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Maternal Calcium Contribution to Glochidial Shells in Freshwater Mussels (Eulamellibranchia: Unionidae)

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ABSTRACT Previous work has demonstrated that calcium concretions, located in the gills of freshwater mussels, disappear during the reproductive season. While calcium is being mobilized, there is no increase in calcium content in the blood. The current study documents the passage of maternal calcium to glochidia, for the production of calcium carbonate shells while the larvae are brooding in the water channels of the lateral gills; ^{45}Ca was injected into *Anodonta grandis* 2 mo before the onset of reproduction and the label was distributed through the animal but was concentrated largely in the calcium concretions. Following glochidia development, ^{45}Ca was found incorporated in the shell of the embryos. To determine the extent of the maternal passage of calcium to the embryos, *Ligumia subrostrata* containing embryos undergoing shell formation were exposed to ^{45}Ca, either by injection into the blood or by inclusion of label in the bathing medium. The amount of label found in the developing glochidia is proportional to the ^{45}Ca content in the blood of the maternal animal. Isolated glochidia directly exposed to label will adsorb ^{45}Ca onto their shells, but there is little addition of label to glochidia in intact maternal gills during the bath exposure experiments. The results indicate that the maternal contribution of ^{45}Ca is over 90%, with direct contribution from the bathing medium accounting for less than 8%. These results indicate that the branchial water channel in which the embryos are developing is isolated from bath ^{45}Ca. This study presents morphological evidence that the physiological isolation of glochidial shells from bath ^{45}Ca is due to a rearrangement of water channel morphology, and that the chamber, in which the embryos develop, has no contact with the bathing medium.

Freshwater unionid mussels brood their larvae to a fully shelled glochidial stage in the water channels of marsupial gills. Individual females of some species brood as many as 600,000 larvae during a single reproductive period (Silverman et al., '85). Mature larvae, recovered from a single *Anodonta grandis*, contain as much as 400 mg of calcium in their collective shells. The source of calcium used by these embryos for the production of a calcium carbonate shell has not been studied previously.

Several obvious sources of calcium are available to these embryos while they are present in the marsupial demibranch. There are two large crystalline stores of calcium in the maternal animal: the maternal shell and extracellular calcium concretions contained in the gills of these mussels. Both of these maternal sources could contribute calcium to the glochidia if a mechanism for mobilization of calcium exists. Finally, although the freshwater environment is a relatively poor source of calcium (0.02–0.04 mM) at our collection sites (Silverman et al., '87), ventilation of sufficient water across the marsupial gill could account for the calcium found in the shells of glochidia. However, flow would have to be rapid, directed toward the larvae, and extraction of calcium by larvae very efficient. Morphological indications are that the marsupial gill does not ventilate large amounts of water through the brood chamber enclosing the larvae.

We have shown recently, using a variety of morphological techniques, that calcium concretions in the gills of freshwater unionid

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mussels disappear during the reproductive period (Silverman et al., '85). The present study demonstrates that a ^{45}Ca label injected into maternal animals 2 mo before reproduction can be mobilized and transferred to the shell of glochidial larvae. This passage of calcium appears to occur without an increase in calcium in the gonad of the maternal animal at any time during the study period. The conclusion is that the maternal animal is a source of at least part of the calcium used to produce glochidial shells. Short-term radiolabel experiments indicate that at least 90% of the calcium acquired by the larvae is from maternal sources and that larvae obtain less than 8% of their Ca directly from the bathing medium.

MATERIALS AND METHODS

All the unionids we have examined contain gill calcium concretions, and for this study, either *Anodonta grandis* or *Ligumia subrostrata* were used as representative species in all experiments. Animals were collected from ponds near Baton Rouge, LA. The animals were maintained briefly in the laboratory in artificial pondwater (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, 0.05 mM KCl).

Long-term ^{45}Ca-labeling studies

Anodonta grandis were weighed (200–450 gm), and numbered by scraping the periostracum on one of the valves. Fifty animals were injected with 3×10^6 cpm ^{45}Ca in H_2O (ICN, 1 mg $CaCl_2$ = 9.16 mCi) on August 1, 1984. The mussels' valves were gently pried apart using blunt forceps and 10 μl ^{45}Ca injected directly into the foot using Hamilton syringe. An additional eight mussels received 10 μl containing 5×10^6 cpm ^{45}Ca injected into the pericardium on September 25, 1984. In some of the animals, whose valves had been pried, there was evidence of mantle damage. Healing of the mantle was rapid, and only 4 of 58 animals died during the period of the experiment. The animals were placed on pond sediment in the bottom of a large volume of water (a 1.5-m-diameter plastic tank). The sediment and water were monitored for the entire period of the study, and no label above background was allowed to build up at any time during the experiment.

