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TESTICULAR HISTOLOGY OF *Mustelus schmitti* SPRINGER, 1939 (ELASMOBRANCHII, TRIAKIDAE)

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Resumen
Mustelus schmitti presenta unos testiculos de desarrollo diametrico en la que espermatocistos forma redonda muestran una disposición zonal. Cada zona tiene espermatocistos que contienen células germinales en una sola fase de desarrollo. Los especímenes adultos mostraron seis etapas diferentes que van desde espermatogonias a espermatozoides maduros. Las mismas etapas morfológicas se encuentran en los animales jóvenes, mientras que algunas etapas adultas se han encontrado en animales inmaduros entre las espermatogonias primarias o secundarias. Etapas similares en el desarrollo espermático se encontraron las descritas para *M. manazo, M. griseus* y otros elasmobranquios

Palabras clave: estuario de Bahía Blanca, Selachii, Carcharhiniformes, desarrollo gonadal, espermatogenesis

Abstract
The testicles of *Mustelus schimitti* from a temperate estuary in the South Western Atlantic have a diametric development in which round-shaped spermatocysts show a zonal arrangement. Each zone had spermatocysts containing germ cells in a single development phase. Adult specimens showed six different stages from spermatogonia to mature sperm. The same morphological stages were found in young animals, while some adult stages have been found in immature animals among the spermatogonia, whether primary or secondary. Spermatic stages were found in similar sequences as described for *M. manazo, M. griseus* and other elasmobranchs.

Key words: Bahia Blanca estuary, Selachii, Carcharhiniformes, gonadal development, spermatogenesis

INTRODUCTION

The elasmobranchs are characterized by their slow growth, late sexual maturity, a long gestation period, and a low fecundity (Camhi et al., 1998; Frisk et al., 2001). An outcome of these biological traits is that they show a low potential for reproduction and population growth. Therefore, these species are highly vulnerable to intensive fishing (Hoenig & Gruber, 1990; Sidders et al., 2005).

Some shark species are being exploited to a higher level than their recovery level (Chiaramonte, 1998; Chiaramonte & Pettovello, 2000; Massa & Lasta, 2000; Massa et al., 2000, 2001, 2003a, 2003b, 2004). Twenty percent of the 547 cartilaginous fish species included in the 2008 IUCN Red List of Threatened Species (IUCN, 2008). are endangered.

Mustelus species occurring in warm waters around the world are commercially exploited (Francisco & Mace, 1980). In Argentina, *Mustelus schmitti* (Patagonian smoothhound) is the most common species of this genus, while *M. fasciatus* and *M. canis* are rare (Cousseau, 1986; Menni et al., 1986; Cousseau and Perrotta, 1998). *Mustelus schmitti* lives in the South Western Atlantic between 22º S (Brazil) and 47º 45' S (Argentina) in coastal areas and up to 120m depth (Menni & García, 1985; Cousseau, 1986; Cousseau & Perrotta, 1998; Chiaramonte & Pettovello, 2000).

In this study, the testicles of *Mustelus schmitti* collected at the Bahía Blanca estuary (Argentina) are histologically described with the aims of determining their morphological changes during development and characterizing the stages of spermatogenesis.

Bahía Blanca estuary is known as a birth and nursery zone for this species (López Cazorla, 1987, 2004). From August on (late winter-spring), adult specimens 40 to 81 cm long enter the estuary; females bear embryos at a late developmental stage that are delivered in the estuary, so locally-born immature specimens of 23 cm long are caught during summer (López Cazorla, 2004).

The testicles of *Mustelus* sharks are pair organs, long round and a little flatenned dorsoventraly. Macroscopic observations reveal a pre-germinal layer, sometimes located along the testicles, which are the place of origin of the spermatogenesis sequence (Pratt, 1988). They are covered by a lymphomyeloid tissue of haematopoietic function named epigonal gland. In mature males, the haematopoietic tissue is limited to the mesorchium and it is separated from the testicle blisters by a thin, apparently glandular layer (Galíndez & Aggio, 2002). The structural and functional unit of the elasmobranch testicles is the spermatocyst, as defined by Callard (1991). During the spermatogenesis process, spermatocysts are produced by associating sperms located in lines embedded on the apical portion of the Sertoli cells (Girard et al., 2000). Elasmobranch testes are classified into three types according to the model of origin and spread of spermatocysts, which may be either radial, diametric, or compound (Pratt, 1988).

MATERIALS AND METHODS

Sampling area

Forty one specimens of *Mustelus schmitti* were caught by fishing between 2004 and 2007 aboard the ship "Sierra de Lima" of Prefectura Naval Argentina, at the Bahía Blanca estuary (38º 45' to 39º 45' S - 61º 30' to 62º 30' W: Canal de la Vieja, Cabeza de Buey, and La Lista). The system used to capture individuals was the dual system wide hooks

This ecosystem is characterized by a dense network of canals of different dimensions delimiting numerous islands and lowland muddy and sandy plains that are regularly covered by the tides (Aliotta & Lizasoain, 2004). This estuary is one of the most important scenarios of the Buenos Aires province coast, in terms of size and use as a protected area (López Cazorla, 2004).

