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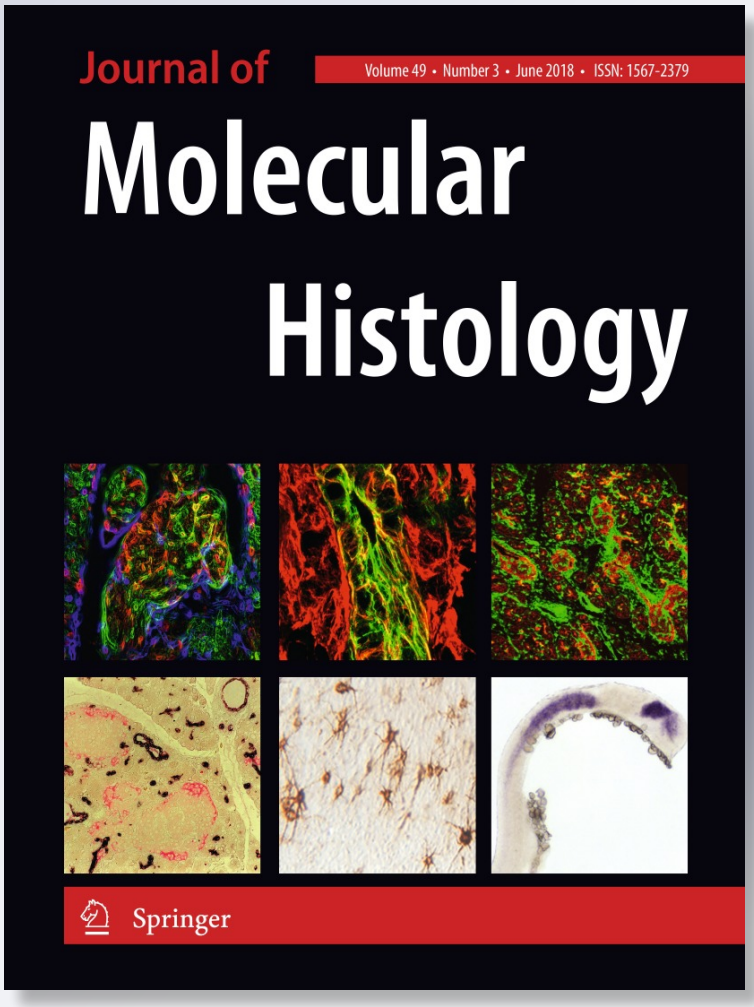

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ORIGINAL PAPER

Endoscopy, histology and electron microscopy analysis of foetal membranes in pregnant South American plains vizcacha reveal unusual excrescences on the yolk sac

Mariela Giacchino[1,2] · Pablo I. F. Inserra[1,2] · Fernando D. Lange[3] · María C. Gariboldi[1,2] · Sergio R. Ferraris[3] · Alfredo D. Vitullo[1,2]

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Abstract

The South American hystricognathe *Lagostomus maximus* is a fossorial rodent whose females show unique reproductive characteristics. They have a 155-day long gestation, show massive polyovulation and a selective process of embryonic resorption in the first half of gestation. In order to explore and perform an in-situ characterization of the reproductive tract, we visualized internal structures through ultrasonography and video-endoscopy in pregnant and non-pregnant females. We describe the finding of protruding structures that lie on the yolk sac and their histological and ultrastructural characterization. The placenta was covered with whitish, small pearl-shaped structures. These structures were also seen on the extra-embryonic space, being the amnion and the umbilical cord free of them. Pearl-shaped structures were composed with loose connective tissue, lacked blood vessels, and showed collagen fibers organized in a spiral form. They were anchored by pedicles to the villous surface of the extraembryonic membrane. We discuss the biological and evolutionary meaning of the pearl-shaped structures that relate *L. maximus* to the African origin of the South American hystricognathe fauna.

Keywords Ultrasound · Endoscopy · Pregnancy · Placenta · Mesoderm excrescences · Vizcacha

Introduction

The South American plains vizcacha, *Lagostomus maximus*, is a hystricomorph rodent whose geographical distribution ranges from the Pampean region in Argentina to Bolivia and Paraguay (Llanos and Crespo 1952; Cabrera and Yepes 1960; Cabrera 1961; Jackson et al. 1996). This fossorial rodent shows colonial and nocturnal habits and remains active throughout the whole year (Branch et al. 1993). Plains vizcacha is of considerable ecologic and economic regional interest. The species modify the matrix that surrounds the burrow system altering nutrient cycles through grazing and extracting some plants but not others, and interfering with agricultural and livestock activities (Jackson et al. 1996). Moreover, the species exhibits unusual anatomical and physiological characteristics encouraging its use in basic biomedical research (Dorfman et al. 2016).

Females display several unique reproductive traits. They show massive polyovulation, releasing up to 800 oocytes per estrus cycle (Weir 1971a). Polyovulation arises from an unusual constitutive tissue-specific suppression of apoptosis that greatly decreases intra-ovarian oocyte dismissal by inhibiting oocyte attrition and follicular atresia in foetal and adult life, respectively (Jensen et al. 2006, 2008; Leopardo et al. 2011; Inserra et al. 2014). Despite the large number of oocytes released only 10–12 blastocysts implant equally distributed in each uterine horn (Roberts and Weir 1973) after an 18-day long pre-implantation development. Embryos show an interstitial pattern of implantation with an anti-mesometrial orientation (Leopardo and Vitullo 2017). Soon after implantation, between 26 and 70 days post-coitum (dpc), a selective process of embryonic resorption

✉ Alfredo D. Vitullo
vitullo.alfredo@maimonides.edu

1 Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico (CEBBAD), Universidad Maimónides, Hidalgo 775, C1405BCK Buenos Aires, Argentina

2 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

3 Centro de Investigación y Desarrollo en Medicina Experimental (CIDME), Universidad Maimónides, Buenos Aires, Argentina

takes place initiating at the proximally implanted conceptuses (Weir 1971a). Only the embryos implanted nearest the cervix in each uterine horn escape resorption and develop to term after a 155-day gestation time (Weir 1971b). At mid-gestation, from 70 dpc onwards, following a decrease in circulating progesterone that enables the activation of the hypothalamic-pituitary-gonadal axis, an ovulatory process that adds a considerable number of accessory corpora lutea occurs (Dorfman et al. 2013; Fraunhoffer et al. 2017). The addition of newly developed corpora lutea helps to recover progesterone levels and rescue the only two surviving foetuses (Jensen et al. 2008).

