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Glochidium morphology of the freshwater pearl mussel (*Margaritifera margaritifera*) and glochidiosis of Atlantic salmon (*Salmo salar*): a study by scanning electron microscopy

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The morphology of glochidia of the freshwater pearl mussel *Margaritifera margaritifera* L. and the development of the cyst for 50 days after glochidial infection of wild and hatchery-reared Atlantic salmon (*Salmo salar* L.) parr were studied using scanning electron microscopy. The microvillar surface of the inner epithelium of the glochidium, which may function in nutrition, respiration, and osmoregulation, is described. Glochidia were found to have five sensory tufts as well as marginal ciliary bands which are believed to function in directing water currents. After artificial infection of salmon parr, cyst formation was the result of shape change and migration of gill epithelial cells, but not hyperplasia. The process of cyst formation lasted from 9 to 12 h. No mortality of salmon parr occurred during the experiment. Based on our study, glochidia appear to have a negligible effect on the gills of Atlantic salmon, its preferred fish host. These results are discussed in the context of the possibility of a mutually beneficial coexistence of Atlantic salmon and pearl mussels in northern European rivers such as the Varzuga River in Russia.

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La morphologie des glochidies de la moule d'eau douce (*Margaritifera margaritifera* L.) et le développement du kyste jusqu'au jour 50 après l'infection chez des tacons du Saumon atlantique (*Salmo salar* L.) élevés en nature ou en pisciculture ont été étudiés au microscope électronique à balayage. La surface des microvillosités de l'épithélium interne de la glochidie, qui joue peut-être un rôle dans la nutrition, la respiration et l'osmorégulation, est décrite. Les glochidies comportent cinq touffes sensorielles de même que des bandes ciliaires marginales qui servent probablement à orienter les courants d'eau. Après infection artificielle des tacons, il a été constaté que la formation des kystes est le résultat du changement de forme et de la migration des cellules épithéliales branchiales, mais il ne se produit pas d'hyperplasie. Le processus de formation des kystes dure 9–12 heures. Aucun tacon de saumon n'est mort au cours de l'expérience. D'après les résultats de notre étude, les glochidies semblent avoir un effet négligeable sur les branchies du Saumon atlantique, l'hôte de prédilection de la moule. Les résultats sont examinés en regard de l'hypothèse d'une relation de bénéfice mutuel entre le Saumon atlantique et la moule d'eau douce dans les rivières du nord de l'Europe, tels la rivière Varzuga en Russie.

[Traduit par la rédaction]

Introduction

The freshwater pearl mussels *Margaritifera margaritifera* L. (Bivalvia: Margaritiferidae) inhabit Holarctic salmonid rivers where they are an important component of the ecosystem (Clarke 1981; Young and Williams 1983; Ziuganov et al. 1988). The larvae, glochidia, are gill parasites of fishes, specifically salmonids (Meyers and Millemann 1977; Young and Williams 1984; Bauer 1987). In northern Europe, as well as in eastern Canada, the most common host of the glochidia is the Atlantic salmon, *Salmo salar* L., particularly the juvenile (parr) stages.

The general parasite–host relationship between the two species has been studied previously (Ziuganov et al. 1990; Cunjak and McGladdery 1991). However, little is known about the morphology of the glochidia and their effects on the host's gill tissue despite the wide distribution of the pearl mussels and the commercial importance of salmon. Being the distributive stage in the life cycle of the mussel, the glochidia are highly modified, with a number of special features that adapt it for attachment to the host. The fine structure of these modifications has not been well described. Previous studies (Awakura 1968; Fustish and Millemann 1978; Young and Williams 1984) have used light microscopy to describe the general parasitic stages of glochidia. However, descriptions of changes in the host's gills during glochidiosis are few and sometimes contradictory (Karna and Millemann 1978; Bruno et al. 1988). Such information is necessary for a better understanding of the principles in the host–parasite relationship.

An objective of this study was to describe the fine structure of the glochidium, giving special attention to the surface structures presumed to function in attachment to the host. A second objective was to detail the process of cyst formation and development on the gills of Atlantic salmon. To meet these objectives, we used scanning electron microscopy (SEM) after artificial infection of salmon parr with glochidia.

