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Ultrastructural evidence for nutritional exchange between brooding unionid mussels and their glochidia larvae

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Abstract. The life history of unionid bivalve molluscs includes retention of developing embryos within the gills of parental mussels. This brooding behavior may facilitate nutrient transfer to the glochidia larvae, i.e., matrotrophy. To address this possibility, morphological relationships between brood chambers and developing larvae of *Pyganodon cataracta* and *Utterbackia imbecillis* were examined with TEM, and larval shells were observed with SEM, for features that could be associated with the uptake of dissolved materials. Early in brooding, glochidia are enclosed in a vitelline membrane that physically contacts numerous cilia and microvilli of the epithelial cells lining the brood chamber (marsupium). The vitelline membrane subsequently disappears. Lamellar tissues of parental mussels initially have large deposits of glycogen that diminish during the course of brooding. Septa separating brood chambers from adjacent secondary water tubes have numerous mitochondria and microvilli, suggesting the potential for active transport of materials into or out of the marsupia. Since punctae (pores) in the larval shells become filled with an organic matrix early in brooding, they are unlikely to be involved in nutrient exchange. Ultrastructure of the brood chamber and physical contact between the parental mussel and larvae are consistent with a nutritive role for retention of glochidia in the marsupia.

Additional key words: Bivalvia, *Utterbackia, Pyganodon,* matrotrophy, gills

In the freshwater molluscan family Unionidae, fertilization occurs within the gills of the female, and resulting embryos are sequestered within primary water tubes of the demibranchs that then become marsupia or brood chambers. Development results in the formation of a shelled larva, the glochidium. When mature, the glochidia are discharged from the marsupia and typically require attachment to a host fish for further development and metamorphosis to a juvenile mussel (Kat 1984; Watters 1997; Watters & O'Dee 1998).

The region of the gill involved in larval brooding undergoes extensive morphological change in accommodating 300,000–3,000,000 glochidia for a few weeks to several months (Coker et al. 1921). As the gill becomes packed with maturing glochidia, it swells and darkens (Richard et al. 1991; Tankersley & Dimock 1993a). In marsupial demibranchs, a pair of secondary water tubes develops at the lateral and medial margin of each primary water tube which becomes capped dorsally, isolating the glochidia from water circulating within the mantle cavity (Tankersley & Dimock 1993a; Tankersley 1996). The secondary water tubes serve as temporary lumina that compensate for the loss of irrigation through the primary water tubes, which ceases during the brooding season (Tankersley & Dimock 1992). Brooding adults of *Pyganodon cataracta* (SAY 1817) have been shown to be less efficient than non-brooders with respect to the capture and retention of particles during suspension feeding (Tankersley & Dimock 1993b).

In *P. cataracta,* as in other anodontine mussels (Heard 1975; Richard et al. 1991), the interlamellar septa in the brooding demibranchs are far more numerous than those in the non-brooding demibranchs of females, or the gills of males (Tankersley & Dimock 1992). The additional septa in the outer demibranch constitute the only sexually dimorphic feature of this dioecious species.

These septa greatly increase the internal surface area in the brooding gill, and their epithelia reportedly are infiltrated with leukocytes and other blood cells, and contain large stores of organic materials (Lefevre & Curtis 1912). The arrangement of the larvae within the

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brood chambers appears to include contact with parental tissues (Tankersley & Dimock 1992), providing a physical surface for potential physiological and nutritional exchange. In addition, both the internal and external surfaces of glochidial shells have numerous pores or punctae (Giusti 1973; Robertson & Coney 1979; Castilho et al. 1989; Lasee 1991). Whether the punctae remain open or are sufficiently permeable to facilitate the uptake and use of materials from the parental mussel has not been fully examined.

Wood (1974) showed that ^{14}C from labeled algae ingested by a brooding mussel subsequently appeared in the developing glochidia. Similarly, calcium stored within parental gills prior to the reproductive season is transferred to glochidia, accounting for 90% of the total calcium in the fully developed glochidial shell (Silverman et al. 1985, 1987). Since the gills of various bivalves also can take up dissolved organic materials from the ambient medium (Jørgenson 1976; Pequignat 1973; Wright et al. 1989), translocation of various molecules via the gills could supplement the nutritional requirements of glochidia.

The present study examines aspects of brooding in two unionid mussels, *Utterbackia imbecillis* (SAY 1829) and *Pyganodon cataracta*. The structure and function of marsupial chambers and their intimate association with the larvae have been examined to explore the possibility of physiological or nutritional exchange between parental mussels and developing glochidia, that is, the occurrence of matrotrophy.

