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MACROSCOPIC AND MICROSCOPIC DESCRIPTION OF ALPACA (*Vicugna pacos*) OVARIES DURING THE FETAL STAGE

Descripción macroscópica y microscópica de ovarios de alpaca (*Vicugna pacos*) durante la etapa fetal

Eliana Vilca Ypanaqué[1]*, Miluska Navarrete Zamora[1], Gilberto Santillán Altamirano[2], Alexander Chávez Reategui[1], Francisco Santos Rueda[1]

[1] Laboratory of Animal Anatomy and Wildlife; [2] Laboratory of Animal Histology, Embryology and Pathology, Faculty of Veterinary Medicine – Universidad Nacional Mayor de San Marcos, Lima-Perú.

* Corresponding author: Eliana Vilca, Av. Circunvalación s/n cuadra 28 San Borja, 15021, Perú. e-mail: eliana.vilca@unmsm.edu.pe

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ABSTRACT

The aim of this work was to describe macroscopically and microscopically the ovaries of alpacas during the fetal stage. We worked with 18 female fetuses collected at the Huancavelica Municipal Slaughterhouse, from alpacas destined for human consumption. The process and analysis of the collected samples was performed at the Faculty of Veterinary Medicine - UNMSM, in Lima, Peru. Fetal age was calculated by measuring the biparietal diameter and divided into three groups: Group I (60 - 150 days), Group II (151 - 239 days) and Group III (240 - 335 days). The weight of the ovaries was 0.02 ± 0.01, 0.03 ± 0.01 and 0.03 ± 0.01 grams in the first, second and third group, respectively. The ovaries were paired, oval-shaped, with a smooth, cream surface, located in the sublumbar region at the level of the 6th and 7th lumbar vertebrae. The cortex and medulla were visible from the third month. Microscopically, in Group I, we observed oogonium, the distinction between cortex and medulla, unilaminar primary follicles, atresia and segmentation of the germ cells. In Group II, we observed preantral primary follicles. In Group III, we observed preovulatory follicles. We concluded that, from day 68 of the fetal stage of the alpaca, ovaries have macroscopic characteristics similar to those of an adult alpaca. Microscopically, they presented ovogonia, primordial follicles, unilaminar primary follicles, preantral follicles, preovulatory follicle, as well as the degeneration of the germ cells.

Keywords: alpaca, fetus, ovaries, preovulatory follicle.

RESUMEN

El objetivo de este trabajo fue describir macroscópica y microscópicamente los ovarios de alpacas durante la etapa fetal. Se trabajó con 18 fetos hembra recolectados en el Matadero Municipal de Huancavelica, provenientes de alpacas con destino al consumo humano. El proceso y análisis de las muestras recolectadas se realizó en la Facultad de Medicina Veterinaria - UNMSM, en Lima, Perú. La edad fetal se calculó midiendo el diámetro biparietal y se dividió en tres grupos: Grupo I (60 - 150 días), Grupo II (151 - 239 días) y Grupo III (240 - 335 días). El peso de los ovarios fue de 0,02 ± 0,01, 0,03 ± 0,01 y 0,03 ± 0,01 gramos en el primer, segundo y tercer grupo, respectivamente. Los ovarios eran pares, de forma ovalada, de superficie lisa color crema, ubicados en la región sublumbar a nivel de la 6ª y 7ª vértebra lumbar. La corteza y la médula eran visibles a partir del tercer mes. Microscópicamente, en el Grupo I, observamos oogonio, distinción entre corteza y médula, folículos primarios unilaminares, atresia y segmentación de las células germinales. En el Grupo II, observamos folículos primarios preantrales. En el Grupo III, observamos folículos preovulatorios. Concluimos que, a partir del día 68 de la etapa fetal de la alpaca, los ovarios presentan características macroscópicas similares a las de una alpaca adulta. Microscópicamente presentaron ovogonia, folículos primordiales, folículos primarios unilaminares, folículos preantrales, folículo preovulatorio, así como la degeneración de las células germinales.

