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Full Paper

Epitheliophagy: Intrauterine cell nourishment in the viviparous alpine salamander, *Salamandra atra* (Laur.)

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Summary. The intrauterine nourishment of the viviparous alpine salamander, *Salamandra atra,* has been demonstrated to consist of two phases: 1) oophagy – after hatching from the jelly membrane, the developing embryo ingests the remaining disintegrated, unfertilized egg mass. 2) Epitheliophagy – a special cranial portion of the uterus wall, the zona trophica, is stimulated by the presence of the embryo. After the yolk mass has been exhausted, the developing embryo is supplied with epithelial cells as nourishment until the end of pregnancy. The epithelial cells of the zona trophica are released into the uterus lumen by partial necrosis of the underlying connective tissue. Regeneration and detachment of the uterine epithelium occur simultaneously in different regions of the zona trophica. A special dentition enables the embryo, according to its position in the uterus, to feed directly on the zona trophica.
Key words. Salamandra atra; viviparous salamander; epitheliophagy; zona trophica.

Introduction

The female alpine salamander, *Salamandra atra,* produces about 100 eggs[1,8]. However, in general, in each uterus (the most caudal portion of the oviduct) only a single egg (according to Czermak[1] the so-called embryonic egg) is surrounded by a jelly membrane, is fertilized and develops into an embryo[8,21,25,32], in contrast to the

situation in the fire salamander, *Salamandra salamandra*, where all eggs give rise to an embryo[8,9]. The remaining embryotrophic eggs disintegrate to form a nutrient yolk mass[1,2,9,30,32]. It has been considered that intrauterine cannibalism (adelphophagy) occurs, resulting in a single survivor[9,10,21]. Following ingestion of all the egg mass the embryo, which measures 25–42 mm in length, remains in the uterus for an additional 1–2 years of an overall pregnancy period of 2–4 years[2,8,24]. During this gestation period the embryo grows approximately another 10 mm and needs a further nutritional supply; at birth the embryo is 40–50 mm in length. Wiedersheim[30] interpreted the presence of extravascular blood mixed with epithelial cells as being a source of oxygen supply as well as nourishment. This could not be confirmed by other investigators[8,10,24]. In addition, it has been suggested that a secretory product of the uterus, called uterine milk, is absorbed by the embryonic gills[8,24,26]. Finally, a special cranial region of the uterus, the zona trophica, whose epithelial cells exhibit a high mitotic activity with a moderate secretory function, is thought to be directly taken up by the embryo[2]. In view of these contradictory results we demonstrate by morphological and physiological analysis the nutritional significance of the zona trophica during the pregnancy of the alpine salamander.

Materials and methods

Salamanders were collected from the Alps in Sernftal, Glarus, Switzerland, (altitude 850–1600 m) and kept in plastic boxes (0.4 m × 0.6 m × 0.35 m) filled with soil at 18 °C in the laboratory. The animals were fed on *Deroceras* sp., crickets and earthworms ad libitum.

Electron microscopy. Females, non-pregnant or pregnant at different stages, were treated with an overdose of MS 222 (Sandoz). Dissections were done at various times during the year. The uterine tissue was fixed at 4 °C in glutaraldehyde (1.8% in 0.05 M phosphate buffer pH 7.4)[15]. Samples were postfixed with 2% OsO_4 in phosphate buffer (0.15 M, pH 7.4) at 4 °C overnight, then the tissues were washed once in the same buffer and several times in double distilled water. Following dehydration with acetone, specimens were embedded in a mixture of Spurr and Epon (1:1)[11].

Thin sections were cut with a Reichert On-U3 microtome, stained with 1% uranyl acetate followed by a lead mixture according to Sato[20] and examined with a Siemens 102 Elmiskop operating at 80 kV. Semi-thin sections were stained with a filtered solution of toluidine blue (0.2% in sodium carbonate 2.5%). For scanning electron microscopy specimens were fixed and dehydrated as noted above, then critical point dried with CO_2 as intermediate medium, sputter-coated with gold-palladium and observed with a Cambridge S-4 Stereoscan.

Hormone treatment. Hormone application to vitellogenic females was performed by i.p. injection of 5 µg progesterone (Merck) or estrogen (Sigma) every 2nd day. The hormones were dissolved in acetone and diluted (1:200) with Holtfreter's saline solution (3.5 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $NaHCO_3$ per liter). As a control, an equivalent amount of acetone diluted with Holtfreter's saline solution was applied. The uterine walls of 6 separate groups, treated as in table 1, were analyzed by scanning electron microscopy.

X-ray examinations were kindly performed by Dr med. E. Suter, Zürich. Prior to examination, animals were anesthetized in a solution of MS 222 (1:100). In order to detect displacements of the embryo in the uterus, the same animals were reanesthetized and reexamined after several hours. After several days the animals were dissected.

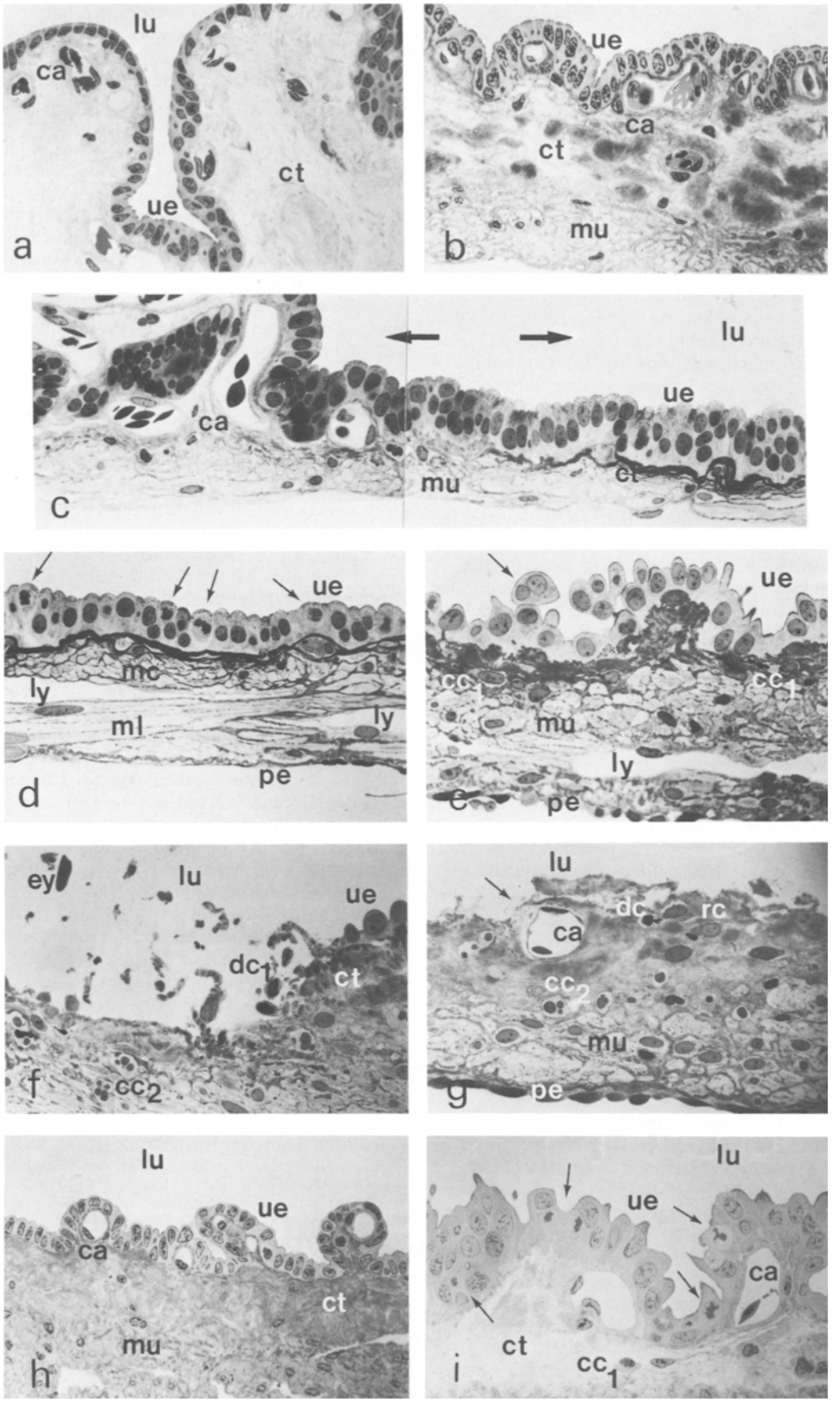
Morphometry. Embryos fixed in ethanol (70%) were measured under a dissecting microscope using a goniometer and a scaled ocular.