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Materials and methods

Adult pearl mussels were collected from the Varzuga River (Kola Peninsula, Russia; 66°42′N, 36°00′E) in mid-September 1991, during their breeding period (Ziuganov et al. 1988). Mussels were placed in 1-L glass containers to stimulate the discharge of glochidia (Meyers and Millemann 1977).

In preparation for SEM, glochidia were collected from the bottom of the container with a glass pipette. They were rinsed in several changes of fresh water, relaxed by incubation for 15 min in a 0.2 M solution of $MgCl_2$, and fixed by rapid addition of an equal volume of 4% OsO_4 in 0.05 M cacodylic buffer (pH 7.4). This procedure ensured that glochidia were fixed with shell valves open. After fixing for 2 h at 10°C, glochidia were rinsed in the same buffer and dehydrated in progressive concentrations of ethanol, then stored in 70% ethanol prior to transfer to acetone and drying through liquid CO_2. Next, glochidia were mounted on SEM specimen stubs with double-faced adhesive tape and covered with approximately 15 nm of gold in a Bio-Rad Polaron SEM Coating System. Observations and micrographs were made using a JEOL JSM-T330 electron microscope.

For artificial infection, 90 Atlantic salmon parr (age 2+) were obtained from the Umba Salmon Hatchery (Umba River, Kola Peninsula) and 12 wild parr of the same age (2+) were captured in the Krivetz River (a tributary of the Varzuga River) by electrofishing in mid-September. As *M. margaritifera* were absent from both the Krivetz and Umba rivers, it was unlikely that these parr had previously been in contact with glochidia. The salmon parr measured 80–110 mm in fork length; no glochidia were found on the gills.

Glochidia from each of 3 or 4 female mussels were collected in 1-L glass containers and rinsed in several changes of fresh water. To determine the concentration of glochidia, ten 10-μL samples of the suspension were withdrawn immediately after stirring, and the glochidia were counted under the microscope.

Parr were exposed to glochidia by being placed in 20-L aerated plastic tanks (20 fish per tank) containing a suspension of 100 000 glochidia/L of water for 10 min. Control fish (20 specimens) were treated the same way except that no glochidia were present in the tanks. All parr (control and treated) were then transferred to floating plastic cages (500 × 80 × 70 cm) in the Umba River, where they were retained for 10 d (average water temperature 10°C).

Prior to being sacrificed, fish were anesthetized rapidly in 0.4% 2-phenoxyethanol. Three parr were killed at each of the following time intervals postexposure: 0.5, 1, 2, 3, 5, 8, 12, 18, and 24 h and 2, 3, 10, 20, 30, 40, and 50 d (for wild fish, 2, 5, 12, and 24 h only). The parr sampled between 10 and 50 d postexposure were retained in the hatchery, in 1500-L oval aquaria filled with filtered tap water of an ionic concentration similar to that in the river. The transfer from the river cages was necessary to control water temperature, which was declining steadily in the river. A flow rate of 13 cm · s^{-1} and a water temperature of 14°C were maintained in the aquaria. Fish were fed live oligochaetes once every second day.

The gills were dissected from the parr, rinsed in fresh water, and fixed in 2.5% glutaraldehyde with 0.05 M cacodylic buffer (pH 7.4) for 2 h at 10°C. The specimens were then dehydrated and prepared for SEM as described above.

A portion of each gill specimen was also prepared for examination under a light microscope. After fixation, these were transferred to 10% glycerin in distilled water, kept at 50°C overnight to evaporate the water, embedded in glycerin, and examined.

Results

The glochidium

The terminology used to describe the glochidium structures follows that of Lefevre and Curtis (1912) and Coker et al. (1922).

Glochidia of *M. margaritifera* are small, measuring approximately 60 × 70 μm (Fig. 1). Each valve of the shell is obliquely rounded in outline. In living glochidia, the maximum angle between the valves, when opened, is about 120° and glochidia flap the valves approximately 1 or 2 times/min. No hooks or spines are present.

At high magnification, the outer surface of the valve is found to have numerous minute protuberances but no pores (Fig. 2). The shell consists of two layers. The outer layer extends beyond the valve border, where it folds towards the inside of the shell and forms a curved edge projecting over the inner, ventral surface of the valve (Fig. 3).

