Ovarian Foliculogenesis in the Smallnose Fanskate *Sympterygia bonapartii* (Müller & Henle, 1841) (Chondrichthyes, Rajidae)

Foliculogénesis Ovárica en la Raya Marmorada, *Sympterygia bonapartii* (Müller & Henle, 1841) (Chondrichthyes, Rajidae)

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SUMMARY: Chondrichthyes have become an important economic resource in the last decades, with Argentina as one of the countries that exploits more sharks and skates, even at levels that exceed de limits of many species. However, there is a scarce knowledge of the reproductive biology of this group, particularly from species inhabiting the Southern hemisphere. This work shows the most relevant facts during folliculogenesis in *Sympterygia bonapartii*. Results show that germinal cells are present in immature and maturing females. The most important facts that vary along de follicular development are the number of types and layers of follicular cells, the establishment of thin projections from the follicular cells and the degree of development of the thecae. Follicular cells are, at least, of two different types and both of them emit projections that break through the zona pellucida. The outer theca shows signs of synthetic activity. Atretic follicles of different sizes are present in exemplars of all the reproductive stages. These results are discussed in a physiological and adaptive context.

KEY WORDS: Elasmobranchs; Reproduction; Ovary; Folliculogenesis; *Sympterygia bonapartii.*

INTRODUCTION

Chondrichthyes represent the oldest surviving jawed vertebrates. However, they have developed a diversity of reproductive modes (Wourms, 1977; McEachran & Dunn, 1998). They are either oviparous (egg laying) or viviparous (live bearing), with different modes of viviparity based on the source of fetal nutrition (Koob & Callard, 1991; Musick & Ellis, 2005). In spite of this highly diversificated reproductive strategies, the group is characterized by a low growth, an extensive period of gestation and a scarce number of large offspring, resulting in a low reproductive potential (Hoenig & Gruber, 1990).

The genus Sympterygia belongs to the Rajidae family and is endemic to temperate waters of South America (Ebert & Compagno, 2007). It is represented in the Argentinean Sea by two species: *S. bonapartii* Müller & Henle, 1841 and *S. acuta* Garman, 1877 (Menni, 1976). *S. bonapartii* (smallnose fanskate) is found from Southern Brazil to Argentina from shoreline down to 100 m depth (Menni & Stehmann, 2000) and is one of the major skates caught along the Buenos Aires coast. This species is single oviparous (external) as all Rajids, (Musick & Ellis) and produces large eggs with considerable yolk supplies. These eggs are encapsulated in morphologically complex cases produced by the oviducal gland and laid in muddy or sandy areas, where they adhere to small pieces of rock, shell or seaweed.

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In the last years there has been an increase in the fishing effort in the Southwestern Atlantic, mainly due to larger fishing vessels, that has resulted in a decline in the abundance of skates in this region (Massa *et al.*, 2004). This fact places the species under a high fishing pressure (Massa *et al.*, 2003). Cedrola *et al.* (2005) reported that the frequency of rajids as bycatch in tows in the Patagonian red shrimp fishery was nearly 90%. Although in this fishery, rajids are not processed on board and discarding occurs immediately after opening the nets, post-capture mortality is unknown. Nevertheless, the UICN (2009) Red List for threatened species includes this species considering it as "data deficient".

In spite of the evolutionary and economic importance of chondrichthyans, there are a scarce number of investigations with regards to the ovary structure and they were performed mainly in species from intensive fishing zones (Pratt, 1988; Chatchavalvanich & Visuttipat, 1997; Hamlett *et al.,* 1999; Hamlett & Koob, 1999; Andreuccetti *et al.*, 1999; Prisco *et al.*, 2001, 2002a, 2002b, 2007; Storrie, 2004; Barone *et al.*, 2007). Only one reference exists about the ovary from species of the genus Sympterygia (Díaz Andrade *et al.*, 2009).

The aim of this study was to describe the features that characterize the main stages of folliculogenesis in the ovary of S. bonapartii, with some notes on the reproductive biology of this economically important species.