Methods

Collection and maintenance of animals

For histological and microscopic examination the hermaphroditic mussel *U. imbecillis* (6.5 cm mean shell length) was collected from Davis' Pond (Mecklenburg County, NC), Litcher's Pond (Forsyth County, NC), and Shore's Pond (Yadkin County, NC) between October 1996 and January 1998. Males and females of *P. cataracta* (15.1 cm mean shell length) were collected from Meyer's Pond (Forsyth County, NC) and Shore's Pond (Yadkin County, NC) during the same period. All animals were used within 2 wk of collection and were held at seasonally appropriate temperature and photoperiod.

Preparation of tissue for TEM

Excised gills were placed in aqueous 2% glutaraldehyde with 0.1 mM EDTA to decalcify glochidial shells and facilitate sectioning, and were kept at 4°C overnight before being washed in 0.2 M Sorenson's phosphate buffer (Meek 1976). Tissues were post-fixed in 2% osmium tetroxide for 1 hour before dehydration in a graded series of ethanols. For one procedure requiring the negative staining of proteins, 1% phosphotungstic acid in EtOH was added to the last dehydration step as outlined by Meek (1976). All tissues were held overnight in Spurr's resin (1:1 by vol with absolute EtOH) to begin infiltration. The resin mixture then was changed twice over a 6 h period with pure resin and cured overnight at 60°C. Tissue was sectioned on a Reichert-Jung Ultracut-e ultramicrotome at 100 nm, stained with uranyl acetate and lead citrate, and examined on a Phillips 400 transmission electron microscope.

Preparation of tissue for light microscopy

Dissected gills were fixed overnight in ethanol-formalin-acetic acid or Bouin's fixative. Samples fixed with Bouin's were cleared with multiple changes of 70% EtOH. All tissues were subsequently dehydrated in a graded series of ethanols, cleared with xylene or cedar wood oil, and embedded in paraffin (Paraplast; MP 56°C). Serial frontal sections were cut at 8–10 μm, mounted on glass slides using Haupt's adhesive or Meyer's Albumin, and were stained with hematoxylin and eosin or Mallory's triple stain, as outlined by Humason (1962).

