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Scanning electron microscopy of glochidia and juveniles of the freshwater mussel, *Hyriopsis myersiana*

KOVITVADHI UTHAIWAN[1], KANNIKA CHATCHAVALVANICH[1], NAPAVARN NOPARATNARAPORN[2] and JORGE MACHADO[3*]

[1]*Department of Zoology;* [2]*Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[3]*Departamento de Produção Aquática, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Centro de Investigação Marítima e Ambiental (CIMAR), Porto 4099–003, Portugal*
Tel. +351 (22) 206-2294; Fax +351 (22) 206-2232; email: jmachado@icbas.up.pt

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Summary

The ultrastructure of early stages of the mussel, *Hyriopsis (Limnoscapha) myersiana* (Lea, 1856), was observed by scanning electron microscopy from the glochidial period until the onset of the juvenile stage 10 days later. Further observations were performed for an additional 13 days to assess juvenile development. Glochidia extracted from the brood chambers have a hookless, semi-oval and equivalve calcareous shell with numerous pores in the internal surface, pits in the external surface and cuticular spines in the ventral region. Keratin fibers with a random arrangement in the cuticle of the glochidial shell were also detected. The appearance of the foot within 10 days of *in vitro* glochidial culture was considered the main feature of metamorphosis to the juvenile stage. Another change during the following 13 days was the formation of a new periostracum exhibiting growth lines under the old glochidial shell. This development occurs mainly in the anterior region and is followed by hardening of the periostracum matrix by calcium deposition. Periostracum growth gradually became apparent in the lateral and posterior regions at the end of this period. The retraction of spines and the alteration of the external surface of the old shell are also described. It is speculated that transcuticular filaments identified in the juvenile stage may have sensory or metabolic exchange functions. The prominent foot, gradually covered by long dense cilia, shows rhythmical movements which suggest a role in feeding. Similarly, cilia present in the mantle may also be involved in the capture of food, while microvilli may facilitate absorption of dissolved materials. Longer cilia, sparsely distributed in the mantle, may function as chemo- or tactile sensors.

Key words: Mussels, glochidia, juvenile, ultrastructure, culture *in vitro*

Introduction

The life cycle of the Unionaceae is atypical among bivalves since it includes both free-living adult and short-lived obligatory ecto-parasitic larval (glochidia) phases. This was well documented for several North American, African and European species in a sequence of studies (Lillie, 1895; Lefevre and Curtis, 1910, 1912; Ortmann, 1910; Coker et al., 1921; Arey, 1924;

*Corresponding author.

Fryer, 1961; Yokley, 1972; Wood, 1974; Kat, 1984). During the reproductive cycle, the outer demibranchs — but sometimes the inner or even both demibranchs — usually function as marsupial chambers depending on the species (Baker, 1928; Kat, 1984; Bauer, 1994; Byrne et al., 2000).

In the marsupial demibranchs of a gravid female, major morphological changes take place to accommodate the large number of developing larvae. After several weeks or months of ctenidial incubation, the young are released when fishes pass by and the larva adheres to its scales, fins or gills and becomes a temporary ectoparasite (Reuling, 1919; Arey, 1932). A cyst is formed by the host fish as the result of chemical, histological and immunological reactions (Kirk and Layzer, 1997). Once inside, the glochidia initiate certain morphological transformations (Coker et al., 1921; Kat, 1984). There is, as yet, insufficient information on the mussel-host fish relationship. However, glochidial metamorphosis depends on the blood composition of the fish and on its immune responses, which is consequently the determining factor in mussel-host specificity (Reuling, 1919; Arey, 1932; Kirk and Layzer, 1997). The parasitic phase of development constitutes a critical period due to presence of specific antibodies to the glochidia (Kirk and Layzer, 1997). Only a small percentage of glochidia reach the juvenile stage due to the difficulty in finding and surviving in a suitable habitat (Kirk and Layzer, 1997). The post-infestation phase begins with juveniles being released from the fish to the bottom of habitat where they are exposed to new hazards (Keller and Zam, 1990; Uthaiwan et al., 2001). Studies of glochidia cultured *in vitro* to the juvenile stages have already been undertaken with success by Isom and Hudson (1982), Keller and Zam (1990) and Uthaiwan et al. (2001), with different species. This method may provide excellent conditions for observing glochidial metamorphosis.

