

Placentation in Garter Snakes. II. Transmission EM of the Chorioallantoic Placenta of *Thamnophis radix* and *T. sirtalis*

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ABSTRACT Transmission electron microscopy was used to examine the ultrastructure of the allantoic placenta of garter snakes during the last half of gestation. This placenta occupies the dorsal hemisphere of the egg and is formed through apposition of the chorioallantois to the inner lining of the uterus. The uterine epithelium consists of flattened cells with short, irregular microvilli and others that bear cilia. The lamina propria is vascularized and its capillaries lie at the base of the uterine epithelial cells. The chorionic epithelium consists of a bilayer of squamous cells that are particularly thin superficial to the allantoic capillaries. Neither the chorionic epithelium nor the uterine epithelium undergoes erosion during development. Although a thin remnant of the shell membrane intervenes between fetal and maternal tissue at mid-gestation, it undergoes fragmentation by the end of gestation. Thus, uterine and chorionic epithelial are directly apposed in some regions of the allantoic placenta, forming continuous cellular boundaries at the placental interface. During development, capillaries proliferate in both the uterine and chorioallantoic tissues. By late gestation, the interhemal diffusion distance has thinned in some areas to less than 2 μm through attenuation of the uterine and chorionic epithelia. Morphologically, the allantoic placenta is well adapted for its function in gas exchange. However, the presence of cytoplasmic vesicles, ribosomal ER, and mitochondria in the chorionic and uterine epithelial cells are consistent with the possibility of additional forms of placental exchange. *J. Morphol.* 256:171–186, 2003.

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Placentas are universal in viviparous lizards and snakes and exhibit remarkable diversity in structural and functional attributes. All live-bearing squamates have placentas that function in gas exchange and water provision, and many (if not all) exhibit placental transfer of nutrients. Major differences between species lie in the quantity and types of nutrients that females provide to their embryos by placental means (Yaron, 1985; Blackburn, 1992; Stewart, 1992; Stewart and Thompson, 2000; Thompson et al., 2000). In most viviparous reptiles, such as thamnophine snakes, fetal nutrition appears to be relatively lecithotrophic. Thus, placental provision of organic and inorganic nutrients are sup-

plemental to nutrients provided by the ovulated yolk (Stewart and Castillo, 1984; Stewart, 1989a; Stewart et al., 1990). In contrast, in certain scincid lizards nutrition is highly placentotrophic. Placental sources in these lizards account for most of the nutrition for embryonic development (Blackburn et al., 1984; Blackburn and Vitt, 1992; Flemming and Branch, 2001; Thompson et al., 2000). Placental membranes of such lizards are correspondingly specialized (Ghiara et al., 1987; Blackburn, 1993a; Stewart and Thompson, 1996; Flemming and Branch, 2001; Jerez and Ramírez-Pinilla, 2001; Blackburn and Vitt, 2002).

Although much attention in the recent literature has focused on specialized placentotrophic squamates (e.g., Blackburn, 1992, 1993a; Flemming and Branch, 2001; Swain and Jones, 2000; Blackburn and Vitt, 2002), lecithotrophic species are also of interest from functional and evolutionary standpoints. Being reproductively more generalized, these species are likely to exhibit reproductive features broadly associated with the origin and subsequent evolution of viviparity (Stewart, 1989a, 1992). One of the challenges in understanding placentation in such forms is to explain, in functional and evolutionary terms, the differences between various placental types within and between species. Placentas of a variety of types occur among squamates, including those formed from the chorion, chorioallantois, omphalopleure, and omphalallantoic membrane (Stewart and Blackburn, 1988; Stewart, 1993; Blackburn and Callard, 1997). Although the chorioallantoic placenta (allantoic placenta) is presumed to function in gas exchange, specific sites of placental transfer have not been determined for various

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nutrients, such as calcium, magnesium, sodium, and amino acids. Stated concisely, we are seeking functions for known structures, as well as structures for known functions.

From a morphological standpoint, one important factor contributing to our incomplete understanding of placentation in squamates is the absence of detailed information on cytology and ultrastructure. Since the advent of biological electron microscopy, transmission and scanning EM (TEM, SEM) have been used in numerous descriptive and experimental studies on mammalian placentas (Ramsey, 1975; Mossman, 1987). However, in squamates virtually everything known about the microscopic anatomy of placentas comes from histology of paraffin-embedded tissues. Published work that draws on TEM is confined to species in two reptilian genera (Hoffman, 1970; Ghiara et al., 1987; Angelini and Ghiara, 1991; also see Stewart, 1989b) and detailed SEM studies are also scarce (Blackburn and Vitt, 2002; Blackburn et al., 2002).

The present study uses TEM and light microscopy of resin-embedded tissues to reveal the ultrastructure of the chorioallantoic placenta in garter snakes of the genus *Thamnophis*. A companion study describes the ultrastructure of the omphalallantoic placenta, an organ with distinctly different structural and functional attributes (Blackburn and Lorenz, 2003). Garter snakes are of particular interest reproductively because their placentas have previously been studied through histology, histochemistry, and SEM (Hoffman, 1970; Blackburn et al., 2002). Furthermore, investigations of *Thamnophis* have quantified placental provision of water and inorganic ions (Stewart et al., 1990) and have revealed experimental evidence of maternal-fetal transfer of nutrients (Hoffman, 1970). Other studies have examined effects of reproductive hormones on the female reproductive tract (Mead et al., 1981; Whittier, 1992) and demonstrated physiological features that enhance gas exchange across the placental membranes (Berner and Ingermann, 1988; Ingermann et al., 1991a,b). We anticipate that studies on placental ultrastructure will contribute to the growing, integrative understanding of reproduction and placentation in *Thamnophis*, as well as in other viviparous squamates.

MATERIALS AND METHODS

Pregnant female *Thamnophis radix* and *T. sirtalis* from Wisconsin, USA, were obtained from a commercial supplier in July. Snakes were housed in aquaria with water bowls and rocks at about 24°C, with a 14:10 L:D light cycle. Pregnant reproductive tracts were harvested in mid to late gestation in late July and early August. At sacrifice, each snake was cooled for about an hour at refrigerator temperatures and given a lethal dose of Nembutal injected intraperitoneally. Uterine oviducts were removed from a mid-ventral incision and cut transversely into segments, each containing one to three developing eggs.

For fixation, whole eggs and the surrounding oviduct tissue were placed into a modified Karnovsky's solution (3% paraformal-

dehyde, 1.5% glutaraldehyde, in 0.1 M sodium cacodylate buffer) at 4°C. After a variable period (about 35 min), the uterus and attached fetal membranes were incised in the equatorial plane to ensure full penetration of fixative. Eggs were fixed for 2 h, washed in buffer with 7% sucrose, and refrigerated overnight. Fetuses were dissected free and placed into neutral buffered formalin for 2 days and stored in 70% ethanol. Tissue samples for TEM were treated with 2% osmium tetroxide in cacodylate buffer, rinsed in successive washes of buffer and of deionized water, and then stained in 2% aqueous uranyl acetate for 2 h. Tissue was dehydrated in a graded ethanol series, followed by propylene oxide, and embedded in Embed 812 resin (Electron Microscopy Sciences, Fort Washington, PA). Tissue blocks were sectioned on glass knives at 0.5 µm (thick sections) and 80 nm (thin sections), using Sorvall Porter-Blum MT-2B microtomes. Thick sections were collected on glass slides and stained with Azur II/methylene blue. Slides were photographed on T-Max 100 professional film using an Olympus BH-2 compound microscope and 35mm camera. Thin sections were cut using a diamond knife, collected on copper mesh grids and formvar-coated slot grids, and stained conventionally using lead citrate and 3% uranyl acetate (adapted from Reynolds, 1963). The sections were examined and photographed using a Zeiss EM900 transmission EM. Fetal development was assessed according to the Zehr (1962) system.

RESULTS

General Observations

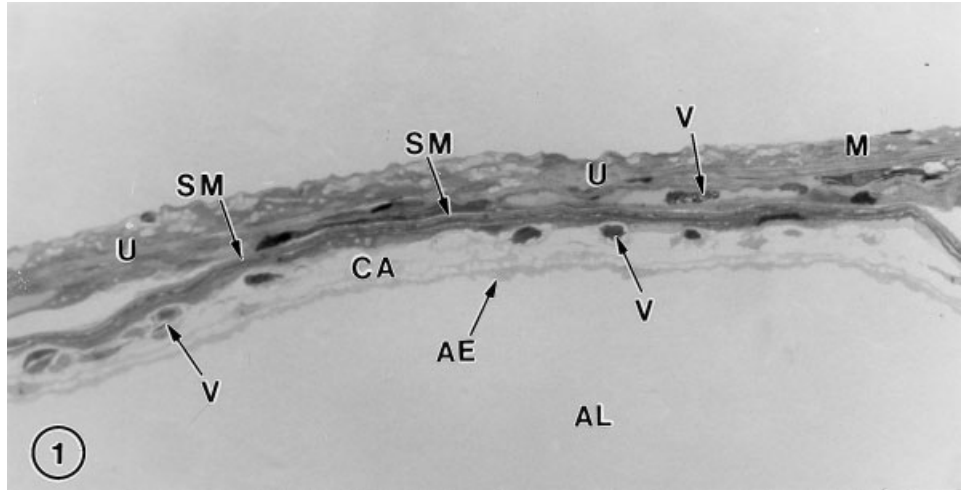
Snake embryos in this study included developmental stages 30–31, 36, and 37 in the Zehr (1962) system of classification. The oldest embryos represent the stage just prior to parturition. Accordingly, we shall refer to the stages as mid-gestation (stage 30–31) and late gestation (stages 36 and 37). In terms of placental morphology, no differences were observed between *Thamnophis radix* and *T. sirtalis*.

The allantoic placenta (=chorioallantoic placenta) is formed through apposition of the chorioallantois to the inner lining of the uterine oviduct. As revealed through dissection and microscopic examination, the allantoic placenta occupies the entire dorsal (mesometrial) hemisphere of the egg and extends past the equatorial plane into the abembryonic hemisphere. The chorioallantoic and uterine components lay in very close contact, but with care could be peeled apart with microforceps. During dissection a very thin remnant of the shell membrane was sometimes visible lying between the fetal and maternal tissues.

