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Description of the glochidium of *Margaritifera auricularia* (Spengler 1793) (Bivalvia, Unionoidea)

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The glochidium of *Margaritifera auricularia* is described for the first time by using light microscopy, scanning electron microscopy and histological techniques. The larval mantle is formed by only two layers of cells; the inner one being much thicker, with microvilli. All cell masses of the glochidium are temporary aggregations that are the rudiments of organs of the subsequent juveniles which will be released after metamorphosis in the host tissues. In the glochidium there are three main masses of cells: (i) the muscle, which is in an anterior position; (ii) the oral plate in the centre of the larva; and (iii) the more ventrally and posteriorly situated ventral plate, or foot rudiment, flanged with lateral pits all bearing dense cilia. No rudimentary organs such as the pericardium, the kidney, the heart or nerve ganglia have developed. There are no visible hooks in the valve margins, but by using light microscopy we observed minute teeth covered by a rim of the periostracum. Near the margin of the shell there are two pairs of sensory hair tufts only observable by scanning electron microscopy. The glochidium of *M. auricularia* is the largest of the family Margaritiferidae and intermediate between the glochidium of the known species of this family and those of Unionoidea.

Keywords: *Margaritifera auricularia*; Unionoidea; glochidium; anatomy; Spain

1. INTRODUCTION

Freshwater bivalves of the superfamily Unionoidea contain a parasitic stage in the reproductive cycle that typically includes a fish host and a modified larva, the glochidium. The glochidia develop from fertilized eggs that are maintained in the gills of the female or the hermaphrodite parents and are released into the water where they must attach to the gills or fins of, in some cases, specific fishes to develop a parasitic stage. Ortmann (1911) and Lefevre & Curtis (1912) were pioneers in the study of larvae of North American species, whereas the glochidia of most European unionid species were described by Pekkarinen & Englund (1995*a,b*). Harms (1907, 1909), Smith (1976), Young & Williams (1984) and recently Nezlin *et al.* (1994) and Pekkarinen & Valovirta (1996) described the glochidium of *Margaritifera margaritifera* (Linnaeus 1758), a Holarctic species and one of the few representatives of the old (Upper Cretaceous) genus *Margaritifera*. With the exception of *M. margaritifera* and the vanishing Irish population of the putative species *Margaritifera durrovensis* (Phillips 1928), there have been no records of living specimens of the other European species *Margaritifera auricularia* since 1917 (Haas 1917), and no data are available on its host fish, reproduction, development or the morphology of its glochidium. As was supposed by Lefevre & Curtis (1912), with only one exception (see Bauer 1994), the type of glochidium is constant for each genus (Pekkarinen & Englund 1995*a*) and therefore may be useful for species identification and classification (Giusti 1973). However, it

has not been described in all species of the genus *Margaritifera*. In their recent paper, Pekkarinen & Valovirta (1996) stated differences between descriptions of the glochidium of different populations of *M. margaritifera*, mostly whether there were teeth present (Harms 1909; Smith 1976) or absent (Young & Williams 1984; Nezlin *et al.* 1994; Pekkarinen & Valovirta 1996). This indicated either that glochidia of the same species may present morphological differences among different geographical locations or that these microscopic structures have been understood in different ways by different authors.

The discovery of a relict population of *M. auricularia* (Araujo & Ramos 1996) has allowed us to study the reproductive cycle of this species. By using optical and electronic microscopy and histological techniques, we describe here the anatomy and morphology of the glochidium of *M. auricularia*. The specialized structures of this larva are described and compared with those of other unionacean species.

2. MATERIALS AND METHODS

Specimens of *Margaritifera auricularia* were collected in February 1996 in an irrigation channel of the Ebro River in Zaragoza, Spain. Animals were transported live to the laboratory in a net inside a portable refrigerator with ice. They were kept in an aquarium at 19 °C.