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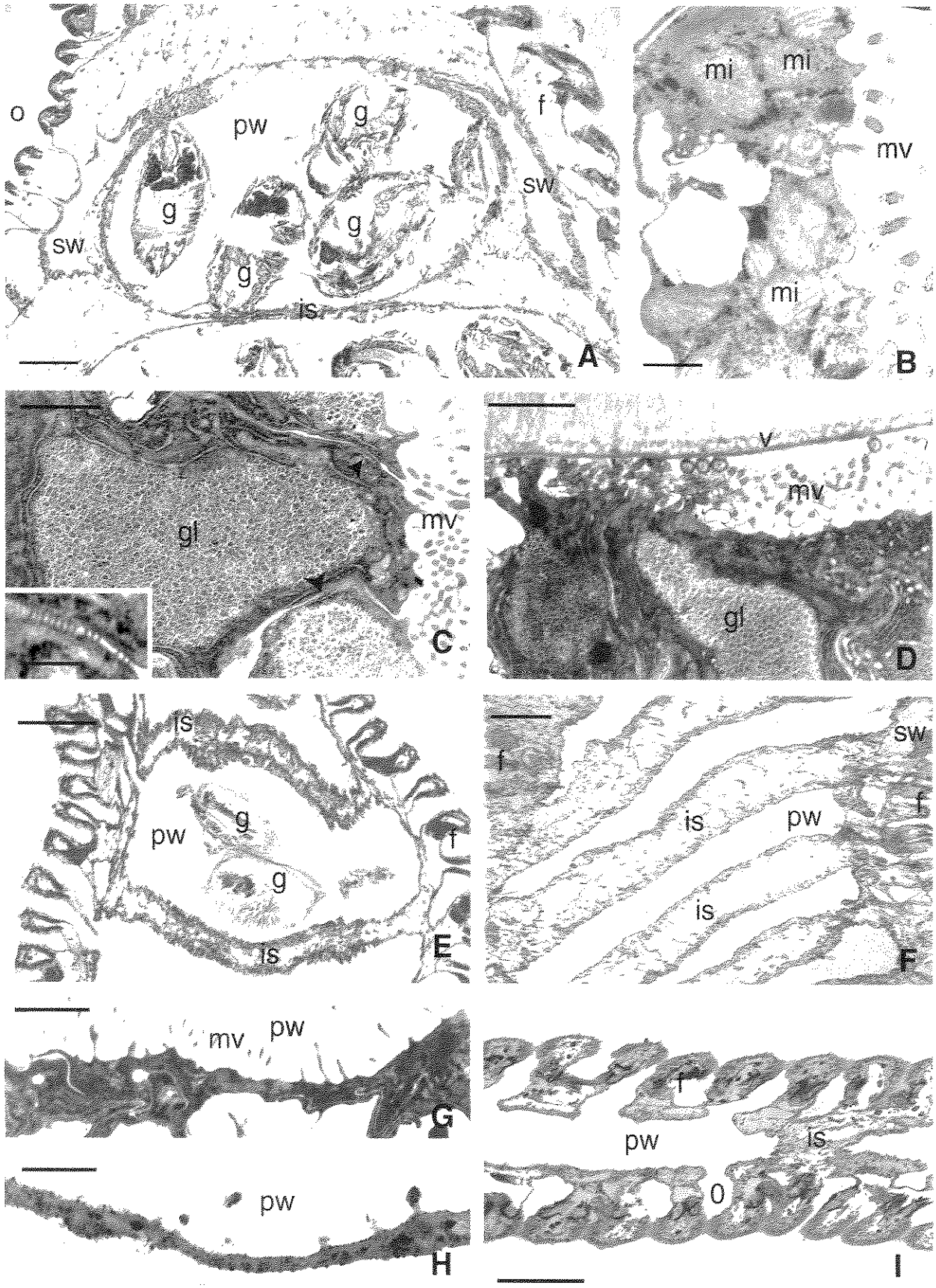
Fig. 1. Light microscopy and TEM of inner and outer demibranchs of *Utterbackia imbecillis*. f, filaments; g, glochidium; gl, glycogen; is, interlamellar septum; mi, mitochondrion; mv, microvilli; o, lumen of ostium; pw, primary water tube; sw, secondary water tube; v, vitelline membrane; arrow heads, septate junction. **A.** Light micrograph of gravid mussel. Frontal section. Scale bar, 50 μm. **B.** TEM of the secondary water tube septum showing microvilli (mv) and mitochondria (mi). Scale bar, 0.3 μm. **C.** TEM of an interlamellar septum cell with microvilli (mv) and glycogen deposits (gl). Scale bar, 1 μm. Inset: septate junction. Scale bar, 0.2 μm. **D.** TEM showing contact between interlamellar septal tissues and the vitelline membrane (v) of the glochidium. Scale bar, 5 μm. **E.** Frontal section of the outer demibranch at the time of release. Note presence of hemocytes between interlamellar septa. Scale bar, 100 μm. **F.