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# Histomorphometric and immunohistochemical study of the goat omasum during prenatal development

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**Summary.** This work studies the morphological changes taking place in the goat omasum during prenatal development, using scanning electron microscope, light microscopy and immunohistochemical analysis. A total of 140 goat embryos and fetuses were used, from the first stages of prenatal life until birth. Differentiation of the omasum as a separate compartment of the primitive gastric tube was observed at 35 days of prenatal life ([crown-rump length (CRL)] 3 cm, 23% gestation). By 38 days (CRL 4.3 cm, 25% gestation) the omasal wall comprised three layers: an internal epithelial layer, a middle layer of pluripotential blastemic tissue and an external layer or serosa. Omasal laminae appeared in the following order: primary at 38 days (CRL 4.3 cm, 25% gestation), secondary at 50 days (CRL 7.7 cm, 33% gestation), tertiary at 59 days (CRL 12 cm, 39% gestation) and quaternary at 64 days (CRL 13.5 cm, 43% gestation). Neuroendocrine cells were detected by synaptophysin (SYP) at 52 days (CRL 8 cm, 35% gestation), while glial cell markers (glial fibrillary acidic protein - GFAP, and vimentin-VIM) were observed at 64 days (CRL 13.5 cm, 43% gestation) and 38 days (CRL 4.3 cm, 25% gestation), respectively. Sympathetic and parasympathetic nerve fibers and nerve bodies were detected via neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) at 95 days (CRL 20 cm, 63% gestation). In conclusion, prenatal development of the omasum - like that of the rumen - appears to take place somewhat earlier in goats than in sheep or cattle, but at a similar stage to that reported in deer.

**Key words:** Forestomach, Goat, Immunohistochemisty, Prenatal development

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## Introduction

Although all ruminants share the ability to digest grass, and transform it into products of great nutritional value, Hofmann (1973) categorized them by their feeding habits into grass-roughage eaters, intermediate feeders and concentrate selectors. The goat is classed as an intermediate feeder, able to make use of marginal pasturelands. These differences in feeding habits are linked to differences in digestive-tract anatomy and histology among ruminant species (Hofmann and Schnorr, 1982).

Morphological examination of the ruminant digestive tract suggests that the forestomach - and particularly the omasum - is structurally adapted to feeding habits (Hofmann and Schnorr, 1982). The omasum contains laminae of varying sizes, which are composed of a thin muscular sheet enclosed by connective tissue and covered by a non-glandular mucosa whose surface is studded with papillae (Yamamoto et al., 1994). Omasal laminae are classified as first-, second-, third- or fourth-order, as a function of their size (Yamamoto et al., 1994; Dyce et al., 2002). The conical papillae studding the surface of the laminae vary in size and shape, depending on species and breed: different morphometric findings have been reported for cattle, sheep (Stafford and Stafford 1993), goats (Yamamoto et al., 1994; Green and Baker, 1996), deer (Mathiesen et al., 2000; Lentle et al., 1998; Kamler, 2001) and musk oxen (Clauss et al., 2006). The relationship between omasal structure and function has long been a matter of debate. While some authors have suggested that its cornified laminae serve as a grinding mill (Ellenberger, 1881), others prefer to see the omasum as a sieve that allows only small particles to pass on to the lower digestive tract (Bost, 1970). More recently, however, it has been widely accepted that the omasum is primarily an organ for the absorption of volatile fatty acids, minerals and electrolytes, due to the surface area provided by its laminae (Phillipson, 1982).

Although there has been considerable research into the ruminant stomach, focusing particularly on cattle (Vivo et al., 1990), sheep (Wardrop, 1961; Franco et al., 1992, 1993a-c; Redondo et al., 1997; Regodón et al., 1996), and deer (Franco et al., 2004a,b, 2011; Redondo et al., 2005, 2011; Masot et al., 2007a,b), few studies have addressed the development of the forestomach in goats (Mcsweeny, 1988; Ramkirshna and Tiwari, 1979; Green and Baker, 1996; Nwaogu and Ezseaor, 2008; El-Gendy et al., 2010; Garcia et al., 2012).

Ruminant animals are born with a stomach that is structurally and functionally similar to non-ruminant animals. During prenatal life, the stomach of ruminant animals suffers some morphological changes to suit its function in postnatal life. In the omasum, the morphological changes are the growth and development of the omasal laminae covered with conical papillae in order to play a role in the digestive process. For this reason, the aims of the present study were as follows: (1) to describe the histological evolution of goat omasum during prenatal life; (2) to determine the morphometric changes in the omasal wall during intrauterine life; (3) to detect by immunohistochemistry the presence of neuroendocrine cells with synaptophysin (SYP), the glial cell with glial fibrillary acidic protein (GFAP) and vimentin (VIM) and markers of peptidergic innervation such as neuropetide Y (NPY) and vasoactive intestinal peptide (VIP); (4) to analyze the surface of the omasal mucosa by scanning electron microscopy from the early embryonic stages until birth.

## Materials and methods

### *Animals*

A total of 140 goat (*Capra hircus*) embryos and fetuses, ranging from the first prenatal stages to birth, were sampled. Specimens were divided into 5 sequential groups, according to major histomorphogenic characteristics: group I (crown-rump length[CRL] 1.5-4.3 cm, age 13-38 days, 1-25% gestation), group II (CRL 4.4-8 cm, 39-52 days, 25-35% gestation), group III (CRL 9-17.5 cm, age 53-75 days, 35-50% gestation), group IV (CRL 18-32 cm, 76-112 days, 50-75% gestation), and group V (CRL 33-47 cm, 113-150 days, 75-100% gestation). Embryos and fetuses were all obtained at a municipal slaughterhouse in Caceres (Spain) from pregnant females. These pregnant females were slaughtered by the usual process in the slaughterhouse, where the embryos and fetuses were obtained after opening the abdominal cavity and uterus. These actions were carried out in accordance with the regulation required for the protection of animals at the time of slaughter in slaughterhouses (Spanish Royal Decree 54/1195). Gestational age was estimated following Evans and Sack (1973) and Sivachelvan et al., (1996), as well as in the light of age classifications previously reported for sheep (Franco et al., 1992) and deer (Franco et al., 2004a,b).

### *Sampling and processing*

Once the omasum had been separated, small pieces of tissue were dissected for analysis. Tissues for histological examination were fixed in 4% buffered formaldehyde for 24 h, routinely processed and embedded in paraffin. Sections 5 $\mu$m thick were stained with Hematoxylin-Eosin (H-E), Masson's Trichrome and Gomori's reticulin.