The body of the glochidium is covered with outer and inner epithelia (outer and inner mantle layers, respectively, according to Karna and Millemann 1978), each of one cell layer (Fig. 4). The outer epithelium (next to the shell) consists of squamous cells; the inner epithelium consists of cells that are cuboidal in the opened glochidium, becoming columnar in shape when the valves are closed. Between the two epithelia is a cavity with a bundle of smooth muscle and scattered cells of varied shapes (rounded, spindle, and stellate). The entire apical surface of the inner epithelium is covered with numerous nonbranching microvilli of similar size (Fig. 5). The ends of the microvilli are fastened together by an extracellular matrix to form a dense extra-epithelial layer 0.1–0.2 μm in thickness (Fig. 5). No openings were found in the epithelium.

There are two pairs of sensory (ventral) tufts situated adjacent to the distal tip of each valve (Figs. 1, 6). A fifth, smaller (medial) tuft is located near the middle of the body (Fig. 7). Each tuft arises from a single polyciliated cell. Mediolaterally, there is a lateral fossa with a ciliated band along its outer margin (Fig. 8). A tuft of very long cilia originates from the centre of the band, with shorter cilia on each side (Figs. 1, 8). This tuft undulates continuously in living glochidia.

Infection of Atlantic salmon parr

No difference in the process of glochidial infection and encapsulation was detected between wild and hatchery-reared parr of *Salmo salar*. No mortalities resulted from the infection in either the hatchery-reared or wild parr over the 50-d duration of the study.

Immediately after salmon parr were exposed to the glochidial suspension (i.e., within 0.5 h), glochidia attached to the host by clasping the gill epithelial tissue between their valves (Fig. 9). The average number of glochidia per gill filament was 1.16 (range 0–4). Shortly thereafter (within 2 h), gill epithelial cells adjacent to the glochidium changed shape and became rounded (Fig. 10), so that the microridges on their surface were no longer obvious. No significant differences in the number of glochidia and terms of their encystment were detected between different regions of the gills.

Cyst formation seemed to be the result of gill epithelial cells moving over and encapsulating the glochidium (Figs. 10–13). There was no evidence of hyperplasia. The entire process of cyst formation took 9–12 h but was not synchronous for all glochidia, even in the same fish (see Fig. 13). Very little secretion of mucus was detected around glochidia during encystment (Fig. 12).

Once encystment was complete, the glochidium was fully enveloped by the gill epithelial tissue (Fig. 13). One to three adjoining gill lamellae could be involved in the cyst. Several hours later, epithelial cells became squamous in shape, which made the surface of the cyst appear flattened (Fig. 14). A complex pattern of microridges, typical of gill epithelium (Hughes 1979) but absent during the earlier stages of