Animals from the first labeled group were collected and monitored weekly, starting in the last week of August. Control animals, which received no label but resided in the same water, also were sampled periodically. The following tissues were routinely monitored for ^{45}Ca: blood, kidney, gill, gonad, and pieces of shell. Tissue preparation was as follows. Blood (1 ml) was collected from the pericardium by heart puncture and centrifuged at 10,000g to remove particulates; 0.5 ml of supernatant was placed in a scintillation cocktail. Small pieces (5–10 mg dry weight) of gill, kidney, and gonad were quickly dissected, weighed, and dried in a hot air oven (90 °C) to constant weight. Approximately 5 mg of dry tissue was digested in 0.2 ml concentrated HNO_3; scintillation cocktail was added and ^{45}Ca radioactivity determined.

For concretion isolation, a lateral and medial demibranch were separately digested in 1 N NaOH at 60°C for several hours. The soft gill tissue dissolved, leaving only calcium concretion and calcified chitinous rods. The concretions were collected by low speed centrifugation (Silverman et al., 1983). Concretions were rinsed in deionized H_2O, dried to constant weight, and 5 mg digested in 0.2 ml HNO_3, scintillation cocktail added, and ^{45}Ca radioactivity determined.

In October, when lateral (marsupial) demibranchs contained embryos, the embryos were isolated from the gill tissue. The ventral edge of the gill was cut, and the embryos extruded free of the gill tissue. The presence of calcified shell matrix was determined by polarization microscopy. Embryos were treated in two ways for analysis of ^{45}Ca. Some samples were dried intact and handled as described above for isolated concretions. For the older, more developed embryos, samples also were placed in 1 N NaOH at 80°C for removal of all soft tissue and contaminating "byssal" threads. Such preparations were examined using light microscopy and harvested after digestion of the soft embryonic tissues. The remaining glochidial shells were then rinsed in distilled H_2O and dried to constant weight in an oven. Glochidial shells were dissolved in HNO_3, and ^{45}Ca radioactivity determined as described above. This preparation allows for the identification of ^{45}Ca associated only with the larval shell.

Radioactivity analyses for ^{45}Ca were made using a liquid scintillation counter corrected for quench using an external standard and for radioactive decay to the day of injection.

Short-term ^{45}Ca studies

A set of experiments was conducted on gravid *Ligumia subrostrata* females. Three experimental groups were set up with the

animals in each group exposed to 10^6 cpm ^{45}Ca for 4 hr. In two groups animals received an injection (10 μl) into the foot muscle (ICN, $^{45}CaCl_2$ in H_2O), specific activity 1 mg $CaCl_2$ = 9.16 mCi). For the third group, each animal was placed into individual 100-ml baths of pondwater containing the 10^8 cpm ^{45}Ca. In one group receiving injected label, each animal was placed in a 100-ml flow-through bath system (flow rate = 5 ml/min) to wash out radio-label and maintain a low level (<50 cpm/ml) in the bath. The other group of injected animals were placed in 100-ml baths for the 4 hr. Radio-label in the bath was monitored over the 4-hr exposure time, and label in the shell, soft maternal tissue, and glochidia were monitored at the end of the exposure period. Soft tissue and glochidial shell ^{45}Ca radioactivity assays were as described above. Adult shell radioactivity was determined after digesting the shell at 60°C for several hours in concentrated HNO_3. The digest was diluted and counted as described for the glochidial shell.

The leakage of radioactivity from injected animals over the 4-hr time period amounted to a maximum of 200 cpm/ml of bath in the static bath, and the flow-through experiment yielded less than 50 cpm/ml of bath. The results of tissue and blood comparisons of these two groups were not significantly different from one another, and thus the data were combined. Each experimental group consisted of at least eight animals.

To assess the ability of glochidia to take up label from the bathing medium, additional experiments were completed in ^{45}Ca labeled pondwater containing 10^6 cpm/100 ml. Gravid lateral gills were separated from the maternal animals, as undamaged as possible, with only the proximal attachment being severed, and exposed to labeled bath for 4 hr. Also, isolated glochidia either as a cluster from a single water channel, or as isolated embryos separated by mechanical agitation, were exposed to labeled bath. To determine shell adsorption of ^{45}Ca, maternal shells, with all tissue removed, were exposed to label for the 4-hr period.