MATERIAL AND METHOD

A total of 41 females of S. bonapartii were collected by line fishing in the inner and medium zones of the Bahía Blanca estuary (61º30'- 62º30' W, 38º45'-39º30' S). The total length and disc width of each specimen were measured at the nearest millimeter. Each animal was terminated by blunt trauma to the cranium and then dissected. Once opened the ventral cavity, the sexual maturity stages was determined following the Braccini & Chiaramonte (2002) criteria. This standard technique, common on board, is based on the macroscopic aspect of the reproductive organs.

For each specimen, at least one ovary was cut in small pieces for histology. This material was fixed in Bouin's fixative in seawater, dehydrated through a graded series of alcohols and embedded in paraffin wax. 5-10 mm-thick sections were stained by Masson's trichromic stain, hematoxylin-eosin, alcian blue technique at 2.5 pH level (AB pH 2.5) and periodic acid Schiff reaction (PAS). The sections were photographed using an Olympus BX51 light microscope equipped with an Olympus C-7070 digital camera.

RESULTS

Macroscopic description of the ovary: Both ovaries were elongated and slightly compressed dorsoventrally (Fig. 1). They were associated with the epigonal organ that extended beyond the length of the ovary. In a maturing or mature ovary (stages II and III of maturation scale) follicles of various sizes were present at any one time, supported by loose connective tissue. Follicles with diameters below 3 mm were translucent or white, meanwhile those bigger than that were yellow. Even though follicles were intermingled in different degrees of maturation, it could be externally recognized an increase in their size from cranial to caudal and from dorsal to ventral.

Microscopic description of the ovary: In the immature animals (stage I of maturation scale), the ovary appears as a small structure at the medial cranial side of the epigonal organ (Fig. 2). As ovary development goes on (stages II and III of maturation scale), the epigonal organ withdraws and becomes restricted to a small posterior outline and to the space between oocytes.

The ovary is surrounded by a folded simple or seudostratified columnar ciliated epithelium. Beneath the epithelium, there are dense connective tissue, smooth muscular cells and blood vessels (Fig. 3). Follicles extend all over the ovary, with no evidence of an ovarian matrix.

Folliculogenesis

Oogonia: Clusters of germinal cells and individual oogonia (Fig. 4) were present just beneath the ovaric epithelium, in the immature (stage I) and maturing exemplars (stage II), but not in the mature ones (stage III). Each germ cell (~20 mm) has an eosinophilic cytoplasm and a big euchromatic nucleus with an evident nucleolus.

The basic structures forming a follicle from inside towards outside are the oocyte and its plasmalema, the zona pellucida, the follicular epithelium and the theca.

Primordial follicles: These follicles are formed by germinal cells surrounded by simple layer of squamous follicular cells and a few plane thecal cells. At this stage of development, the oocyte shows a large nucleus containing a single nucleolus and lampbrush chromosomes, and the zona pellucida appears as a diffuse and discontinuous structure. The larger primordial follicles (~ 47 mm diameter) show some enlarged globed-shaped cells (6-25 mm diameter) between the squamous follicular cells (Fig. 5).
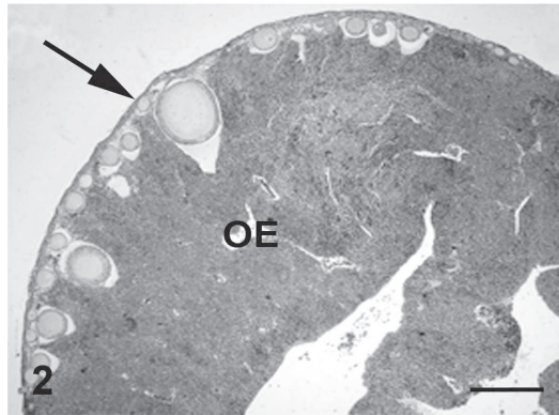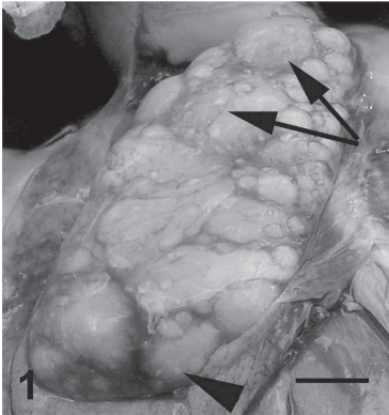
Fig. 1. Macroscopic ventral view of the mature ovary. Arrow indicates a fully developed follicle near the ovulation size; arrowhead depicts the smallest ova visible at naked eye. Scale bar = 2 cm.