The mussel of interest in the present work is a native, *Hyriopsis (Limnoscapha) myersiana* (Lea, 1856) (Order Unionoida, Family Amblemidae, Brant 1974), living in the Maeklong River in Kanchanaburi Province, east Thailand. It is a freshwater pearl mussel (Nagachinta et al., 1986; Panha, 1990) which spawns from October to May releasing glochidia 13–25 times during this period. The spawning season reaches its peak from December to January (Nagachinta and Meejui, 1998). In the gravid female the hookless type of glochidia with two valves and a larval thread develop inside the outer demibranchs for a short period of time. Subsequently, the larva needs to parasitize a host fish in order to develop into a juvenile form. This transformation also occurs rapidly. Wood (1974) gave a detailed description of the structure of the glochidium of *Anodonta cygnea*, and other observations expanded our knowledge to other species (Pekkarinen and Englund, 1955a; 1955b; Brondiewicz, 1968, Castilho et al., 1989; D'Eliscu, 1972; Giusti, 1973; Clarke, 1981, 1985; Jeong, 1989; Kwon et al., 1993; Pekarinen and Valovirta, 1996; Pekarinen, 1996; Jupiter and Byrne, 1997; Hoggart, 1999). The glochidial morphology of several Thai species have been described by Panha and Eongrapornkeaw (1995).

The microscopic morphology associated with glochidial metamorphosis and juvenile growth has not been investigated in *H. (L.) myersiana* under conditions of artificial culture of glochidia and juveniles. Thus, it is of interest to observe ultrastructure changes by scanning electron microscopy (SEM) and aspects of behavior from the larval stage until the complete development of the juvenile form.

Materials and Methods

One of the natural habitats of mussel *H. (L.) myersiana* is in the Maeklong River in Kanchanaburi Province, Thailand. However, this species has been cultured for more than 10 years in the Vajiralongkorn Reservoir, close to the river, mainly for research use. Gravid mussels were collected from the latter during brooding periods, i.e., the pre-gravid (white and yellow marsupia) and gravid (pale brown and brown marsupia) periods.

The juveniles were obtained from glochidia cultured in an artificial medium according Keller and Zam (1990), as modified by Uthaiwan et al. (2001). Each culture dish contained 2 ml of M199, 1 ml of common carp fish plasma and 0.5 ml of antibiotics/antimycotic agents. Glochidial activity was observed by light microscopy (×100), and those showing opening and closing shell movements were selected. The active glochidia were collected from the gravid mussels using a sterilized 1 ml syringe with 18 gauge needles. They were then washed in sterile distilled water and transferred to the artificial medium under sterile conditions. The glochidia were cultured in a low temperature incubator at 23±2°C with a constant supply of 5% CO_2 and room humidity. The medium was changed on the 5th and the 9th days of culture, and 1 ml sterile distilled water was added to each culture dish. After glochidia developed into juveniles, based on complete foot formation (within 10 days), early