Preparation of marsupia for morphological analysis

Since the physiological data suggested brooding glochidia were isolated from the external pondwater bathing media, a series of morphological studies was conducted in an attempt to determine a morphological basis for the functional isolation. A series of *Anodonta* gills was fixed from late September through November 1985 for examination of the structural changes in the gill during the time of calcium transfer in adult females. Whole demibranchs were excised from the animal and fixed in 2% glutaraldehyde (pH 7.8) containing 1 mM EDTA. Gills were rinsed briefly in 30 mM Tris buffer, cut in sections, parallel to the major septa running between gill Lamellae. These tissue blocks were postfixed in 1% OsO_4, dehydrated, and embedded in Spurr's resin. Some blocks, from tissue containing very advanced glochidia, were initially decalcified before osmium fixation by incubation (3–24 hr) in 10 mM EDTA, pH 3.0. Blocks were sectioned with glass knives at a thickness of 1–3 μm. Sections were stained with toluidine blue and examined using either bright field or polarization light microscopy. A few sections were cut at 0.5 μm and examined using a Joel 100 CX electron microscope adjusted for low magnification imaging.

RESULTS

Localization of a single injection of ^{45}Ca

Large single injections of ^{45}Ca are rapidly incorporated into the tissues of freshwater mussels. All tissues monitored contained label 12 hr following injection (Table 1). The adult shell also shows significant accumulation of the label, with the leading edge showing more ^{45}Ca accumulation than the umbo. The pattern of label accumulation in the shell is similar to what has been reported previously for other molluscs (Dillaman and Ford, '82).

The majority of the label localized in the gill is associated with extracellular calcium phosphate concretions (Table 1). Based on a concretion weight of 50 mg in the gills of *Ligumia subrostrata* (Silverman et al., '83), concretions account for about 10% of the total injected label after 12 hrs, and about 30% by 48 hrs.

Long-term distribution of ^{45}Ca following a single injection of label

The concretions account for approximately 50% of the dry weight of the gill in *Anodonta grandis* (Silverman et al., '85), and incorporate ^{45}Ca very rapidly. The label that is bound to the concretions remains there for a long period of time, as indicated by Figure 1. All of the other tissues examined contain little label a week following injection. Label in all tissues declined during the period of the long-term study, with only the gill cal-

TABLE 1. cpm in tissue fractions of Ligumia subrostrata *12 hr after injection of 4.4 × 10^6 ^{45}Ca*

Tissue	Units	^{45}Ca Activity
Blood	cpm/ml	22,665 ± 2,487 (8)[a]
Mantle		
Pallial Edge	cpm/mg dry wt	1,673 ± 126 (8)
Central Region	cpm/mg dry wt	1,823 ± 244 (8)
Gonad	cpm/mg dry wt	1,936 ± 228 (8)
Whole Gill		
Medial	cpm/mg dry wt	1,088 ± 186 (8)
Lateral	cpm/mg dry wt	1,083 ± 211 (8)
Gill Concretions	cpm/mg dry wt	7,162 ± 933 (8)
Shell		
Edge	cpm/cm^2	583 ± 70 (8)[b]
Umbo	cpm/cm^2	330 ± 55 (8)
After 48 hr:		26,173 (2)
Isolated Concretions	cpm/mg dry wt	

[a]Mean ± SEM (N).
[b]Shell values were obtained by taking a piece of shell (1 cm^2) from the appropriate region and placing it nacre side down in 0.1 M succinic acid for 3 min. The acid was then counted for activity. The same piece of shell was then soaked in acid for another 3 min and less than 2% of the original activity was present in the acid.

cium concretions showing any long-term activity more than 20% above background (Fig. 1).

The shell also contained label in quantities similar to those indicated in Table 1, reflecting rapid uptake. Radioactivity at the edge of the shell (the site of shell growth) a few weeks after injection was between 50–100 cpm, indicating significant label storage in the shell, since the shell weighs 10^2–10^3 more than the concretions. Concretions isolated from the medial gills of females and from males in late November still contained some of the label bound after the single August injection (Fig. 1). Sperm release from the males occurred during the third week in September 1984. Embryonic development occurs in October and early November in *A. grandis* from southern Louisiana; ^{45}Ca is found in glochidia developing in mussels given a single injection of label, while no ^{45}Ca is found in uninjected controls residing in the same environment. To standardize for variation in label found in each mussel and across the two experimental protocols, data have been standardized and expressed as a percentage of the label found in isolable medial concretions. These data are shown in Table 2 and indicate that 18.7% of the label found in calcium concretion stores within the maternal animal is found in the glochidia residing in the gill water channel. Further, the data indicate a transfer of maternal calcium directly to the embryos in the water channel, since gonadal label is minimal and does not show any increase from the nonreproductive condition (Table 2).