In order to perform an in-situ characterization of the reproductive tract of *L. maximus*, especially during gestation, we explored internal structures with minimum invasion through ultrasonography and video-endoscopy in both pregnant and non-pregnant females. Here, we describe the finding of protruding structures that lie on the yolk sac, their histological and ultrastructural characterization, and we discuss their biological meaning that relates them to the African origin of the South American hystricognathe fauna.

Materials and methods

Ethics

All experimental protocols concerning animals were conducted in accordance with the guidelines published by the National Institutes of Health (NIH) (NIH 1985) and the Guide for the Care and Use of Laboratory Animals (CCAC 2002, 2003), and were reviewed and approved by the Institutional Committee of Use and Care of Laboratory Animals (CICUAL) from Facultad de Veterinaria, Universidad de Buenos Aires (Res. N° 2014/5), and the Institutional Committee on Use and Care of Experimental Animals (CICUAE) from Universidad Maimónides, Argentina. Appropriate procedures were performed to minimize the number of animals used. Capture, handle and transport of animals were approved by the Buenos Aires Province Government authorities (Dirección de Flora y Fauna, Ministerio de Asuntos Agrarios).

Animals

Adult female plains vizcachas, 2.5–3.0 kg body weight; 2–2.5 years old determined by the dry crystalline lens weight according to Jackson (1986), were captured from a resident natural population at the Estación de Cría de Animales Silvestres (ECAS), Villa Elisa, Buenos Aires, Argentina, employing live-traps located at the entrance of burrows. In order to obtain females at different reproductive stages, captures were planned according to the natural reproductive cycle which extends from April to August as described by Llanos and Crespo (1952) and our own field expertise (Jensen et al. 2006, 2008; Dorfman et al. 2011; Leopardo et al. 2011; Inserra et al. 2014). Non-gestating females (n = 5) were captured in February, when reproductive season starts. Mid-pregnant (n = 19) and late-pregnant (n = 16) females were captured in July and August, respectively. Gestational age was estimated on the basis of the capture time and fetal development, according to Leopardo et al. (2011).

All individuals were anesthetized by the intramuscular injection of 10 mg/kg body weight of ketamine chlorydrate (Holliday Scott S.A., Argentina) and 1 mg/kg body of weight xylazine chlorhydrate (Richmond Laboratories, Argentina). Transabdominal ultrasonographies were performed to check the developmental stage of the foetuses (see below). Some animals from each developmental stage were selected for video-endoscopy whereas the others were sacrificed by trained technical staff with an intracardiac injection of 0.5 ml/kg body weight of Euthanyl (sodium pentobarbital, sodium diphenylhydantoin, Brouwer S. A., Argentina).

Ultrasonography and video-endoscopy

Non-pregnant (n = 5) and mid- (n = 8) and late-gestating (n = 5) females were studied by transabdominal ultrasonography. All ultrasound examinations were conducted under general anaesthesia. The equipment employed was an ESAOTE MyLab TM GOLD 30 connected FP to a lineal transducer set to 10 MHz (ESAOTE Model LA 523 F; 4–13 Mhz). After shaving the abdomen, animals were positioned lying on their backs (supine position). Ultrasound gel was applied and pregnant or non-pregnant stage was ascertained. The viability of the foetuses was determined employing a Doppler colour tool. Representative images were taken along the procedure.

The reproductive tract of both pregnant and non-pregnant females was explored by means of video-endoscopy under general anaesthesia. Entering from the vagina, the horns were insufflated with CO_2 in order to clear the cavity. According to the size and anatomy of the vizcacha, an optic fiber (Dyonics 30 degree 4 mm Arthoroscope) was employed. The optical fiber was attached with a camera (Karl Storz Telecam DX 202330/20), a light source (Karl Storzendoskope—halogen) and an image digitizer (AVID technology—IDX-video). Animals were positioned lying on their backs in a tilt table to adjust the position of the organs during the procedure. The evaluation was carried out in each uterine horn and video recorded along the whole tract.

The choice of appropriate instrumentation both for ultrasound and endoscopy were based on Divers (2010).

Tissue collection

The uterine horns of pregnant females were exposed and the foetuses were immediately removed for measuring and weighing. After scoring foetal measures, both foetuses and accompanying membranes were fixed in 10% formaldehyde for 24 h and further processed for standard histology (see "Optical microscopy"). Then, uterine horns and placenta were removed, fixed in 10% formaldehyde and processed for optical or electron microscopy as indicated below.

Optical microscopy

Formaldehyde-fixed tissue samples of mid- (n = 5) and late-gestating (n = 5) females were dehydrated through a gradual series of ethanol (70, 96 and 100%) and embedded in paraffin. Paraffin-embedded samples were entirely cut into serial sections (5 µm thick) and mounted onto coated slides. Mounted sections were dewaxed in xylene and rehydrated through a decreasing series of ethanol. Selected sections of each specimen were used to perform classical hematoxylin and eosin (H&E), periodic acid of Schiff (PAS) and Masson's trichrome staining for general description. Representative images were captured with an optic microscope (BX40, Olympus Optical Corporation, Tokyo, Japan) fitted with a digital camera (390CU 3.2 Megapixel CCD Camera, Micrometrics, Spain) and the image software Micrometrics SE P4 (Standard Edition Premium 4, Micrometrics, Spain).

