

## HISTOLOGICAL AND ULTRASTRUCTURAL CHANGES IN THE BLASTOCYST AND REPRODUCTIVE TRACT OF THE ROE DEER, *CAPREOLUS CAPREOLUS*, DURING DELAYED IMPLANTATION

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(Received 14th August 1972)

**Summary.** The ultrastructure of four roe-deer blastocysts at different stages of embryonic development were studied. During delayed implantation, the outer surface of the trophoblast possessed numerous microvilli and periodic invaginations or caveolae. There was a marked lack of organelles such as mitochondria or endoplasmic reticulum in the cytoplasm of the trophoblast cells, though many lipid droplets, granular inclusions and a lamina of fine fibrillae were present. Elongation of the blastocyst was associated with a decrease in the size and number of the microvilli, the disappearance of lipid droplets and granular inclusions, a reduction in the amount of fibrillar material and a dramatic increase in the development of mitochondria, granular endoplasmic reticulum, ribosomes and Golgi apparatus.

The histology of the ovaries and uterus was studied in thirty-one roe deer. No prominent changes occurred in the ovaries at any stage of development; all ovaries possessed active CL and showed signs of follicular growth and atresia. Changes in the degree of mitotic activity, epithelial cell height, endometrial vascularity and stromal oedema were observed in the uterus throughout the period of delayed implantation and during the phase of rapid embryonic growth. Elongation of the embryo was associated with a marked decline in the height of the glandular epithelium and an increase in endometrial vascularity.

The most important ultrastructural changes in the uteri of six roe deer were observed in the endometrial glands. Delayed implantation was associated with the accumulation of numerous supranuclear vesicles derived from the Golgi apparatus, while the resumption of embryonic growth was correlated with their sudden disappearance. When elongation had

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been completed, there was a sudden decrease in the cellular activity of these endometrial glands.

## INTRODUCTION

The roe deer, *Capreolus capreolus*, mates in late July or early August. After fertilization, the cleaving egg passes into the uterus, develops into a blastocyst, and loses the zona pellucida before the end of August. The blastocyst then remains free in the uterine lumen for 5 months, increasing in size during this time from  $0.27 \times 0.28$  mm to  $3.3 \times 1.7$  mm (Bischoff, 1854; Keibel, 1902; Sakurai, 1906). This is followed towards the end of December or the beginning of January by a period of rapid embryonic growth, which immediately precedes implantation. The conceptus eventually forms a villous cotyledonary attachment to each of the uterine caruncular ridges and the young are born in May.

The histology of the uterus and CL has already been described (Short & Hay, 1966). The CL of the roe deer appears to be fully active during delay, as judged by its size, histological appearance and progesterone content, and by the development of the endometrium (Short & Hay, 1966). This is in contrast to the situation in other mammals with delayed implantation: the CL is relatively inactive during delay in marsupials (Sharman, 1963), in mustelids such as the mink (Enders & Enders, 1963) and in the badger (Canivenc, Short & Bonnin-Laffargue, 1966); in such animals, the termination of delay is associated with an increase in luteal activity. Short & Hay (1966) described the roe-deer endometrium during delay as progestational, the uterine glands being well developed with some containing PAS-positive material. Histologically, no change could be detected in the endometrium at the time of elongation of the blastocyst.

The purpose of this study was, firstly, to describe the ultrastructural features of the blastocyst during delayed implantation and at the time of rapid elongation and, secondly, to carry out a more detailed histological and ultrastructural examination of the ovaries and endometrium to see if there were any changes which might account for this sudden resumption of blastocyst growth.

## MATERIALS AND METHODS

### *Animals*

Thirty-one roe does were shot by Forestry Commission staff in Thetford Chase, Suffolk, between August 1969 and January 1970. The five animals used for the ultrastructural study of the endometrium were shot between October 1970 and January 1971. Ages were estimated according to the degree of molar tooth wear in the lower jaw and by the number of annular deposits of cementum between the roots of the first molar tooth (R. J. Aitken, unpublished observations). Animals were judged to have had a previous pregnancy if the uterine arteries were well developed (Short & Hay, 1966).

### *Ultrastructure of the blastocyst*

After an animal had been shot, the uterus and ovaries were removed within a few minutes and placed on ice for transport to a field laboratory. The uterine

horns were separated from each other and the contents of each horn were flushed into a glass dish from the tubal end with 20 ml of 0.9% saline. The recovered blastocysts were transferred within 20 min of death to phosphate-buffered osmium tetroxide and fixed at 2° C for 1 to 3 hr. In the laboratory, they were subsequently dehydrated through a series of cold aqueous ethyl alcohol solutions before being allowed to warm up to room temperature in absolute alcohol. They were then placed in epoxy-propane for 30 min before embedding in Araldite. Sections were cut on a Huxley microtome, using either diamond or glass knives. The sections were stained for contrast with uranyl acetate and lead citrate before examination on a Philips E.M. 75 or a Siemens 1 electron microscope.

#### *Histology of the uterus and ovaries*

After the uteri had been flushed to recover the blastocysts, segments from the centre of each uterine horn and both ovaries were transferred either to 10% formaldehyde or to Bouin's fluid for fixation. The material was dehydrated, embedded in paraffin wax, sectioned at 6 to 8  $\mu$ m and stained with Meyer's haematoxylin and eosin. The heights of the luminal, caruncular, ductal and glandular epithelia were estimated in each section using a calibrated eye-piece graticule, and means were calculated from thirty to sixty observations.

#### *Ultrastructure of the endometrium*

Within 1 hr of death, segments were cut from the middle of each horn for electron microscopy.

After fixation in phosphate-buffered glutaraldehyde for 1½ hr at 2° C, the uterine samples were transferred to phosphate buffer and subsequently post-fixed in 1% osmium for 1½ hr. The tissues were then dehydrated and embedded in Araldite using standard procedures. Silver-grey sections were cut on a Huxley Cambridge microtome and stained with uranyl acetate followed by lead citrate. Grids were viewed under an AEI 6B electron microscope.