Pregnancy stages were determined according to Schwalbe[21]: stage I – the developing egg is still within the jelly membrane; stage II – the hatched embryo is surrounded by disintegrated eggs; stage III – all the trophic egg mass has disappeared.

Table 1. Hormone application to vitellogenic female salamanders

Group	Number of animals	Mode of application	Dissection in days after application
1	3	Control	8, 16, 24
2	6	4 doses of estrogen (5 µg each)	8, 16
3	3	1 dose of estrogen (20 µg)	4, 6
4	11	4 doses of estrogen (5 µg each) followed by 8 doses of progesterone	24, 32, 40
5	5	8 doses of progesterone (5 µg each)	16, 24, 32
6	4	16 doses of progesterone (5 µg each)	32, 40, 48

Figure 1. Light microscopic view of the uterus wall at different stages of pregnancy, showing general organization of the tissue. × 200. *a* Cross section of the middle part of the uterus of a non-pregnant female. Note the heterochromatic stain of the epithelial cell nuclei and the loosely organized collagen fibers. *b* Cranial part of the uterus of a pregnant female, at stage II according to Schwalbe. The fibers of the connective tissue layer are organized in compact bundles. *c* Transition from the middle (right) to the cranial (left) part of the uterus where the zona trophica is located. Pregnant female, at Schwalbe stage III. The connective tissue layer becomes thicker and more compact. The nuclei of the epithelial cells stain more euchromatically. *d* Newly regenerated zona trophica of a pregnant female, at Schwalbe stage III. Mitosis occurs abundantly (see arrows). Note the two muscular layers and th lymphatic spaces. *e* Zona trophica (stage III). A section showing the differentiated epithelium with the characteristic cell forms. Note the cell with two nuclei (arrow). *f* Zona trophica (stage III). The section demonstrates a cell-free area and the loss of connective tissue material. *g* Zona trophica (stage III). In the cell-free area regenerating cells are visible in the connective tissue, and also a damaged capillary (arrow). *h* Cranial portion of the right uterus of the female shown in fig. 5c. The uterus was only extended by an undeveloped embryonic egg and the embryotrophic yolk mass, no differentiated zona trophica was seen. *i* Differentiated zona trophica of the left uterus of the same female. The uterus was bearing a normal embryo. Note that the capillaries are wider and the nuclei of the epithelial cells are much larger than those in the right uterus. Ca, capillary; cc1, connective tissue cell type I; cc2, connective tissue cell type II; dc, dead connective tissue cell; dc1, dead connective tissue cell type I; ct, connective tissue; ey, erythrocyte; lu, lumen of the uterus; ly, lymphatic space; mc, layer of muscle cells, crosswise oriented; ml, layer of muscle cells, lengthwise oriented; mu, layer of muscle cells; pe, peritoneal epithelium; rc, regenerating cell; ue, uterine epithelium.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Results

1. General tissue organization of the uterus, especially in non-pregnant animals

The uterine wall consists of a monolayer of epithelial cells lining the lumen, a connective tissue layer and a layer of smooth muscle cells oriented in a more or less crosswise and lengthwise array (fig. 1). Lymphatic spaces are common between the muscles. The outermost enclosure of the uterus is formed by a flat peritoneal epithelium (fig. 1d, e, g). Capillaries lie just beneath the uterine epithelium embedded in the connective tissue (figs 1a–c, 3e). In both the muscle layer and the connective tissue, bundles of axons are abundant. At the non-pregnant, vitellogenic stage the uterine epithelium appears to be folded lengthwise with short crossfolds (fig. 2a). On the tips of the folds the epithelial cells appear rather flattened and (see inset in fig. 2a, fig. 1a) express a moderate secretory activity, particularly in the distal part of the uterus. Occasionally, ciliated cells occur (not shown). The nuclei of the uterine cells exhibit a tortuous surface and intense heterochromatic staining (fig. 3a, b). The same is true for the light cells scattered among the epithelium[5]. In the uterus of non-pregnant animals, no difference was found between cranially and more caudally located cells. A well-pronounced basement membrane (fig. 3b) separates the epithelium from the connective tissue layer which, in general loosely formed, appears in older animals to be cranially more compact.

2. Epithelial feature in early pregnancy at Schwalbe stages I–II

After ovulation, the uterus becomes enlarged by the embryotrophic egg mass and probably by water uptake (fig. 5a, b). During stage II the growing embryo expands the different uterine layers so that the folds virtually disappear (fig. 1d) and the uterus becomes translucent. Stacks of secretory granules differing in shape and electron density appear to be more abundant in the apical part of the epithelial cells[5]. Microvilli-like projections are formed on the surface of these cells which, in combination with the secretory granules, give rise to a light appearance in scanning electron micrographs. The nuclei stain less heterochromatically at stage II than at the non-pregnant stage (fig. 1a, b); their volumes, like those of the cells, are increasing. The nuclear shape at late stage II is less invaginated, resulting in a round form.

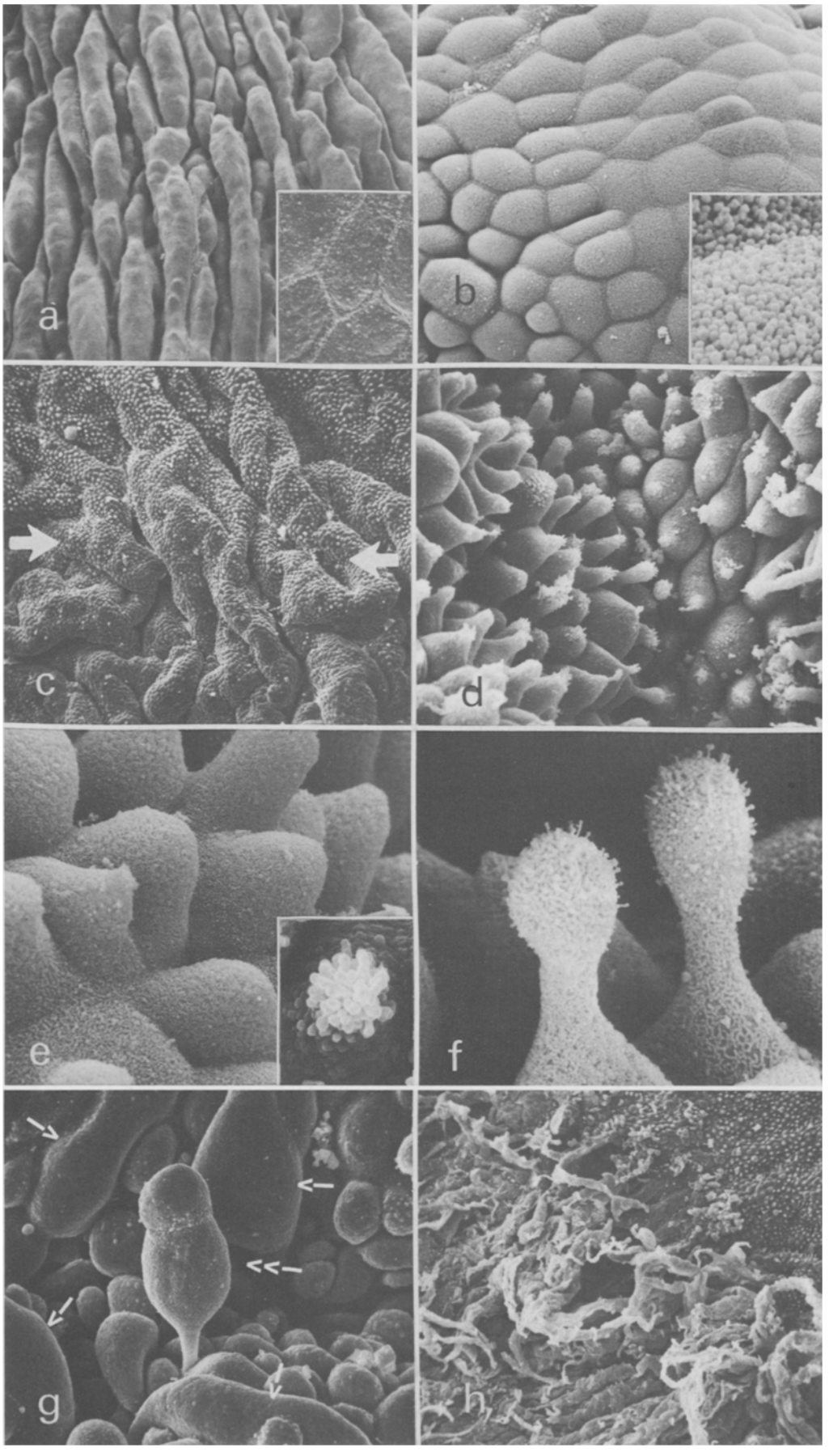
3. Uterus during Schwalbe stage III, zona trophica, and characteristics of the connective tissue