Determination of developmental stages

The criteria of Bass et al. (1975)., Stevens & McLoughlin (1991)., and Watson & Smale (1998) were followed in that three developmental stages were defined on the basis of clasper size and consistency:

Stage 1 (immature): claspers that do not exceed the pelvic fins, flexible and non-calcified.

Stage 2 (young): claspers slightly exceeding the pelvic fins, moderately flexible and to some extent calcified.
Stage 3 (adult): claspers clearly exceeding the pelvic fins, rigid and calcified.

Morphometric analysis

Body size of the specimens was measured (SL: standard length; TL: total length; unit: cm) together with size of the external male organs (CL: claspers length; unit: mm). Relationships between these variables were evaluated by regression analysis (Sokal & Rohlf, 1981). order externally determine the maturity of individuals

Material processing

Eight specimens of stages 1 (immature), five of stage 2 (young), and seven of stage 3 (adult) were used for histological study. The animals were sacrificed by cervical dislocation.
After macroscopic observation samples were obtained for histological analysis of maturation state. Samples were fixed in Bouin fluid prepared with sea water, to ensure maintenance of osmolarity. Routine techniques were performed, including haematoxylin-eosin, Masson's trichromic and PAS staining.

Observation of the samples and measurement of cells were performed under an AFM Nikon microscope. Photographical records were obtained with a digital camera Olympus Camedia C-7070 Mod, angular zoom, placed on an optic microscope Olympus BX 51.
Five spermatocysts from mature specimens 15 individuals were used were used to measure different types of cells; 25 cells of each type and 25 cysts were measured for each animal stage.

RESULTS

Our results reveal that testes of *Mustelus schimitti* have a diametric development in which round-shaped spermatocysts or spermatocytes show a zonal arrangement. Six different stages were identified from spermatogonia to mature sperms in a similar sequence as described for other *Mustelus* species in the literature. The testicular morphological stages were compared to that of young and immature animals, allowing a comprehensive description of the gonadal cycle of this economically significant species.

Clasper morphometry

In Fig. 1, plotting the total length versus the length of clasper with a coefficient of correlation R =0.7748

Macroscopic study

Males belonging to stage 1 (immature), besides having claspers not reaching the pelvic fin tips, had very slender and filiform, underdeveloped, genital ducts. In stage 2 (young), the genital ducts are easily visible and slightly twisted. In stage 3 (adult), the claspers are longer than the pelvic fins, rigid and calcified; testes are well developed and the genital ducts are very coiled.

Histological study

Immature specimens (stage 1) showed slightly developed testes in front of the epigonal gland (Fig.3).

There is a germinal zone (Fig. 3) and some cysts at stages I, II and III (Fig. 4) from the spermatogenic development described in adults, with mitosis in both spermatogonia and Sertoli cells (Fig. 4).

Between spermatocysts II and III, there is an area of degeneration similar to that found in adults and juveniles (Fig. 4).

Near the germinal zone, the intratesticular ducts were lined by a simple cuboidal epithelium (Fig. 5 and 6).

In juveniles (stage 2), the testicles are more developed morphologically (Fig. 7)and the epigonal organ is smaller. Histologically, all stages of the spermatogenic series have been found, similar to those of the adult specimens (Figs. 7, 8, 9, 10, and 11).
In adult animals (stage 3), the epigonal organ is very small and confined to an area between the two testicles (Fig. 13.

In a cross section of the testicle, a development of diametral type was observed (Fig. 14). Each spermatocyst had germ cells at a single stage of development (Fig. 14).

The germinal area is located along the laterodistal wall of the testicle, in front of the mesorchium (Fig. 14), in a small area that is sometimes visible as a small projection on the surface of the testicle cysts that radiates from it in a sequence of maturation along the diameter of the testicle. The spermatogenesis sequence was observed from the germinal area of the mesorchium, i.e., from the lateral to medial wall (Fig. 13). Six different stages have been identified in the development of the spermatogenesis. Cell size of the different types of cells along the spermatogenesis is shown in Tables 1 and 2.

Stage 1. Spermatogonia

This stage corresponds to the formation of spermatocysts, whose development begins in the germinal zone (Fig. 15), from an association of a Sertoli cell with spermatogonia, without a basal membrane surrounding it. In this zone, parenchymal cells are also interspersed with spermatogonia (Fig. 15).

Because of mitotic division the number of Sertoli's cells and spermatogonia increase to form small cysts bearing Sertoli cells in the centre covered by a few espermatogonia and a basal membrane. Sertoli cells are big, their nuclei are voluminous, euchromatic, with visible nucleoli (Fig. 15, 16 and 17). Spermatogonia nuclei are oval with thick granules of chromatin (Fig. 15 and 16). The cysts, intratesticular ducts are underlined by a simple cubic epithelium (Fig. 6).