#### *Histochemistry of the endometrium*

In order to detect the presence of lipid in the endometrium, sections were stained with Sudan III using standard histochemical procedures.

## RESULTS

#### *Ultrastructure of the blastocyst*

The details of the animals used in this study are given in Table 1.

*The blastocyst during delayed implantation.* The blastocysts examined during the phase of delay were those flushed from Animals 1 and 2. The trophoblast formed a single layer of uniform squamous cells whose outer surface was covered by numerous slender microvilli. Membrane-bound granules could occasionally be found on the surface of the microvilli. Periodic invaginations or caveolae were present in the outer membrane, beneath which a region containing numerous microvesicles could be seen. Both caveolae and microvesicles were bounded by a membrane lined by a thin layer of electron-dense material.

The striking feature of the trophoblast cells during delayed implantation was

Table 1. Details of experimental animals used in the ultrastructural study of the roe-deer blastocyst

Animal no.	Date shot	Age (years)	Parity	Position and no. of CL		No. and location of embryos	Embryo size
				Left ovary	Right ovary		
1	24 Nov. 1969	3	Non-parous	1	1	Right uterine horn; one blastocyst Left uterine horn; one blastocyst	1.2 mm diameter
2	1 Dec. 1969	2	Parous	1	1	Right uterine horn; two blastocysts	1.3 mm diameter
3	6 Jan. 1970	—	Parous	1	1	Left uterine horn; one elongated blastocyst	1.5 mm diameter
4	14 Jan. 1970	7	Parous	1	1	Right uterine horn; one elongated conceptus Left uterine horn; one elongated conceptus	15 mm long 50 mm long approximately 50 mm long approximately

PLATE 1

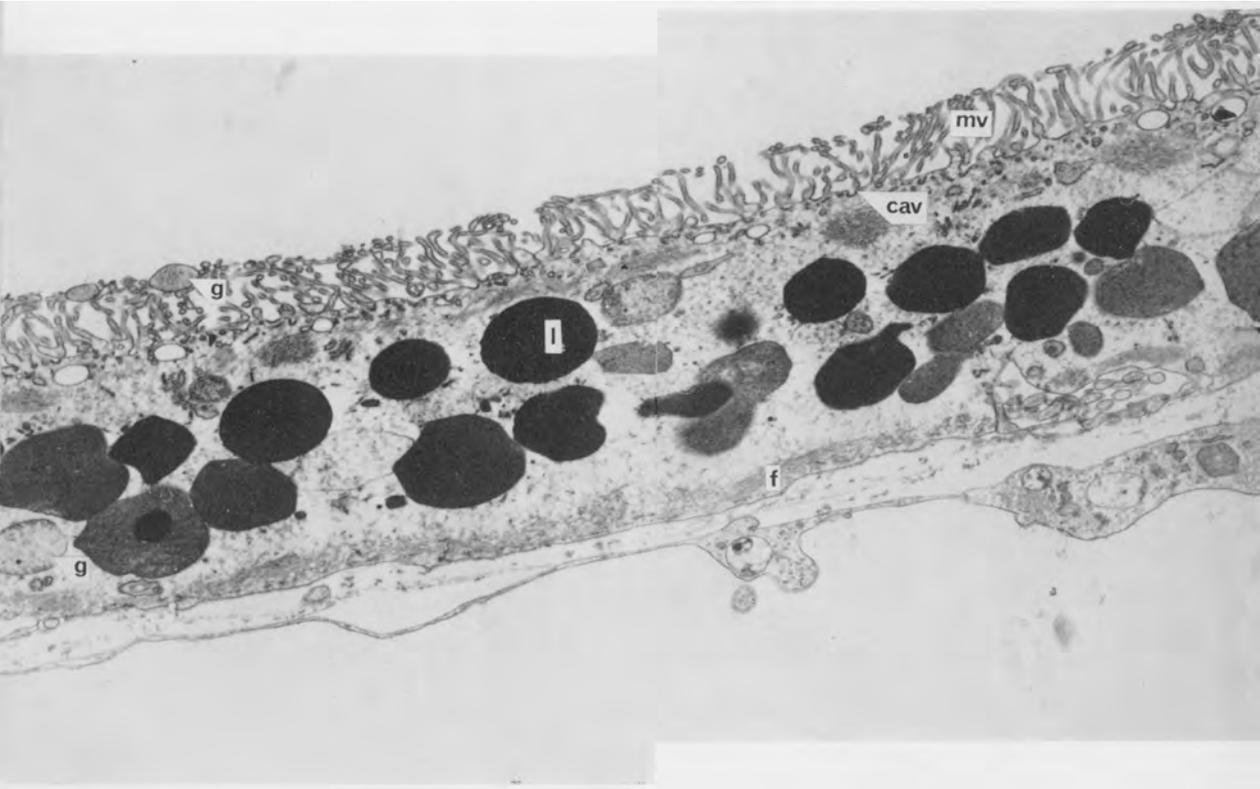


FIG. 1. Electron micrograph of a roe-deer blastocyst during delayed implantation. Caveolae (cav) and large numbers of microvilli (mv) are present on the outer surface of the trophoblast while the cytoplasm contains numerous lipid droplets (l) and membrane-bound granules (g). Occasionally, membrane-bound granules may be found on the surface of the microvilli. A lamina of fine fibrillae (f) can also be seen running round the inner margin of the cells. Note the striking lack of cytoplasmic organelles.  $\times 7200$ .