At Schwalbe stage III the uterus is enormously extended by the growing embryo which fills the whole lumen (fig. 5c, d). The yolk mass is exhausted. The cells covered by numerous granules (figs 2b, 3d) still form a flat epithelium in the caudal and middle part of the uterus wall, but cranially a region of 4–8 mm in width can be distinguished by deep boundaries between the cells (fig. 2c–f). Club-shaped and giant cells (fig. 2f, g) characterize the zona trophica, as mentioned by Fachbach[2]. Using the scanning electron microscope, the zona trophica is distinguishable by its light appearance resulting from microvilli-like projections on the top of the cell surface (fig. 2d, f). The giant cells are 3–4 times larger than the surrounding cells. They were not found in other parts of the uterus except in the zona trophica at stage III. In contrast to the long, tortuous lateral boundaries of the more caudally situated cells, they become in general shorter and less branched in the cranial region. This difference was not observed in vitellogenic or stage II animals. The nuclei in the cells of the zona trophica stain euchromatically (figs 1e, i, 3c) and are variably situated in the cells. Their swollen condition suggests a higher activity than that in other parts of the uterus. The basement membrane is not continuously detectable (figs 3f, 4b). Large vacuoles may be present in the cytoplasm of the trophic cells (fig. 3f), suggesting cell death. Mitoses were seen in uterine epithelium at all stages, but were especially abundant in the cranial portion of the uterus at stage III (fig. 1d, i).

Changes in cellular features appear to be even more striking in the connective tissue. A clear-cut boundary is established between the trophic zone and the remaining uterine epithelium (fig. 1c). Thick collagen bundles, mostly directed lengthwise, underly cells of the zona trophica, which become separated from the muscular layers by loosely organized collagen fibers (fig. 4b). Connective tissue cells are more abundant in the compact upper layer, and can be divided into two types. The so-called type I cells are characterized by ovoid nuclei that lack prominent invaginations (figs 1e, f, 4a, b). The cell bodies have an elongated form and inclusions of filament bundles are abundant. Their endoplasmic reticulum appears to be very active. In general, type I cells are tightly enclosed by collagen fibers (fig. 4a, b). Therefore it is thought that some cells of type I are fibroblasts. The type II cells can be distinguished by their folded nuclei (fig. 1f, g) and round cell bodies with various inclusions, e.g. mast cells are recognized by their typical granules. Mitoses have never been observed in type I or in type II cells. Independent of the embryo's position, areas of the uterine epithelium occur devoid of cells (figs 1f, g, 2h, 4d). In the case where the embryo is in a rump position (fig. 5d), collagen bundles protrude into the lumen (fig. 2h) and cell-free areas of the zona trophica, which occur in characteristic tracks, may amount to up to 50% of the total wall surface of the zone trophica.

When the embryo is in a head position, how ever, only about 10–20% cell-free area can be observed, resulting in a more patchy appearance.

Figure 2. Scanning electron micrographs (SEM) of the uterus epithelium, especially the zona trophica, at Schwalbe stage III. *a* Uterus wall of a vitellogenic female. × 60. Inset showing the cell surface at a higher magnification. × 3000. *b* Epithelium from the middle part of the uterus of a pregnant female, at Schwalbe stage III. × 1300. Inset shows the cell surface with granules. × 14,000. *c* Transition from the middle part of the uterus epithelium to cranially located zona trophica. Note the sharp boundary indicated by arrows. × 130. *d* Differentiated cells of the zona trophica at Schwalbe stage III. × 1300. *e* Zona trophica at Schwalbe stage III. The cells establish deep intercellular boundaries after regeneration. × 3300. Inset illustrates the microvilli-like projections on the top of the differeniated cells. × 6300. *f* Pyramid-like cells from the zona trophica at Schwalbe stage III. × 6300. *g* Giant cells (single arrow) and a cell with an unusual form (double arrow), from the zona trophica, Schwalbe stage III. × 1300. *h* Cell-free area of the zona trophica with collagen tracks protruding into the uterus lumen. The embryo was in the rump position. × 130.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4. Fate of the cells of the zona trophica and feeding mechanism during the embryonic stages

To determine the fate of the disconnected epithelial cells we examined the luminal content of the uterus and of the stomachs of embryos. Scanning electron micrographs of broken stomachs revealed lipid droplets embedded in a proteinaceous mass in stage II embryos (fig. 6a), whereas stage III embryos had clearly ingested cellular material including erythrocytes (fig.6b). Therefore blood vessels of the uterus wall must be temporally damaged (fig. 1g). The embryo ingests the egg mass by suck-snapping[7]. Most probably, the mouth floor is lowered before opening, producing a negative pressure. Lip borders, hanging down from the upper jaw, cover the mouth laterally to effect the suction, a mechanism similar to that described for the tritons[14]. The lip borders disappear at early stage III. Weight measurements of isolated stomachs indicate that these serve as a storage organ at least during stage II. Theoretically, the embryo could continuously ingest and digest the egg mass at the transition from stage II to III; but the relative weight of the stomach measures up to one quarter of that of the embryo itself. During stage III the relative stomach weight drops down to 4–7% of the total weight (table 2). No differences were found between embryos in head or rump position relative to stomach weight and content. However, only embryos in rump position can feed directly on the zona trophica, since no change of position is possible for the embryo in the uterus at least after the middle of stage II (figs 5b, 7). A special dentition is developed (figs 8, 9) in the alpine salamander during the oophagous stage. Several rows of teeth grow out, at an angle of up to 190° to the lower jaw, and form a tooth plate at an angle of up to 50° relative to the latter (table 3). Each time the embryo opens its mouth, the uterine epithelium becomes stretched between the jaws, not in a tangential plane, as suggested by the mathematical model (fig. 10). but rather as a saddle area. In this manner the epithelium may be held by the teeth of the upper jaw while the tooth plate of the lower jaw scrapes the cells, leaving collagen tracks protruding into the lumen (fig. 2h), and occasionally bundles of damaged axons. Therefore the opening angle may be much smaller than that suggested by the simplified mathematical approach (fig. 10, table 3). The form of the the uterus towards the cloacal opening is a more conical one, so that when the embryo is in the head position the caudal epithelium cannot be reached by the teeth (fig. 5). Cell free areas were never found caudally. Despite the fixed position and number of embryos, i.e. twins or even triplets, their stomachs are filled with cellular materials. Therefore mechanisms other than direct scraping must exist in order to ensure nourishment of the fetus.

5. Necrotic process in the connective tissue of the zona trophica

As already pointed out, the parts of the zona trophica devoid of cells remain smaller in the head position than in the rump position, and only a few protruding collagen bundles were found. The epithlial cells become detached from the connective tissue without being influenced by the dentition; even parts of the underlying connective tissue lose their integrity. Type I cells of the connective tissue die and leave behind free floating cellular debris with highly condensed nuclei (figs 1g, 4h), and event rarely observed in type II cells. The remaining dead cells with overlying collagen fibers are mainly sequestered into the lumen of the uterus. The first sign of the cell death process is an enormous production of vesicles which are probably lytic in nature and which appear to be related to the Golgi apparatus in the formation of large vacuoles (fig. 4c). The mitochondria disappear. An additional feature indicating cell death is the swollen endoplasmatic reticulum; its membranes are about to disrupt (fig. 4e). The nucleus may or may not be condensed at the time of cell disruption (fig. 4f). This autonomous cell death characterized by dissolved membranes and emptying of the cell contents into the connective tissue is known as necrosis[33]. However, the condensed appearance of some connective tissue cells suggests that in these cells apoptosis[33] leads to cell death (fig. 4g). The necrotic process was usually observed as a patchwise event, never affecting the whole zona trophica at one time. Regenerating parts showing already mature trophic cells co-exist with cell-free areas. The connective tissue and at least the type I cells behave in a cyclic manner, as does the epithelium of the zona trophica (fig. 11).