Stage 2. Spermatogonia and Sertoli cells migration

As spermatogenesis progresses, spermatogonia migration is a rearrangement, in which Sertoli cells occupying a central position in the cysts migrate to the periphery, at various stages of displacement (Fig. 17). At first, cysts formed by a single layer of cells around a lumen were seen (Fig. 18); then, the two cell types separated into two concentric layers, with Sertoli cells and spermatogonia in a basal position, which has increased in size (Fig. 19). Spermatogonia continue dividing by mitosis, increasing the number of epithelial layers. The presence of flat myoid cells was also observed around the cysts (Fig. 19).

Stage 3. Spermatocytes

Cysts considerably increased in size. Spermatogonia became primary spermatocytes by mitosis and have a large nucleus, typically spherical. They divide by meiosis to produce secondary spermatocytes. These spermatocytes are smaller than primary ones and have an spherical nucleous with condensed chromosomes.
The nucleus of Sertoli cells are in a basal position. Mature cysts consist almost exclusively of secondary spermatocytes.

Stage 4. Spermatids

Cysts with spermatids were observed at different stages of maturation. The youngest cysts contain round cells with spherical nuclei (spermatids type I). In a later stage (spermatids type II), they have an oval to pear shaped nucleus. Both types are radially aligned according to the light of the cyst containing older spermatids (type III) and are grouped into loose bunches with the head embedded in Sertoli cells. Basophilic heads are directed towards the basal membrane and the flagellum filiform, eosinophil are oriented towards the light.

Stage 5. Sperm

Spermatids differentiate into sperm, which are grouped in conical compact packages, attached to Sertoli cells. In some cysts, sperm flagella are arranged in a spiral form.

Stage 6. Degeneration area

This stage is between phase II and III. In the degeneration area of spermatocysts, cellular rests are observed; some of these are completely full, while others contain a few germinal cells at various stages of degeneration. In the space between cysts, few small Leydig cells were observed, with round nuclei and eosinophilic cytoplasm.

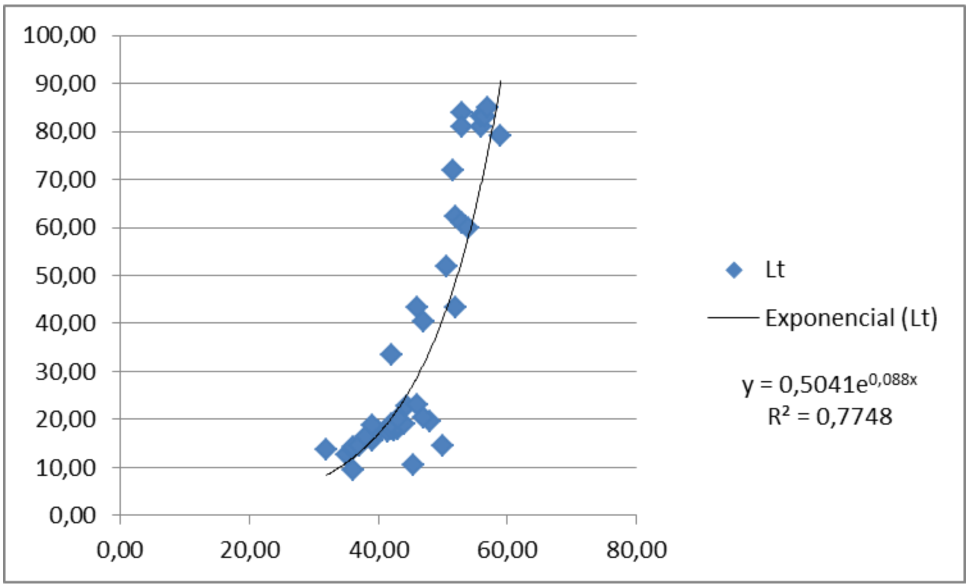


Fig.1. In the y-axis in mm and total length in the axis of the abscissae the long clasper in mm

Table 1. Measure the cells of the testis of *Mustelus schmitti*

Cell type	Mean	Standard deviation	Range
Spermatogonia	10.41	± 0,5	8-15
Spermatocyte 1	7.52	± 0,6	6-10
Spermatocyte 2	6.16	± 0,34	5-7
Spermatid type 1	6.80	±0,12	4-11
Spermatid type 2	6.64	±0,12	4-14
Spermatid type 3	20.92	±0,11	15-25

Table 2. measure of the cyst and Sertoli cells of adult of *Mustelus schmitti*

	Cysts			Sertoli cells		
	Mean	Standard deviation	Range	Mean	Standard deviation	Range
stage 1	57,65	±1	(37,5-87)	19,31	±1	(19-19,8)
stage 2	68,9	±0,5	(50-82,7)	19,79	±0,5	(18-21,1)
stage 3	139,66	±0,2	(65-250)	20,19	±0,2	(18,8-20,7)
stage 4	215,63	±03	(62-239,5)	20,75	±03	(20-22)
stage 5	178,38	±0,2	(120-269)	21,32	±0,2	(19-25)
stage 6	XXXXX	XXXXX	XXXXX	21,64	±0,1	(19-27)