Mid-Gestation

Light microscopy. At Zehr stage 30 the chorioallantois and uterus form very thin vascularized membranes (Fig. 1). In thick sections stained with Azure II/methylene blue a shell membrane is visible at the placental interface. The membrane appears to be uniform, with no breaks or substructure visible; however, its thickness varies moderately between specimens. In some areas the shell membrane is separated from the uterine lining and remains adherent to the outer surface of the chorioallantois. Whether the separation is artifactual is unclear, since dissection reveals that the chorioallantois and

Fig. 1. Allantoplacenta, *Thamnophis radix* (embryonic stage 30). The shell membrane (SM) lies at the interface of the fetal and maternal tissues; it is more distinct on the specimen than in the micrograph. Blood vessels (V) can be seen in the uterus (U) and chorioallantois (CA). AE, allantoic endoderm; AL, allantoic lumen; M, uterine muscle. Azur II/methylene blue. $\times 530$.



uterine lining are not tightly apposed throughout their length.

The uterine tissue is so attenuated that histological details are difficult to discern. Capillaries and other small blood vessels are scattered throughout the tissue; many contain nucleated erythrocytes (Fig. 1). No glands are apparent. The uterine epithelium is low cuboidal to squamous. In at least some areas, thin extensions of the epithelial cells appear to extend over the capillaries, separating them from the uterine lumen, although an epithelial covering to the capillaries could not always be discerned. Nuclei of the epithelial cells are displaced to the sides of blood vessels. The uterus is bounded externally by a thin band of smooth muscle cells.

The chorioallantois stains much more lightly than does the uterine tissue. Most of its width is made up of a sparse, irregular connective tissue with isolated mesenchymal elements (Fig. 1). Small blood vessels are sparsely scattered throughout the connective tissue, but generally lie at the base of the overlying epithelial cells. Their lumens are generally open, making them readily visible. Some contain erythrocytes and leukocytes. The squamous epithelium of the chorion forms a lightly stained border of cells next to the shell membrane. The cells send thin extensions over the capillaries and their nuclei lie displaced to the sides of the vessels. However, as with the uterine tissue, whether the capillaries are invariably covered by epithelium could not be determined with light microscopy. In some areas the capillaries bulge towards the lumen, forming small ridges that protrude towards the uterine lumen. The allantois is bounded internally by a thin cellular layer, which constitutes the allantoic endoderm.

TEM. The pregnant uterus consists of three general layers that are readily distinguishable with TEM: an epithelium, lamina propria, and muscularis externa (Fig. 2). Epithelial cells lining the uterine lumen consist of a layer of broad, flattened cells (described in more detail below). A homogeneous

basal lamina separates the epithelium from the underlying layers. The lamina propria contains collagen fibers, interspersed with fibroblasts and blood vessels, but it lacks evidence of glands (Figs. 3, 4). The fibroblasts are elongated and oriented transversely, roughly in parallel with the luminal surface and with cells of the muscularis externa. Each fibroblast contains a single elongate nucleus, oriented lengthwise in the cell. The nuclei exhibit euchromatin intermixed with heterochromatin. Cytoplasm of the fibroblasts contains an abundance of rough endoplasmic reticulum (RER), with scattered mitochondria. The surrounding collagen fibers of the lamina propria are oriented roughly in parallel with the fibroblasts. Scattered between patches of collagen fibers is a granular material of uncertain identity; perhaps it represents precipitated ground substance. Deep to the epithelial basal lamina lie small blood vessels, each of which is surrounded by a monolayered endothelium. The endothelial cells exhibit irregularly shaped nuclei and a granular cytoplasm with sparse mitochondria, RER, and free ribosomes (Figs. 2, 3, 7). Small electron-lucent vesicles lie in the endothelial cell cytoplasm. The muscularis externa consists of several layers of smooth muscle cells, interspersed with patches of extracellular granular material like that present in the lamina propria. Cytoplasm of the muscle cells contains myofibrils, scattered mitochondria, and vesicular invaginations. The muscle cells are surrounded by a basal lamina of uniform thickness; in some regions, adjacent cells appear to be linked by gap junctions.

The uterine epithelial cells range in shape from low cuboidal to tall squamous (Figs. 2–6). Two populations of cells are present: electron-dense cells with cilia and lightly staining (electron-lucent) cells that exhibit sparse microvilli (Fig. 2). A single layer of cells appears to be present, although overlap of the cells often gives the impression of a cell bilayer. Nuclei of the epithelial cells contain both euchromatin and heterochromatin and occupy a central to

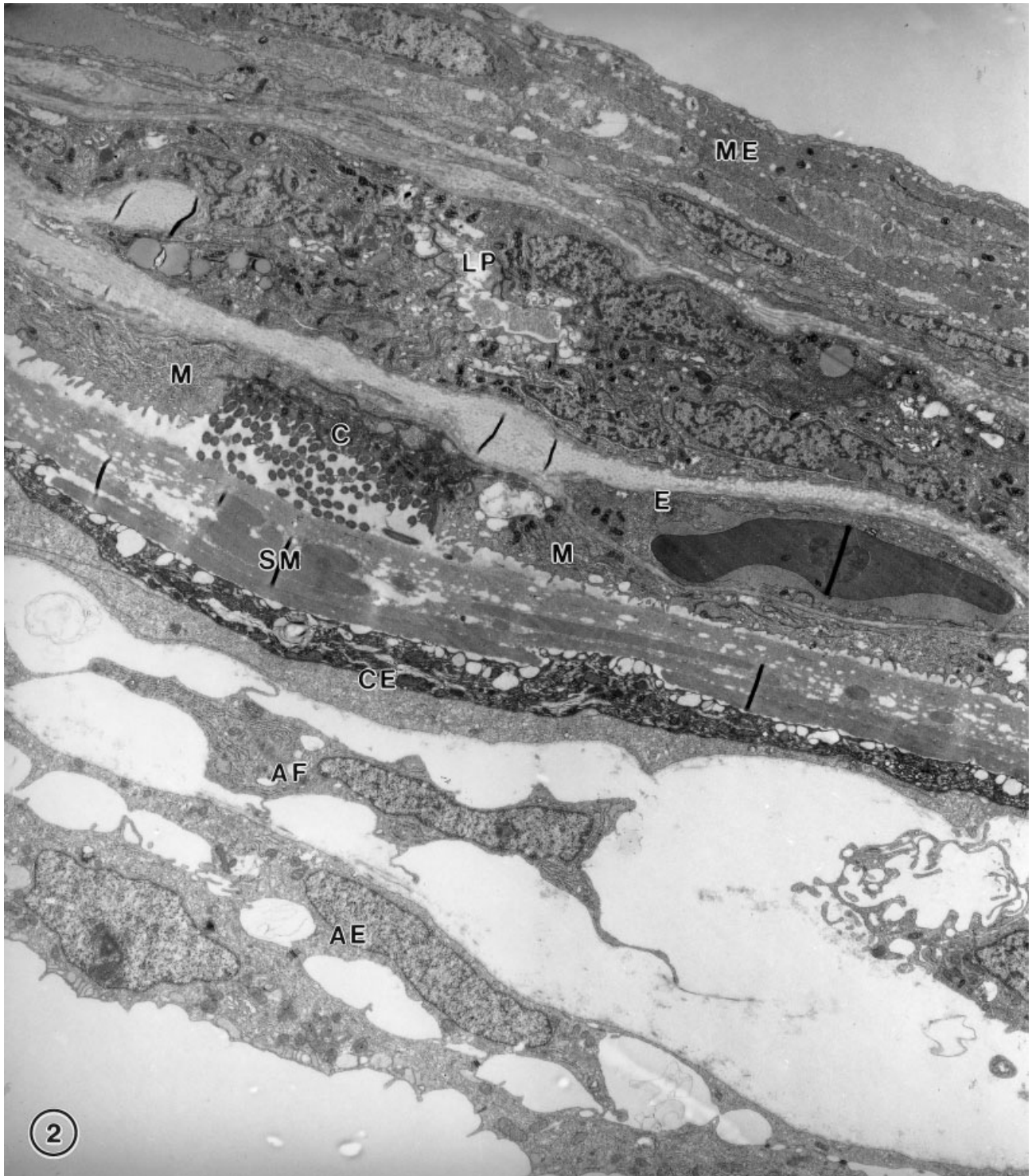


Fig. 2. Allantoplacenta, *Thamnophis radix* (embryonic stage 30). The shell membrane (SM) lies interposed between tissue of the uterus (top half of micrograph) and chorioallantois (bottom of micrograph). The uterine tissue exhibits an epithelium of ciliated cells (C) and microvilliated cells (M), a lamina propria (LP) with fibroblasts, and a muscularis externa (ME). The chorioallantois consists of a bilayered chorionic epithelium (CE), allantoic connective tissue with fibroblasts (AF), and cells of the allantoic endoderm (AE). E, endothelium of uterine capillary. $\times 7700$.

basal location, with their longest dimensions roughly parallel to the basal surfaces of the cells. These nuclei are very irregular in outline; conse-

quently, in section they sometimes appear to surround regions of cytoplasm (Fig. 3). Cytoplasm of the epithelial cells is granular, with an abundance of