6. Regeneration of the trophic tissue

Since the uteri of females after the birth of the offspring show intact epithelia in the area of the zona trophica, the origin of regenerative cells had to be determined. Two different regeneration patterns were observed. A new tissue layer grows out form already established epithelium due to mitosis, or, less frequently, from single cells growing out from the connective tissue (fig. 12a), giving rise to a new epithelium. These single cells are characterized by a large nucleus surrounded by mitochondria and a small cell body (figs 1g, 3g, h); filopods and lamellipods develop (fig. 12b, c), and in the latter, filamentous structures are abundant; secondary lysosomes occur as inclusions (fig. 3g). Both the shape and the abundance of filaments suggest amoeboid movements of these cells. The endoplasmatic reticulum becomes more active if the cells are no longer covered by collagen fibers. It is not known whether the single regenerative cells are derived from the

Table 2. Stomach weight relative to total embryo weight

Stage of embryo according to Schwalbe	Relative weight of stomach	N
Early stage II	10–20%	5
Middle stage II	17–25%	4
Transition from stage II to stage III	20–25%	7
All stage III	4–7%	4

Table 3. Values of mouth opening angle obtained according to the mathematical proposal shown in figure 10

Stage	Tooth angle	Jaw quotient b/a	Tooth plate angle	Opening angle	N
Transition stage	100–190°	0.87	35–50°	88–115°	7
Early stage III	100–150°	0.89	20–40°	105–142°	12

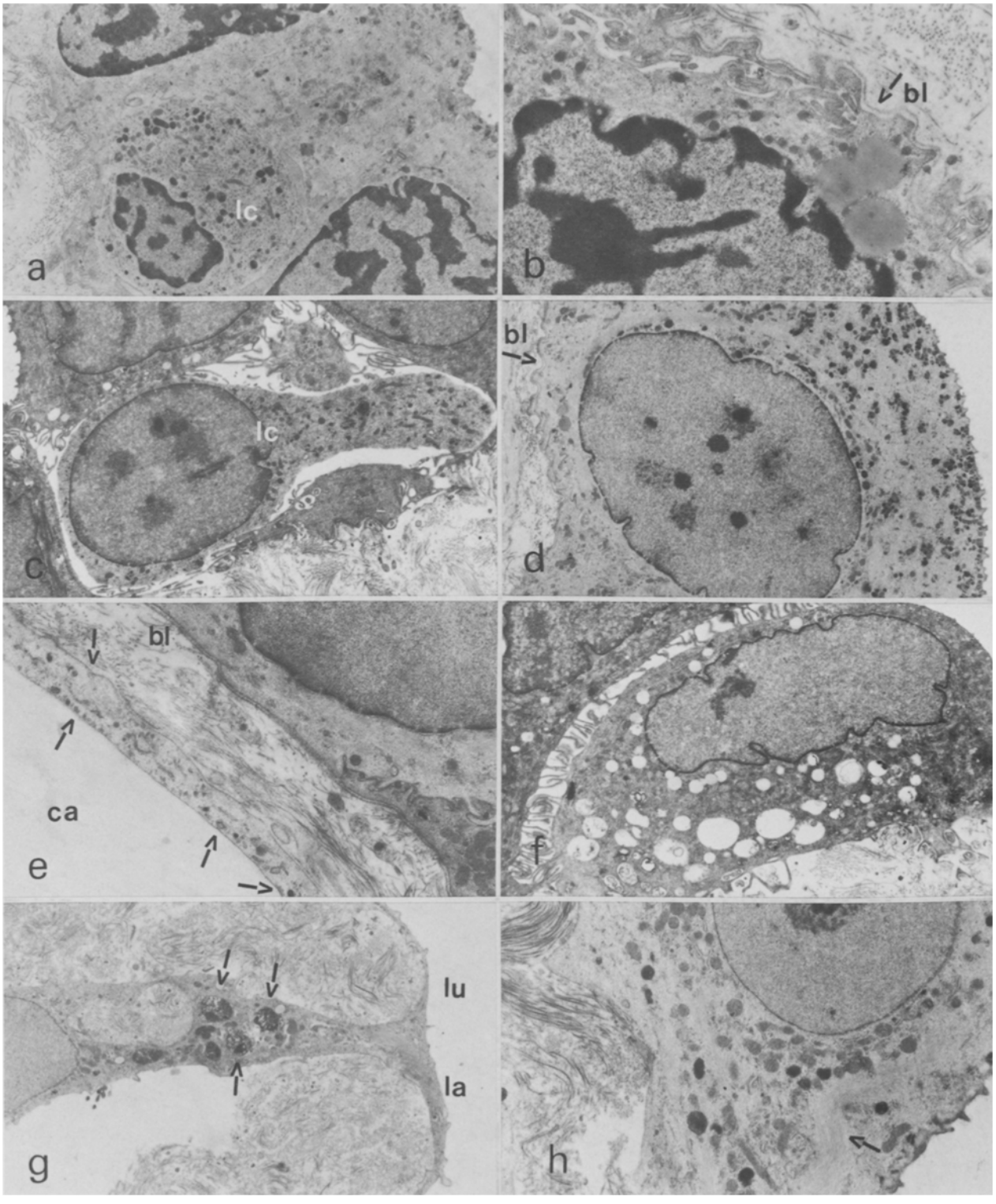


Figure 3. Transmission electron micrographs (TEM) of the uterus at different stages of pregnancy. *a* Epithelial cells from the middle part of the uterus of a vitellogenic female with a light cell in between. × 3000. *b* Epithelial cell with basal lamella (arrow) from the middle part of the uterus of a vitellogenic female. × 8000. Micrographs *c–f* illustrate the fine structure of the uterine epithelium of pregnant females at Schwalbe stage III. *c* Epithelial cells from the middle part of the uterus with a light cell in between. Note the euchromatic staining properties of the nuclei. × 3000. *d* Epithelial cell with basal lamella (arrow) from the middle part of the uterus. Note the microvilli-like projection and the granules in the apical part of the cell. × 3000. *e* Capillary with small vesicles (arrows) in the endothelium underlying the epithelium. × 8000. *f* Epithelial cell from the zona trophica with numerous vacuoles, suggesting cell death. × 3000. *g* Regeneration cell of the zona trophica with bundles of filaments in the lamellipods. Note the secondary lysosome-like inclusions (arrows). × 3000. *h* Undifferentiated epithelial cell of the zona trophica with bundles of filaments (arrow). × 5000. bl, basal lamella; ca, capillary; la, lamellipod; lc, light cell; lu, lumen of the uterus.