### *Morphometric analysis*

Ten specimens were selected for each group for histology and morphometric analysis. In group I of these 10 individuals, 2 belonged to the age of 13 days, 3 of 35 days and 5 of 38 days; in group II, 3 fetuses of 39 days, 3 of 46 days and 4 of 50 days were selected; in group III, 2 fetuses of 53 days, 3 of 62 days, 3 of 70 days and 2 of 74 days were chosen; in group IV, 3 fetuses of 76 days, 3 of 84 days, 2 of 95 days and 2 of 112 days were selected; in group V, 2 fetuses of 113 days, 3 of 125 days, 3 of 138 days and 2 fetuses of 150 days were chosen. Specimens were embedded in paraffin, stained with H-E, and viewed through a microscope (NIKON Eclipse 80i) equipped with a digital video camera (NIKON DXMI200F). The computerized image was analyzed using the Nis-Element 2.30 software package. The variables studied were the height of various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis and serosa) and total wall thickness. One hundred measurements were made in each tissue layer (epithelium, lamina propria and submucosa, tunica muscularis and serosa) of each of the selected individuals from each group.

### *Statistical analysis*

The results are shown as mean ± SE. Data was subjected to analysis of variance (ANOVA). Wherever ANOVA revealed significant differences, a post-hoc (Tukey) analysis was carried out to test for significant differences between tissue strata and groups. A value of $P<0.05$ was considered significant.

Tissue growth models were created using a personal computer and a statistics program (Statistica Six Sigma, 2006). Graphs represent the averages of real growth values together with the adjusted line of regression. The correctness of fit of this adjustment was measured using the rate of determination, $r^2$. In all cases, embryo body length (crown-rump length, CRL, in centimetres) was used as the independent variable; the thickness of each tissue stratum (epithelium, lamina propria and submucosa, tunica muscularis and serosa) served as the dependent variable.

### *Immunohistochemical analysis*

The UltraVision One HRP polymer (polymer conjugated to horseradish peroxidase) was performed on

deparaffinized tissue from the omasum to detect the neuroendocrine cell marker with synaptophysin (SYP), the glial cell marker with glial fibrillary acidic protein (GFAP) and vimentin (VIM) and markers of peptidergic innervation such as neuropetide Y (NPY) and vasoactive intestinal peptide (VIP). Tissues were deparaffinized and hydrated. Recovery of antigens was performed with citrate and microwave. Blocking of endogenous peroxidase activity was done with 0.5% hydrogen peroxide for 30 min. Non-specific tissue binding sites were blocked by incubation in 1% normal goat serum for 30 min. Samples were incubated with the following primary antisera: 1:10 mouse monoclonal anti-SYP (Thermo Scientific, MA1-35810); ready to use rabbit polyclonal anti-GFAP (Thermo Scientifc, RB-087- R7), ready to use mouse monoclonal anti-VIM (Thermo Scientific, MS-129-R7), 1:50 rabbit polyclonal anti-NPY (Thermo Scientific, PA1-41576) and 1:50 rabbit polyclonal anti-VIP (AbD serotec, 9535-0204) for 30 min at room temperature. Sections were finally incubated with polymer conjugated to horseradish peroxidase (Thermo Scientific, UltraVision ONE HRP Polymer, TL-015-PHJ) for 30 minutes at room temperature and without exposure to light. After that, the diaminobenzidine was applied in the tissue (Thermo Scientific, DAB Plus Chromogen TA-001-HCX and DAB Plus Substrate TA-015-HSX) for 5-15 minutes, depending on the desired stain intensity. Finally, the reaction was contrasted with Mayer's hematoxylin. The specificity of the staining reaction was determined in control experiments involving either substitution of the primary antibody by PBS or normal goat serum 1:100, or omission of both primary and secondary antibodies. Absorption controls were obtained by incubating sections adjacent to those above with antiserum that contained 25 $\mu$g of Ag/ml of diluted antiserum. No staining was found in structures on the sections which served as absorption controls.

### *Scanning electron microscopy*

For scanning electron microscopy (SEM) small pieces of omasum were fixed in 2.5% buffered glutaraldehyde for 24 hours, dehydrated through graded ethanol and amyl acetate, and dried in a critical-point dryer. Sections were covered with coating materials including gold and examined at various tilt angles, at a magnification of 10 to 800x.

## Results

### *Gross findings*

At 35 days (CRL 3 cm, 23% gestation), the omasum was observed as a separate compartment of the primitive gastric tube, appearing as an oval, laterally-compressed cavity lined by a beige mucosa with small surface elevations corresponding to rudimentary omasal laminae. By 75 days (CRL 17.5cm, 50% gestation), primary, secondary, tertiary and quaternary laminae had started to protrude into the omasal cavity. At 112 days (CRL 32 cm, 75% gestation), conical papillae were apparent as small prominences arising from the lateral surfaces of laminae. Finally, by 150 days (CRL 47 cm, 100% gestation), all four orders of laminae were clearly apparent; they varied in length, and their surfaces were studded with numerous conical papillae.

### *Omasal histomorphogenesis*

Group I (CRL 1.5 to 4.3 cm, 13-38 days, 1-25% gestation)

At 35 days (CRL 3 cm, 23% gestation), the omasum became apparent as a separate compartment of the primitive gastric tube. The omasal wall comprised an internal epithelial layer and an external layer of pluripotential blastemic tissue (Fig. 1a).

At 38 days (CRL 4.3 cm, 25% gestation), the omasal wall comprised three layers: an internal epithelial layer, a middle layer of pluripotential blastemic tissue and an external layer, or serosa. Rudimentary primary omasal papillae were visible as small protrusions from the omasal wall.

The stratified epithelium (163.58±5 $\mu$m) was divided into lighter luminal zones composed of globose cells with rounded apical nuclei, and inner darker zones comprising cylindrical germinal cells with ovoid basal nuclei.

The pluripotent blastemic tissue layer (936.36±11 $\mu$m) was composed of abundant ground substance interspersed with pluripotent mesenchymal stem cells.

The external serosa (507.14±7 $\mu$m) was formed by a slender layer of connective tissue covered by flat cells.

Group II (4.4 to 8 cm CRL, 39-52 days, 25-35% of gestation)

This stage of prenatal development was marked by histological changes affecting each stratum of the omasal wall.

The stratified epithelium (236.28±11 $\mu$m) was clearly divided into a stratum basale formed by 2-4 layers of germinal cells with dark cytoplasm and a central nucleus, and a thicker external stratum granulosum comprising 6-10 layers of globose cells with light cytoplasm (Fig. 1b).

