



REVIEW

Structure and Function of Placental Exchange Surfaces in Goodeid Fishes (Teleostei: Atheriniformes)

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ABSTRACT The species of the family Goodeidae have evolved reproductive strategies involving intraovarian gestation, early evacuation of nearly yolk-exhausted embryos from the ovigerous tissue into the ovarian cavity, placental matrotrophy during intraluminal gestation, and the birth of highly developed fry. The inner ovarian lining becomes hypervascularized during gestational periods and functions as the maternal component of the placental association. Embryotrophic liquid is secreted by the inner ovarian epithelium into the ovarian cavity. Comparative electrophoretic analyses of embryotrophe and maternal blood serum provide evidence for the transfer of maternal serum proteins into the embryotrophe. Trophotaeniae, proctodaeal processes of the embryos, provide a surface for nutrient absorption. Endocytic activity was demonstrated by ingestion of unspecific tracer proteins in various species. Moreover, the trophotaenial absorptive cells (TACs) in *Ameia splendens* ingest various proteins or random copolymers conjugated to colloidal gold as well as radioiodinated proteins in a way that satisfies the criteria of receptor-mediated endocytosis. Several aminopeptidases (APs) on the surface of TACs were identified as protein binding sites as evidenced by inhibition of binding and uptake of marker proteins in the presence of AP substrates or AP inhibitors. Morphological adaptations of the embryonic circulatory system pertaining to nutrient and gas exchange were characterized. The embryonic epidermis comprises two layers of squamous cells closely underlain by a dense capillary net. Efficient gas exchange is facilitated by a thin embryotrophe-blood barrier of both the embryonic skin and the intraovarian lining. *J. Morphol.* 276:991–1003, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: Goodeidae; ovarian gestation; trophotaenial placenta; embryotrophe; receptor mediated endocytosis; cutaneous gas exchange

INTRODUCTION

Goodeidae, a family of viviparous cyprinodont teleosts endemic to the Central Plateau of Mexico have evolved unique reproductive peculiarities first described by Turner (1932). Most conspicuously, the embryos develop proctodaeal appendages, so called trophotaeniae, after which the name trophotaenial placenta was coined (Figs. 1 and 2). The maternal component of the placental association consists of specializations of the

goodeid ovary during gestation. A broad description and classification of the reproductive tract in goodeid females was given by Turner (1933), Hubbs and Turner (1939), and Mendoza (1965). As in viviparous teleosts generally, goodeids possess a simple fused ovary serving a dual function: i) production of germinal cells. ii) Substitution for a uterus. The ovary is suspended dorsally in the coelomic cavity by a short mesentery. It is continuous with the gonaduct that opens to the exterior at the genital pore. In the absence of a uterus, gestation in goodeid fishes is intraluminal, that is, the ovary is a hollow organ, the cavity of which is partitioned by a midseptum into two chambers accommodating the embryos (Fig. 3). Goodeid embryos are harbored in the ovarian cavity during most of the gestation period. The scarce yolk supply of the zygote is exhausted within short span of time. Henceforth, the embryos exclusively depend on matrotrophic strategies facilitated by placental associations. Because there is no fusion of embryonic and maternal tissues, embryonic requirements are met through transfer of nutritive and gaseous substances across placental exchange surfaces adapted for either sequestration of embryotrophic liquid, nutrient absorption, or respiratory function. As such, the inner lining of the ovarian cavity, trophotaeniae, and embryonic skin are highlighted in this brief review.

INNER OVARIAN LINING: THE MATERNAL COMPONENT OF THE TROPHOTAENIAL PLACENTA

In gravid females, the ovarian tissue is confined to a narrow band subjacent to the serous membrane and the septum. Germinal tissue is located

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Fig. 1. *Ameca splendens*: Near term embryo with appending trophotaeniae. Bar = 1 cm.

in folds of the ovarian lining that protrude into the ovarian lumen. The stromal connective tissue cells can have very thin plate-shaped cellular processes that are interconnected to form irregularly delineated, variously sized, electron-lucent loculi (Schindler, 1990; Schindler and Radda, 1990).

Ultrastructurally the relatively electron-dense inner lining of the ovarian cavity is in relief against the stroma. It is composed of the inner ovarian epithelium (IOE), dense-meshed capillaries, and connective tissue with varying amounts of collagen fibrils (Fig. 4). Ultrastructural studies of the IOE in gravid goodeid females were undertaken with *Ameca splendens* (Lombardi and Wourms, 1985a), *Xenoporus captivus* (Schindler et al., 1988), *Xenotoca eiseni*, *Girardinichthys viviparus* (Schindler, 1990), *Skiffia bilineata* (Schindler and Kujat, 1990) as well as *Allodontichthys robustus*, *Zoogoneticus quitzeoensis*, and *Goodea atripinnis* (Hollenberg and Wourms, 1995). In *A. splendens*, the IOE was described as a simple cuboidal epithelium, overlying a well-vascularized bed of connective tissue. The apical cytoplasm contains abundant well-developed granular and agranular endoplasmic reticula and large vesicles filled with electron-dense material. Thus, it has been speculated that proteins may be synthesized in the IOE serving as a nutritive source for embryonic development (Lombardi and



Fig. 2. *Girardinichthys viviparus*: Near term embryo with appending trophotaeniae. Bar = 1 cm.



Fig. 3. *Xenotoca eiseni*: Cross section of the gravid ovary. The ovarian cavity is divided by a median septum (arrowheads) into two chambers each harboring three embryos. Sections of trophotaeniae (asterisks) are abundant in the nutritive liquid that circumsfuse the embryos, 13.5 \times . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Wourms, 1985a). In *X. captivus* the structure of the IOE changed during pregnancy from highly columnar to very attenuated cytoplasmic sheets overlying capillaries that were pressed toward the surface. The IOE contains a well-developed vacuolar apparatus. Rough endoplasmic reticulum and ribosomal clusters were moderately distributed in the cytoplasm (Schindler et al., 1988).

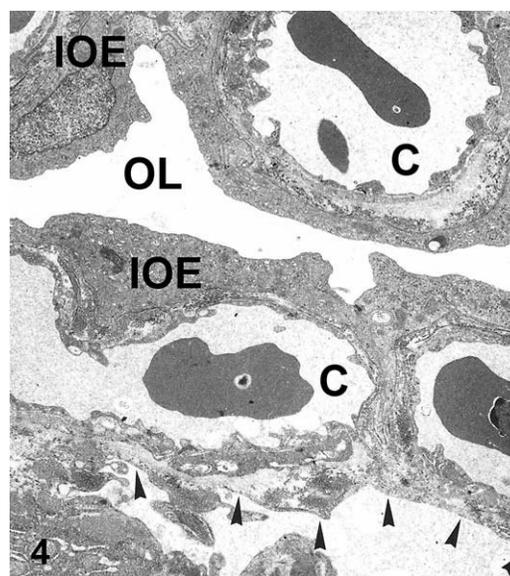


Fig. 4. *Xenotoca eiseni*: Intraovarian epithelium (IOE) in a gravid ovary covering opposing folds on the ovarian septum. Capillaries (C) covered by attenuated cytoplasmic sheets take up a location close to the apical surface. The demarcation between the subepithelial zone and the multilocular core of the ovarian folds is indicated by arrowheads. OL, ovarian lumen, 6.400 \times .