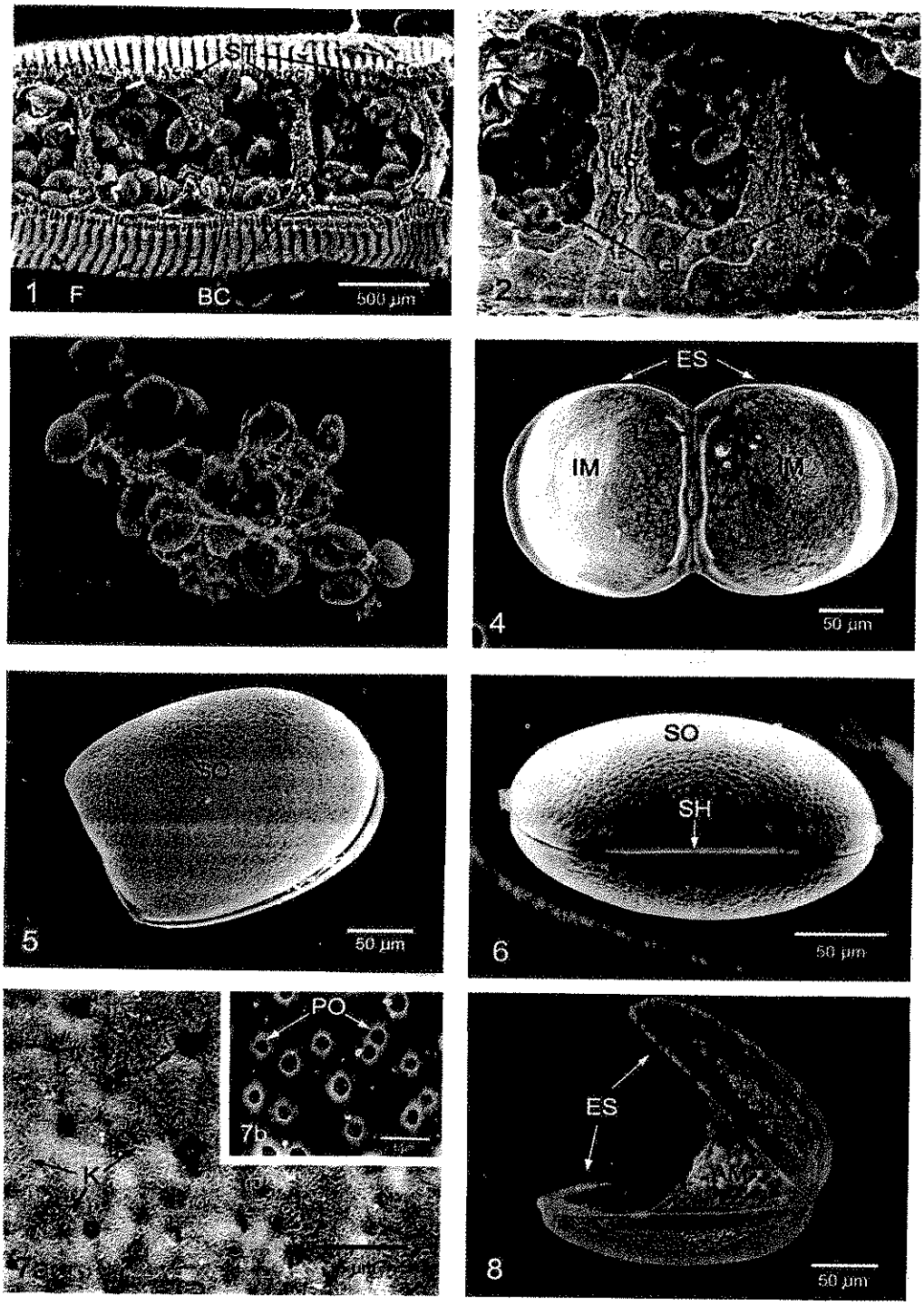
juveniles together with the medium were transferred to a beaker and the total volume was diluted with an equal volume of sterilized dechlorinated water. This medium was further diluted in the same manner five times over. Then, half of the diluted medium was removed and replaced with equal amount of sterilized dechlorinated water every 2 h, three times over. Juveniles were fed in a glass container with a mixture of three phytoplankton species, namely, *Chlamydomonas* sp.; *Monoraphidium* sp.; *Navicula* sp., for a period of 13 days more. This algal mixture was added daily to the juveniles at a ratio of 1:1:1 (v/v) after decanting half the volume of the medium. Juvenile ultrastructure was observed using scanning electron microscopy (SEM) and comparing with that of glochidia.

For SEM observations, marsupia were dissected from mussels and the central part of each was cut to facilitate the visualization of the glochidia within the brooding channels. Sections approximately 2–5 mm in width and 5–10 mm in length were fixed in 10% buffered formalin for 2 h and preserved in 5% buffered formalin for 24 h The specimens were then dehydrated in a graded series of ethanol and critically point dried. An identical procedure was employed with both glochidial and juvenile samples. Before fixation, glochidia and juveniles were anesthetized in 2% chloral hydrate to observe the foot and the mantle. The organic structures of some larvae were removed by allowing bacterial decomposition for one week and cleaned by distilled water in order to observe the internal calcareous layer of the shell. All samples were mounted on SEM specimen stubs with conductive silver paint and coated with gold and observed with a Jeol JSM-35 CF scanning electron microscope operated at 25 kV. The assessment of hardening of the new juvenile periostracum was based on relative calcium deposition analysis by energy dispersive X-ray spectroscopy (EDS), with a spectrum collection time of 60 s.