Primary omasal laminae were larger and more numerous. By 50 days (CRL 7.7 cm, 33% gestation), secondary omasal laminae had become visible as small elevations between primary laminae, growing out from the wall towards the lumen (Fig. 1b). Both primary and secondary laminae appeared as outfoldings of the epithelial stratum basale.

The middle layer of blastemic tissue (853.14±16 $\mu$m) was formed by highly-cellular connective tissue and sparse ground substance, and the first signs of differentiation into lamina propria and submucosa were

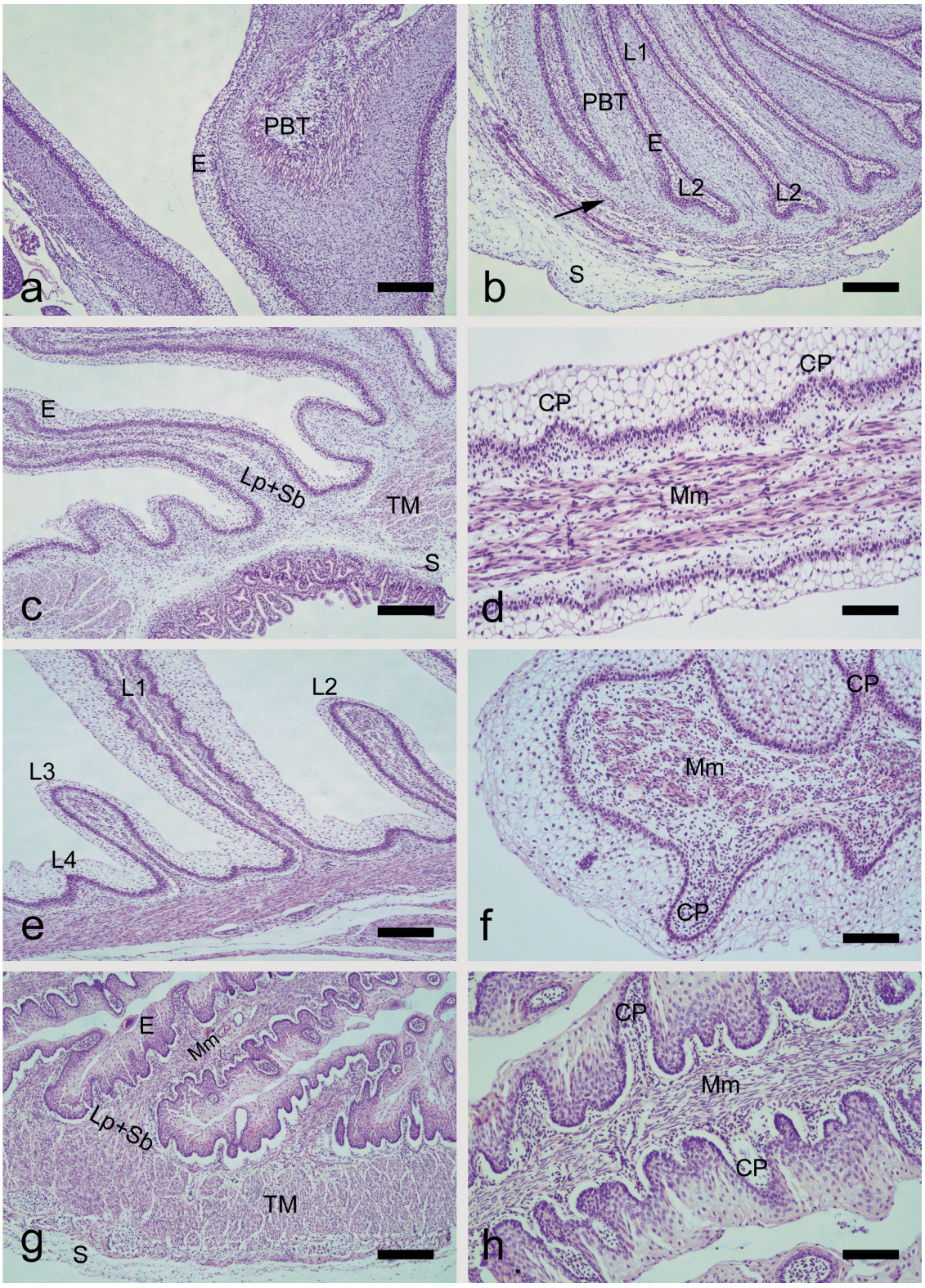


**Fig. 1.** Histomorphogenesis of goat omasal wall (35 to 150 days, 23-100% gestation). **a.** Photomicrograph of section of the differentiated omasum (35 days, 23% gestation). The wall comprises two layers: epithelium (E) and pluripotential blastemic tissue (PBT). **b.** Photomicrograph of section of omasal wall (52 days, 35% gestation). The wall is composed of three layers: epithelium (E), pluripotential blastemic tissue (PBT) with spindle-shaped myoblastic cells (arrow) and serosa (S). Primary (L1) and secondary laminae (L2) are visible. **c.** Photomicrograph of section of omasal wall (64 days, 35% gestation). The wall comprises four layers: epithelium (E), lamina propria and submucosa (Lp+Sb), tunica muscularis (TM), and serosa (S). Myoblastic fibers of the tunica muscularis (TM) infiltrating laminae to constitute muscularis mucosae. **d.** Photomicrograph of section of omasal laminae (70 days, 46% gestation). Muscularis mucosae (Mm) constituting the skeleton of omasal laminae. Conical papillae (CP) visible in primary laminae. **e.** Photomicrograph of section of the omasal wall (80 days, 53% gestation). Four orders of laminae now visible: primary (L1), secondary (L2), tertiary (L3) and quaternary (L4). **f.** Photomicrograph of section of omasal laminae (101 days, 67% gestation). Fully-developed muscularis mucosae (Mm) occupying the center of laminae. Larger conical papillae (CP) visible within stratified epithelium. **g.** Photomicrograph of section of omasal wall (113 days, 75% gestation). Wall formed by epithelium (E), lamina propria and submucosa (Lp+Sb), tunica muscularis (TM) and serosa (S). Conical papillae visible in primary, secondary and tertiary laminae. **h.** Photomicrograph of section of omasal laminae (150 days, 100% gestation). Muscularis mucosae (Mm) visible within omasal laminae. H-E. Bar: a, b, 30 $\mu$m; c, e, f, 25 $\mu$m; d, h, 20 $\mu$m.

observed. Both tissue layers protruded towards the epithelium, becoming involved in the formation of omasal laminae. By 50 days (CRL 7.7 cm, 33% gestation), a rudimentary tunica muscularis was visible in the form of a slender layer of myoblasts enclosed by blastemic tissue.