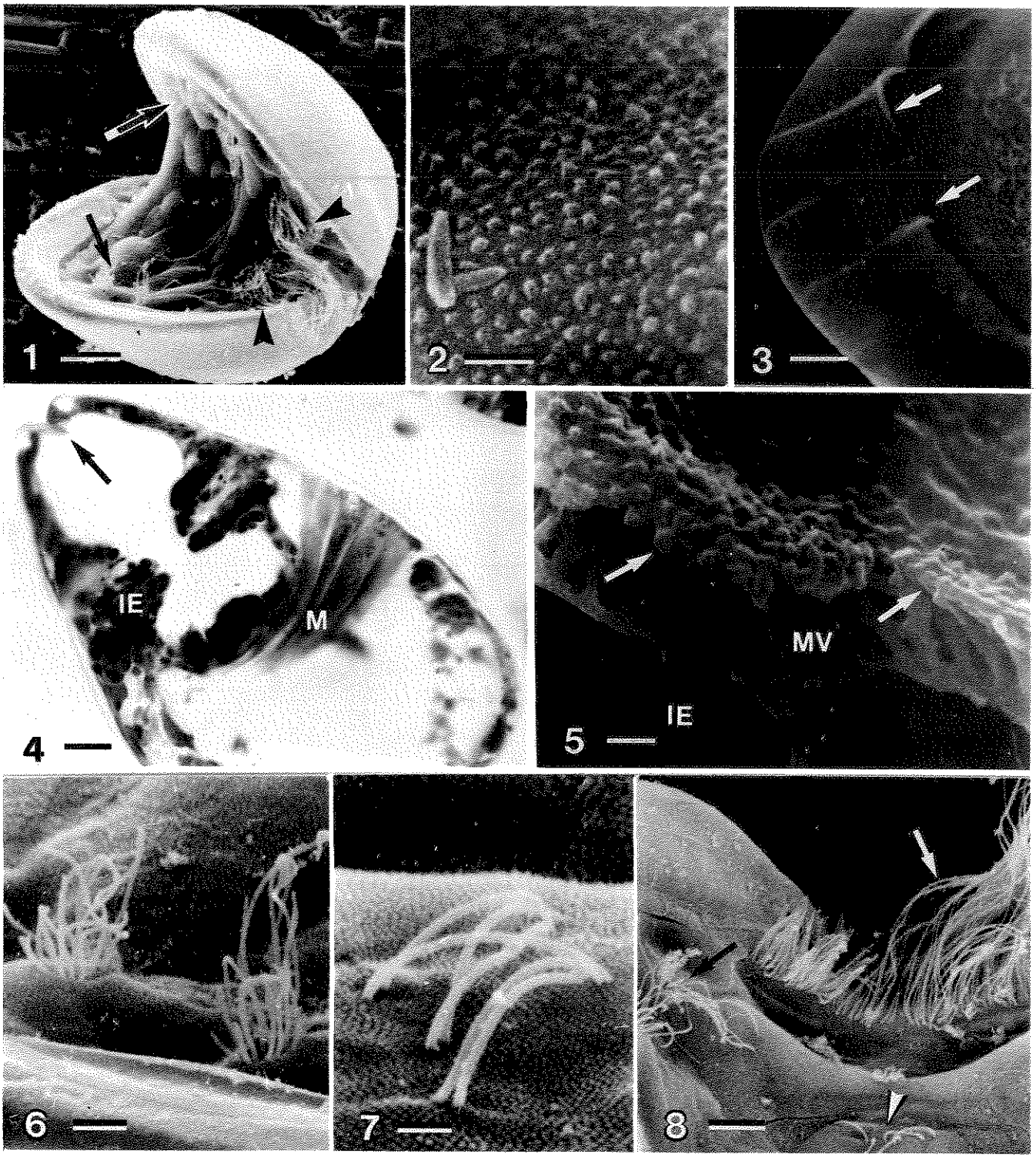

FIG. 1. Open glochidium of *Margaritifera margaritifera*, view of whole animal. Arrows indicate ventral sensory tufts; arrowheads indicate the marginal ciliary band. Scale bar = 10 μm. FIG. 2. External surface of shell of glochidium showing microprotuberances. Scale bar = 0.2 μm. FIG. 3. Ventral aspect of the shell margin showing the sharp flange (arrows). Scale bar = 2 μm. FIG. 4. Cross section through the midline of a closed glochidium. The 1 μm thick section was cut from the Araldite-embedded specimen and stained with toluidine blue. IE, inner epithelium; M, muscle; the arrow indicates the cleft between the valves. Scale bar = 5 μm. FIG. 5. Fractured cross section through the apical portion of the inner epithelium (IE), approximately in the middle of the body. Note the distal portions of the microvilli (MV), which are fastened together to form an extracellular matrix (arrows). Scale bar = 0.2 μm. FIG. 6. Lower valve from Fig. 1 at higher magnification, showing a pair of ventral sensory tufts. Scale bar = 2 μm. FIG. 7. Medial sensory tuft. Scale bar = 1 μm. FIG. 8. Lateral fossa with marginal ciliary band. The white arrow indicates long central cilia; the arrowhead indicates a medial sensory tuft; the black arrow indicates a ventral sensory tuft. Scale bar = 5 μm.