** Frontal section of outer demibranch post release. Scale bar, 100 μm. **G.** TEM of the interlamellar septa tissue post release. Note the loss of glycogen deposits. Scale bar, 4 μm. **H.** TEM of the interlamellar septum in a non-brooding demibranch. Scale bar, 2.2 μm. **I.** Light micrograph of the non-brooding demibranch. Scale bar, 50 μm.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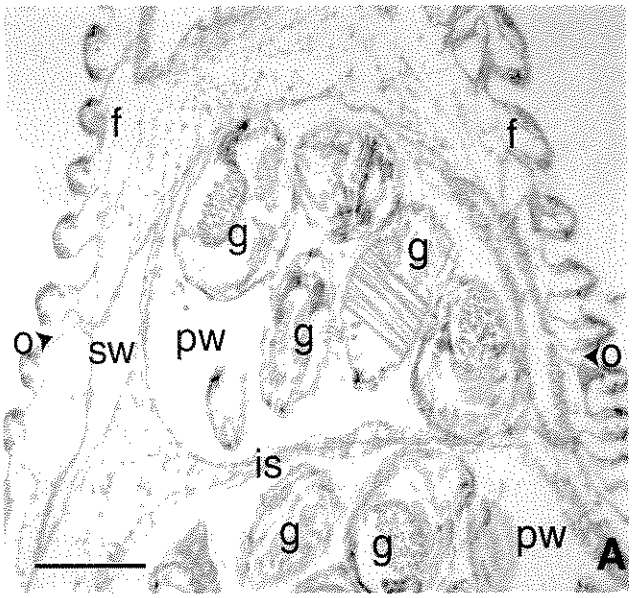
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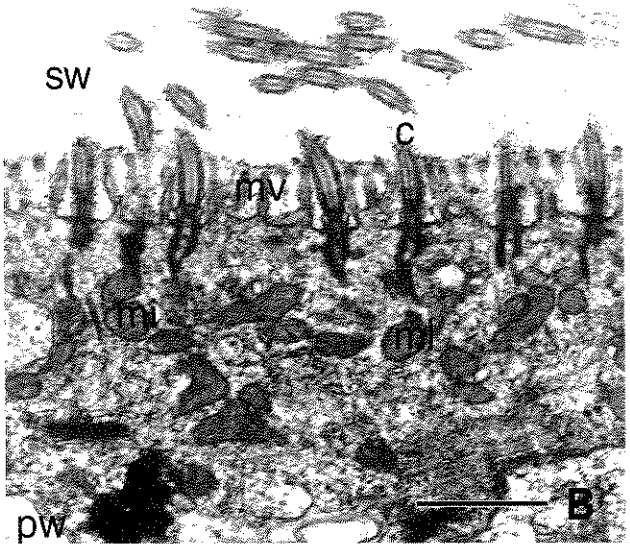
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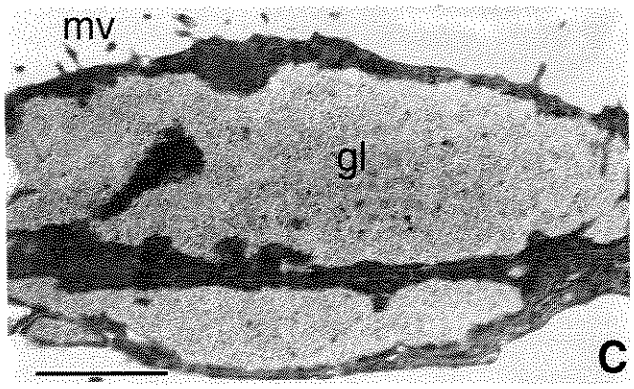
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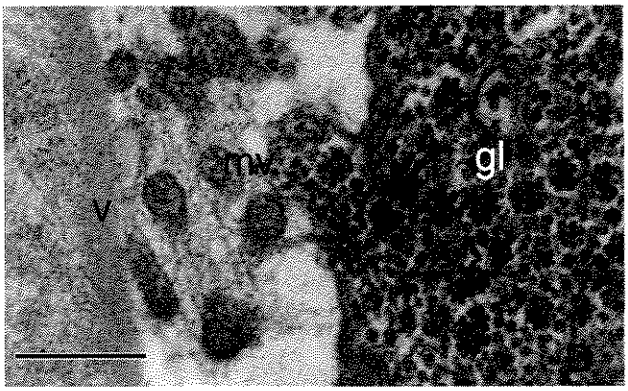
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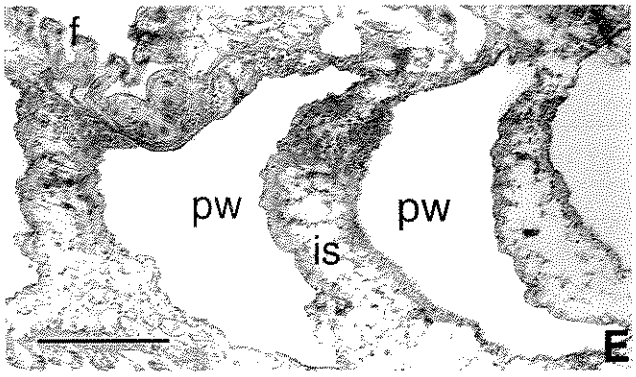
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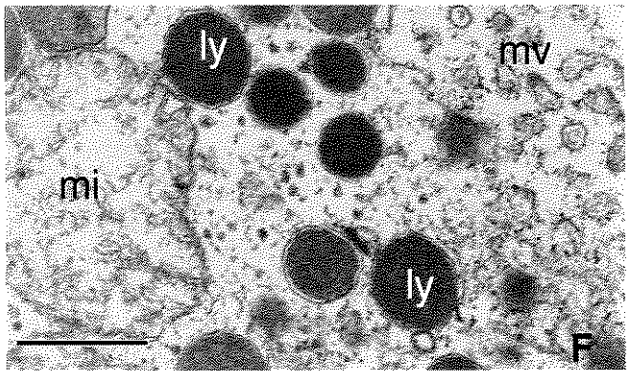
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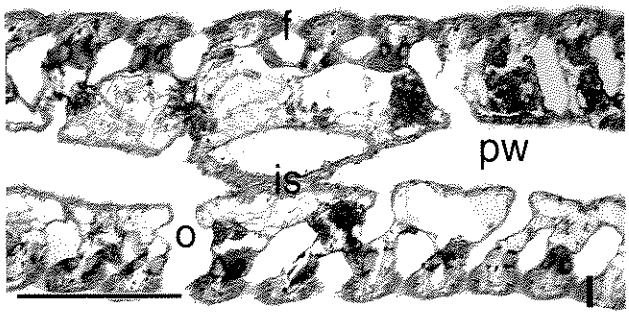
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Preparation of glochidial shells for SEM