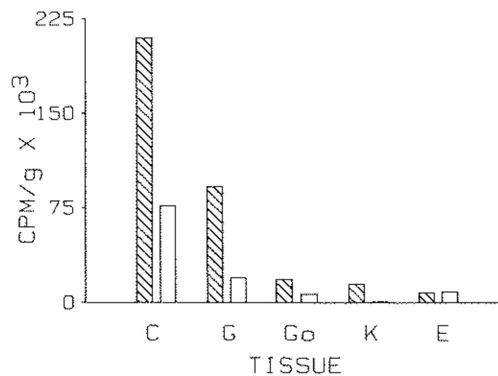


Fig. 1. Histogram representing average tissue levels of ^{45}Ca in gill concretions (C), in gill tissue (G), in gonadal tissue (Go), in kidney tissue (K), and in developing glochidia (E) on the day of collection. The averages are for values collected over a 2-mo period, so error bars are not presented. The trends established in this histogram for tissue relationships hold for every animal examined, independent of the absolute amount of label incorporated by the animal. Values are expressed as 10^3 × cpm/gm dry tissue weight.

The direct transfer of Ca from mother to embryo is further supported by short-term label experiments in which gravid mussels, containing glochidia in the process of shell production, were exposed to label either in the pondwater or by direct injection into the

TABLE 2. ^{45}Ca label incorporated into tissues of reproductive and nonreproductive Anodonta grandis *tissues. Data expressed as percent of cpm/mg dry weight of medial gill concretions. All values are means ± SEM (N).*

	Whole Gill	Gonad	Kidney	Medial Concretion Isolated	Lateral Concretion Isolated	Glochidial Shells
Nonreproductive	47.0 ± 5.5(17)	7.3 ± 1.5(13)	10.9 ± 3.1(11)	100(17)	117 ± 11(15)	—
Reproductive	56.7 ± 14.5(7)	7.3 ± 1.4(7)	10.6 ± 2.7(7)	100(7)	—	19 ± 7 (7)

TABLE 3. Radio-label uptake by Ligumia subrostrata *tissues after 4-hr exposure to 10^6 cpm ^{45}Ca either in the 100-ml pondwater bath or by direct injection into the pericardial region (N = 8).*

	Exposure method	
Tissues	Injection	Bath
Blood cpm/ml	14,116 ± 1,571[a]	2,157 ± 400
Soft Tissue cpm/gm dry wt	696,186 ± 7,518	96,115 ± 14,050
Shell cpm/gm dry wt	16,243 ± 809	80,176 ± 5,032
Demibranch cpm/gm dry wt	402,755 ± 46,553	65,280 ± 9,510
Glochidia cpm/gm dry wt	42,565 ± 4,074	8,915 ± 1,183

[a]Mean ± SEM. When label was added to the bath 83% of the label remained in the bath after 4 hr.

TABLE 4. Uptake of ^{45}Ca by tissue components of Ligumia subrostrata. *Exposure to 10^6 ^{45}Ca was either by addition to pondwater or by injection (10 μl) into the foot of intact animals. In some cases tissue components were isolated and directly exposed to pondwater. All radioactivity expressed as cpm/gm dry wt.*

	Exposure method	
Tissue treatment	Bath	Injection
Glochidia, whole animal	8,915 ± 1,183(8)	42,565 ± 4,074(8)
Glochidia, isolated gill	25,794 ± 3,929(4)	—
Glochidia, isolated	78,424 ± 12,559(7)	—
Shell, maternal animal	80,954 ± 4,676(8)	18,238 ± 772(8)
Shell, empty	270,191 ± 25,683(4)	—

foot muscle. The animals used for these experiments were smaller *Ligumia subrostrata* (30–70 gm). The accumulation of label by the animals is shown in Table 3. Maternal animals placed in ^{45}Ca labeled bath transported calcium into the blood during the 4-hr time span. Glochidia in these animals accumulated only 9×10^3 cpm/gm, while in animals injected with label, glochidia have 4×10^4 cpm/gm. The maternal shell shows modest incorporation of calcium in the injected animals, indicating actual addition to the shell from maternal blood. Significant adsorption to the surface of the shell occurs in the animals in the labeled bath (Table 3), and this is supported by the additional adsorption of ^{45}Ca to isolated shells, which have twice the surface area exposed to label (Table 4). The glochidia from animals injected with ^{45}Ca show roughly five times more label than those from animals sitting in labeled bath. These results suggest that glochidia have little or no access to free-flowing pondwater when they are developing in the marsupial gills.

In support of this hypothesis are the data obtained from isolated glochidia exposed to labeled pondwater (Table 4). These glochidia accumulate the same label as appears to be adsorbed to maternal shell. This indicates that the glochidia would at least adsorb label if they were in direct contact with a free-

flowing labeled pondwater. Interestingly, the glochidia in intact isolated gills do not show the massive increase in label that the isolated glochidia do, indicating they are still isolated from the label in the pondwater.