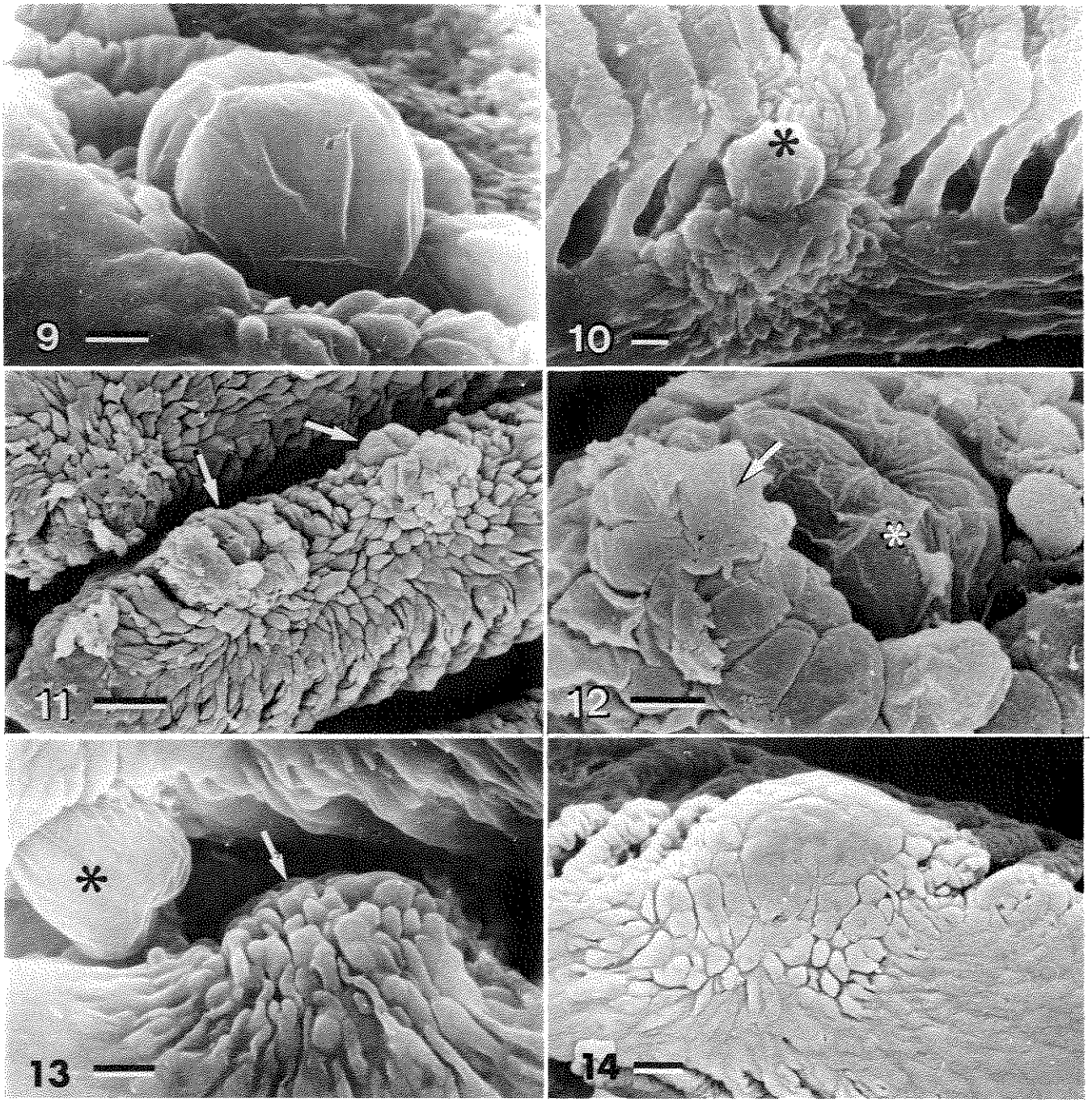

FIG. 9. Glochidium, 0.5 h after infection (AI) of Atlantic salmon parr, attached to the gill lamella. Note the flat surface of the gill filament to the left of the glochidium. Scale bar = 10 μm. FIG. 10. Two hours AI. Note that epithelial cells of the gill filament adjacent to the glochidium (asterisk) have become rounded in shape. Scale bar = 20 μm. FIG. 11. Five hours AI. Glochidia (arrows) are semi-submerged in the epithelium. Note the rounded and stretched shape of the adjoining epithelial cells. Scale bar = 50 μm. FIG. 12. Detail of Fig. 11 at higher magnification, showing the glochidium (asterisk) surrounded by epithelial cells (note their rounded and smooth shape). The arrow points to mucus. Scale bar = 10 μm. FIG. 13. Two glochidia, 12 h AI. One glochidium is fully submerged (arrow) in the epithelium; the other is not encysted (asterisk). Scale bar = 20 μm. FIG. 14. Cyst, 24 h AI, involving three gill lamellae. Scale bar = 20 μm.

encystment (e.g., Fig. 12), reappeared on the surface of the cysts (Fig. 15).