Glochidia were placed in 0.01 N NaOH overnight to remove soft tissue as suggested by Calloway & Turner (1978). Shells were washed in several changes of distilled water, dehydrated in a graded series of ethanols, and dried in a Pelco CPD-2 critical point drier. They were mounted on aluminum stubs, sputter-coated with gold-palladium (Pelco Model SC-4), and examined on an Amray 1810 scanning electron microscope operated between 20–30 kV.

Results

Changes in gill morphology

Glochidia larvae of *U. imbecillis* and *P. cataracta* develop in the primary water tubes of the entire outer demibranchs. Each primary water tube has a secondary water tube at its lateral and medial margin, the septa of which serve to enclose glochidia within the resulting marsupial (brood) chamber (Figs. 1A, 2A). Early in development, the glochidia of both species were ~120–130 μm in diameter and were surrounded by a vitelline membrane and egg cytoplasm. By about midway in development, the vitelline membrane and egg cytoplasm disappeared, and the glochidium had a fully formed shell. Mature glochidia had a shell length of ~250 μm for *U. imbecillis* and ~380 μm for *P. cataracta.*

In all stages of the brooding period that were examined, the septa of the secondary water tubes of both species had a high density of mitochondria, and the surface facing the lumen of the secondary water tube was covered with microvilli (Figs. 1B, 2B). In *P. cataracta,* this surface included multiciliated cells (Fig. 2B). When the glochidia of *P. cataracta* were mature, this septum also contained numerous putative primary lysosomes (Fig. 2F).

In both *U. imbecillis* and *P. cataracta,* the cells of the interlamellar septa forming the transverse boundaries of the brood chambers are held tightly together by septate junctions, and early in brooding were filled with large deposits of glycogen (Figs. 1C, 2C). The septa were covered with numerous branched and unbranched microvilli and some cilia (Figs. 1D, 2D) that increased the surface area and appeared to provide physical contact between glochidia and parental tissues (Figs. 1D, 2D).

Following release of the glochidia, the interlamellar septal tissues became greatly distended and infiltrated with hemocytes (Figs. 1E, 2E). In animals that were examined at ~1 wk post-release, the glycogen deposits that previously had been extensive in this tissue were greatly reduced (Figs. 1F, 2G). In contrast to the outer demibranchs, the non-brooding inner demibranchs did not undergo morphological changes during the brooding season and had not accumulated glycogen (Figs. 1G, H, 2H, I).

Changes in glochidial shell structure

In both *U. imbecillis* and *P. cataracta,* the glochidial shell is subtriangular and has a straight hinge line (Figs. 3A, B, C, 4A, B, C). Shells of *P. cataracta* are somewhat more inflated than those of *U. imbecillis.* Hooks with cuticulae are present on the ventral margin of each valve and slide past each other as the shells close (Figs. 3A, C, 4A, C). Numerous punctae cover both the interior and exterior surface of the shell (Figs. 3D, E, 4D, E). Early in development these pores appeared to be open except for a thin pellicle on the outer surface of the shell (Fig. 3F, 4F). The punctae on the exterior surface at the dorsal margin of the valves seemed to be associated with the hinge ligament (Fig. 3G). When shells of mature glochidia were negatively stained with phosphotungstic acid and decalcified, an organic matrix was seen to extend from the overlying pellicle through each puncta to the interior surface of the shell, where it continued as a sheet of organic material (Fig. 4G).

Discussion

In both *Utterbackia imbecillis* and *Pyganodon cataracta,* the entire outer demibranch is utilized for lar-

