VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 68, No. 4, pp. 277–286

ISSN 0015–5659
www.fm.viamedica.pl

Histomorphology of prepuberal ovaries in the South American fur seal (*Arctocephalus australis* Zimmerman, 1783)

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[Received 14 May 2009; Accepted 21 September 2009]

*The South American fur seal reproductive histophysiology is scarcely described. This study provides a histological description of prepuberal South American fur seal (*Arctocephalus australis*) ovaries as well as three-dimensional reconstructions of subcapsular crypts and primordial follicles. Ovaries from fresh dead animals were processed for histology and sliced into serial sections. A portion of the superficial cortex was photographed, and the images were processed using BioVis3d software in order to generate 3-dimensional reconstructions.* A. australis *prepuberal ovaries conform to the basic structure of pinnipedian species, with a subcapsular crypts system made up of interconnecting cisternae and tubules with multiple openings to the surface. Generally, the primordial follicles were arranged in a monolayer beneath the tunica albuginea and were closely associated with subcapsular crypts. The large number of interstitial cells distributed throughout the cortex was the main histological feature in comparison with previous reports in other seals. Three-dimensional reconstructions modelled the subcapsular crypts microarchitecture and showed the close spatial relationship between the crypts and the primordial follicles. Despite the fact that the general ovarian histological structure was similar to that of other pinnipeds, the large number of interstitial cells is a distinctive feature that raises the question about the origin and function in* A. australis *with regard to the steroidogenic activity reported in other seal species.* (Folia Morphol 2009; 68, 4: 277–286)

Key words: *Arctocephalus* reproduction, ovary, subcapsular crypts, interstitial cells, three-dimensional reconstruction

INTRODUCTION

The South American fur seal, *Arctocephalus australis* (Zimmerman, 1783), is a pinniped (fam. Otariidae) native to the Atlantic and Pacific coasts of South America [37]. Uruguayan reproductive colonies are located on different islands (Isla de Lobos 35°01'S-54°52'W, islets in Cabo Polonio 34°24'S, 53°46'W) [36] and are considered the largest colonies in the continent: in 2004, its population was estimated at 340,000––360,000 individuals, with an annual growth rate of 2% [29].

A. australis females are seasonal monoestrous breeders; previous reports showed that births and mating occurred between late November and the

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second week of December (with a 21-day period synchrony [31]) but most recent data showed a longer breeding period (35 days) with peaks occurring on the 15th of December [14]. Five to eight days post-partum they present oestrus and mate [31]. Gestation period is 11 to 12 months and includes embryonic diapause [4] as described in other pinnipeds [37]. Embryonic diapause had only been determined by the absence of an embryo in the uterine horns in the presence of corpus luteum development after the reproductive season (Páez E. pers. com.). Females reach puberty when they are three to five years old [37], having attained 80% of adult length [23]. Although ethological and reproductive pattern studies have been done formerly in this species, information about their reproductive histophysiology is scarce.

There has been increasing interest in the reproductive biology of pinnipeds for different reasons, in particular due to a decline in population numbers in several species because of reproductive failure. There are several hypotheses including bioaccumulation of toxic compounds in the food chain that interfere with hormone dynamics [3, 38] and nutritional stress [35]. Although uruguayan *A. australis* population numbers are stable [29], the Peruvian population suffered a huge decline (72%) in the 1997–1998 period after the "El Niño" phenomenon caused low food availability; its recovery is still difficult to evaluate [9]. In view of this situation, the need of normal histophysiological parameters would be the base to evaluate possible negative interactions that affect fertility, birth rates, and population dynamics in *A. australis* as well as in other wild species.

Ovaries from Pinnipedia, Proboscidea, and some Carnivora present subcapsular/subsurface crypts [39], which are invaginations of the superficial epithelium through the tunica albuginea into the ovarian cortex. They have been described in several pinnipedian species from the early stages of embryonic development (16 cm foetuses in *Leptonychotes weddellii,* [19]), in newborns of several Phocidae species [2, 20], and in sexually mature females (*L. weddellii*, [20], *Otaria flavescens,* [6]). Mülling et al. [26] described three types of subcapsular crypts in harbour seals (*Phoca vitulina vitulina)*, according to their location and extension. Another histological feature in mammals is ovarian interstitial cells. They are located at the cortex or medulla, depending on the age of the animal. Their organization, number, and activity vary according to species, age, and reproductive status. They are more developed in wild carnivores than in domestic ones [39]. In adult females, the ultrastructure and histochemical and biochemical features of interstitial cells are typical of steroid-producing cells, the function of which appears to be for progesterone and androgens synthesis [25]. Their origin varies between foetal and postnatal immature animals (mesenchyme-like cells) and mature females (hypertrophy of theca interna of large atretic follicles, interfollicular stromal cells) [12, 16, 17, 25]. In some pinnipeds, their number changes according to age and reproductive status or season [4, 7, 19, 26, 28, 33, 34] although the functional significance of this is still unknown. In *P. vitulina*, there is strong evidence that the main function of subcapsular crypts is the formation of cell cords only during the first year of life; these cell cords are activated and seem to differentiate in interstitial cells after puberty [26].

