Summary. This work studies the morphological changes taking place in the Dama dama rumen during prenatal development using histomorphometrics, surface microstructure and immunohistochemistry analysis as well as carrying out a comparative analysis of this species with other wild (red deer) and domestic-type ruminants. A total of 25 fallow deer embryos and fetuses were used, from the first stage of prenatal life until birth. The appearance of the rumen from the primitive gastric tube was observed at 51 days of prenatal life (CRL 3 cm, 21% gestation). By 57 days (CRL 4.3 cm, 24% gestation) the ruminal wall comprised three layers: an internal epithelial layer, a middle layer of pluripotential blastemic tissue and an external layer or serosa. Ruminal pillars were visible at 72 days (CRL 6 cm, 30% gestation), and by 85 days (CRL 7.2 cm, 35% gestation) ruminal papillae were starting to appear. Under scanning electron microscopy, by 80 days (CRL 7 cm, 33% gestation) small ruminal papillae were observed protruding from the surface. Morphometric results showed accelerated growth of the epithelial layer and the tunica muscularis at 180 days (75% gestation). By contrast, the growth-rate of the lamina propria and submucosa declined from the early embryonic stages until birth. The serosa maintained a steady rate of growth until birth. Neuroendocrine cells (synaptophysin) were detected at 85 days (CRL 7.2 cm CRL, 35% gestation), while glial cell markers (glial fibrillary acidic protein and vimentin) were found at 108 days (CRL 31 cm, 45% gestation) and 63 days (CRL 4.4 cm, 26% gestation) respectively. Neuropeptide Y and vasoactive intestinal polypeptide were detected immunohistochemically at 180 days (CRL 33 cm, 75% gestation) and 192 days (CRL 35 cm, 80% gestation) respectively. In comparison to other wild and domestic-type ruminants, histomorphogenesis of the rumen in Dama dama was similar to that reported in red deer and goats, but rather slower than that observed for sheep or cattle.

Key words: Fallow deer, Histology, Prenatal development, Stomach, Ruminal

Introduction

The fallow deer (Dama dama) is a cervid species native to the Mediterranean. Gestation lasts eight months, and a single fawn is born in spring (Baker et al., 2017).

The ruminant stomach is particularly remarkable for its ability to transform low-quality forage into products of great nutritional value (Lombardi, 2005). It is subdivided into four compartments: rumen, reticulum, omasum and abomasum. The rumen, reticulum and omasum form the forestomach, where the tunica mucosa is lined with a squamous, keratinised stratified epithelium (Vivo et al., 1990). Each compartment is characterised by certain unique gross and histological features (Schummer and Nickel, 1975) reflecting its...
morphological and functional adaptation to the ingestion, processing and digestion of plant material. The rumen plays a key role in the breakdown and microbial digestion of cellulose, an essential nutrient for ruminants (Vivo and Robina, 1990).

The rumen was subjected to a number of morphological studies, including immunohistochemical and morphometric analysis in both wild (Franco et al., 2004, 2017) and domestic-type ruminants (Vivo and Robina, 1990; Vivo et al., 1990; Franco et al., 1992, 2011; García et al., 2012). Comparative analysis of the forestomach mucosa in red deer (Masot et al., 2007) confirms that prenatal development of the red deer rumen is similar to that reported in goats (García et al., 2012), but somewhat slower than that recorded for sheep (Franco el al., 1992, 2011) and cattle (Vivo and Robina, 1990; Vivo et al., 1990).

The ontogenesis of the stomach in Dama dama had not previously been charted. This study has been carried out in an extensive fallow deer farming system, without food supplements, and the objectives of the study were as follows: (1) to describe histological (scanning electron microscopy, light microscopy and immunohistochemistry) and histomorphometrical phenomena that occur during prenatal development of the rumen and (2) to carry out a comparative analysis of this species with other wild (red deer) and domestic-type ruminants (cattle, sheep and goats).

Material and methods

Embryos and fetuses

Dama dama embryos and fetuses (n=25) were sampled from the first prenatal stages to birth. To obtain embryos and fetuses at various stages of development, a total of 95 laparotomies were performed on the same number of dead females. The females were hunted in legal shootings in eight hunting grounds on extensive unenclosed estates in the Sierra de San Pedro (in the north-east of Cáceres Province, Extremadura, Spain). Animals were raised on a natural feeding regimen with no added food supplements. Samples of rumen were collected at the shooting site on the same day. Fetal age was estimated from Crown Rump Length method (CRL) proposed by Evans and Sack (1973) also taking into account age classifications previously reported for sheep (Franco et al., 2017), goats (García et al., 2012) and red deer (Masot et al., 2007). Crown-rump length (CRL) refers to the distance from the top of the head to the base of the tail. Specimens were divided into 5 sequential groups, according to major histomorphogenetic characteristics: Group I (crown-rump length [CRL] 1.4-3.6 cm, age 30-60 days, 1-25% gestation), Group II (CRL 4.5-7.2 cm, 63-85 days, 26-35% gestation), Group III (CRL 8-19 cm, age 86-120 days, 36-50% gestation), Group IV (CRL 21-33 cm, 123-180 days, 51-75% gestation), and Group V (CRL 36-40 cm, 183-240 days, 76-100% gestation).