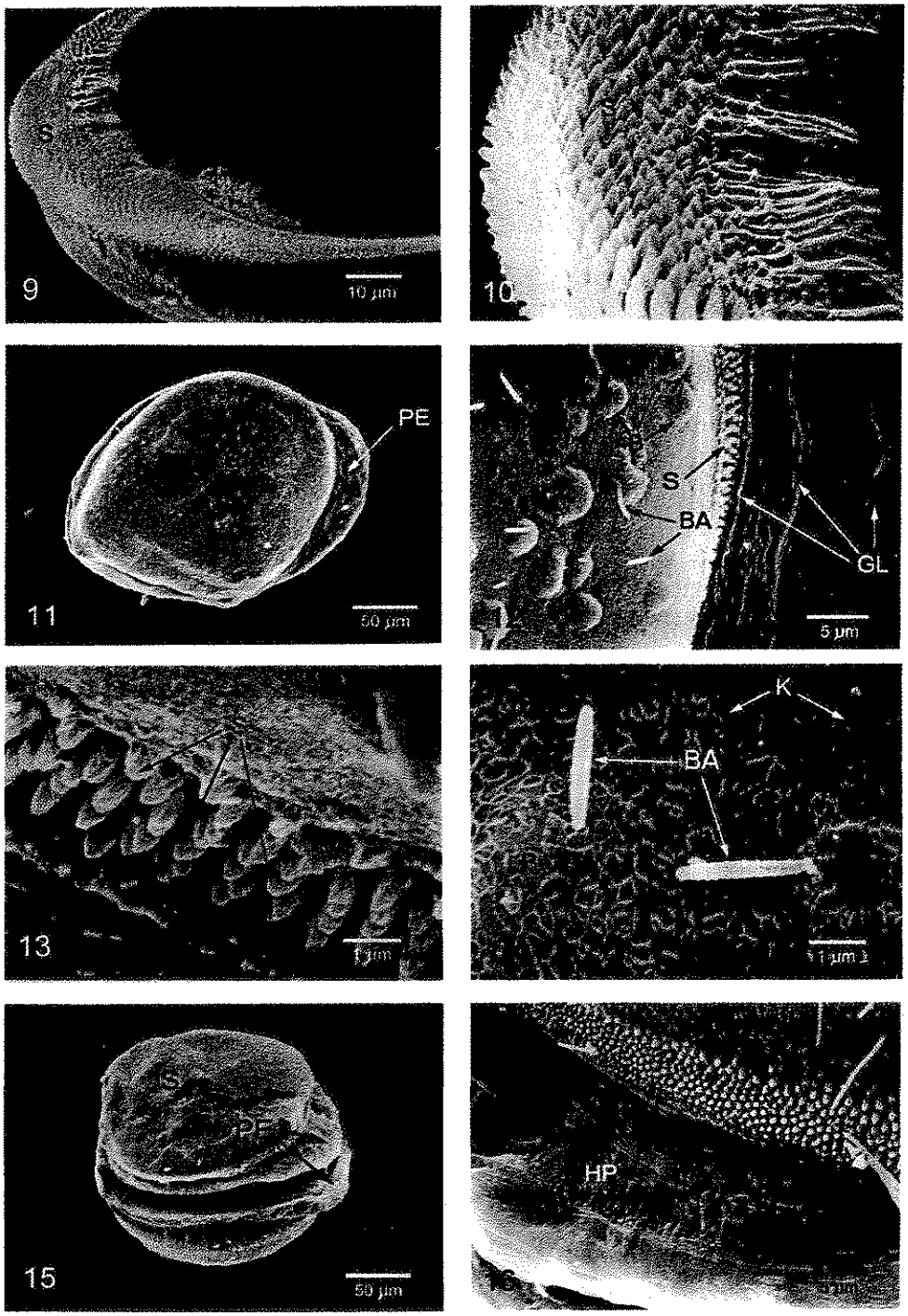
Results

The marsupial gills of female *H. (L.) myersiana* are located in the outer demibranchs. Non-fecund marsupial gills are thin, but after fertilization they expand greatly on account of glochidial development. The appearance of marsupia coincided with the formation of secondary water tubes, whereas the primary water tubes housed the embryos. The color of marsupia changed depending on their stage of development, from transparent yellow with early embryos to dark brown before glochidial parturition. This change was accomplished at 5–9 days post-fertilization. A fragment of marsupial gill from a gravid female with well-develop glochidia in the brood chamber is shown in Figs. 1 and 2. Gill filaments and an interlamellar septum can also be distinguished. The larvae were released from brood chambers in clumps enveloped in mucus (Fig. 3). The glochidia have semi-oval and equivalve shells with an equilateral valve. The glochidial shell has a length of around 200 μm, is about 150 μm in width and 75 μm maximum in thickness. Shells do not possess hooks in this species (Figs. 4–6). The glochidial thread whose role is attachment to the host fish is present. The valves are joined by a straight hinge and the oval areas present in their internal surfaces correspond to the insertion regions of the adductor muscle (Figs. 4 and 6). The internal surface of glochidial valves shows numerous pores while the external surface is covered with a fibrilar organic matrix (Figs. 6 and 7a, b). The valves are connected by a strong adductor muscle (Fig. 8). The external edges of the valves have a different texture due to the absence of the pores (Figs.5, 6 and 8); the rim of the valves is lined by rows of spines (Figs. 9 and 10).