There are only brief descriptions of *Arctocephalus spp.* ovarian histology regarding *A. australis* [7] and *A. pussillus* [32], and most of them are done in adult animals. Until now, no detailed histological data has been provided about prepuberal *A. australis* ovaries, the characterization of subcapsular crypts and interstitial cells, and the possible changes that the ovaries could suffer before sexual maturity. Information about the basic ovarian structure would be useful, to compare it with sexually mature females as a way to improve our understanding of the reproductive physiology in this pinniped species.

On other hand, three-dimensional (3D) reconstruction is a modern tool for studying images obtained from serial histological or anatomical sections [10, 18], in order to allow a better understanding of organs, tissue structures, and stereoscopic relationships, and eventually it can provide information regarding their function and pathological changes. Kimura et al. [21, 22] made three-dimensional reconstructions of whole equine ovaries as a way to understand the anatomical arrangement and follicular development. No 3D reconstruction has been performed in pinnipedian ovaries.

Therefore, the objectives of the present work were to perform a detailed histological description of prepuberal *A. australis* ovaries, and to create a 3D model of the capsule, the subcapsular crypt system, and the primordial follicles in the superficial ovarian cortex.

Table 1. Morphometric data from prepubescent female *A. australis*

Age range (months)	Body length [cm]	N	Subjective body condition classification according to blubber thickness [cm]		
			Very thin to emaciated (0–0.5) or BC 1	Moderate (0.6–1.5) or BC 2	Good condition (1.6–3) or BC 3
1–7 (infant)	60–80	9	6	–	–
8–10 (weaned)	68–94	12	1	–	10
17 (juvenile)	90 and 115	2	–	1	–
Total		23	7	1	10

NOTES: 5 animals without data about blubber thickness
Subjective Decomposition Scale (SDS): 1 — recently dead or known time of death; 2 — fresh aspect, bright eyes/cornea, in *rigor mortis*; 3 — advanced autolysis: insufflated, opaque cornea, hair detachment; 4 — too advanced autolysis: insufflated, skin without hair, change in muscle and organ coloration); BC — body condition

MATERIAL AND METHODS

Ovarian samples were obtained from recently dead prepuberal *A. australis* females (n = 23) found on Cabo Polonio beaches (Rocha, Uruguay, 34°24′01′′S, 53°46′06′′W). Permission for animal sample collection for scientific purposes was obtained (No. 584/2006) from the National Direction of Aquatic Resources — Ministry of Livestock, Agriculture, and Fisheries (DINARA-MGAP), Uruguay. Necropsies were done following standard methods [11] and classified according to a subjective decomposition scale (SDS) from 1 (fresh) to 4 (advanced autolysis) (Table 1); only samples from 1 or 2 SDS were used for histology. Age was estimated by body morphometry, which takes into account body length [23] and sampling date. Body condition (BC) was estimated subjectively by measuring blubber thickness at the level of the xiphoid process (Table 1). The animals were grouped into three categories according to estimated age range: 1) infant or lactating, 2) weaned, and 3) juvenile (Table 1); nearly half of the pups sampled belonged to the weaned category (weaning occurs at around eight to ten months of age).

Field and laboratory work

Reproductive organs were sampled *in toto* together with the urinary bladder after performing an incision at the pubic symphysis. Ovaries were dissected from their ovarian bursae, sliced along the longitudinal axis, and fixed by immersion in 10% formalin solution. Histology was performed in both ovaries from twenty females. Ovaries were dehydrated in increasing concentrations of ethanol, immersed in chloroform, embedded in paraffin blocks, cut into serial sections (6–7 μm thickness), and stained either with haematoxylin and eosin or periodic acid-Schiff (PAS) and counterstained with haematoxylin and a modified Van Giesson trichrome technique in order to see details about PAS positive cell or cytological components and stomal structure.

