MORPHOLOGICAL DEVELOPMENT OF GLOCHIDIA IN ARTIFICIAL MEDIA CULTURE THROUGH JUVENILE OF FRESHWATER PEARL MUSSEL, *Hyriopsis (Hyriopsis) bialatus* SIMSON, 1900

INTRODUCTION

Hyriopsis (Hyriopsis) bialatus Simpson 1900 is a native freshwater pearl mussel of Thailand. They are important economically as multi-utility aquatic animals and widely distributed in the bottom of the reservoirs and rivers in the central, northern and north-eastern parts of Thailand (Brandt, 1974; Kovitvadhi and Kovitvadhi, 2002). The nacreous mussel shell can be used for inlaying pearl furniture, ornaments, kitchen utensils and souvenirs, as well as for bone-implantation (nacreous implantation) in the patients (Lopez *et al*., 1994; Haihong *et al*., 2002). The mussels can also produce freshwater pearl while the meat is a source of protein for humans and animals. Mussels are filter-feeder and river-keeper by siphoning nutrients from water column while antioxidant enzymes or biotransformation enzymes in their digestive gland can detoxify substances in water. These activities contribute to maintaining clean river and stream ecosystem (Pennak, 1989; Birmelin *et al*., 1999). Moreover, mussels can be used as a bioindicator, especially at their juvenile stages for toxicity testing of the river and stream (Keller and Zam, 1990; Keller *et al*., 1998).

The life cycle of freshwater pearl mussels is quite complex. Fertilized eggs develop into larvae (or glochidia) within the gills of female mussels called marsupial demibranch. There are major morphological changes of demibranch of the mature female to accommodate the large number of these larvae. When glochidia are released from the female gills they may come in contact with a passing fish and attach themselves to the gills, fins or body of the fish. After a few days to several weeks, glochidia could leave the host, drift to the bottom substrate and begin their lives as juvenile mussels. It may take several years before juveniles become matured and can reproduce as an adult (Gary, 2004).

The problem arises from the fact that the number of freshwater mussels has been declining drastically due to environmental deterioration resulting in water quality problems and reduction of fish hosts while commercial demand for mussel use continue to increase. Attempts have been made to replenish the number of mussels through artificial culture techniques (Isom and Hudson, 1982, 1984; Hudson and Isom, 1984; Keller and Zam, 1990; Kovitvadhi *et al*., 2001; Kovitvadhi *et al*., 2002). However, morphological changes of the freshwater pearl mussel, *H. (H.) bialatus*, have never been thoroughly studied, especially from glochidia period until the juvenile stage which are the most crucial and vulnerable stages of their lives. The histological knowledge of these developing stages could give us more information and understanding about the larval organogenesis.

OBJECTIVES

1. To observe the morphological development of glochidia to the juvenile stage of freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus*.

2. To identify the sequences of organogenesis from glochidia to juvenile of freshwater pearl mussel, *H. (H.) bialatus*, with histological techniques.

LITERATURE REVIEW

Hyriopsis (Hyriopsis) bialatus Simpson, 1900 is one of the freshwater mussels that is economically important considering from its high protein content (up to 41%) in mussel which can be used as food while the shells are valuable for pearl production (Kovitvadhi *et al*., 2002).

The taxonomy of freshwater pearl mussel *Hyriopsis (Hyriopsis) bialatus* Simpson, 1900 is as follows:-

Phylum Mollusca
  Class Bivalvia
    Subclass Schizodontida
      Order Unionoida
        Superfamily Unionacea
          Family Amblemidae
            Subfamily Hyriopsinae
              Genus Hyriopsis
                Subgenus *Hyriopsis*
                  Species *Hyriopsis bialatus*

1. *Morphology and biology of freshwater mussels*

The shell's main component is calcium carbonate and is formed by the deposition of crystals of this salt in an organic matrix of the protein, conchiolin. Three layers make up the shell: (1) a thin outer periostracum of horny conchiolin, often much reduced due to mechanical abrasion , fouling organisms, parasites or disease, (2) a middle prismatic layer of oragonite or calcite, a crystalline form of calaium carbonate, and (3) an inner calcereous (nacreous) layer, that is either of dull texture or iridescent mother-of-pearl, depending on species.

At very early in larval development an area of ectodermal cells in the dorsal region of the developing embryo secretes the first larval shell. The secretion of the second larval shell by the mantle follows soon after. The secretion of the adult shell begins after metamorphosis. The third layer of shell is more heavily calcified, has different pigmentation and more conspicuous sculpturing than the larval shell. The outer mantle fold secretes the periostracum and prismatic layer, while the inner nacreous layer is secreted by the general mantle surface (Gosling, 2003).

Major bivalve characteristics include (1) a hinged shell, the two sides of which are closed by one or two adductor muscles; a springy ligament springs the shell valves apart when the adductor muscles relax, (2) lateral compression of the body and foot, (3) a spacious mantle cavity. The hinged portion of a bivalved shell is dorsal. The shell valves, then, are on the animal’s left and right sides. The shell opens ventrally. A conspicuous bulge in the shell is frequently seen on the dorsal surface, adjacent to the hinge. This bulge, termed the umbo, is composed of the earliest shell material doposited by the animal. Distinct growth lines typically run parallel to the shell’s outer margin. The foot projects ventrally and anteriorly, in the direction of movement, and the siphons, when present, project posteriorly (Pechenik, 2005). On the central part of the shell edge is cape, called posterior wing and on the anterior part is a small anterior wing.

The mantle consists of connective tissue with haemolymph (blood) vessel, nerves and muscles that are particularly well developed near the mantle margins. It is attached to the shell by muscle fibers in the inner fold; the line of attachment, the pallial line, runs in a semicircle a short distance from the edge of the shell. Cilia on the inner surface of the mantle play an important role in directing particles onto the gills and in deflecting heavier material along rejection tracts towards the inhalant opening, the entry point on the mantle for incoming water.

In mussels the mantle contains most of the gonad. Gametes proliferate within the mantle and are carried along ciliated channels to paired gonoducts that discharge into the mantle cavity. After mussels have released their gametes the mantle is thin and transparent. The mantle is not only the site of gametogenesis but is also the main site for the storage of nutrient reserves, especially glycogen. The mantle plays a role in the bioaccumulation of metal and organic contaminants in mussels, although the gills, kidney and digestive gland are considered more important. Accumulated organic contaminants are actively metabolized and eliminated through the kidney, while heavy metals are sequestered by a specialized group of protein called metallothionines in gill, mantle and digestive gland tissue, or by lysosomes in digestive gland and kidney cells (Gosling, 2003).