Glochidia were collected in the aquarium with a pipette directly from the mussels' exhalant apertures. The study was made with live and fixed glochidia by using stereomicroscope, light microscope and scanning electron microscope (SEM) techniques. Images of live glochidia

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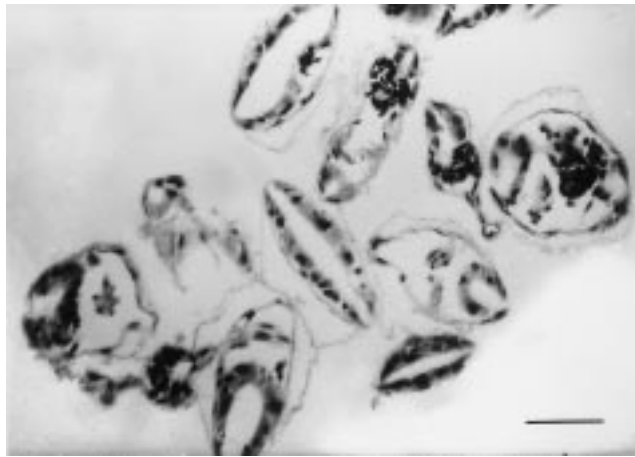


Figure 1. Light micrograph of the conglutinate of larvae of *M. auricularia*. Scale bar: 75 μm .

were obtained with a stereomicroscope, light microscope, and video.

For a histological investigation, the sample was fixed in Bouin's fluid, dehydrated in a graded ethanol series (70, 80, 90 and 100%), submerged in Benzil-benzoate for 30 min, then in Benzil-benzoate and Paraplast (1:1) for 15 min and embedded in Paraplast. Sections were made of 3–5 μm with a microtome (Leitz Model, 1512).

For SEM, samples were fixed for 2 h 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 then coated with gold in a Bio-Rad SC515 sputter-coating unit, 20 nm thick. Observations were made in a Philips XL20 SEM at accelerating voltages of 20–30 kV.

Once the release of glochidia had started, some juvenile specimens of the fish *Accipenser cf. baeri* (sturgeon) were introduced into the aquarium to test its susceptibility to glochidial exposure. (The terminology used in describing glochidial anatomy is based on Harms (1907, 1909) and Wood (1974).)

3. RESULTS

In the aquarium the mussels released white masses of eggs, several larval stages and glochidia. The stages

immediately prior to mature glochidia, which are always covered by the vitelline membrane, form the conglutinate (see figure 1) that may be found moulded into the shape of the cavity of the marsupium; it is not shaped like marsupial water tubes as in other species (Lefevre & Curtis 1910, 1912) because *M. auricularia* does not have a marsupium divided into water tubes. The results we describe here are for mature glochidia only. The study of the development from fertilized egg to glochidium will be published later.

All cell masses in the glochidium are temporary aggregations that are the rudiments of organs of subsequent juveniles to be released from host gill tissues after metamorphosis. At this stage, the glochidium of *M. auricularia* is very poorly developed, as only diffuse cell aggregations are detected.

(a) General morphology

The glochidia of *M. auricularia* (figure 2*a,b*) are microscopic (table 1) (mean length = 134 μm ; s.d. = 4.96; variation coefficient (v.c.) = 0.03; $n = 25$; mean height = 126 μm ; s.d. = 5.52; v.c. = 0.04; $n = 22$; mean width = 62 μm ; s.d. = 5.09; v.c. = 0.08; $n = 15$), white or light-coloured and very thin. At high magnification the shell surface (figure 2*c*) has no pores, nor particular sculpture, and only small depressions are observable. These depressions disappear at the valve border where the periostracum folds towards the inside of the shell. The general shape is similar to the D-shape of other bivalve larvae (i.e. *Corbicula fluminea*) and the glochidia of *M. margaritifera*: with a straight hinge and a very rounded ventral margin.

No hooks were observed on the margins of the valves, but minute teeth, covered by the rim of the periostracum, could be seen under a light microscope (see figure 3). Under SEM observation, the ventral aspect of the shell margin is readily apparent, as is the position of the covered teeth in open glochidia.

The shell is so thin that the single adductor muscle is easily visible through it. The adductor is attached to the anterior side of the inner surface of each valve (figure 4). Contraction of this muscle depresses the valve, so that a dent appears in it (figure 5). Spontaneous contractions of the adductor muscle make the valves snap.