Follicular population

Ovarian follicles were classified morphologically according to Lundy et al. [24] and Grimes et al. [15] where the following elements were taken into account: height and number of layers of granulosa cells, the presence or absence of a zona pellucida, thecal membrane, antrum formation, and degree of atresia of different follicles. The Nomina Histologica Veterinaria [27] was used for the histological nomenclature.

Three-dimensional reconstruction of subcapsular crypts and primordial follicles

To perform the 3D reconstruction of subcapsular crypts and primordial follicles, serial sections (n = 80 to 160/ovary, 6–7 μm thickness) where obtained from the left ovary of four females of the same age (approximately nine months old as determined by body length and sampling data) and good body condition (BC = 3). Eight 50%-overlapping-colour pictures from a random region of the superficial ovarian cortex were taken from every slide in the series with an image analysis system: light microscope (BX50, Olympus, Tokyo, Japan) connected to a video camera (SSC-C158P, Sony, Tokyo, Japan) and a personal computer with Image Pro Plus™

(Media Cybernetics, Silver Spring, MA, USA) software. Images were stored in .jpg format at a final magnification of 200×. They were then processed with BioVis3d, a newly-developed software (Institute of Electric Engineering, University of Uruguay, http://www.biovis3d.com). Digital reconstructions were done as follows: 1) mosaic building, by overlapping approximately 50% the eight images/slide in all four series, 2) registration of mosaics, i.e. correcting the relative position of mosaics to allow their correct overlapping/positioning along the series, 3) manual outlining of capsule, subcapsular crypts, and primordial follicles (from two to sixteen primordial follicles per section) — each feature was outlined with different colour lines and stored as distinct objects, so that the program could group them and rebuild properly each object, and 4) automatic digital reconstruction of histological structures (previously outlined features).

Finally, microanatomical arrangements of *A. australis* ovarian components from the superficial cortex were described.

RESULTS

The age of the female fur seals ranged from 1–17 months (Table 1), as estimated from body length, sampling data, and breeding season (we consider 15th of December as the date of birth [14]). Most seals had a good physical condition according to blubber thickness (BC = 3, media 1.61 ± 0.34 cm, n = 18, Table 1).

Macroscopic appearance

Ovaries were flattened, ovoid in shape, and completely covered by an ovarian bursa. The periovarian space was connected to the peritoneal cavity near the uterine horn through a small opening.

To the naked eye, the ovaries presented a smooth surface with no corpora lutea, corpora albicans, or antral follicles protruding to the surface.

Microscopic appearance

Topographically, the ovaries had an outer cortex (zona parenchymatosa) and inner medulla (zona vasculosa). The ovarian cortex was made up of connective tissue stroma which is continuous with the tunica albuginea that surrounds the ovary. The cortex contained follicles in different developmental stages, large numbers of interstitial cells (*endocrinocytus interstitialis*), and blood vessels (Fig. 1). The tunica albuginea was covered by the superficial epithelium, which invaginated and formed subcapsular crypts (Figs. 1, 2). The medulla contained large blood vessels and nerves (Fig. 3).

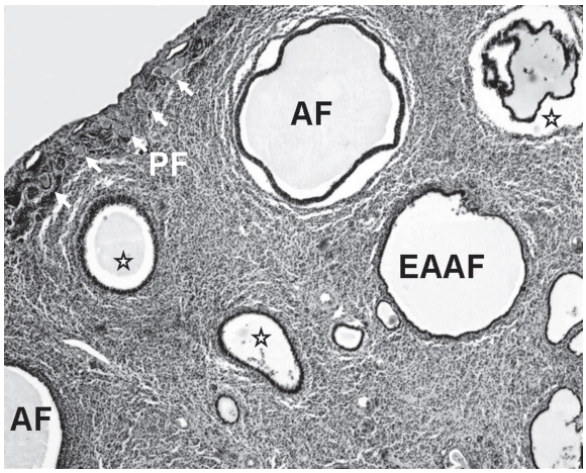

Figure 1. Panoramic view from the ovarian cortex: several primordial follicles in monolayer rows along the capsule; large antral follicles and early atretic antral follicles (white stars) at different depths in the cortex; large number of interstitial cells (H–E, bar = 10 μm); AF — antral follicle; EAAF and white star — early atretic antral follicle; PF — primordial follicles.