2. *Life cycle*

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) phase. This type of life cycle is commonly found in the mussels of North American, African and European species (Lillie, 1895; Lefevre and Curtis, 1910, 1912; Ortmann, 1910; Coker *et al*., 1921; Arey, 1924; Fryer, 1961; Yokley, 1972; Wood, 1974; Kat, 1984; Skinner *et al*., 2003). 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). 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 incubation, the young are released when fishes pass by and the larva adheres to its scales, fin or gills and becomes a temporaly ectoparasite (Reuling, 1919; Arey, 1932a). A cyst is formed by the host fish as the result of chemical, histological and immunological reaction (Kirk and Layzer, 1997). Once inside, the glochidia initiate certain morphological transformation (Coker *et al*., 1921; Kat, 1984). There is still 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 spesificity (Reuling, 1919; Arey, 1932a; Kirk and Layzer, 1997). The parasitic phase of development constitutes a critical period due to the presence of specific antibodies to 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 Layer, 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; Kovitvadhi *et al*., 2001). It may take several (2-9) years before juveniles mature and can reproduce as an adult. Adults may live 60 to 70 years if conditions are suitable (Gary, 2004).

3. Fertilization

In most bivalves, eggs and sperm are shed directly from the genital ducts into the water column where fertilization takes place. Sperm penetration of the egg is facilitated by the release of a substance capable of lysing the vitelline membrane around the egg. Meiosis is completed in the egg once fertilization has taken place. In the mussel *Mytilus edulis*, eggs that are unfertilized for 6-4 hours at 18 ^{o}C are not capable of developing further, and sperm lose their motility after 1-2 hours at this temperature (Bayne, 1976). According to the histological examination of the foot of *Anodonta cygnea*, the specimens collected in early summer showed most individuals contain both eggs and sperms. The gametes were apparently mature and probably develop simultaneously in different regions of the same gonad (Wood, 1974). In gravid mussels, mature eggs pass from the gonad into the interlamellar spaces. The sperm, however, pass from the gonad, through the exhalant siphon into the water, and are fan into the water tube where the mature eggs are localized (Gosling, 2003).

The conditions for successful fertilization vary depending on the species. In *M. edulis* fertilization occurs successfully at temperatures from 5 to 20 ^{o}C and salinity between 5 and 40 psu (Bayne, 1965). However, for Genus *Hyriopsis*, there is no report on the suitable conditions for successful fertilization.

4. Larval development

In scallops, the fertilized eggs rapidly divide to become balls of cells that begin to swim once cilia appear some 4-5 hours after fertilization. A ciliated trochophore stage is reached about 24 hours after fertilization. A shell gland begins to secrete the first larval shell, the prodissoconch I. The shell is D-shaped in outline and the larva, now a veliger (100-120 μm shell length), immediately starts to secrete the second larval shell, or prodissoconch II. This shell is secreted by the mantle and exhibits growth lines. Veliger larvae possess a velum, a circular lobe of tissue bearing a ring of cilia, which serves as both a swimming and feeding organ. Small particles (1-2 μm diameter) caught by the cilia are swept towards the mouth and onwards into a simple gut. In *Mytilus edulis*, this stage lasts several weeks and is characterized by rapid growth from 120 μm to 250 μm shell length (Bayne, 1976). As metamorphosis approaches pigmented eyespots and an extensible ciliated foot appear. The larva, now known as a pediveliger, is between 210 and 300 μm in length and is characterized by Bayne (1971) and Lutz and Kennish (1992) to have a large velum for swimming and feeding, ciliated pulp that sorts food particles, a few pair of gill filaments, mouth, oesophagus, stomach with style sac and large digestive gland, simple intestine, thin mantle that secretes shell, foot used in crawling and byssus secretion, cerebral, pedal and visceral ganglia, sensory, spots and pedal statocysts.

Larvae vary in their response to the light, gravity and pressure. During the veliger stage larvae are positively phototrophic and sensitive to pressure. These responses tend to keep the shelled larvae in the surface waters. On the other hand, pediveligers are positively geotrophic and insensitive to pressure which encourages them to the bottom in preparation for settlement (Gosling, 2003).

In freshwater pearl mussels, for exaple, *Hyriopsis myersiana* studied by Kovitvadhi *et al*. (2001) is similar to the observation in *Anodonta californiensis* as observed by D'Eliscu (1972) and in *Anodonta cygnea* as observed by Wood (1974), the larvae are released from brood chambers enveloped in mucus at 5-9 days post-fertilization. The glochidia have semi-oval and equivalve calcareous shells with numerous pores in the internal surface, pits in the external surface and cuticalar spines in the ventral region. 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. 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. After 10 days of glochidial development, 3-day-old juveniles have a soft periostracum under the glochidial valves. The new shell growth is marked by addition of parallel, slender lines initially in anterior region. A strong foot is also present and expands rhythmically outside the intrapallial cavity. It was the main feature of metamorphosis to the juvenile stage. The shell thickness has increased by additional calcium deposition and 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 (Kovitvadhi *et al*., 2001).

5. *Factors affecting larval growth*

The important factors in larval development are the same factors influencing bivalve growth in general, namely temperature, salinity, and food ration.

5.1 Temperature and salinity

Temperature has a marked effect on larval growth. In the oyster *Ostrea edulis* development from early cleavage to 160 μm shell length took between 13-15 days at 14 ^{o}C, but was reduced to 5 days at 24 ^{o}C (Walne, 1974). This strong positive correlation between temperature and larval growth has also been reported for other bivalve species.

Bayne (1965) reported the effect of salinity on larval growth in *M. edulis* larvae from two populations where the parents experienced different salinity regimes. Larvae from North Wale, UK, did not grow at 19 psu and showed retarded growth at 24 psu, but at 30-32 psu growth was normal. In larvae from Denmark, close to the Baltic sea, growth occurred even at 14 psu, indicating that there is a genetic component to salinity (and probably temperature) tolerance. Moreover, shelled larva show a greater tolerance to salinity and temperature change than do the embryonic larval stage (Gosling, 2003).

5.2 Food ration

The planktotrophic eggs of bivalves have reserves of lipid, protein and glycogen that fuel the early developmental stage. Feeding commences shortly after the development of the shell and velum. Although the diet of larvae in the wild is not known for certain, they probably depend on a ration of phytoplankton cells, dissolved organic material (DOM), detritus and bacteria for successful growth and development (Lutz and Kennish, 1992). As for the diet, the most widely used species for bivalve culture are the diatom, *Phaeodactylum tricornutum*, *Chaetoceros calcitrans*, *Thalasiosira pseudonana*, *Skeletonema costatum* and the flagellates *Isochrysis galbana* (Tahitian strain) and Tetraselmis suecica. A mixture of these species is also known to give larvae that are significantly larger and greater than giving one species alone (Walne, 1974).