Fig. 2. includes tables 1 and 2

Fig.3. General image of the testicle of a juvenile of *Mustelus schmitti* Ref. **T** testis; **OE** epigonal organ

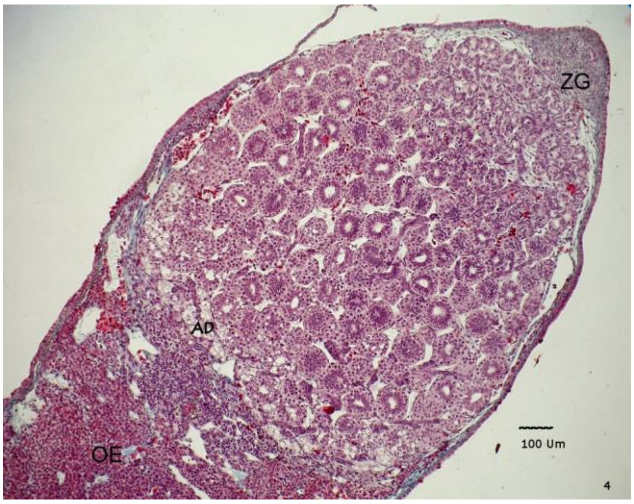


Fig.4. Testicle detail showing the germinal zone (ZG), the organ epigonal (OE) and degeneration area (AG)

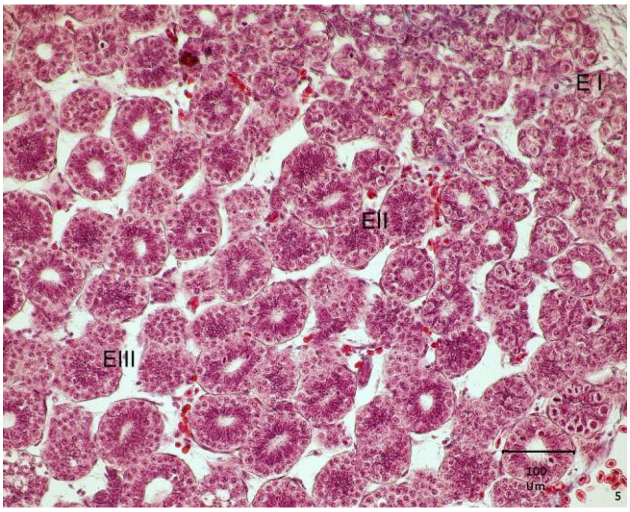


Fig. 5. Spermatocysts in detail the different stages. Ref. **EI** stage I, **EII** stage II, **EIII** stage III

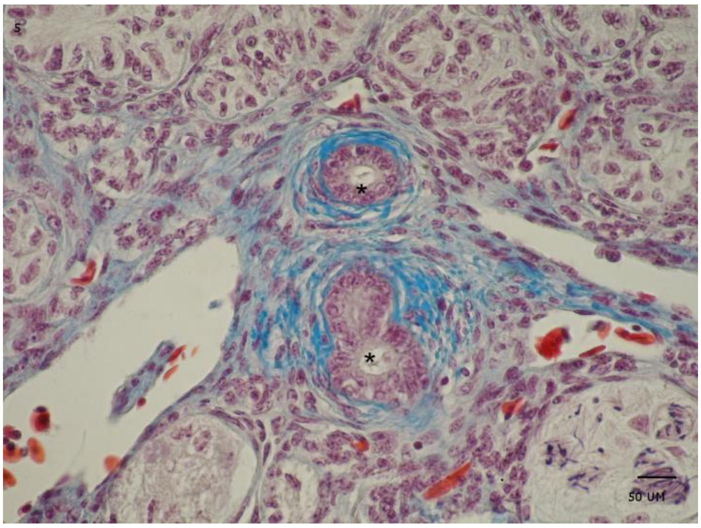
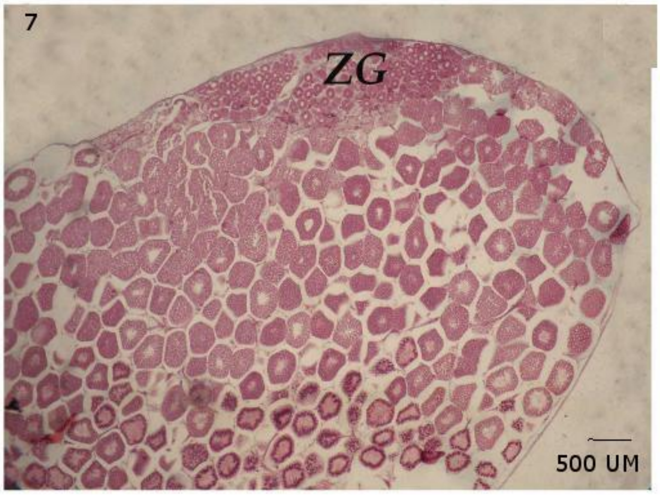