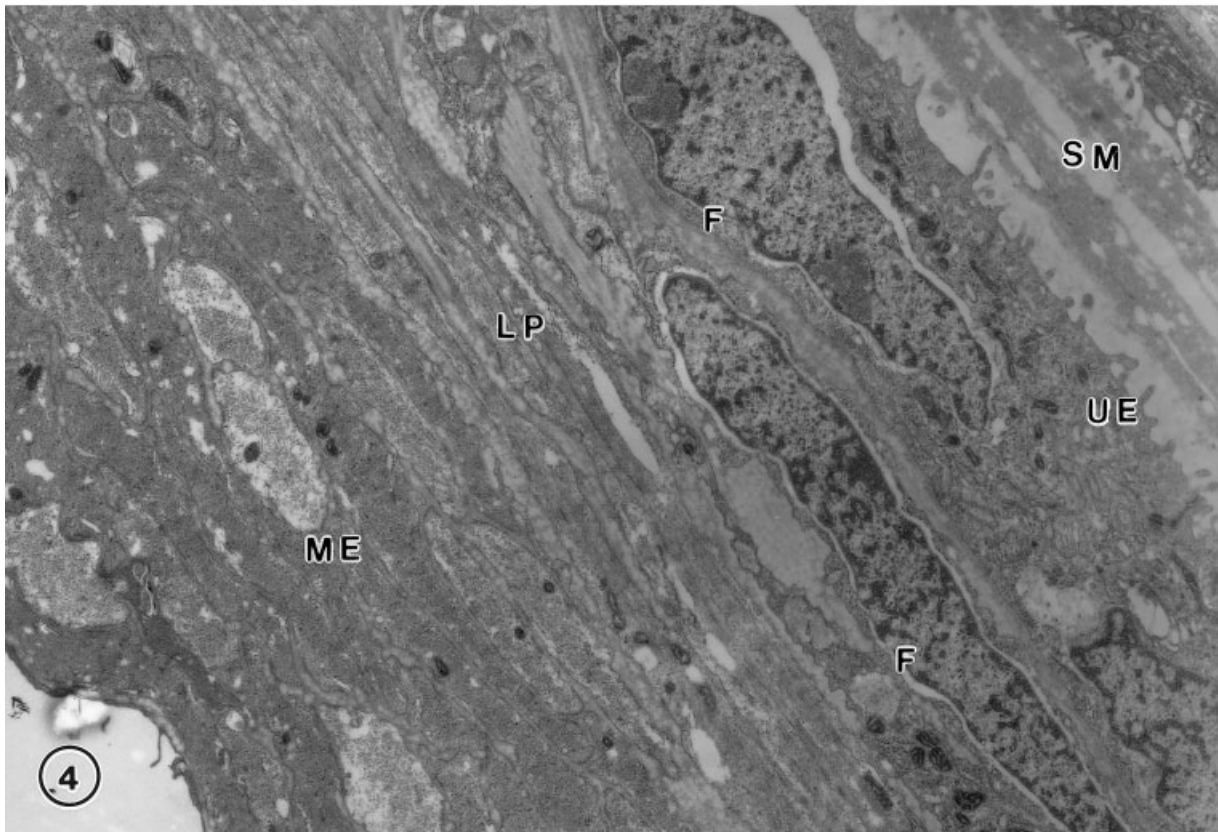
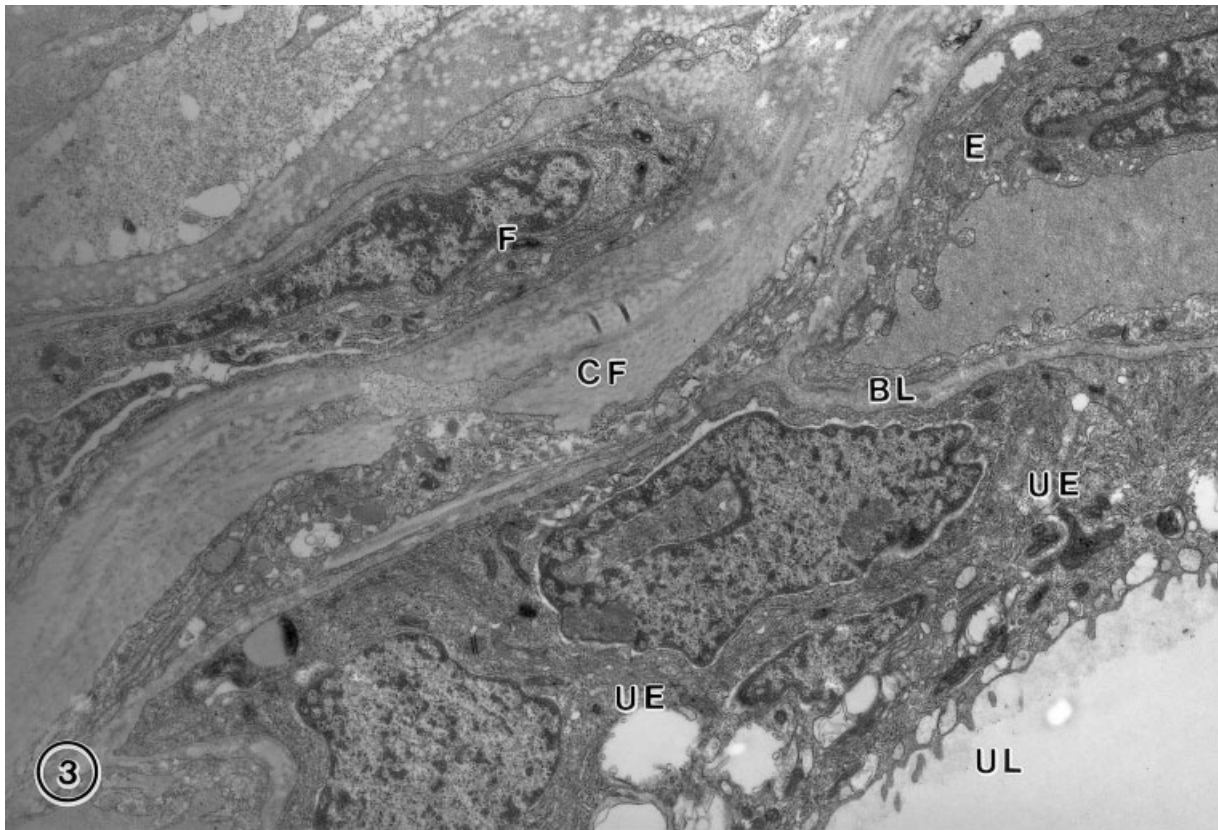


Fig. 3. Uterine component of the allantoplacenta, *Thamnophis radix* (embryonic stage 30). The uterine tissue exhibits a uterine epithelium (UE), which is separated by a basal lamina (BL) from the underlying lamina propria, the latter of which contains fibroblasts (F) and collagen fibers (CF). Note the vesicles in the apices of the uterine epithelial cells, bordering the uterine lumen (UL). E, endothelial cell. $\times 9600$.

Fig. 4. Uterine component of the allantoplacenta, *Thamnophis radix* (embryonic stage 30). UE, uterine epithelium; F, uterine fibroblast; LP, lamina propria; ME, muscularis externa; SM, shell membrane. $\times 11,100$.

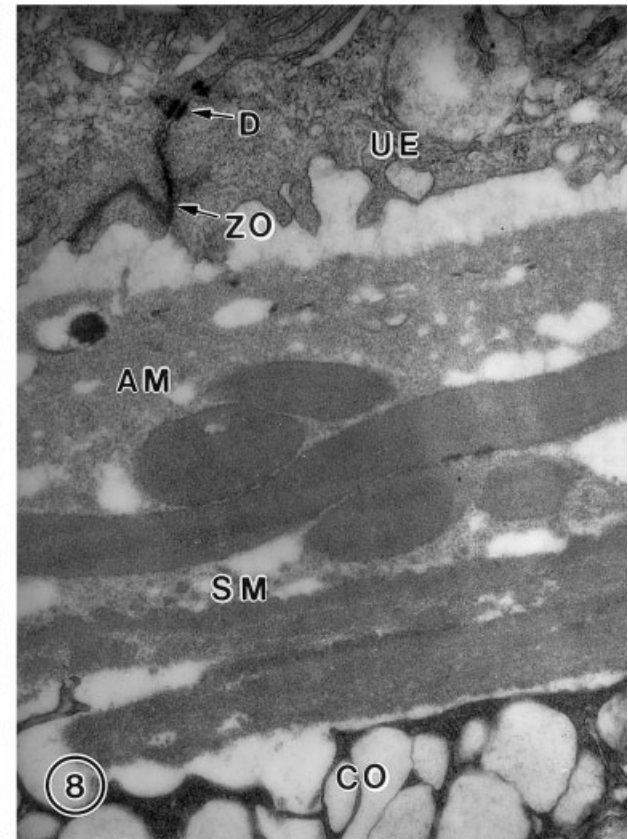
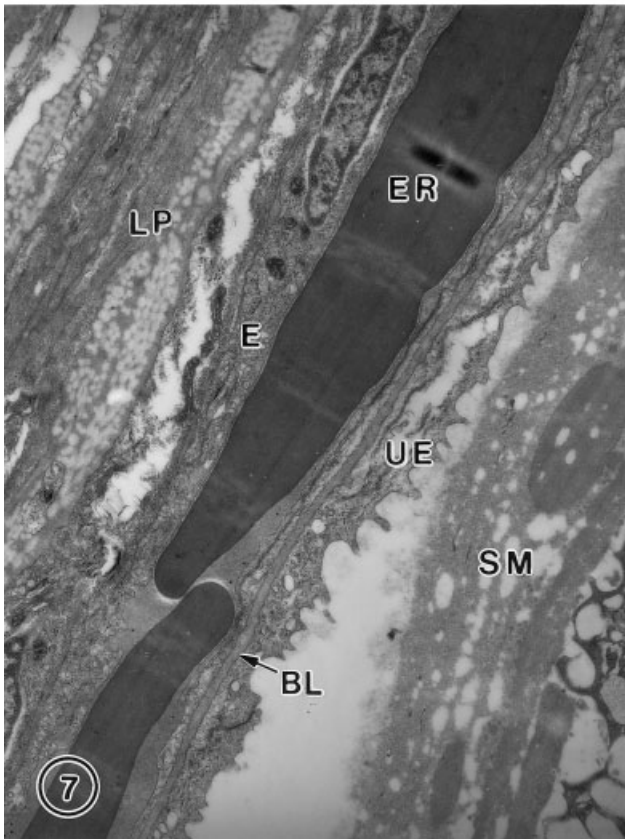
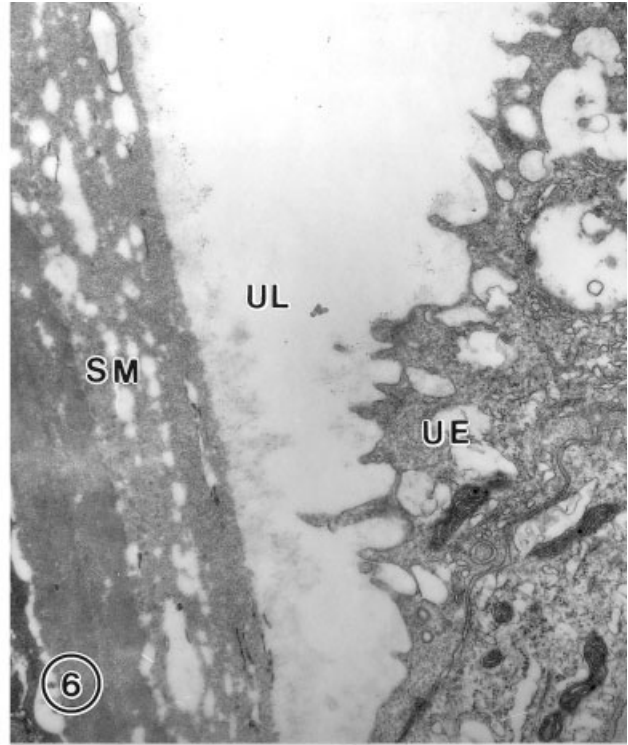
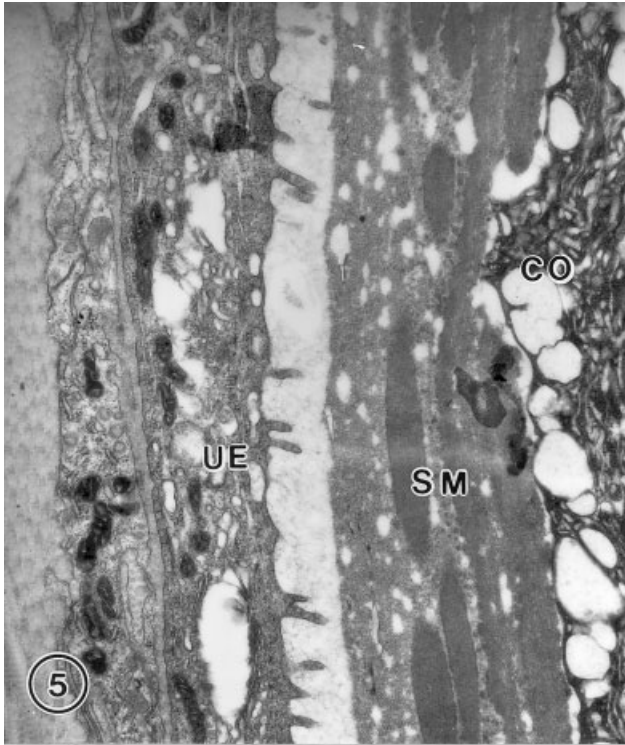


