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Glochidium Metamorphosis in the Endangered Freshwater Mussel *Margaritifera auricularia* (Spengler, 1793): A Histological and Scanning Electron Microscopy Study

R. Araujo,* N. Cámara, and M.A. Ramos

Department of Biodiversity and Evolutive Biology, Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain

ABSTRACT The metamorphosis of the glochidium of the critically endangered *Margaritifera auricularia* in the gills of a host is studied here for the first time. Siberian sturgeon, *Acipenser baeri,* were infected with glochidia and regularly inspected using scanning and optical microscopy. The mature glochidia immediately attach to the epithelium of the sturgeon gill filaments, piercing the secondary lamellae and the connective tissues, blood cells, and vessels within the lamellae. Once the epithelium is pierced, overlapping host lamellae cover the glochidium and form a cyst. Metamorphosis takes place inside the cyst. Sixteen days after infection the glochidium becomes spherical in shape and the larval muscle is reabsorbed. The two adductor muscles of the juvenile are observed 34 days after infection at 16–20°C. Metamorphosis is complete in approximately 51 days at 18–22°C and in 65 days at 16–17°C. Released juveniles have a spherical shell with a thin rim of new shell material and a finely ciliated foot. Juvenile mean measurements are: length = 190 μm, width = 193 μm, and height = 210 μm. J. Morphol. 254: 259–265, 2002.

KEY WORDS: *Margaritifera*; glochidium; metamorphosis; freshwater mussels

Currently, the giant freshwater pearl mussel *Margaritifera auricularia* is only found in rivers of southern Europe and North Africa (Araujo and Ramos, 2000a), although at one time it was common in large rivers throughout western Europe (Preece et al., 1983). Like other Unionoidea species, its larval stage, the glochidium, must infect a vertebrate host in order to metamorphose into a benthic juvenile. In the case of the critically endangered (IUCN, 1996) *M. auricularia,*, the Atlantic sturgeon *Acipenser sturio* L. (Araujo and Ramos, 2001) appears to be the preferred host, although *M. auricularia* glochidia can also successfully metamorphose in the river blenny *Salaria fluviatilis* (Asso) (Araujo, et al., 2001).

Araujo and Ramos (1998) recently described the morphology of *Margaritifera auricularia* glochidium, but little is known of the larval changes and the modifications suffered by host tissues during glochidial metamorphosis.

Metamorphosis of *Margaritifera auricularia* glochidium in the gills of a host is studied here for the first time. We used both scanning electron microscopy (SEM) and histological techniques to investigate the internal and external changes that occur during this process. A similar SEM study of another species of the genus, *M. margaritifera,* was recently published (Nezlin et al., 1994). It is important to note that since the first pioneering articles of Arey (1921, 1924, 1932a,b,c), no significant advances have been made in the study of Unionoid glochidial metamorphosis.

MATERIALS AND METHODS

Once the glochidia attach to the host they are enclosed by host tissue; therefore, we use the term "encapsulation" rather than "encystment," following Hoggart and Gaunt (1988). Nevertheless, we retain the term "cyst" and use it in the sense of "capsule."

Infection of *Acipenser baeri* Brandt with glochidia of *Margaritifera auricularia* (Spengler) was carried out in laboratory aquaria at the Museo Nacional de Ciencias Naturales over a period of 3 years: 1996, 1997, and 1999. Sturgeon specimens were maintained in aquaria at different temperatures (16–20°C in 1996, 18–22°C in 1997, the experiment began at 22°C and finished at 18°C and 16–17°C in 1999) and fed mosquito larvae prior to being artificially or naturally infected with glochidia. Aquaria pH levels were maintained at 7.5–7.7.

For artificial infection, glochidia were collected from the exhalant aperture of the gravid mussels with a pipette and rinsed with aerated water in a glass jar containing the isolated fish. Natural infection occurred when fish were placed in contact with gravid mussels in the aquarium. Once infected, the fish were isolated in individual aquaria until inspected. Prior to being sacrificed, specimens were anesthetized with 3-aminobenzoic acid ethyl ester (MS222) to examine the infected gills. Gill samples were excised and fixed following two different protocols.