Fig. 2. Microscopic general image of the ovary at stage I of development. Look at the follicles disposed below the ovaric epithelium (arrow), most of them intermingled with the epigonal organ (OE). Scale bar = 200 µm.
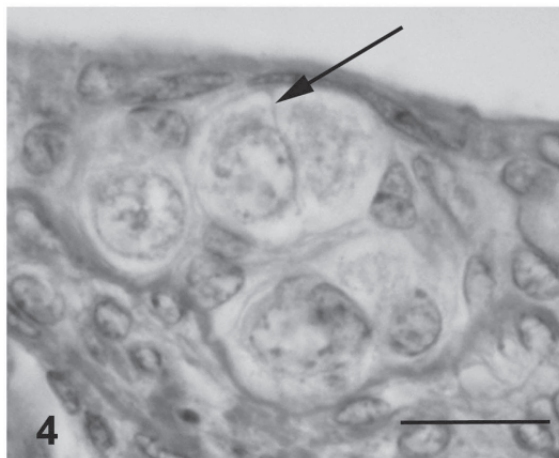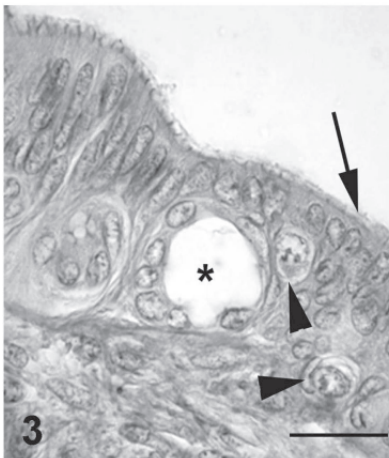
Fig. 3. The ovary epithelium. Arrow indicates cilia and arrowhead make points prefollicular germ cells; asterisk depicts an "empty" follicle. Scale bar = 25 µm.

Fig. 4. High magnification of small nest of oogonias, some of them in cellular division (arrow). Scale bar = 25 µm.
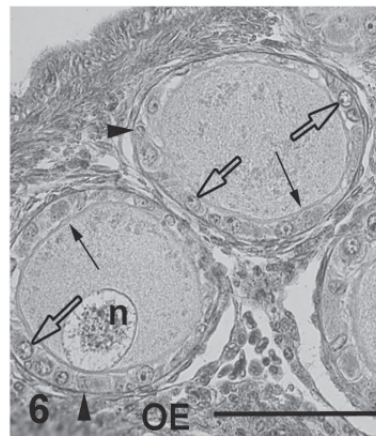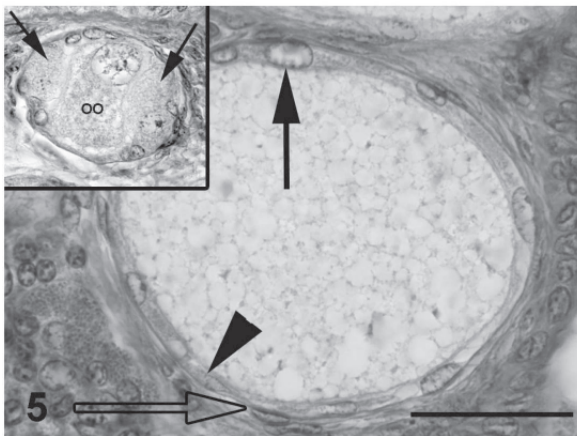
Fig. 5. Primordial follicle. Arrowhead points squamous follicular cells and arrow indicates the incipient follicular globet cell; the outlined arrow depicts the thecal cells. Insert shows a smaller primordial follicle with two big globet cells (arrows). Scale bar = 25 µm.