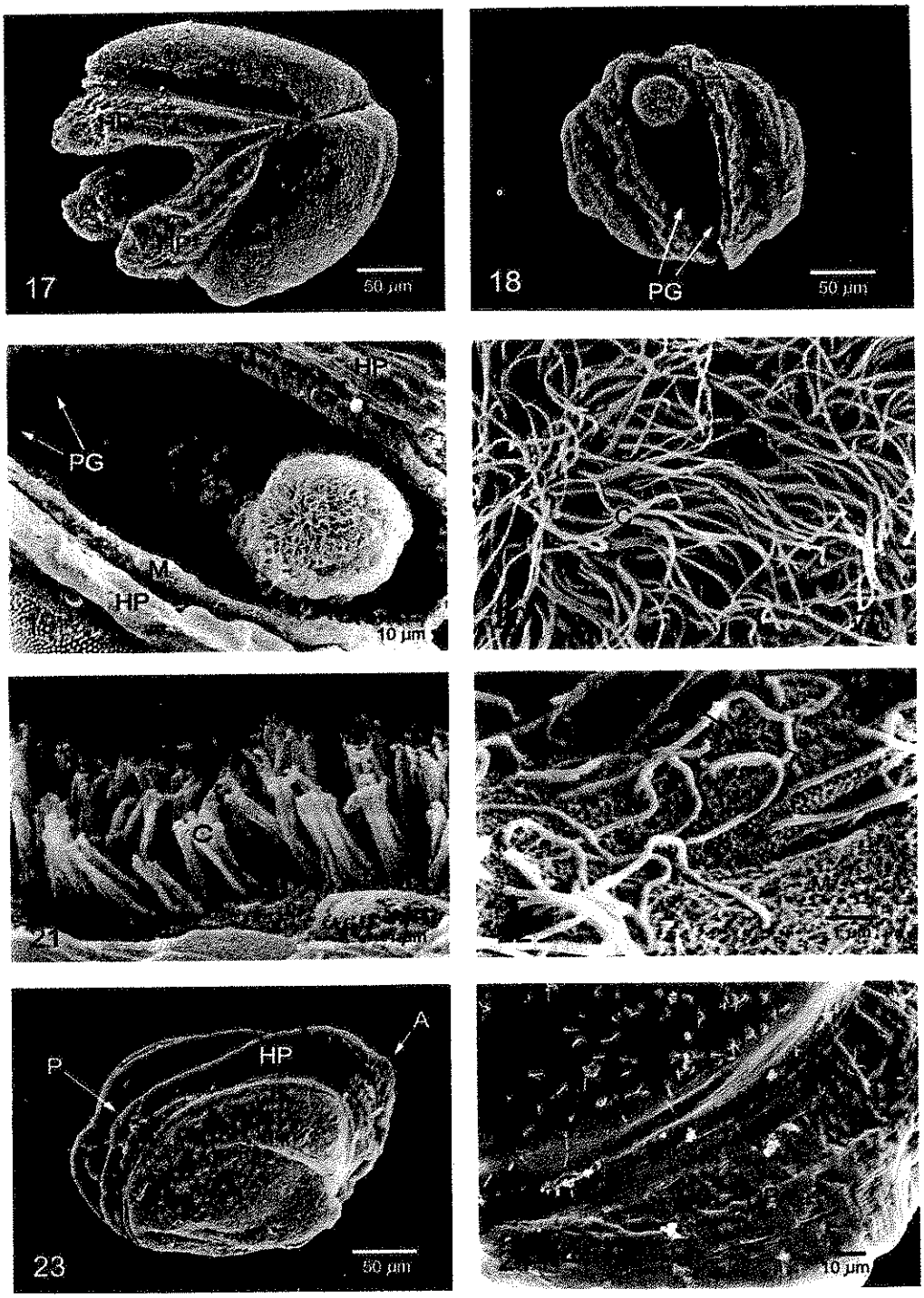
Following on from 10 days of glochidial development, 3-day-old juveniles were observed to have a soft periostracum under the glochidial valves (Figs. 11 and 12). The new shell growth is marked by the addition of parallel, slender lines initially in the anterior region (Fig 12). A strong foot is also present and expands rhythmically outside the intrapallial cavity. The spines on the glochidial shell gradually become reduced, and bud structures appear in the cuticle matrix with filamentous expansions 2 μm in length. (Figs. 12–14). Five-day-old juveniles (Figs. 15 and 16) show an increase of about 25 μm in the width of the new periostracum in the anterior region. This region is also hardened due to initial calcium deposition. The subsequent morphological changes are readily observed after 8 days of juvenile life (Figs. 17–22). In fact, the anterior shell edge reaches around 50 μm in width and its thickness has increased by additional calcium deposition. The prominent foot is entirely covered with long cilia around 4–6 μm in length (Figs. 19 and 20). The foot actively contracts and expands by muscular activity; the valves are kept open facilitating foot movements The expanded foot can reach double the size of the shell. The mantle bordering the new shell and the primitive gills at the base of the foot are both covered with cilia about 2 μm in length (Fig. 21). Scattered longer and thicker cilia around 4–5 μm in length are also present in the mantle tissue (Fig. 22). Finally, by 13 days of juvenile life