*Correspondence to: Rafael Araujo, Department of Biodiversity and Evolutive Biology, Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, José Gutiérrez Abascal 2, 28006 Madrid, Spain. E-mail: rafael@mncn.csic.es

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Histology

In the experiment of 1996, sturgeon were sacrificed at different intervals after infection: 5, 13, 34, and 60 days. In 1997, sturgeons were sacrificed at: 1, 2, 3, 4, and 5 h, and 2, 3, 16, 34, 51, and 55 days after infection. In 1999 the day intervals were: 6, 28–43, 29–44, 32–47, 35–50, 36–51, 37–52, 53, and 65 days. Fish gill samples were fixed in Bouin's fluid, dehydrated in a graded ethanol series (70, 80, 90, and 100%), submerged in benzyl-benzoate for 30 min, then in benzyl-benzoate and paraplast (1:1) for 15 min, embedded in paraplast, and serial-sectioned (6 μm) with a Leica (RM 2045) microtome. All slides were stained with hematoxylin-eosin (modified from the Carazzi method). A total of 300 slides were prepared.

Scanning Electron Microscopy

In 1996 samples were obtained from fish 5 and 13 days after infection. In 1997, fixation intervals were: 1, 2, 3, 4, and 5 h, and 2 and 3 days. In 1999: 29–44, 35–50, 37–52, 53, and 65 days. Fish gill samples were fixed in glutaraldehyde, dehydrated in a graded ethanol series (30 min at 30, 50, 70, 90, 96, 100, and 100%), and transferred to acetone for 30 min. They were critical-point-dried with liquid CO_2 in a Polaron E-3000 unit and coated with gold in a Bio-Rad (Richmond, CA) SC515 sputter-coating unit, 20 nm thick. Observations were made with a Philips XL20 SEM at accelerating voltages of 20–30 kV.

In the 1999 experiment, sturgeon had been in previous contact with gravid molluscs and as a result may have already harbored encapsulated glochidia in their gill filaments; therefore, the time intervals between infection and inspection were not as precise as in other years.

RESULTS

Immediately after making contact with the sturgeon, the mature glochidia attach to the epithelium of the gill filaments. They puncture the secondary lamellae (the gas-exchange area) at any point along the length of the filament (Fig. 1A), but are especially prone to pierce distal areas of the filament. The pierced gill epithelium, along with host connective tissue, blood cells, and vessels, are incorporated into the body of the glochidium and are visible between the glochidial valves (Fig. 1B).

The glochidial shell is not visible in histological sections, possibly due to decalcification. The periostracum, which originates in the ventral larval mantle folds, appears as a dense, thin layer, and completely covers the glochidial shell (Fig. 1C).

The adductor muscle occupies the anterior and dorsal regions of the glochidium. It connects the two glochidial valves and consists of unicellular fibers with elongated nuclei (Fig. 1D). The mantle consists of a simple epithelium of thin, flat cells. Internally, the ventral and oral plates resemble two large hollow masses formed by a layer of large cuboidal cells with dark nuclei. The ventral plate (Fig. 1D) is composed of thick ciliated cells and is located near the ventral edge of the glochidium. The oral plate, located posteriodorsally, is smaller than the ventral plate.

The successive stages of metamorphosis described below are based mainly on the experiment conducted in 1997 at 22–18°C. Nevertheless, some figures and comments correspond to the other experiments and in those instances the temperature at which the experiment was conducted is shown in brackets.

Once punctured by the glochidia, the host epithelium begins to form a capsule (= cyst) (Fig. 1E) that covers half of the glochidia in approximately 4 h (Fig. 1F). Five hours after attachment the cyst is nearly completely grown and in 48 h the entire glochidium is enclosed by several layers of cells made up of overlapping lamellae (Figs. 2A–C). The width of the cyst wall at this stage is approximately 25 μm. During the first 13 days of metamorphosis the cyst undergoes no further external changes.

With the exception of a small size increase of the ventral and oral plates and some slight cell proliferation, there are no internal changes within the cyst during the first 5 h. Organogenesis has not yet begun and the ventral plate retains its ciliated epithelium.

Three days after infection the cells of the ventral plate begin to proliferate and invaginate, forming what will be the foot and gill (anlage). Five days after infection (at 17°C), two rudimentary gills form and surround the foot (Fig. 2D). A cavity appears within the oral plate that will become the digestive and reno-pericardial systems (Fig. 2E).