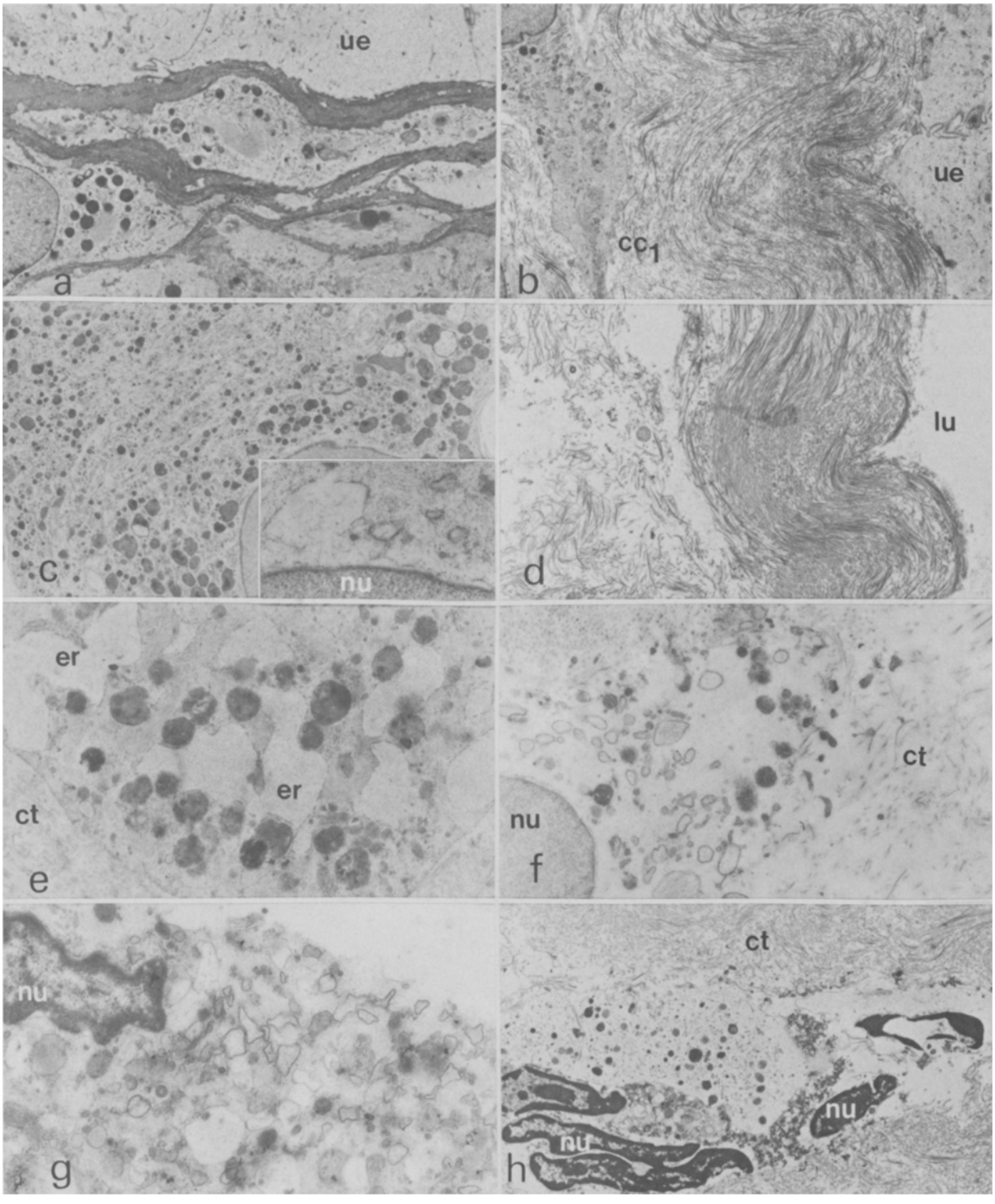


Figure 4. TEM of the connective tissue cells in both the undifferentiated (a) and differentiated (b–h) zona trophica of pregnant females at Schwalbe stage III. *a* Type I connective tissue cell surrounded by tightly packed collagen fibers, in undifferentiated zona trophica. × 3000. *b* In differentiated zona trophica type I connective tissue cells surrounded by more loosely organized collagen fibers which form typical tracks oriented to the lumen. × 3000. *c* Type I connective tissue cell filled up with various, possibly lytic, vesicles. × 3000. Inset: heavily damaged nuclear membrane of a type I connective tissue cell in differentiated zona trophica. × 15,000. *d* Cell-free area with disintegrated collagen fibers. Note the thick track oriented towards the lumen. × 3000. *e* Swollen endoplasmatic reticulum of a type I connective tissue cell in differentiated zona trophica. × 8000. *f* Type I connective tissue cell with released contents. × 8000. *g* A dead type I connective tissue cell resulting largely from the process of apoptosis. × 10,000. *h* Condensed nuclei of connective tissue cells in differentiated zona trophica. × 3000. ccl, connective tissue cell, type I; ct, connective tissue; er, endoplasmatic reticulum; lu, lumen of the uterus; nu, nucleus; ue, uterine epithelium.

epithelium submerged into the connective tissue or from a cell population of another origin. The newly regenerated epithelium remains flat (fig. 1d); no grooves occur between the cells until a higher cell density is achieved by mitosis. The epithelium is protected by its flatness against damage from the embryo's activity. The differentiation of the regenerated epithelium results in the same appearance as that described above. Type I cells are considered to migrate into the damaged connective tissue, since their number is low in areas that had shed the epithelium and increases during regeneration and differentiation (fig. 11). But mitosis has never been observed in the connective tissue. Type I connective tissue cells are surrounded by packed and unstructured collagen (fig. 4a), and later on, during the differentiation process of the epithelium, the collagen appears clearly structured and more loose, with the typical thick tracks oriented towards the lumen (fig. 4b, d). Type I connective tissue cells undergo a similar cycle parallel to the epithelial cells of the zona trophica during stage III. Intact nerve bundles are present in the newly regenerated connective tissue.

7. Induction of the zona trophica during pregnancy

The formation of the zona trophica during the transition from stage II to stage III could be triggered by a hormone. Subsequent to ovulation, corpora lutea are formed and persist during pregnancy, but their number and volume decrease. Their function is thought to maintain the nutritional secretory activity of the uterus and to control oogenesis through the influence of progesterone[9, 16, 24, 26].

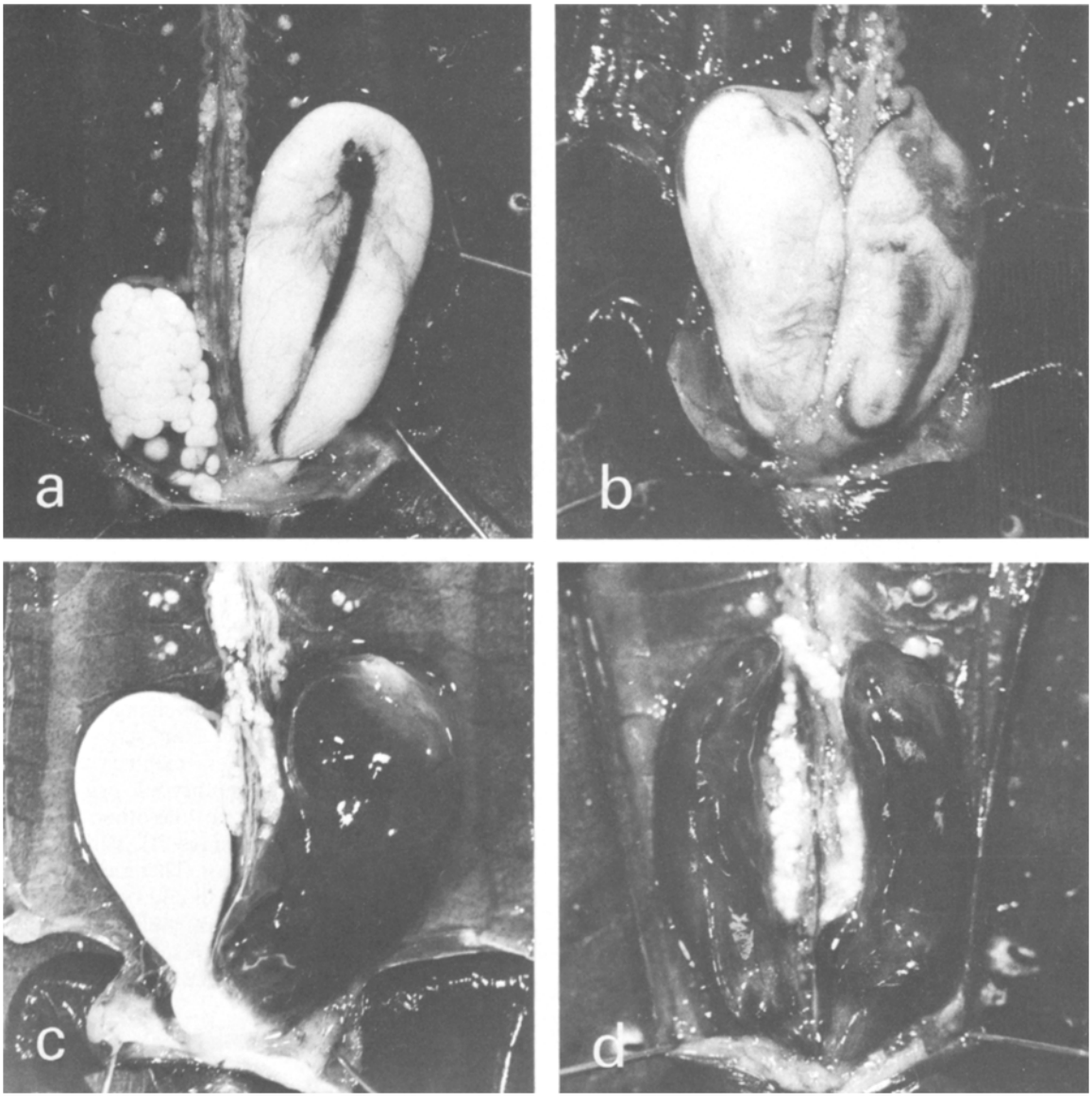
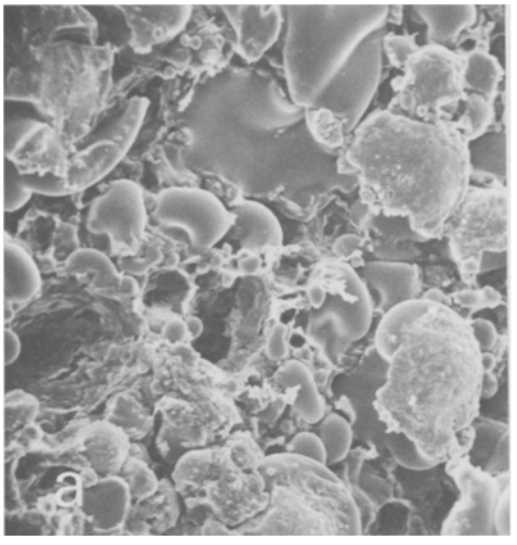