In *S. binineata*, the cells of the IOE contain only small amounts of rough endoplasmic reticulum scattered throughout the cytoplasm. The epithelial cells are distinct by the presence of large myelin bodies on postfixation with $\text{OsO}_4/\text{K}_4\text{Fe}(\text{CN})_6$. Most conspicuous were coated vesicles and medium-sized electron lucent vacuoles (Schindler and Kujat, 1990). The inner ovarian epithelial cells of gravid females in *A. robustus*, *Z. quitzeensis*, and *G. atripinnis* were unanimously described as cuboidal in shape with mostly dome-shaped apices. Their cytoplasm was rich in small rounded or flattened vesicles and free ribosomes. On the other hand, there were only small amounts of rough endoplasmic reticulum prompting the authors to conclude that there is little, if any, protein synthetic activity in the IOE of all three species (Hollenberg and Wourms, 1995).

EMBRYOTROPHIC OVARIAN LIQUID: SDS-PAGE AND ELECTROFOCUSING OF BLOOD SERUM AND EMBRYOTROPHE

Embryotrophic proteins in the ovarian liquid conceivably may be imported from the maternal blood serum by macromolecular transport or, alternatively, may be synthesized in the IOE. Ultrastructural evidence for synthesis and secretion of proteinaceous nutrient substrates was found in the IOE in *A. splendens* (Lombardi and Wourms, 1985a). In contrast, studies of the IOE in diverse other goodeid species revealed considerable morphological heterogeneity, with no conclusive evidence for protein synthesis (Schindler, 1990; Hollenberg and Wourms, 1995). Therefore, it would be premature to draw a conclusion on the source of embryotrophic proteins by taking into account the morphological evidence alone. However, there is circumstantial evidence for the intra-ovarian lining being involved in transepithelial transport of maternal serum proteins into the embryotrophe (Schindler et al., 1988; Schindler, 1990; Hollenberg and Wourms, 1995). The IOE in *X. eiseni* and *G. viviparus* was shown to exhibit morphologic features consistent with transepithelial vesicular transport of macromolecules (Schindler, 1990). Moreover, retrograde trafficking of cationized ferritin (CF) by the inner ovarian lining in *X. eiseni* and *G. viviparus* was traced experimentally. Diluted NaCl/CF saline was injected into the ovary of anesthetized female fish late in gestation. After 6 h of exposure to CF, tracer molecules ingested by invaginated coated pits on the luminal surface of the IOE were sequestered in multivesiculated vacuoles of the lysosomal apparatus. Entrapped in small vesicles, a small share of the ingested CF was translocated to the basal cell membrane and exocytosed into the extracellular matrix. Such clusters of tracer protein were transcytosed across the endothelium to the luminal

surface of capillaries in the inner ovarian lining (Schindler, 1990).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electropherograms of both maternal blood serum and embryotrophe in *S. bilineata* showed numerous bands, each covering the range between approximately 15 and 100 kD (Schindler and Kujat, 1990). Some components of higher molecular weight may also be present in small amounts as indicated by faint mobilities. In another study, comparative SDS-PAGE of ovarian liquid and blood serum obtained from nongravid females of *A. robustus* and *G. atripinnis* each produced similar but not identical patterns covering a wide range of molecular weights (Hollenberg and Wourms, 1995). Ovarian liquid of gravid *A. robustus* did not contain detectable amounts of proteins during midgestation and late gestation. This phenomenon was attributed to efficient protein absorption by the trophotaeniae in this species. In the ovarian liquid of *G. atripinnis*, conversely, serum proteins were present at midgestation and were dramatically reduced at late gestation. For this, a tentative explanation was offered in that the trophotaeniae of *G. atripinnis* are largely the nonendocytosing type, and that nearly fully developed embryos might imbibe the proteinaceous substances (Hollenberg and Wourms, 1995).

Isoelectric focusing was used to compare the protein patterns of maternal blood serum and embryotrophe in *X. captivus* (Schindler et al., 1988), *S. bilineata* (Schindler and Kujat, 1990), *X. eiseni* (Schindler and Hamlett, 1993), and *Ataenobius toweri* (Schindler and Kujat, unpublished). In each case, both samples produced a qualitatively similar band pattern between maternal blood and embryotrophe (Fig. 5).

Ovarian liquid of *X. captivus*, *A. splendens*, and *S. bilineata* was analyzed with respect to proteolytic activity (Schindler and Kujat, unpublished). Using bovine hemoglobin bound to ultrasensitive cellulose acetate substrate films (Kujat, 1987) peptic digestion of embryotrophe was assayed. In embryotrophe samples protein degradation was only detected in *X. captivus* (Fig. 6). Quantitative analyses of 16 amino acids in the maternal blood serum and ovarian liquid of a gravid female of *A. splendens* resulted in distinctly lower values of all 16 amino acids in the embryotrophic liquid (Schindler and Greven, unpublished).

TROPHOTAENIAE: PROCTODAEAL APPENDAGES SPECIALIZED FOR NUTRIENT ABSORPTION

Trophotaeniae are ribbon- or rosette-like structures that extend from the perianal lips of goodeid embryos (Turner, 1937). There is considerable between-species variation in trophotaenial size, number of processes, mode of branching, shape,

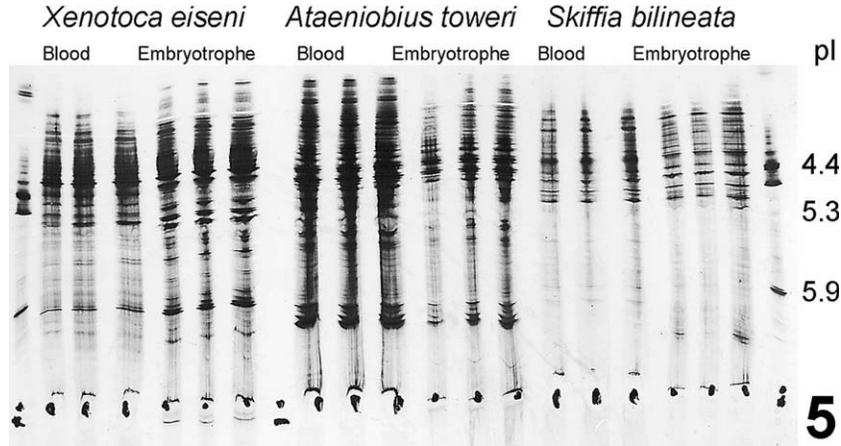


Fig. 5. *Xenotoca eiseni*, *Ataeniobius toweri*, *Skiffia bilineata*: Ultrathin-layer polyacrylamide gel isoelectric focusing of blood and embryotrophe. Anode at top, cathode at bottom. pI = isoelectric point.

regularity, and symmetry. Since their discovery, the trophotaeniae were attributed to play pivotal roles in both nutritive and respiratory functions (Turner, 1933). Meanwhile, diverse issues associated with nutritional protein absorption have been extensively studied. There has been, however, much less effort to study exchange surfaces of goodeid embryos with regard to adaptations for gas exchange.