Transmission electron microscopy

Samples from mid- (n = 3) and late-gestating (n = 3) females were initially fixed in 3% glutaraldehyde in 0.1M phosphate buffer (PBS) for 24 h and then transferred to fresh PBS for 48 h. A second round of fixation was performed with 1% osmium tetroxide for 60 min at 0 °C. Samples were washed twice with distilled water for 15 min each. A last round of fixation was performed incubating samples overnight with 5% uranyl acetate followed by two 15 min-washing rounds with distilled water. Samples were then dehydrated through a graded series of ethanol followed by two 10 min immersions in acetone. Samples were embedded in Durcupan resin and polymerized at 60 °C for 72 h. Ultrathin sections (50 nm thick) were obtained by means of an ultramicrotome (Reichert Jung Ultracut E, Wien, Austria). Finally, sections were mounted on copper grids and counterstained with 5% uranyl acetate and 2.5% lead citrate. Representative images were captured with a transmission electron microscope (Zeiss EM 109T, Oberkochen, Germany).

Scanning electron microscopy

Samples from mid- (n = 3) and late-gestating (n = 3) females were fixed in 4% formaldehyde for 24 h, dehydrated through a graded series of ethanol (25, 50, 75 and 100%) and preserved in ethanol 100% for critical point drying in an EMS 850 Critical Point Dryer (Electron Microscopy Sciences, USA). Samples were refrigerated to 5 °C and dehydrated increasing their temperature up to 40 °C. Finally, samples were mounted in steel based slides and metalized with a Mini Sputter Coater (SC7620, Quorum Technologies, UK). They were introduced in an Electronic Microscope Swept (Philips XL 30 SEM, New York, USA) to 20 KV. Representative images were taken.

Image analysis

Adobe Photoshop software (Adobe Photoshop CS5, Adobe Systems Inc., Ontario, Canada) was used for digital manipulation of brightness and contrast when preparing the shown images.

Results

General anatomy of the placenta of *L. maximus*

A general macroscopic view of the placenta at mid-gestation is shown in Fig. 1. As gestation progressed, the membrane covering the parietal face of the placenta was covered with whitish small pearl-shaped structures of approximately 1 mm in diameter (Fig. 1). However, pearl-shaped structures

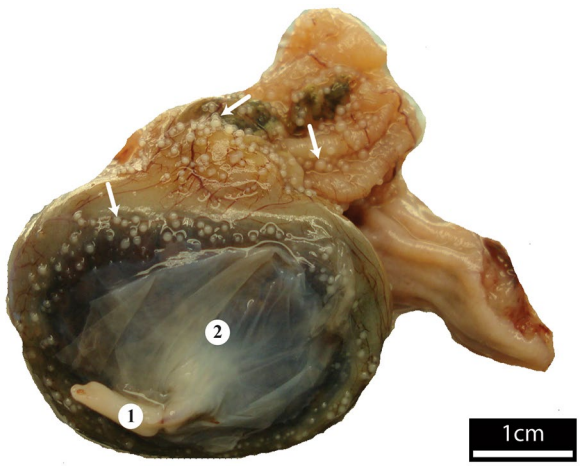


Fig. 1 General morphology of the placenta of *L. maximus*. From mid-gestation whitish, small pearl-shaped excrescences (arrows) are found distributed over the deciduas and the foetal face of placenta. Note the absence of excrescences in the remaining of umbilical cord (1) and the amnion (2)

were observed neither in the amnion nor in the umbilical cord (see below).

Ultrasonographic and videoendoscopical inspection of the reproductive tract of *L. maximus*

Ultrasonographic examination of the genital tract in non-, mid- and late-pregnant females is illustrated in Fig. 2. All pregnant animals examined showed a single foetus in each uterine horn. Pearl-shaped structures were not detected using ultrasonography (Fig. 2a–d). Foetal viability was ascertained by means of heart rate and frequency (Fig. 2e). Doppler analysis of the placenta revealed a heterogeneous ecotexture and a conspicuous vasculature intimately related to the foetus (Fig. 2f).

Video-endoscopy in non-pregnant females (Fig. 3) enabled the distinction of two anatomical regions of the genital tract: a cranial region with a conspicuous medial septum (Fig. 3a, b) and a posterior region lacking the septum (not shown). The vagina bifurcates into the two uterine horns (Fig. 3c) whose mucosa showed transversal hyperemic folds that blocked the light (Fig. 3d, e). The mucosa of the oviduct presented a tortuous disposition with longitudinal folds (Fig. 3f).