Not all glochidia that initially attached to gill filaments became encysted. From 2 to 5% of the glochidia remained on the gill surface, with no significant reaction in the surrounding epithelial cells. These glochidia disappeared in 1 or 2 days.

No visible changes were detected in the appearance of cysts for the first 15 d following encystment. No hyperplasia was observed (Fig. 16). After approximately 20 d, the cysts started to increase in size; 40 d after infection, they measured 500–600 μm in diameter (Fig. 17) and contained fully developed juvenile molluscs (Fig. 18). At this time, empty cysts with no

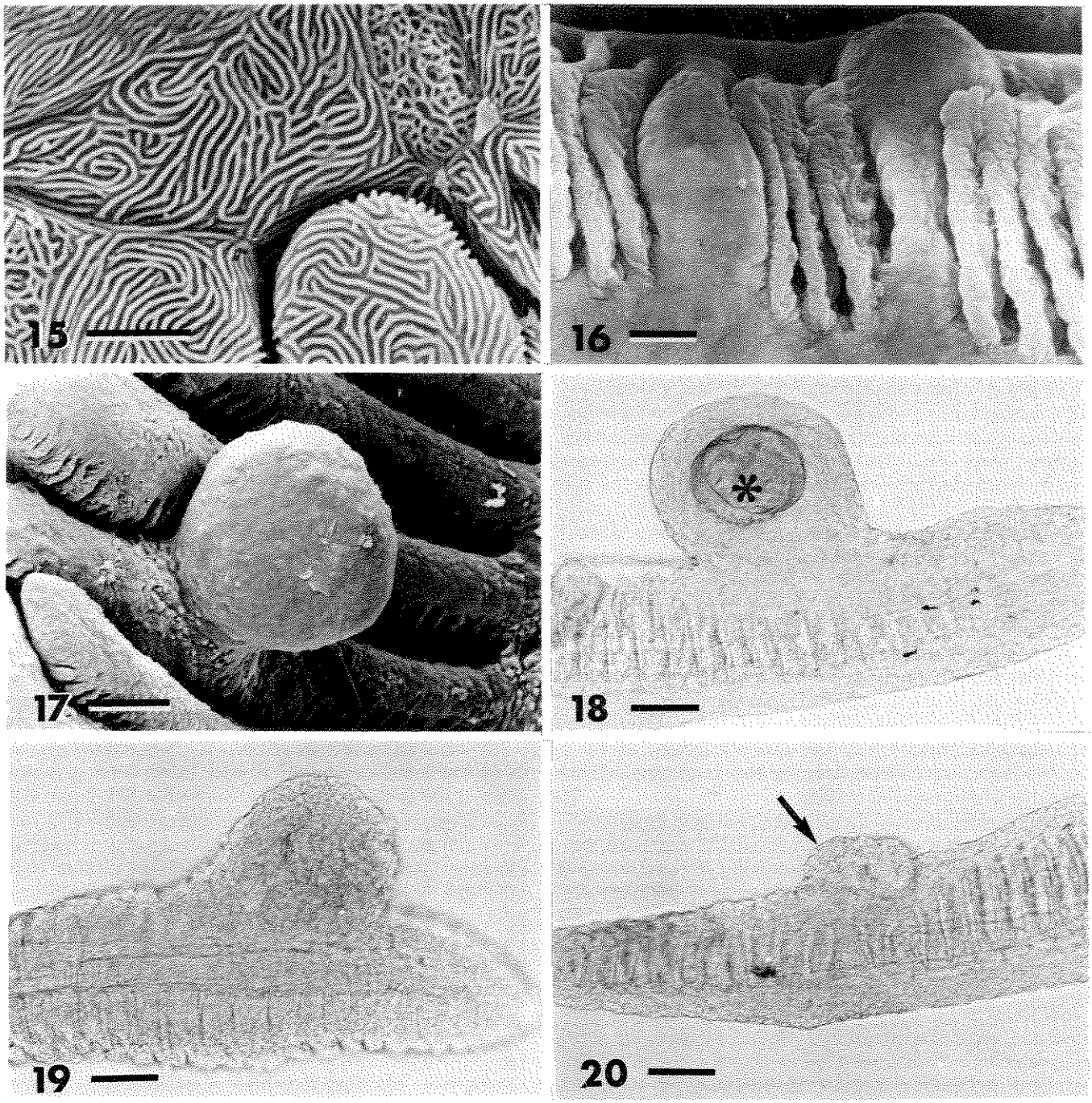

FIG. 15. Detail of cyst from Fig. 14 at higher magnification. Note the complex pattern of microridges on the cyst surface. Scale bar = 5 μm. FIG. 16. Two cysts, 10 d after infection (AI), involving one (right) and two (left) gill lamellae. Scale bar = 50 μm. FIG. 17. Whole view of cyst, 40 d AI. Scale bar = 200 μm. FIGS. 18–20. Glycerin-embedded gill lamellae. FIG. 18. Forty days AI. Fully developed juvenile mollusc (asterisk) within the cyst. Scale bar = 200 μm. FIG. 19. Fifty days AI. Cyst several hours after release of the mollusc. Scale bar = 200 μm. FIG. 20. Fifty days AI. Disappearing cyst (arrow) several days after release of the mollusc. Scale bar = 200 μm.

glochidia inside were detected (Fig. 19). The size of these empty cysts decreased rapidly, and 50 d after infection they looked like small protuberances on the gill filament (Fig. 20).

Discussion

Although glochidia have been studied since the last century (Lillie 1895), the fine structure of the shell has been described only for large so-called "hooked-type" glochidia, such as those of the genera *Anodonta, Pseudoanodonta*, and *Unio* (Giusti 1973; Rand and Wiles 1982; Kinzelbach and Nagel 1986; Antonova and Starobogatov 1989; Antonova et al. 1990). Structural details of the smaller, simple glochidia of pearl mussels (family Margaritiferidae), the "hookless type," are practically unknown.

In contrast to the observations of Coker et al. (1922) we found no larval threads in the several hundred glochidia of

Margaritifera margaritifera that were examined. Also, we found no pores on the shell surface. Pores perforating both the valve and mantle have been described in all other molluscan glochidia examined (Lefevre and Curtis 1912; Coker et al. 1922; Atkins 1979; Rand and Wiles 1982; Kinzelbach and Nagel 1986; Antonova and Starobogatov 1989), and a respiratory (gaseous exchange) or nutritional role has been ascribed to them (Rand and Wiles 1982; Antonova et al. 1990). The absence of the pores in pearl mussel glochidia is probably a function of their relatively smaller size, or the function of the pores in other glochidia may differ from the one suspected.