Figs. 1–8. Scanning electron microscopy (SEM) of glochidia of *H. myersiana* collected from gills of the gravid female and kept *in vitro* for 10 days. Figs. 1, 2: Fragment of marsupial gill with glochidia (GL) in brood chamber (BC); secondary water tubes (ST); gill filaments (F); interlamellar septum (ILS). Figs. 3–8: Glochidia (GL) surrounded by mucus (Mu); hookless equivalve shell (ES); semi-oval valves (SO); shell ligament (L); pits (P); insertion area of adductor muscle (IM); keratin fibers in external surface layer of the shell (K); pores in the internal calcareous layer (PO); adductor muscle (AM); spines in ventral region of the shell (S).



Figs. 9, 10. Scanning electron microscopy (SEM) of glochidia of *H. myersiana* collected from gills of the gravid female and kept *in vitro* for 10 days. Abbreviations as above. Figs. 11–16. SEM of juvenile *H. myersiana* after 10 days of glochidial development, and additional periods of juvenile life. Abbreviations as below. Figs. 11–14: Three-day-old juveniles: appearance in anterior region of new soft periostracum formed by organic matrix bands under the old cuticle; filamentous expansions, strong foot and retraction of spines were observed. Figs. 15, 16: Five-day-old juveniles: new periostracum growth and its hardening by calcium deposition.



Figs. 17–24. SEM of juvenile *H. myersiana* after 10 days of glochidial development, and additional periods of juvenile life. Figs. 17–22. Eight-day-old juveniles: growth of juvenile periostracum, presence of prominent foot covered with long cilia, internal mantle surface with numerous cilia and scattered longer cilia. Figs. 23, 24: Thirteen-day-old juveniles: growth of new periostracum mainly in the lateral and posterior regions and subsequent calcium deposition. Initial glochidia shell (IS); periostracum of new shell in anterior region (PE); growth lines of periostracum (GL); cuticle buds (B); cuticle spines (S); stick filament (SF); keratin fibers (K); foot (F); primitive gills (PG); mantle (M); cilia (C); microvilli (MI); long solitary filaments (LF); anterior (A) and posterior (P) region of periostracum; hard periostracum (HP).

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