Fig. 6. Primary follicle. n: nucleus; OE: epigonal organ; Arrows indicate the zona pelúcida, arrow heads make point on the cuboidal follicular cells and outlined arrows display the globet cells. Scale bar = 40 µm.

Primary follicles: At this stage, the oocyte is surrounded by cuboid follicular cells arrayed in one layer, with numerous globed-shaped cells between them. The oocyte plasmalema folds densely against the zona pellucida and appears as a fluted refracting zone. The zona pellucida is a homogeneous hyaline smooth line of ~2 mm thick, PAS (+) and AB pH 2.5 (+). The thecal layer begins to differentiate in an inner theca of squamous cells with plane euchromatic cleared ends nucleus and eosinophilic cytoplasm, and an outer theca of squamous cells with plane, fusiform, heterochromatic nucleus and slightly basophilic cytoplasm (Fig. 6).

Developing follicles: As oocyte grows, follicular cells become columnar. When follicles reach ~450 mm diameter this epithelium begins to stratificate. Columnar cells are eosinophilic with heterochromatic nucleus. Between them, there are enlarged basophilic cells with euchromatic nucleus, some of them with a pyriform-like shape (Fig. 7). The
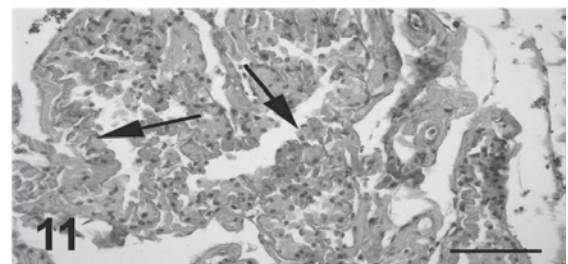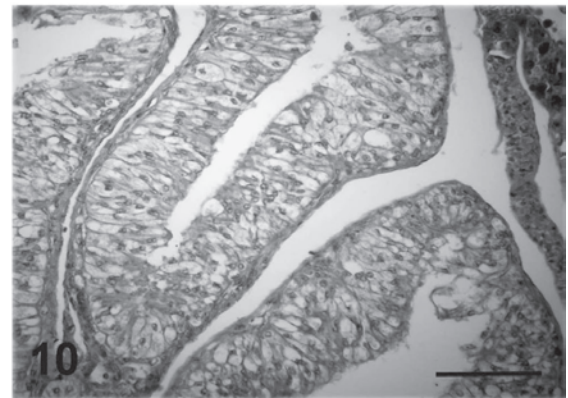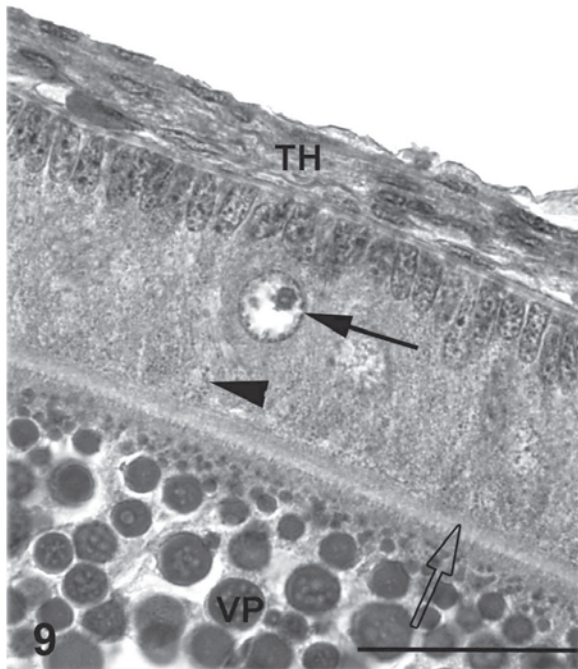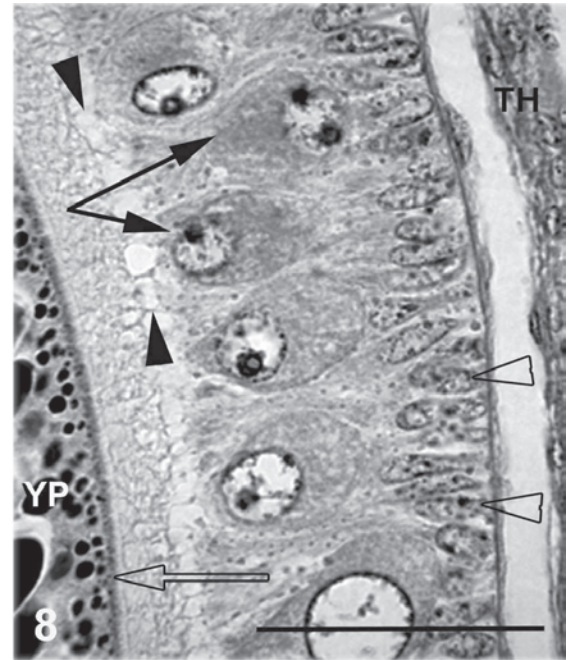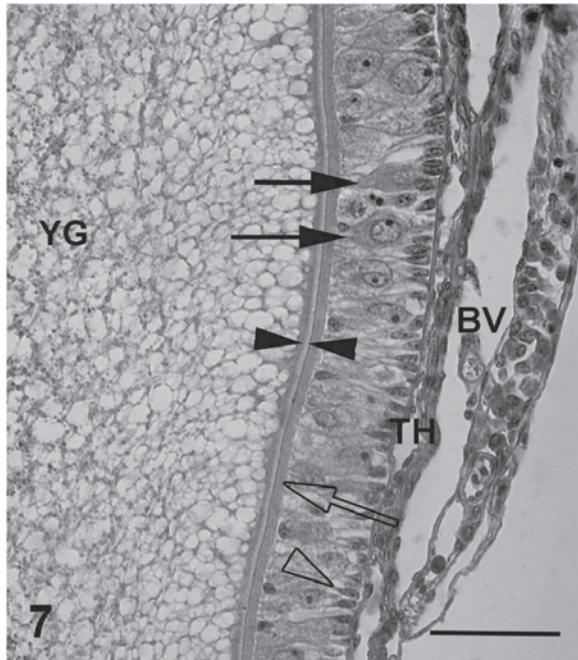
Fig. 7. Follicle in development. YG: yolk granules; BV: blood vessel; TH: Thecas. Arrows indicate the pyriform-like cells; arrow heads depict the striated oolema; outlined arrow points the cellular projections of follicular cells and outlined arrowhead shows the nucleus of prismatic follicular cells. Scale bar = 60 µm.