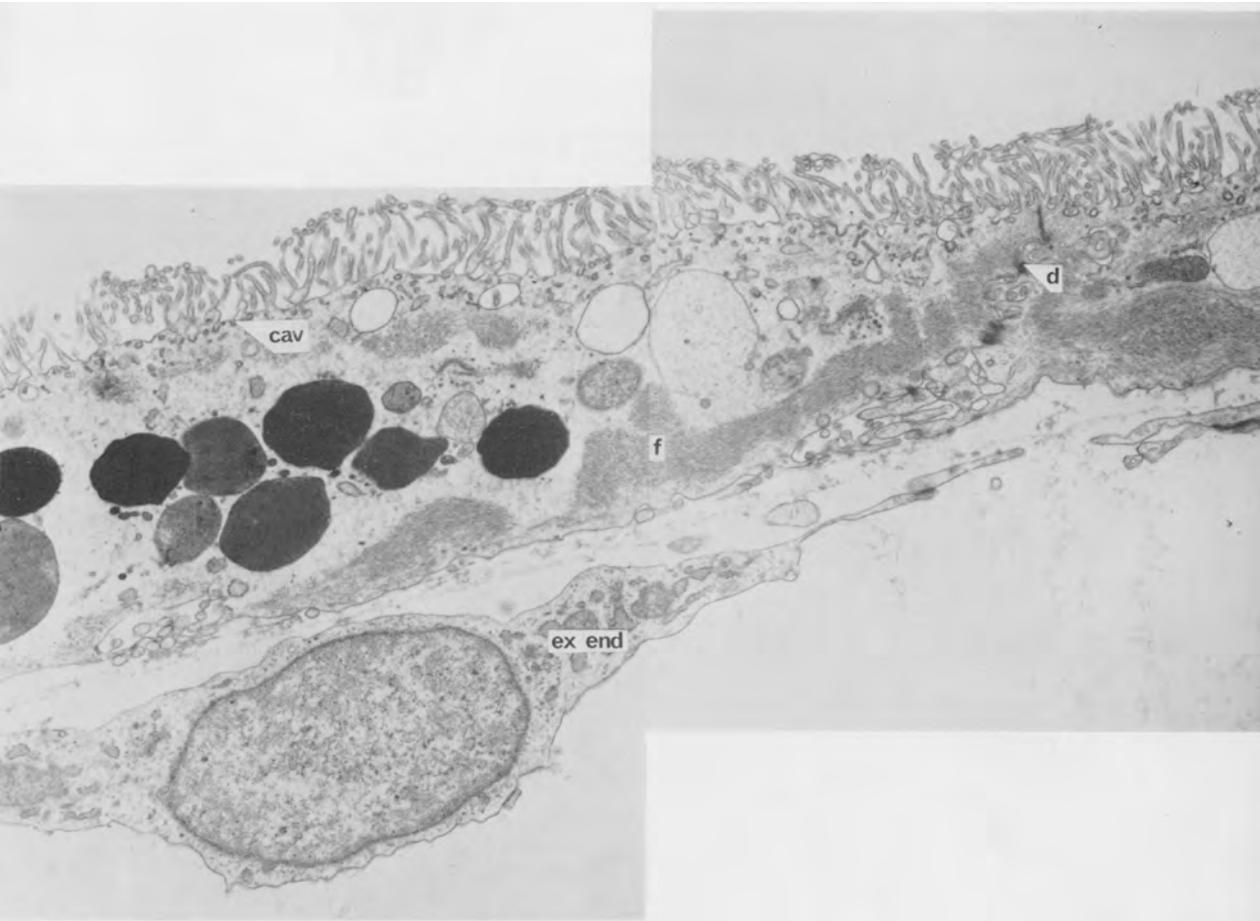


FIG. 2. Electron micrograph of a roe-deer blastocyst during delayed implantation, again illustrating the marked lack of cytoplasmic organelles. Note the way in which the lamina of fine fibrillae makes contact with the desmosomes (d) of the lateral cell wall, and the presence of the extra-embryonic endoderm (ex end) inside the trophoblast.  $\times 7200$ .