Palabras clave: alpaca, feto, ovarios, folículo preovulatorio.

INTRODUCTION

South American Camelids are one of the few species able to adapt to the hostile high-altitude areas in the Peruvian territory, thus becoming a valuable economic and productive resource to most Andean families. They represent between 70 to 80% of the annual family income, with their high-quality fiber and meat (FAO, 2008). Peru has the biggest population of alpaca in the world, with 85% of the total world population distributed in 17 provinces (MINAGRI, 2015).

Despite this high relevance, there are not complete studies that describe their fetal development. In this process, the fetus achieves its sexual differentiation at the second month of gestation (Bravo, 2002), which lasts for approximately 345 days (Fernández-Baca, 1993).

The reproductive system is the fundamental piece for the reproductive function. This system consists of primary organs or gonads (ovaries), accessory organs (oviduct, vagina, greater and lesser vestibular glands) and copulatory organs and external genitalia (vagina, clitoris and vulva) (Sato y Montoya, 1990).

Ovaries are glandular organs located inside the abdominal cavity (Sato and Montoya, 1990). They produce and develop follicles through all their stages, being the last preovulatory follicle the one that provides the oocyte (Illera M et al., 1994), and corpus luteum (Bravo, 2002). The ovaries are also responsible of secreting sexual hormones, such as estrogens and progesterone. After the oocyte is formed it may be fertilized by the sperm, thus conceiving a new being.

It is extremely important to assure a good reproductive level. This would increase the number of offspring per herd and per year (FAO, 1996). Therefore, this study aims to describe the ovaries during the fetal stage of the alpaca, both macroscopically and microscopically, in order to amplify the knowledge on its anatomy and physiology.

MATERIALS AND METHODS

Samples were obtained at the Huancavelica Municipal Slaughterhouse, located at the Huancavelica district, Huancavelica province, Huancavelica region, with an altitude of 3660 MASL. This slaughterhouse is authorized by SENASA (National Agrarian Health Service) for the slaughter of South American Camelids.

Sample processing and analysis was done at the Morphological Investigation area of the Animal Anatomy and Wildlife of the Faculty of Veterinary Medicine of the San Marcos University (FMV – UNMSM) in Lima. Samples were granted and financed by the project 025-2016-FONDECYT, which was approved by the ethics committee, N° 018-004.

Methods

18 female Huacaya fetuses obtained from pregnant alpacas destined for human consumption were divided into 3 groups, with 6 fetuses in each group. The first group included fetuses between 60 – 150 days, the second group included fetuses between 151 – 239 days and the third group included fetuses between 240 – 335 days of age. The fetus with the shortest time of gestation had 68 days of development, whereas the fetus with the longest time of gestation had 324 days. This was determined using the formula described by Gazitua et al., 2001, in which we use the biparietal diameter. We used fetuses starting from 60 days of gestation, as described by Bravo in 2002.

A. Macroscopic study

The attributes, location and relationship towards other organs was observed in situ. The size (length and width) and weight were determined using a 0.01 grams analytical balance. Afterwards, the right and left ovary was separated and preserved in small plastic containers with 10% formaldehyde for subsequent histological processing.

B. Microscopic study

The ovaries were reduced with a longitudinal section in the middle of the organ. Following this, the organ was embedded with 5 um paraffin for subsequent Hematoxilin – Eosin (H-E) staining to observe the basophile nucleus and acidophile cytoplasm, and Masson's trichrome stain to observe the connective tissue as blue, and the muscular tissue as red.
Histological description of the ovaries was done by recognizing the microscopical characteristics of its components: ovarian epithelium, tunica albuginea, cortex (with the observation of all the stages of follicle development), medulla (with its blood vessels) and connective tissues, using both H – E and Masson's trichrome stains.

C. Data Analysis

Collected data, such as weight, width, length of fetuses and ovaries in all three groups, was analyzed through statistical measures such as mean and standard deviation.
Macroscopic and microscopic observations were stored as photographic images.