Fig. 5. Maternal–fetal interface of the allantoplacenta of *Thamnophis radix* (embryonic stage 30). The uterine epithelium (UE) is attenuated and contains an abundance of vesicles. The outer cell layer of the chorionic epithelium (CO) is vesicular. Note the heterogeneous appearance of the shell membrane (SM). $\times 17,000$.

Fig. 6. Uterine lining of the allantoplacenta, *Thamnophis radix* (embryonic stage 30). Cells of the uterine epithelium (UE) contain apical vesicles and small, irregular microvilli. SM, shell membrane; UL, uterine lumen. $\times 20,000$.

Fig. 7. Uterine lining of the allantoplacenta, *Thamnophis radix* (embryonic stage 30). Very thin extension of cells of the uterine epithelium (UE) extend over the capillaries. BL, basal lamina; E, capillary endothelium; ER, erythrocyte; LP, lamina propria; SM, shell membrane. $\times 12,000$.

Fig. 8. Allantoplacental interface, *Thamnophis radix* (embryonic stage 30). The shell membrane (SM) consists of fibers and an amorphous material (AM). Cells of the uterine epithelium (UE) are joined by zonula occludens (ZO) and desmosomes (D). CO, chorionic epithelium (outer layer). $\times 30,000$.

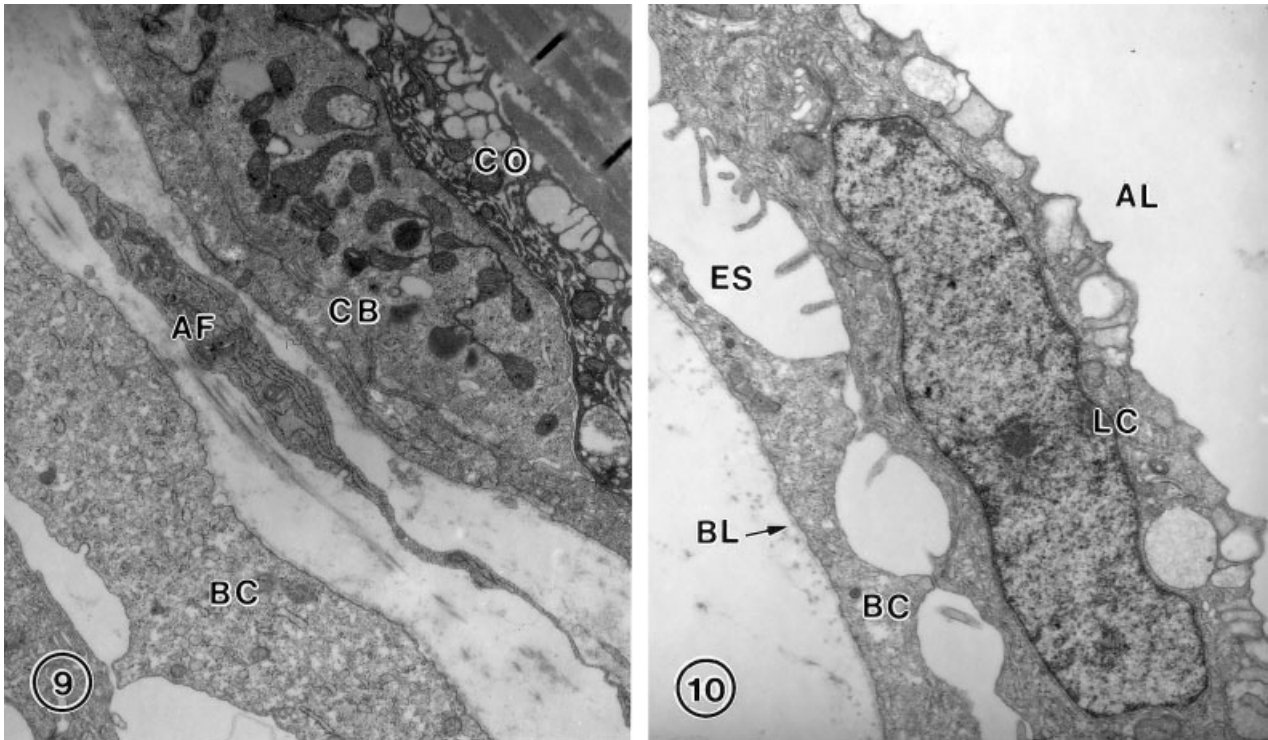


Fig. 9. Chorioallantois, *Thamnophis radix* (embryonic stage 30). The chorionic epithelium consists of an outer layer (CO) and a basal layer of cells (CB). AF, allantoic fibroblast; BC, basal cells of the allantoic endoderm. $\times 12,000$.

Fig. 10. Allantois, *Thamnophis radix* (embryonic stage 30). The allantoic endoderm contains a layer of basal cells (BC) adjoining the basal lamina (BL) and a layer of luminal cells (LC) that line the allantoic lumen (AL). ES, extracellular space. $\times 12,000$.

RER, free ribosomes, and moderate numbers of mitochondria (Figs. 3, 5, 6). The microvilli are sparse, short, and irregular. The microvilliated cells contain electron-lucent vesicles that range markedly in size. Over the capillaries, the epithelial cells consist of an attenuated layer that in some regions is as thin as $0.5 \mu\text{m}$ (Fig. 7). Adjacent epithelial cells are linked along with desmosomes and, apically, with tight junctions (Fig. 8).

A shell membrane, approximately $1.2\text{--}3 \mu\text{m}$ in thickness, forms a continuous barrier between fetal and maternal tissues at the placental interface (Figs. 2, 5). It consists of elongated, thick fibers of medium electron density (Figs. 5, 8). On the uterine side of the shell membrane and interspersed between the fibers lies an amorphous granular substance; it is somewhat more electron-lucent than the fibers.

The chorioallantois consists of three layers: an external chorionic epithelium, the vascularized allantoic connective tissue, and the allantoic endoderm, which lines the allantoic lumen (Fig. 2). The chorionic epithelium is squamous and consists of an external cell layer and a basal layer. The external cell layer, which lies apposed to the shell membrane, is electron-dense and highly vesicular (Figs. 5, 8). It contains mitochondria and membrane profiles, but most of its structure is made up of electron-lucent vesicles surrounded by thin strands

of cytoplasm. Thin extensions of the cells protrude towards the shell membrane. Although the peculiar appearance of these cells in our samples may well be artifactual, surrounding cells show no evidence of artifact beyond the extraction of vesicular contents. The basal epithelial layer consists of lightly stained, squamous cells (Figs. 2, 9). They contain a homogeneous, granular cytoplasm with free ribosomes and little RER. Some of the cells exhibit an abundance of oddly shaped mitochondria (Fig. 9). The epithelium is separated from the underlying allantoic components by a basal lamina.

The allantoic connective tissue contains scattered fibroblasts lying in a very sparse matrix of collagen fibers and blood vessels (Figs. 2, 9). The fibroblasts exhibit mitochondria and an abundance of prominent RER (Fig. 9) and their nuclei are ovoid, with both euchromatin and heterochromatin (Fig. 2). The cells appear to be in contact with one another by means of long, thin cellular extensions.