Figure 5. Pregnancy stages and positions of embryos in the uterus. *a* Left uterus: The embryo at Schwalbe stage II is in the rump position. Note that the embryonic gills are visible through the uterus. Right uterus: Only the undeveloped embryonic egg is surrounded by a jelly coat. *b* Schwalbe stage II, left uterus: Rump position of the embryo; right uterus: Embryo in head position. The embryos are already fixed in their positions. *c* The left uterus bears an embryo in head position at stage III. The right uterus contains only the disintegrated embryotrophic egg mass. *d* The two uteri each bear an embryo in rump position at stage III. Note the transparent uterus wall.



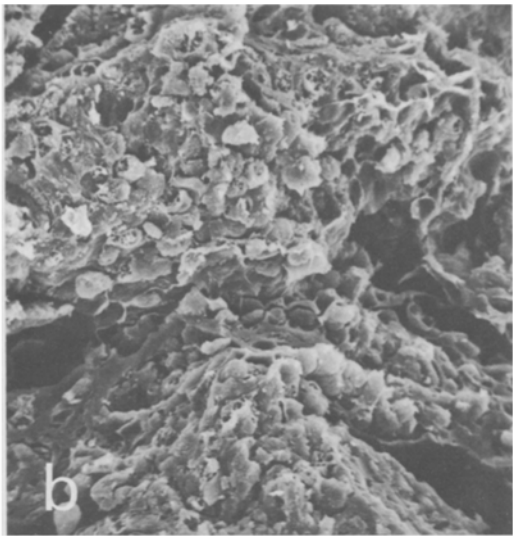


Figure 6. Stomach contents of the embryos at different stages of pregnancy. *a* Stomach contents with lipid droplets from the oophagous embryonic stage. × 250. *b* Stomach contents from the epitheliophagous stage. Note the conglomerate of ingested cells. × 630.

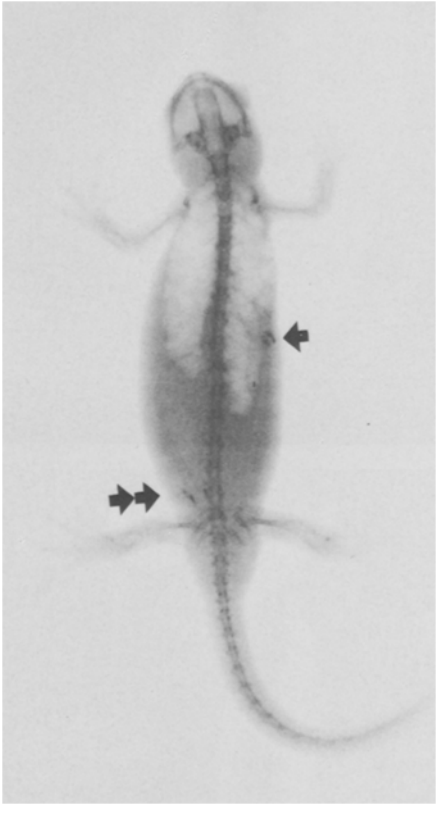


Figure 7. Reversed copy of an x-ray picture. Right uterus; embryo in rump position (single arrow). Left uterus: Embryo in head position (double arrow). Transition from stage II to stage III. The embryos are already fixed in their positions. × 1.

Figure 8. Side-view of the mouth of an embryo at transition from Schwalbe stage II to stage III. Note the projecting teeth (arrows).

No significant changes in the appearance of the uterine epithelium or the connective tissue could be demonstrated by hormonal treatment (table 1). But estrogen application resulted in a swelling of the oviduct, a typical reaction for ovulating females. The secretory cells of the oviduct were filled with granules, but no free jelly was found in the lumen. It is thought that the jelly is released from the secretory cells only through the pressure of a passing egg. Estrogen treatment followed by progesterone application, or treatment with the latter hormone alone, did not result in any obvious change as revealed by the scanning electron microscope and semi-thin sections. Two females did not form zona trophica in the uterus extended by an undeveloped embryonic egg and the embryotrophic egg mass, in spite of the other uterus carrying a developing embryo of stage III, with well-differentiated trophic tissue (fig. 1h, i). Theoretically, the hormonal influence on both uteri should be equal. The two females with the described anomalies of pregnancy (fig. 5c) were considered to be healthy and no macroscopic abnormalities of the ovaries or other genital structures could be observed.

Discussion

1. General tissue organization of the uterus during different stages of pregnancy

The rather dark appearance of the epithelial cells, including the light cells, in vitellogenic animals reflects the rest-

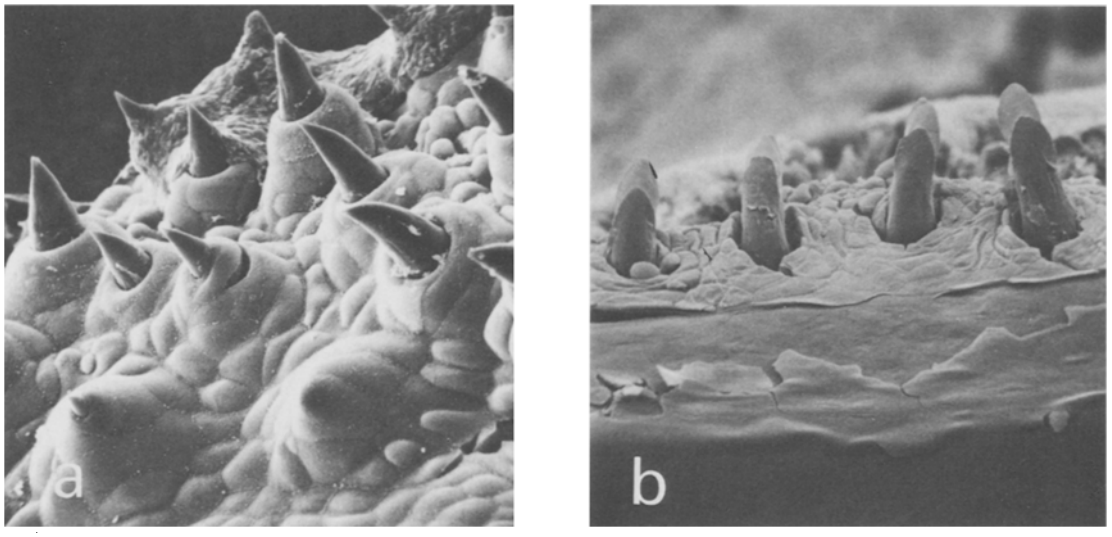


Figure 9. Dentition of the salamander embryo during development. *a* Monocuspid dentition: tooth plate of an embryo at transition from Schwalbe stage II to stage III. × 630. *b* Biscuspid dentition of a metamorphosed juvenile. × 250.