6. Culture of glochidia in artificial media

Efforts are being made to find freshwater mussel culture techniques for mass production and conservation. Generally, mussel culture tends to mimic natural culture, because in the larval stage, the glochidia need to parasitise (glochidiosis) fish or some amphibians prior to transformation into the early juvenile stage (D'Eliscu, 1972; Seshaiya, 1941; Watters and O'Dee, 1998; Kraemer and Swanson, 1985). However, high juvenile mortality in nature is evident due to the disturbance caused by bacteria, protozoa and contaminating fungi. These complex culture processes can scarcely contribute to the maintenance of the mussel population. It is also possible to use artificial media for glochidia culture to achieve high production as well as preventing contamination as Isom and Hudson (1982, 1984a,b) and Keller and Zam (1990) developed simple culture techniques for glochidia in artificial media resulting in a high percentage of survival.

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 Kovitvadhi *et al*. (2001). These culture techniques were adapted to the local species *of H. myersiana* (L.) using the plasma from the exotic fish, Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758). Moreover, Kovitvadhi and Kovitvadhi (2002) and Kovitvadhi *et al*. (2003) have successfully cultured *Hyriopsis (Limnoscapha) myersiana* from glochidia to juvenile stage in 9-10 days with 100% transformation rate in the artificial medium containing a mixture of M199, fish (*Oreochromis niloticus*) plasma, and antibiotics or antimycotics. However, their morphological changes and organogenesis development in the transition period have not been observed in any species of these freshwater pearl mussels before.

MATERIALS AND METHODS

1. Glochidia culture in artificial media

1.1 Preparation of glochidia

Adult *Hyriopsis (Hyriopsis) bialatus* were collected from the Mun River Basin in Srisaket province, Thailand. These individuals had an average weight of 37.51 ± 6.49 g, width of 1.88 ± 0.20 cm, length of 8.28 ± 2.80 cm and height of 6.28 ± 0.30 cm. The mussels were sexually identified by microscopic observation of sperms and eggs in fluid suctioned from gonad. They were cultured in circle net (45 cm in diameter and 30 cm in height) in an earthen pond at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The mussels were fed with natural food in the pond. To prepare the mussels for collection of glochidia, soil and algae were thoroughly removed from the outside shell of gravid mussels with dechlorinated tap water. Only gravid mussels with completely brown marsupia were selected for glochidia culture (Kovitvadhi *et al*., 2001).

1.2 Glochidia culture

Glochidia were cultured in tissue culture dishes (60x15 mm). Each culture dish contained 3.5 ml artificial media with fish (*Cyprinus carpio*) plasma and 50–100 glochidia. The culture media were not circulated or even changed during culture until glochidia reached the juvenile stages. Antibiotic and antimycotic compounds were added to the media at the ratio suggested by Kovitvadhi *et al*. (2002, 2003) to prevent contamination from symbiotic organism.

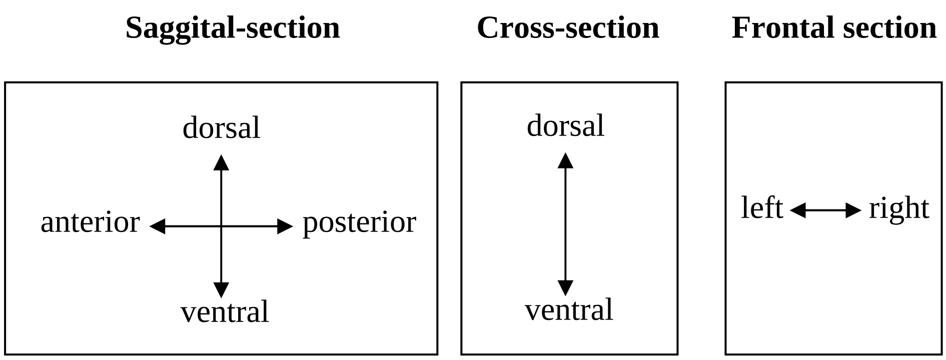
All culture dishes were placed in plastic boxes inside an incubator with a constant supply of 5% CO_2 and room humidity. The internal temperature in the box was kept at 25 ^{o}C and the medium was added on days 5 and distilled water was added for the transformation of glochidia to an active juvenile stage on days 9. The foot extended outside the shell is observed as an indicator for the transformation (Kovitvadhi *et al*., 2001)

2. Preparation of glochidia samples for light microscopic observations

Larvae (glochidia) samples were collected on days 0, 2, 4, 6, 8 and 10 of glochidia development. Then, the glochidia samples were fixed in fixative solution containing 10% neutral buffered formalin for 2 h, and stored in 5% neutral buffered formalin for further process. Glochidia were then placed in a decalcifying solution (5% EDTA) for 3 h to soften the tissues. The specimens were through washed under running water for 30 min, and placed in 10% ethanol. Paraffin technique was done by dehydrating the specimen using a series of ethanol at 10%, 30%, 50% and 70% for 10 min each, in 95% ethanol for 10 min twice, in absolute ethanol for 15 min twice. Clearing was done with xylene for 5 min twice while infiltration was done twice in melted paraplast for 15 min at 60 °C in the hot air oven, and finally the samples were embeded in melted paraplast. Tissue blocks were cut at 5-6 μm thick using the rotary microtome. Sections were attached onto the glass slides and stained with Harris hematoxylin and eosin (Sheehan and Hrapchak, 1980). The specimens were examined under a light microscope.

The subsequent serial sections were stained with Masson’s trichrome and periodic acid Schiff - hematoxylin (PAS-H) for light microscopic observation.

In the results, there are three sectioning planes are defined as the saggital-sections or cross-sections or frontal sections. The position of glochidia in each section plan is defined as follows.



RESULTS

Morphological development of glochidia to juvenile

The successful transformations in the glochidium of *Hyriopsis (Hyriopsis) bialatus* occurred within 10 days in culture media. During the transformation process, the larvae retained their semi-oval shape and did not significantly change their size until the last day of transformation.

However, there were several distinctive changes in the main developing structures leading to foot formation, gill bars, digestive tract, anterior and posterior juvenile adductor, and mantle. On the contrary, rudimentary organs such as pericardium, kidney, heart or nervous ganglia were not observed in glochidia or early juvenile.

Mature glochidia (day 0 glochidia)

Mature glochidia of *H. (H.) bialatus* were collected from the outer demibranch of gravid mussel. The glochidia were semi-oval in shape and contained an equivalved shell that completely enclosed the body. These two valves were equilateral and hookless type. The rim of glochidia valves, particularly in the ventral region, showed different arrangements with numerous spines which were eosinophilicly stained. The average size of these valves (n=50) was 190 ± 0.02 μm in length, 230± 0.02 μm in height, and 61± 0.86 μm in width. The valves were joined by straight hinge and had a muscle bundle called larval adductor extended transversely between the valves (Figure 1, 2). Larval adductor consisted of several smooth muscle cells, each cell was spindle-shaped with an elongate concentric nucleus. All the soft parts of glochidia were enclosed within the larval shell. There were two layers of larval mantle cells lining the internal glochidial shell surface. The outer layer was very thin and inconspicuous. It directly adhered to the shell by a connective tissue. The inner layer, on the other hand, comprised of low columnar cells containing concentric nuclei and numerous eosinophilic granules in the cytoplasm. Between the inner shell surface and outer layer of larval mantle, a thick conspicuous band of mucopolysaccharide was found as a supporting structure positively stained with PAS and Masson's trichrome (Figures 3, 4, 5, 8, 9). Between the inner mantle cells of each valve a mantle cavity was found (Figure 2). The mantle was composed of three types of cell masses: the ventral plate, the lateral pits and the oral plate (endodermic sac). These cell masses were further developed into foot rudiment, the gill buds and digestive tract rudiment, respectively.