Lining the allantoic cavity, the allantoic endoderm consists of a bilayer of attenuated, broad cells (Figs. 2, 9, 10). They are separated from the allantoic connective tissue by a basal lamina. The two cell layers will be distinguished here by the terms "basal" (the layer adjacent to the basal lamina) and "luminal" (the layer immediately adjacent to the allantoic lumen). Cells of these two layers are in contact via cell extensions that are attached by

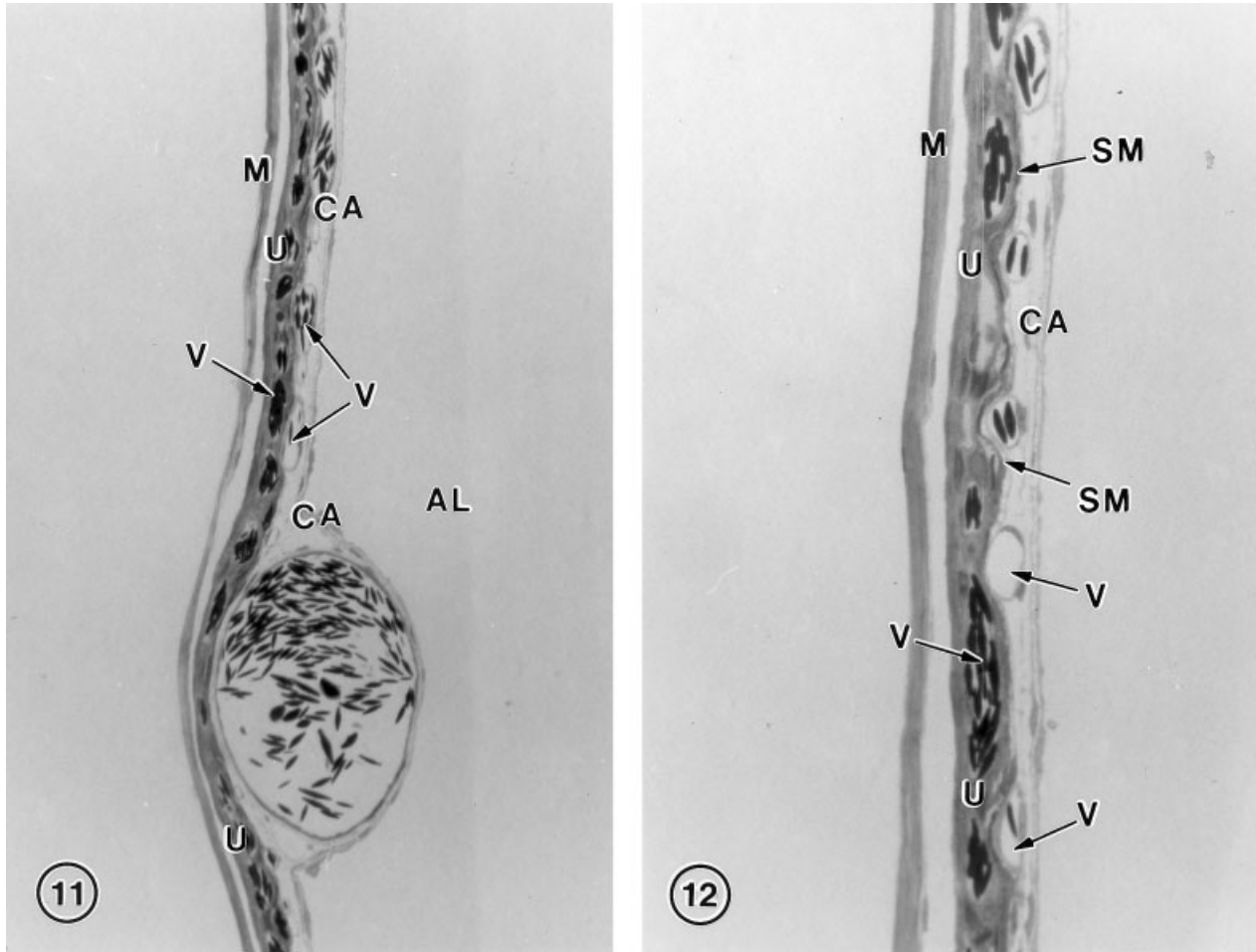


Fig. 11. Allanto-placenta, *Thamnophis sirtalis* (embryonic stage 37). The thin chorioallantois (CA) lies closely apposed to the inner lining of the uterus (U) throughout its length. Blood vessels (V) are more abundant than in mid-gestation. AL, allantoic lumen; M, uterine muscle (the separation is artifactual). Azur II/methylene blue. $\times 260$.

Fig. 12. Allanto-placenta, *Thamnophis sirtalis* (embryonic stage 37). The shell membrane (SM) is represented as thin pieces of variable thickness, lying between tissue of the uterus (U) and the chorioallantois (CA). Whether uterine and chorionic epithelia are intact is difficult to determine from light microscopy. M, uterine muscle; V, blood vessels. Azur II/methylene blue. $\times 520$.

desmosomes. However, over most of their length the cells are separated by large areas of extracellular space (Fig. 10). The luminal cells contain RER and mitochondria; their nuclei are large, ovoid, and heterochromatic. Vesicles of various sizes are located in the cytoplasm adjacent to the allantoic lumen (Fig. 10). The luminal cells are linked to one another apically by tight junctions. Laterally, the cells interdigitate extensively with one another by means of elongate protrusions (Fig. 9). Cell extensions also protrude into the extracellular space towards the basal cells (Fig. 10). In the basal cells, the cytoplasm contains occasional mitochondria and sparse amounts of RER.

Late Gestation

Light microscopy. At Zehr stages 36 and 37, the chorioallantoic placenta appears much like that of mid-gestation. The chorioallantois and uterus are

thin, vascularized membranes that lie closely apposed to one another (Figs. 11, 12). A thin squamous epithelium lines the inner surface of the uterus and the external surface of the chorion. However, as in the earlier developmental stage, whether the chorionic and uterine epithelium forms a continuous lining over the capillaries could not always be determined through light microscopy. The membranes exhibit two differences from the allanto-placenta at mid-gestation. First, the shell membrane is so thin as to be very difficult to discern and, in some areas, it appears to be discontinuous. Second, both the uterus and the chorioallantois appear more vascular than at the earlier developmental stage. In particular, the uterus shows a greater abundance of capillaries and other small blood vessels.

TEM. Ultrastructural examination of the allanto-placenta near the end of gestation confirms that it has undergone significant changes. The shell mem-

brane is broken into fragments and no longer forms a continuous barrier between fetal and maternal tissues (Figs. 13, 14). As a consequence, the uterine epithelium and chorionic epithelium lie directly apposed in some regions (Fig. 15). The shell membrane fragments vary markedly in length and thickness. However, in general they are thinner than the shell membrane at mid-gestation, having been reduced to pieces that are 0.3–0.7 μm in thickness.

In terms of components and overall organization, the uterine portion of the allantoaplacenta appears much like that described at the earlier developmental stage (Fig. 13). The uterine epithelium is flattened and forms a continuous lining to the uterine lumen. Deep to the epithelium lies a lamina propria that contains fibroblasts, collagen fibers, and blood vessels. The external wall of the uterus is formed by the muscularis externa, which consists of a few layers of smooth muscle cells.

The uterine epithelium in late pregnancy contains both microvilliated cells and ciliated cells (Fig. 14). As described previously, the cells that bear cilia appear more electron-dense than the microvilliated cells; however, both cell types exhibit ribosomes, RER, and mitochondria. Single, sparse microvilli extend into the uterine lumen towards the shell membrane (Figs. 14, 15). The epithelial cells are linked to one another at their apical borders by tight junctions and, laterally, by desmosomes. The plasma membranes of adjacent epithelial cells interdigitate extensively. Nuclei and most of the cytoplasm of the epithelial cells lies lateral to (rather than superficial to) the blood vessels. The epithelial cells send very thin cellular extensions over the capillaries (Figs. 13, 14). These cellular extensions are separated from the capillary endothelium by a thin basal lamina. Thus, the barrier to diffusion between the capillaries and the uterine lumen consists of a thin endothelium, basal lamina, and thin epithelial cell extensions (Fig. 16).

The chorionic epithelium is attenuated, but forms a continuous boundary to the fetal component of the allantoaplacenta (Fig. 15). Two layers of chorionic epithelial cells are present, the most superficial of which consists of highly vesicular cells with dark-staining cytoplasm. Nuclei of the epithelial cells lie basally and are located lateral to (rather than on the luminal side of) the blood vessels. Cells of both layers send thin extensions over the capillaries and the allantoic tissue in general (Fig. 16). At high magnification these two layers are represented as two very thin cell projections lying superficial to the basal lamina (Fig. 17). Because the uterine and chorionic epithelia are so thin, the diffusion distance between fetal and maternal capillaries in some regions is less than 2 μm . The allantoic connective tissue is sparse and contains scattered fibroblasts that lie surrounded by collagen fibers (Figs. 13, 17).

DISCUSSION

Placental Anatomy

TEM has revealed several details about morphology of the placental and extraembryonic tissues that are not entirely evident from light microscopy, and has clarified discrepant interpretations raised through previous studies. Features of particular concern have to do with the nature of the placental interface, the location and abundance of capillaries, and the nature of the shell membrane. Ultrastructural examination has also provided insight into developmental changes and functional attributes of the placental membranes. Each of these features will be considered below.

Maternal-fetal interface. Ultrastructural examination demonstrates that the uterine epithelium is thin but intact during the last half of gestation. Very thin extensions of the cells extend over the uterine capillaries (Figs. 7, 16), but they are so attenuated as to be difficult or impossible to see with light microscopy. Epithelium of the chorion is also attenuated. Although the chorionic epithelium consists of two cell layers, both are extremely thin superficial to the allantoic capillaries (Figs. 2, 7, 13, 16). Consequently, where fetal and maternal tissue lie in apposition, the arrangement can be described as “epitheliochorial” (*sensu* Grosser, 1927).

Some studies on thamnophine snakes have inferred that epithelia at the allantoaplacental interface disappears in some places, exposing the underlying capillaries; however, these studies lack corroborative micrographs. For example, investigations of *Thamnophis sirtalis* inferred that the chorionic epithelium is eroded, as in the mammalian cotyledonary placenta (Gibson, 1934; Clark et al., 1955). Other work suggested that the uterine capillaries are exposed to the lumen in the thamnophiine snake *Storeria occipitomaculata* (Rahn, 1939). Yet another study described the allantoaplacenta of the related species *Nerodia sipedon* as “endothelial-endothelial” (Conaway and Fleming, 1960), implying the loss of both uterine and chorionic epithelia. Similar claims have been made of distantly related, viviparous snakes (Weekes, 1929; Kasturirangan, 1951a,b; Parameswaran, 1962) as well as lizards (ten Cate-Hoedemaker, 1933). However, reexamination of the allantoaplacenta in some of these species (i.e., *Chalcides chalcides*, *Nerodia sipedon*) has shown that the epithelium is not eroded and that the arrangement is more accurately characterized as epitheliochorial (Blackburn, 1993a, 1998; Blackburn and Callard, 1997).