The sharp flange along the ventral border of the shell may have a function similar to that of the hooks of other types of glochidia. That is, it may cut into the host's epithelial tissue during attachment and thus fix the glochidium to the gills of the host.

Two possible functions are suggested for the dense microvilli covering the soft body of glochidia. First, the microvillar surface may serve for respiration and nutrition, as previously suggested (Arey 1932). Second, such a complicated arrangement in the epithelium (i.e., the distal portions of the microvilli are interconnected to form an extracellular matrix) may act as supplementary protection against the low osmotic pressure of the water, because glochidia have no defined organs of osmoregulation. Unfortunately, there is no information on the structure of the glochidial epithelium of other species for comparison.

Ciliary tufts found in the glochidia of *M. margaritifera* are similar to those found in other species, although the number differs. All workers agree that their function is probably sensory (Lillie 1895; Lefevre and Curtis 1912; Coker et al. 1922; Rand and Wiles 1982). The cilia of the marginal band probably direct water currents along the body of the glochidium, facilitating respiration and feeding.

Once glochidia attach to the gills, the process of cyst formation is very rapid. Lefevre and Curtis (1912) suggested that glochidia release a substance that causes an epithelial reaction in the host tissue. These authors attempted to stimulate the gills with glochidial extracts but found no increase (or proliferation) of epithelial cells. Our results support the contention that glochidia secrete some substance that stimulates gill epithelium cells to envelop the parasites. Within 2 h of successful attachment, adjoining epithelial cells changed their shape and surface structure and seemed to move towards the glochidium. Proliferation of epithelial cells would be improbable in this time frame, as it takes longer to complete the cell cycle (Prescott 1976). More likely, the glochidium produces some factor(s) stimulating migration, rather than proliferation, of epithelial cells in specific hosts. Immediately following the completion of encystment (approximately 12 h), the surfaces of the epithelial cells reverted to their normal structure of microridges.