The ventral plate was found at the posterior end, adjacent to the adductor muscle (Figures 13, 14, 16, 17). The ventral plate was characterized by 1-2 layers of cuboidal cells that were darkly basophilic stained. The plate was invaginated to become a pair of grooves known as "lateral pits" (Figure 14). The oral plate was near the hinge of shell (Figures 13, 15, 16, 17). These irregular-shape cell masses were formed by loose aggregation of cells.

A few sensory hair cells containing several cilia were found on the larval mantle (Figures 6, 7). They were somewhat rounded cells containing concentric nuclei and also had several cilia on their free ends.

An external thread and a thread gland were found in the central region of the ventral plate (Figures 8, 9). In addition, an internal thread was found located around the larval adductor muscle (Figures10, 12). Both external and internal threads were non-cellular structures. They were stained magenta with PAS and stained blue with Masson's trichrome. The thread gland, on the other hand, consisted of a few polyhedric cells (Figure 15).

Development of digestive tract

“Mouth” was the first distinctive digestive organ found on day 2 of developing glochidium *H. (H.) bialatus* cultured in artificial media. Subsequently, esophagus, intestine and rectum appeared on day 4, while stomach and digestive glands were clearly observed on day 6. The early juvenile was completely developed by day 10 (Table 2). The detail process of digestive tract development was as follows:

The proximal digestive tract (mouth) was formed at day 2 by invagination of the oral plate (Figures 20, 28). At day 4, the digestive tract was extended and the wall became thicker along the hinge line. At day 6, the epithelial tissues of digestive tract contained several cilia stained pink with eosin (Figure 41). At day 8, stomach, digestive gland, style sac and intestine became clearly seen (Figures 50, 51, 52). At day 10 (early juvenile stage), the digestive system was completely developed showing the mouth opened into a ciliated esophagus, leading to the stomach, style sac, intestine, and terminated at a rectum. However, a crystalline style was not observed at this stage (Figure 53).

Development of adductor muscle

The larval adductor muscle began to degenerate during the first two days of glochidia development as seen from the deterioration of muscle cell and the remnants which still remained attached to the larval mantle layer (Figures 18, 19, 28). The larval adductor muscle was completely disintegrated within four days causing the mantle cavity to become larger in size (Figure 20). The muscle bundle at this point migrated to the anterior end and degenerated (Figure29).

The anterior and posterior adductor muscles began to develop within six days. They comprised of densely aggregated smooth muscle cells and located at the corner of hinge line. The anterior adductor muscle was near the foot, whereas the posterior adductor muscle was closed to the gill bud or gill bar (Figures 38, 44, 45). Between eight to ten days, two adductor muscles were formed at the anterior and posterior of the shell. The anterior adductor muscle was larger than posterior adductor (Figures 43, 53, 54, 55, 63). The muscle cells were spindle-shaped and had concentric nuclei. These muscles cells stained red with Masson's trichrome, and they were surrounded by groups of cells which were darkly stained with hematoxylin.

Development of gill

The lateral pits were composed of several cell layers. The cells that lined the mantle cavity side had numerous cilia (Figures 24, 25, 26). Gill buds began to form, consisting of darkly hematoxylin stained cells (Figure 27). The boundary of these cells was not clearly identified. They were protruding into the mantle cavity on the dorso-posterior shell within 2 days of glochidial development, and became distinctive gill buds at day 4 (Figures 33, 34, 35, 36). At day 6, gill buds were larger in size and continued to elongate to form gill bars. The cells boundary was distinctively seen. These cells were cuboidal in shape lining parallel to both side of the foot (Figures 38, 42). At day 8-10, gill bars were extended towards the mid–body and located next to the mantle (Figures 48, 49, 62) closed to the posterior adductor muscle (Figures 45, 63).

Development of foot

At day 2, the cells near the ventral plate region were highly proliferated and became elongated. These cells were darkly basophilic stained (Figures 22, 23). At day 4, the mid-region of ventral plate was invaginated to form two lobes known as "foot lobe" (Figures 31, 32, 36, 37). The foot lobes continued to develop and extended into the mantle cavity. At day 6, the sizes of foot lobes became larger and fused to form a single foot situated between a pair of gill bars (Figures 41, 42). This foot lobe caused the mushroom body to move towards the shell (Figure 40). From day 8 on, the foot became distinctively seen located in the middle of two gill bars (Figures 49, 58). The distal foot was covered with stratified ciliated cuboidal epithelium (Figures 50, 53, 59, 61). Inside the foot region, some part of the intestine (Figures 55, 56), and also smooth muscle cells which were stained red with Masson's trichome (Figure 45) were observed.

Development of mantle

At days 2-4, the inner larval mantle cells were gradually hypertrophied and protruded into the mantle cavity (Figures 21, 30). These cells are grouped together and appeared as a mushroom body when viewed from frontal-section at the end of day 4 (Figure 30). The larval mantle cells were clearly seen at day 6. These cells were club-shaped, extended into the mantle cavity. Their nuclei were found near the base of the cell (Figure 39). At days 8-10, the inner larval mantle cells start to change their shape from club-shaped to low columnar with condensed nuclei (Figures 46, 47, 48, 60, 62). Some mushroom body cell debris was also found in the mantle cavity (Figure 57).

Figure 1 Mature glochidia at day 0

ELT	=	External Larval Thread
ILT	=	Internal Larval Thread
LAM	=	Larval adductor muscle
LS	=	Larval shell

(Wet mount: bar = 25 µm)

Figure 2 Cross-section of the mature glochidia at day 0

EG	=	Eosinophilic granule
IMC	=	Inner mantle cells
LAM	=	Larval adductor muscle
LS	=	Larval shell
MC	=	Mantle cavity
OMC	=	Outer mantle cells
S	=	Spines

An arrowhead indicates the single elongate nucleus of smooth muscle cell.