Sampling and processing

Sampling and processing were performed as previously described (Franco et al., 2004). Once the rumen had been separated, small pieces of tissue were dissected from the primitive gastric tube (before differentiation of the rumen) or from medial region of the dorsal and cranial sacs. Tissues for histological, histomorphometric and immunohistochemical examinations were fixed in 4% buffered formaldehyde for 24 h, routinely processed and embedded in paraffin. Sections 5 µm thick were stained with Hematoxylin-Eosin (H-E), Masson’s Trichrome (MT) and Gomori’s reticulin (GR). For scanning electron microscopy small pieces of rumen at all stages of development were immediately fixed in 2.5% buffered glutaraldehyde for 24 hours.

Histomorphometric analysis

Histomorphometric analysis was performed as previously described (Franco et al., 2004; García et al., 2012).

Specimens for morphometric analysis were viewed through a microscope (NIKON Eclipse 80i) equipped with a digital video camera (NIKON DXMI200F). Digital images were analysed 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 for each tissue layer (epithelium, lamina propria plus submucosa, tunica muscularis, serosa and total thickness of the rumen wall) of each of five selected individuals from each group.

Statistical analysis

The results are shown as mean ± SE. Data were 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. Graphs represent the averages of real growth values together with the adjusted line of regression.

Immunohistochemical analysis

Immunohistochemical analysis was conducted as previously described (Franco et al., 2017). Using an UltraVision Quanto Detection System HRP DAB (Thermo Scientific, Fremont, USA, #TL-060-QHD), and following the manufacturer’s directions, the polymer detection method was applied to deparaffinised and hydrated forestomach sections to detect the neuroendocrine cell marker synaptophysin (SYP), glial cell markers glial fibrillary acidic protein (GFAP) and vimentin (VIM), and peptidergic innervation markers.
Neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) sections. For antigen retrieval, sections were heated in a microwave for 5 minutes in buffer citrate solution (0.01M, pH 6) at 800 watts. Endogenous peroxidase activity was blocked by 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 for 30 min at room temperature with the following primary antibodies: 1:10 mouse monoclonal anti-Spy (Thermo Scientific, MA1-35810); anti-non neuronal enolase (AbD Serotec, 6880-0410); ready-to-use rabbit polyclonal anti-GFAP (Thermo Scientific, 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). Sections were then incubated with horseradish peroxidase-conjugated polymer (Thermo Scientific, UltraVision ONE HRP Polymer, TL-015-PHJ) for 30 minutes at room temperature, without exposure to light.

Diaminobenzidine was applied to tissues (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. Sections were contrasted with Mayer's haematoxylin. The specificity of the staining reaction was determined in control experiments in which the primary antiserum was replaced by PBS, or both primary and secondary antibodies were omitted. Absorption controls were obtained by incubating sections adjacent to the sample section in a solution containing 25 mg Ag/ml diluted antiserum. The antigens used were Spy (33R-6191, Fitzgerald), GFAP protein (30R-AG009 Fitzgerald), VIM (30R-2137, Fitzgerald), NPY (22873, AnaSpec) and VIP (22465, AnaSpec). No immunostaining was observed on sections used as absorption controls.

Immunolabelled sections were analysed using the Nis-Element 2.30 software package. The immunostaining surface was measured for various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis, serosa) and for the whole wall. Optimal intervals were determined statistically, and four immunoreactivity classes were established as described by García et al. (2014a,b): no immunoreactivity; no surface staining; low immunoreactivity: stained surface bellow 200 μm²; moderate immunoreactivity: stained surface between 200 and 400 μm²; intense immunoreactivity: stained surface over 400 μm². Measurements, expressed in μm², are shown as mean ± SE. Data were subjected to statistical analysis as described above for Histomorphometric analysis.

Scanning electron microscopy

Fixed rumen samples were dehydrated using graded ethanol and amyl acetate, and dried in a Balzers critical-point dryer Mod CPD 030. Sections were covered with coating materials including gold, and examined and photographed at various tilt angles, at a magnification of 10 to 800x using a JSM-6300 scanning electron microscope (García et al., 2012).