The serosa (420.72±7 $\mu$m) comprised a subserosa of loose connective tissue underlying a mesothelial layer of flat cells.

Group III (CRL 9 to 17.5 cm, 53-75 days, 35-50% gestation)

At this stage, the omasal wall was formed by four distinct layers: mucosa (composed of epithelium and lamina propria), submucosa, tunica muscularis and serosa.

The epithelium (422.8±12 $\mu$m) comprised the stratum basale and stratum granulosum. An outer stratum corneum - formed by a single layer of flat, anucleate cells - was also visible.

The lamina propria and submucosa (598.15±13 $\mu$m) were composed of fibroblast-rich connective tissue interspersed with sparse ground substance. The tunica muscularis (379±18 $\mu$m) comprised a circular internal layer and a longitudinal external layer. By 64 days (CRL 13.5 cm, 43% gestation), the muscularis mucosae was visible at the centre of omasal laminae, arising from the inner circular layer of smooth muscle fibers of the tunica muscularis (Fig. 1c).

Primary and secondary laminae were taller and thicker. At 59 days (CRL 12 cm, 39% gestation), tertiary laminae were apparent in the spaces between primary and secondary laminae. By 64 days (CRL 13.5 cm, 43% gestation), quaternary laminae were also visible (Fig. 1e). These laminae were composed of all tissue layers (epithelium, lamina propria, submucosa and muscularis) except the serosa.

At 70 days (CRL 15 cm, 47% gestation), conical papillae started to arise from the surface of omasal laminae (Fig. 1f). These were first apparent as elevations of the epithelial stratum basale in primary laminae, and by 76 days (CRL 16 cm, 51% gestation) were also visible in secondary laminae.

The serosa (184±12 $\mu$m) displayed no differences with respect to earlier stages of development.

Group IV (CRL 18 to 32 cm, 76-112 days, 50-75% gestation)

During this stage, the omasal wall was composed of the same tissue layers observed earlier.

The epithelium (579.08±17 $\mu$m) comprised four strata: a stratum basale underlying a stratum granulosum, as noted earlier, and an overlying stratum spinosum composed of elongated cells; finally, the stratum corneum - formed by flat, anucleate cells - was in contact with the lumen.

Abundant conical papillae, visible in primary and secondary laminae, were filled with connective tissue from the lamina propria and the submucosa (376.18±15 $\mu$m). Both layers were apparent inside omasal laminae and conical papillae, forming the skeleton.

Both layers of the tunica muscularis were thicker (752.36±21 $\mu$m); the internal fiber layer had thickened sufficiently to give rise to the muscularis mucosae. At 101 days (CRL 21 cm, 67% gestation), a fully-formed muscularis mucosae was visible within omasal laminae.

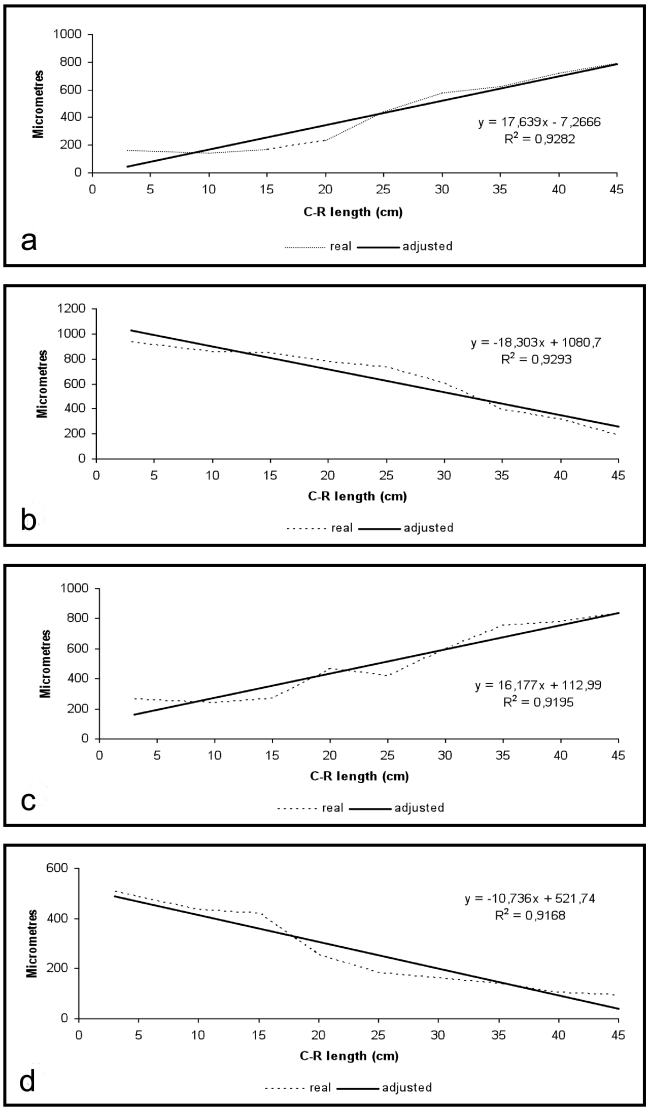


**Fig. 2.** Mathematical growth models for omasal tissue layers. **a.** Mathematical model of omasal growth (epithelium). **b.** Mathematical model of omasal growth (lamina propria and submucosa). **c.** Mathematical model of omasal growth (tunica muscularis). **d.** Mathematical model of omasal growth (serosa).