Fig.6. Detail of the testis of *M. schmitti* intratesticular ducts are observed. Ref. * intratesticular ducts



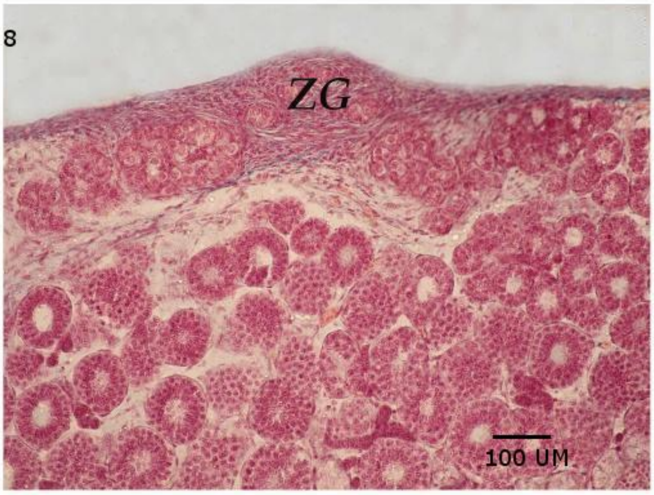
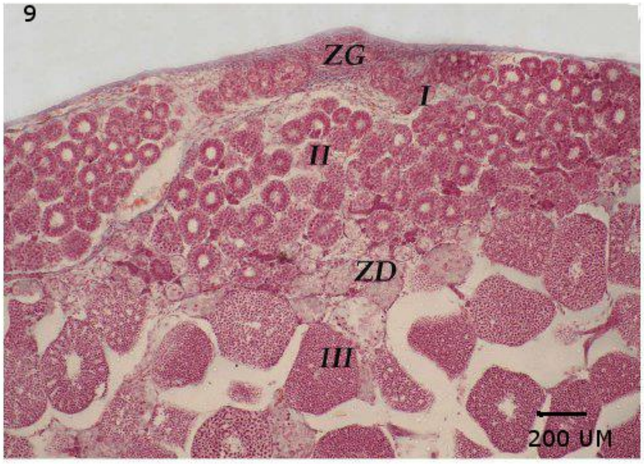


Fig.7. Detail of the testis of juvenile of *M. schmitti.* Ref. **ZG** Germinal Zone

Fig.8. Detail of germinal zone of testis of juvenile of *M. schmitti.* Ref. **ZG** Germinal Zone



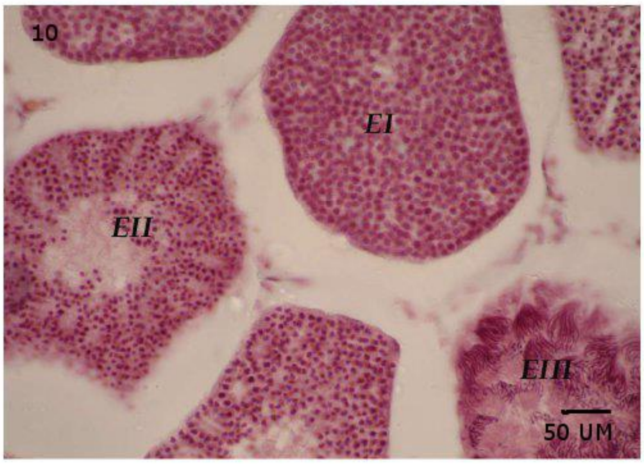
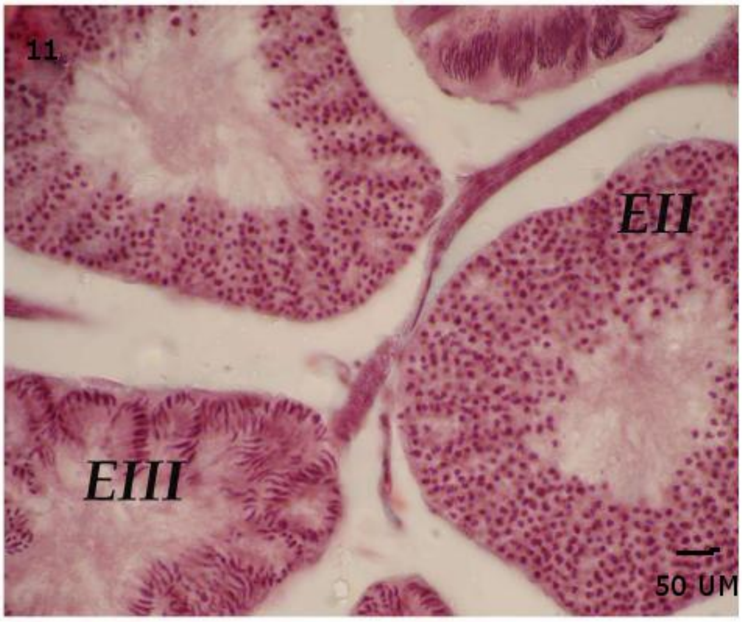


Fig.9. Detail of a juvenile testis of *M. schmitti* who was seen in the area of degeneration.