Most importantly, the data indicate that the label appearing in glochidial shells in both sets of experiments is directly related to the radio-label accumulated into the blood (Fig. 2). The relationship between ^{45}Ca uptake by glochidia and maternal blood ^{45}Ca content was analyzed by least squares linear regression. Uptake of ^{45}Ca by glochidia in the water channels of intact animals is closely related to ^{45}Ca in the maternal blood and the regression of glochidial ^{45}Ca on maternal blood-^{45}Ca yields a slope of 3.4 (cpm/g)·(cpm/ml)$^{-1}$ with an R^2 value of 0.988. The regression has an intercept that is slightly greater than zero (153 cpm/gm) probably due to the minor contribution (153/2157 = 7.1%) of ^{45}Ca directly from the bathing medium to glochidial shell calcium over the 4-hr study period. Analysis of these data using a multiple linear model including maternal shell, glochidial shell, and blood as tissues and method of label application as the treatment also shows an excellent fit (F ratio 110; R^2= 0.92; with an unexplained error of 7.1%). The significant relationship is completely lost when glochidial shell/blood covariance is factored out. Thus, maternal sources are responsible for at least 90% of the glochidial ^{45}Ca uptake over the 4-hr study period, and that the bath contributes directly only about 7% of the total incorporated calcium.

Morphology of the water channels containing the developing glochidia

The morphological changes occurring in the marsupial gill during reproduction are extensive and have been described only by early workers using light microscopic techniques (Ridewood, '03; Ortmann, '11). We have therefore reexamined the structure of unionid marsupia, particularly the gross water channel morphology and water channel packing by developing glochidia. The structural implications for the lack of environmental water flow over the glochidia are consistent with the physiological isolation of the glochidia as suggested above.

The water channels in animals preparing for reproduction expand greatly in preparation for receiving fertilized eggs (compare Figs. 3 and 4 with 5 and 6). This expansion is such that a lateral gill weighs 0.5–1 gm in

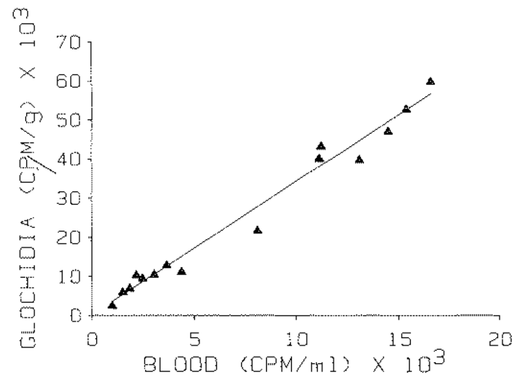


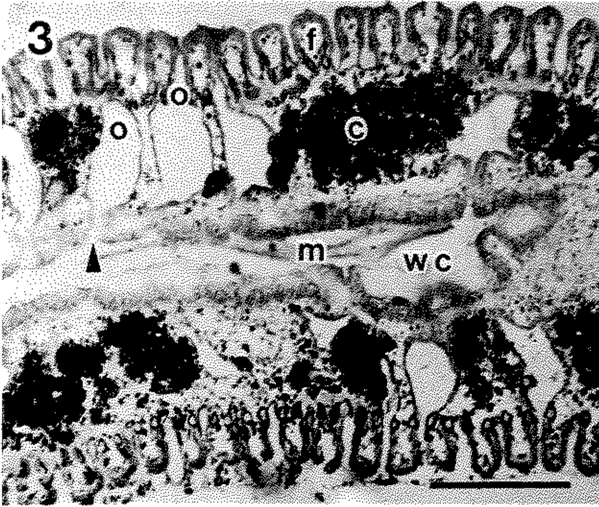
Fig. 2. Regression of blood ^{45}Ca (cpm/ml) on glochidia shell ^{45}Ca (cpm/g), after exposure to 10^6 cpm ^{45}Ca. Data from both the bath exposure and injection experiments are combined and analyzed in this regression. The slope of the regression line is 3.4 (cpm/g)·(cpm/ml)$^{-1}$ with an r^2 value of 0.988; the intercept is 153 cpm/gm.

Fig. 3. Cross section of *A. grandis* lateral gill from a nonreproductive animal. This gill section is a 20-μm-thick section of freeze-dried gill and shows the masses of concretions (c) lying between the gill filaments (f) and the water channel (wc). The water channel in this section contains some mucus (m) in the lumen. The water channel in nonreproductive animals is continuous with the environmental water flow through a series of ostia, openings at the base of the filament, which connect to a series of canals (o) leading to the water channel. The opening of one of these canals into the water channel is denoted by the arrowhead. Bar = 500 μm.

Fig. 4. Thin (2 μm) section of *A. grandis* lateral gill from a nonreproductive animal. This section matches the thickness of the reproductive sections and serves as the appropriate comparison with these sections. The masses of gill concretions are visible (c), and many water canal (o) openings into the water channel are denoted by arrowheads. Bar = 700 μm.