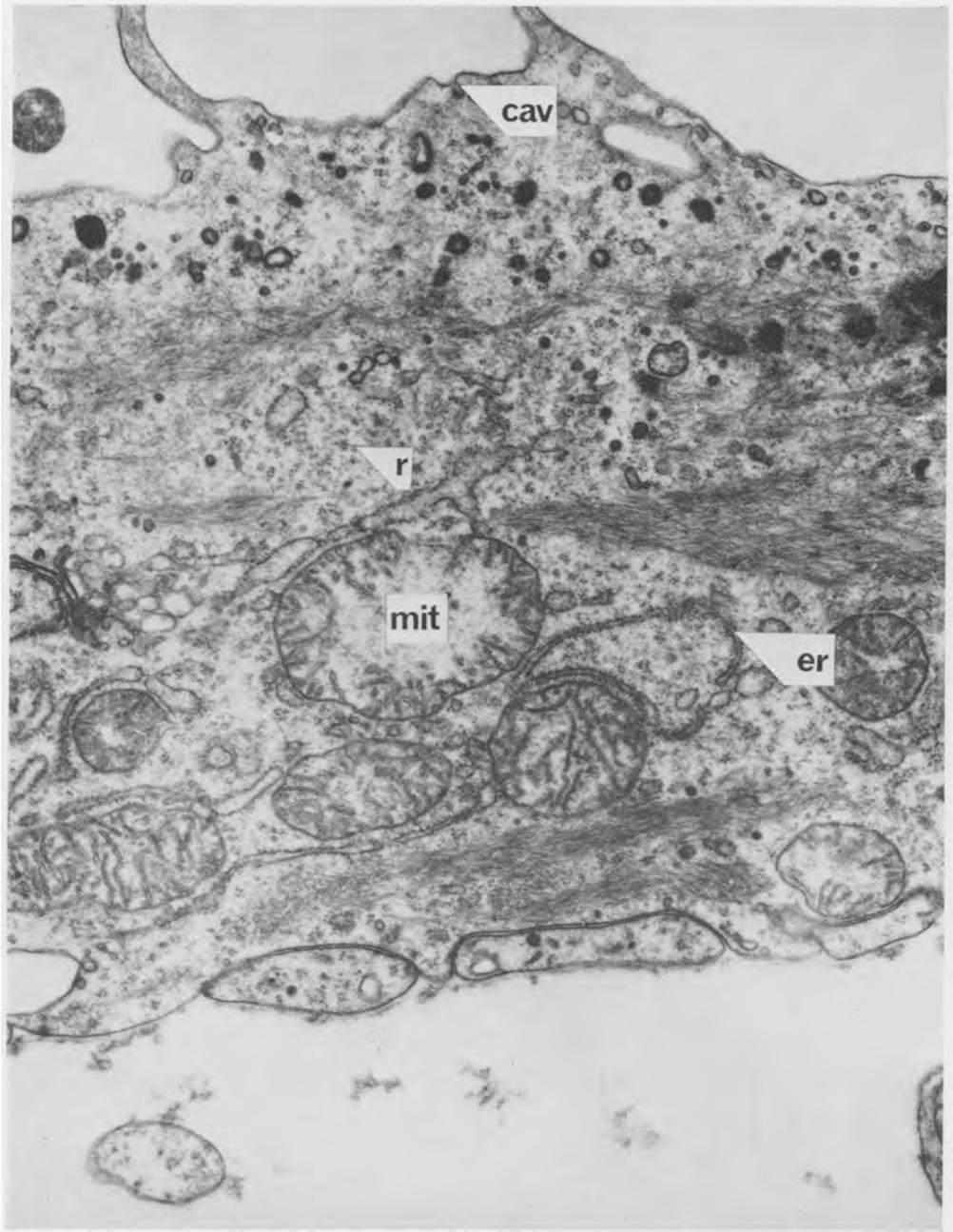


FIG. 3. Electron micrograph of an elongating roe-deer blastocyst. Elongation of the trophoblast is associated with a dramatic increase in the number of cytoplasmic organelles. Mitochondria (mit), endoplasmic reticulum (er) and ribosomes (r) are abundant. A few caveolae (cav) are still present in the outer membrane but the number of microvilli lipid droplets and membrane-bound granules is severely reduced.  $\times 22,300$ .

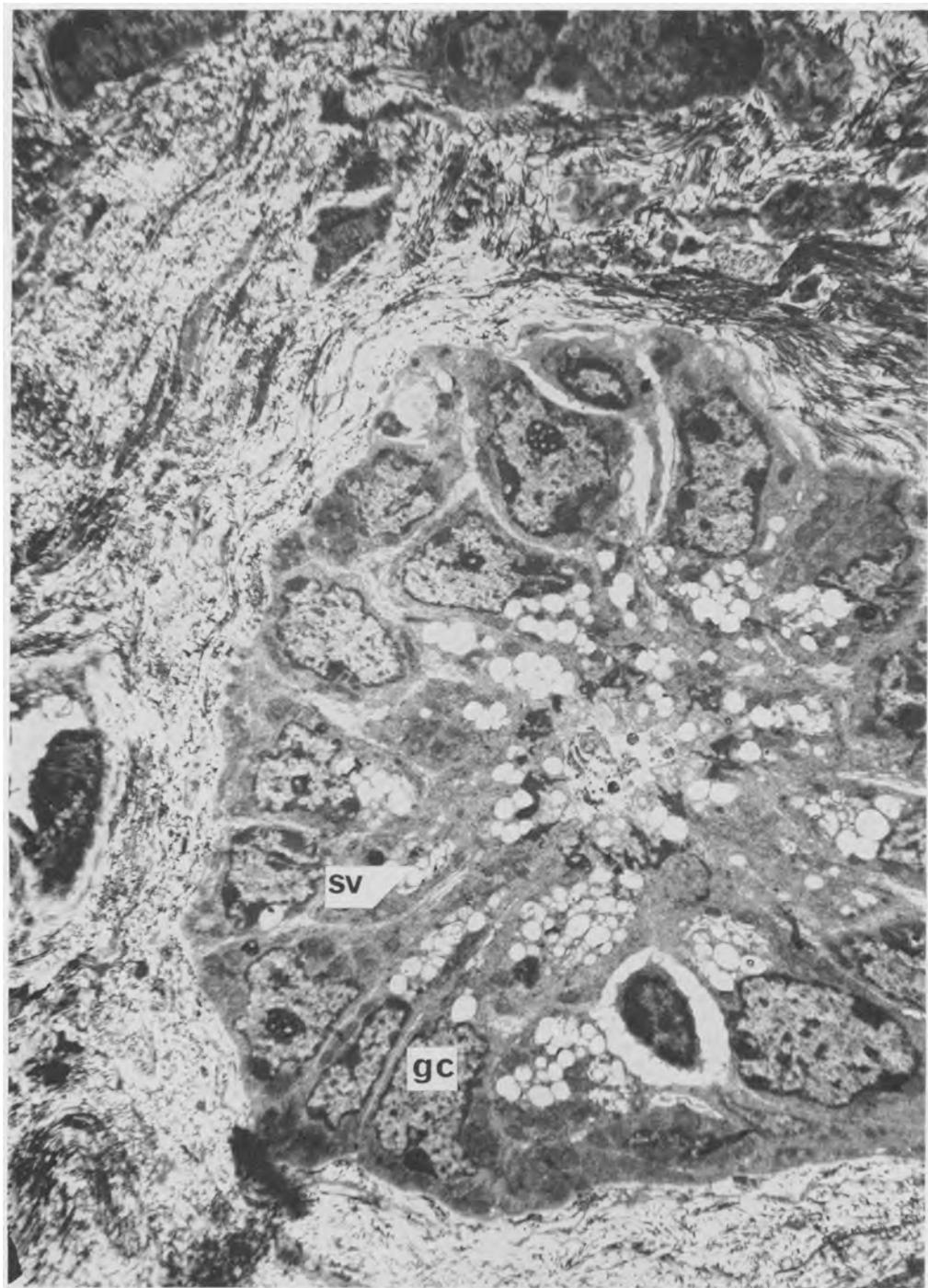


FIG. 4. Electron micrograph of a roe-deer endometrial gland during delayed implantation. Note the accumulation of supranuclear vesicles (sv) in the gland cells (gc).  $\times 3350$ .