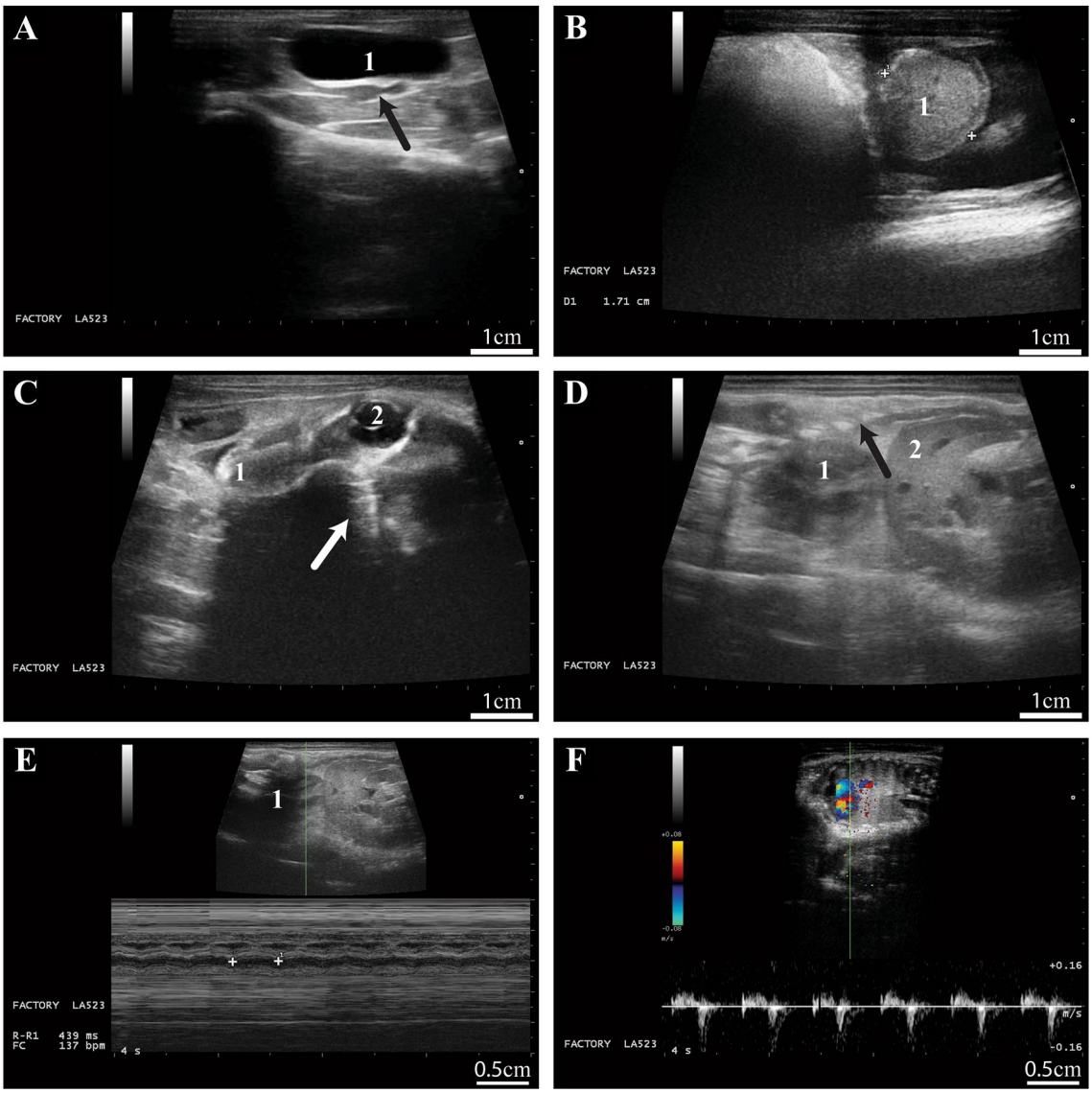


Fig. 2 Ultrasound and Doppler diagnosis of foetal development in *L. maximus*. **a** Ultrasound of a non-pregnant female showing the anechoic bladder (1) and the empty uterine horn (arrow) underneath. **b** Transverse mid-pregnancy foetal image with measuring of abdominal diameter (1). **c** Cranial ultrasound of a late-gestation foetus; nostril (1), ocular cavity (2) and trachea (arrow) are observed. **d** Longitudinal view of a late-gestation foetus; note the spinal cord (arrow), heart (1) and liver (2). **e** Ultrasound cut at cardiac level (1) in a late-gestation foetus. **f** Doppler ultrasonogram indicating the heartbeat and liveliness of the foetus