Fig. 5. Cross section of an early reproductive gill from *A. grandis*. The water channels proper become packed with developing embryos (e) and are labeled as brood chambers (bc). The brood chambers are no longer connected via water canals to the outside; instead a new set of chambers develop on either side of the brood chamber (vertical arrowheads) which are connected to the outside by the water canals (horizontal arrowheads). Also note the loss of concretion content from the gill. Bar = 440 μm.

Fig. 6. Cross section of an early reproductive gill of *A. grandis* from which the embryos have been lost during preparation. Note the extensive development of the secondary chambers (wc) on either end of the brood chambers (bc). Bar = 700 μm.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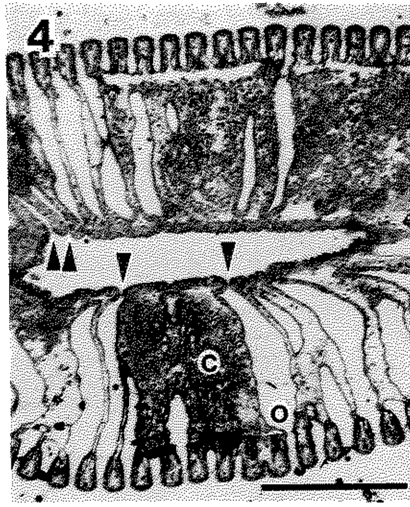
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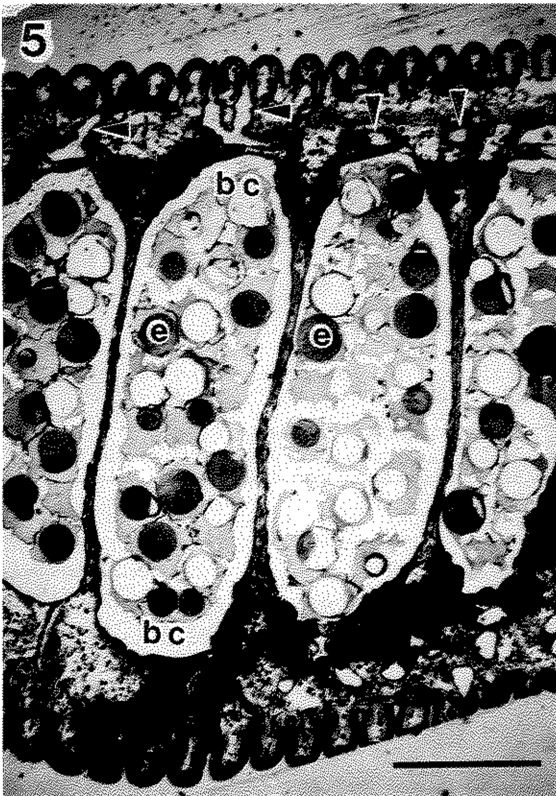
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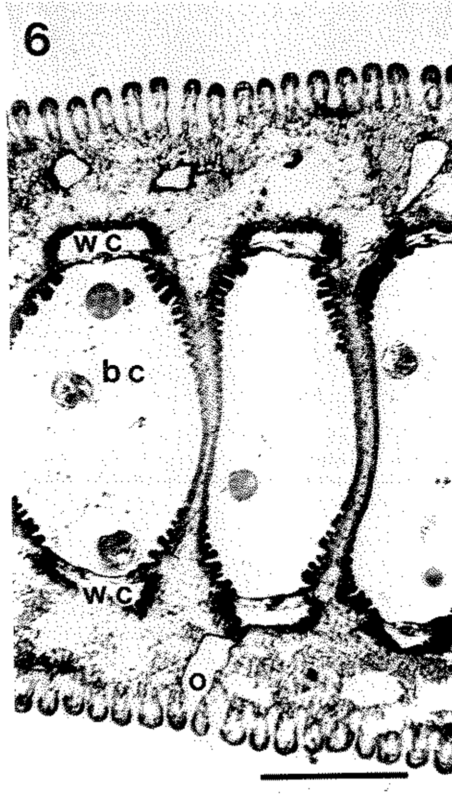
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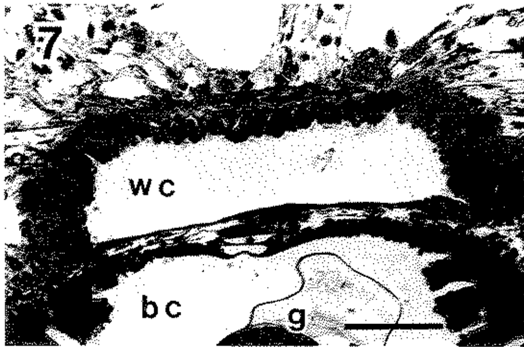