Results

Rumenal histomorphogenesis

Group I: (CRL 1.4-3.6 cm, 30-60 days, 1-25% gestation)

At 45 days (CRL 2.8 cm; 19% gestation), the early stomach was apparent as a single cavity in the spindle-shaped primitive gastric tube. The gastric wall comprised an internal non-ciliated, stratified epithelium layer of cylindrical cells and an external layer of pluripotential blastemic tissue. This occupied most of the wall and was composed mainly of irregularly oriented cells of varying morphology, surrounded by numerous capillaries.

Differentiation of the rumen from the primitive gastric tube was first observed at 51 days (CRL 3 cm, 21% gestation) (Fig. 1A). By 57 days (CRL 4.3 cm, 24% gestation), the ruminal wall comprised three clearly defined layers. The internal epithelial layer (100±19 μm) displayed a stratified epithelium composed of cylindrical cells with basal nuclei and an apical band of cytoplasm. This epithelial layer was separated from the middle layer by a well-defined basement membrane (Fig. 1B). The middle layer of pluripotential blastemic tissue (133±12 μm) contained evidence of a rudimentary tunica muscularis (longitudinally arranged, spindle-shaped mesenchymal cells). The middle layer comprised two well-differentiated areas: an internal area containing abundant cell elements and an external layer in which ground substance predominated (Fig. 1B). The external layer or serosa (55±5 μm) was formed by a single layer of flat cells (mesothelium) supported by loose connective tissue (Fig. 1B).

Group II: (CRL 4.5-7.2 cm, 63-85 days, 26-35% gestation)

At 70 days (CRL 6 cm; 30% gestation) the ruminal mucosa comprised the three layers observed in Group 1: an epithelium of considerably greater thickness (123±18 μm), pluripotential blastemic tissue and serosa (Fig. 1B). Papilliform projections were observed growing from the epithelial layer into the lumen to form rudimentary ruminal pillars (Fig. 1C).

By 79 days (CRL 7.2 cm; 33% gestation), the first evidence was observed of differentiation of the epithelium into two bands: a darker basal area and a lighter band of cytoplasm, marking the start of stratification (Fig. 1D). Pluripotential blastemic tissue was highly vascularised, and the first signs of differentiation into lamina propria and submucosa were observed (Fig. 1D). Ruminal pillars were increasingly evident due to the appearance of a rudimentary tunica muscularis composed of two layers of...
Fig. 1. Histomorphogenesis of the *Dama dama* rumen (30-240 days, 1-100% gestation). Crown rump length (CRL).

A. Photomicrograph of a section of the rumen, which appears as a single cavity of the early stomach (51 days, CRL 3 cm, 21% gestation). The wall comprises two layers: epithelium (E) and pluripotential blastemic tissue (PBT).

B. Photomicrograph of a section of the ruminal wall (57 days, CRL 4.3 cm, 24% gestation). The wall is composed of three layers: epithelium (E), pluripotential blastemic tissue (PBT) with longitudinally arranged spindle-shaped myoblastic cells (arrow) which are beginning to differentiate, and an external layer or serosa (S).

C. Photomicrograph of a section of the ruminal wall (72 days, 30% gestation). Outline of ruminal pillars (Rpi) as the epithelial layer (E) projects into the ruminal lumen. H-E.

D. Photomicrograph of a section of the ruminal wall (85 days, 35% gestation). The wall is composed of four layers: epithelium (E), lamina propria and submucosa (Lp + Sb), tunica muscularis (Tm) and serosa (S). Histodifferentiation of lamina propria, submucosa and tunica muscularis from pluripotential blastemic tissue. H-E.

E. Photomicrograph of a section of the ruminal wall (86 days, 36% gestation). Epithelium stratified into two distinct areas: a basal area or stratum basale (Eb) and an apical area or stratum granulosum (Eg). Outline of ruminal pillars (Rp). Capillaries were also visible (arrowheads). Considerable development of the tunica muscularis (Tm), comprising an internal circular bundle (I) and an external longitudinal bundle (E). H-E.

F. Photomicrograph of a section of the ruminal wall (103 days, 43% gestation). Tunica muscularis (Tm) entering pillars to form the muscular body (arrow). Thinning of epithelium and lamina propria + submucosa (Lp+Sb). Numerous blood vessels in serosa (S). H-E.

G. Photomicrograph of a section of the ruminal wall (123 days, 51% gestation). Stratified epithelium: stratum basale (Eb), stratum granulosum (Eg), stratum spinosum (Es). Ruminal papillae (Rp) visible as elevations of the stratum basale towards the stratum granulosum, reaching half the height of the epithelium. H-E.