The response of gill tissue to glochidial infection varies greatly among the different fish species being parasitized. Meyers and Millemann (1977) found coho salmon (*Oncorhynchus kisutch*) to be the most resistant to infection from *Margaritifera falcata*, whereas chinook salmon (*O. tshawytscha*) were the most susceptible. In an earlier paper, we demonstrated that experimental infection of a nonspecific fish host (the minnow *Phoxinus phoxinus*) with *M. margaritifera* resulted in extensive hyperplasia followed by mortality of the glochidia (Ziuganov et al. 1990). The hyperplasia observed in the minnow was very similar to that described in coho salmon (Fustish and Millemann 1978). After infecting Atlantic salmon parr, its most common fish host, with glochidia of *M. margaritifera*, Bruno et al. (1988) detected extensive hyperplasia. Hyperplasia has also been observed in the gills of rainbow trout (*O. mykiss*) infested with the parasitic copepod *Salmincola californiensis* (Sutherland and Wittrock 1985). These results suggest that proliferation of gill epithelial cells is a common response to gill ectoparasites (Kat 1984).

In contrast to the above findings, few pathological changes were detected in the gills of Atlantic salmon parr from the Varzuga River system after infection with glochidia of *M. margaritifera*. We observed no hyperplasia. The ultrastructure of both the cyst and the adjoining gill lamellae was very similar to that of intact, uninfected gills (Hughes 1979). Histopathological changes to gill tissue such as those resulting from the impact of suspended wood debris (Magor 1988) or from low pH water (Lacroix et al. 1990) were not detected in the present study.

The development and metamorphosis of the glochidia on the gills of Varzuga River salmon parr were similar to those described previously (Awakura 1968; Fustish and Millemann 1978; Karna and Millemann 1978; Young and Williams 1984). Although the duration of the parasitic stage varies among populations, it is generally the case that the glochidia of *M. margaritifera* metamorphose at approximately 400 μm shell diameter. Our findings correspond to such a size threshold. The first empty cysts indicating release of juvenile mussels appeared when the shell diameter was approximately 450 μm. Thereafter, the size of the empty cysts gradually decreased and no marked changes to the gill epithelium were detected.

It is our opinion that the relationship between the glochidia of *M. margaritifera* and the parr of *Salmo salar* is a highly evolved and adaptive parasite–host relationship. We base this conclusion on several findings. As noted in the present study, Atlantic salmon parr were highly susceptible to glochidial infection. The successful glochidial metamorphosis on the gills and the lack of mortality attributable to experimental infection provide evidence of the specificity of *S. salar* as the preferred fish host for *M. margaritifera*. During a field study by Cunjak and McGladdery (1991) in eastern Canada, it was similarly noted that the condition of young-of-the-year Atlantic salmon parr parasitized by *M. margaritifera* was not deleteriously affected, except for the most heavily infected salmon. Meyers and Milleman (1977) found that Atlantic salmon were the least susceptible (among several salmonid species) to mortality from exposure to experimental infections of *M. margaritifera* glochidia. Further, Cunjak and McGladdery (1991) found that the abundance of glochidia on the gills of parr did not vary significantly over the course of the parasitic phase. In contrast, Young and Williams (1984) found that only 5% of the glochidia of *M. margaritifera* on the gills of wild brown trout (*Salmo trutta*) in a Scottish stream survived from September to May.

In previous papers (Ziuganov et al. 1988; Nezlin et al. 1989), we suggested that Atlantic salmon and pearl mussels may display a mutually beneficial coexistence in rivers of northern Russia. That is, by filtering and clarifying the typically dark-coloured water of rivers like the Varzuga, the vast colonies of adult mussels create an environment that is ideal for visual feeding species such as Atlantic salmon; in return, glochidial parasitism of the gills provides a source of

nutrition and development as well as a means of dispersion throughout the river system. The high specificity of the host–parasite relationship and the negligible impact of glochidiosis found in the present study provide further support for this theory.

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