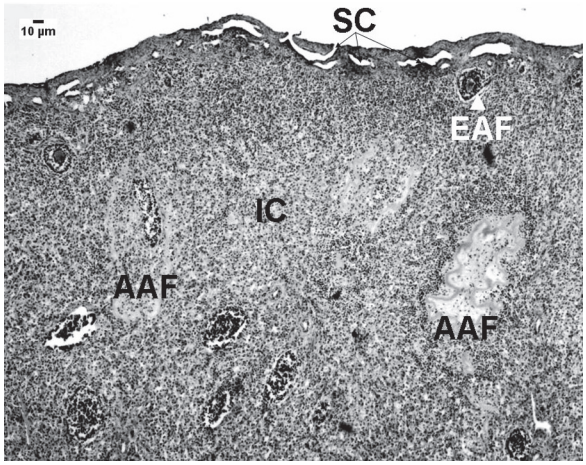

Figure 2. Panoramic view from the ovarian cortex; subcapsular crypts labyrinth with several openings to the surface; small early atretic follicles and advanced atretic follicles (with zona pellucida scars) located at different depths within the cortex; large number of interstitial cells (H–E, bar = 10 μm); SC — subcapsular crypts; AAF — advanced atretic follicle; IC — interstitial cells; EAF — early atretic follicle.

The particular features of each region and tissue are described as follows.

Stroma

The ovarian stroma was organized in a tunica albuginea and an internal network of septa running through the cortex and medulla. The tunica albuginea and medulla were formed by dense connective

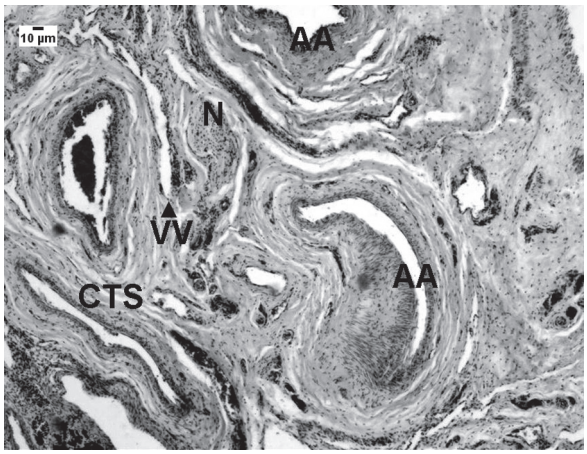

Figure 3. Cortico-medullary limit. The medulla is made up of connective tissue giving support to large arteries, veins and nerves (H–E, bar = 10 μm); AA — artery; VV — vein; CTS — connective tissue septa; N — nerve.

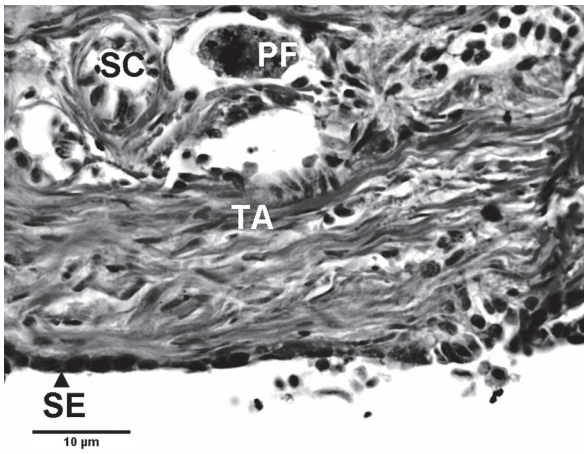

Figure 5. Ovarian capsule: superficial epithelium covers the tunica albuginea made up of connective tissue fibres which support the subcapsular crypts and some branching fibres surround a primordial follicle (modified van Giesson trichrome, bar = 10 μm); SC — subcapsular crypts; PF — primordial follicles; SE — superficial epithelium; TA — tunica albuginea.