H. Photomicrograph of a section of the ruminal wall (240 days, 100% gestation). Lamina propria and submucosa (Lp + Sb) visible within papilla, forming the papillary body. Numerous blood vessels (arrow) between the internal (I) and external bundles (E) of the tunica muscularis (Tm). Ruminal papillae (Rp) reaching the apical third of the epithelium (E). H-E. Scale bars: A, B, G, 30 μm; C, 25 μm; D, 40 μm; E, H, 35 μm; F, 20 μm.
myoblasts, a circular internal layer and a longitudinal external layer (Fig. 1D).

The serosa (34±5 µm) comprised a flat mesothelium and a highly cellular subserosa projecting into the pillar parenchyma. This indicated a role – albeit minor – in pillar formation (Fig. 1D).

Group III: (CRL 8-19 cm, 86-120 days, 36-50% gestation)

At 86 days (CRL 10 cm; 36% gestation), the ruminal wall was composed of four distinct layers: mucosa, submucosa, tunica muscularis and serosa.

The epithelial layer of the mucosa was thickened (136±21 µm) due to an increase in both the size and the number of cells. The epithelium displayed two clearly differentiated bands. Most of the cell space was occupied by a basal band, or stratum germinativum, composed of 3-5 layers of oval cells containing basophilic cytoplasm and large central nuclei. An apical band of polyhedral cells with light cytoplasm formed the stratum granulosum (Fig. 1E). This early epithelial stratification was accompanied by slight elevations of the stratum germinativum into the stratum granulosum to form rudimentary ruminal papillae.

The lamina propria and the submucosa were visible as two distinct layers derived from pluripotential blastemic tissue, adjacent to the epithelial layer (Fig. 1E). The lamina propria, composed of stellate cells surrounded by a very small amount of ground substance, was visible within elevations of the epithelial stratum germinativum, forming nascent papillae (Fig. 1E). The submucosa, adjacent to the tunica muscularis, contained fewer cell elements and a larger amount of ground substance. These two layers had a joint thickness of 130±15 µm.

The tunica muscularis (67±13 µm) comprised two interwoven bundles of smooth muscle fibre: an internal circular bundle and an external longitudinal bundle. A dense network of capillaries was also visible. The thickness of the muscularis was greater in the area of the ruminal pillars, for which it provided muscular support (corpus muscularis).

The serosa (29±6 µm) was lined with a flat epithelium, while the mesothelium was supported by a subserosa rich in amorphous ground substance and reticulin fibres.

By 120 days (CRL 17 cm; 50% gestation), ruminal pillars were considerably more developed (Fig. 1F). Three distinct layers were visible: a thin germinative epithelium, a thick tunica muscularis projecting into the pillars, and a basal layer separating the tunica muscularis from the serosa.

Group IV: (CRL 21-33 cm; 123-180 days, 51-75% gestation)

By this stage of intrauterine development, the epithelium (445±56 µm) displayed a greater degree of stratification than at earlier stages. The basal stratum germinativum was composed of strongly staining cells, while the overlying stratum granulosum comprised polyhedral cells with lightly staining cytoplasm. The uppermost stratum corneum was formed by a single layer of flat cells and was in contact with the ruminal lumen. A transitional stratum lucidum-spinosum was visible in the form of intercellular bridges.

At 123 days (CRL 21 cm; 51% gestation), the first ruminal papillae were visible as small elevations of the basal area towards the ruminal lumen, involving the basement membrane, the lamina propria and the submucosa. By this stage, the papillae had reached half the height of the epithelial layer (Fig. 1G).

The lamina propria was highly vascularised, especially in the papillae and not as thick. The lamina propria and the submucosa were more clearly differentiated and their joint thickness was 80±12 µm.

The tunica muscularis was significantly thicker than at earlier stages (104±18 µm), the increase being most pronounced in the internal bundle. Blood vessels and nerve fibres were observed within intramuscular and perimuscular connective tissue (Fig. 1G).

The serosa (23±3 µm) displayed no differences with respect to earlier stages of development (Fig. 1G).

Group V: (CRL 36-40 cm; 183-240 days; 76-100% gestation)

This stage was marked by a considerable increase in the thickness of the ruminal wall. The squamous stratified epithelium comprised: a basal stratum germinativum composed of small palisaded cells; a stratum granulosum similar to that observed at the previous stage; a stratum lucidum-spinosum with discernible intercellular bridges and a stratum corneum in direct contact with the ruminal lumen. Numerous undulations of the epithelial surface were visible, coinciding with the tips of the most developed papillae.