Fig.10. Detail of the Spermatids type 1, 2, and 3 in a juvenile of *M schmitti*
Ref **I:** Stage I**, II**: Stage II, **III**: Stage III, **EI:** spermatids type 1 **EII** : spermatids type 2 **EIII**: spermatids type 3, **ZD**: Zone of degeneration, **ZG**: germinal zone.



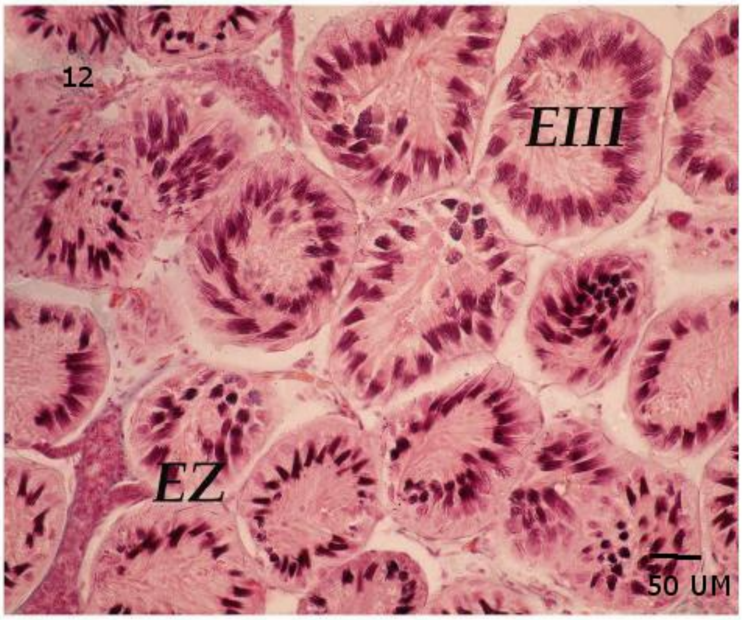
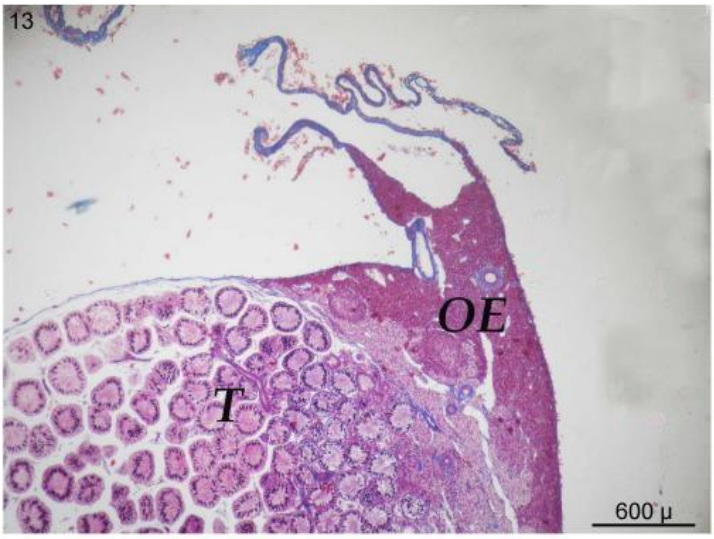


Fig.11. Detail of the spermatids type 2 and 3 of a juvenile of *M. schmitti*

Fig.12. Detail of the type 3 spermatids and sperm in a juvenile.of *M. schmitti*
Ref.: **EII**: spermatids type 2 **EIII**: spermatids type 3, **EZ**: Sperm



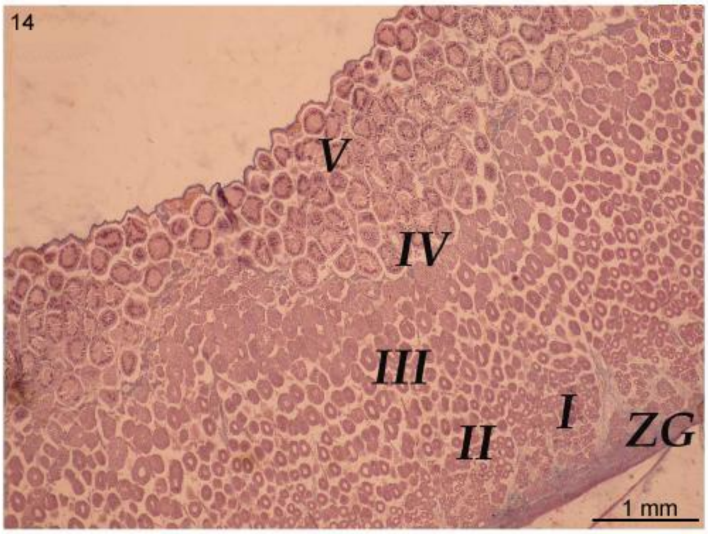


Fig.13. Overview of the testis and epigonal body of an adult of *M. schmitti*

Fig.14. Overview of the testis of an adult in longitudinal section.of *M. schmitti*
Ref.**: I:** Stage I, **II**: Stage II, **III**: Stage III, **IV**: Stage IV, **V:** Stage V, **OE**: Body epigonal, **T**: Testis, **ZG**: germinal zone.