(H&E: bar = 25 µm)

Figure 3 Saggital-section of the mature glochidia at day 0

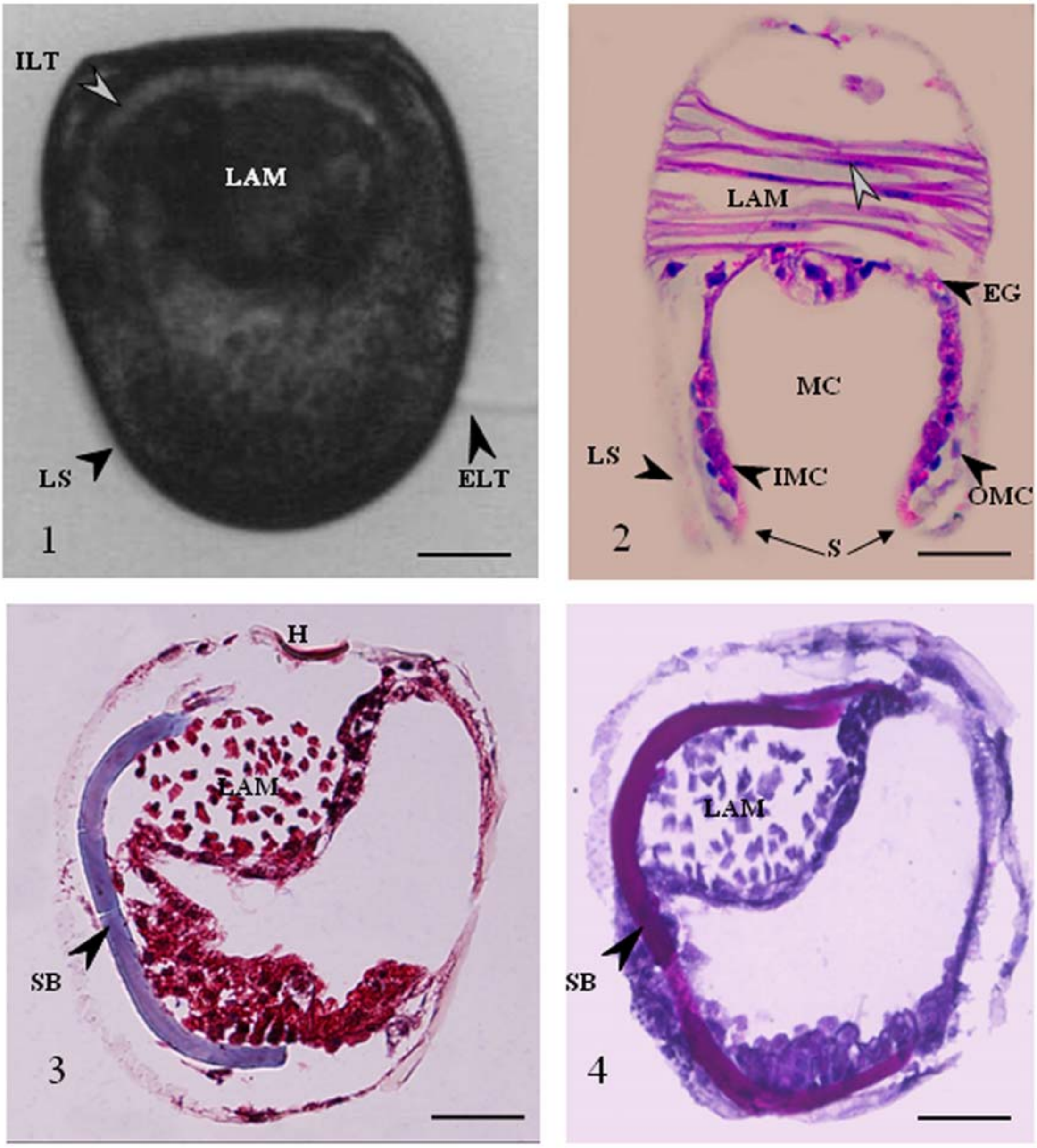
H	=	hinge
LAM	=	Larval adductor muscle
SB	=	Supporting Band

(Masson's trichrome: bar = 25 µm)

Figure 4 Cross-section of the mature glochidia at day 0

LAM	=	Larval adductor muscle
SB	=	Supporting Band

(PAS-H: bar = 25 µm)


ILT
LAM
LS
ELT
1
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MC
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LAM
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Figure 5 Cross-section of the mature glochidia

SB = Supporting Band

(Masson's trichrome: bar = 25 μm)

Figure 6 Cross-section of the mature glochidia

SHC = Sensory hair cell

(H&E: bar = 25 μm)

Figure 7 Saggital-section of the mature glochidia

SHC = Sensory hair cell

LAM = Larval adductor muscle

(H&E: bar = 10 μm)