Thirteen days after infection the gills are substantially more developed and symmetrical (Fig. 2F). Sixteen days after infection an increase in glochidial size makes the glochidium spherical in shape (Fig. 3A). The cyst remains spherical until the end of metamorphosis (Fig. 3B). The muscle at this stage has been reabsorbed and the cyst wall is becoming progressively thinner. An increase of the space between the glochidium mantle and the cyst, probably an artifact, is observed in many cases.

Thirty-four days after infection the encapsulated glochidium is a sphere with a diameter of approximately 150 μm. The cyst wall is now very thin (Fig. 3C) and composed of large cells (Fig. 3D). In contrast, at day 34 in the 1996 experiment (16–20°C), the cyst wall still consisted of several layers of cells (Fig. 3E).

Inside the glochidium, organogenesis of new structures continues. Anterior to the foot, rudiments of the digestive glands appear as two pink masses of globular cells (Fig. 3F). The gills are more developed at this stage and continue to surround the foot. Thirty-four days after infection at 16–20°C (Fig. 4A), the two adductor muscles are present in the juvenile and a long cavity (dorsal) formed of vacuolated cells that will become the future kidney and pericardium appears (Fig. 4B). These structures were not developed 16 days after infection in the experiment at 18–22°C (1997). Metamorphosis is complete in approximately 51 days at 18–22°C and in 65 days at 16–17°C.

The released juveniles have spherical shells with a thin rim of new shell material (Fig. 4C) and a