Table 1. Comparison of margaritiferaid glochidial dimensions according to tables in Bauer (1994) and Pekkarinen & Valovirta (1996) (See text for detailed data on *M. auricularia*.)

species	length (μm)	height (μm)	width (μm)	reference
<i>Margaritifera auricularia</i>	127–144	120–142	54–71	This study
<i>M. margaritifera</i>	47.5	—	—	Harms (1909)
<i>M. margaritifera</i>	50	—	—	Bykhovskaya-Pavlovskaya <i>et al.</i> (1964)
<i>M. margaritifera</i>	60	80	—	Smith (1976)
<i>M. margaritifera</i>	60	80	—	Young & Williams (1984)
<i>M. margaritifera</i>	60	70	—	Nezlin <i>et al.</i> (1994)
<i>M. margaritifera</i>	70	—	—	Bauer (1994)
<i>M. margaritifera</i>	66–71	74–80	—	Pekkarinen & Valovirta (1996)
<i>M. falcata</i>	50–60	—	—	Murphy (1942)
<i>M. falcata</i>	70–73	75–80	—	Karna & Millemann (1978)
<i>M. laevis</i>	70–90	—	—	Awakura (1968)
<i>Cumberlandia monodonta</i>	55	—	—	Howard (1915)

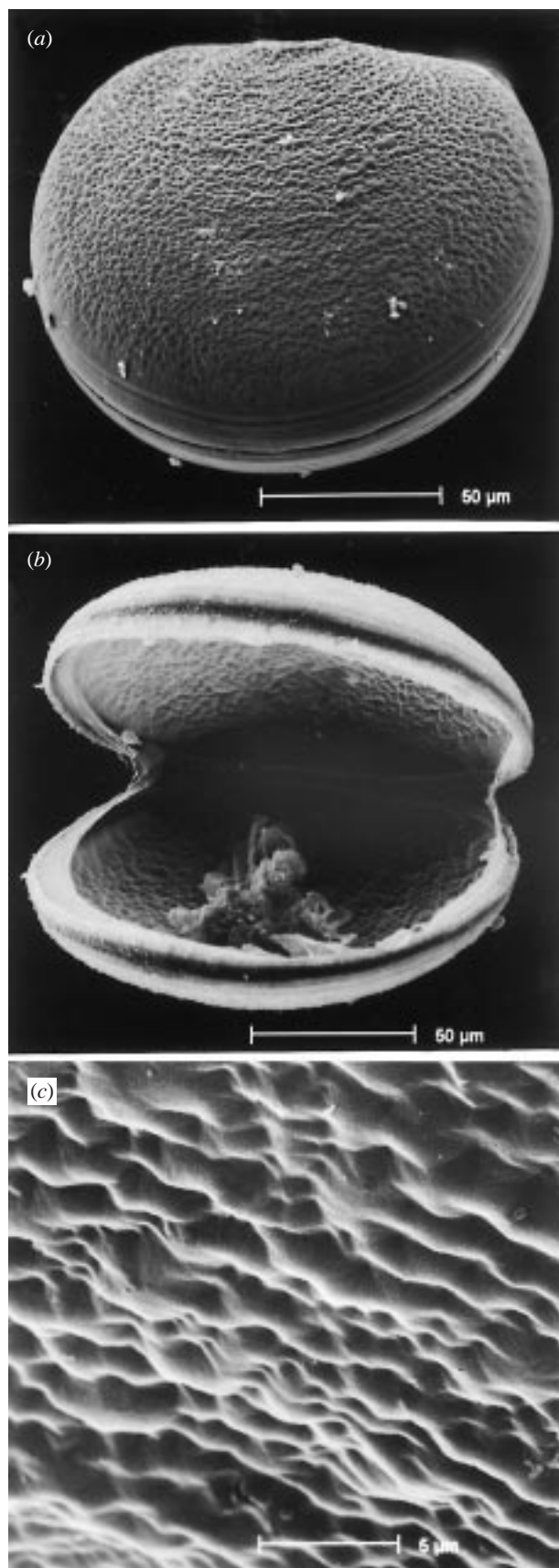


Figure 2. (a, b) SEM micrographs of the glochidium of *M. auricularia*. (c) Outer surface of the larval shell.