D. Histological reading

Histological sections were observed with an optical microscope Leica DM 750, which had an incorporated digital camera ICC50W and measurement software Las4.12 Leica Macrosistems.

Microscopic characteristics were observed following Junqueira, 2013:

- Lining Epithelium: a single layer of flat or cubical cells
- Tunica albuginea: dense connective tissue
- Cortex: consisting of ovarian follicles

Ovarian follicles:

- Primary follicle: oocytes surrounded by flat cells known as follicular cells
- Unilaminar primary follicles: consist of an oocyte surrounded by a single layer of cuboidal follicular cells
- Multilaminar primary follicles: Also known as Preantral follicles, they consist of oocytes surrounded by two or more layers of follicular cells, now called granulosa cells.
- Secondary follicles: Also known as antral follicles, observed as oocytes surrounded by granulosa cells that have small spaces containing follicular fluid
- Preovulatory follicle: Also known as Graafian follicle, consists of a single follicular cavity of great dimensions and thinner layers of granulosa cells
- Medulla: Loose connective tissue with an abundancy of blood vessels

RESULTS

Macroscopic observations

Sex can be determined by macroscopic observation at 68 days of fetal development. Ovaries were located at the pelvic cavity corresponding with the caudal abdominal region, in a sub-lumbar position between 6th and 7th lumbar vertebra, caudal to the kidneys. The right ovary was more in a more cranial position than the left. Fetal ovaries are paired organs, with a smooth surface and a cream color (Figure 1A). Both ovaries were anchored to the cavity by the mesovarium, part of the broad ligament (Figure 1B). After a longitudinal section, two areas were observed. An external one, named cortex, and an internal one, named medulla, which presented a darker color in fetuses older than three months of fetal age (Figure 1C). Uteri were thin and elongated in fetuses younger than three months of fetal age. After this age, uteri were thicker and began turning cranially. At four months of fetal age a complete cranial turn was observed, with the ovaries in a sub-lumbar position (Figure 1D).

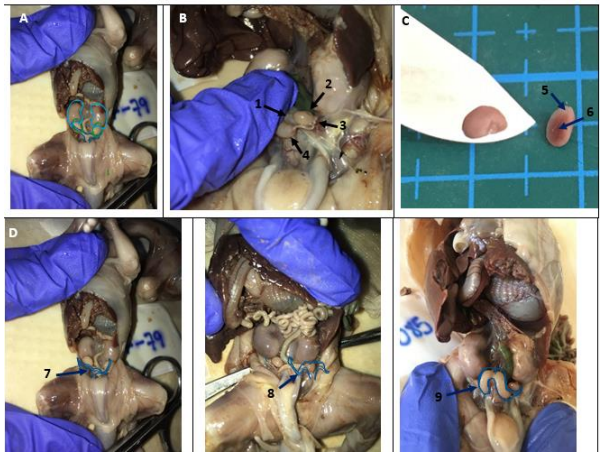

Figure 1. A. Caudal abdominal cavity of a female alpaca fetus of 2 and a half months. Ovaries, uterus and kidneys are delimited with the colors green, blue and light blue, respectively. B. Anchoring structures of the ovaries in an alpaca fetus of 4 months: 1. Mesovarium 2. Ovary 3. Oviduct 4. Uterine horn C. Longitudinal section of the ovary of an alpaca fetus: 5. Cortex 6. Medulla. D. Caudal abdominal cavity of female alpaca fetuses. Thickening and turning of the uterus can be observed in fetuses of 2 and a half months (7), 3 months (8) and 4 months (9).

Microscopic observations

Descriptions were categorized by groups and stains, beginning with H-E and finishing with Masson's Trichrome.

First group (68-138 days)

Lining epithelium was completely developed starting at day 102. Lining epithelium was observed as a single layer of both cuboidal and columnar epithelial cells, with ovoid nuclei of different sizes, some of them filling almost the whole cell, located in a distal position (Figure 2A).