Trophotaenial development involves externalization of the embryonic hindgut epithelium through growth-based dilation and eversion of the perianal lips. In *A. splendens* and *G. atripinnis* the whole process has been subdivided into five phases: i) Formation of the anus. ii) Dilation of the anus, enlargement of the perianal lips, differentiation of the absorptive epithelium of the hindgut, and formation of the trophotaenial peduncle. iii) Marked hypertrophy and lateral expansion of the perianal lips giving rise to the formation of short trophotaenial processes. iv) Continued outward expansion of the inner mucosal surface of the trophotaenial peduncle resulting in its eversion and lobulation. v) Elongation and branching of the trophotaenial processes. Formation of ribbon- or rosette-like trophotaeniae is a consequence of the degree of axial elongation during the fifth phase (Lombardi and Wourms, 1988). Trophotaeniae are of ectodermal and endodermal origin as reflected in the composition of the trophotaenial surface of both brush border cells and epidermal cells (Fig. 7). Scanning electron micrograph studies showed varying proportions of epidermal epithelium among and between species (Schindler and de Vries, 1986a,b, 1987a). The majority of cases indicated that the predominant component was gut-derived epithelium.

Trophotaeniae are present in all goodeid species except for *A. toweri* (Hubbs and Turner, 1939). The absorptive epithelium has been described as simple columnar, cuboidal or rarely squamous. On the other hand, sometimes there are basal cells which do not seem to reach the surface. They were not seen to undergo cell cycles. Thus, the epithelium may correctly be termed pseudostratified. Electron microscope studies of *X. eiseni* (Mendoza, 1972), *A. splendens* (Lombardi and Wourms, 1985b), *G. viviparus* (Schindler and de Vries, 1986a), *X. captivus* (Schindler and de Vries, 1987a), *S. bilineata*

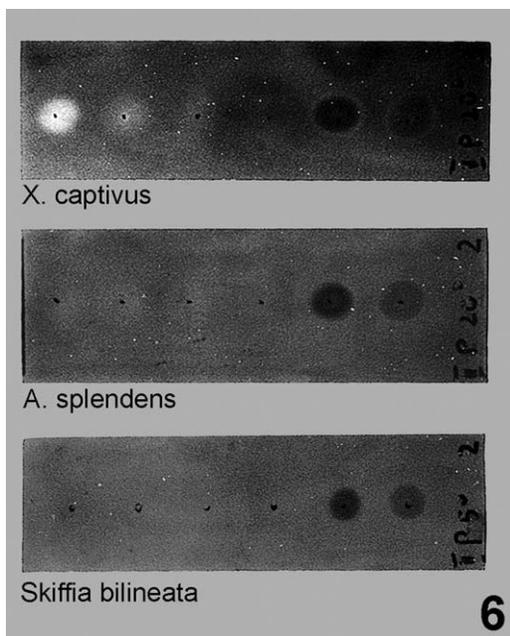


Fig. 6. *Xenophorus captivus*, *Amea splendens*, *Skiffia bilineata*: Protease detection in embryotrophe using cellulose acetate substrate films. Lysis of the immobilized substrate (hemoglobin) by proteases in the embryotrophe is indicated by white spots on the films stained with Coomassie Brilliant Blue. The embryotrophe in *Xenophorus captivus* exhibited protease activity, whereas that in *Amea splendens* and *Skiffia bilineata* did not.

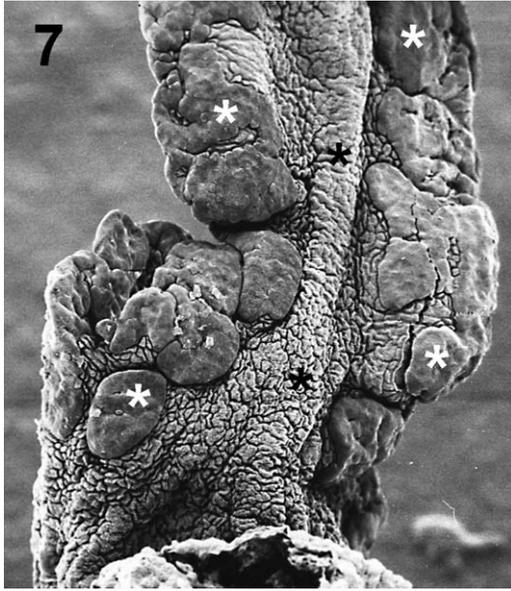


Fig. 7. *Xenoophorus captivus*: Basal section of a trophotaenia showing areas of both brushborder epithelium (white asterisks) and epidermal epithelium (black asterisks), 170 \times .

(Schindler and Kujat, 1990), *Allodontichthys zonistius* (Schindler and Radda, 1990), as well as *Ilyodon furcidens*, *G. atripinnis*, *Z. quitzeoensis*, and *Allophorus robustus* (Hollenberg and Wourms, 1994) revealed the existence of two fundamental types of trophotaenial absorptive cells (TACs). The one represented in *G. viviparus* and *G. atripinnis* is characterized by abundant mitochondria in the apical cytoplasm (Fig. 8). Tubular and vacuolar organelles are conspicuously absent. In the basolateral cytoplasm profiles of lamellar membranes remotely resembling extended, less dissected profiles of smooth endoplasmic reticulum predominate. Often the lamellae were seen contiguous to or looping around mitochondria. The lumina of the intracellular lamellae and the intercellular spaces communicate via lamellar processes that are connected to the lateral cell membrane. The lamellae were stained by incubation with ruthenium red (Wichtrup and Greven, 1986).

The second absorptive cell type is represented, inter alia, by the TACs in *X. eiseni* (Mendoza, 1972), *A. splendens* (Lombardi and Wourms, 1985b), and *X. captivus* (Fig. 9; Schindler and de Vries, 1988). Its morphological characteristics are determined by a prominent vacuolar apparatus indicative of high endocytic activity in the apical cytoplasm beneath the brush border. The endocytic complex is particularly well pronounced in the three aforementioned species. However, typical features of macromolecular transport are also present in the TACs of *A. robustus*, *I. furcidens*, and *Z. quitzeoensis* (Hollenberg and Wourms, 1994).

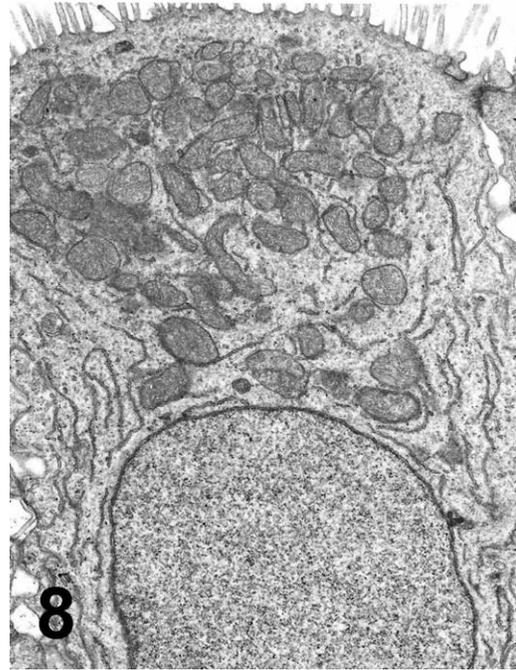


Fig. 8. *Girardinichthys captivus*: Trophotaenial absorptive cell (TAC) which appears endocytotically relatively quiescent. The supranuclear cytoplasm is crowded with mitochondria. In the lateral cytoplasm membranous lamellae are oriented in parallel to the plasma membrane, 8,200 \times .