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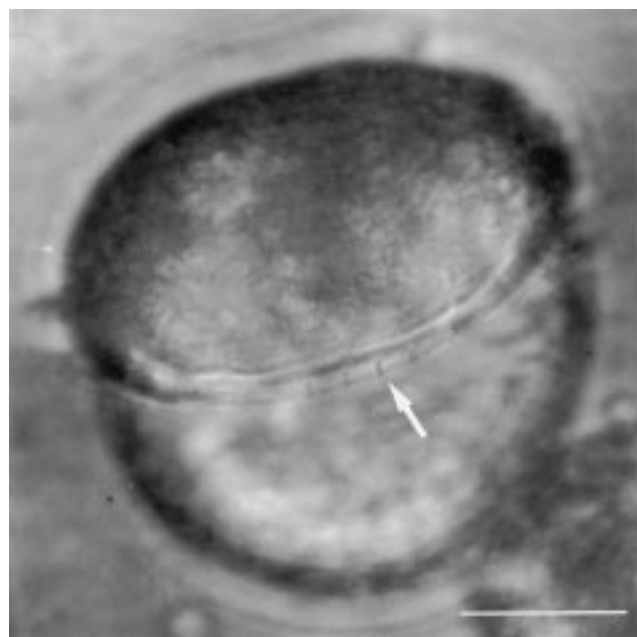


Figure 3. Light micrograph of the teeth (arrow) in the shell ventral margin of the glochidium. Scale bar: 50 µm.

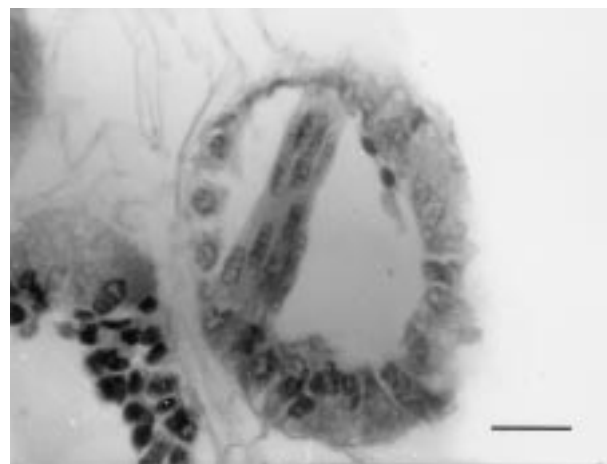


Figure 4. Cross-section of a glochidium showing the fibres of the adductor muscle. Scale bar: 3 µm.

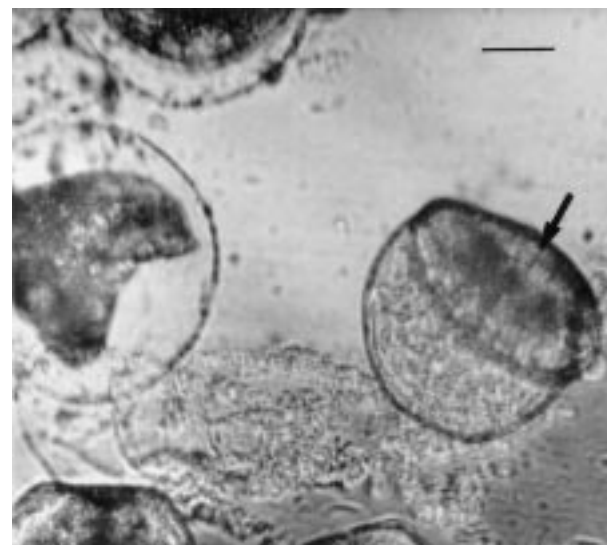


Figure 5. Light micrograph of a glochidium. The arrow shows the dent produced in the shell by the muscle contraction. Scale bar: 50 µm.