No distinction of cortex and medulla was observed until day 106. A great amount of germinative cells named oogonium were observed in the ovarian stroma (Figure 2B). These are ovoid shaped cells with different sizes of nuclei, some as big as to fill the whole cell, decreasing the size of the cytoplasm; some of lesser size located at the middle or the end of the cells. Many oogonium were found in clusters, going from a distal towards a central position. They varied in quantity during the whole development, diminishing in number towards the end and locating in the periphery of the ovary.

Degeneration of some germ cells was evidenced, in a process named atresia (Figure 2B), observable in all three groups and in follicles of different growth stages. Cellular segmentation was observed, in addition to oocytes surrounded by flat follicular cells, named Primary follicles (Figure 2C) which were surrounded by stromal cells. In the middle of this group there were oocytes surrounded by a single layer of cuboidal follicular cells, named unilaminar primary follicles (Figure 2D), located mainly in the medulla, near the blood vessels.

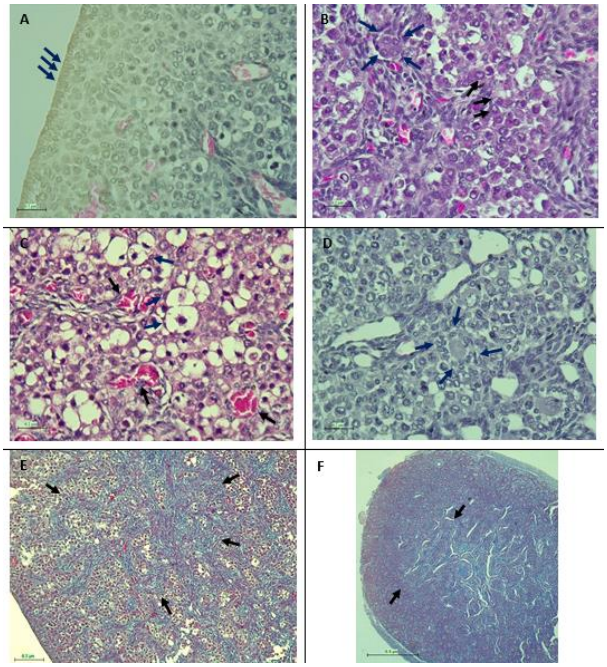

Figure 2. A. Right ovary of an alpaca fetus of 106 days of gestation. Germinal epithelium can be observed, with columnar and cuboid cells, with big ovoid nucleus in a distal position. B. Left ovary of an alpaca fetus of 68 days of gestation. Four oogonium (blue arrows) and germ cells (black arrows) in a degenerative process (atresia) can be seen. C. Ovary of an alpaca fetus of 102 days of gestation. Primary follicles surrounded by stromal cells (blue arrows) and blood vessels (black arrows). D. Right ovary of an alpaca fetus of 106 days of gestation. One primary follicle can be seen. H-E Stain, 40X. E. Right ovary of an alpaca fetus of 68 days of gestation. Masson's Trichrome Stain, 10X. F. Left ovary of an alpaca fetus of 138 days of gestation. Connective tissue can be seen in a blue color (black arrows). Masson's Trichrome Stain, 4X.

Second group (151 – 218 days)

Observation of the connective tissue was made possible by Masson's Trichrome stain. It was observed as a blue mesh-like structure, distributed in the whole ovary starting at a distal position towards the middle. There were fusiform cells with an ovoid or flat nucleus, with fibers pointing at all directions. Germ cells, blood vessels and follicles in different stages were

observed surrounding the structures. Connective tissue was found in both ovaries, in varying amounts (Figure 2E and 2F).