The intermicrovillar plasma membrane of endocytosing TACs becomes invaginated at intervals to form deep pits, the bulbous ends of which pinch off as vesicles. The invaginated membrane and pinched-off vesicles are mostly clathrin coated. The external or luminal surface of invaginated membrane and vesicles is coated with amorphous fuzzy material. A large population of small vesicles and tubules occupies the apical endocytic complex. The latter form thick-walled elongate structures varying in length from short stub-like fragments to long bent tubules up to 1.5 μm in length (Schindler and de Vries, 1988). On simultaneous fixation with paraformaldehyde, glutaraldehyde, and osmium tetroxide electron dense proteins lining the inner tubular membrane produce a diagonally oriented striped pattern similar to that in several other absorptive epithelia (Hatae et al., 1986). Such dense tubules were seen in contact with small endosomal vacuoles (Fig. 10). Pleomorphic endosomes (300–800 nm in diameter) with electron-lucent lumina are located deeper in the endocytic zone. Heterogeneous electron opaque lysosome-like bodies predominantly occur in the nuclear vicinity of TACs.

Ultrastructural studies of protein uptake by trophotaeniae were initially performed using unspecific markers such as horseradish peroxidase (HRP) and CF. Whole embryos of *A. splendens* with appending trophotaeniae were incubated in

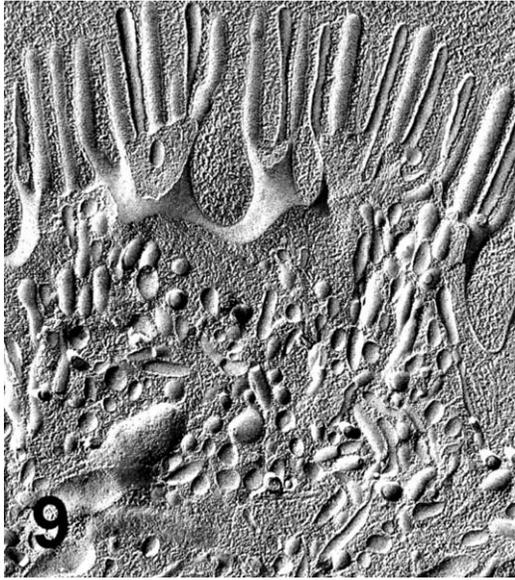


Fig. 9. *Xenoophorus captivus*: Freeze-fracture replica of the apical endocytic zone of a TAC. Deep to the prominent microvillar border a zone corresponding to the terminal web is visible. It is almost devoid of intracellular endocytic organelles yet it is penetrated by invaginated apical plasma membrane. The cytoplasm beneath the terminal web zone contains abundant tubules, vesicles, and vacuoles, 22,000 \times .

HRP-containing media for various periods of time. The tracer protein ingested by micropinocytosis was lysosomally degraded within 3 h (Lombardi and Wourms, 1985c). Exposure of trophotaeniae in *X. captivus* to the electrostatic marker CF resulted in the labeling of TACs at the microvilli tips and the cell membrane in endocytic invaginations. Incubation of 1 min resulted in labeling of pinocytotic vesicles. After 1 h CF was partly segregated within lysosome-like bodies. Incubation of trophotaeniae for 10 min in media containing both CF and HRP followed by 50 min chase in pure buffer resulted in segregation of CF and HRP in separate endosomal vacuoles (Schindler and de Vries, 1988). Experiments incubating so-called nonendocytosing trophotaeniae of *G. viviparus* with CF or

HRP nonetheless resulted in endocytic uptake of the unspecific tracer substances, though the ingestion rate was distinctly lower than in TACs possessing a well-developed endocytic apparatus (Schindler and de Vries, 1987b). The trophotaeniae of *G. atripinnis* did not ingest HRP (Hollenberg and Wourms, 1995).

Incubation studies with trophotaeniae obtained from *A. splendens* embryos revealed the capacity of the trophotaeniae to ingest proteins by a mechanism that satisfies the criteria of receptor-mediated endocytosis. Exposure of trophotaeniae to native ferritin (NF) resulted in labeling of clathrin-coated endocytic invaginations (Wichtrup and Greven, 1985). After internalization, the protein marker was transported via tubular and vesicular elements to the endosomal compartment and segregated into lysosomes (Fig. 11). The results of the latter study were somewhat astounding, since adherence of NF to the cell surface similar to receptor binding or attraction by electrostatic forces is unusual, as the marker normally enters pinocytotic vesicles in the fluid phase. Still more surprising was the finding that addition of excess bovine serum albumin (BSA), human transferrin (HTF), or other random proteins to incubation media with NF abolished NF binding to TACs (Schindler, 2003a, 2005). Thus, protein binding sites on TACs of *A. splendens* were postulated. This notion was scrutinized by incubations with diverse protein-gold complexes such as Au-BSA (Fig. 12) and Au-HTF (Schindler and Greven, 1992). Following binding to the TAC surface and internalization via clathrin-coated pits and vesicles the several ligands were routed to the lysosomal pathway with transit through the endosomal compartment. Prolonged incubation intervals led to massive intracellular accumulation of gold beads. As with NF, binding of protein-gold complexes to TACs was inhibited by the presence of excess unlabeled protein irrespective of the competitive protein chosen (Schindler and Greven, 1992). Protein binding and endocytosis in the trophotaeniae of *A. splendens* were further

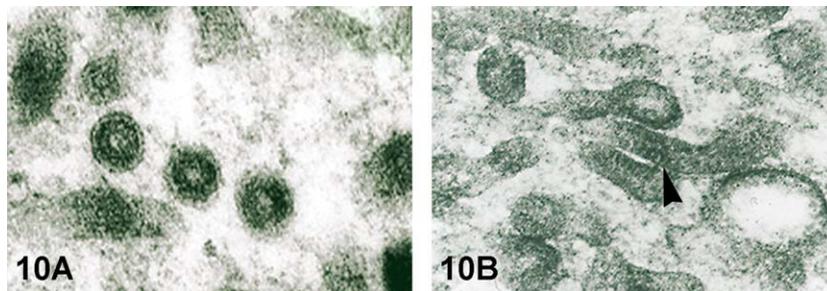


Fig. 10. *Xenoophorus captivus*: (left) Cross sections of thick-walled apical tubules. (right) Thick-walled apical tubules after simultaneous fixation with paraformaldehyde, glutaraldehyde, and osmium tetroxide show a periodical, diagonally oriented striped pattern (arrowhead), 83,000 \times . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