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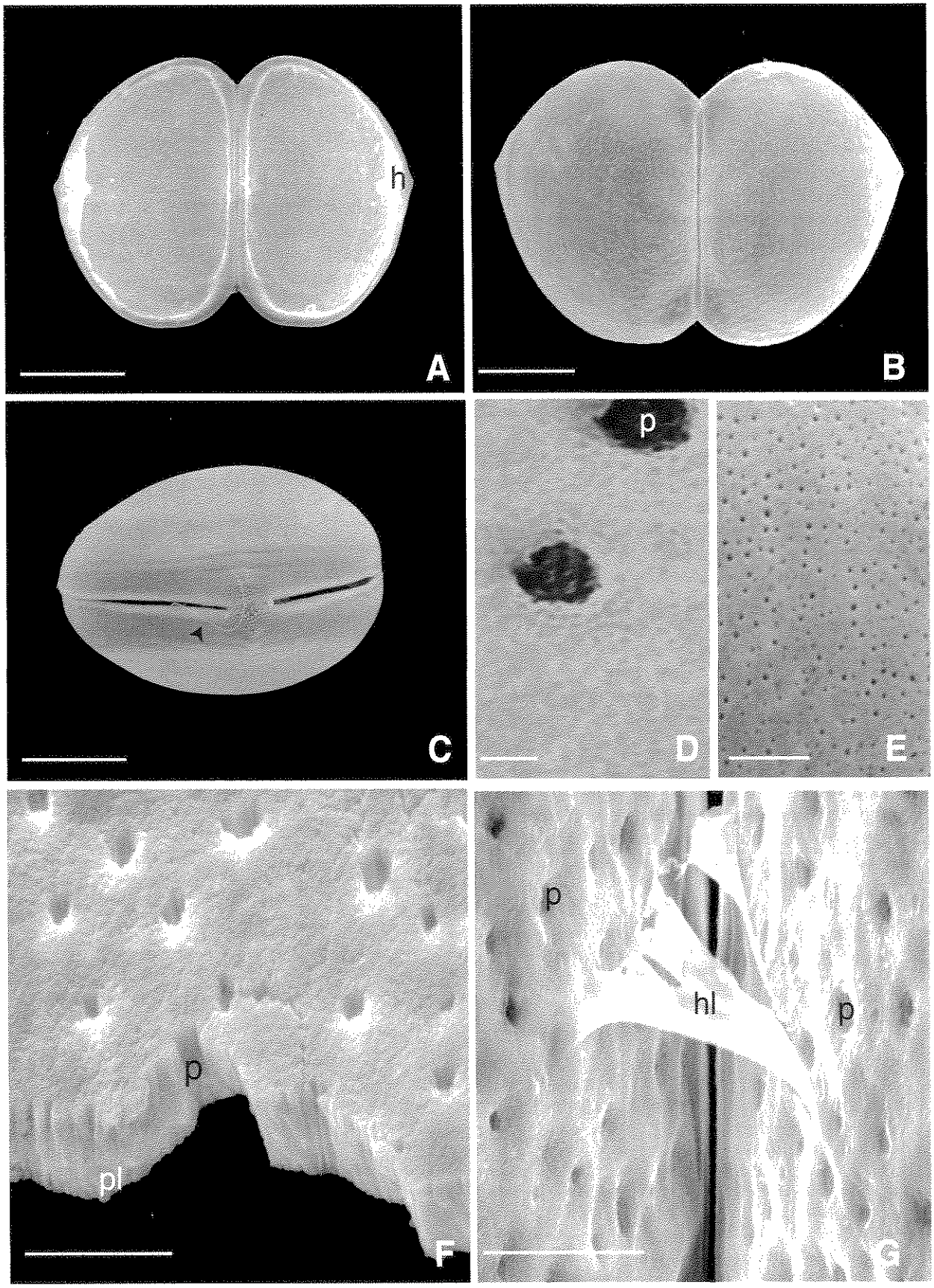
Fig. 2. Light microscopy and TEM of inner and outer demibranchs of *Pyganodon cataracta.* c, cilium; f, filaments; g, glochidium; gl, glycogen; is, interlamellar septum; ly, lysosome; mi, mitochondrion; mv, microvilli; o, lumen of ostium; pw, primary water tube; sw, secondary water tube; v, vitelline membrane. **A.** Frontal section of outer demibranch. Scale bar, 50 μm. **B.** TEM of the secondary water tube septum showing microvilli (mv), cilia (c) and mitochondria (mi). Scale bar, 1 μm. **C.** TEM of two interlamellar septum cells with microvilli (mv) and glycogen deposits (gl). Scale bar, 5 μm. **D.** TEM showing contact between interlamellar septal tissues with their glycogen deposits and the vitelline membrane (v) of the glochidium. Scale bar, 1 μm. **E.** Frontal section of outer demibranch post release. Scale bar, 50 μm. **F.** TEM of the interlamellar septa tissue post release. Note the loss of glycogen deposits. Scale bar, 0.25 μm. **G.** TEM of the interlamellar septum in the non-brooding demibranch. Scale bar, 5 μm. **H.** TEM of the interlamellar septum of the non-brooding demibranch. Scale bar, 2.2 μm. **I.** Light micrograph of the non-brooding inner demibranch. Scale bar, 50 μm.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val brooding in addition to the routine functions of the gill for respiration, suspension feeding, and ion exchange. Speculation on the functional significance of brooding in the reproductive biology of unionid bivalves has included a mechanism for embryonic nourishment, shielding developing larvae from environmental stress, and the retention of mature larvae until conditions are favorable for their release (Sellmer 1967; Wood 1974; Kat 1984; Silverman et al. 1987; Richard et al. 1991; Tankersley & Dimock 1992). Davis & Fuller (1981) suggested that brooding was an adaptation facilitating the transition of bivalves from marine to freshwater habitats by preventing larvae from being swept downstream.