Fig. 8. More advanced developing follicle. TH: theca; YP: yolk plates; outlined arrowhead shows the nucleus of prismatic follicular cells; outlined arrow indicates the striated oolema; arrows make point in the pyriform-like cells and arrowhead depicts the intercellular bridges. Scale bar = 60 µm.

Fig. 9. At term follicle. TH: theca; arrow indicates a pyriform-like cell and the arrowhead depict the intercellular vitelline granules. Scale bar = 50 µm.

Fig. 10. Medium magnification of the corpora lutea. Look at the folded epithelium. Scale bar = 85 µm.

Fig. 11. Atretic follicle. Arrows indicates the folded cords of infiltrated dense connective tissue. Scale bar = 60 µm.

cytoplasm of follicular cells contain small granules AB (+) and PAS (+). At this point of development, thin projections establish between both types of follicular cells and ooplasm, crossing the zona pellucida. As a result, this region becomes thicker and irregular (Fig.8). The oocyte plasmalema shows no changes. Inner and outer thecae are clearly differentiable, with a high blood irrigation of the prime. When follicles reach ~1.2 mm of diameter they begun to accumulate granules of yolk slightly stained. This granules increase in size as development continues to finally form elliptic plates (Fig. 8).

Pre-ovulatory follicles ("at term"): As development goes on, both types of follicular cells return to their one-layer disposition and the zona pellucida becomes thinner with no projections. The inner theca stratifies and the outer theca is inconspicuous (Fig. 9).