The present study leaves no doubt that in the *Thamnophis* species examined epithelia of the chorion and uterus remain intact at the site of the allantoaplacenta. Thus, although the epithelial boundaries become extremely thin, capillaries are not exposed to the uterine lumen (Figs. 2, 13, 16). This observation is consistent with recent findings

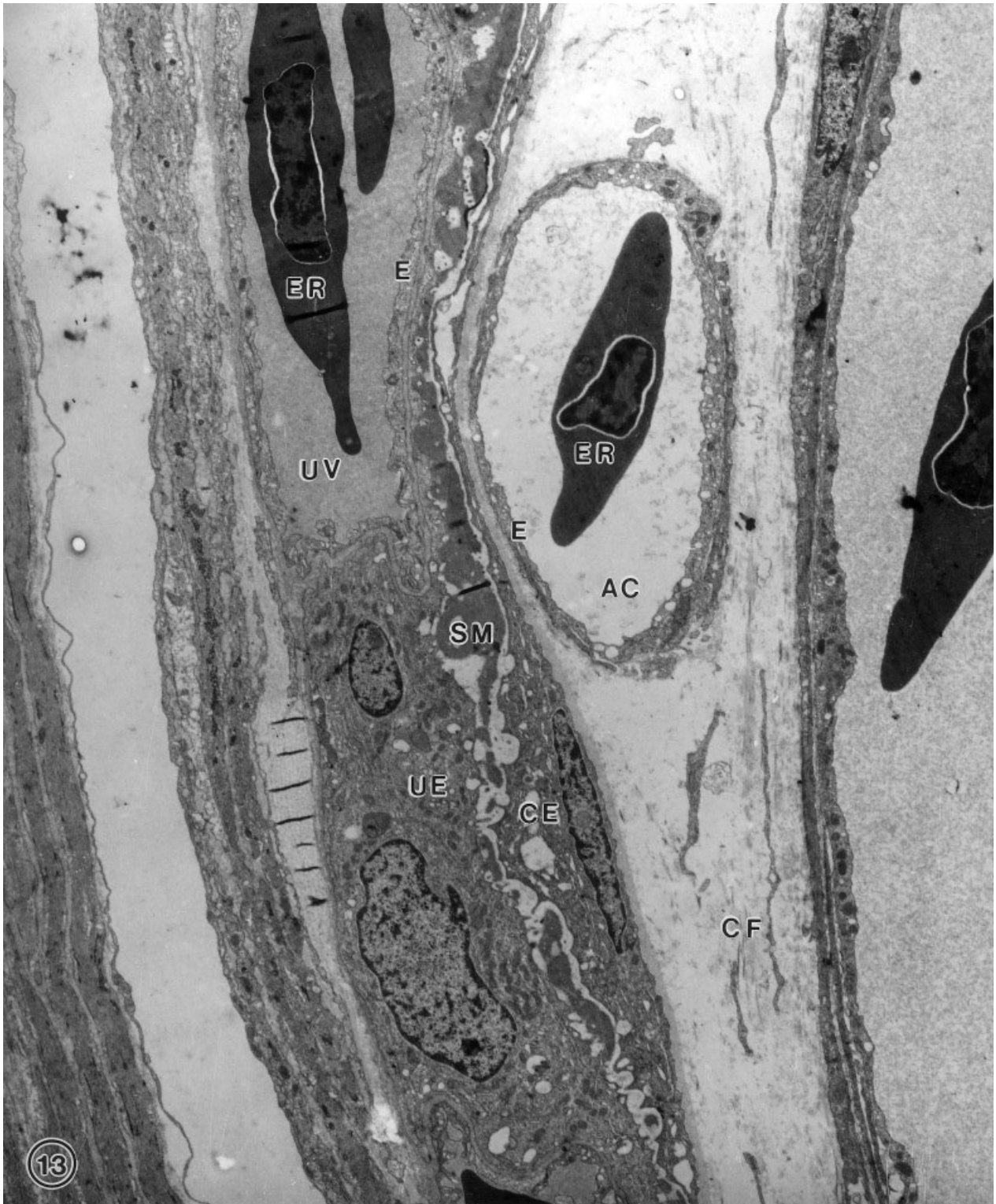


Fig. 13. Allantoplacenta, *Thamnophis sirtalis* (embryonic stage 37). Lying at the interface of uterine and chorionic tissue, the shell membrane (SM) is discontinuous, and represented by clumps of material. AC, allantoic capillary; CE, chorionic epithelium; CF, collagen fibers; E, endothelium; ER, erythrocytes; UE, uterine epithelium; UV, uterine blood vessel. A large allantoic vessel occupies the right side of the micrograph. $\times 7700$.

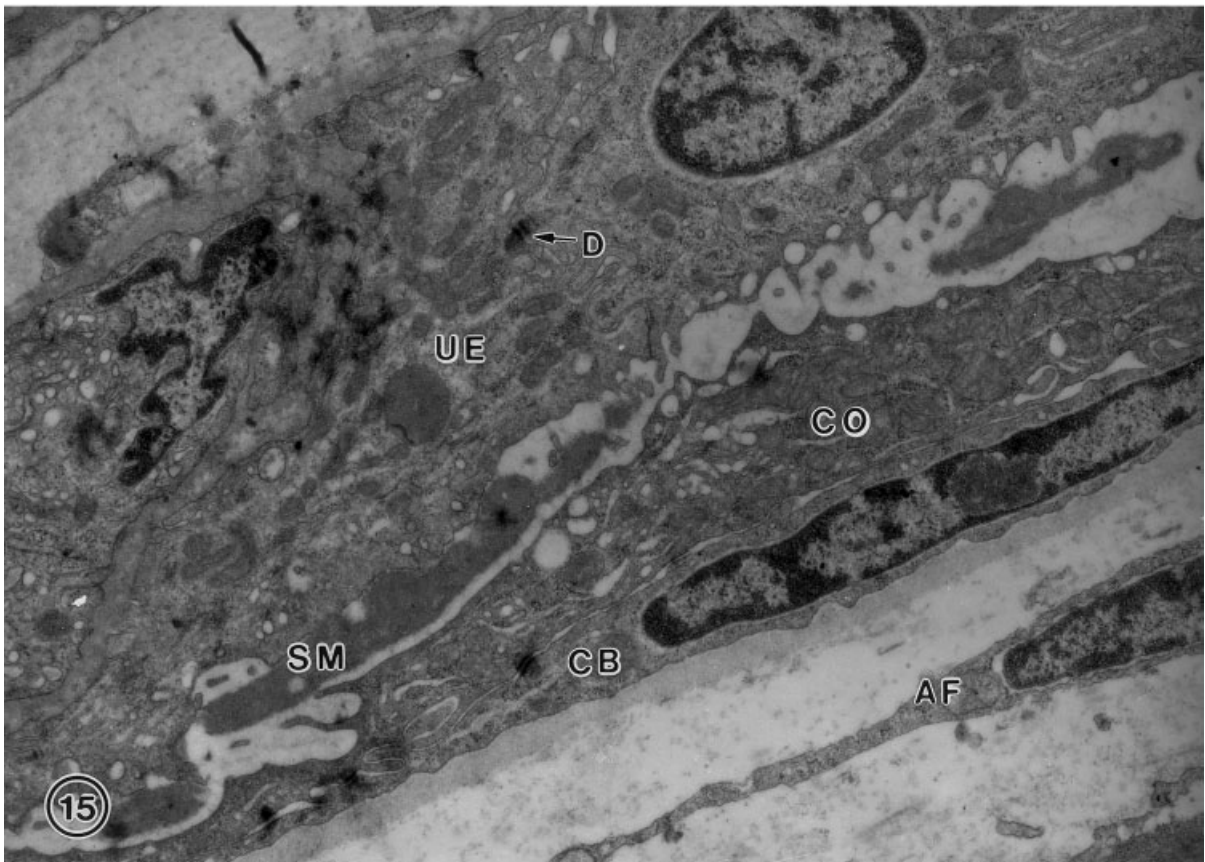
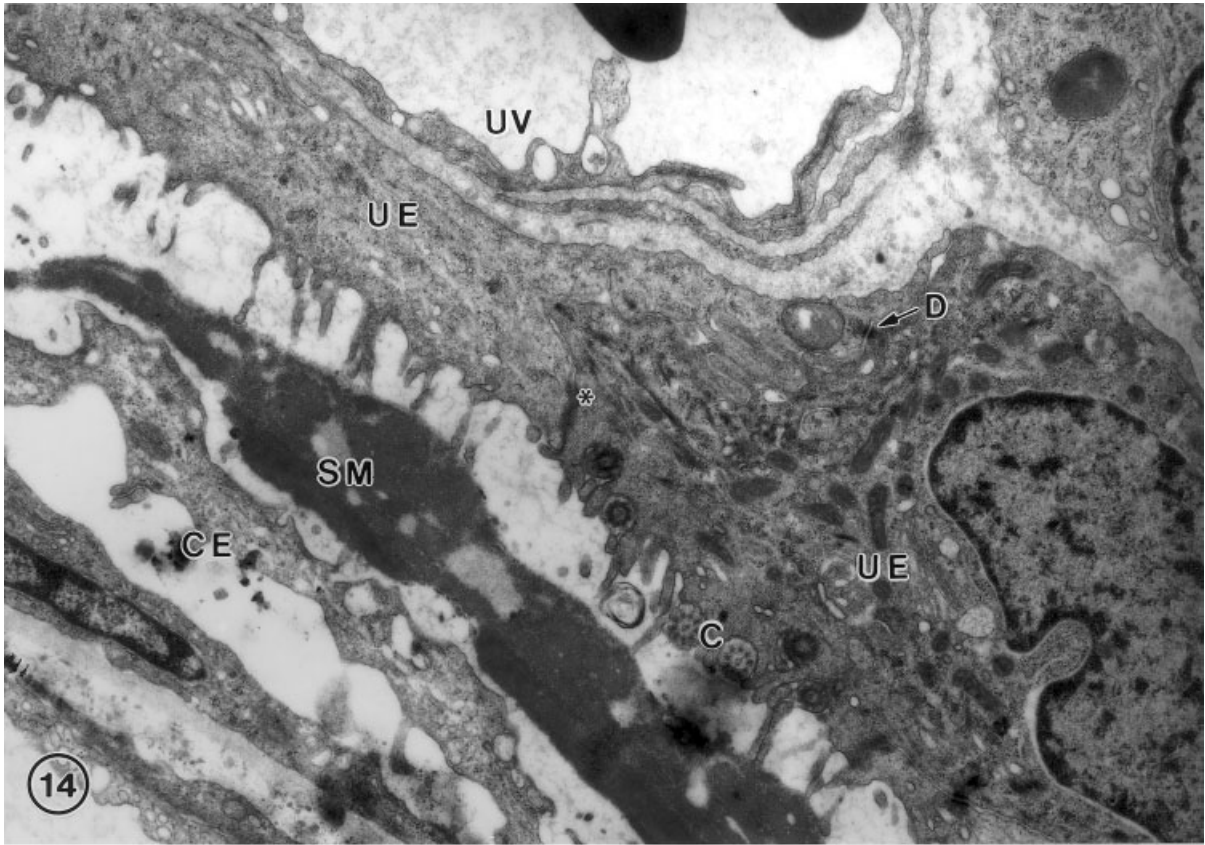