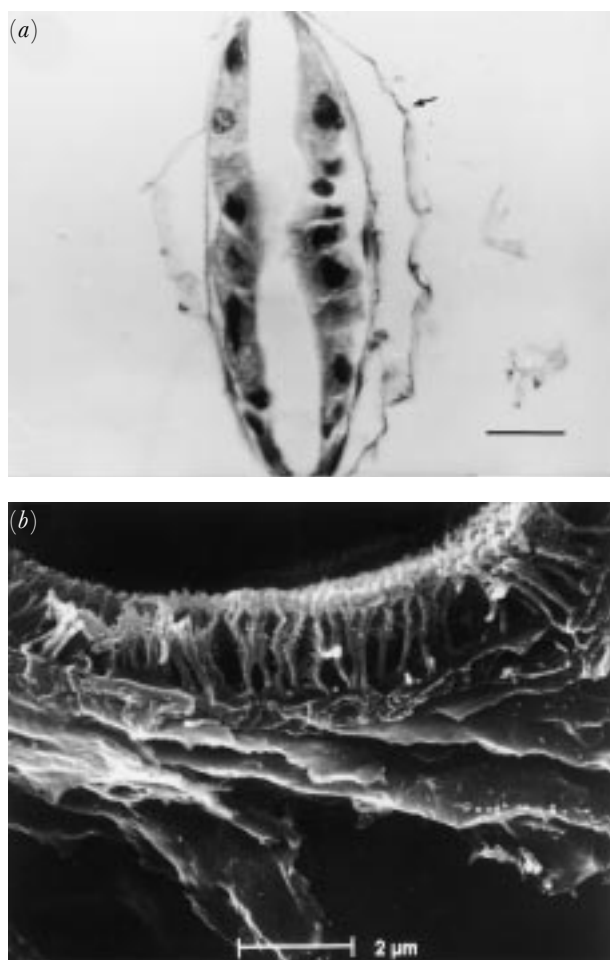


Figure 6. (a) Cross-section of a glochidium showing the two mantle layers. The arrow shows the periostracum. Scale bar: 3 µm. (b) SEM micrograph of a section of the mantle. Inner layer with microvilli upwards.

The movement of the cilia and sensory cells can also be seen under a light microscope, but their morphology will be described in the following section. No threads were seen inside the glochidia of study samples, but sometimes fine thread-like structures or 'hairs' were visible between the eggs in the conglutinate.

(b) Anatomy

All the soft parts of the glochidia are enclosed within the larval shell. Immediately below this shell are two layers of cells, the inner layer is much thicker and has microvilli (figure 6a,b). Cells in the outer layer are very flat and separated by a wide space (which becomes narrower towards the anterior and posterior sides) of the polyedric and vacuolated inner cells. The latter have a highly stained large central nucleus.

The mantle is comprised of three main masses of cells: the muscle, the oral plate and the ventral plate. The single adductor muscle indicates the anterior end of the glochidium. It consists of fibres with a single nucleus (see figures 4a and 7a) connecting the two valves of the shell. On the opposite side is the ventral plate (figure 7b–f) or foot rudiment, flanged with the lateral pits, which will be the future gills; all bear dense cilia. The next cell mass immediately posterior to the muscle and in the centre of the larva is the oral plate (figure 7g,h) or endodermic sac.

The cilia of the ventral plate (figure 8) move very vigorously, causing the glochidium to rotate; they are easily visible in live glochidia. No rudimentary organs such as pericardium, kidney, heart or nervous ganglia are present.

Near the shell margin are two pairs of sensory hair tufts (figure 9a), only observable by SEM. They are located very near the mantle edges, rising from holes in the microvilli of the mantle and formed by hairs of different lengths (figure 9b). No larval thread or thread gland was observed.

We examined the fish 24 h after they were exposed to the glochidia and the gill filaments were seen to be packed with glochidia. No glochidia were found on the fins or tail of exposed sturgeons.

4. DISCUSSION

The general appearance of the glochidium of *M. auricularia* resembles that of other unionoid larvae, but is most similar to the glochidium of *M. margaritifera*. Both are colourless, delicate and shaped 'like the bowl of a very blunt spoon', a description given for all hookless glochidia by Lefevre & Curtis (1912). Compared with the glochidium of other European unionoids, it is intermediate in size between *M. margaritifera* and species of Unionidae (genus *Unio* and *Psilunio*) (see table 1; Bauer 1994). In the scheme of Davis & Fuller (1981) the *M. auricularia* glochidium is between the small and medium classes, being the largest of the genus *Margaritifera*.