Corpora lutea: Once oocytes are released follicular cells begin to degenerate. At first, they are tall eosinophilic cells with irregular apical surfaces (Fig. 10). Between these cells there are few globed-shaped cells that apparently undergo in the first stages apoptosis. As corpora lutea development continues, dense connective tissue infiltrates this structure and follicular cells become cubic with a vacuolated aspect. At most advanced stages, a dense conjunctive structure encloses the cellular fragments.

Atretic follicles: Atretic follicles of different sizes were present in the ovaries of immature, maturing and mature females. They are characterized by the presence of follicular apoptotic cells, with eosinophylic foamy cytoplasm. At a more advanced stage, cells completely lose their structure and the atretic follicle is infiltrated by dense connective tissue, forming a scar-like body (Fig. 11)

DISCUSSION

The basic organization of the ovary in *S. bonapartii* is similar to that seen in other Batoids (Prisco *et al.,* 2007; Díaz Andrade *et al.*), so as their relationship with the epigonal organ during sexual development (Koob & Callard, 1999; Galíndez & Aggio, 2002, Storrie). The features of the ovary in this species agree with the "external" type defined by Pratt (1988).

The ovarian epithelium in the smallnose fanskate is similar to that of *S. acuta* (Díaz Andrade *et al.*) and *Raja asterias* (Barone *et al.*). The presence of cilia in the epithelium could be related with the translation movement of the ovum from the ovary to the oviduct.

During development, primordial germ cells originate at the endodermic layer of the yolk sac and migrate to the germinal ridge where they associate with follicular cells (Matova & Cooley, 2001). Prisco *et al.* (2001) have shown that in *Torpedo marmorata* the oogenesis occurs early in development and continues even after birth. They observed clusters of oogonia and prefollicular oocytes only in newborn specimens and concluded that this feature was concordant with the low fecundity of the species. Even though the results of this work suggest that in *S. bonapartii* the oogenesis may extend longer in time, a more detailed study is needed to make certain conclusions.

The follicular wall constitution in the smallnose fanskate agrees with the observed in most Chondrichthyes (Guraya, 1978; Hamlett *et al.*; Díaz Andrade *et al.*). The basic structure of the follicular epithelium at the first stages of the folliculogenesis is similar to that found in other cartilaginous fishes, and even in Osteichthyes and other Vertebrates (Guraya, 1986; Andreuccetti *et al.*; Storrie). However, the developmental dynamics of the follicular wall varies between species. With respect to this point, the stratification of the follicular epithelium in this skate is common in all the studied Rajiforms (Guraya 1986; Pratt; Prisco *et al.*, 2002a; Díaz Andrade *et al.*), but shows a different model from that found in some Carcharhiniformes (Guraya, 1986; Wourms, 1993; Storrie; Díaz Andrade *et al.*). In spite of this diversity, there is no consistent pattern between the basic structure of the follicular wall, the phylogeny, the reproductive mode or even the maximum size reached by the follicle (Storrie; Díaz Andrade *et al.*).

The presence of different follicular cell types is a common feature in all studied Rajiforms (Guraya, 1978; Pratt; Prisco *et al.*, 2002a; Barone *et al.*). Prisco *et al.* (2002a) and Barone *et al.* have differentiated, at an ultrastructural level, three cell types in *T. marmorata* and *Raja asterias*. Though in this work we have observed enlarged cells of different shapes (globed-shaped and pyriform-like cells) we cannot assure they constitute different cell types.

Follicular cell projections are common in Chondrichthyes (Koob & Callard, 1999; Hamlett *et al.*; Prisco *et al.*, 2002a; Storrie). Hamlet *et al.* observed digital projections of the small follicular cells in *Urolophus jamaicensis* and name them as transosomes. On the other hand, Prisco *et al.* (2002a) describe the presence of intercellular bridges in *T. mamorata* and suggest their need for follicular cell differentiation. This pattern seems to be the same in *R. asterias* (Barone *et al.*) and could probably be the case of the *S. bonapartii*. On the other hand, the presence of microvilli on the oocyte surface is common in

no mammalian vertebrates (Guraya, 1978; Andreuccetti *et al.*; Hamlett & Koob; Prisco *et al.*, 2002a) and the smallnose fanskate is not an exception. Their presence, together with the follicular cell projections, may probably facilitate the transfer of material between the oocyte and surrounding tissues and the entry of nutrients and yolk precursors (Prisco *et al.*, 2002b).