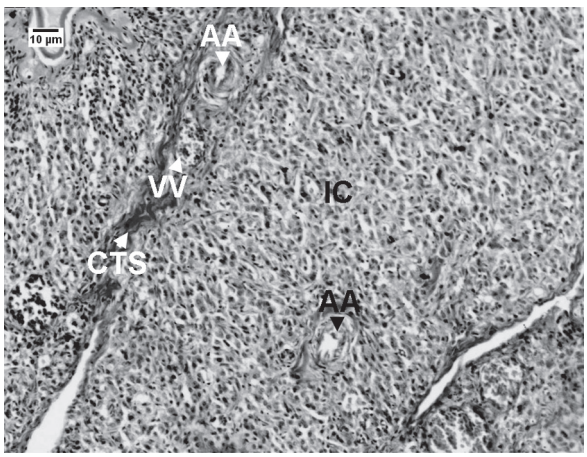

Figure 4. Connective tissue septa which give support to blood vessels (arteries and veins) through the cortex; large amount of interstitial cells (modified van Giesson trichrome, bar = 10 μm); AA — artery; VV — vein; IC — interstitial cells; CTS — connective tissue septa.

tissue (mainly collagen fibres) that gave rise to septa supporting blood vessels (Fig. 4). Collagen fibres in the tunica albuginea were oriented parallel to the surface and surrounded subcapsular crypts (Fig. 5). In a few places the collagen fibres also surrounded primordial follicles, small blood vessels, and groups of interstitial cells. The tunica albuginea formed a thin band in most of the ovarian surface but thickened near the hilum (Fig. 5).

Ovarian medulla, blood vessels, and nerves

The ovarian medulla accounted for a small part of the ovarian volume. It contained large veins, arteries, and nerve fibres (Fig. 3). Blood vessels gave rise to smaller branches that reached the superficial cortex beneath the tunica albuginea via connective tissue septa (Fig. 4). In a few animals, interstitial cells were also present in the medulla organized in small groups lined by thin bands of connective fibres.

Ovarian germinal epithelium

The ovarian germinal or superficial epithelium was a typical single layer of cells the height of which varied from flat to columnar (Fig. 5). The epithelial cells gradually diminished their height at the site where the epithelium invaginated and formed the subcapsular crypts.

Subcapsular or subsurface crypts

Subcapsular crypts were invaginations from the surface epithelium into the tunica albuginea, and ran parallel to the capsule. They were made up of interconnecting cisternae and tubules with multiple openings into the ovarian surface (Figs. 2, 6). The wall was lined by three types of epithelial cells which were either tall columnar, cubical, or squamous cells. Squamous cells were usually restricted to the superficial region of the crypts or to crypt entrances, but this pattern was not constant. The middle and deep parts of the crypts were lined by an epithelium with alternating columnar and cubical cells. Near the ovarian hilum, the crypts went deeper into the ovarian cortex and were surrounded by fibroblasts; their walls were

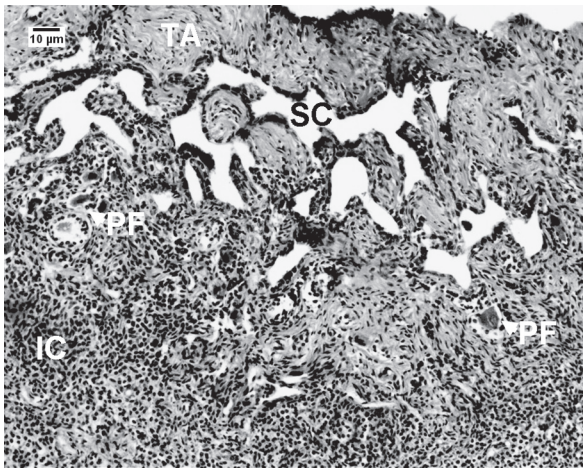

Figure 6. Superficial cortex near the hilum: deep subcapsular crypts labyrinth within a thick tunica albuginea and small group of primordial follicles; large number of interstitial cells (H–E, bar = 10 μm); SC — subcapsular crypts; PF — primordial follicles; TA — tunica albuginea; IC — interstitial cells.

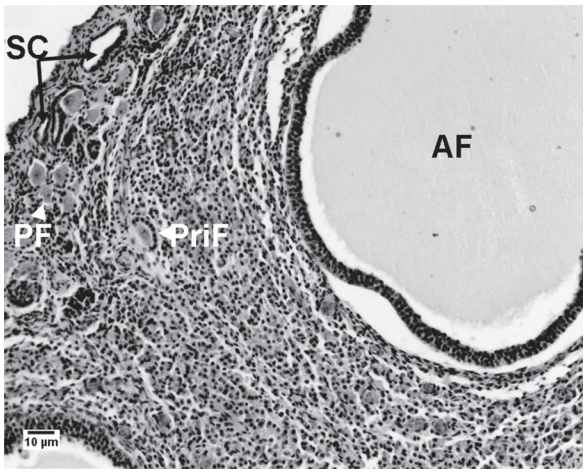

Figure 7. Superficial cortex showing a small group of primordial and a primary follicles associated to subcapsular crypts; large antral follicle whit a thin thecal membrane and large number of interstitial cells (H–E, bar = 10 μm); AF — antral follicle; PriF — primary follicle; SC — subcapsular crypts; PF — primordial follicles.

made up of columnar and cubic cells lining a central lumen (Fig. 6).