Fig. 14. Allantoplacenta, *Thamnophis sirtalis* (embryonic stage 37). The uterine epithelium (UE) contains both ciliated and microvilliated cells. Adjacent cells are linked by tight junctions (asterisk). C, cilia; CE, external cells of chorionic epithelium; D, desmosome; SM, shell membrane; UV, uterine blood vessel. $\times 17,000$.

Fig. 15. Allantoplacental interface, *Thamnophis sirtalis* (embryonic stage 37). This micrograph is from the same section in Figure 13. The chorionic epithelium is bilayered, consisting of an outer layer (CO) and basal layer (CB). AF, allantoic fibroblast; D, desmosome; SM, shell membrane; UE, uterine epithelium. $\times 17,000$.

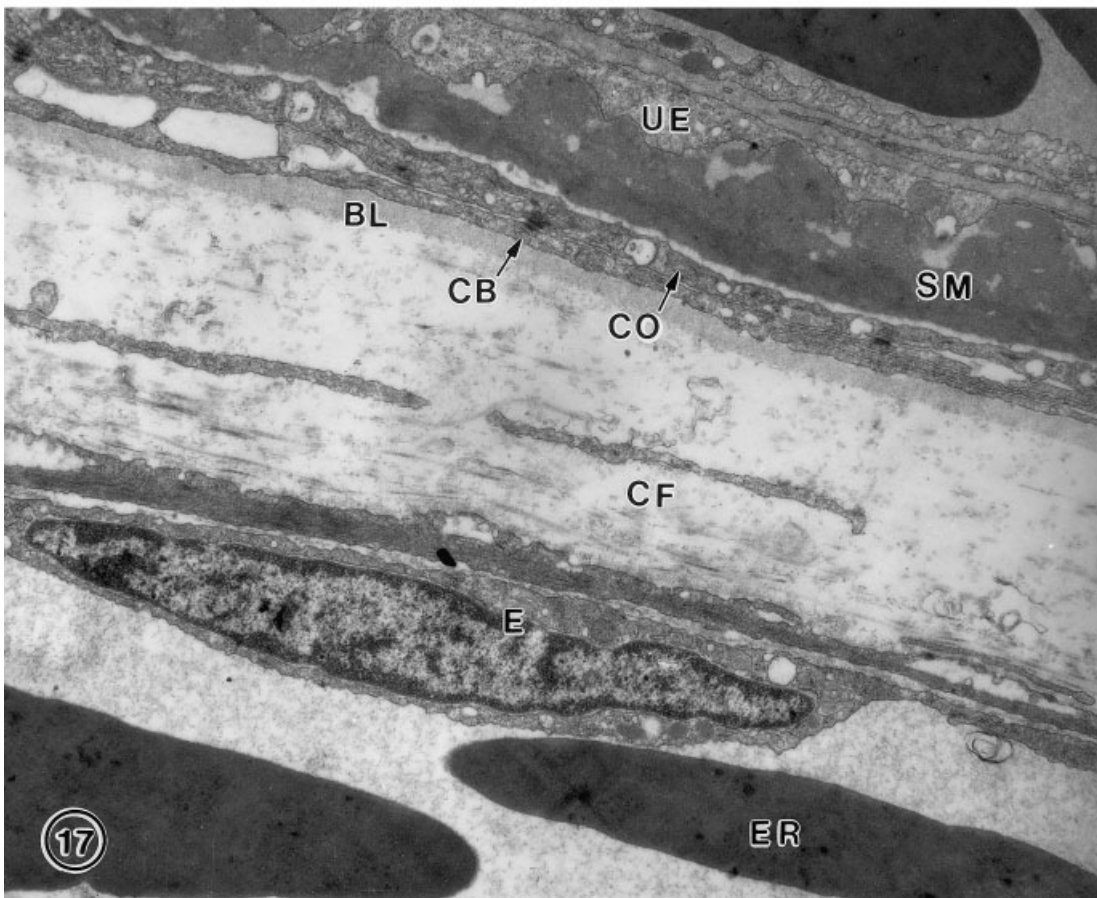
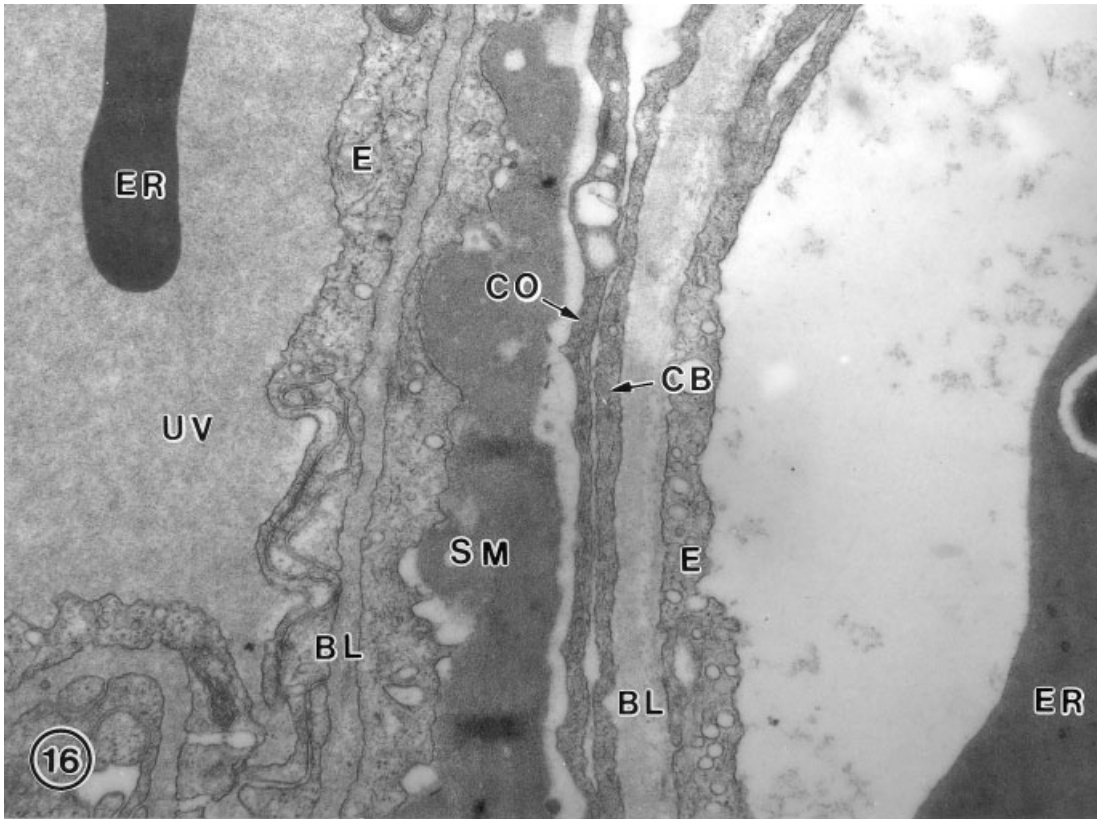


Fig. 16. Allantoplacental interface, *Thamnophis sirtalis* (embryonic stage 37). BL, basal lamina; CB, chorionic epithelium, basal cell; CO, chorionic epithelium, outer cell layer; E, endothelium; ER, erythrocyte; SM, shell membrane; UV, uterine blood vessel. $\times 27,000$.

Fig. 17. Allantoplacental interface, *Thamnophis sirtalis* (embryonic stage 37). BL, basal lamina; CB, chorionic epithelium, basal cell layer; CF, collagen fibers; CO, chorionic epithelium, outer cell layer; E, endothelium; ER, erythrocyte; SM, shell membrane; UE, uterine epithelium. $\times 16,000$.

from SEM (Blackburn et al., 2002), as well as inferences from light microscopy (Hoffman, 1970; Blackburn, 1998). Intact epithelia have also been demonstrated with EM in thamnophine snakes of the genera *Regina* (Attaway, 2000) and *Virginia* (Stewart, 1989b). In fact, epithelial erosion at the site of the allanto-placenta has never been firmly documented in any viviparous squamate (Blackburn, 1993b). Claims for its occurrence require corroboration from SEM or TEM in order to be accepted as definitive.

A previous study of *Thamnophis sirtalis* concluded from light microscopy that the uterine epithelium contains no ciliated cells in the region of the allanto-placenta (Hoffman, 1970). We find ciliated cells to be present (Figs. 2, 13) but not abundant. Examination of *T. sirtalis* and *T. ordinoides* using SEM has also revealed ciliated cells in the uterine oviduct (Blackburn et al., 2002). In *T. radix*, the proportion of ciliated cells is considerably less during pregnancy than in the pre-ovulatory period (Stearns, 1986), probably accounting for their having been overlooked in other work. Our observations also show no evidence that the uterine epithelium is syncytial. A syncytial epithelium does develop in the uterus of some eutherian mammals (Mossman, 1987).