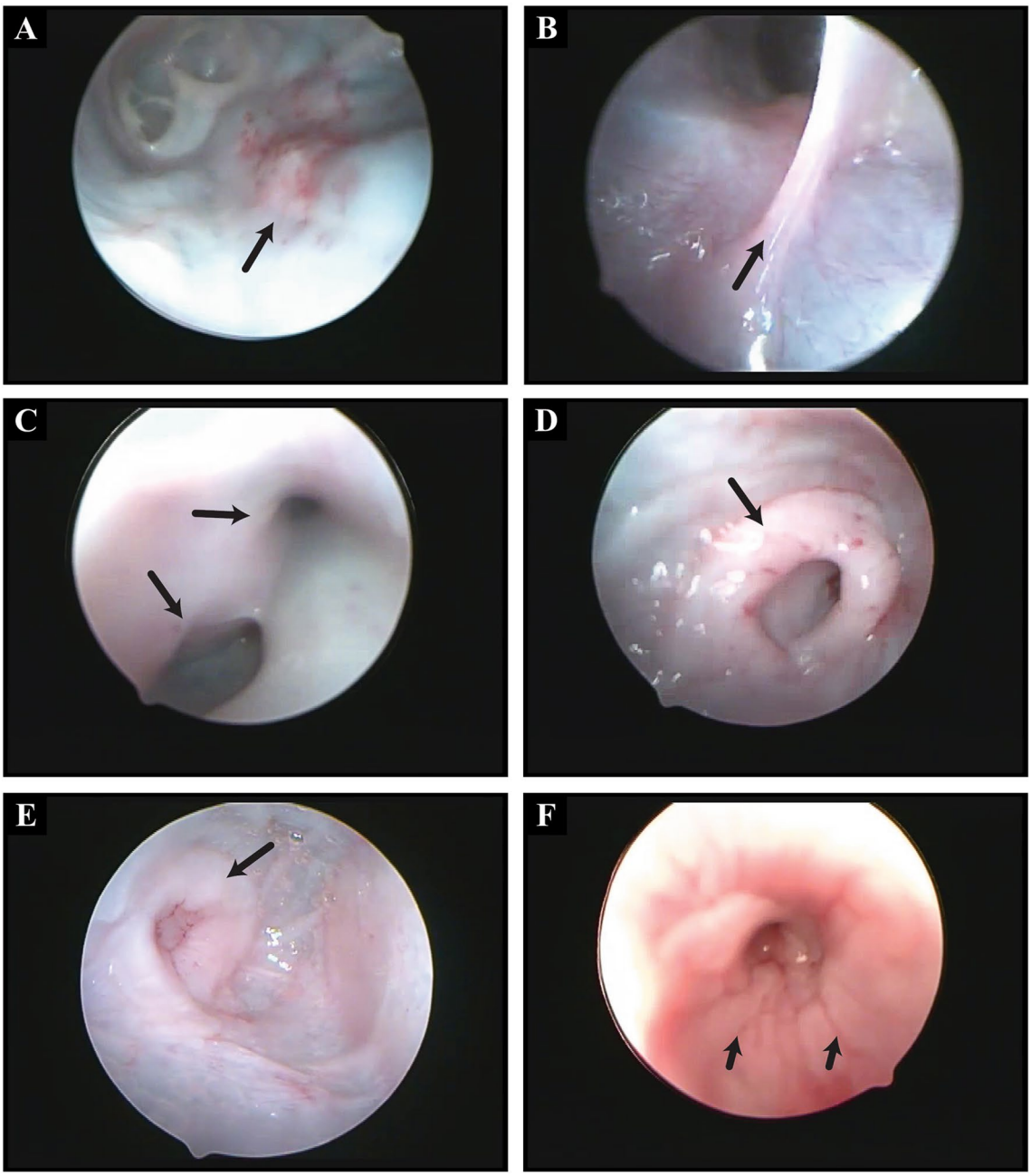
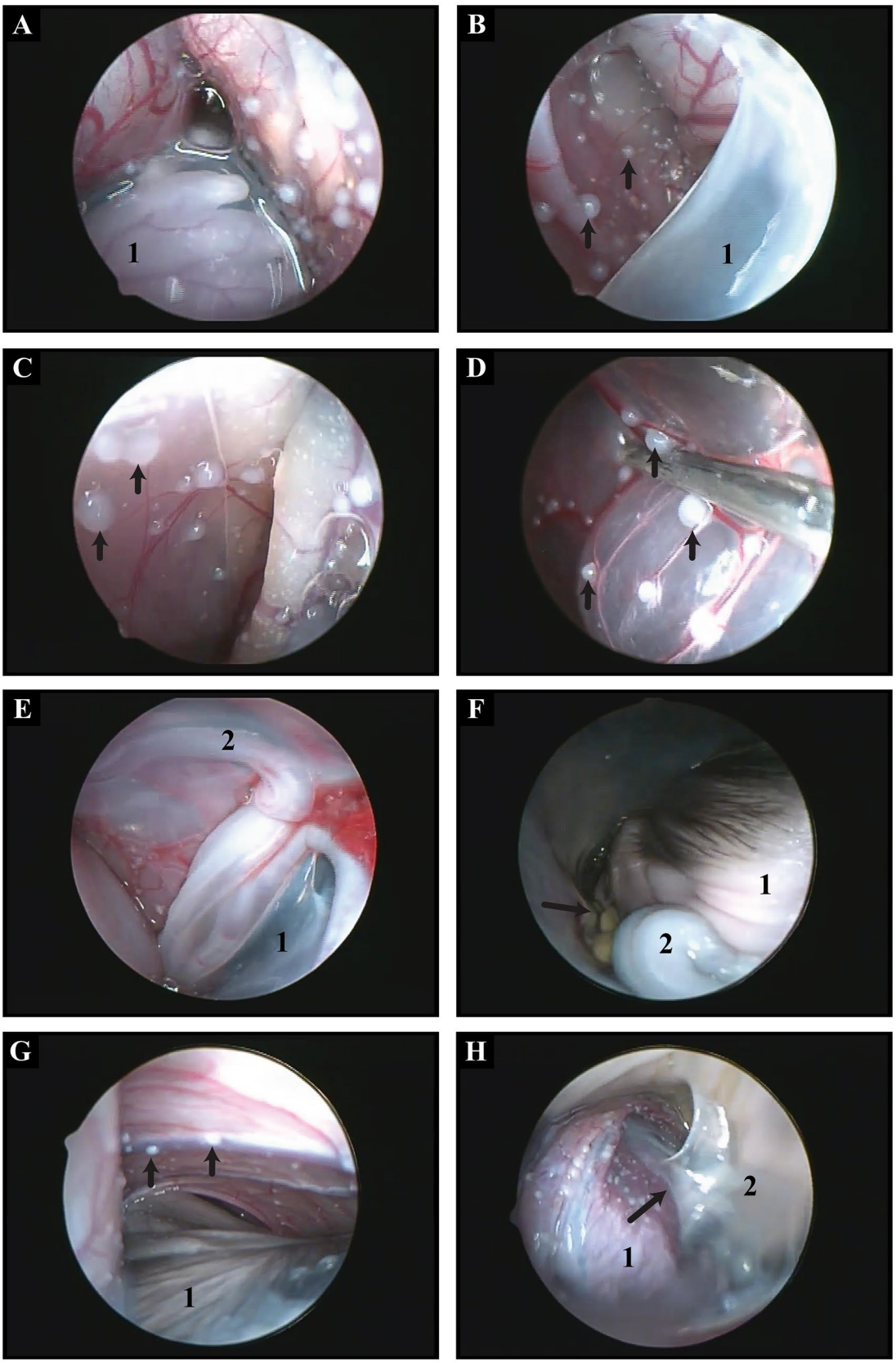


Fig. 3 Endoscopical analysis of the reproductive tract in non-pregnant female of *L. maximus*. **a, b** Presence of a pseudo-septum (arrows) in the cranial vagina. **c** Visualization of the entrance to both uterine horns (arrows). **d, e** Expansions of the intracornual wall of *L. maximus* toward light (arrows). **f** Mucous of the oviduct with multiple longitudinal folds (arrows)

Video-endoscopy in advanced mid- (Fig. 4a–e) and late-pregnant females (Fig. 4f–h) revealed whitish pearl-shaped structures in the extracoelomic space fixed to the extraembryonic membranes, following the circuit of foetal blood vessel. These structures were also partially found in the foetal face of the placenta, stacked to the membrane covering the placenta (Fig. 4h). The amnion (Fig. 4a, b, g, h) and the umbilical cord (Fig. 4e, f) were free of these structures. Early-gestating animals did not show these protruding structures (not shown).