With the aid of BioVis3D software, we could obtain a 3D colour model of subcapsular crypts (white), primordial follicles (pink) distribution, and of the ovarian capsule (green) (http://www.dmd.fvet.edu.uy/histologia/helenita/ene2009/). In general, subcapsular crypts formed a labyrinth network with multiple interconnections and openings to the ovarian surface. Some crypts were small and isolated and were surrounded by a few primary follicles whereas most ovarian crypts formed an extended, irregular, interconnected system associated with many primordial follicles. The pattern of distribution and arrangement was similar in all animals studied.

Ovarian follicles

Ovarian follicles appeared at different developmental stages and were located at different depths within the cortex. According to follicle morphology [15, 24], they were classified as follows:

Primordial follicles (type 1) were surrounded by a single sheet of flattened granulosa cells (Fig. 5). Usually they were distributed in irregular, mono-layered rows along the capsule and mostly associated with subcapsular crypts (Fig. 1), but in some cases they formed groups containing between four and thirty follicles (Figs. 6, 7). Three-dimensional reconstruction showed them distributed in small-monolayer groups close to the crypts within the superficial cortex. No follicles were seen inside the crypts.

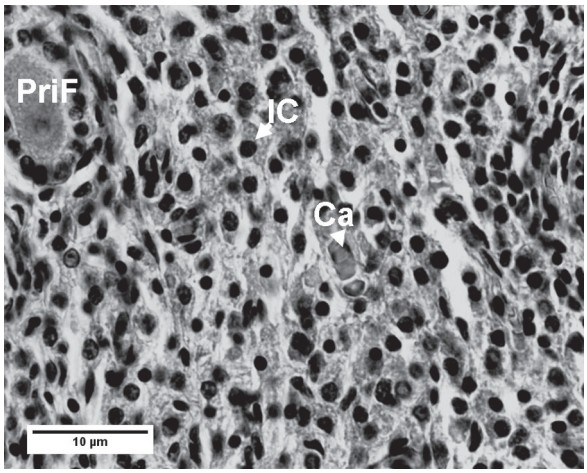

Figure 8. Interstitial cells with typical polyedric shape, round eccentric nuclei and pale cytoplasm in strong association with capillaries; primary follicle surrounded by a single layer of granulose cells and thin band of fibroblasts (H–E, bar = 10 μm); PriF — primary follicle; Ca — capillary; IC — interstitial cells.

Primary follicles (type 2) were covered by one layer of cubical granulosa cells, surrounded by fibroblasts and connective tissue fibres and located in the superficial cortex (Figs. 7, 8).

Some primordial and primary follicles were lined by a thick layer of fibroblasts and connective fibres originating from the tunica albuginea.

Small and large preantral follicles (types 3 and 4) presented more than two complete layers of cubical granulosa cells with emerging antral spaces.

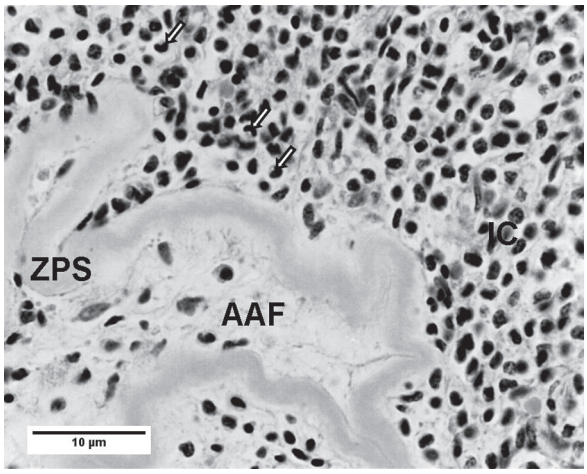

Figure 9. Advanced atretic follicle (with zona pellucida scars) surrounded by fibroblast and several macrophages in the stroma; large number of interstitial cells (H–E, bar = 10 μm); AAF — advanced atretic follicle; IC — interstitial cells; ZPS — zona pellucida scars; black border arrow: macrophages.