ing stage of the uterus. No special cell activities were encountered in either the epithelium or the connective tissue; these results, and the general features of the uterus wall are in agreement with the observations reported by Greven[5]. In contrast to the findings of Harms[9], the lymphatic spaces did not disappear during gestation and no glandular complexes were recorded. The secretion of the epithelial cells, which consists of PAS-positive substances[5], was observed to increase during pregnancy. The function of secretion is thought to be responsible for the maintenance and regulation of the intrauterine milieu, e.g. correction of the surface tension during pregnancy, rather than to serve as a nutrient supply for the embryo, as the amount of secretion is obviously insufficient for the latter purpose. Probably an anti-coagulant or some substances with a similar effect are present since coagulated blood has never been detected in the uterus. The grade of vascularization seems not to alter during gestation, but the lumen of the capillaries is enlarged in pregnant animals and differs between the two uteri, only one of which bears an embryo (fig. 1h, i). The extended lateral processes of the epithelial cells no doubt represent a surface enlargement and suggests a transport function for the uterine epithelium. A similar function related to the regulation of electrolytes and water transport has been applied to this epithelium in the fire salamander[6]. The changes of shape, volume and stain properties of the epithelial cells, including the light and connective tissue cells (fig. 3a, c), indicate an altered metabolic activity of the uterus during late pregnancy. These changes coincide with the formation of the zona trophica and roughly with the decline in volume and numbers of the corpora lutea[24,26].

2. Induction of the zona trophica

The presumptive zona trophica region of the uterine wall in the vitellogenic and early pregnancy stage animals most probably exhibits more characteristics and properties than just the observed thicker collagen layer, in order to differentiate into a functional trophic system. It seems that this specialized cranial portion of the uterus responds by its competence to a sequence of appropriate triggers. The fact that the trophic function of the epithelium was neither expressed after hormone treatment, nor in the uterus merely extended by undeveloped eggs in a female where the other uterus carried a normal embryo at stage III, strongly suggests that the development of zona trophica is not exclusively controlled by hormone(s) of the corpora lutea. Hence, we postulate that an inductive effect depends essentially on the presence of an embryo. Admittedly, whether the difference of extension between the two uteri is a critical one remains unknown.

The inductive action of the embryo may consist of: 1) secretion of substances during a specific developmental stage from the skin, the oral epithelium or other embryonic tissue; this later case is known in the gastric brooding

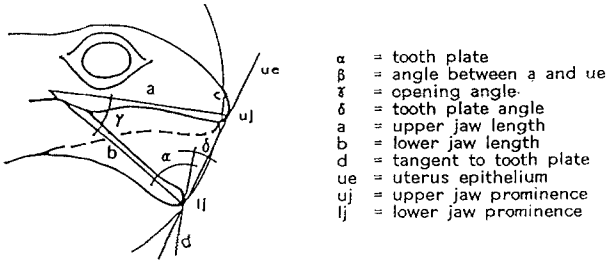


Figure 10. A mathematical proposal for estimating the required mouth opening angle for the best feeding position. Conditions for the opening angle γ are sought, under which the tooth plate is parallel to the uterus epithelium, which leads to the best feeding position. For an orientation of the tooth plate parallel to the uterus epithelium the following condition has to be true: 1. $\delta = 0$. A triangle is defined with the sides: 2. a, b, lj, uj. and the angles: 3. α, β, γ. It follows from the sine rule that: 4. $\sin\beta = (b \times \sin\alpha)/a$. Further the sum of the angles has to be 180° in a triangle: 5. $\alpha+\beta+\gamma = 180°$. Therefore we have the equation for γ: 6. $\gamma = 180° - (\alpha + \beta)$. Equation 4 substituted in equation 6 yields the condition for the opening angle that we require: 7. $\gamma = 180° - (\alpha + \sin^{-1}[b \times \sin\alpha/a])$. The tangent (d) to the tooth plate does not join exactly to the lower jaw prominence; this error was neglected.

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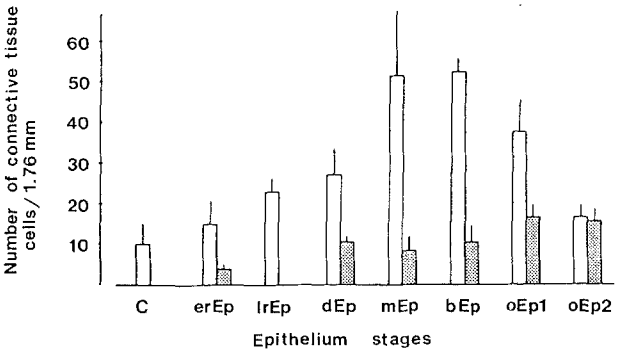

Figure 11. Quantitative estimation of healthy (open column) and dead (dotted column) connective tissue cells in the uterus wall of a pregnant female at Schwalbe stage III. The embryo was in head position. The values were determined by counting 10 semi-thin sections out of 40 on each slide, including 5 slides for each stage. The standard deviation (n = 5) is added on top of each column. C, normal uterus epithelium as control; erEp, early regenerating epithelium; lrEp, late regenerating epithelium; dEp, differentiating epithelium; mEp, mature trophic epithelium; bEp, beginning of cell detachment; oEp1 and oEp2, uterus wall free of epithelial cells.

frog, *Rheobatrachus silus*[23], whose tadpoles sequester prostaglandin as a controlling agent; 2) excretion of products acting at a critical level or rate resulting from a distinct physiological response of the embryo; 3) reduction of the remaining egg mass; 4) mechanical stimulation of the epithelium by simple movements of the embryo or by the scraping action of the dentition. It should be emphasized that in the case of the head position a direct action of the dentition on the cranial portion of the uterus is no longer possible after transition from stage II to stage III, because the embryo becomes fixed in