Following fertilization and until attachment to a fish host, glochidia would have to be lecithotrophic unless there were additional nutritional resources in the brood chamber environment that could be utilized. Evidence presented here suggests that brooding may provide nutritional and physiological support for larvae within the marsupium, and isolates them from potentially unfavorable conditions presented by the freshwater environment. The presence of secondary water tubes provides an avenue for irrigation of the outer demibranchs, and their septa contribute to isolation of the developing glochidia from direct exposure to mantle cavity water (Silverman et al. 1987; Richard et al. 1991; Tankersley & Dimock 1992). This has been shown to protect glochidia from pollutants (Jacobson et al. 1997), and may also protect them from osmotic stress or other environmental factors such as fluctuating pH.

The surface of the septum facing the lumen of the secondary water tube is covered with microvilli, and the cells of this tissue contain a high density of mitochondria (Figs. 1B, 2B). This morphology is consistent with a role of the septum in active transport, a well known function of the gills of freshwater mussels (Dietz 1978; Pynnonen 1991) and for which the requisite enzymes have been demonstrated in gills of the unionid *Ligumia subrostrata* (SAY 1831) (Kays et al. 1990). This septum also might take up hydrogen ions to activate primary lysosomes (Fig. 2F) in the degradation of the septum, following release of the glochidia as the demibranch returns to its non-brooding morphology.

Although absent from the septa of the non-brooding demibranchs (Figs. 1G, H, 2H, I), large deposits of glycogen were present in the interlamellar septa of both *U. imbecillis* and *P. cataracta* until midway through brooding (Figs. 1C, 2C). The abundant microvilli on the surface of these septa (Figs. 1D, 2D) provided extensive contact between the glochidia and parental tissues and could facilitate nutritional exchange. Fluctuation of glycogen and lipid content in the gills of freshwater mussels is well documented (Nagabhushanam & Lomte 1971; Jadhav & Lomte 1982; Pekkarinen 1997), with concentrations generally decreasing during the brooding season. Since physiological mechanisms required for the transfer of glycogen to gametes have been identified in some marine bivalves (Bayne et al. 1982; Crespo & Espinosa 1990), comparable mechanisms may mobilize glycogen in the nutritional physiology of developing glochidia.

The uptake of any nutrients by glochidia within the brood chamber likely would involve transport across the epithelium of the larval mantle, as has been suggested by Pekkarinen (1996) who demonstrated the presence of microvilli and acid phosphatase activity in the mantle epithelium of two species of glochidia. This role for the larval mantle would be consistent with its subsequent involvement in the nutrition of encysted glochidia on a fish host (Blystad 1923; Arey 1932; Fukuhara et al. 1990).

The role of punctae in the biology of glochidia is not clear. SEM of the glochidial shells showed that the pores did not penetrate the pellicle, a condition that has been described for all other glochidia examined (Lefevre & Curtis 1912; Coker et al. 1921; Atkins 1979; Rand & Wiles 1982) except in *Margaritifera margaritifera* LINNAEUS 1758 (Nezlin et al. 1994). Although Rand & Wiles (1982) suggested that punctae could be involved in nutrition or gaseous exchange, their being covered externally by a pellicle (probably quinone-tanned protein) or being filled with an organic matrix (Fig. 4G), makes such transfer unlikely. Robertson & Coney (1979) observed that the mantle epithelium projected into the pores from the inside of the