Histology of pearl-shaped excrescences of foetal membranes in *L. maximus*

Pearl-shaped structures consisted of loose connective tissue lacking blood vessels, with fibers organized in a spiral



◂**Fig. 4** Endoscopy of the reproductive tract of pregnant females of *L. maximus*. **a**–**e** Mid-pregnancy, **f**–**h** late-pregnancy. **a** Foetal hand (1) within the amnion. **b** Amnion (1) free of pearly structures (arrows). **c** Extra-embryonic membrane, backed up on the uterine horn, very irrigated with presence of pearly structures (arrows). **d** Gas is insufflated between the corneal wall and the highly vascularized membrane covered with pearls (arrows). **e** Placenta in situ (1) showing the umbilical cord (2) coming out. **f** Observation inside the amnion: part of the foetus (1), umbilical cord (2) and meconium (arrow). **g** The foetus with hairy mantle covered by amnion (1) is observed underneath and on top of the uterine horn, coated by extra-embryonic membrane and whitish structures (arrows) following the trajectory of blood vessels. **h** Placenta in situ (1) showing extra-embryonic membrane (2) covering the foetal face; amnion adhesion to the placenta (arrow)

form (Fig. 5a, b) and small fibroblast nuclei were scattered between the fibers (Fig. 5c). The center of these structures showed abundant collagen fibers. The entire structure was surrounded by a cuboideal epithelium (Fig. 5a, b). Some pearl-shaped structures were found free in the proximity of the yolk sac (Fig. 5d), most likely as a result of the histological technical manipulation, whereas others were anchored to the parietal yolk sac (Fig. 5e). Pearl-shaped structures were PAS negative (not shown).

Sub-cellular organization of pearl-shaped excrescences of foetal membranes in *L. maximus*

Using scanning electron microscopy (Fig. 6a–d), pearl-shaped structures were found anchored to the villous surface of the extraembryonic membrane by pedicles. They showed a round or oval morphology of different sizes (Fig. 6a) and 2–3 pedicles of variable length (Fig. 6b–d). Transmission electron microscopy (Fig. 6e, f) revealed cells with abundant rough endoplasmic reticulum and extensive and complex cisterns of Golgi apparatus (Fig. 6e). Excrescences consisted of conjunctive tissue in development, with great quantity of fibroblasts actively synthesizing collagen. Fibers of lax connective tissue were unorganized and fibrils showed the characteristic pattern of transverse striation (Fig. 6f).

Discussion

Except in very advanced stages, the detection of pregnancy in the vizcacha by external palpation is difficult. The successful development of ultrasound techniques reported here enabled to diagnose pregnancy as early as mid-gestation, 70 dpc onwards, in living individuals and to ascertain foetal viability through cardiac activity. This adds a useful tool for field studies for this species since non-invasive ultrasound just requires a moderate anaesthesia.

Video-endoscopy of the female reproductive tract of *L. maximus* showed an anatomical organization of both the vagina and the uterine horns concordant with previous histological descriptions (Weir 1971a). Transition from the uterine horn into the oviduct occurred through an abrupt decrease in diameter without any evident intra-uterine portion of the oviduct wall as expected from previous histological reports (Flamini et al. 2014).

The general inspection of mid-gestation placenta revealed that it was covered for the most part, except for the amnion and the umbilical cord, with abundant whitish excrescences. The histology of the placenta in *L. maximus* was extensively described by Flamini et al. (2011) showing that it is a discoid, chorioallantoic and hemomonochorial placenta, with great subplacenta development and a complex labyrinth structure separated by interlobular tissue and marginal trophoblast. A similar type of placenta is found in other South American hystricognathe such as the capybara (*Hydrochoerus hydrochaeris*), the agouti (*Dasyprocta aguti*), the paca (*Cuniculus paca*), the chinchilla (*Chinchilla lanigera*) and the guinea pig (*Cavia porcellus*) (Enders 1965; Miglino et al. 2002, 2004; Rodrigues et al. 2006; Kanashiro et al. 2009; Franco de Oliveira et al. 2012). Nevertheless, the presence of excrescences in foetal membranes was not described in any of these species. Roberts and Perry (1974) first noticed the excrescences in the placenta of *L. maximus*, which they called pustules, but neither a morphological nor a functional analysis were performed. The term "pustule" refers to a small elevation of the skin containing cloudy or purulent material associated with an infectious process. Our histological analysis shows that excrescences in *L. maximus* are not associated with an infectious process but rather with a physiological event during pregnancy, making the term pustule inappropriate.

Excrescences in foetal membranes were described in a variety of mammals, such as the horse (Naaktgeboren and Zwillenberg 1961), the African elephant (*Loxodonta africana*) (Amoroso 1952; Amoroso and Perry 1964; Allen et al. 2003), the aardvark (*Orycteropus afer*) (Taverne and Bakker-Slotboom 1970), the hippopotamus (*Hippopotamus amphibious*, Linnaeus) (Amoroso et al. 1958) and the whales (Naaktgeboren and Zwillenberg 1961). In hystricomorph rodents, excrescences of foetal membranes were described in the African greater cane rat (*Thryonomys swinderianus*) (Oduor-Okelo 1979; Oduor-Okelo and Gombe 1982). Although the structures described in *T. swinderianus* are morphologically similar to those found in the vizcacha, they are located differently. In the cane rat, the excrescences are found in the amnion and the umbilical cord (Oduor-Okelo 1979; Oduor-Okelo and Gombe 1982) whereas both embryonic structures are free of them in the vizcacha.