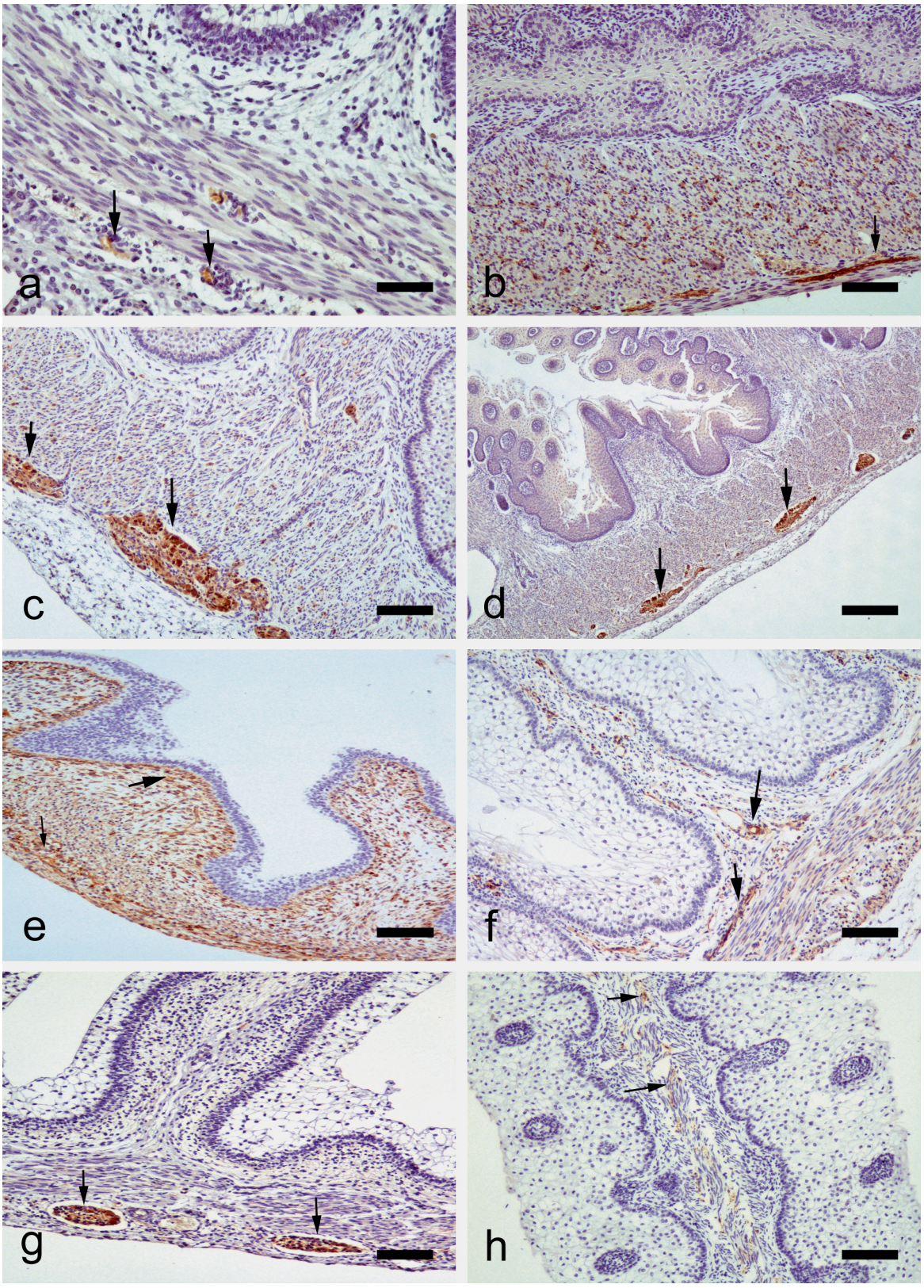


**Fig. 3.** Immunohistochemical findings in goat omasum. **a.** Photomicrograph of section of omasal wall (53 days, 35% gestation). SYP+ staining in lamina propria + submucosa, tunica muscularis and myenteric plexus (arrows). **b.** Photomicrograph of section of omasal wall (70 days, 75% gestation). SYP+ staining in lamina propria + submucosa, tunica muscularis and myenteric plexus (arrow). **c.** Photomicrograph of section of omasal wall (70 days, 46% gestation). GFAP+ cells (arrow) in lamina propria + submucosa, tunica muscularis and myenteric plexus. **d.** Photomicrograph of section of omasal wall (113 days, 60% gestation). GFAP+ staining in lamina propria-submucosa, tunica muscularis and myenteric plexus (arrow). **e.** Photomicrograph of section of omasal wall (38 days, 26% gestation). VIM+ cells (arrow) in mesenchymal layer of pluripotential blastemic tissue. **f.** Photomicrograph of section of omasal wall (84 days, 53% gestation). VIM+ staining in myenteric plexus, tunica muscularis and intervascular connective tissue in lamina propria-submucosa of laminae (arrow). **g.** Photomicrograph of section of omasal wall (95 days, 75% gestation). VIP+ staining (arrow) in lamina propria + submucosa and myenteric plexus. **h.** Photomicrograph of section of omasal wall (113 days, 80% gestation). NPY+ staining in connective tissue of lamina propria and submucosa of laminae (arrow). HRP. Bar: a, g, h, 20 $\mu$m; b-d, 30 $\mu$m; e, f, 25 $\mu$m.