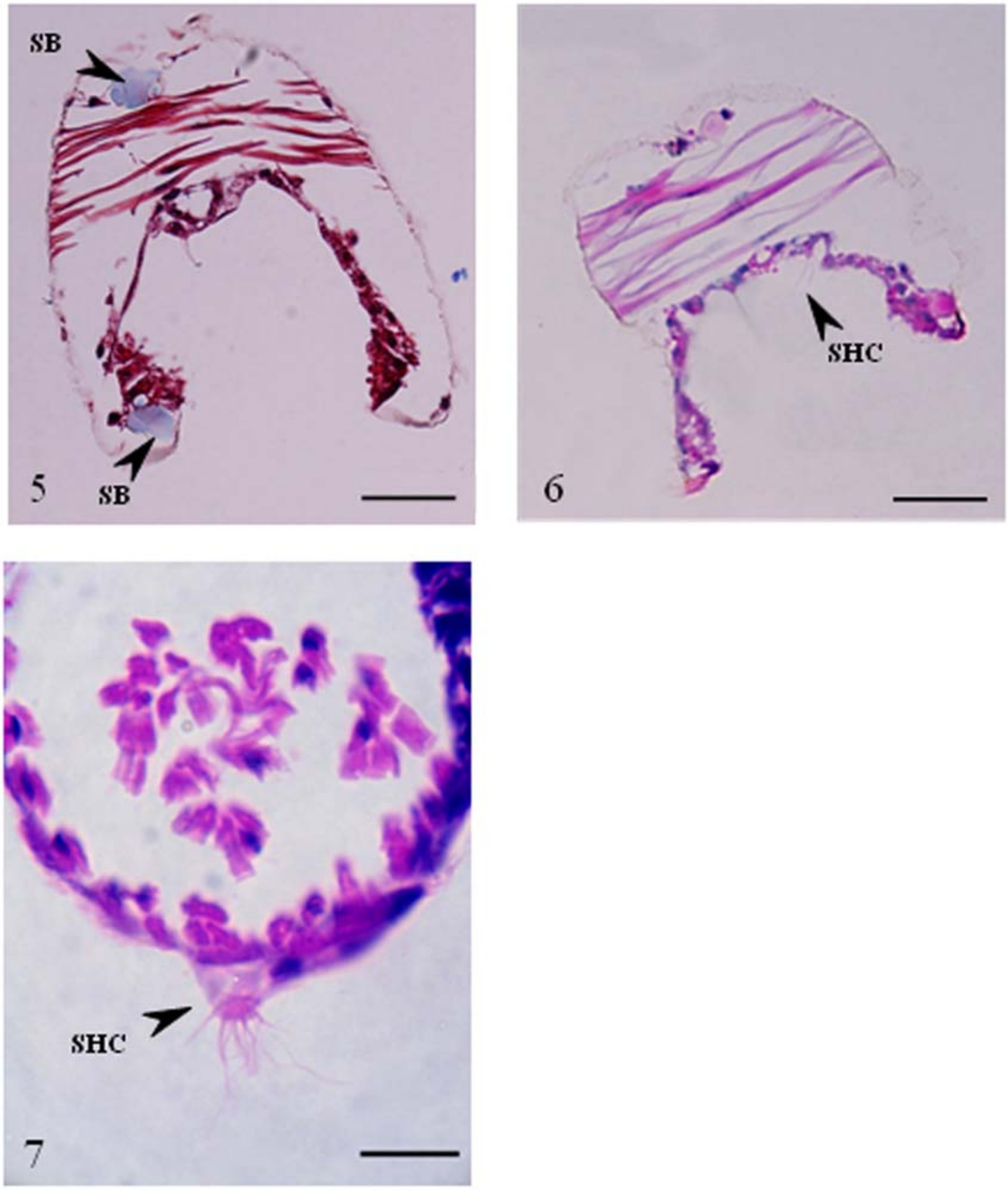

SB
SB
5
6
SHC
SHC
7

Figure 8 Cross-section of the mature glochidia

LAM = Larval adductor muscle

ELT = External larval thread

SB = Supporting Band

TG = Thread gland

(PAS-H: bar = 25 μm)

Figure 9 Cross-section of the mature glochidia

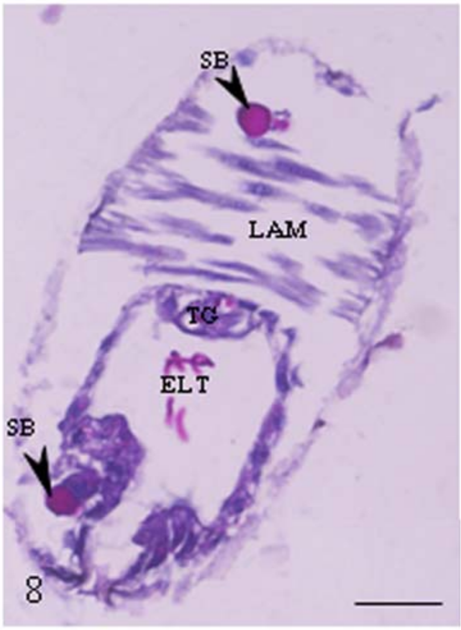
LAM = Larval adductor muscle

ELT = External larval thread

SB = Supporting Band

TG = Thread gland

(Masson's trichrome: bar = 25 μm)


SB
LAM
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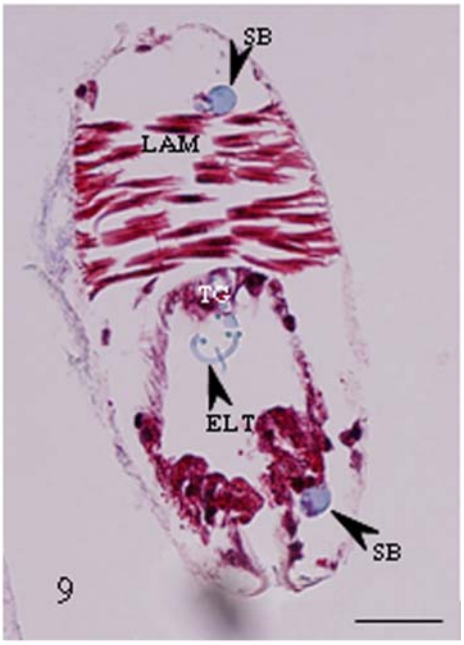
SB
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SB
9

Figure 10 Saggital-section of the mature glochidia

ILT = Internal larval thread

LAM = Larval adductor muscle

(PAS-H: bar = 25 μm)

Figure 11 Saggital-section of the mature glochidia

ILT = Internal larval thread

LAM = Larval adductor muscle

(Masson's trichrome: bar = 25 μm)

Figure 12 Saggital -section of the mature glochidia

LAM = Larval adductor muscle

ELT = External larval thread

ILT = Internal larval thread

(Masson's trichrome : bar = 25 μm)

Figure 13 Saggital-section of the mature glochidia

VP = Ventral plate

OP = Oral plate

LAM = Larval adductor muscle

(H&E: bar = 25 μm)