Our histological and electron microscopy analysis revealed that in vizcacha these protruding excrescences are anchored by pedicles to the yolk sac and consist of loose connective tissue, with fibers organized in a spiral form, lacking blood vessels. Hence, the vizcacha is the only

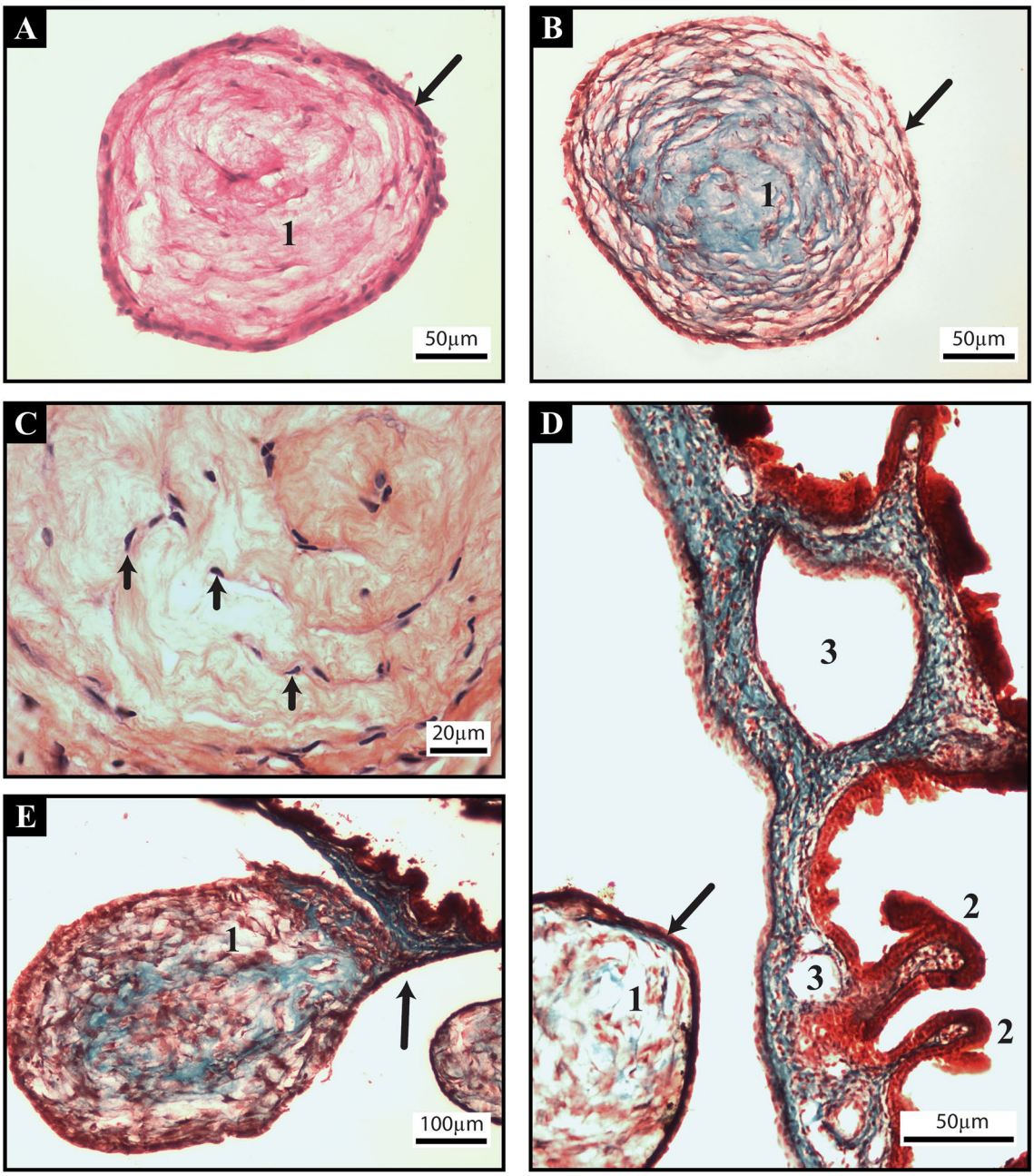


Fig. 5 Optic microscopy of pearl-shaped structures in fetal membranes of *L. maximus*. **a**–**b** Isolated pearl-shaped structure, organized in spiral layers of fibrous tissue (1), surrounded by a simple cuboidal epithelium (arrow); no blood vessels are found inside the pearl. **c** Detailed image of the inside of the pearl-shaped structure showing fibroblasts with small flattened nuclei (arrows) and collagen fibers. **d** Loose pearl-shaped structure (1), surrounded by simple cuboidal epithelium (arrow) facing the inverted yolk sac with villous (2) that contact the maternal tissue and the mesothelium with abundant blood vessels (3). **e** Pearl-shaped excrescence (1) anchored to the extra-embryonic membrane (arrow). **a**, **c** Hematoxilin and eosin. **b**, **d**, **e** Masson's trichrome stain

species so far described with these protruding structures lying only on the yolk sac.

Despite the description of excrescences in several mammals, its role and constitution are still unknown. The presence of pearly-shaped white structures found in the stomach of the common eland foetus (*Taurotragus oryx pattersonianus*) was related with a nutritional function, since they show glycogen in their interior (Oduor-Okelo 1979). However, the protruding structures of the vizcacha yolk sac are PAS negative, presenting a fibrous constitution and the absence of glycogen granules. Hence, a nutritional function seems not suitable in this case.