A transition can be seen at the lining epithelium. In samples with fewer days of gestation there were only columnar shaped cells with different sizes of ovoid nuclei, and in some parts of the epithelium germ cells could be observed (Figure 3A), whereas in samples with more days of gestation there were columnar and cuboid cells. In the ovarian cortex, a proliferation of follicular cells could be observed in samples with fewer days of gestation. In the right ovary there was an invagination with simple columnar epithelium in the inferior portion (Figure 3B). In samples with more days of gestation there was a higher quantity of preantral follicles (follicles with two or more layers of follicular cells) (Figure 3C) in the left ovary than in the right ovary.

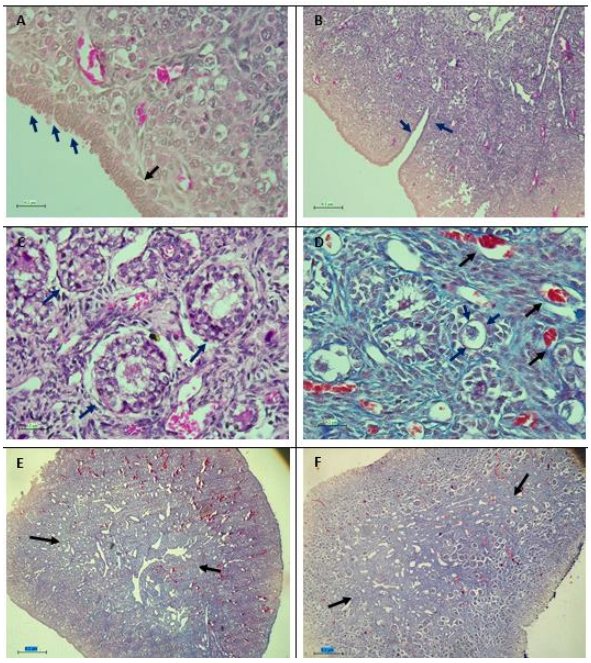

Figure 3. A. Right ovary of an alpaca fetus of 151 of gestation. Germinal epithelium can be observed with a single row of columnar epithelial cells (blue arrows) and a germ cell (black arrow). B. Right ovary of an alpaca fetus of 151 days of gestation. An invagination towards the center can be observed in the germinal epithelium (blue arrows). H-E Stain, 10X. C. Left ovary of an alpaca fetus of 218 days of gestation. Primary preantral follicles can be observed, with a proliferation of follicular cells (blue arrows). H-E Stain, 40X. D. Right ovary of an alpaca fetus of 187 days of gestation. Several unilaminar primary follicles can be observed, one with its zona pellucida (blue arrows). Some blood vessels can be seen (black arrows). Masson's Trichrome Stain, 40X. E. Right ovary of an alpaca fetus of 151 of gestation. Masson's Trichrome Stain, 4X. F. Right ovary of an alpaca fetus of 218 days of gestation. Connective tissue can be seen in a blue color. Masson's Trichrome Stain, 4X.

In the ovarian medulla, an organization of great quantities of different sizes of blood vessels could be observed in the right ovary. A better visualization of an unilaminar primary follicle with its zona pellucida was achieved with Masson's Trichrome Stain (Figure 3D). In the left ovary there was a size reduction in blood vessels and an increase in unilaminar and preantral primary follicles. Both ovaries end with large amounts of blood vessels.

With Masson's Trichrome Stain, connective tissue was observed as a blue mesh-like structure distributed in the whole ovary starting at a distal position towards the middle. In all samples there were oogoniums in strings, blood vessels and developing follicles. Connective tissue was found in both ovaries, in varying amounts (Figure 3E and 3F).

Third Group (240 – 324 days)

A single layer of columnar, cuboid and squamous cells was observed in the lining epithelium.

In the ovarian cortex a great amount of unilaminar and preantral primary follicles was observed, and a decrease of oogonium and primary follicles. Three wide invaginations towards the center were observed in the right ovary, and one in the left ovary, with a simple epithelium of columnar and cuboid cells.

A higher amount of preantral follicles was observed at the cortico-medullary junction, each of them with their respective theca and blood vessels (Figure 4A). A higher amount of unilaminar and preantral primary follicles was observed in both ovaries as fetal development progressed (Figure 4B).