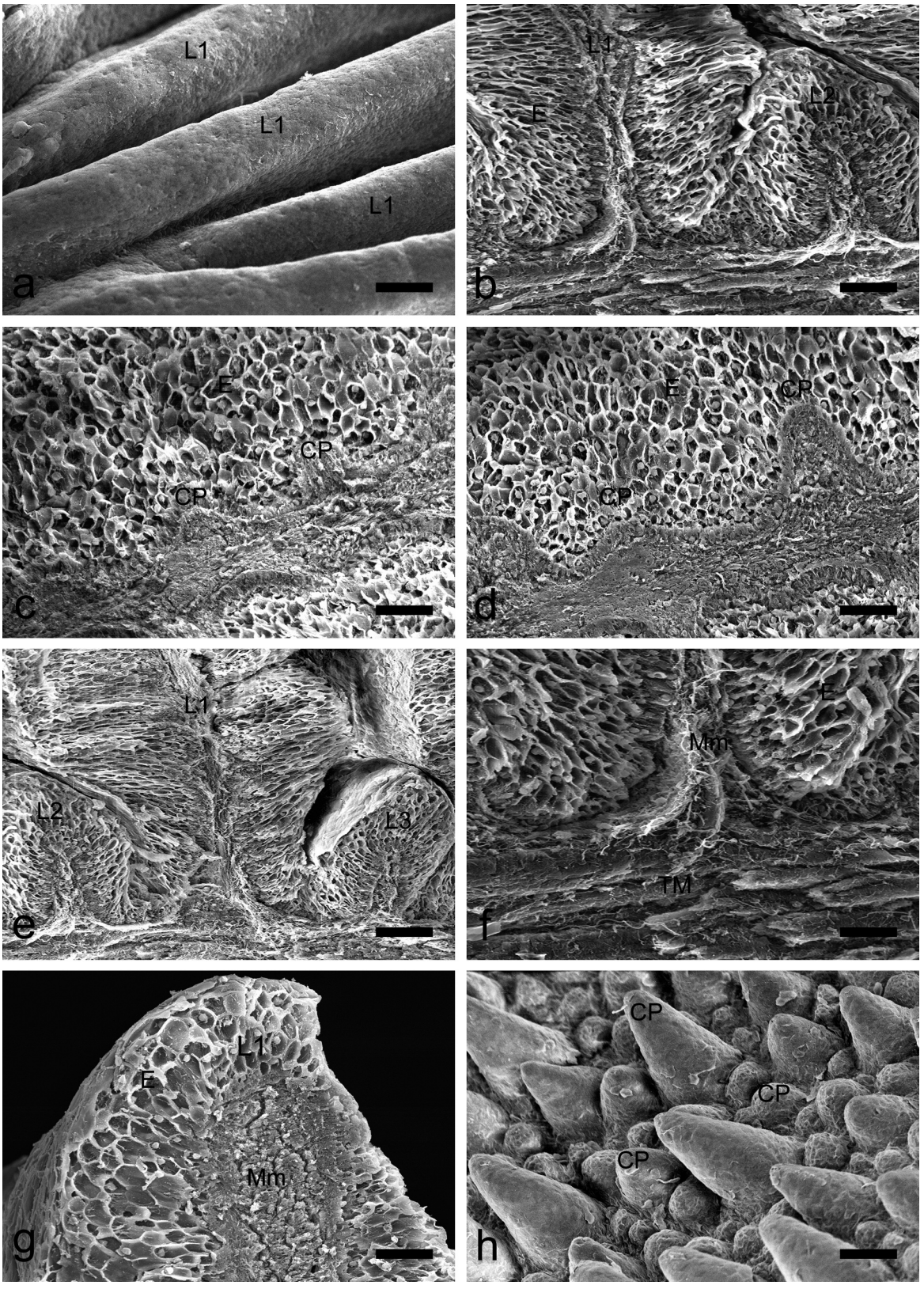


**Fig. 4.** Scanning electron microscopy of the goat omasum (38 to 150 days, 25-100% gestation). **a.** Photomicrograph of section of omasal wall (38 days, 25% gestation). Superficial longitudinal folds were observed at the surface of the omasum. These constituted the primary omasal laminae (L1). **b.** Photomicrograph of section of omasal wall (52 days, 35% gestation). Tunica muscularis (TM) and lamina propria-submucosa (Lp+Sb) visible in omasal wall. Secondary laminae were visible between primary laminae. **c.** Photomicrograph of section of omasal wall (70 days, 46% gestation). Conical papillae (CP) infiltrating epithelium (E) of omasal lamina. **d.** Photomicrograph of section of omasal wall (85 days, 55% gestation). Increase in size and number of conical papillae (CP) in omasal lamina. **e.** Photomicrograph of section of omasal wall (100 days, 66% gestation). Secondary (L2), tertiary (L3) laminae visible in spaces between primary laminae (L1). **f.** Photomicrograph of section of omasal wall (112 days, 74% gestation). Inner layer of muscular fiber of tunica muscularis (TM) infiltrating omasal lamina to form muscularis mucosae (Mm). **g.** Photomicrograph of section of primary omasal laminae (L1) (125 days, 82% gestation). Muscularis mucosae (Mm) occupying inside of omasal lamina. **h.** Photomicrograph of section of omasal wall (150 days, 100% gestation). Conical papillae (CP) of different sizes on the surface of the primary laminae. SEM. Bar: a-d, 6 $\mu$m; e, f, 4 $\mu$m; g, h, 2 $\mu$m.

Group V (CRL 33 to 47 cm, 113-150 days, 75-100% gestation)

By this late stage of prenatal development, all tissue layers were fully evident in the omasal wall. The epithelium was thicker (719.15±16 $\mu$m), and the stratum corneum comprised a larger number of layers of flat cells.

Four orders of omasal laminae of varying thickness were apparent, all studded with numerous conical papillae (Fig. 1g).

The skeleton of omasal laminae and conical papillae was formed by the lamina propria and submucosa (316±27 $\mu$m), composed of connective tissue, together with the muscularis mucosae.

The tunica muscularis (839.43±23 $\mu$m) comprised two layers of smooth muscle fibers, an inner circular layer and an outer longitudinal layer. The muscularis mucosae was fully developed, and occupied virtually the whole thickness of omasal laminae (Fig. 1h).

The whole stomach compartment was lined by an external serosa (94.88±7 $\mu$m) formed by a subserosa of connective tissue and an overlying mesothelium.

### *Morphometric observations*

Changes in the thickness of omasal wall tissue layers during prenatal development are shown in Table 1.

Mean epithelial growth in group I was significantly lower than in groups II to V (P= 0.004). Pluripotential blastemic tissue started to decline in thickness as the first signs of differentiation into lamina propria + submucosa and tunica muscularis were observed. Growth of the lamina propria and submucosa in group III differed significantly from that observed in groups IV and V (P=0.003). The tunica muscularis increased in thickness throughout prenatal development, a significant difference in growth-rate being noted between groups III/IV and V (P=0.003). The serosa became more slender as fetal development progressed: mean serosa thickness was significantly greater in group I than in groups II to V (P=0.002).

Mathematical growth models were constructed for each tissue stratum, with the corresponding growth equations and correlation coefficients. The epithelial layer grew progressively throughout prenatal development, and reached a maximum thickness at CRL 30 cm (105 days, 70% gestation) with the formation of the four epithelial strata: basale, granulosum, spinosum, and corneum (Fig. 2a). The growth rate of the lamina propria and submucosa declined from the early embryonic stages until birth (Fig. 2b). The tunica muscularis grew progressively throughout development. The greatest thickness of the tunica muscular was reached at CRL 35 cm (120 days, 80% gestation; Fig. 2c). The growth rate of the serosa diminished steadily from the early stages until birth (Fig. 2d).

### *Immunohistochemical observations*

The results of immunohistochemical staining of the

**Table 1.** Morphometrical findings of the tissue layer thickness in the omasum of goat during prenatal development ($\mu$m).

| | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| E | 151±5 | 280±8[a] | 442±13[a] | 639±21[a] | 791±33[a] |
| Lp + Sb | pbt* | pbt | 602±14 | 396±24[b] | 316±37[b] |
| TM | pbt | pbt | 419±22 | 778±27[b] | 946±41[b] |
| S | 420±6 | 269±12[a] | 139±13[a] | 103±8[a] | 95±7[a] |

Group I (1.5-4.3 cm CRL, 26-38 days: 1-25% gestation); Group II (4.4-8 cm CRL, 39-52 days: 25-35% gestation); Group III (9-17.5 cm CRL, 53-75 days: 25-35% gestation); Group IV (18-32 cm CRL, 76-112 days: 50-75% gestation); Group V (33-47 cm CRL, 113-150 days: 75-100% gestation). E: Epithelium; Lp+Sb: Lamina propria and submucosa; TM: Tunica muscularis; S: Serosa; pbt: pluripotential blastic tissue. * The pluripotent blastic tissue, which will later give rise to the lamina propria-submucosa and tunica muscularis was not statistically compared owing to the fact that one structure will give rise to various others. [a]P<0.005 vs Group I; [b]P<0.005 vs Group III.