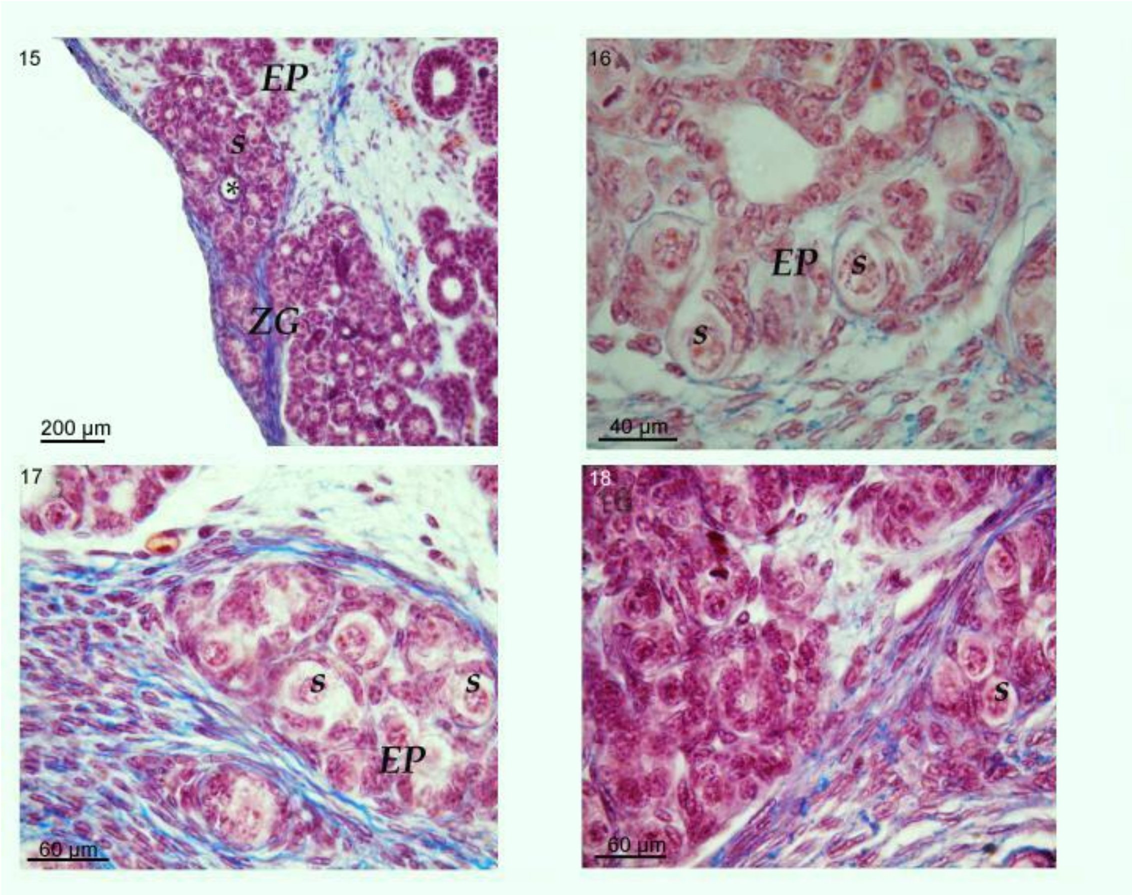


Fig.15. General view an adult testis with spermatogonia and Sertoli cells of *M. schmitti*
Fig.16. Details of the germinal zone of the testicle of an adult.Spermatogonia are observed free. of *M. schmitti*
Fig.17. Details of the germinal zone of an adult. Sertoli cells are observed in position central. of *M. schmitti*
Fig. 18. Adult testis, there are spermatocysts I. of *M. schmitti*
Ref.: **EP**: Spermatogonia; **s**: Sertoli cells; **ZG**: germinal zone; *****: intratesticular ducts.