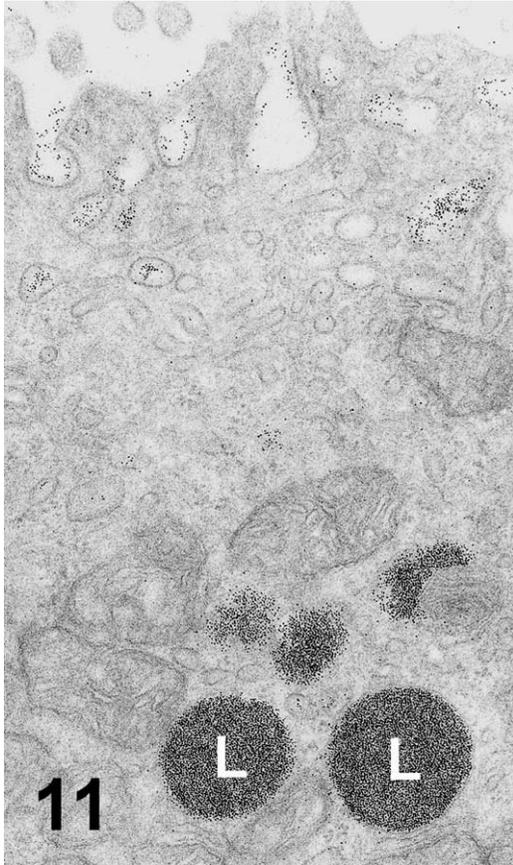


Fig. 11. *Ameca splendens*: Binding and uptake of native ferritin by a TAC after 1 h incubation. L, lysosome. No counterstain, 48,000 \times .

characterized using radioiodinated proteins and radioiodinated random copolymers (Schindler, 2003a). Radiolabels such as ^{125}I -BSA, ^{125}I -HTF, and ^{125}I -poly (Glu,Lys,Tyr) bound to the trophotaeniae in a specific saturable manner. Scatchard analysis of concentration-dependent ^{125}I -BSA binding revealed both low- and medium-affinity binding sites with apparent dissociation constants, K_{ds} , of 3.4×10^{-5} and 2×10^{-7} M, respectively (Fig. 13). Protein binding to TACs in *A. splendens* was dependent on calcium ions in the incubation media and $\text{pH} \geq 7$.

The scavenging of proteinaceous substances by TACs in *A. splendens* from circumambient fluid satisfies the criteria of receptor-mediated endocytosis. In the effort to identify the cellular receptor molecules several membrane-bound aminopeptidases (APs) became the focus of attention (Schindler, 2003b). The presence of aminopeptidase A (APA) (Fig. 14), aminopeptidase N (APN), γ -glutamyltransferase, dipeptidyl aminopeptidase IV, and neutral endopeptidase on and in TACs was shown by cleavage of, respectively, Glu- α -(4M β NA), Ala-(4M β NA), Glu- γ -(4M β NA), Gly-Pro-(4M β NA), and Gl-(Ala) $_3$ -(4M β NA). Reaction product was localized to the apical and basolateral

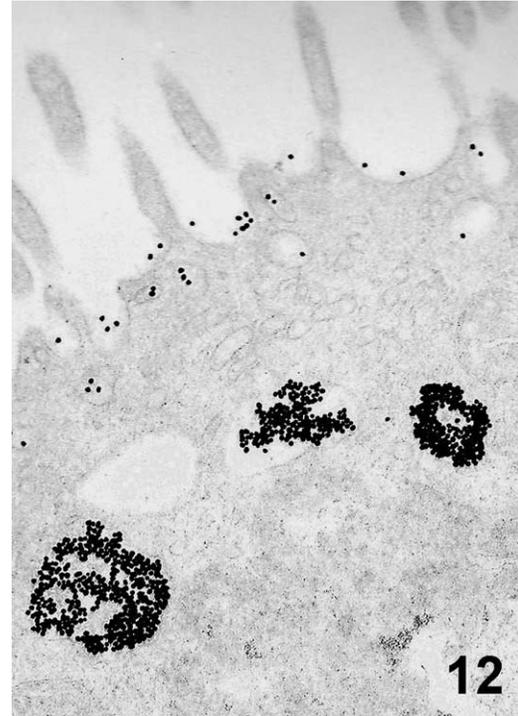


Fig. 12. *Ameca splendens*: Binding and uptake of 15 nm colloidal gold beads conjugated to bovine serum albumin (BSA) by a TAC. No counterstain. No counterstain, 43,000 \times .

plasma membrane as well as to vacuolar structures in the cell. The involvement of APs in the ingestion of proteins was evidenced by inhibition of binding and endocytosis of labeled proteins by addition of unspecific proteinase inhibitors such as diisopropylphosphofluoride, phenylmethanesulfonylfluoride, antipain, 1.10-phenylanthroline, and dithiothreitol to the incubation media. Moreover, a combination of the specific microbial AP inhibitors amastatin, bestatin, and phosphoramidon efficiently suppressed protein binding to TACs. Similarly, the protein binding to TACs was significantly reduced in the presence of 4-methoxy- β -naphthylamide (4M β NA) assay substrates of APs. The tissue-associated AP activities were modulated by the presence of proteins. Trophotaeniae were incubated in rising substrate concentrations in the absence and presence of either BSA or HTF by a method that enables results of each incubation series to be obtained from a single trophotaenia by passing it on through a series of ascending substrate concentrations. Linear transformation of the results by the method of Lineweaver and Burk yielded rectilinear relationships between reciprocal values of the velocities of AP-catalyzed reactions and the substrate concentrations. The resultant K_{m} values were 205 and 625 μM for APA and APN, respectively (Fig. 15; Schindler, 2003b).

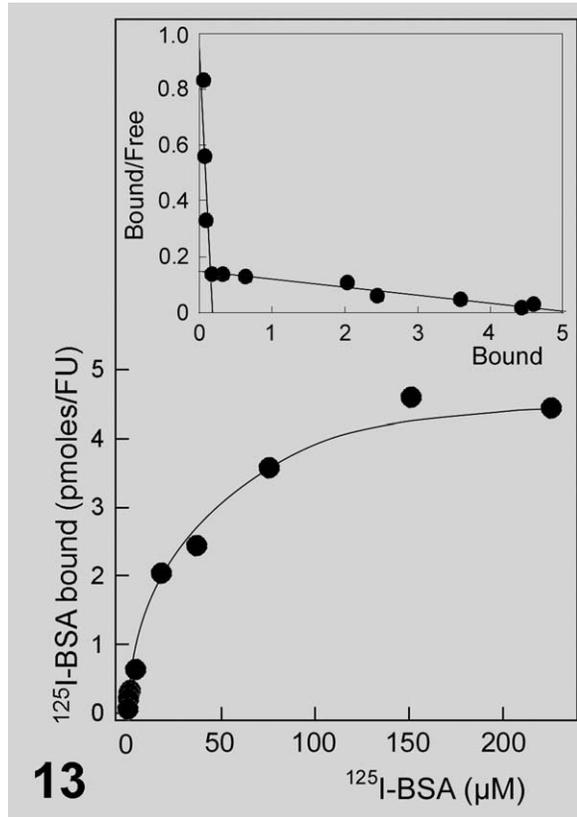


Fig. 13. *Amea splendens*: Saturation of overall ^{125}I -binding to TACs. Trophotaeniae were incubated for 20 min in buffered incubation saline (BIS) at 20°C to strip protein binding sites off adherent ligands. Thereafter, the trophotaeniae were incubated for 2 h at 0°C with a series of ascending concentrations of ^{125}I -BSA. Trophotaenia-associated radioactivity was assayed by gamma counting after rinsing in three charges (3×20 sec) of BIS. Inset: Scatchard plot analysis of the binding data. Abscissa pmoles/FU (arbitrary fluorescence unit). Ordinate $\mu\text{l}/\text{FU}$.

