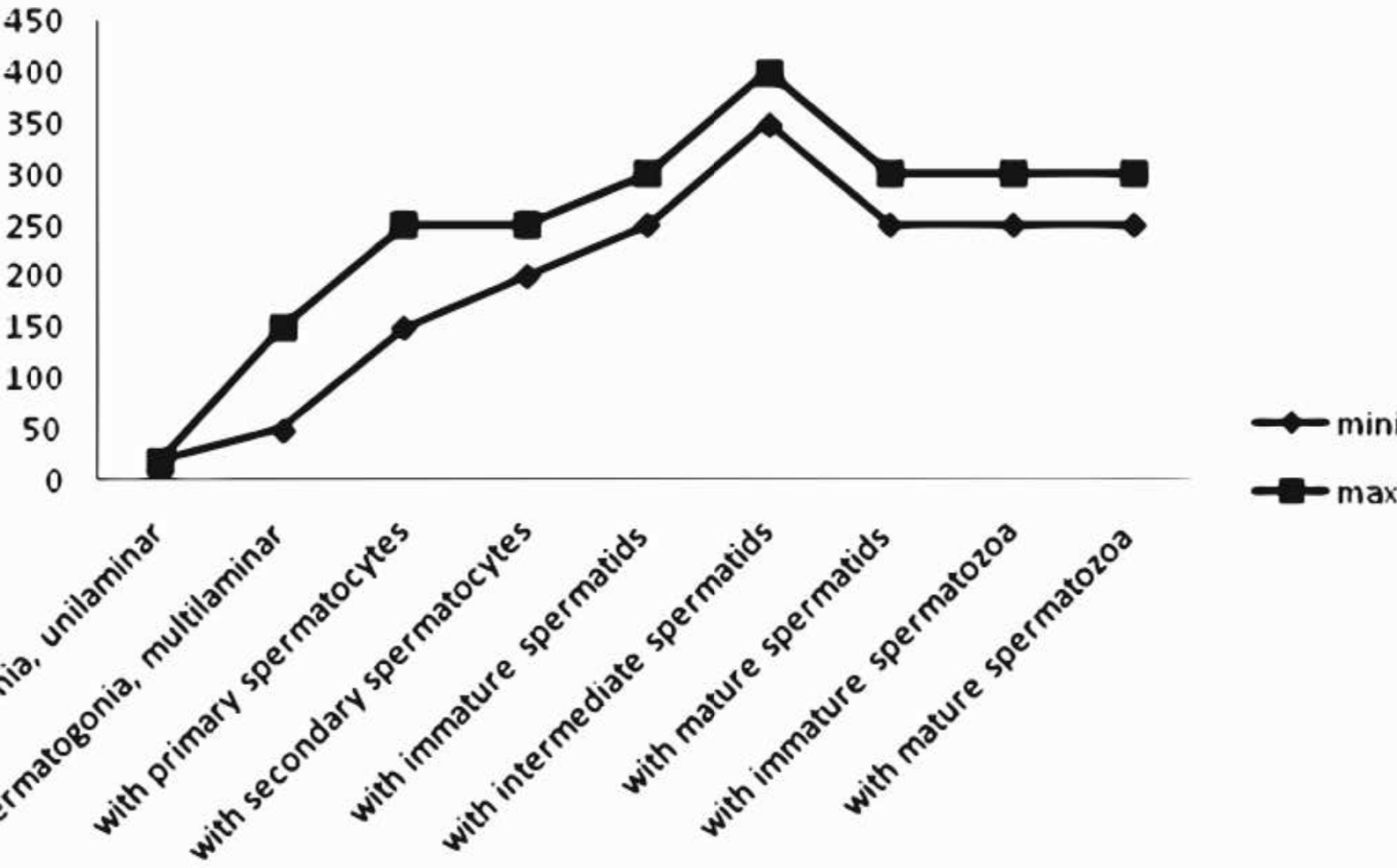
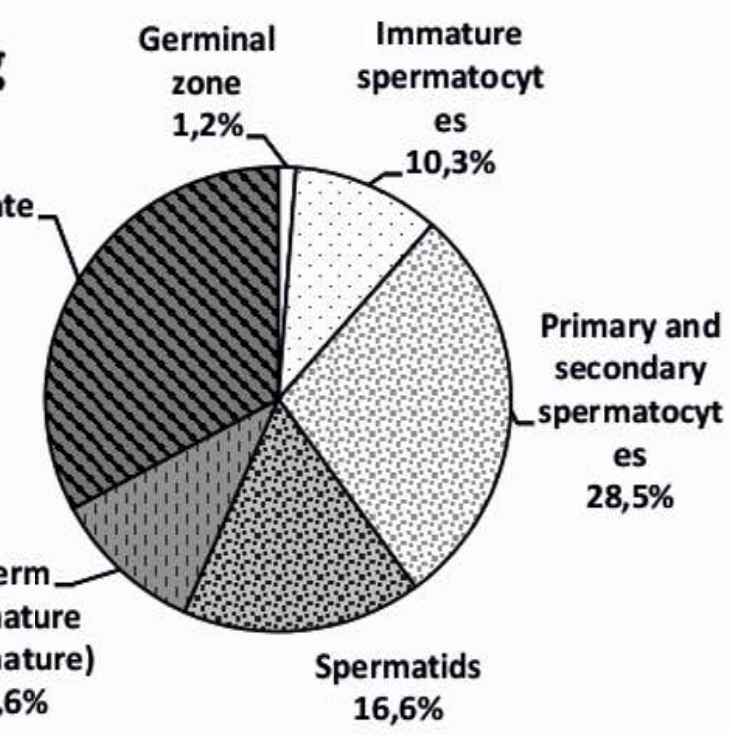
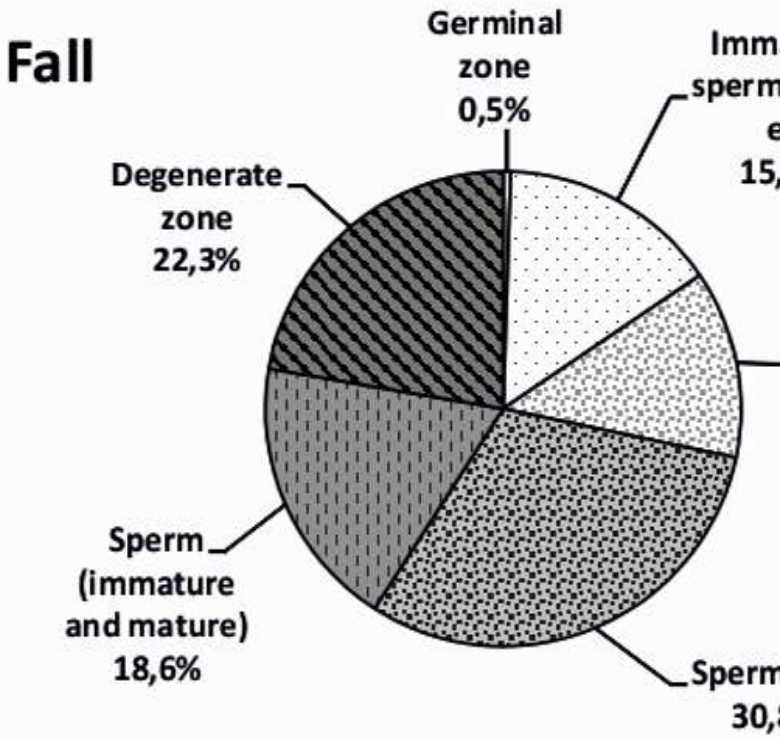

18
16
14
12
10
8
6
4
2
0
Frequency (%)
Fall (%)
Spring (%)
< 30
30-32
33-35
36-38
39-41
42-44
45-47
48-50
51-53
54-56
57-59
60-62
63-65
66-68
69-71
72-74
75-77
78-80
Total lenght classes (cm)

2


60
50
40
30
20
10
0
Frequency (%)
Fall
Spring
Immature
Maturing
Mature
Maturity Stage


450
400
350
300
250
200
150
100
50
0
ia, unilaminar
rmatogonia, multilaminar
with primary spermatocytes
with secondary spermatocytes
with immature spermatids
with intermediate spermatids
with mature spermatids
with immature spermatozoa
with mature spermatozoa
mini
max


Germinal zone 1,2%
Immature spermatocyt es 10,3%
Primary and secondary spermatocyt es 28,5%
Spermatids 16,6%


Fall
Germinal zone 0,5%
Degenerate zone 22,3%
Sperm (immature and mature) 18,6%