At 183 days (CRL 36 cm; 76% gestation), the ruminal papillae were much longer, reaching the apical third of the epithelium. The corpus papillaris, lined by the basal stratum, contained highly cellular connective tissue, as well as small amounts of collagen and reticulin, forming the papillary skeleton. At 240 days (100% gestation), these fully-developed papillae had reached the epithelial surface (Fig. 1H).

The submucosa was composed of loose connective tissue containing abundant blood vessels and occasional nerve fibres. There was no clear boundary between the submucosa and the lamina propria (Fig. 1H), which comprised dense, fibrous connective tissue. This was particularly apparent in the papillary skeleton of the ruminal papillae. The joint thickness of the two layers was 73±10 µm.

The tunica muscularis (175±22 µm) was composed of two layers of smooth muscle tissue. The fibres of the inner layer were arranged in a circular pattern, while those of the outer layer were arranged longitudinally.
Histomorphological development of Dama dama rumen

The very slender serosa (22±3 µm) was composed of loose connective tissue (subserosa) with a mesothelial lining, containing small amounts of collagen and elastin, together with occasional liposomes, blood vessels and nerve tissue (Fig. 1H).

Histomorphometric observations

Changes in the thickness of ruminal wall tissue layers during prenatal development are shown in Table 1 and Fig. 2.

Mean epithelial growth in Group I (Table 1) was significantly lower than in Groups II to V (P= 0.003). The epithelial layer grew slowly until 120 days (CRL 19 cm; 50% gestation), after which growth was faster until 180 days (CRL 33 cm; 75% gestation), coinciding with a greater degree of stratification. From 183 days onwards (CRL 36 cm; 76% gestation), growth rates remained stable until the end of gestation (Fig. 2A).

Growth of the lamina propria and submucosa in Group III (Table 1) differed significantly from that observed in Groups IV and V (P=0.003). Growth rates declined over the early stages, until around 120 days (CRL 19 cm; 50% gestation), recovering slightly thereafter and remaining stable until the perinatal stages (Fig. 2B).

Growth of the tunica muscularis in Group III differed significantly from that observed in Groups IV and V (P=0.004). Exponential growth of the tunica muscularis continued from the early embryonic stages until birth (Fig. 2C).

Mean growth of the serosa was significantly greater in Group I (Table 1) than in Groups II to V (P=0.004). The growth rate of the serosa declined from the early embryonic stages until around 160 days (CRL 27 cm; 67% gestation), stabilising thereafter until birth (Fig. 2D).

Total ruminal wall thickness differed significantly between Group III (Table 1) and Groups IV and V (P=0.003). The growth rate of the luminal wall remained steady from the early embryonic stages until around 110 days (CRL 15 cm; 46% gestation). A subsequent phase of exponential growth lasted until 168 days (CRL 30 cm; 70% gestation). Thereafter, growth remained steady until birth (Fig. 2E).

Immunohistochemical observations

The results of immunohistochemical staining SYP, GFAP, VIM, NPY and VIP in the rumen during prenatal development are shown in Table 2 and Fig. 3.

Neuroendocrine cells were first detected by SYP staining at 85 days (CRL 7.2 cm, 35% gestation) in the lamina propria, submucosa, tunica muscularis, myenteric plexus, and intervascular and perivascular connective tissue. At 180 days (CRL 33 cm, 75% gestation) neuroendocrine cells (SYP+) were observed in the epithelial layer (Fig. 3A,B).

Positive staining for GFAP was detected from 108

### Table 1. Morphometrical analysis of tissue layer thickness in Dama dama rumen during prenatal development. mean ± SE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Epithelium (µm)</th>
<th>Lp+Sb (µm)</th>
<th>Tm (µm)</th>
<th>Serosa (µm)</th>
<th>Wall (µm)</th>
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<tr>
<td>I</td>
<td>100±19*</td>
<td>Pbta</td>
<td>Pbta</td>
<td>55±5</td>
<td>328±24</td>
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<tr>
<td>II</td>
<td>123±18*</td>
<td>130±15*</td>
<td>67±13</td>
<td>34±5*</td>
<td>315±20</td>
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<tr>
<td>III</td>
<td>136±21*</td>
<td>80±12**</td>
<td>104±18**</td>
<td>29±6*</td>
<td>314±21</td>
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</table>
| IV      | 445±56*        | *P<0.005 vs Grupo I; **P<0.005 vs Grupo III. Group I (age 30-60 days of prenatal life, 1-25% gestation), Group II (age 63-85 days of prenatal life, 26-35% gestation), Group III (age 86-120 days of prenatal life, 36-50% gestation), Group IV (age 123-180 days of prenatal life, 51-75% gestation) and Group V (age 183-240 days of prenatal life, 76-100% gestation).