**Table 2.** Immunohistochemical findings in the omasum of goat during prenatal development.

| | Group I | | | | Group II | | | | Group III | | | | Group IV | | | | Group V | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E | Lp+Sb | TM | S | E | Lp+Sb | TM | S | E | Lp+Sb | TM | S | E | Lp+Sb | TM | S | E | Lp+Sb | TM | S |
| SYP | - | - | - | - | - | + | + | - | - | ++ | ++ | - | - | ++ | ++ | - | - | +++ | +++ | - |
| GFAP | - | - | - | - | - | - | - | - | - | + | + | - | - | ++ | ++ | + | - | ++ | ++ | + |
| VIM | - | - | - | - | - | + | + | + | - | '+ | + | + | - | ++ | ++ | ++ | - | +++ | +++ | +++ |
| VIP | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | ++ | + | + |
| NPY | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | ++ | + | + |

Group I (1.5-4.3 cm CRL, 26-38 days: 1-25% gestation); Group II (4.4-8 cm CRL, 39-52 days: 25-35% gestation), Group III (9-17.5 cm CRL, 53-75 days: 25-35% gestation), Group IV (18-32 cm CRL, 76-112 days: 50-75% gestation); Group V (33-47 cm CRL, 113-150 days: 75-100% gestation). E: Epithelium, Lp+Sb: Lamina propia and submucosa, TM: Tunica muscularis, S: Serosa. SYP: synaptophysin, GFAP: glial fibrillary acidic protein, VIM: vimentine, VIP: vasoactive intestinal peptide, NPY: neuropeptide Y. -, non immunoreactivity; +, low immunoreactivity; ++, moderate immunoreactivity; +++, high immunoreactivity.

omasum during prenatal development for synaptophysin (SYP), glial fibrillary acidic protein (GFAP), vimentin (VIM), neuropeptide Y (NPY), and vasoactive intestinal polypeptide (VIP) are shown in Table 2.

Neuroendocrine cells were first detected by SYP staining at 52 days (CRL 8 cm, 35% gestation; fig. 3a), in the lamina propria, submucosa, tunica muscularis and myenteric plexus. Positive-staining cells were more abundant at these sites by 64 days (CRL 13.5 cm, 43% gestation; fig. 3b).

Cells staining positive to GFAP were first observed at 64 days (CRL 13.5 cm, 43% gestation), in myenteric and submucosal plexuses, and scattered throughout the lamina propria-submucosa and tunica muscularis (Fig. 3c); they were more abundant at these locations by 113 days (Fig. 3d). VIM-positive glial cells were observed at 38 days (CRL 4.3 cm, 25% gestation) in the lamina propria, submucosa, tunica muscularis and serosa (Fig. 3e). By 101 days (CRL 21 cm, 67% gestation), VIM-positive cells were observed in myenteric plexuses and scattered throughout the lamina propria and submucosa of omasal laminae (Fig. 3f).

Sympathetic and parasympathetic nerve fibers and nerve bodies were detected by immunolabeling with neuropeptides NPY and VIP at 95 days (CRL 22.5 cm, 63% gestation). These were observed in the lamina propria and submucosa, tunica muscularis and serosa (Figs. 3g, h). The number of positive-staining cells increased as prenatal development progressed.

Scanning electron microscopy

During the early embryonic stages, the omasum displayed superficial longitudinal folds arising from the epithelium towards the omasal lumen; these constituted the primary omasal laminae (Fig. 4a).

At 50 days (CRL 7.7 cm, 33% gestation), primary omasal laminae were longer, and small folds were starting to develop in the spaces between them, to form secondary laminae (Fig. 4b).

Midway through gestation, at 70 days (CRL 15 cm, 47% gestation), the first conical papillae emerged on the lateral surface of omasal laminae (Fig. 4c). Thenceforth, papillae grew in size and number, horizontal to the omasal wall (Fig. 4d).

As development progressed, tertiary and quaternary laminae gradually arose from the omasal wall. By 85 days (CRL 20 cm, 57% gestation) all four orders of laminae were visible (Fig. 4e).

At 112 days (CRL 32 cm, 75% gestation), the two smooth muscle fiber layers forming the tunica muscularis were clearly visible. The inner layer gave rise to the muscularis mucosae, which gradually penetrated omasal laminae (Fig. 4f). The fully-developed muscularis mucosae extended along the inside of the omasal lamina, constituting the skeleton (Fig. 4g).

By the final stages of prenatal development, the omasal surface comprised four orders of omasal laminae of varying sizes, primary to quaternary (Fig. 4h).

## Discussion

Differentiation of the goat omasum took place at 35 days (23% gestation); this is somewhat later than the 28 days (19% gestation) reported by Mutoh and Wakuri (1989) in goats. Omasum differentiation in sheep takes place at a similar stage, reported at 33 days (22% gestation) by Franco et al. (1993a), whereas in cattle this differentiation has been observed at 30 days (11% gestation) by Vivo et al. (1990). In wild ruminants, including deer (Redondo et al., 2005), it takes place at around 67 days (25% gestation), i.e. at a similar stage as that noted here.

During the early stages of embryo development, rudimentary primary laminae were visible as slight elevations of the omasal wall, which comprised an internal epithelium, a middle layer of pluripotent blastemic tissue, and an external serosa. A similar structure has been reported in the Nigerian goat fetus by Nwaogu and Ezeasor (2008) at 60 days (40% gestation). Primary omasal laminae were observed here at 38 days (25% gestation), somewhat later than in sheep, where they have been reported at 33 days (22% gestation) by Franco et al., (1993a), at 21% gestation by Lubis and O'Shea (1978) and Fath-El Bab et al., (1983), and at 24% gestation by Del Rio Ortega (1973).