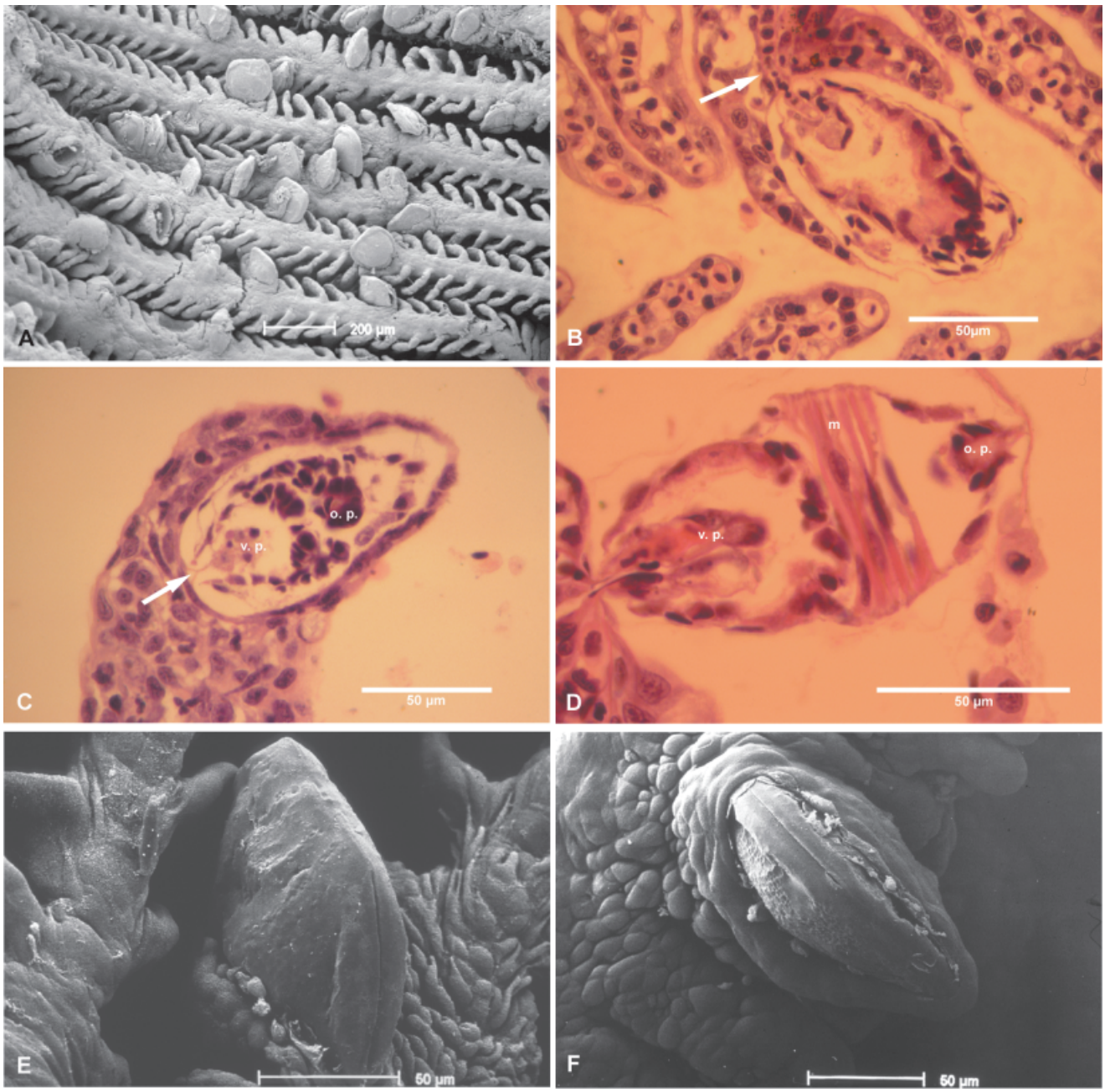

Fig. 1. Metamorphosis of *Margaritifera auricularia* glochidia in *Acipenser baeri* gill lamellae. **A**: Gill lamellae 5 h after infection; SEM. **B**: Histological medial transverse section of the glochidium 1 h after infection; arrow indicates host blood vessel pierced by the glochidium. **C**: Medial transverse section 3 days after infection; arrow indicates the periostracum of the glochidium. o. p., oral plate; v. p., ventral plate. **D**: Anterior transverse section one hour after infection. m, muscle; o. p., oral plate; v. p., ventral plate. **E**: Growing cyst 3 h after infection; SEM. **F**: Cyst growth 4 h after infection.

finely ciliated foot (Fig. 4D). Mean measurements of these juveniles are: length = 190 µm (n = 1), width (from the umbo to the ventral border) = 193 µm (n = 4), and height = 210 µm (n = 4).

DISCUSSION

In Scottish streams, Young and Williams (1984) found that only 5% of *Margaritifera margaritifera* glochidia survived in the gills of its preferred host, *Salmo trutta*. In contrast, Cunjak and McGladdery (1991), working with the same mussel species in South River (Nova Scotia, Canada), found no glochidia loss in salmon parrs (*Salmo salar*) during the parasitic phase. In our study, the abundance of *M. auricularia* glochidia found in the gills of *Acipenser baeri*, both at the beginning (Fig. 1A) and at the end of metamorphosis (more than 300 glochidia in each