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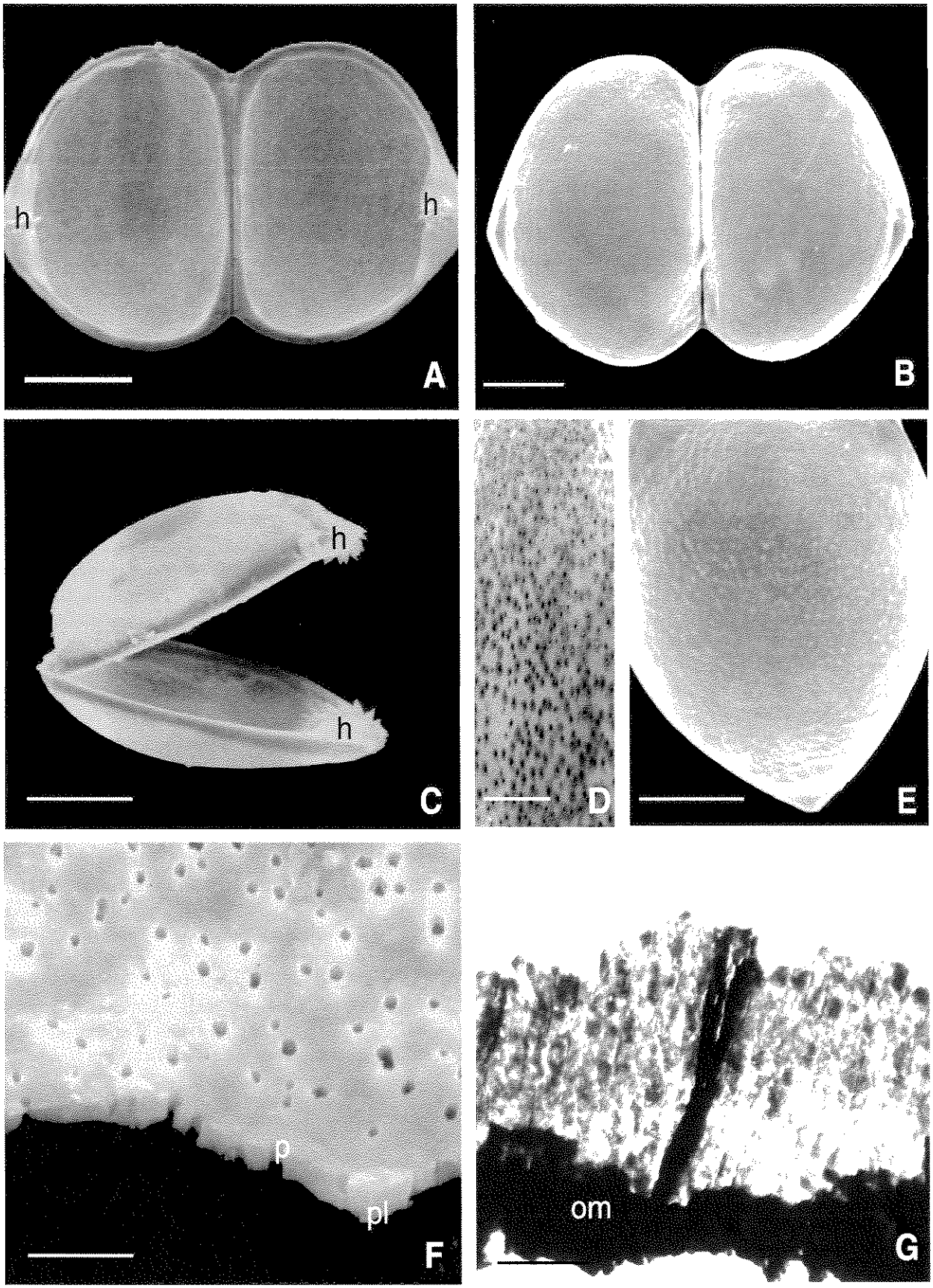
Fig. 3. SEM of glochidial shells of *Utterbackia imbecillis*. h, hooks; hl, hinge ligament; p, punctae; pl, pellicle, arrow head, larval thread. **A.** Interior of surface of shell. Scale bar, 100 μm. **B.** Exterior surface of shell. Scale bar, 100 μm. **C.** Ventral SEM of the closed glochidium showing larval thread (arrow head). Scale bar, 100 μm. **D.** SEM of punctae on the interior surface the shell. Scale bar, 1 μm. **E.** High magnification of external surface of shell. Scale bar, 10 μm. **F.** SEM of a fractured shell showing internal surface of shell and pellicle (pl). Scale bar, 10 μm. **G.** SEM of the dorsal margin of the shell showing the hinge ligament. Scale bar, 10 μm.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shell in a pea clam (*Musculium*), and suggested that pores and associated tissue were involved in an excretory or secretory function. Perhaps formation of the shell or of the hinge involves secretion through punctae early in shell formation. The presence of such holes could contribute to producing a light-weight yet fairly strong structure (Robertson & Coney 1979; Kat 1984).

Brooding often occurs in association with harsh or variable environments, a condition that is presented to freshwater mussels (Mackie 1984). The loss of a planktonic larva and the development of a brooded glochidium were perhaps instrumental in the invasion of freshwater by unionoid bivalves. Brooding may have been accompanied by supplemental nutrition of the larvae through matrotrophy. Although definitive evidence for matrotrophy should include a comparison of the dry organic mass of the egg with that of the mature dispersal stage (Frick 1998), the increase in the size of the larvae of an Australian hyriid mussel during development within the brood chamber (Jupiter & Byrne 1997) may indirectly indicate such an increase in mass.

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Fig. 4. SEM and TEM of glochidial shells of *Pyganodon cataracta*. h, hooks; om, organic matrix; p, punctae; pl, pellicle. **A.** SEM of the interior surface of shell. Scale bar, 100 μm. **B.** SEM of the exterior surface of shell. Scale bar, 100 μm. **C.** SEM of glochidia shell. Scale bar, 100 μm. **D.** Pores of the interior surface the shell. Scale bar, 50 μm. **E.** Punctae on exterior surface of the shell. Scale bar, 100 μm. **F.** SEM of a fractured shell. Scale bar, 10 μm. **G.** TEM of mature glochidial shell decalcified and stained with phosphotungstic acid showing organic matrix (om) extending through shell. Scale bar, 30 μm.

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