The outer shell surface differs between both margaritifera species, consisting of numerous small protuberances in *M. margaritifera* (Nezlin *et al.* 1994; Pekkarinen & Valovirta 1996) and numerous minute hollows in *M. auricularia* glochidia.

In the glochidium of *M. auricularia*, and probably *M. margaritifera*, the microscopic teeth of the shell margin are covered by a rim of the periostracum. Only Harms (1907, 1909) has cited the presence of six or seven minute teeth, while other authors (Nezlin *et al.* 1994; Pekkarinen & Valovirta 1996) have reported an absence of spines or teeth. Such a condition in which a flange of cuticle (periostracum) is present along the ventral border of the shell was cited by Lefevre & Curtis (1912) for North American hookless glochidia and by Giusti (1973) for the glochidium of the European species *Potomida littoralis* (Lamarck 1801). The occurrence of minute teeth in the glochidia of these two species of the genus *Margaritifera* may be a useful character in resolving the taxonomic position of the genus (Davis & Fuller 1981; Smith & Wall 1984).

The poor differentiation of larval organs in the glochidium of *M. auricularia* is similar to the anatomy of the larval stage in *M. margaritifera* described by Harms (1907, 1909). In both, the adductor muscle and the ciliated organs (ventral plate and lateral pits) are the most conspicuous cell masses. The embryonic mantle is formed by polyhedric and vacuolated cells flanged by very flat cells below the shell. No respiratory organs have developed, so the cilia of the lateral pits (future origin of the gills) and ventral plate (origin of the foot) probably aerate the larva. The oral plate will be the future estomodeum and the mid-gut (endodermal). The nervous system is completely lacking in the glochidia of the two species of *Margaritifera*, whereas in *Anodonta* the cerebral ganglion and sometimes the visceral ganglion are already