A preovulatory follicle was observed in the left ovary. This follicle features a single antral cavity, where follicular fluid occupies most of its area. A cumulus – oocyte complex points towards one end, and the oocyte is surrounded by its zona pellucida and the corona radiata, both surrounded by a subset of granulosa cells, called cumulus oophorus. This structure connects to the rest of granulosa cells, surrounding everything previously described in a thin layer. In the outer portion of this follicle theca interna and theca externa can be observed, which are separated from the follicle by the basal lamina (Figure 5).

Ovarian Medulla

A great amount of blood vessels of different sizes was observed. A better visualization of a developing follicle and its parts was achieved with Masson's Trichrome Stain. It also made possible to observe the division of the follicle with the follicular theca by the basal lamina in a clear way (Figure 4C and Figure 4D). In later samples, the right ovary was observed filled with developing follicles that reduce the amount of blood vessels. A great amount of lymphatic and blood vessels was observed in the left ovary.

With Masson's Trichrome Stain, connective tissue was observed as a blue mesh-like structure distributed in the whole ovary starting at a distal position towards the middle. In all samples there were oogoniums in strings, blood vessels and developing follicles. Connective tissue was found in both ovaries, with a higher quantity in the right ovary (Figure 4E and 4F).

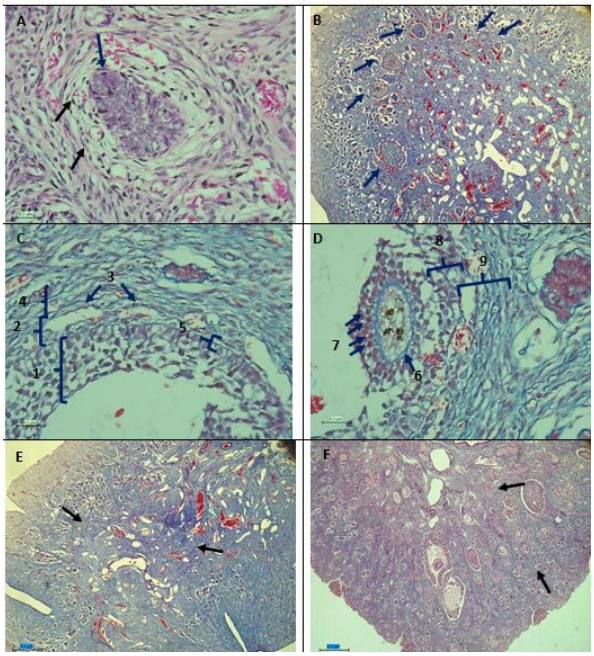

Figure 4. A. Left ovary of an alpaca fetus of 279 days of gestation. A preantral follicle is observed (blue arrow) with follicular theca and blood vessels (black arrows). B. Ovary of an alpaca fetus of 292 days of gestation. Blood vessels can be observed organized surrounding the growing follicles at the cortico-medullary junction (blue arrows). Masson's Trichrome Stain. 10X. C. Left ovary of an alpaca fetus of 303 days of gestation. The following structures can be observed: 1. Zona granulosa (layers of granulosa cells). 2. Theca interna. 3. Blood vessels. 4. Theca externa. 5.Basal lamina separating the follicle from follicular theca. Masson's Trichrome Stain. 40X. D. Left ovary of an alpaca fetus of 303 days of gestation. The following structures can be observed. 6. Zona pellucida in a blue color. 7. Corona radiata. 8. Zona granulosa. 9. Follicular theca. Masson's Trichrome Stain. 40X. E. Right ovary of an alpaca fetus of 240 days of gestation, 4X. F. Left ovary of an alpaca fetus of 324 days of gestation. Connective tissue can be observed in a blue color (black arrows). Masson's Trichrome Stain. 4X.