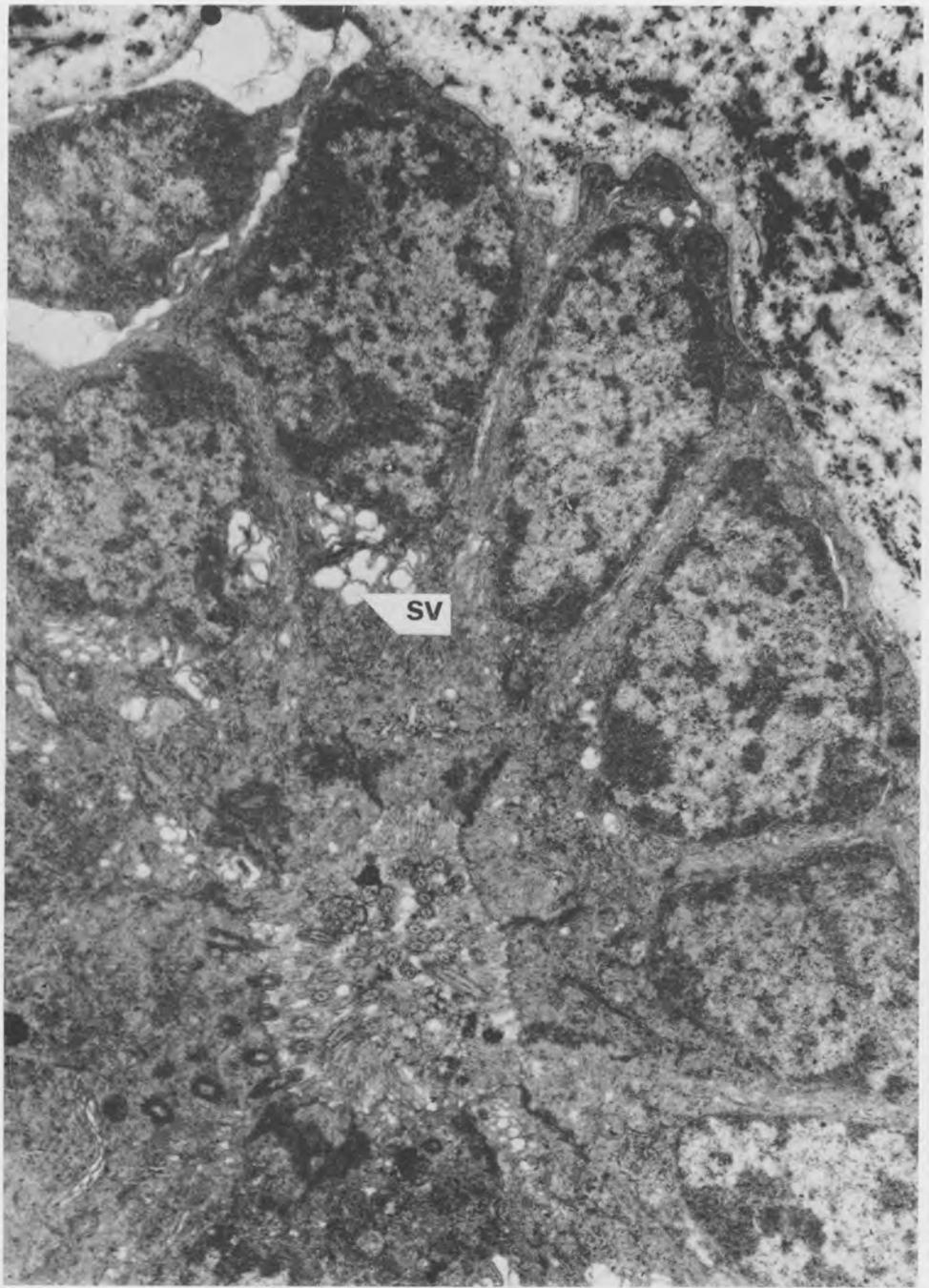


FIG. 5. Electron micrograph of a roe-deer endometrial gland after elongation of the embryo had reached an advanced stage. Note the marked decline in the number of supra-nuclear vesicles (sv) and cell height.  $\times 11,000$ .

the lack of cytoplasmic organelles. Mitochondria, when present, were circular but neither endoplasmic reticulum nor Golgi membranes were conspicuous (Pl. 1, Fig. 1).

The trophoblast cells did contain numerous large electron-dense droplets, probably consisting of lipid, which occupied the central region of the cytoplasm. These droplets did not appear to be bounded by a membrane: when close to an organelle, their shape was adapted accordingly.

Interspersed between these droplets were inclusions containing granular material of variable electron density. These inclusions often occupied extensive areas of the cytoplasm and were surrounded by a discontinuous membrane which was invaginated in certain places, the invaginations being lined by a layer of darkly staining electron-dense material. Round the inner margin of the cells was a lamina of fine fibrillae. The fibrillae occupied extensive areas of the cytoplasm and communicated with the filaments of the desmosomes of the lateral cell borders (Pl. 2, Fig. 2).

Inside the trophoblast, a layer of cells with much attenuated cytoplasm was present, forming the extra-embryonic endoderm. The endodermal cells did not form a continuous band round the inner surface of the blastocyst at this stage. The cells of the inner cell mass were not studied in these specimens.

*The elongating blastocyst.* A blastocyst in the process of elongation was recovered from Animal 3. The conceptuses from Animal 4 consisted of two 5-mm long embryos with accompanying membranes which had already elongated to approximately 50 mm.

By this stage, the trophoblast cells had become irregularly cuboidal in shape and the number of microvilli on the outer surface had decreased. The microvilli were shorter and fatter than during the period of delay, were randomly orientated, and contained microvesicles. Microvesicles and caveolae were still present in the outer regions of the cells in large numbers.

In association with the process of rapid embryonic growth, a dramatic increase in the number and size of the cytoplasmic organelles was observed within the trophoblast cells. The shape of the mitochondria was oval or cigar-like or occasionally branched. Rough-surfaced endoplasmic reticulum and ribosomes were present in large quantities and the Golgi apparatus had undergone considerable development (Pl. 3, Fig. 3).

The growth of the embryo was also accompanied by a sudden decrease in the number of lipid droplets and granular inclusions. The fibrillar material was also less evident though fibrils could still be seen making contact with desmosomal filaments at the intercellular junctions.