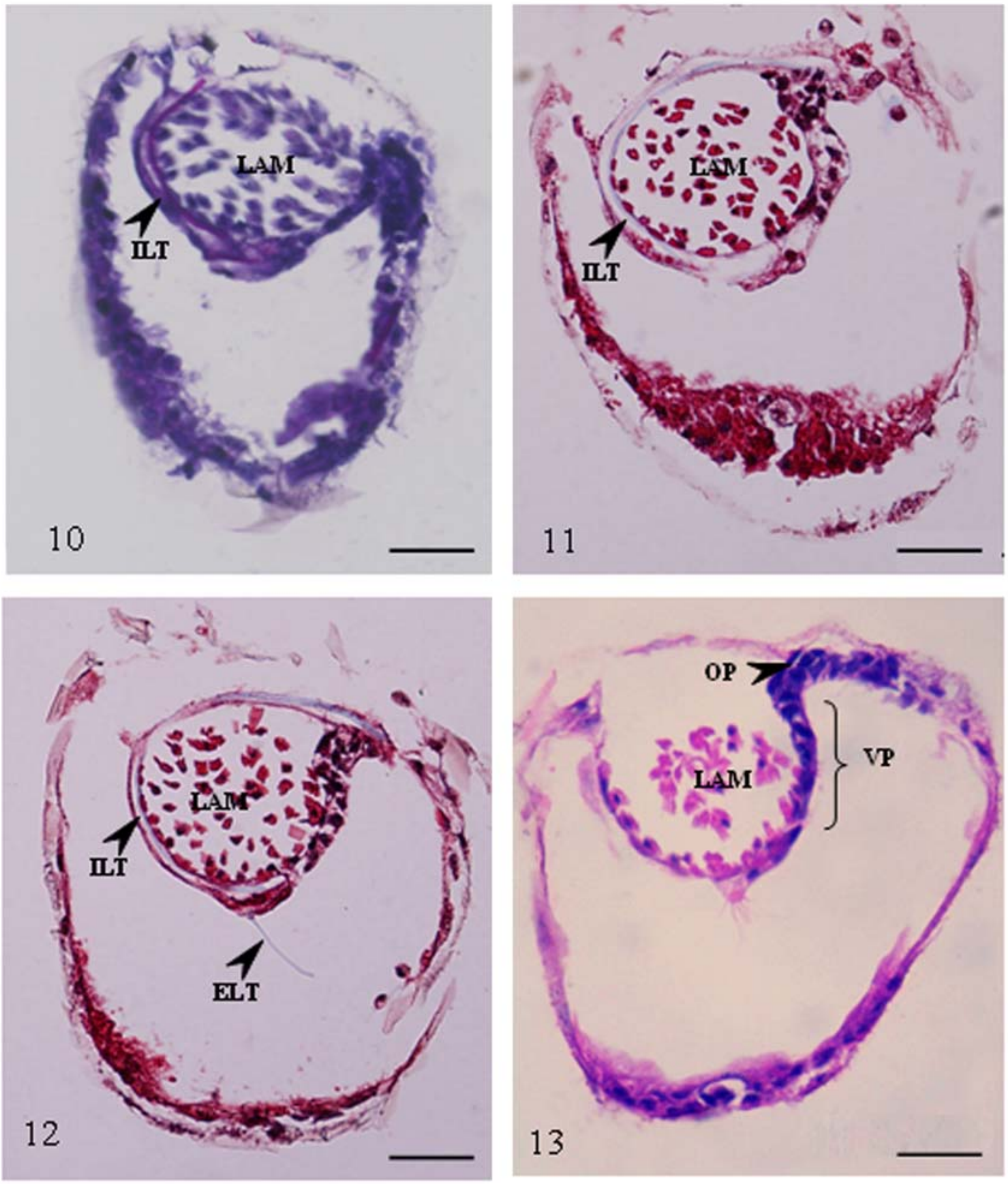

LAM
ILT
10
LAM
ILT
11
LAM
ILT
ELT
12
OP
VP
LAM
13

Figure 14 Cross-section of the mature glochidia

VP = Ventral plate

(H&E: bar = 25 μm)

Figure 15 Saggital-section of the mature glochidia

LAM = Larval adductor muscle

OP = Oral plate

TG = Thread gland

(Masson's trichrome: bar = 25 μm)

Figure 16 Oblique section of the mature glochidia

LAM = Larval adductor muscle

OP = Oral plate

VP = Ventral plate

(Masson's trichrome: bar = 25 μm)

Figure 17 Oblique section of the mature glochidia

LAM = Larval adductor muscle

OP = Oral plate

VP = Ventral plate

(PAS-H: bar = 25 μm)

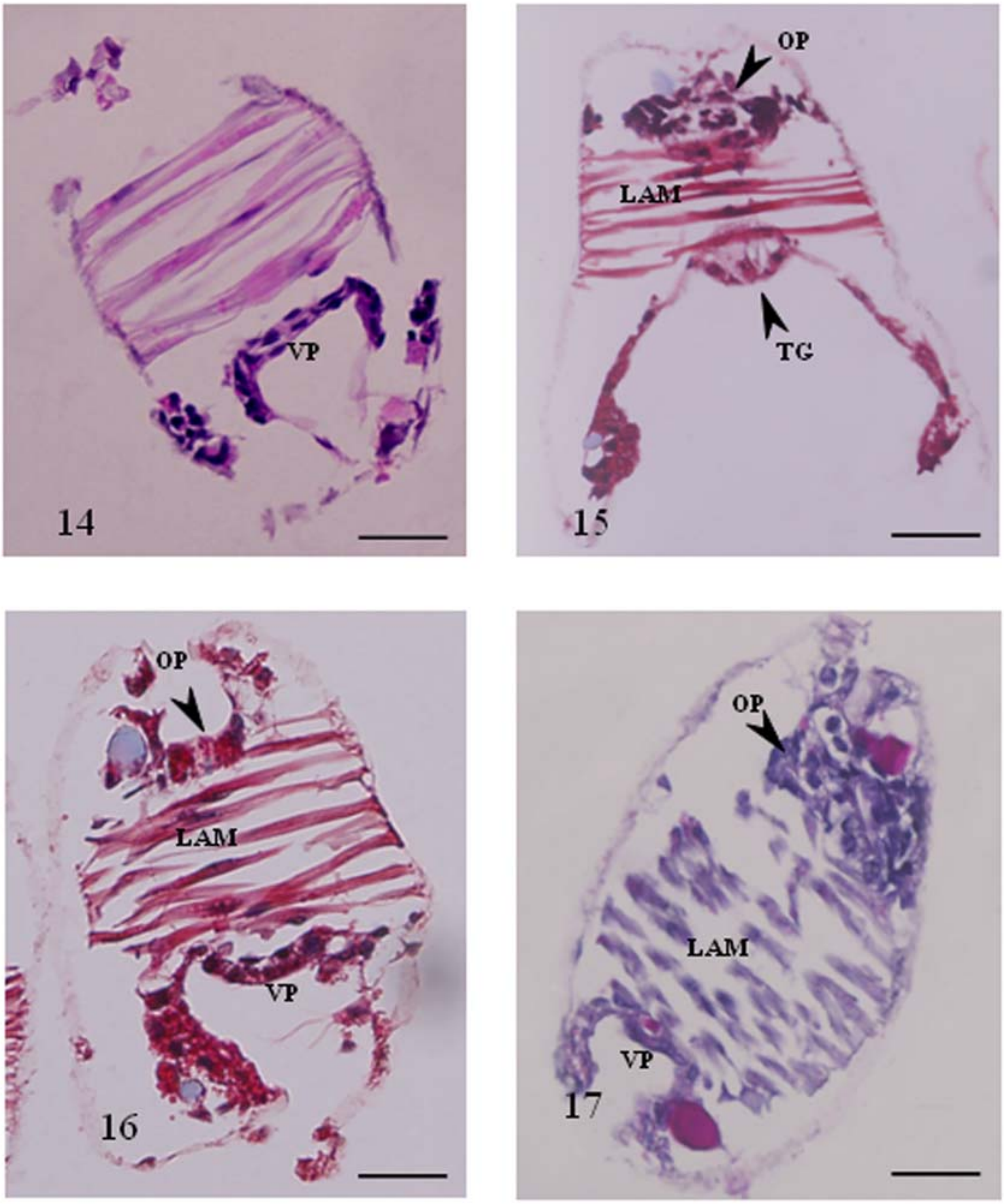

VP
14
OP
LAM
TG
15
OP
LAM
VP
16
OP
LAM
VP
17

Figure 18 Cross-section of day 2 glochidia

LAM = Larval adductor muscle

(H&E: bar = 25 μm)

Figure 19 Cross-section of day 2 glochidia

DMC = Degenerating muscle cells

(H&E: bar = 25 μm)

Figure 20 Saggital -section of day 2 glochidia

LAM = Larval adductor muscle

FL = Foot lobe

An arrowhead indicates the larval mouth formed by invagination of the oral plate.