The origin of the zona pellucida has not been conclusively determined for chondrichthyans, it seems like it is produced by the follicular epithelium and the oocyte as in other animal groups (Hamlett *et al.*; Storrie; Modig *et al.*, 2007). The histochemical reaction to PAS observed in this coat indicates the presence, at least in part, of mucopolysaccharides as in other species (Guraya, 1986; Koob & Callard, 1999; Storrie; Barone *et al.*). However, the presence of acid mucopolysaccharides evidenced by the AB pH 2.5 reactivity in *S. bonapartii* has only been recorded before in S. acuta (Díaz Andrade *et al.*). Some of the functions that have been attributed to the zona pellucida, at least in bony fishes, are the contribution to the acrosomic reaction, prevention of polyspermy, and mechanical, bacterial and fungal protection of the oocyte (Ravaglia & Maggese, 2003; Modig *et al.*). It is likely that this structure fulfills the same functions in cartilaginous fishes.

There are many studies about the stereidogenic capacity of the theca in Chondrichthyes and there are differences among species referring to which thecal layer has this capacity (Koob & Callard, 1999). The characteristics of the outer theca in the smallnose fanskate are consistent with synthetic activity, and this is similar to the observed in other species, like S. acuta (Díaz Andrade *et al.*), Scyliorhinus canicula (Guraya, 1978) and *T. marmorata* (Prisco *et al.*, 2002a). Furthermore, the high blood irrigation of the inner theca in *S. bonapartii* may constitute an adaptation to increase the flow of nutrients to the oocyte and the delivery of products.

There is no information about the ovulatory process in cartilaginous fishes. However, the results of this work indicate that, once produced, follicular cells persist in the ovary and transform in a transient endocrine gland. The lifespan of these structures is correlated with the reproductive mode, persisting only a short period of time in oviparous species (Hamlett & Koob). On the other hand, it is known that atresia is a highly frequent event in Chondrichthyes (Hamlett & Koob; Hamlett *et al.*). However, there are no quantitative studies about that and the exact mechanism that triggers this process remains unknown. It seems likely that non-viable oocytes are eliminated by this way, though this hypothesis does not fully explain the presence of atretic follicles of sizes close to ovulation. Probably, other environmental or physiological events contribute in triggering this response.

Elasmobranches constitute a valuable resource, not only economically but because of their diversity and phylogenetic position. Unfortunately, those features that make them so interesting and special are the same that put them in serious danger. Is in this context that morphophysiological studies regarding to its reproduction are extremely necessary to evaluate and implement effective management and preservation policies for this valued resource.

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RESUMEN: Los Condrictios se han convertido en un recurso económico importante en las últimas décadas, siendo Argentina uno de los países que más explota tiburones y rayas, incluso a niveles que exceden los límites de varias especies. A pesar de esto, es poco lo que se conoce sobre la biología reproductiva de este grupo, particularmente en especies del Hemisferio Sur. En este trabajo se estudian los estadios más relevantes de la foliculogénesis en *Sympterygia bonapartii*. Los resultados muestran que las ovogonias están presentes tanto en ejemplares inmaduros como subadultos. Las características más importantes que varían a lo largo del desarrollo folicular son el número de capas y tipos celulares que constituyen el epitelio folicular, el desarrollo de proyecciones de las células de la granulosa y el grado de desarrollo de las tecas. Las células foliculares son, al menos, de dos tipos y ambos emiten proyecciones que atraviesan la zona pelúcida. La teca externa presenta características compatibles con la actividad sintética. Folículos atrésicos de distintos tamaños están presentes en ejemplares de todos los estadios de madurez sexual. Estos resultados se discuten en un marco fisiológico y adaptativo.

PALABRAS CLAVE: Elasmobranquios; Reproducción; Ovario; Foliculogénesis; *Sympterygia bonapartii.***

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