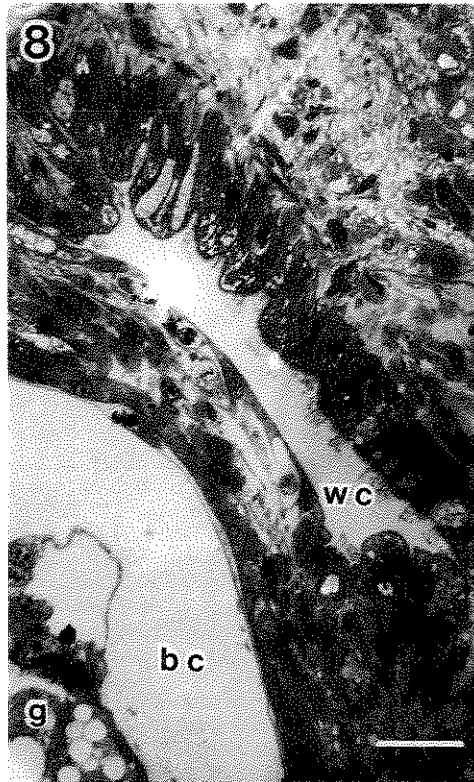

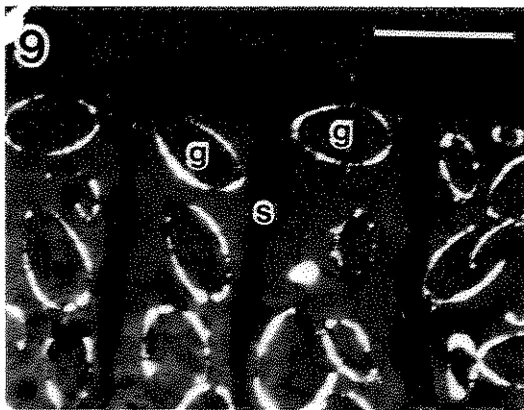

Fig. 7. High-magnification cross section of the junction between the secondary water channel (wc) and the brood chamber (bc). The brood chamber contains a developing glochidium (g). Note that the tissue which forms the junctional separation between the two chambers appears to be complete. Bar = 100 μm.

Fig. 8. Transmission electron micrograph of a similar area to the one depicted in Figure 5. The separation between the two chambers (brood (bc) and the secondary water (wc)) is complete, with both chambers being lined by their own epithelia. The brood chamber contains a developing glochidium (g). Bar = 50 μm.

Fig. 9. Cross section of *A. grandis* gill viewed under cross-polarized light. The gill is in the late reproductive stage and glochidia (g) have developed fully calcified valves. Septa (s) between water channels are visible, and the filaments can be seen in their regular orientation at the top of the figure. Bar = 500 μm.

a nonreproductive *A. grandis* and as much as 30 gm in a mature gravid female. The connective tissue septa separating the adjacent water channels become thickened and the water channels themselves are subdivided by connective tissue into lateral and medial water tubes surrounding a greatly enlarged central marsupial chamber (Figs. 6, 7, and 8). This central marsupial region comprises the majority of the water channel's volume (Figs. 5 and 6). The central channel is filled by a set of embryos and their embryonic investment coats which occupy the entire volume of this region (Fig. 5).

Initially, as the embryos are being transferred into the marsupia, there are masses of calcium concretions in the connective tissue underneath the gill filaments (Fig. 3). During the early cleavage stages these concretions remain evident, although their numbers are declining. By the time the embryos begin to demonstrate birefringence in polarized light, ie, the presence of calcium carbonate shell material, calcium concretions are much reduced in the marsupial gill. These results are similar to quantitative measurements done using concretion isolation procedures on early marsupia (Silverman et al.,

'85). Development of calcified glochidia within the brood chamber is documented with polarized light in Figure 9.

DISCUSSION

Data from the ^{45}Ca labeling studies indicate that Ca from a maternal source is deposited in the glochidial shells forming in the water channel of the gills. In a typical *Anodonta*, 400 mg of calcium must be available for shell formation of 600,000 glochidia (Silverman et al., '85). The two obvious sources of calcium are the calcium deposits present in the maternal animal and environmental calcium which might be sequestered by the embryos in the water channel. The present study clearly indicates that the maternal animal is the major calcium source.

Passage of ^{45}Ca from maternal animal to glochidia in the long-term experiments indicates that Ca is passed to the embryo from a maternal source, but does not indicate either the mechanism or the exact sources of calcium. Maternal calcium could be passed to the embryo in the gametes and their investment coats, as has been demonstrated for several gastropod species (Tompa and Wilbur, '77; Fournie and Chetail, '82, '84). The data presented in this study suggest that this is not a major transfer route for the unionids. At no time during the sampling of labeled animals was there an increase in the amount of label found in the gonads, and gonadal ^{45}Ca activity was always similar to ^{45}Ca found in the kidneys.