### Table 2. Immunohistochemical analysis of Dama dama rumen during prenatal development.

<table>
<thead>
<tr>
<th>GROUP I</th>
<th>E</th>
<th>Lp+Sb</th>
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<th>GROUP II</th>
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E. epithelium; Lp+Sb, lamina propria + submucosa; Tm, tunica muscularis; S, serosa. -, no immunoreactivity; no surface staining; +, low immunoreactivity; stained surface ≤200 µm²; ++, moderate immunoreactivity; stained surface between 200 and 400 µm²; ++++, intense immunoreactivity; stained surface ≥400 µm². Group I (age 30-60 days of prenatal life, 1-25% gestation), Group II (age 63-85 days of prenatal life, 26-35% gestation), Group III (age 86-120 days of prenatal life, 36-50% gestation), Group IV (age 123-180 days of prenatal life, 51-75% gestation) and Group V (age 183-240 days of prenatal life, 76-100% gestation).
days (CRL 15 cm, 45% gestation) until birth in the lamina propria, submucosa, tunica muscularis and serosa (Fig. 3C,D). The same layers stained positive for VIM at 63 days (CRL 4.4 cm, 26% gestation; Fig. 3E,F).

Positive staining for NPY and VIP was observed at 180 days (CRL 33 cm, 75% gestation) and 192 days (CRL 35 cm, 80% gestation) respectively in both the lamina propria and the submucosa (Fig. 3G,H).

**Scanning electronic microscopy (SEM)**

At 60 days (CRL 4.3 cm, 25% gestation), the ruminal wall was visible as a smooth surface displaying no evidence of keratinisation or desquamation (Fig. 4A). Cells were well defined, with clear outlines and intercellular borders (Fig. 4B).

Small ruminal papillae started to protrude from the surface at 79 days (CRL 7.7 cm, 33% gestation) (Fig. 4C), acquiring clearer outlines by 100 days (Fig. 4D).

At 130 days (CRL 24 cm, 54% gestation), ruminal papillae were circular in shape and varied in length (Fig. 4E). By 180 days (CRL 33 cm, 75% gestation) papillae had started to assume a conical shape and were of uniform size (Fig. 4F).

![Graphs showing mathematical models for rumen tissue layers.](image)
Fig. 3. Immunohistochemical findings in Dama dama rumen (30-240 days, 1-100% gestation). A. Photomicrograph of a section of the ruminal wall (113 days, 47% gestation). SYP+ staining in epithelial cells (arrow). EAS. B. Photomicrograph of a section of the ruminal wall (180 days, 75% gestation). SYP+ staining in lamina propria + submucosa, myenteric plexus (arrows) and intervascular and perivascular connective tissue (arrows). EAS. C. Photomicrograph of a section of the ruminal wall (108 days, 45% gestation). GFAP+ cells in lamina propria + submucosa and myenteric plexus (arrow). EAS. D. Photomicrograph of a section of the ruminal wall (180 days, 75% gestation). GFAP+ staining within the papillary body, in intervascular connective tissue in the lamina propria + submucosa. EAS. E. Photomicrograph of a section of the ruminal wall (63 days, 26% gestation). VIM+ cells (arrow) in myoblastic fibres and myenteric plexus (arrow). EAS. F. Photomicrograph of a section of the ruminal wall (180 days, 75% gestation). VIM+ staining within the papillary body and in connective tissue of lamina propria + submucosa. EAS. G. Photomicrograph of a section of the ruminal wall (192 days, 80% gestation). VIP+ staining in the connective tissue of lamina propria and submucosa and myenteric plexus (arrow). EAS. Scale bars: A, 40 μm; B, G, H, 30 μm; C, D, E, F, 25 μm.
Fig. 4. Scanning electron microscopy of the *Dama dama* rumen (30-240 days, 1-100% gestation). A. Photomicrograph of the ruminal wall (60 days, 25% gestation). Smooth surface, with no evidence of keratinisation or desquamation. Intercellular borders are observed. B. Photomicrograph of the ruminal wall (60 days, 25% gestation). Well-defined cells with clear outlines and intercellular borders. C. Photomicrograph of the ruminal wall (85 days, 33% gestation). Incipient ruminal papillae starting to protrude into the epithelial surface. D. Photomicrograph of the ruminal wall (100 days, 42% gestation). Differentiation of ruminal papillae, separated by clear boundaries. E. Photomicrograph of the ruminal wall (123 days, 51% gestation). Circular ruminal papillae of varying lengths. F. Photomicrograph of the ruminal wall (180 days, 75% gestation). Conical ruminal papillae of uniform size. G. Photomicrograph of the ruminal wall (240 days, 100% gestation). Development of lamina propria + submucosa. Tunica muscularis composed of internal and external bundles. H. Photomicrograph of the ruminal wall (240 days, 100% gestation). Leaf-shaped ruminal papillae, showing signs of surface keratinisation. Scale bars: A, E, 200 μm; B, 50 μm; C, 300 μm; D, G, 100 μm; F, 75 μm; H, 150 μm.
Histomorphological development of Dama dama rumen

Immediately before birth (230 days, CRL 47 cm, 96% gestation), ruminal papillae were leaf-shaped (Fig. 4G) and showed signs of surface keratinisation (Fig. 4H).