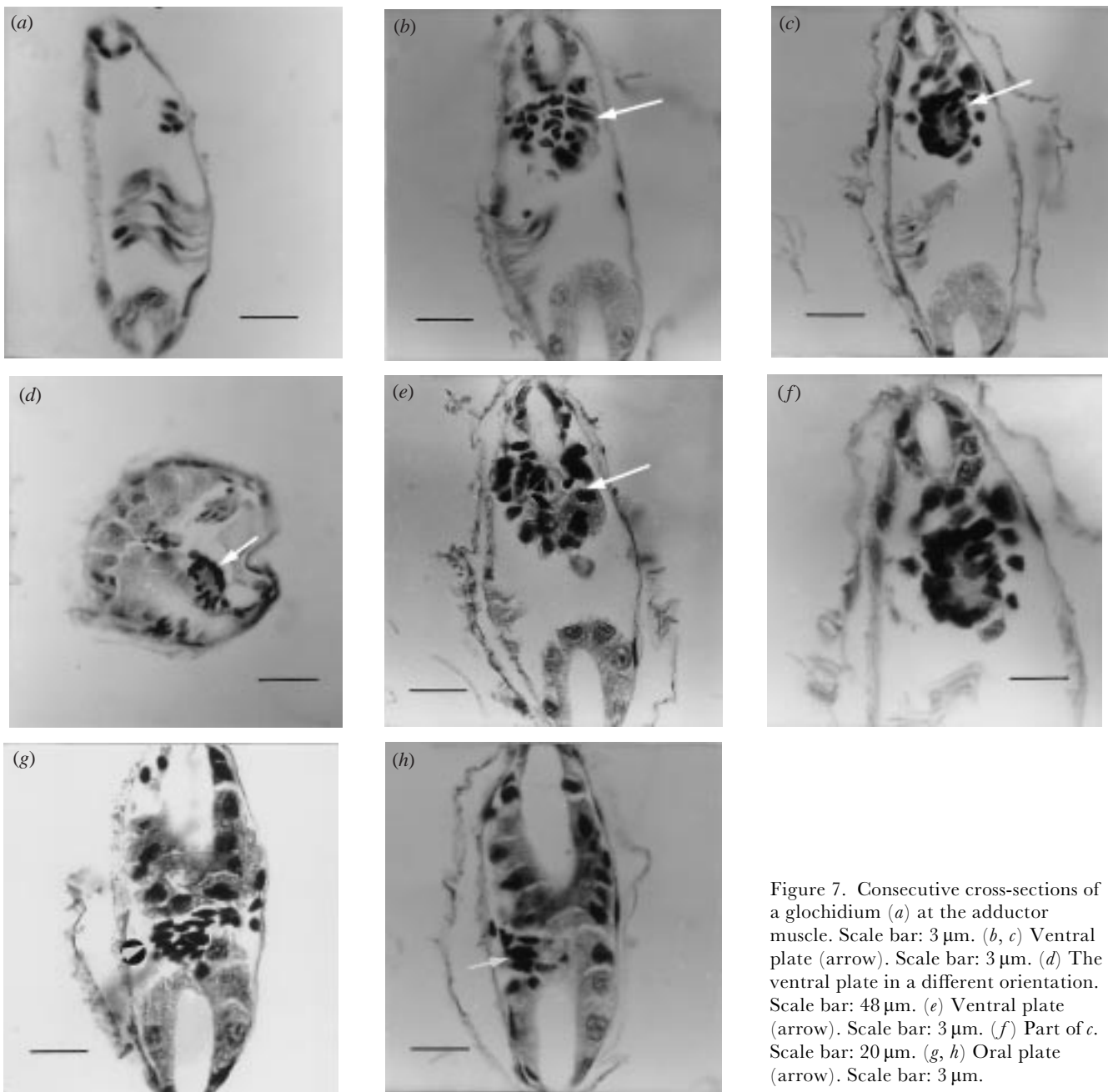


Figure 7. Consecutive cross-sections of a glochidium (a) at the adductor muscle. Scale bar: 3 μ m. (b, c) Ventral plate (arrow). Scale bar: 3 μ m. (d) The ventral plate in a different orientation. Scale bar: 48 μ m. (e) Ventral plate (arrow). Scale bar: 3 μ m. (f) Part of c. Scale bar: 20 μ m. (g, h) Oral plate (arrow). Scale bar: 3 μ m.

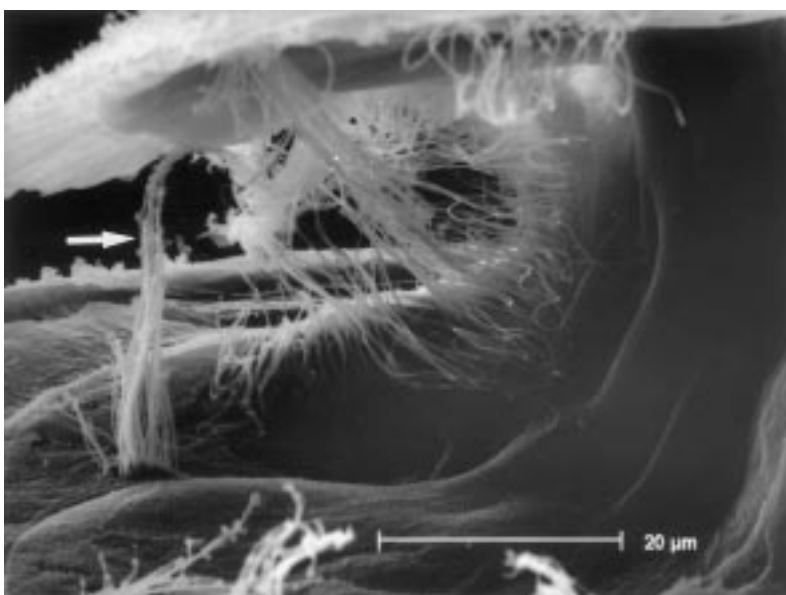


Figure 8. Scanning electron micrograph of the cilia of the ventral plate. The arrow shows a ventral sensory tuft.