A major contribution from environmental sources to the glochidia is ruled out by both morphological findings and the short-term ^{45}Ca experiments on brooding *Ligumia subrostrata*. In order to sequester calcium, which is present at 0.02–0.04 mM/L in the natural freshwater environment, 100 L of water must pass over the glochidia and an efficient extraction mechanism that removed all calcium from the water would be necessary. Such water flow through the water channel is not unreasonable for nonreproductive mussels, but does not occur in reproductive females. The water channel of brooding females becomes greatly expanded, but is packed with glochidia larvae (Figs. 5 and 9). Flow of fluid through such an area should be limited. Further, brooding females actually have water channels divided into three portions (Ortmann, '11; Fig. 6): a central area storing glochidia and two peripheral portions divided from the central region by tissue septa lined by epithelia. Thus, water for respiratory purposes may flow through the unobstructed channels, but such flow is excluded from the region holding glochidia (see Figs. 7 and 8). Such an arrangement virtually precludes uptake of environmental calcium by glochidia. The same arrangement may, however, allow some minor contact of glochidia with environmental calcium, allowing for a small portion of the embryonic calcium supply.

The short-term radio-label experiments using ^{45}Ca in brooding *L. subrostrata* confirm the lack of environmental water flow across the developing embryos. Glochidia exposed directly to labeled bath adsorb ^{45}Ca, but glochidia present in the brood chamber do not. This suggests that water flow in the brood chamber is minimal. Further, the strong relationship between maternal blood ^{45}Ca and glochidial shell incorpration over a wide range of blood ^{45}Ca content indicates that the label in the glochidia comes almost exclusively from maternal sources, and not directly from pondwater. Analyses of data from the short-term experiments suggest that less than 8% of the label is accounted for from sources other than blood label.

Blood calcium concentration in these mussels is about 4 mM and remains so or decreases slightly during glochidial development (Silverman et al., '85). During the reproductive season, marked changes in the morphology of the gill occur, including a loss of the majority of the calcium concretions found in nonreproductive animals (Silverman et al., '85), modification in water channel epithelia structure (Silverman, unpublished), and enlargement and septation of the water channels (see Fig. 3). The concretions compose over 50% of the gills' dry weight in nonreproductive *Anodonta grandis* and are composed of 20–25% Ca by weight (Silverman et al., '83, '85). Thus, large quantities of calcium are being liberated with no measurable increase in blood calcium. In the long-term studies reported here, the concretions become rapidly labeled with ^{45}Ca (Fig. 1, Table 2) and retain the label until analysis months later. The only other important storage site is the maternal shell. Thus, the indirect evidence suggests that the concretions may be the major source of maternal calcium transferred to the glochidia. The data do not, however, eliminate the possibility that the maternal shell also contributes calcium to the larvae.

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LITERATURE CITED

Dillaman, R.M., and S.E. Ford (1982) Measurement of calcium carbonate deposition in molluscs by controlled etching of radioactively labeled shells. Mar. Biol., *66:*133–143.

Fournie, J., and M. Chetail (1984) Calcium dynamics in land gastropods. Am. Zool. *24:*857–870.

Fournie, J., and M. Chetail (1982) Evidence for a mobilization of calcium reserves for reproduction requirements in *Deroceras reticulum* (Syn: *Agriolimax reticulatus*) (Gastropoda: Pulmonata). Malacologia, *22:*285–291.

Ortmann, A.E. (1911) A Monograph of the Najades of Pennsylvania. Mem. Carnegie Museum, *4:*279–347.

Ridewood, W.G. (1903) On the structure of the gills of *Lamellibranchia.* Trans. R. Soc. B (London), *195:*147–284.

Silverman, H., W.L. Steffens, and T.H. Dietz (1983) Calcium concretions in the gills of a freshwater mussel serve as a calcium reservoir during periods of hypoxia. J. Exp. Zool., *227:*177–189.

Silverman, H., W.L. Steffens, and T.H. Dietz (1985) Calcium from extracellular concretions in the gills of freshwater unionid mussels is mobilized during reproduction. J. Exp. Zool., *236:*137–147.

Silverman, H., J.W. McNeil, and T.H. Dietz (1987) Calcium concretion interaction with the trace metals Zn, Cd, and Mn in the gills of the freshwater mussel, *Anodonta grandis*. Can. J. Zool., in press.

Tompa, A.S., and K.M. Wilbur (1977) Calcium mobilization during reproduction in snail *Helix aspersa.* Nature, *270:*53–54.