EMBRYONIC RESPIRATORY SURFACES: MORPHOLOGICAL EVIDENCE OF CUTANEOUS GAS EXCHANGE (SCHINDLER AND GREVEN, 2010)

The gestation of goodeids lasting several weeks renders the embryos highly dependent on maternal-embryonic gas exchange. For this purpose, many vertebrates have evolved placentae that simultaneously accommodate both absorptive and diffusive functions. Accordingly, trophotaeniae have been posited to serve a dual function in nutrient transfer and gas exchange (Turner, 1933; Mendoza, 1937).

On the other hand, the functional surface area of trophotaeniae is relatively small owing to the widely spaced subepithelial capillary network (Fig. 16). Moreover, the long diffusion distance across cuboidal to columnar brush border epithelium presumably does not allow of effective gas exchange. Given the morphological evidence it may be inferred that the function of trophotaeniae

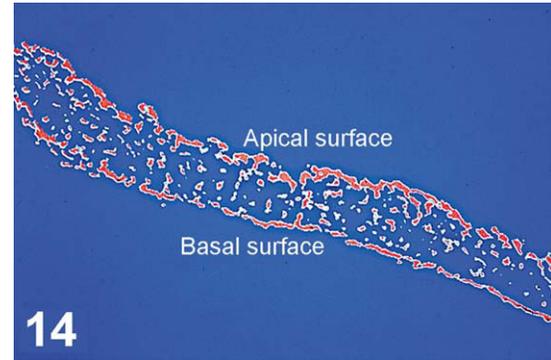


Fig. 14. *Amea splendens*: Immunohistochemical localization of aminopeptidase A activity associated with the trophotaenial epithelium. Reaction product covers both the brushborder (above) and basolateral (below) cell membrane. In addition, intracellular vacuoles are stained. Computerized image analysis, $770\times$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

is principally absorptive and that trophotaenial gas exchange does not make a major contribution to the embryos overall respiratory demands. This notion is further supported by the smallness of trophotaeniae in some species or their absence during most of the gestation period in *A. toweri*.

Cutaneous gas exchange is well characterized in several air-breathing fishes (Mittal and Datta Munshi, 1971; Grizzle and Thiyagarajah, 1987) and antarctic white-blooded fishes (Jakubowski, 1982). High respiratory capacity of scaled skin is indicated by the presence of a dense subepidermal capillary plexus on the outer surface of imbricate scales embedded in the skin. Moreover, cutaneous gas exchange is initially obligatory in vertebrate ontogeny (Rombough, 1988) and remains functional during most of gestation in several viviparous teleost embryos. With this in mind, we were prompted to study embryonic vascular and circulatory adaptations that are likely to be associated with cutaneous gas exchange (Schindler and Greven, 2010).

The basic structure of embryonic skin is similar to that in adult fish. The epidermis is a stratified epithelium of squamous filament-containing cells. It comprises two cell layers on the dorsolateral body surface increasing to several cell layers mid-ventrally. In the filament-containing cells bundles of intermediate filaments converge at numerous desmosomal junctions interconnecting cells within and between the cellular layers. Occasional chloride cells lie embedded in bulbous epidermal cavities around the vent. The cavities are connected with the free surface via narrow epidermal pores. In the epidermis of *I. furcidens* embryos unicellular glands were occasionally observed (Schindler, unpublished).

Similar to the skin of adult fish, embryonic skin is strengthened by the basement lamella, an

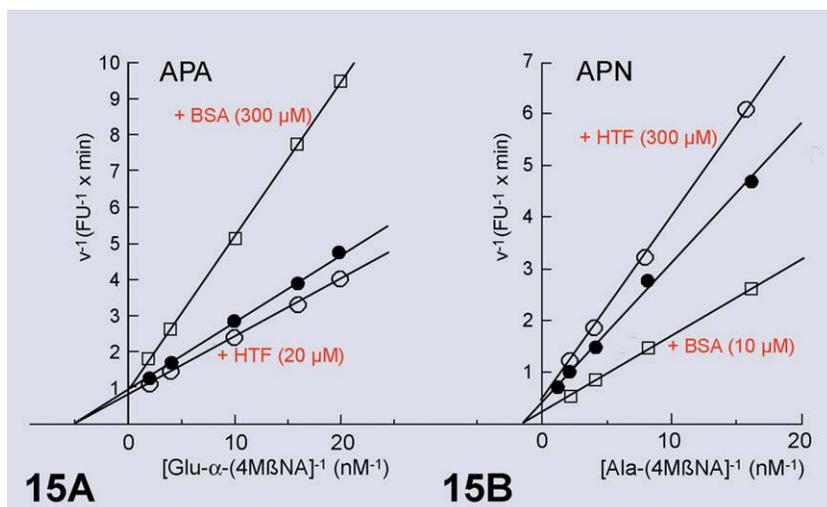


Fig. 15. *Amea splendens*: **A** and **B**, Dose-response experiments demonstrating the effect of respectively trophotaenial aminopeptidase A activity on increasing concentrations of Glu- α -(4M β NA) (filled circles) and of trophotaenial aminopeptidase N activity on increasing concentrations of Ala-(4M β NA) (filled circles) in the absence and presence of BSA, (hollow squares) and human transferrin (HTF, hollow circles). One trophotaenia was each passed through 500 μ l portions of media. The starting media were prepared in duplicate and the first measurement discarded. Incubation intervals were 10 min, with a 30-second intermediate rinsing in buffered incubation saline (BIS). The medium was constantly agitated and the temperature maintained at 20°C. Aliquots (10 μ l) of the incubation media were diluted 100fold with BIS and then the content of the 4M β NA reaction product determined fluorometrically. The data are plotted following the method of Lineweaver and Burk. FU = standard fluorescence units. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acellular compact dermal stratum of orthogonally arrayed collagen fibrils. The basement lamella of fish skin was originally described as plywood-structured, implying the layers of collagen fibrils to lie parallel to the epidermal basement membrane. However, this theory was challenged by the results of a morphological study of the skin in developing stages of *Fundulus heteroclitus*, the findings of which were in favor of a "shingle" theory. Collagen fibril layers were observed to descend at a slight angle (Nadol et al., 1969).