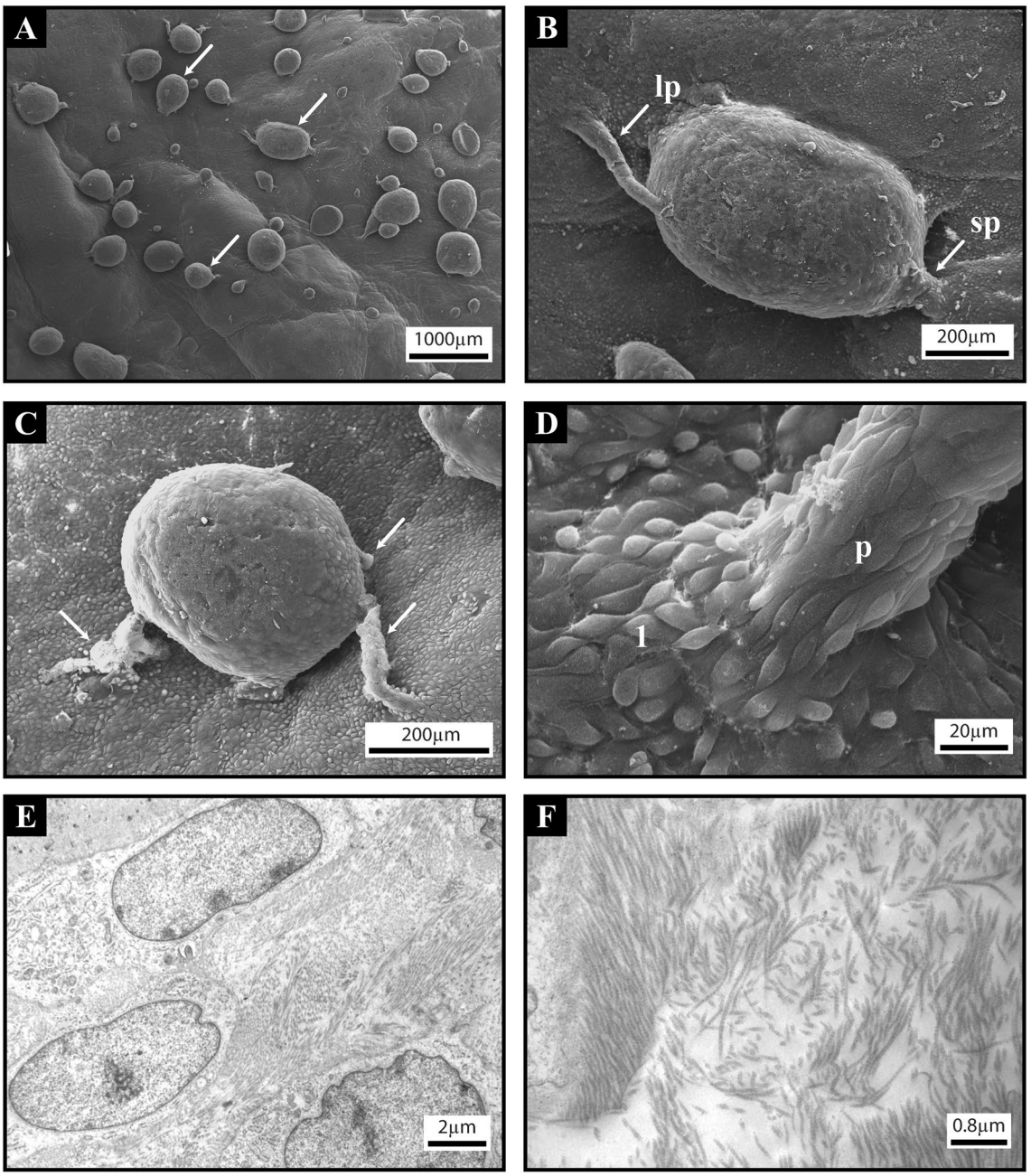


Fig. 6 Scanning and transmission electron microscopy of mesodermal excrescences of the fetal membranes in *L. maximus*. **a** Panoramic view of multiple oval excrescences (arrows) of variable size. **b** Detailed view of a pearl-shaped excrescence anchored by two pedicles to villous surface: long pedicle (lp) and short pedicle (sp). **c** Pearl-shaped structure anchored by three pedicles (arrows). **d** Detail of the anchored pedicle (p) to the villous surface (1). **e** Fibroblasts with well developed organelles that could indicate that cells are synthesizing collagen actively. **f** Fibers of collagen randomly arranged. **a**, **d** Scanning electron microscopy. **e**,**f** Transmission electron microscopy

In the African elephant, excrescences were observed by ultrasound in the allantois starting from day 126 of gestation (Hildebrandt et al. 2007). However, in the vizcacha these pearl-shaped structures were not detected by this technique, probably due to their small size, approximately 1 mm or less. On the other hand, video-endoscopy proved to be an adequate tool for the detection of these excrescences. Moreover, from late mid-gestation (90 days of gestation) the placenta of the vizcacha is progressively covered with these mesoderm excrescences and therefore they can be used as a gestational dating parameter for the species.

The hystricognathe rodents of the New World, the Caviomorpha, reached South America during the Eocene from Africa (Simpson 1983; Voloch et al. 2013). Molecular phylogenetic analysis relates the Caviomorpha with the African Phiomorpha, especially with the families Thryonomyidae, Petromuridae and Bathyergidae (Huchon and Douzery 2001). Although a general agreement on the African origin of Caviomorpha exists, the possible North American origin proposed by Wood (1980) still persists. Morphological traits greatly contribute to settle controversies in molecular phylogenies. For instance, the enamel structure of Caviomorpha teeth is similar to some species of Phiomorpha (Martin 2005) advocating in favour of an African origin. In the same way, the finding described in this report of mesoderm excrescences in the yolk sac in the South American *L. maximus*, comparable to those found in the African phiomorph *T. swinderianus*, adds new support to the African origin of the New World hystricognathe rodents which also share a common middle ear structure with the hystricognathe African fossil *Diamantomys* (Lavocat 1967).

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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