Thecal membranes were thin and formed by one or two layers of flattened cells. Oocytes were surrounded by a thin PAS positive zona pellucida.

Small and large antral follicles (small type 5 and type 5) had a clearly defined antral space and three to four complete layers of cubical granulosa cells. Oocytes were big and surrounded by a well-defined zona pellucida and corona radiata. The thecal membrane was well defined and made up of a few cellular layers. In large antral follicles, thecal cells presented oval nuclei with a fibroblastic aspect, and the external layer contained an evident capillary network. These follicles were located at varying depths within the cortex (Figs. 1, 7).

Most follicles were monovular, but we found a few preantral and antral follicles containing two oocytes.

Atretic follicles were abundant in the superficial cortex (Figs. 1, 2). Different stages of atresia were observed in all types of follicles described but were more evident in antral ones. Early atretic antral follicles showed detachment of granulosa cells into the antral cavity (Fig. 1) and pycnotic bodies in the granulosa layer. In most cases, oocyte nuclei either presented evident degenerative changes or were absent; the zona pellucida remained intact. On the other hand, the advanced atretic follicles had completely degenerated or missing oocytes (Fig. 2); scars were present as remnants of the zona pellucida (Fig. 9). Degenerated granulosa cells completely invaded the antral cavity; such granulosa cells were either small cells with pycnotic nuclei or round big cells with oval or round euchromatic nuclei, an evident nucleolus, and a pale cytoplasm similar to interstitial cells. Thecal cells formed an external layer (with fibroblastic appearance) which closely followed the general shape changes of the follicle into a big irregular branching or ovoid structure. Macrophages were also evident in the surrounding stroma (Fig. 9). In some cases, thecal connective fibres were continuous with the tunica albuginea.

Interstitial cells *(endocrinocytus interstitialis)*

In a panoramic view, interstitial cells were distributed homogenously in large numbers throughout the cortex (Figs. 1, 2, 4, 6). At higher magnification they were organized in round, long, or irregular groups limited by thin bands of connective tissue or just loosely between follicles (Fig. 8). Haematoxylin-eosin staining showed interstitial cells as big, polyhedral cells with pale eosinophilic cytoplasm and round eccentric euchromatic nuclei (Figs. 8, 9). At the ovarian medulla, these cells formed small groups between fibres and blood vessels. A very dense capillary network accompanied them (Fig. 8). No epithelial cell cords were observed near subcapsular crypts.

DISCUSSION

According to our knowledge, this is the first detailed histological description of prepuberal *A. australis* ovaries.

The basic cortico-medullary structure with a cohort of follicles in different developmental stages was similar to other mammals and immature seal ovaries [30, 33, 39]. Skinner and Westlin-Van Aarde [33] described ovaries from mature Ross seals (*Ommatophoca rosii*) in which primordial and primary follicles were always included in the tunica albuginea. This was rarely seen in prepuberal *A. australis* ovaries, which in turn presented those follicles beneath the tunica albuginea in monolayer rows, as seen in other seals [34].

Subcapsular crypts of *A. australis* prepuberal ovaries were similar, in general, to other seal species and were present in very young animals (as young as one month of age) [2, 13, 26, 39]. Three--dimensional reconstruction images confirmed the irregular branching pattern of crypts beneath the capsule and the close vicinity to primordial follicles, which were located next to them but not included within their lumina as mentioned by Craig [8]. Although morphometry had not been performed,

ovarian crypts did not show apparent differences in depth associated with animal age during the prepuberal period. In *P. vitulina*, crypts were short and narrow in juvenile animals, whereas in adult females they were long, branched, stretched, or dilated; the epithelium height (from flat to cylindrical) and crypt complexity were more evident during the reproductive season when the activity of the ovary [26] and hormonal influences (estradiol 17β, 350 pmol/L, [5]) are Highest. On the other hand, crypts were rarely seen in adult *O. rossii* ovaries during the period of free blastocyst stage (diapause) [33]. Both reports show that crypt structure reflects changing ovarian activity at different moments in the reproductive cycle of seals. In *P. vitulina*, the main function of subcapsular crypts is the formation of a cell pool (cell cords) during the first year of life, which become activated and apparently differentiate in steroid hormone synthesizing interstitial cells after puberty [26]. In other domestic carnivore species (*Canis familiaris*), there is a subcapsular system (cortical tubules) [1], which is a source of interstitial cells [17]. We did not find these cell cords in prepuberal *A. australis* ovaries; therefore, we cannot assume the same functions as subcapsular crypts in other species.