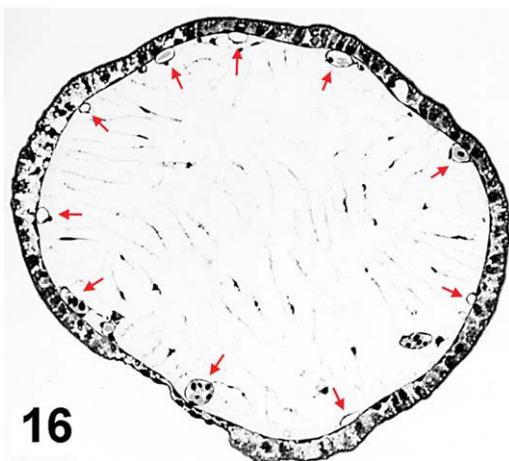


Fig. 16. *Xenoophorus captivus*: Cross section through a trophotaenia exhibiting a relatively widely spaced capillary network (arrows) beneath the cuboidal epithelium, 80 \times . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The epidermis in goodeid embryos excepting the genus *Girardinichthys* is in immediate contact with a dense-meshed capillary plexus (Schindler and Hamlett, 1993; Schindler and Greven, 2010; Fig. 17). Similarly, in various species of seaperch undergoing intraluminal gestation a dense capillary bed is formed closely apposing the epidermis. It is maintained as long as scales are absent. With the formation of scales, however, the near-surface vascularization disappears (Webb and Brett, 1972). By contrast, in goodeid embryos the capillarization outside the imbricate scales does not regress in the whole gestation period. Even in adult fish remnants of it continue to exist deep to the then multilayered epidermis (personal observation). Highly attenuated cytoplasmic processes of filament containing cells that are almost devoid of organelles cover the capillaries. More voluminous segments of the cells harboring the nucleus and most organelles are recessed between the meshes of the capillary net. The genus *Girardinichthys* is distinguished from other genera by lack of dermal scales until birth. Moreover, their dermal capillary plexus lies deep to the basement lamella, which is in close contact to the epidermal basement membrane (Fig. 18).

The blood-embryotrophe barrier in scaled skin comprises the flattened cytoplasm of epidermal filament containing cells, the capillary endothelium, and the basal lamina common to both epithelia, resulting in a minimum diffusion distance less than 1 μ m, equal or less than that of the pulmonary blood-air barrier in amphibians, reptiles, and

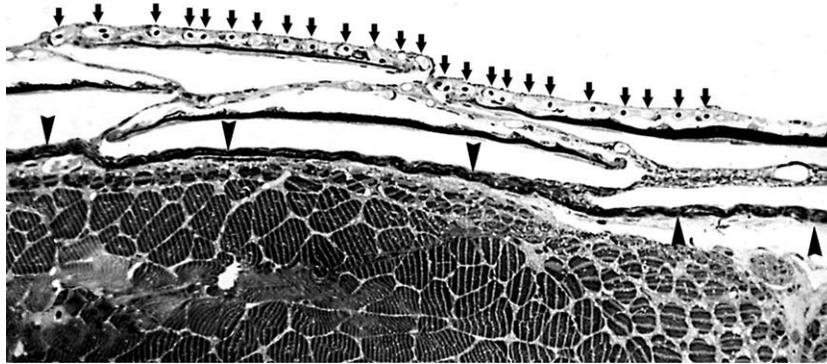


Fig. 17. *Ataeniobius toweri*: Scaled skin on the lateral body wall. Very thin epidermis covers a close-meshed microvascular network (arrows). The basement lamella (arrowheads) lies deep to the scales, 300 \times .

mammals, inclusive humans (Meban, 1980). In *Girardinichthys* spp. the collagenous basement lamella adds to the thickness of the blood-embryotrophe barrier, resulting in a diffusion distance of $1.9 \pm 0.5 \mu\text{m}$ in most of the skin.

The rate of oxygen consumption by the embryos increases steadily during prolonged gestation of approximately 7 weeks. However, profusely perfused blood vessels in a near-surface location inevitably entail trade-offs. Exposure of delicate blood vessels to environmental hazards necessarily contrasts with the skin's fundamental functions such as protection from mechanical injury, thermoregulation, and prevention of pathogen incursion. Incurring threats must, therefore, be offset by substantial survival advantage, and this may well be the case in goodeids, as being born in an advanced developmental stage is associated with the reduction of death rate during a risky start to free-living in the water.

EMBRYONIC VASCULAR SYSTEM: ADAPTATIONS PERTAINING TO PLACENTAL EXCHANGE FUNCTION (SCHINDLER AND GREVEN, 2010)

The bulk cardiac output is distributed from the ventral aorta by way of afferent branchial arteries to the gill arches. These vessels lead into the blood channels of the primary and secondary lamellae that were not fully developed at the embryonic stages examined. On serial sections of *I. furcoidens* embryos the ventral aorta was followed beyond the branchial bifurcations. This aortic extension tapered to a relatively small blood vessel that ramified within the mandibular skin giving rise to a net of subepidermal microvessels.

The bilateral efferent branchial arteries unite ventrally to form the dorsal aorta. Vessels that branch off from the dorsal aorta traverse the

muscular body wall and feed the embryonic skin with blood. Side branches of these arteries ramifying in the musculature were only rarely observed. Red blood cells could be localized in the strands of filmy connective tissue between adjacent muscular compartments at infrequent intervals.

Trophotaeniae integrate tissues of endodermal and ectodermal origin. Thus, the blood supply of the processes is derived both from the intestinal and the cutaneous circulation. Trophotaenial arteries constitute the terminal branches of the mesenteric artery, which branches off from the common coeliaco-mesenteric artery a short distance downstream from its origin at the confluence of the bilateral efferent branchial arteries (Mendoza, 1937). Terminal branches of the mesenteric artery penetrate the muscular coat of the gut near the anal opening and traverse the trophotaenial peduncle embedded in submucosal tissue. The ramifications of these trophotaenial arteries form a relatively wide-meshed sinusoidal network underlying both epithelial types of the processes.

At the coeliaco-mesenteric artery orifice intimal flaps form a valvular structure (Fig. 19). It may curtail blood flow to the digestive system of the embryos that is quite inactive. The common coeliaco-mesenteric artery as well as the ventral aorta is distinct from all other arteries by possessing a thin muscular layer.

Both the valvular device and the muscular coat may act to shunt blood away from the coeliaco-mesenteric pathway, thus enhancing the blood flow to the cutaneous circulation. This notion was substantiated on histological inspection: the common coeliaco-mesenteric artery actually appeared rather devoid of red blood cells. Due to the very thin embryotrophe-blood barrier in the embryonic skin, increasing the blood flow through the subepidermal capillaries may be an appropriate means to improve respiratory gas exchange.

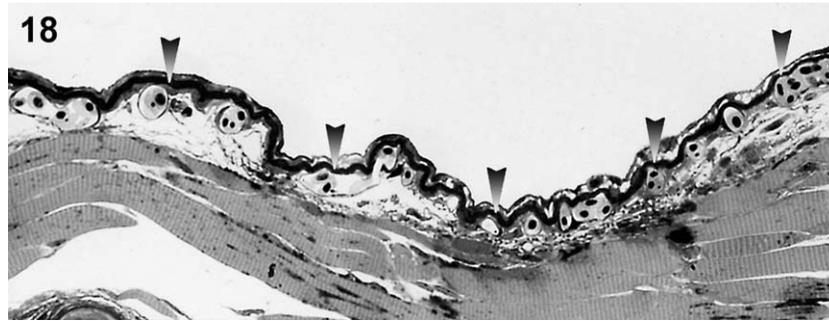


Fig. 18. *Girardinichthys viviparus*: Cross section of embryonic skin at the level of the hind gut. The collagenous basement lamella (arrowheads) is situated outside a dense meshed capillary plexus. Scales are totally absent, 370 \times .

Blood is also supplied to the trophotaeniae from the capillary plexus underlying the ventral epidermis. Extensions of cutaneous microvessels pass through the trophotaenial peduncle immediately beneath the epidermal surface (Fig. 20). The cutaneous and trophotaenial microvessels blend into each other. In this way, partly deoxygenated blood from the trophotaenial arteries becomes enriched by freshly oxygenated blood from the skin. The augmented oxygen content of trophotaenial blood may result in improved utilization of absorbed nutritive substances by the embryo.