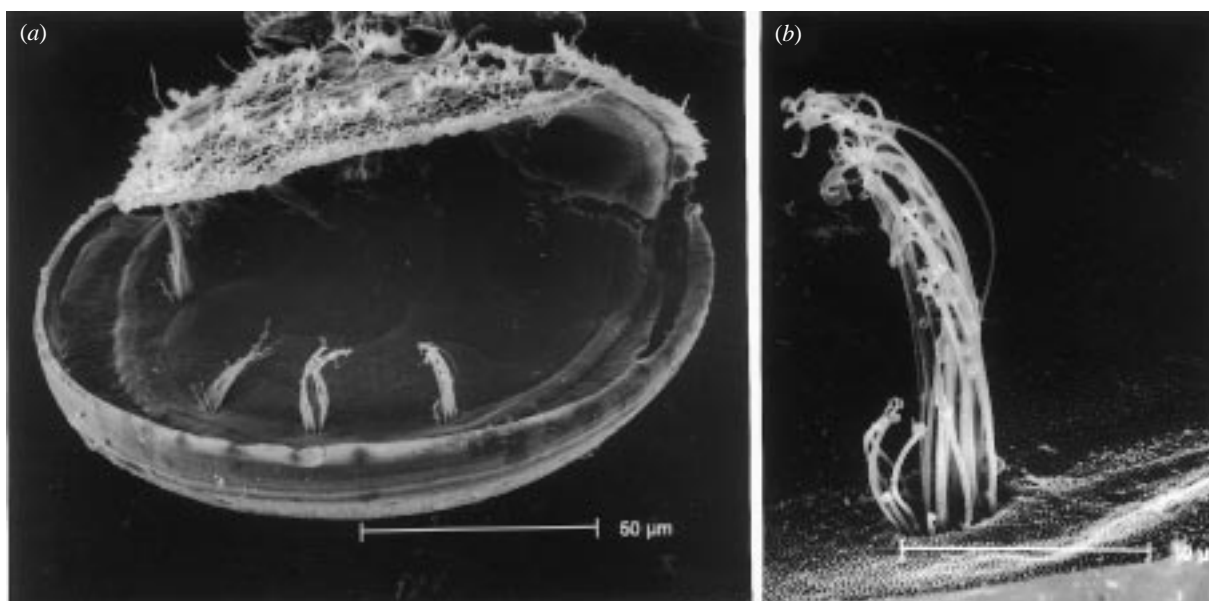


Figure 9. (a) Opened glochidium with the two pairs of sensory tufts. (b) Part of one tuft.

developed at this stage (Harms 1909). Owing to the absence of a nervous system the contraction of the adductor muscle may be explained as a tactile response transmitted by the hair tufts, as Pekkarinen & Valovirta (1996) suggested for the glochidium of *M. margaritifera*.

Our observation of a lack of any kind of larval thread in the mature glochidium agrees with Lefevre & Curtis (1912), who, in a study of hookless glochidia, only found larval threads in species of *Unio*: 'we have never seen any sign of such a structure in the ripe glochidia of the other genera which possess hookless glochidia'. Other authors (Schierholz 1889; Conner 1907) have also reported glochidial threads in species of *Unio*. There is no sign of this thread in the glochidium of *M. margaritifera*; however, Harms (1907, 1909) cited a very long filament when the glochidia are surrounded by the vitelline membrane, which is lost in the mature glochidium. As in *M. margaritifera* (Pekkarinen & Valovirta 1996), *M. auricularia* has only two pairs of sensory hair tufts instead of the four pairs of other unionoid glochidia (Lefevre & Curtis 1912; Pekkarinen & Englund 1995b).

Regarding the behaviour and reactions of the glochidia of *M. auricularia*, recorded on more than 10 h of videotape which was then studied, very little may be added to the results of Lefevre & Curtis (1912) concerning hookless glochidia. Glochidia are incapable of locomotion by the spasmodic contractions of the adductor muscle. The shell is so delicate that this contraction causes a depression in the larval shell immediately inside the area of the muscle insertion.

The presence of many glochidia of *M. auricularia* attached to the gill filaments of sturgeon and its absence on tail and fins (Araujo & Ramos 1996) indicates that this glochidium, like that of *M. margaritifera*, is exclusively a gill parasite. The host of the glochidium of *M. auricularia* is unknown. The decline of this species in an area with abundant specimens of *Unio elongatulus* (C. Pfeiffer 1825), *Anodonta cygnea* (Linnaeus 1758) and *Psilunio littoralis* indicates either a high degree of fish specificity, as in North

American unionids (Zale & Neves 1982) and *M. margaritifera* (Ziuganov *et al.* 1994), and/or a greater sensitivity to water quality.

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