Vasculature of the placental membranes. In late gestation both the uterine and chorionic tissues are highly vascular. Our observations indicate that the maternal and fetal components of allanto-placenta both become more vascular during the last half of gestation (Figs. 12, 13). Similar conclusions have been reached through light microscopy (Hoffman, 1970; Gerrard, 1974), from SEM (Stearns, 1986), and from digitized images of whole mounts (Blackburn, 1998). In addition, epithelial layers over the uterine and allantoic capillaries become thinned (Figs. 2, 7), as the vessels push up against the base of the epithelial cells. Appearance of the epithelial cells suggest that their nuclei and cytoplasm may be pushed to the sides of the capillaries as the latter migrate in a luminal direction (Fig. 13). As a consequence, on both sides of the placenta the capillaries lie as close to the uterine lumen as would be possible without erosion of the epithelial tissues (Fig. 14). These features have significant functional consequences, as discussed below.

Shell membrane. The shell membrane is a vestige of the eggshell and appears to be present at some stage of development in most or all viviparous squamates (Blackburn, 1993b, 1998; Stewart, 1993). The structure has elicited attention because its presence bears on the nature of the placental interface and, most likely, aspects of maternal-fetal exchange. Quite commonly the shell membrane undergoes deterioration during development in viviparous squamates (e.g., Weekes, 1929; Kasturirangan, 1951b; Stewart and Thompson, 1994; Blackburn and Callard, 1997), although not in all species (Panigel,

1956; Stewart, 1985; Heulin, 1990). The shell membrane varies in thickness between species and between regions in a single individual. Because it can be difficult to see histologically, inferences of its absence require verification with electron microscopy (Hoffman, 1970).

A light microscopic study of the oviduct inferred that no shell membrane is present in *Thamnophis sirtalis* (Seaman, 1949). However, Hoffman (1970, p. 67) was able to identify a shell membrane in *T. sirtalis* through TEM, characterizing it as a "continuous, bilaminar sheath." According to his description, the membrane consists of a thin, dense inner layer (facing the chorion) and a thicker, less dense outer layer (facing the uterine lumen), in which particulate matter occurs. Hoffman's (1970) study does not specify the region or the developmental stage illustrated; thus, it is not clear whether the allanto-placenta or the omphalallantoic placenta is represented. The presence of a shell membrane in *T. sirtalis* has been verified with histology of appropriately stained tissue (Blackburn, 1998), as well as by means of SEM (Blackburn et al., 2002). Shell membranes have also been reported in *T. radix* (Gerrard, 1974; Stearns, 1986) and thamnophine snakes of the genera *Virginia* (Stewart, 1990), *Regina* (Attaway, 2000), *Nerodia* (Blackburn, pers. obs.), and *Tropidoclonion* (Baxter, 1987).

Our observations confirm the presence of a shell membrane in *Thamnophis* (see Figs. 2, 8), while offering additional detail. We find that the shell membrane thins during development and, in some areas, undergoes deterioration in late gestation (Figs. 14, 15). As a result, chorionic and uterine epithelia become more closely associated and, where the membrane disappears, they are directly apposed (Fig. 15). The membrane may also undergo changes in composition during gestation. In mid-gestation, thick fibers are evident running primarily lengthwise through the membrane. The uterine face of the membrane shows an amorphous material with particulate matter (Fig. 8). By late gestation the shell membrane is more homogeneous, with less evidence of substructure. The portion lying adjacent to the chorion is somewhat more dense than that facing the uterine epithelium (Fig. 17). If breakage of the membrane is accompanied by chemical disruption, such could account for the loss of structural integrity along with the loss of membrane substructure. Most likely the uterine and/or chorionic epithelium plays a role in its breakdown. In regions where uterine epithelial cells protrude into the shell membrane, the latter is thin and appears to be undergoing attenuation (Fig. 16). Whatever the mechanism of its deterioration, thinning and loss of the shell membrane in the region of the allanto-placenta leads to a close association of fetal and maternal tissues, potentially enhancing the potential for transplacental exchange.

Functional Considerations

Placental gas exchange. Three related features of the allantoplacental membranes of *Thamnophis* are especially relevant to a consideration of functional attributes. One is that both the chorioallantois and the uterus are well vascularized and show an increase in capillary density over the course of gestation. The second feature is that the uterine and allantoic capillaries become displaced towards the uterine lumen through thinning of the overlying tissues. Accordingly, any intervening lamina propria is obliterated and the epithelial cells become attenuated. Third, the shell membrane diminishes in thickness during gestation and deteriorates in some regions, allowing direct contact between the fetal and maternal tissues. Thus, the interhemal barrier consists of nothing more than the thin capillary endothelia, the thin uterine and chorionic epithelia and their basal laminae, and any remnant of the shell membrane (Fig. 16). As a result, the transplacental diffusion distance between capillaries is minimal, being as small as 2 μm .

Chorioallantoic placentas of squamate reptiles generally are considered to accomplish gas exchange between maternal and fetal tissues (Weekes, 1935; Yaron, 1985; Blackburn, 1993b). Microscopic anatomy of the placental membranes in *Thamnophis sirtalis* and *T. radix* is entirely consistent with such a respiratory function. Components of the interhemal membrane, as well as deterioration of the shell membrane, appear to be designed to minimize diffusion between fetal and maternal circulations and maximize interhemal exchange. In fact, the diffusion distance between fetal and maternal blood systems is smaller than the width of a single erythrocyte (see Fig. 13). Because the chorioallantois occupies most of the circumference of the egg, and lies adjacent to the uterine tissue throughout, the membranes provide a broad expanse for physiological exchange. Given that the chorioallantois is the only well-vascularized fetal membrane during the last half of development (see Blackburn and Lorenz, 2003), its function in maternal–fetal gas exchange can be considered as well established.

In addition to their morphological features, viviparous snakes exhibit various physiological specializations that enhance transplacental gas exchange. In *Thamnophis elegans*, fetal blood has a higher oxygen affinity than maternal blood (Berner and Ingermann, 1988). The differences are due to raised nucleoside triphosphate (NTP) levels in pregnant females and lowered levels in fetuses, features that significantly affect hemoglobin affinity for oxygen (Ingermann et al., 1991a,b). Maternal NTP levels increase during gestation in *T. elegans*, a response that is modulated by luteal progesterone (Ragsdale et al., 1993). Maternal–fetal differences in blood oxygen affinity occur in distantly related viviparous squamates (Grigg and Harlow, 1981; Birchard et al.,

1984; Ingermann, 1992). However, the means by which the maternal–fetal differences are accomplished vary between species (Blackburn, 2000). The morphological specializations of the allantoplacenta documented herein and elsewhere can be viewed as an important, but not the sole, contribution to maternal–fetal gas exchange during pregnancy.

Other placental functions. Given the close association of uterine and chorioallantoic tissues, the placental transfer of substances other than gases should be considered. Experimental studies have demonstrated the transplacental movement of sodium and glycine (and/or protein) in *Thamnophis sirtalis* (Hoffman, 1970). Comparisons of the composition of eggs and fetuses in *T. ordinoides* indirectly have demonstrated maternal–fetal transfer of sodium and calcium (Stewart et al., 1990). In addition, sex steroids pass between maternal and fetal tissues during pregnancy in *T. radix* (Gerrard, 1974). The close association of uterine and allantoic blood streams offer one potential site of transfer. However, whether such substances are transferred via the allantoplacenta or the omphalallantoic placenta is not known. As shown elsewhere, ultrastructural anatomy of the omphalallantoic membrane strongly suggests absorptive functions (Hoffman, 1970; Blackburn et al., 2002; Blackburn and Lorenz, 2003).

Nevertheless, the allantoplacental membranes show anatomical evidence of physiological activity involving more than hemotrophic exchange. The uterine epithelial cells contain small apical vesicles (Figs. 5, 6); whether they are secretory or absorptive is not clear from their morphology. One might speculate that they are associated with breakdown of the shell membrane or, alternatively, secretion of material of nutritional benefit to the embryo. Cytoplasm of the external layer of chorionic epithelium is full of vesicles, although the nature of their contents in life is unknown. In the basal layer of epithelial cells, mitochondria are abundant (Fig. 9), hardly what one would expect of a cell that simply serves as a passive barrier to diffusion of gases. Therefore, the possibility that the allantoplacenta plays some role in histotrophic transfer and/or regression of the shell membrane deserves consideration in future studies.

The ultrastructure of the allantoic epithelium is also worthy of note. While a complete description of the allantoic components is beyond the scope of this study, at least some of the endodermal cells that line the allantoic lumen contain ribosomal ER, mitochondria, and show abundant apical vesicles (Fig. 10), features suggestive of metabolic activity. Although the allantois is often viewed as a repository of nitrogenous waste, that of the domestic chicken also has osmoregulatory functions (Hoyt, 1979). Viviparity raises additional possibilities; given the incorporation of the allantois into placental structures, nitrogenous wastes conceivably might be transferred to the maternal circulation (Clark and

Sisken, 1956). Whatever its functional role in *Thamnophis*, if contents of the allantoic fluid are regulated such could explain the presence of physiologically active cells surrounding the allantoic lumen, distant from the site of maternal–fetal exchange.

As the first examination of the allanto-placental membranes of garter snakes using TEM, this investigation leaves a number of questions unanswered. Placentas are dynamic organs that can undergo dramatic changes over the course of gestation. While our samples have bracketed the middle and late stages of development, further study is needed to characterize the developmental changes in the epithelia at the placental interface, the abundance and position of capillaries, and the changing character of the shell membrane. Further examination may also clarify the functional roles of the uterine and chorionic epithelia, as well as of the endodermal cells of the allantois. Finally, a full understanding of placentation in *Thamnophis* will require clarification of the differential roles played by the allanto-placenta and the omphalallantoic placenta during the course of gestation.

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This article and its successor (Blackburn and Lorenz, 2003) are dedicated to the late Loren H. Hoffman (1942–1999). Appropriately, Loren's first article, "Placentation in the garter snake, *Thamnophis sirtalis*" was published in the *Journal of Morphology* in 1970; for over 30 years, it has presented unsurpassed standards of quality and breadth in the field of reptile placentation. We thank Ann Lehman for technical assistance during this project.

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