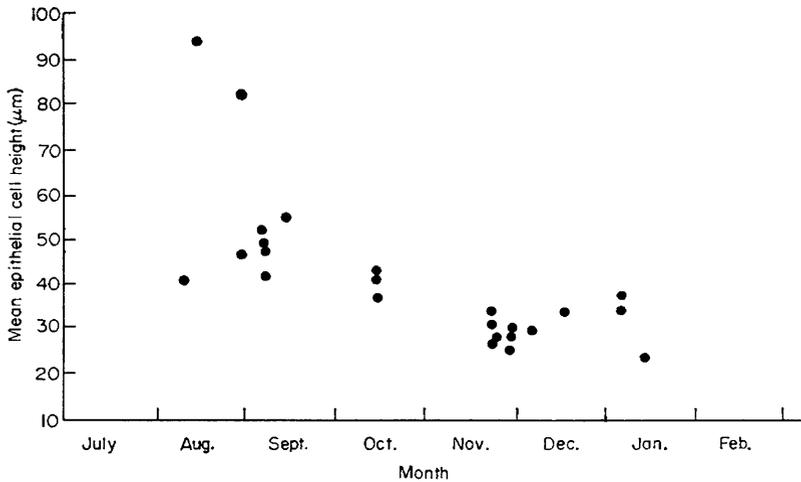
The cells of the extra-embryonic endoderm had increased in size. Their cytoplasm contained numerous mitochondria and dilated rough and smooth endoplasmic reticulum.

The inner cell mass at this stage showed distinctive ectodermal, mesodermal and endodermal layers. A few microvilli were present on the extreme outer and inner surfaces of the cell mass. A small number of caveolae could be seen on the outer surface of the ectodermal cells. All layers were characterized by a loose organization of the cells with numerous intercellular spaces. The cytoplasm of the cells was largely undifferentiated.

*Histology of the ovaries*

The CL of all animals examined appeared to be in an active secretory condition. The cytoplasm of the luteal cells was hypertrophied, and the majority contained aggregations of small eosinophilic granules. The nuclei were spherical and eccentric with prominent nucleoli.

Follicles at all stages of development could be found in the ovarian stroma, and mitotic figures in the membrana granulosa of some follicles indicated that these structures were in an active state of development. Call-Exner bodies were found in the granulosa layer of some follicles. Atretic follicles were present in a majority of ovaries examined. Atresia affected secondary, tertiary and mature follicles alike, but this did not prevent some follicles developing to a very advanced stage.

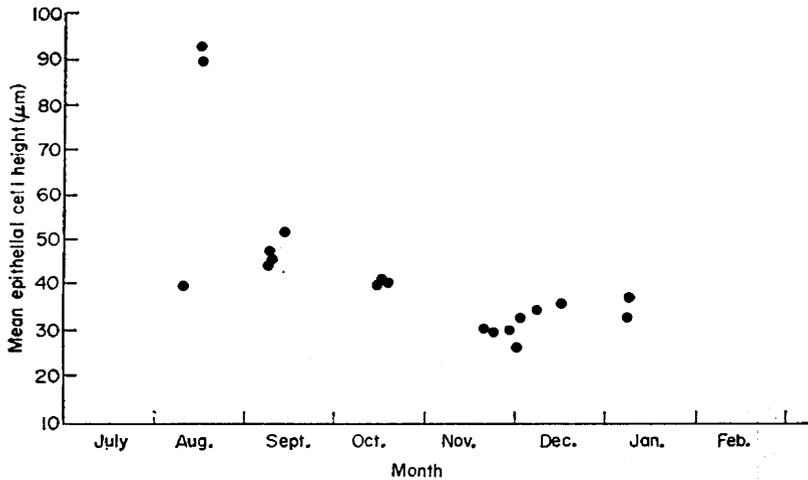


TEXT-FIG. 1. Changes in the mean height of the luminal epithelium of the roe-deer uterus during delayed implantation.

*Histology of the uterine endometrium*

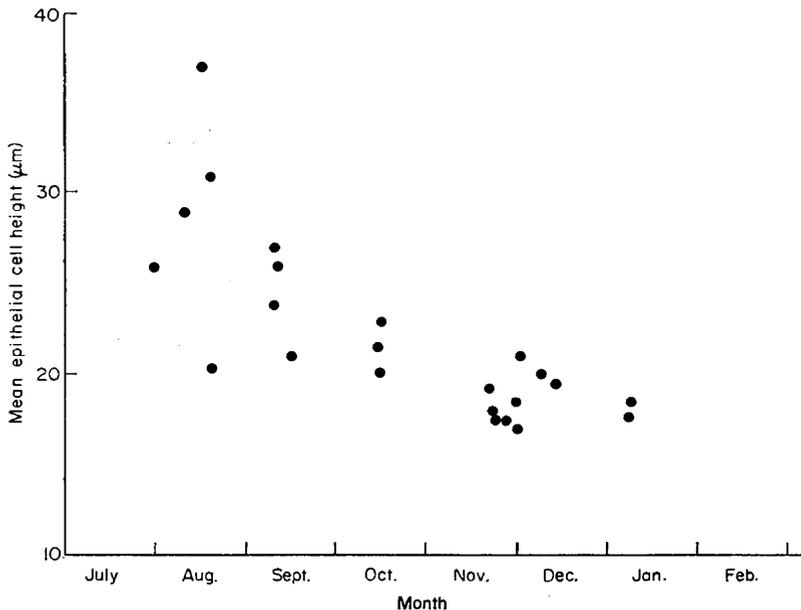
The roe-deer endometrium during delayed implantation was composed of a highly glandular mucosa and about four aglandular caruncular ridges. In transverse sections through the uterine horns, the correspondence of contours in the luminal surface suggested that the uterine lumen was occluded throughout delayed implantation. The stromal connective tissue was particularly dense in the caruncular ridges and immediately beneath the luminal epithelium. The stromal tissue became extremely oedematous early in August; the oedema subsided gradually during the succeeding months, only to increase again at the time of placental attachment.