Discussion

Differentiation of the rumen from the primitive gastric tube in fallow deer was first observed at 51 days (21% gestation) while in red deer, Franco et al. (2004) observed differentiation at 60 days (25% gestation). In domestic ruminants, it takes place at 35 days of prenatal life (23% gestation) in goats (Moliniari and Jorquera, 1988) and at 34 days (22% gestation) in sheep (Del Río Ortega, 1973; Franco et al., 1992), while in cattle (Vivo and Robina, 1990; Vivo et al., 1990) it occurs rather earlier, at 30 days (11% gestation).

By 60 days (25% gestation) the ruminal wall comprised three clearly defined layers: an internal mucosa, an intermediate layer of pluripotential blastemic tissue and an external serosa. In the mucosa, the transition from “epithelium of the embryo” to stratified epithelium was similar, both in morphology and timing of appearance, to that reported in red deer by Franco et al. (2004), in goats by Ramkrishna and Tiwari (1979) and García et al. (2012) and in sheep by Franco et al. (1992). However, Panchamukhi and Srivastava (1979) reported epithelial stratification in buffalo, and Vivo et al. (1990) in cattle though neither mentions specific component strata. The stratum corneum and stratum espinosum were visible at 123 days (51%), i.e. considerably later than reported in red deer (Franco et al., 2004), in sheep (Franco et al., 1992) and in goats (García et al., 2012). The stratum lucidum has hitherto only been reported in buffalo rumen (Osman and Berg, 1981).

The stratum corneum (123 days) was observed in the prenatal ruminal epithelium at a similar stage of development in red deer (Franco et al., 2004). However, findings regarding the appearance of this stratum in other ruminant species vary considerably. In goats, for example, García et al. (2012) observed it at 76 days (50% gestation), while Moliniari and Jorquera (1988) noted it only at 102 days (70% gestation). In sheep, Franco et al. (1992) first identified the stratum corneum at 81 days (54% gestation), whereas Del Río Ortega (1973) observed it only shortly before birth, a finding also reported for buffalo (Panchamukhi and Srivastava, 1979) and cattle (Kano et al., 1981; Vivo et al., 1990).

Epithelial stratification was accompanied by a considerable increase in thickness, and by structural modification in the form of nascent ruminal pillars and papillae. Similar findings have been reported in red deer at 67 days (27%; Franco et al., 2004), in sheep at 39 days (26%; Del Río Ortega, 1973) and 45 days (30%; Franco et al., 1992) and in goats at 42 days (28%; García et al., 2012). By contrast, Vivo et al. (1990) noted pillar formation at a much earlier stage of development at around 44 days (16% gestation).

Ruminal papillae appeared at 86 days (35%) as elevations of the stratum germinativum involving the basement membrane, the lamina propria and the submucosa. Similar findings are noted by Franco et al. (2004) for red deer (142 days, 45% gestation). The appearance of ruminal papillae has been observed in goats at 53 days (35%; García et al., 2012). However, this differs markedly from the 136 days (91% gestation) reported in goats by Molinari and Jorquera (1988) and the 126 days (84% gestation) noted in this species by Ramakrishna and Tiwari (1979). Studies in sheep by Del Río Ortega (1973), Fath-El Bab et al. (1983) and Franco et al. (1992) report the appearance of ruminal papillae at 64 days (43%), 103 days (69%) and 61 days (42%), respectively. In cattle, ruminal papillae are observed only shortly before birth (Arias et al 1978; Amasaki and Daigo, 1987, 1988; Vivo et al., 1990).

The differentiation of lamina propria and submucosa from pluripotential blastemic tissue was first observed at 85 days (35% gestation). In other wild and domestic ruminant species, differentiation has been observed at earlier stages of prenatal development: at 60 days (25%) in red deer (Franco et al. 2004); at 33 days (22%) in sheep (Franco et al., 1992), at 50 days (33%) in goats (García et al., 2012) and at 35 days (15%) in cattle (Vivo et al., 1990).