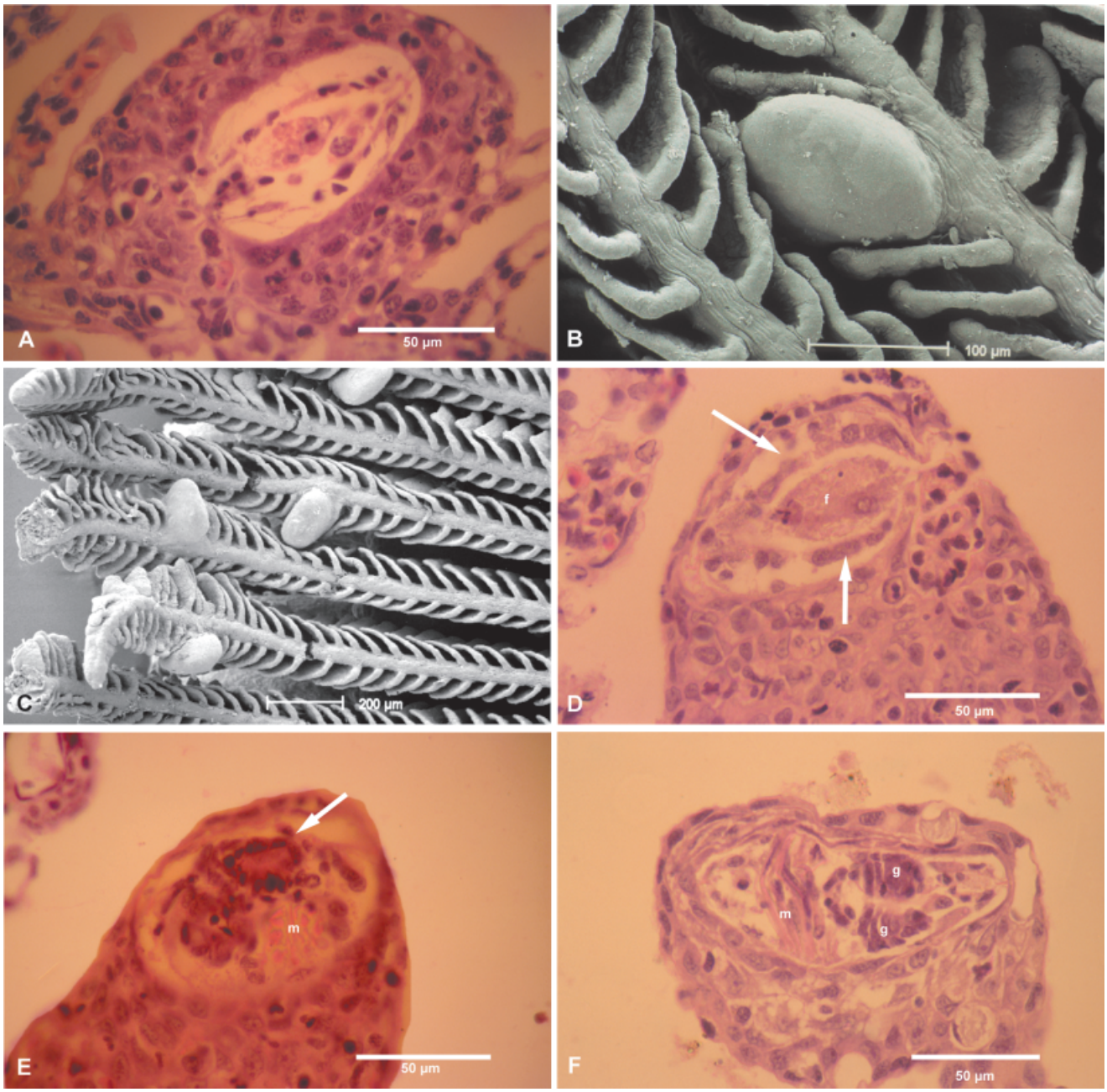

Fig. 2. Metamorphosis of *Margaritifera auricularia* glochidia in *Acipenser baeri*. **A**: Histological section of cyst surrounding the glochidium 3 days after infection. **B**: Gill lamellae with an encysted glochidium; SEM. **C**: Gill lamellae and formation of cysts by overlapping host lamellae; SEM. **D**: Medial transverse section of a glochidium 5 days after infection (16–20°C). The arrows show the rudimentary gills. f, foot. **E**: Medial sagittal section of a glochidium 5 days after infection (16–20°C). Arrow indicates the future digestive and reno-pericardial systems. m, muscle. **F**: Anterior transverse section of an encysted glochidium 12 days after infection (16–20°C). g, gill; m, muscle.

gill lamellae), indicates that the species is highly host-specific (Kat, 1984). Nevertheless, this does not indicate that *A. baeri* is the only suitable host for this endangered mussel species (Araujo et al., 2001).

The formation of cysts is a reparative response of the host gill tissues (Arey, 1921). It was originally believed that a proliferation of host gill epithelial cells created the cyst, but Arey (1932a,b) found this description insufficient and demonstrated that cell migration better explained the encystment process. Similarly, Nezlin et al. (1994) reported that cyst formation in *Salmo salar* parrs infected with *Margaritifera margaritifera* glochidia was the result of migration and shape change of gill epithelial cells, not hyperplasia (the excessive multiplication of normal cells on a tissue). In contrast, our results sug-