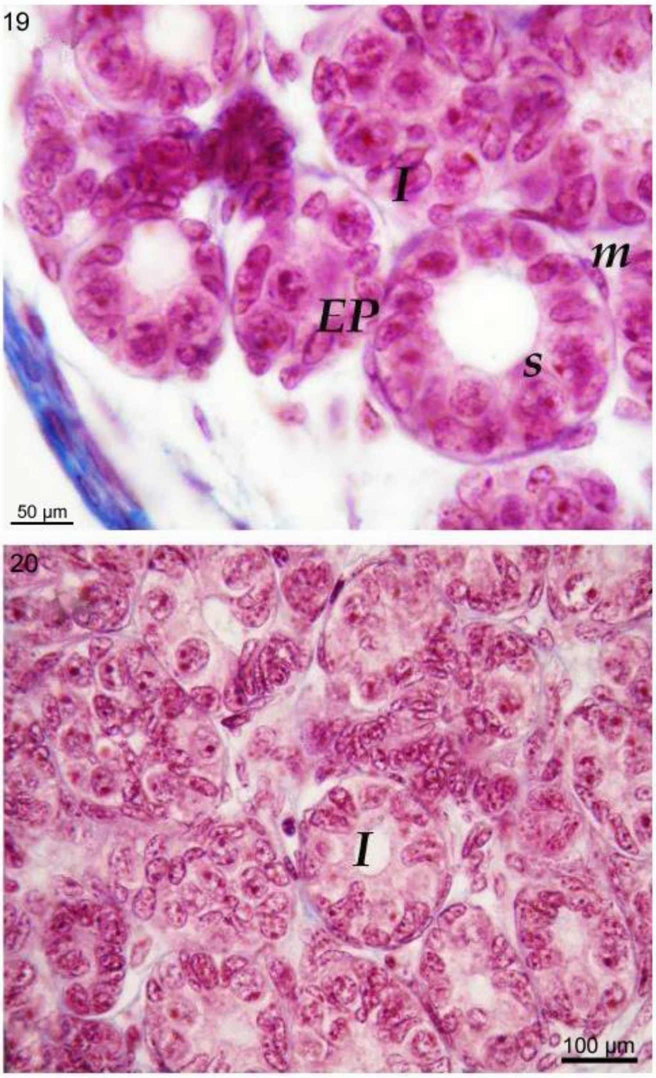


Fig.19. Testis of a mature specimen. Spermatocysts stage I.Sertoli cells are observed in apical position of *M. schmitti*

Fig.20. Mature testis. Spermatocysts stage I. Cysts were observed consisting of a layer cells of *M. schmitti*
Ref.: **I**: Stage I; **EP**: Spermatogonia; **m**: myoid cells; **s**: Sertoli cells.

DISCUSSION

This is the first histological description of the testicles of *Mustelus schmitti*. In the morphometric analysis performed so far, it is easy to see that there is a linear behaviour between the logarithms of total length and the standard length, which is in agreement with an appropriate growth observed in the visual analysis of maturity.

Sexual maturity classes have been established taking into account the length and calcification of the claspers, which is a widely used approach in elasmobranchs (Bass et al., 1975; Stevens & Mc. Loughlin, 1991; Watson & Smale, 1998; Capapé et al., 2006; Sulikowski et al., 2006). This determination has been confirmed taking into account the macroscopic appearance of the genital ducts (Girard et al., 2000). The macroscopic anatomy of the *Mustelus schmitti* testicles provides additional information on the subject in each of the stages of maturation.

The testicles of the immature specimens are recognized because there are not cysts with spermatids or sperm in them. On the contrary, in *Centrophorus squamosus* (Girard et al., 2000), *M. manazo* and *M. griseus* (Teshima, 1981), cysts with spermatids and sperm have been described in their immature stages, showing that, in these species, spermatogenesis can occur in immature animals and that, in these cases, the testicles mature before sharks can reproduce (Girard et al., 2000).

In young *Mustelus schmitti*, all the stages described in the spermatogenesis of mature specimens are observed. This would be similar to the pattern(?) described by Girard et al., (2000) in *Centroscymnus coelolepis*, species in which the cysts containing spermatids and sperm appear only in juveniles; the same happens in *Sphyrna tiburo* (Gelsleichter et al., 2002).

Spermatogenesis is a complex process that involves functional interactions between germ cells and one or more types of somatic cells (Setchell, 1978; Skinner, 1991). In sharks, like in mammals and other vertebrates, the successive stages of development of germ cells are arranged in a strict temporal and spatial order.

There is a spermatocysts' area of degeneration in the testicles of immature specimens as well as in juvenile and adult *Mustelus schmitti*, between the areas occupied by Stages II and III. A similar area has been described in the mature testicle of *Squalus acanthias* (Simpson & Wardle, 1967). and *Scyliorhinus canicula* (Mellinger, 1965). According to Engel & Callard (2005), that area is a band of apoptotic cysts, which appears at the end of winter and reflects a disruption of spermatogenesis. According to these authors, a period of spermatogenic inactivity is normal in seasonally reproducing species.

However, due to the existence of the same area in immature specimens with months of life, is considered that these cysts may reflect a control mechanism of cell division.

The elasmobranchs' testicles may be classified in three different models according to the origin and spread of spermatocysts (Pratt, 1988). In *Mustelus schmitti*, the testicles are of diametric development.

CONCLUSION

As in other Chondrichthyes, three stages of sexual maturity are identified, according to the length and calcification of the claspers. The testicle of *Mustelus schmitti* responds to the diametric model described in other carcharhinoids. In the spermatogenic adult stage, six stages are described, equal as to the ones found in young ones. In the immature animals there are spermatocytes only with primary and secondary spermatogonias and spermatocytes. Both, in the immature testicles as in the adults an area of degeneration of cysts with apoptosis was observed. The interstitial space of the testis Leydig cells.

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