A. australis interstitial cells were abundant and dispersed throughout the ovarian cortex in early stages of postnatal life (from one month of age, the youngest animal sampled). This feature represents one of the main differences with other seals, in which they were first detectable in 2–3-year-old females, are linked to growing follicles (antral follicles), and are organized in groups [26]. In other species, interstitial cells could originate either from subcapsular crypts or fibroblasts (*P. vitulina* [26]) or, apparently, from thecal cells (*O. rossi*, [33]). In prepuberal *A. australis,* due to the absence of cell cords originating from the crypts system, the high amount of atretic follicles, and the abundance of interstitial cells throughout the ovarian cortex, we speculate that interstitial cells might have another origin, perhaps mesenchyme-like cells. However, we do not have direct evidence supporting such a hypothesis. In the newborn seal, the ovarian medulla (medullary substance) acts as a provisional endocrine gland and undergoes hypertrophy (*Leptonychotes weddellii*, [19]; *Phoca groenlandica*, *Erignatus barbatus*, *P. vitulina* [28]; *N. cinerea* [34]). Later on, they suffer regressive changes and interstitial cells are substituted for connective tissue [28, 34]. In contrast with this information, we could not find these kinds of changes in prepuberal *A. australis*. In seals, the transformation process continues as the sexual maturation period approaches and the amount of interstitial cells increases [26, 28]; in adult females, cell numbers change cyclically according to the stage of the oestrous cycle [19]. This last feature has also been described in *A. australis* mature females [7] but there are no details about seasonal or reproductive cycle changes.

Little is known about interstitial cell function in pinnipeds; in Phocidae (*P. groenlandica*, *P. vitulina*, *E. barbatus, L. weddellii*) they are probably a source of sex hormones because of the presence of cytoplasmic lipid inclusions and organelles indicative of steroidogenesis [20, 26, 28]. In adult *C. ursinus,* interstitial cells expressed steroidogenic enzymes (17α-hydroxylase/17,20-lyase and cytochrome *b5*) related to sexual hormone synthesis (androgenesis) [3], which is in strong correlation with high sexual steroid concentrations in serum during the reproductive season (also noted in *P. vitulina* [26]).

CONCLUSIONS

In conclusion, prepuberal *A. australis* ovaries presented similar features to other pinnipeds ovaries. However, the early presence and abundance of interstitial cells is a relevant finding which is apparently characteristic of *A. australis* prepuberal ovaries. Three-dimensional reconstruction software was a useful technique to understand the close spatial relationship of primordial follicles and crypts as part of the ovarian cortical microarchitecture. On the other hand, the histomorphological evaluation of interstitial cells did not provide enough information regarding their origin.

Studies should be continued in order to analyze if there are morphological changes at different ages and stages of the oestrous cycle in *A. australis* ovaries, their relation to crypt development, and the origin and function of interstitial cells. Localization of steroidogenic enzymes will give crucial information about the source of sexual hormones that regulate the *A. australis* reproductive cycle.

ACKNOWLEDGEMENTS

This study relied heavily on the kind support of my friends and colleagues Diana Morgades, Oscar Castro, Oscar Correa, C. Gustavo de Souza and Francisco Gutiérrez for field assistance and Tech. Mónica Viqueira for laboratory aid (Veterinary Faculty, University of Uruguay). Especial thanks to Dra. Graciela Pedrana, who gave great support during the hardest moments throughout academic life. I am

also grateful to: Ing. Juan Cardelino, Martín de los Heros and Javier Preziozi who developed and taught us how to use the Biovist3D software, my seminar students Cortazzo N, Delgado R, Díaz A, Díaz G, Ganuza F, Olivera K, Pais B, Perdomo A, Pintos J, Rodríguez S with which we started the use of 3DR software, Msc. E. Páez, Lic. A. Ponce de León, C. Barreiro, M. Casella, F. Machado and M. Pereira (DINARA-MGAP) for institutional and field support of our activities in Cabo Polonio, Lic. Ma. Florencia Grande (Laboratorio de Mamíferos Marinos, Centro Nacional Patagónico) for Corcuera's thesis. This work was partially financed by CSIC (Comisión Sectorial de Investigación Científica) Uruguay.

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