Primary laminae were observed here at 38 days (25% gestation), secondary laminae at 50 days (33% gestation), tertiary laminae at 59 days (39% gestation) and quaternary laminae at 64 days (43% gestation). These timings differ slightly from those reported for other species. In sheep the four orders of laminae were noted at 24, 28, 32 and 33% gestation, respectively (Del Rio Ortega, 1973), at 21, 26, 33, and 52% (Fath-El Bab, 1983) and at 21, 26, 33 and 40% gestation (Franco et al., 1993a). In cattle, Vivo et al., (1990) observed all four orders of laminae at a rather earlier stage of prenatal development, i.e. around 20% gestation. Emergence of the four omasal laminae in deer has been reported by Redondo et al. (2005) at 25, 30, 35 and 50% gestation, indicating timing similar to that observed here. Other studies in goats noted the presence of all four orders midway through gestation (Nwaogu and Ezeasor, 2008).

Although most studies report laminae of four different sizes in the omasal wall, a number of authors - including Wardrop (1961) in sheep, Ramkrishna and Tiwari (1979) in goats, Osman and Berg (1982) in buffalo, and Vivo et al., (1990) in cattle - highlight a fifth laminar size during prenatal development.

Conical papillae, apparent as small lateral outfoldings of the omasal laminar surface, were observed at 70 days (46% gestation). Similar histological findings have been reported in goats by Ramkrishna and Tiwari (1979), in Nigerian goats by Nwaogu and Ezeasor (2008), in sheep by Franco et al. (1993a) and in deer by Redondo et al. (2005), at 45%, 50%, 45% and

50% gestation, respectively.

The differentiation of lamina propria + submucosa from pluripotent blastemic tissue was first observed at 50 days (33% gestation); there was no histological separation between these two layers, as also reported in the goat rumen (García et al., 2012). Similar timings are reported in sheep (Franco et al., 1993a) and in deer (Redondo et al., 2005).

Differentiation of the tunica muscularis from pluripotent blastemic tissue also took place at 50 days (33% gestation). By 60 days (40% gestation), two layers of smooth muscle fibers were apparent: an inner, circular layer and an outer longitudinal layer. Ramkrishna and Tiwari (1979) observed a fully-formed tunica muscularis in goats at 67 days (45% gestation). Findings for other species differ: in sheep, Duncan and Phillipson (1955) and Franco et al. (1993a) report tunica muscularis formation at an earlier point in development (39 days, 26% gestation), while Redondo et al. (2005) noted it in deer at 67 days (25% gestation).

The muscularis mucosa was observed in omasal laminae at 64 days (43% gestation). The findings of the present study agree with those reported in goats by Ramkrishna and Tiwari (1979), who observed the muscularis mucosae at 67 days (45% gestation), and in cattle by Totzauer and Sinowatz (1990), who reported it at 120 days (43% gestation). By contrast, it has been observed later in sheep (79 days, 53% gestation) by Franco et al. (1993a) and at an earlier stage of development in deer (90 days, 35% gestation) by Redondo et al. (2005).

In our study, omasal lamina was formed by an intermediate layer of smooth muscle and two layers of connective tissue on both sides. According to our observations and those previously referenced by Franco et al. (1993a) in sheep and Franco et al. (2004a,b) in deer, this intermediate layer of smooth fibers corresponded to the muscularis mucosa and originated from the inner layer of the tunica muscularis.

However, some authors have argued that the muscularis mucosa does not arise solely from the internal muscle layer (Gerneke, 1989; Bacha and Wood, 1990; Banks, 1993). Our results do not agree with Yamamoto et al. (1991), who described in the omasal laminae three layers of smooth muscle fibers: an intermediate layer and two lateral layers, but without mentioning that this intermediate layer constituted the muscularis mucosa.

The histological characteristics of the serosa, comprising a subserosa of loose connective tissue with an overlying mesothelium, are similar to those reported in the goat rumen by Garcia et al. (2012).

Immunohistochemical examination of the goat omasum revealed SYP-positive neuroendocrine cells at 53 days (35% gestation) in the lamina propria, the submucosa and the tunica muscularis. Similar findings have been reported for the deer omasum by Redondo et al., (2005), for the sheep rumen and reticulum by Franco et al., (2004a,b) and for the goat rumen by García et al., (2012).

VIM-positive glial cells were detected at 38 days (25% gestation) in the lamina propria, submucosa, tunica muscularis and serosa. Differentiation of tissue layers became more marked as development progressed, allowing more precise location of VIM-positive cells. At 101 days, these cells were also observed in myenteric and submucosal plexuses. GFAP-positive glial cells were first detected somewhat later than VIM-positive cells (64 days, 43% gestation), and in the same locations. A similar time-lag in the immunohistochemical detection of glial cells has also been reported by Redondo et al. (2011) in a comparative study of the omasum in deer and sheep. The difference in glial-cell labeling reflects the fact that VIM is an earlier marker, as noted by Franco et al. (1997) in a study of prenatal pineal-gland development in sheep.

The sympathetic and parasympathetic nerve fibers and nerve cells were detected by neuropeptide Y and vasoactive intestinal peptide (VIP), respectively. Both were detected at 90 days in the lamina propria, submucosa, tunica muscularis and serosa, and also in myenteric plexuses. Wathuta (1986) has also reported VIP-positive nerve fibers in the forestomach, abomasum and small and large intestines of sheep at a similar stage of development. Yamamoto et al. (1994) detected the neuropeptides VIP and NPY in nerve fibers, especially in the inner layer of the tunica muscularis in the omasum of adult sheep. VIP and NPY positivity has also been reported in the myenteric neurons of sheep rumen, reticulum and omasum by Groenewald (1994). In deer, Redondo et al. (2005) observed nerve fibers in the omasum at an earlier stage of development (142 days, 51% gestation).

Structurally, primary omasal laminae were apparent during the earliest stages of prenatal life. As development progressed, the formation and growth of further laminae of varying sizes was observed. Four orders of laminae were recorded here; similar findings have been reported by other authors both in the European goat (Schummer and Nickel, 1979) and in Zarabi goats (El-Gendy et al., 2010). However, other researchers have observed only 3 orders in the omasum of the African goat (Green and Baker, 1996), and 5 orders in the Australian goat (McSweeny, 1988). Inter-breed differences in the number of omasal laminae may reflect the influence of locally-available foliage in different regions (Hofmann, 1973). Omasal laminae were studded with small conical papillae. Here, papillae were structurally apparent at 70 days (47% gestation). These papillae were observed with blunt or pointed conical tips. These have also been reported in goats (Green and Backer, 1996), sheep (Scott and Gardner, 1973) and cattle (Yamamoto, 1994).

Overall, histological, structural and immunohistochemical examination suggests that prenatal development of the omasum - like the rumen - is somewhat faster in the goat than in sheep and cattle, but takes place at a similar pace to that reported in deer.

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