The trophotaeniae are drained by veins that traverse the trophotaenial peduncle peripherally to the arteries. Two major veins enter the rear abdominal cavity ambilaterally, run through the mesenteries of the hind gut and fuse midventrally to form an abdominal vein, which drains blood into the portal circuit. In addition, the portal system receives blood from the body wall. Cutaneous veins that traverse the abdominal musculature and penetrate the parietal serous membrane drain into the trophotaenial/abdominal veins. The embryos in *A. toweri* that lack trophotaeniae, veins collecting blood from the skin of the anterior abdominal wall penetrate the serous membrane and drain into abdominal veins that converge to form the hepatic portal system (schematically simplified in Fig. 21).

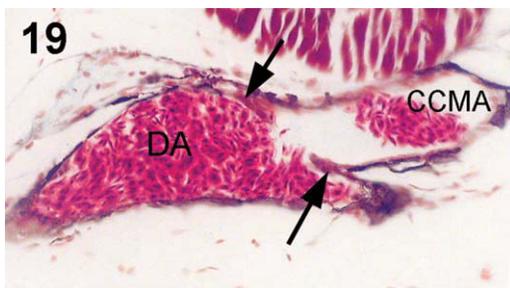


Fig. 19. *Ilyodon furcidens*: At the common coeliac-mesenteric artery (CCMA) orifice intimal flaps (arrows) form a valvular structure. DA, dorsal aorta, 80 \times . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

THE BIOLOGY OF THE TROPHOTAENIAL PLACENTA IN THE GOODEIDAE IS A MULTIFACETED FIELD OF MORPHOLOGICAL AND EXPERIMENTAL RESEARCH

Viviparity is not widespread among teleost fishes. This reproductive mode occurs in no more than 510 of approximately 18,000 species of teleosts (Wourms, 1981). In its broadest sense, viviparity is defined as the birth of life young without regard to the source of nutrients for embryonic development. Wourms divides viviparous fishes between lecithotrophes (embryos exclusively yolk dependent) and matrotrophes (embryos additionally or nearly exclusively supplied with maternal nutrients). The latter remarkably advanced reproductive strategy is found in all species of the family Goodeidae. It is characterized by a trophotaenial placental including secretory, absorptive, and gas exchange surfaces such as the IOE, the trophotaenial absorptive epithelium, and the embryonic integument exhibiting a striking diversity of histological and cytological modifications. In the absence of fusion of embryonic and maternal tissues, there is no close relationship of embryonic and maternal blood circulation systems. Thus, maternal nutrients are transferred via



Fig. 20. *Ilyodon furcidens*: Capillaries derived from the cutaneous vascularization (arrows) traverse the trophotaenial peduncle. T, trophotaenia, 125 \times . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

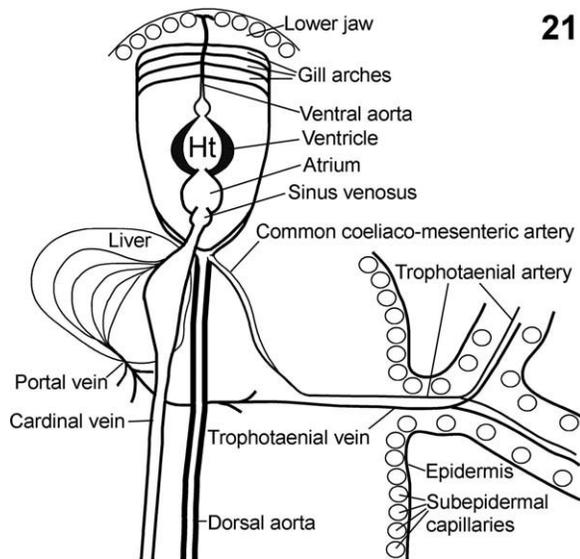


Fig. 21. Schematic representation of vascular anatomy in goodeid embryos pertaining to placental nutrition and gas exchange. The ventral aorta, which takes blood from heart (Ht) to gill arches, extends beyond the branchial bifurcations. In the lower jaw the tapered artery gives rise to a subepidermal microvascular plexus. The arterial supply of trophotaeniae is from the common coeliaco-mesenteric artery, which branches off at the root of the dorsal aorta. In addition to trophotaeniae arteries, microvessels that are continuous with the subepidermal capillary bed enter the trophotaenia peduncle providing supplemental oxygenated blood to the trophotaenia circuit. Most of the venous drainage of trophotaeniae is into the portal circulation via an abdominal vein.

embryotrophe secreted by the IOE and absorbed by the TACs.

The main emphasis of our and other researchers' morphological and experimental work lay on the identification of the source of intact proteins in the nutritive secretion, their utilization by the embryos, and the identification of embryonic sites of gas exchange. These issues were addressed by using light microscopy, transmission and scanning electron microscopy, freeze-fracture techniques, electrophoretic techniques, Au-conjugated tracers, radioiodinated labels, enzyme histochemistry, and fluorescence spectrophotometry. In studying the embryonic integument including the trophotaenia epithelium with regard to morphologic correlates of respiratory function using transmission light and electron microscopy it became evident that goodeid embryos seem to rely primarily on cutaneous gas exchange. This inference is based on the presence of a dense-meshed capillary net underlying the squamous epidermal epithelium resulting in diffusive distances of mostly less than 1 μm (Schindler and Greven, 2010). Ultrastructural characterization of the IOE and even more convincingly electrophoretic analyses indicated the translocation of maternal plasma proteins into the embryotrophe (Schindler et al., 1988; Schindler, 1990; Hollenberg and Wourms, 1995). On the

other hand, it cannot be entirely ruled out that some of the secreted proteins in the embryotrophe are synthesized in the IOE (Hollenberg and Wourms, 1995). The major characteristic of TACs is the presence or absence of an extensive apical endocytic apparatus (Mendoza, 1972; Lombardi and Wourms, 1985b; Schindler and de Vries, 1986a; Schindler and de Vries, 1987a; Schindler and Kujat, 1990). Nonspecific endocytosis of macromolecules may be a universal cellular mechanism. It was shown to occur in TACs even in the absence of a distinct endocytic apparatus (Schindler and de Vries, 1987b; Schindler and Kujat, 1990). On the other hand, extensive endocytic activity (in particular receptor mediated endocytosis) is a process by which cells attain specific functional traits. First and foremost, the absorption of intact proteins by placental exchange surfaces is thought to have an immunological role but has not been shown to serve a nutritive function. This may be different in the trophotaenia placenta (Schindler, 2005). Most intriguing was the finding that the TACs in *A. splendens* indiscriminately absorb a whole host of different proteins bound to APs (Schindler, 2003a,b). Although it is well established that APs, in particular APN, are widely expressed in animals and plants with functions seemingly unrelated to their APs activity, there seems to be little evidence, if any, for interactions of APs with other proteins serving the endocytic uptake of nutritive macromolecules (Mina-Osorio, 2008; Chen et al., 2012). To the best knowledge of the author, this phenomenon is thus far only described in the trophotaenia placenta of *A. splendens*, an issue that warrants further study.

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