Changes were observed in the mean height of the luminal, caruncular and ductal epithelia at different stages of delay and these are shown in Text-figs 1, 2 and 3. Shortly after oestrus, these epithelia were columnar in appearance with regions of pseudostratification in both luminal and caruncular epithelia. A period of intense mitotic activity during early August produced a marked increase in



TEXT-FIG. 2. Changes in the mean height of the caruncular epithelium of the roe-deer uterus during delayed implantation.

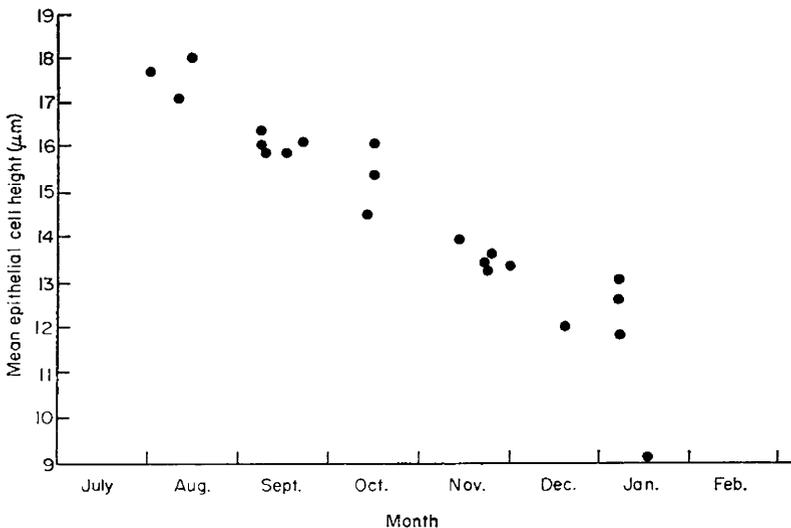
the height of the epithelial cells, together with an increased degree of pseudostratification and an intense folding or corrugation of the epithelia. Thereafter, the height of the epithelial cells declined and the degree of folding and pseudostratification became less marked. Shortly after the termination of delay the epithelium lining the ducts was columnar while the luminal and caruncular epithelia were still pseudostratified.



TEXT-FIG. 3. Changes in the mean height of the duct epithelium of the roe-deer uterus during delayed implantation.

No significant difference was found between the mean heights of the epithelia along the length of a single uterine horn or between the epithelia of contra-lateral horns.

The endometrial glands were composed of coiled fundi lying close to the myometrium which communicated with the uterine lumen through coiled ducts. The ducts appeared patent at all stages of the delay phase and the lumina often contained mucoid droplets or occasionally cellular material. In mid-August, a sudden increase in mitotic activity occurred in the glandular epithelium but thereafter, only occasional mitoses were observed in the glands. Estimations of the mean epithelial cell height in the glandular fundi (Text-fig. 4) revealed a small but significant ( $P < 0.05$ ) fall during the course of delay followed by a very rapid decline early in January at the time of rapid embryonic



TEXT-FIG. 4. Changes in the mean height of the glandular epithelium of the roe-deer uterus during delayed implantation.

growth. During delayed implantation, the cells of the glandular fundi were columnar with round or oval basal nuclei. After the process of embryonic elongation had been completed, however, the entire area of supranuclear cytoplasm had disappeared and the cells were cuboidal with large darkly staining nuclei. This dramatic decline in cell height was accompanied by a reduction in the degree of glandular coiling.

The endometrium appeared to be well supplied with capillaries throughout the period of delay. Larger blood vessels were found lying close to the myometrium and at the base of the caruncular ridges. The dilatation of the uterine capillaries after the end of the delay period appeared to be indicative of an increased blood flow through the uterus at this time.

#### *Ultrastructure of the endometrium*

Details of the animals used in this study are given in Table 2.

*Changes in the glandular epithelium.* In mid-October, the gland cells contained

**Table 2.** Details of experimental animals used in the ultrastructural study of the roe-deer uterus

Animal no.	Date shot	Age (years)	Parity	Position and no. of CL		No. and location of embryos	Embryo size
				Left ovary	Right ovary		
5	20 Oct. 1970	4	Parous	—	2	Right uterine horn; two blastocysts	0.9 × 0.7 mm diameter 1.1 × 0.9 mm diameter
6	19 Oct. 1970	7	Parous	—	3	Right uterine horn; two blastocysts Left uterine horn; one blastocyst	1.6 × 1.7 mm diameter 1.1 × 1.3 mm diameter 1.1 × 1.3 mm diameter
7	6 Jan. 1971	3	Parous	—	2	Left uterine horn; one elongating blastocyst Right uterine horn; one elongating blastocyst	12 × 4.4 mm 9.9 × 3.2 mm
8	12 Jan. 1971	2	—	1	1	Right uterine horn; one elongating conceptus Left uterine horn; one elongating conceptus	7.5 mm C-R length 7.5 mm C-R length
9	22 Jan. 1971	3	Parous	1	—	Right uterine horn; one implanted conceptus	33 mm C-R length

C-R = crown to rump.

several mitochondria, a prominent Golgi apparatus and a few clear vesicles in the supranuclear region.

By mid-November, there had been an extensive proliferation of the Golgi apparatus and an accumulation of clear vesicles in the apical region of the cells. These vesicles were particularly numerous in the deeper portions of the glands (Pl. 4, Fig. 4). There had also been an increase in the number of apical mitochondria and an accumulation of lipid droplets below the irregular nucleus. The presence of basal lipid deposits was confirmed by staining thick sections with Sudan III.

In January, at the time of rapid embryonic growth, the apical part of each gland cell was packed with clear vesicles. The Golgi apparatus at this time was less extensive, the nucleus more irregular in shape, and the basal lipid had almost disappeared. A slight increase in the diameter of the basal mitochondria was also observed at this stage.

By the time elongation had reached an advanced stage (Animal 8), the cells of the glandular epithelium were reduced in height and appeared to be declining in activity. The number of supranuclear vesicles was sharply reduced and both Golgi apparatus and endoplasmic reticulum were less prominent (Pl. 5, Fig. 5). Basal lipid had disappeared and the few remaining mitochondria were swollen in appearance.