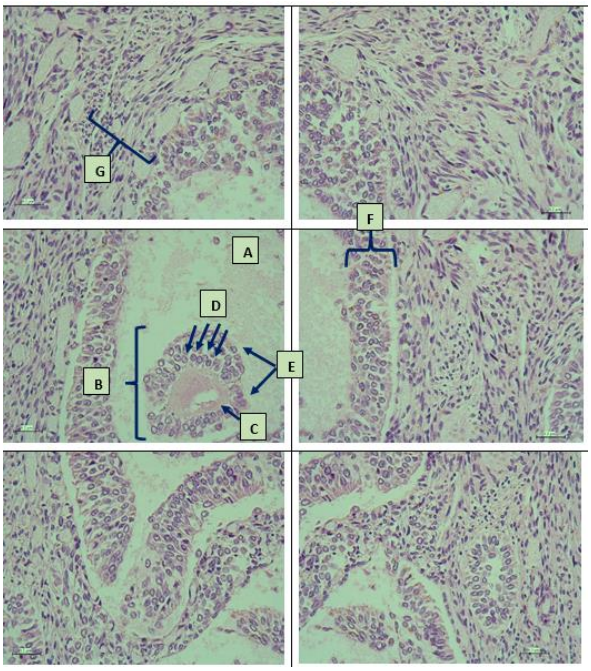

Figure 5. Left ovary of an alpaca fetus of 324 days of gestation. The following structures can be observed. A. One follicle surrounded by its antrum and the follicular fluid. B. Cumulus oocytum complex. C. Oocytum surrounded by its zona pellucida. D. Corona radiata. E. Cumulus oophorus. F. Zona granulosa. G. Theca interna and externa. H-E Stain, 40X.

DISCUSSION

The sample with the lowest gestational age was an alpaca fetus of 68 days. Feminine sex could already be macroscopically distinguished in this fetus. This finding agrees with Ferradas et al., who in 2015 mentions that at 46 days of gestational age sex could already be determined via observation of external genitalia.

Ovaries are paired organs, with ovoidal shape. These observations fit the descriptions of adult ovaries by Sato and Montoya in 1990; they were similar to bovine, ovine and caprine ovaries but different to mare ovaries, which are kidney-shaped due to the presence of a well-defined wedge called the ovulation fossa, described by Hafez B and Hafez E in 2002. This description differs from Sumar and García in 1986 and Sumar in 1996, who mention that ovaries have an irregular shape, similar to sows.

Ovaries have a smooth surface, a cream color and are located caudally to the kidneys. This observation fits the caprine anatomy description mentioned by Kumari et al., in 2017. Both ovaries were anchored by the broad ligament, also known as mesovarium, as mentioned by Sato and Montoya in 1990.

The average of weights measured were 0.02 ± 0.01, 0.03 ± 0.01 y 0.03 ± 0.01 grams in the first, second and third group, respectively. This differs from weights found in caprine fetuses, which were 0.0106 ± 0.0004, 0.0306 ± 0.0015 y 0.0393 ± 0.0014 grams in the first, second and third group, respectively. These observations are statistically different according to Kumari et al., 2017.

Microscopically, lining epithelium could already be distinguished in the first group, with a simple epithelium with cells that vary in shape during all development between columnar, cuboid and squamous, similar to descriptions in dromedaries by Abd-Elrazik et al., who in 2014 mentions that epithelium varies in shape between columnar, cuboid and squamous. These descriptions differ from observations in bovines by Kenngott et al., who in 2013 mentioned that there were sections with simple epithelium and sections with stratified epithelium with thin columnar and cuboid cells.

A great amount of oogonium were observed. These cells were classified as such due to their shape description, however a nucleus evaluation was not performed. They were found in clusters or strings, similar to observations in dromedaries by Abd-Elrazik et al., in 2014; in bovines by Kenngott et al., 2013; in mares by Savastano et al., 2008; in goats by Kumari et al., in 2017 and in sheep by McNatty et al., 1995.