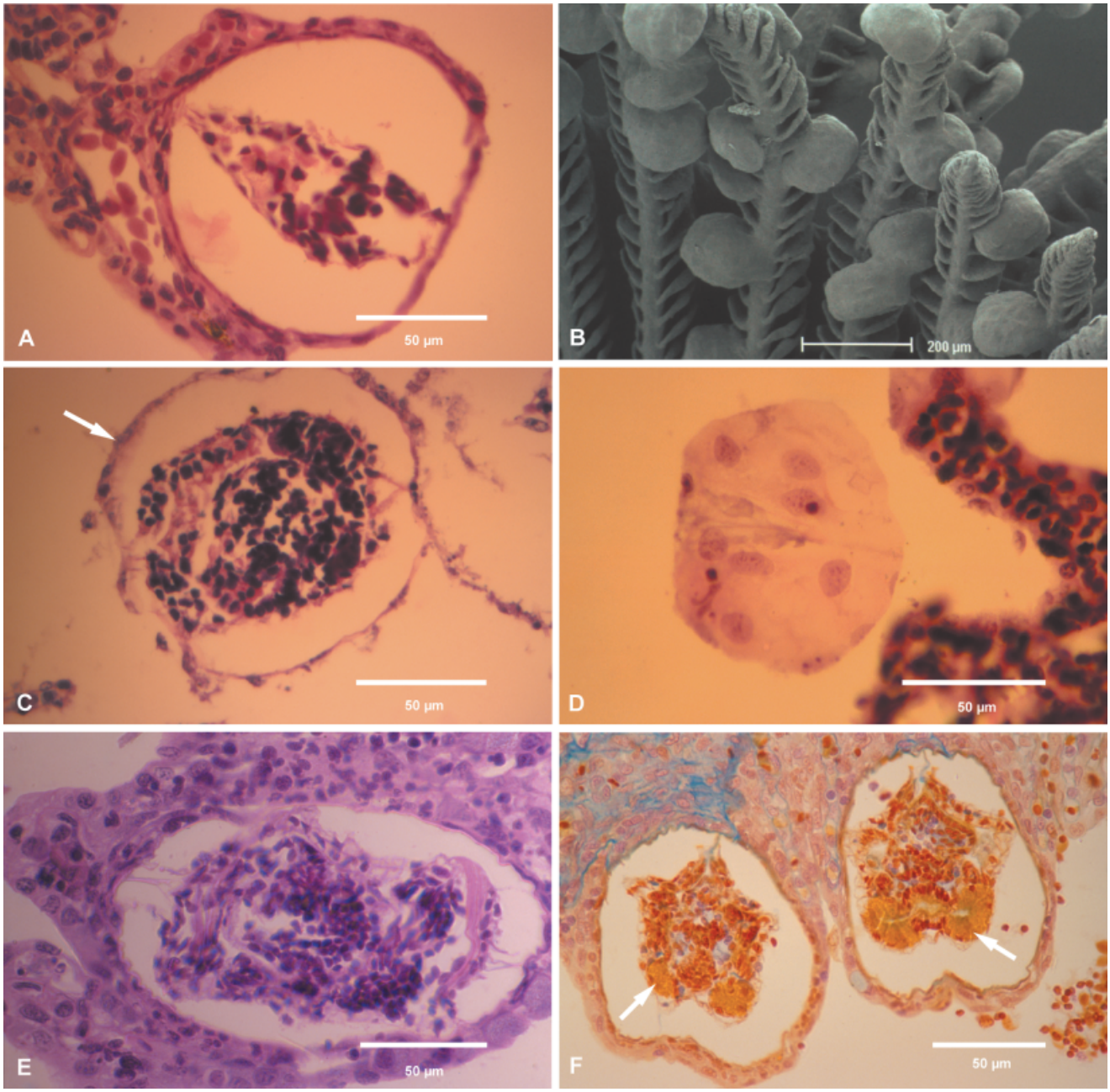

Fig. 3. Metamorphosis of *Margaritifera auricularia* glochidia in *Acipenser baeri*. **A**: Medial transverse section of a glochidium 16 days after infection. **B**: The glochidia close to the end of metamorphosis showing the spherical shape of the cyst; SEM. **C**: Thinning of the cyst wall (arrow) 34 days after infection (18–20°C). **D**: Section of the cells of the cyst wall. **E**: Dorsal horizontal section of an encysted glochidium 34 days after infection (16–20°C). **F**: Medial transverse section of two glochidia. Arrows indicate the future digestive glands 34 days after infection (16–20°C). Heidenhain's Azan.

gest that hyperplasia is the likely cause of cyst formation in the gill filaments of *Acipenser baeri* infected with *M. auricularia* glochidia.