Differentiation of the tunica muscularis – comprising two layers of myoblasts with a clearly-defined orientation – from pluripotential blastemic tissue was also observed at 85 days (35% gestation). By contrast, Franco et al. (2004) noted differentiation at 60 days (25%) in red deer. Similar findings have been reported by García et al. (2012) in goats and by Del Río Ortega (1973) and Franco et al. (1992) in sheep. In contrast, other authors have recorded varying results for different ruminant species. In sheep, Fath-El-Bab et al. (1983) observed differentiation of the tunica muscularis at 52 days (22%), whilst in cattle Vivo et al. (1990) reported it an earlier stage of 35 days (15%). In the present study, differentiation of the tunica muscularis peaked at 123 days (51%), coinciding with increased pillar growth and development. It might thus be surmised that, while the lamina propria is actively involved in ruminal pillar formation, the main role is played by the development of the tunica muscularis, which provides the pillar's muscular body.

Growth and differentiation of the serosa took place at a fairly steady rate from the earliest stages of gestation to birth. Similar findings have been reported for red deer (Franco et al., 2004), sheep (Franco et al., 1992), goats (García et al., 2012) and cattle (Vivo et al., 1990).

Morphometric results showed accelerated growth of the epithelial layer and the tunica muscularis at 180 days (75% gestation), coinciding with greater stratification of the epithelium and involvement of the tunica muscularis in the formation of the muscle body of the ruminal pillars. In contrast, the growth-rate of the lamina propria
and submucosa declined from the early embryonic stages until birth, perhaps due to constraints on expansion resulting from the active growth of the epithelial layer and the tunica muscularis. The serosa maintained a steady rate of growth until birth. Similar morphometric findings have been reported in red deer (Franco et al., 2004), in sheep (Franco et al., 1992) and in goats (García et al., 2012).

Neuroendocrine (SYP-positive) cells were detected at 85 days (35% gestation) in the lamina propria, submucosa and tunica muscularis, and at 180 days (75%) in the epithelium. Similar findings have been reported in red deer (Franco et al., 2004) and goats (García et al., 2012). In contrast, Franco et al. (2011) detected these neuroendocrine cells in sheep at a later stage of development (81 days; 54%).

Gliial fibrillary acidic protein-positive glial cells were observed at 108 days (45% gestation), and VIM-positive cells at 63 days (26%), as also noted in goats (García et al., 2012, 2014b). Gliial fibrillary acidic protein is widely considered a valid marker for the immunohistochemical identification of astrocytes (Franco et al., 2004), while VIM has been identified as an early glial cell marker in the prenatal development of the ruminal mucosa in ruminants (Franco et al., 2011). Gliial cells were detected earlier here than in red deer (180 days; 75% gestation) or sheep (77 days; 51% gestation) (Franco et al., 2004, 2011 respectively).

Vasoactive intestinal polypeptide and NPY were detected in the latter stages of prenatal development, at 180 days (75%) and 192 days (80%) respectively. Similar findings have been observed for red deer (Franco et al., 2004) and goats (García et al., 2012, 2014b). The presence of neuropeptides in the prenatal ruminal epithelium has also been reported in sheep (Vergara-Esteras et al., 1990).

Scanning Electron Microscopy findings suggest that age is a crucial factor in the structural development of ruminal papillae. During the early embryonic stages, the ruminal wall was visible as a smooth surface displaying no evidence of keratinisation or desquamation. Incipient papillae started to protrude from the ruminal surface at 80 days (33%). Similar changes were observed by Scott and Gardner (1973) and García et al. (2012) in sheep and goat rumen respectively. In cattle, Amasaki and Daigo (1988) detected ruminal papillae at later stages of development, in the fifth month of gestation.

By 180 days (75%), ruminal papillae in the mucosa were of uniform size and appearance. McGavin and Morrill (1976) highlighted the relationship between papillary morphology and diet, noting that in calves fed only milk, papillae were slender and tongue-shaped, whereas in calves fed roughage they were smaller and rounded, and showed signs of keratinisation. Similar findings were reported earlier by Nockels et al. (1966). In contrast, both here and in a study of the goat rumen by García et al. (2012), differences were found in the length of ruminal papillae as a function of ruminal wall growth rates, but no difference in morphology was observed.

The results obtained here confirm that prenatal development of the fallow deer rumen is broadly similar to that reported in red deer and in goats, but somewhat slower than that observed in sheep and cattle. Comparative analysis of ruminal mucosa development in different wild and domestic ruminant species raised on a natural feeding regimen or receiving feed (but no protein supplements) suggests that feeding regime does not have a decisive effect on the rate of prenatal development of the rumen in Dama dama.

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Conflicts of interest. The authors declare no competing financial interests.

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