Signs of degeneration were observed in germ cells from samples of the first group, similar to observations in dromedaries by Abd-Elrazik et al., in 2014. This differs from observations in bovines by Kenngott et al., in 2013, who observed these signs in the second group. Mitosis was also found in dromedaries according to Abd-Elrazik et al., 2014; in bovines according to Kenngott et al., 2013 and in sheep, according to McNatty et al., in 1995.
Cortex and medulla could be distinguished in the first group, similar to observations in dromedaries by Abd-Elrazik et al., in 2014; in bovines by Kenngott et al., 2013; in mares by Savastano et al., 2008; but different to observations in goats by Kumari et al., who in 2017 mentions that this could be observed starting from the second group.

Primary follicles were observed in the first group, similar to observations in dromedaries by Abd-Elrazik et al., in 2014; in mares by Savastano et al., 2008; and different to observations in goats and sheep by Kumari et al., in 2017 and McNatty et al., 1995 respectively, who observed them starting from the second group. These follicles were surrounded by stromal cells, similar to observations in bovines by Kenngott et al., 2013.

Unilaminar primary follicles were observed in the first group, similar to observations in dromedaries by Abd-Elrazik et al., in 2014; in mares by Savastano et al., 2008; and different to observations in bovines, goats and sheep by Kenngott et al., 2013, Kumari et al., in 2017 and McNatty et al., 1995 respectively, who observed them starting from the second group. These follicles were surrounded by stromal cells, similar to observations in bovines by Kenngott et al., 2013.

Proliferation of follicular cells started in the second group, similar to observations in dromedaries by Abd-Elrazik et al., in 2014; in bovines by Kenngott et al., 2013; and different to observations in sheep by McNatty et al., who in 1995 mentioned that this proliferation started in the third group; and different to observations in mares by Savastano et al., who in 2008 mentioned that this proliferation started in the first group. Preantral follicles were observed in the second group, similar to observations in dromedaries by Abd-Elrazik et al., in 2014; in bovines and sheeps by Kenngott et al., 2013 and Kumuri et al., 2017 respectively; but different to observations in sheep by McNatty K, who in 1995 mentioned that these follicles were observed in the third group; and in mares by Savastano et al., who in 2008 mentioned that these follicles were observed in the first group.

Lining epithelium was observed as a simple columnar, cuboid and squamous epithelium in the third group. This observation was made in the second group in dromedaries by Abd-Elrazik et al., 2014.

Preovulatory follicles were observed, similar to observations in goats by Kumari et al., 2017 who mention them as antral or Graafian follicles, but in the second group. These observations differ from observations in mares by Savastano et al., 2008 who mention them as follicles with a completely formed antrum, classified as tertiary follicle and observed in the second group. Different from observations in bovines by Kenngott et al., 2013 who mentions them as antral follicles; and different from observations in sheep by McNatty K, 1995 who describes follicles with early antral structures.

Great blood and lymphatic vessels were observed in the medulla, similar to observations in dromedaries in the second group by Abd-Elrazik et al., 2014; in sheep in the third by McNatty K, 1995; and in bovines in the first group by Kenngott et al., 2013.

CONCLUSIONS

Macroscopically, as gestation progresses the ovaries form and locate in a similar way as observed in adult alpacas.

Microscopically, in day 324 of gestation one follicle with its antrum, cumulus oocytum complex surrounded by zona pellucida, corona radiata, cumulus oophorus, zona granulosa, theca interna y externa. This was classified as a preovulatory follicle, which was found and studied in other investigations of adult alpacas. This finding will amplify the knowledge and will serve for future studies.

Conflict of interest
Authors declare no conflicts of interest.

Contribution of authors
E.R.V.Y, M.B. N. Z, G.S.A, A.C. R, F.S.R made the conception and design of the study, data collection, analysis and interpretation. E.R.V.Y wrote the manuscript and approved the definitive version, and M.B.N.Z was the supervisor of all steps.

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