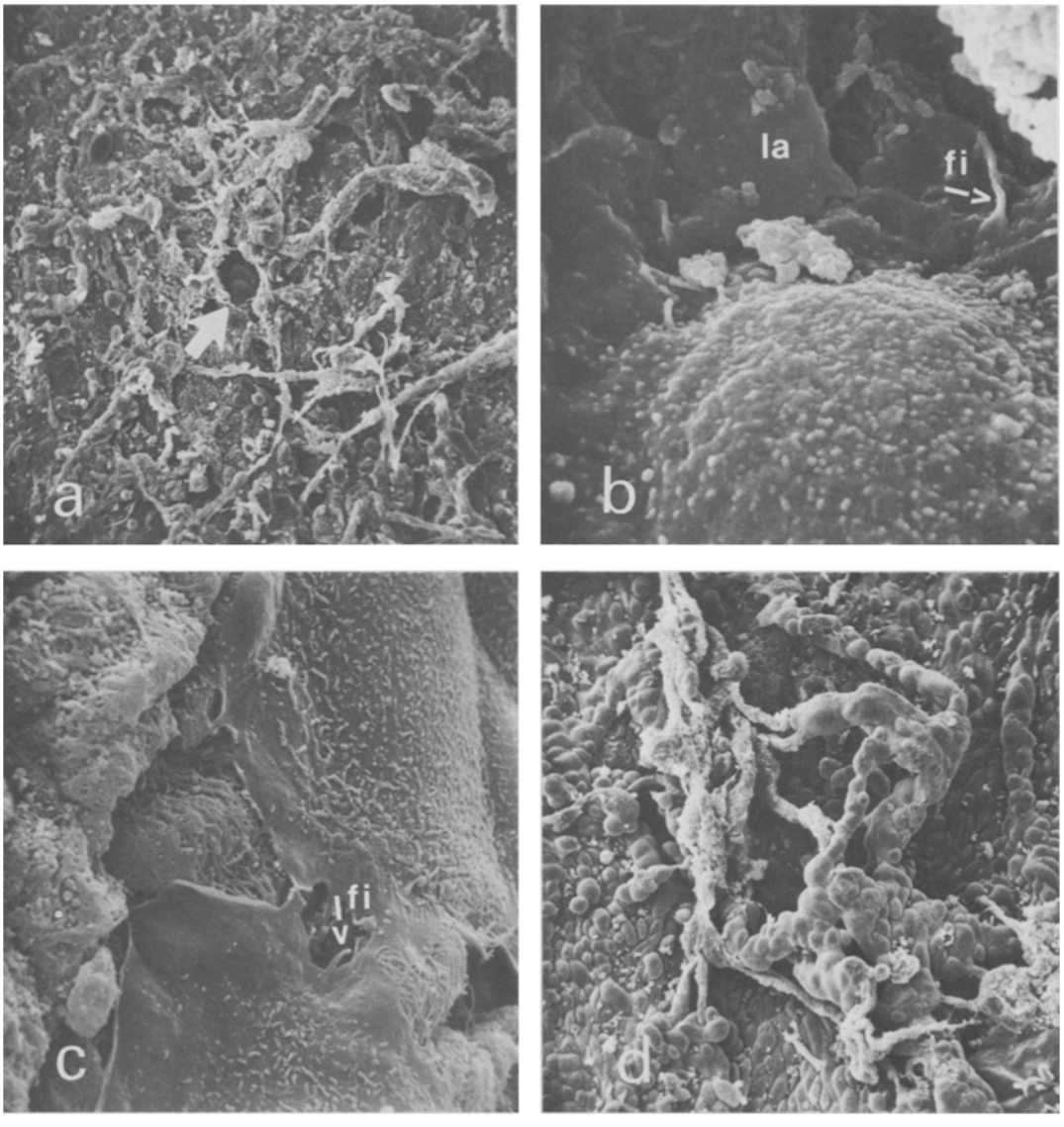

Figure 12. SEM of the zona trophica during regeneration. *a* A group of regenerating cells (arrow), probably derivated from one single cell growing out of the connective tissue. × 250. *b* Detail from 12a showing the lamellipods and the filipods of the regeneration cell. × 6300. *c* Two regeneration cells with various projections making contact in the zona trophica at Schwalbe stage III. × 3300. *d* Overgrown collagen tracks in the zona trophica protruding into the uterine lumen. The embryo was in rump position at Schwalbe stage III. This illustrates that the protruding collagen tracks are not artefacts from the preparation procedure. × 250. fi, filipods; la, lamellipods.

its position at this time or sometimes even earlier. Direct stimulation by the dentition is suggsted for the viviparous caecilians[19,27,28], but in this case it acts on a glandular epithelium[18,29]. According to Haefeli[8] and Wiedersheim[30] there is no preferential position of the alpine salamander embryo, although Schwalbe[21], Hirzel[10] and Fachbach[2] reported that the rump position is more frequent. It has to be pointed out that shortly after the transition from stage II to stage III the embryos show the first signs of metamorphosis. From developmental stage III on, the embryos can be cultured in vitro, reaching the normal juvenile stage by feeding with *Tubifex*. In contrast, embryos from the oophagous stage all died within 3 weeks of culture at 18 °C. Apparently the uterine milieu is essential for embryos of the oophagous stage[7]. We conclude that maintenance of the trophic system, at least in the alpine salamander, must be independent of the embryo's intrauterine position.

3. Connective tissue cell death and detachment of the epithelium

The detachment of the epithelium appears to be unrelated to the embryo's dentition, and may occur by enzymatic processes such as lysis of the contact complex between epithelial cells and connective tissue, since the basement membrane does not remain intact during the cycle. Cavities resulting from dead connective tissue cells affect the mechanical integrity of the connective tissue, and may result in a detachment of collagen bundles caused by an unequal tension between different collagen layers. The release of cell contents into the connective tissue may arise simply from pressure on affected cells. It is possible that the mode of cell death may vary among different cell types of the connective tissue. Damaged connective tissue cells were found with either a highly condensed nucleus or with a swollen, uncondensed nucleus and heavily affected nuclear membranes, reflecting either different types of programed cell death processes and/or particular characteristics of the death of different cell types. The mechanism of control of the patchwise necrotic event is unknown; cell contact between connective tissue cells were not studied, but may play a role in the regulation of necrosis. It is thought that the thick collagen layer protects the muscle layer. In case of strong irritation of the injured connective tissue, more collagen would be produced as a protection. The 'programed inflammation area' would attract connective tissue cells, i.e. cells of the immune system and fibroblasts, leading to 'fibrosis' which could not be healed until the stress on the epithelium is eliminated, after the birth of the embryo. A functional necrosis of the uterus epithelium is known in the viviparous anuran *Nectophrynoides occidentalis,* but it takes place only after parturition of the metamorphosed juvenile. The embryos of *Nectophrynoides occidentalis* feed on a secretion of the glandular uterine epithelium[34,35]. We suggest that evolution towards epitheliophagy was achieved through an oophagous stage, similar to that found in several races of the fire salamander[4,7,12]; further oophagy is known in the shark *Caracharias taurus*[22] (for viviparty and oophagy in fishes see Wourms[31]) and from the Luschan's salamander, *Mertensiella luschani*[17]. Most fire salamander races may not feed until birth after which the larvae leave their jelly capsules. No gain of nutrient substances by the uterine fire salamander larvae was reported by Gasche[3], even though Lostanlen[13] was able to demonstrate the uptake of ^{14}C-lysin. To our knowledge, epitheliophagy occurs only in the alpine salamander, *Salamandra atra*. In agreement with Greven[5] and Lostanlen[13] no preformed epithelial alteration of the uterus similar to the zona trophica was found during pregnancy of the fire salamander. A long life span and frew predators can be predicted for the alpine salamander from its duration of reproduction.

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Short Communications

The effect of unilateral cerebellar pedunculotomy on the vascular development of the neonatal rat cerebellum

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Summary. After cerebellar pedunculotomy the density of the blood vessel network in the cerebellar cortex was not different from that in the control animals. But the pattern of the blood vessels was different, being less organized in the operated animals.
Key words. Cerebellum; pedunculotomy; blood supply; development.

There have been a number of investigations recently that have used the operation of unilateral cerebellar pedunculotomy in the study of the postnatal development of the rat cerebellum[1–8]. One of the consistent findings is that the cerebellar hemisphere on the side of the operation is smaller than the unoperated side. A possible explanation of this finding is that the operation has compromized the normal blood supply to the cerebellar hemisphere thus affecting the normal growth of the neural tissue. Obviously, this would limit the usefulness of pedunculotomy as a tool for investigating the effect of the removal of afferent fibres on the subsequent growth of the cerebellar hemisphere and its constituents.

The blood supply of the cerebellar cortex arises from the vascular network that covers the pia meter[9]. Arterioles supplying the cortex penetrate the surface at 90° and ramify to form a dense plexus in the substance of the cerebellum. The Purkinje cells (PC) have a special network of vessels around them[9] and the molecular layer is the most poorly supplied[10]. The granular layer is supplied from both the pial network and vessels that are derived from the white matter. The white matter and deep nuclei are supplied from blood vessels which arrive via the inferior cerebellar peduncle[11].

The major postnatal growth of the cerebellum occurs in the first 30 postnatal days[12] and is closely paralleled by the developing vasculature[13]. In all cerebellar components there is a general increase in vascularity until the adult pattern is reached at about day 21. An exception to this is in the molecular layer where the density of blood vessels declines between days 5 and 10 and with the adult pattern not being reached until day 90[13]. Recently, Koppel et al.[14] have confirmed Craigie's 1924 result and have also shown that the density of the pial capillary network decreases during the first three postnatal weeks.

The present investigation was undertaken to see what effect, if any, the operation of unilateral cerebellar pedunculotomy had on subsequent cerebellar development. Neonatal inbred Wistar rats aged 1, 3, 5, 7, 10, 15 and 20 days were used. The day of birth was counted as day 0. One litter of 10 pups was used for each of the ages investigated. Under open ether anesthesia half of each litter had a left or right unilateral cerebellar pedunculotomy using a technique previously described[15]. The other half had a sham operation in which the knife was inserted into the 4th ventricle but the peduncles were not cut. After the operation the animals were allowed to recover and survive for 35 days. At the end of this time, using a modification of the technique described by Koppel et al.[14], the animals were perfused with a regime which demonstrates the blood vessels. Under deep ether anesthesia the animals were perfused transcardially with 60 ml each of the following solutions at 37 °C: 0.9% saline, 10% phosphate buffered formalin, 0.9% saline and finally with India ink in 3% aqueous gelatin. The perfusion with buffered formalin meant that the brains did not have to be stored in fixative for several weeks as described by Koppel et al.[14]. The second wash with 0.9% saline ensured that the gelatin was not fixed by the intravascular formalin before the fixation was complete. The India ink solution contained of 3 g of gelatin dissolved at 50 °C in 40 ml of water and 60 ml of India ink. This is a thicker solution than the