Glandular inactivity was even more evident just before implantation (Animal 9). At this time, the glandular cells were much reduced in height and no Golgi apparatus, supranuclear vesicles or smooth endoplasmic reticulum were present. The major part of the cell was now occupied by the regularly shaped nucleus. Basal lipid deposits were absent and the mitochondria appeared swollen. Degenerative changes in certain light-coloured cells in the glandular epithelium were thought to be indicative of a holocrine secretory process.

*Changes in the luminal epithelium.* During October, the epithelial cells lining the uterine lumen bore numerous microvilli on their apical surface. There were large numbers of mitochondria above and below the centrally placed nucleus and considerable basal lipid deposits. Golgi apparatus and smooth endoplasmic reticulum were both present to a moderate degree, but no supranuclear vesicles were observed.

By mid-November, lipid deposits were present throughout much of the cell but in other respects the structure of the luminal epithelial cells was similar to that observed in October.

Little change was observed in the luminal epithelial cells during the rapid elongation of the embryo, with the exception of the apical mitochondria which appeared somewhat swollen and the lipid deposits which had slightly decreased in number.

By the time rapid elongation had been completed, the microvilli had become shorter, the apical mitochondria were fewer in number, and the lipid deposits had almost disappeared.

Immediately before implantation (Animal 8), few microvilli were present on the luminal surface of the epithelial cells. Apical and basal mitochondria were reduced in number, and many of those remaining appeared swollen. The lipid deposits had completely disappeared.

At the time of implantation, little change had occurred in the luminal epithelium of the intercaruncular areas, though a few basal lipid deposits could now be detected. In the caruncular areas, however, many crypts had formed, into which the fetal villi extended. The caruncular epithelium was characterized by the presence of large quantities of lipid and much rough endoplasmic reticulum.

## DISCUSSION

Our studies of the roe-deer uterus suggest that the eventual elongation of the blastocyst is the result of the release of a secretion from the endometrial glands. Secretory vesicles derived from the Golgi apparatus gradually accumulated beneath the apical membrane of the endometrial gland cells during delayed implantation; the sudden disappearance of these apical vesicles from the uterine glands occurred at the time of rapid elongation of the embryo in January. Once the process of elongation had been completed and placental attachment was imminent, the glandular epithelial cells showed a sudden cessation of activity manifested by a rapid reduction in cell height and in the number of cellular organelles.

As a consequence of the resumption of embryonic growth, several very marked changes were observed in the cells of the trophoblast. In particular, a marked increase in the number and size of the mitochondria and ribosomes and a reduction in the amount of lipid were presumably related to the increased metabolic and mitotic activity accompanying elongation.

In common with the blastocysts of many other animals during delayed implantation, the trophoblast of the roe deer possessed numerous lipid droplets, microvilli and periodic invaginations or caveolae on the outer surface (Enders & Schlafke, 1965). The extensive meshwork of fine fibrillae also resembled the fibrillar material in the armadillo blastocyst (Enders, 1962). The fibrillae probably serve to maintain the integrity and structural rigidity of the trophoblast during delayed implantation. The granular inclusions were similar to the membrane-bound inclusions described by Schlafke & Enders (1963) in the rat trophoblast on Day 5 of normal pregnancy and Day 7 of delayed implantation. In both the roe deer and the rat, these inclusions disappear during the later stages of delayed implantation. In the roe deer, indentations of the membrane surrounding some of the granular inclusions were composed of the same electron-dense material as the caveolae and microvesicles of the outer membrane; the inclusions may therefore contain material taken up from the uterine lumen. A reduction in the size and number of microvilli on the luminal epithelium of the uterus and on the trophoblast was seen in the roe deer during the phase of rapid embryonic growth. This has also been observed in the rat (Schlafke & Enders, 1963; Potts, 1969) and the mouse (Reinius, 1967; Potts, 1968, 1969) at the time of implantation and probably facilitates the close apposition and adhesion of the embryo to the uterine epithelium.

No prominent changes were observed in the ovaries at the time of rapid elongation that might have accounted for the changes in endometrial gland activity. This is surprising because studies in progress (R. J. Aitken and T.

Brinck-Johnsen, unpublished observations) have shown a marked increase in blood oestrogen levels during elongation.

Until the end of December, the CL always appear to be active, regardless of whether or not blastocysts are recovered from the uterus. Later, the presence of a functional CL apparently depends on the existence of a viable embryo in the uterus, since we have recently observed degenerating CL in the ovaries of non-pregnant deer shot in January or February. This poses the question of when the ovary first 'becomes aware' of the presence of an embryo in the uterus. It seems probable that the blastocyst is too small to make its presence felt; in sheep or pigs, the maternal 'recognition' of pregnancy only occurs after the blastocyst has elongated (Short, 1969).

Studies in which two roe does were kept in a large enclosure with a vasectomized buck (F. Guinness, T. J. Fletcher and R. V. Short, unpublished observations) show that the doe is monoestrous. Following ovulation, which is spontaneous, the animal enters an obligatory phase of pseudopregnancy (F. Guinness, personal communication), and it seems likely that the CL remains functional for about 5 months, regardless of whether or not the animal is pregnant. If an embryo is present, a change in the uterine environment allows it to resume a normal rate of growth in January, so that it becomes capable of prolonging the functional life of the CL. In the non-pregnant animal, however, the CL is deprived of this embryonic stimulus and regresses.

We have yet to discover the nature of the environmental cue that terminates the delay period, how this brings about the local changes in the uterine environment described in this paper, and how these changes in turn allow the embryo to resume a normal rate of growth.

#### ACKNOWLEDGMENTS

Our sincere thanks are due to the Forestry Commission and in particular to Mr R. Whitta, Mr D. Green, Mr D. Rands, Mr F. Johnson, Mr T. Banham and Mr C. Halls for shooting the deer, and for their help and enthusiasm.

The assistance of Mr W. Mouel and Mr K. Thurley in the preparation of material for electron microscopy is gratefully acknowledged.

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