The relationship between glochidium and host is highly specific, since the formation of a complete cyst and successful metamorphosis only occur when glochidia are attached to appropriate aquatic vertebrate hosts. Evidence also suggests a nutritive relationship exists between glochidium and host (Arey, 1932a; Murphy, 1942). The host tissue and the degenerating larval adductor muscle are important sources of nutrients for glochidia during metamorphosis (Arey, 1932a,c).

In *Margaritifera auricularia,* encystment is completed in a few hours, very similar to the time reported for *M. margaritifera* (9–12 h; Nezlin et al., 1994), *M. falcata* (2–4 h; Murphy, 1942) and *Lampsilis luteola* (2 h; Arey, 1921). Similar to what we

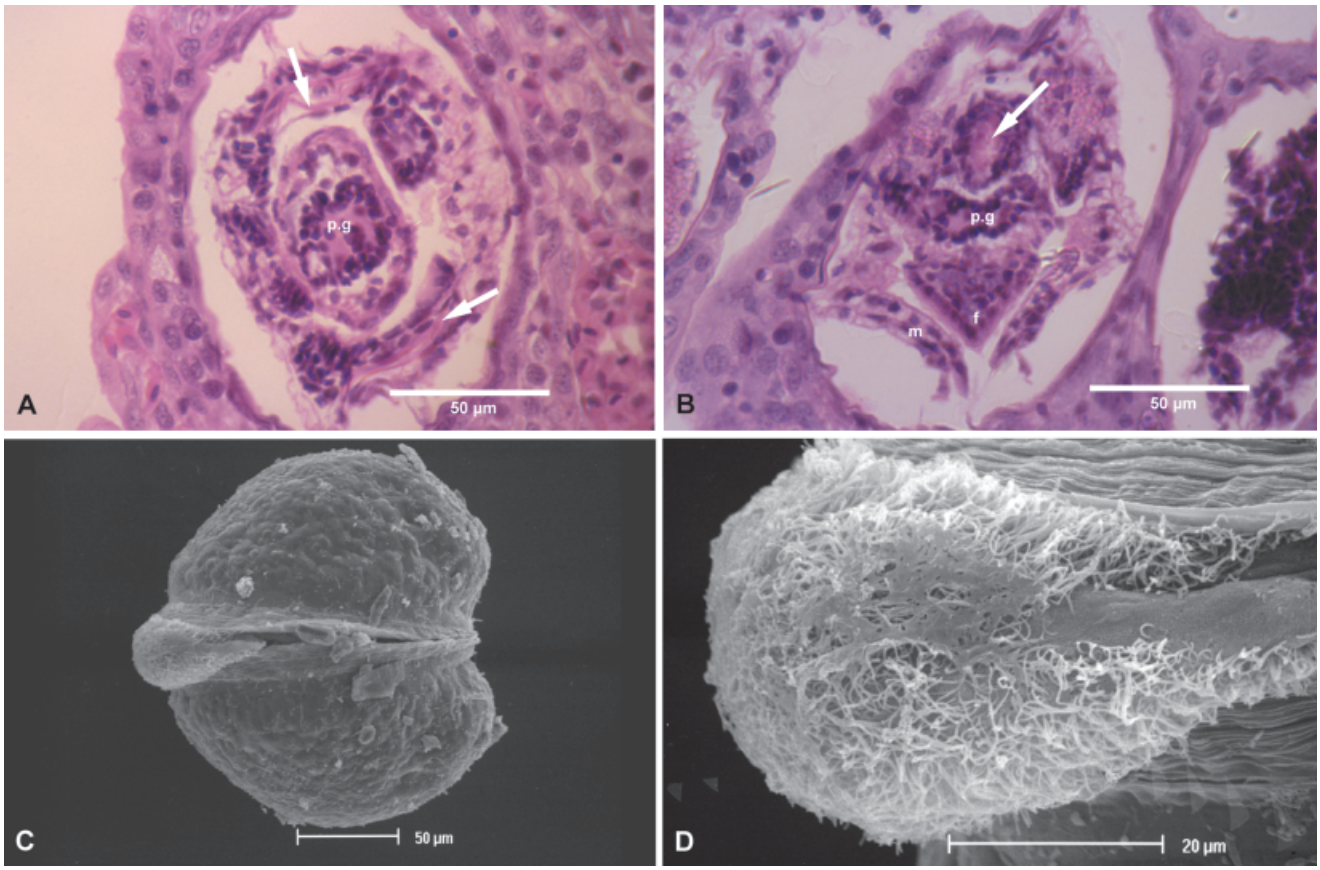

Fig. 4. **A**: Horizontal section of *Margaritifera auricularia* glochidium 34 days after infection (16–20°C); arrows indicate the two adductor muscles. p. g., pedal ganglia. **B**: Medial transverse section 34 days after infection. The arrow shows the future kidney and pericardium. f, foot; m, mantle; p.g., pedal ganglia. **C**: The juvenile *M. auricularia*; SEM. **D**: Detail of the ciliated foot of the juvenile.

observed in *M. auricularia,* cysts formed by overlapping gill lamellae have also been reported by Karna and Millemann (1978) in Chinook salmon (*Oncorhynchus tshawytscha*) with *M. margaritifera* (= *falcata*) and by Nezlin et al. (1994) in Atlantic salmon (*Salmo salar*) with *M. margaritifera*.

Arey (1932a) reported that following the first few days of initial encystment there is no significant change in the cyst until the juvenile is released. In contrast, *Margaritifera auricularia* and *M. falcata* cysts become gradually thinner as metamorphosis progresses.

The rate of glochidial metamorphosis varies among species and is highly dependent on environmental conditions. *Elliptio complanatus* completes metamorphosis in only 18 days in the gills of yellow perch (Matteson, 1948). The duration of metamorphosis of *Margaritifera auricularia* glochidia in *Acipenser baeri* varies, depending on the temperature, but it is likely more constant when this parameter is stable. Araujo and Ramos (2000b) observed that *M. auricularia* metamorphosed in ca. 700 degree-days (30 days at 23–24°C). In the present study, data seem to indicate that more time is required for metamorphosis, i.e., close to 1,000 degree-days in 1997 (the temperature fell to 18°C in the second half of the process and the last sturgeon died on day 51 when juveniles were close to being released) and close to 1,100 degree-days (65 days at 16–17°C) in 1999. Results from the 1996 experiment are not available because no juveniles were found, but in another experiment carried out at 20–21°C in 1998, juveniles were released 31 days after infection (635 degree-days). This suggests that metamorphosis accelerates when water temperature is raised to 20°C. However, raising the temperature several degrees above this does not appear to further accelerate metamorphosis. In a similar experiment with the other known suitable host of *M. auricularia* glochidia, *Salaria fluviatilis* (= *Blennius fluviatilis*), juveniles were released in 42 days at a mean temperature of 19°C (798 degree-days) (Araujo et al., 2001).

Metamorphosis in *Margaritifera falcata* lasted 36 days at 14°C in North America (Murphy, 1942).

Although the glochidia of several unionid species do not increase in size during metamorphosis (see Kat, 1984), size increase is common in species of the

genus *Margaritifera*: glochidia of *M. falcata* increased in length from 0.06–0.42 mm (660%) (Murphy, 1942) and an increase of 500% was cited by Karna and Millemann (1978). In Scotland, European *M. margaritifera* glochidia grew from an initial length of 0.07 mm to 0.40 mm in 9 months (Young and Williams, 1984). A similar glochidial growth rate was reported in Germany (Bauer and Vogel, 1987). The growth rate of *M. auricularia* glochidia during metamorphosis (length, 41%; width, 53%; and height, 238%) is the smallest reported for the genus.

This study presents the only published information to date regarding metamorphosis in *Margaritifera auricularia*. Results suggest that temperature plays a critical role in the metamorphosis process and that surrogate fish species can be used when native sturgeon, their preferred host, is not available. Preservation of this critically endangered mussel species will depend on further research on host fish suitability and regulation of water conditions.

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