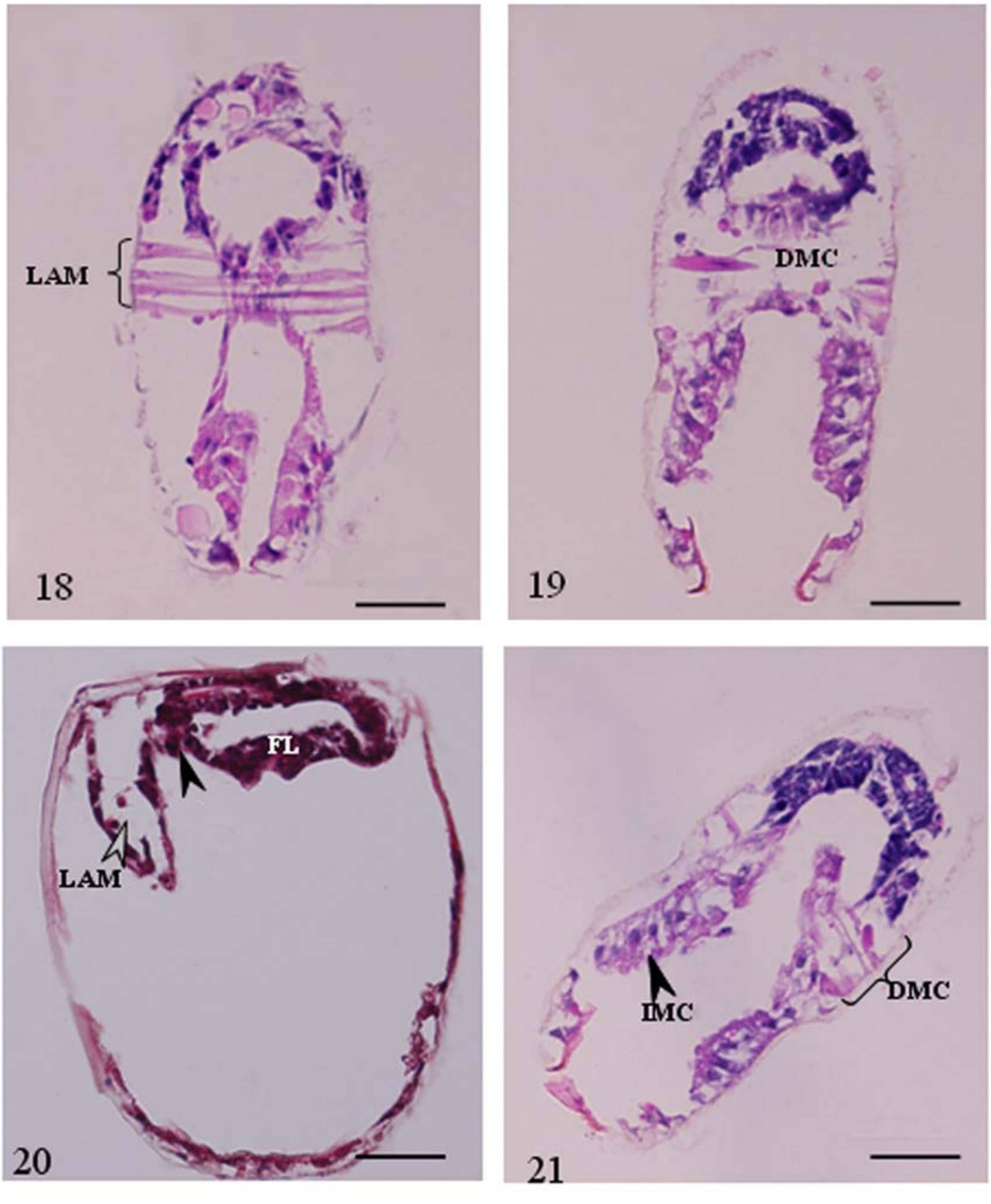
(Masson's trichrome: bar = 25 μm)

Figure 21 Cross-section of day 2 glochidia

DMC = Degenerating muscle cells

IMC = Inner layer of larval mantle cells

(H&E: bar = 25 μm)



LAM
18
DMC
19
FL
LAM
20
IMC
DMC
21

Figure 22 Cross-section of day 2 glochidia

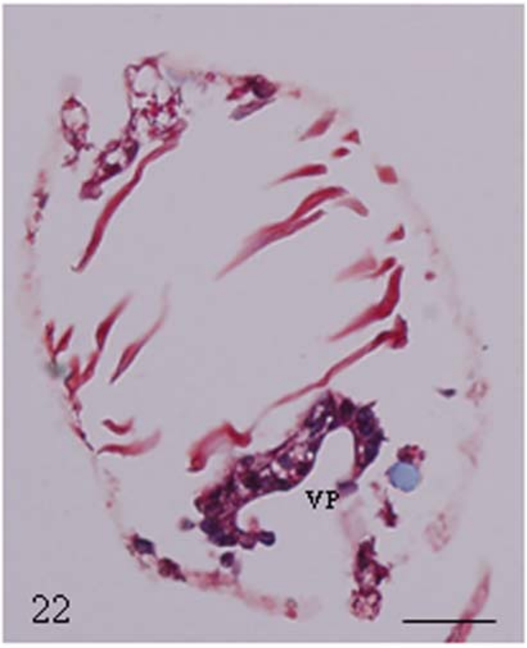
VP = Ventral plate

(Masson' trichrome: bar = 25 μm)

Figure 23 Oblique section of day 2 glochidia

VP = Ventral plate

(Masson' trichrome: bar = 25 μm)


VP
22

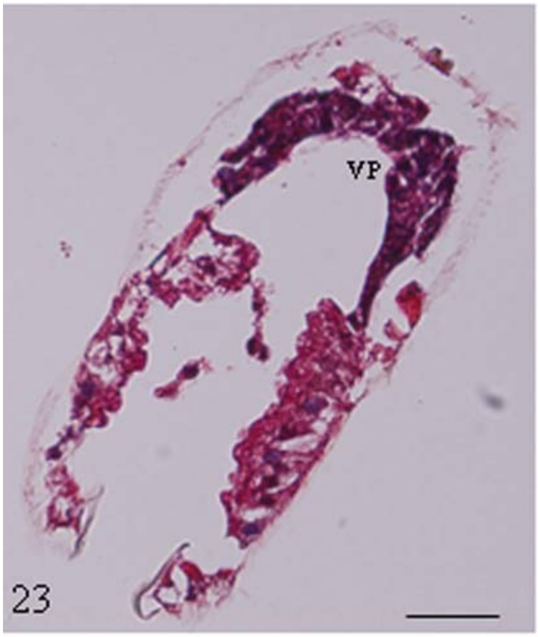

VP
23

Figure 24 Cross-sections from the ventral to the dorsal of day 2 glochidia

DMC = Degenerating muscle cells

LP = Lateral pits

An arrowhead indicates the developing ventral plate.

(Masson' trichrome: bar = 25 μm)

Figure 25 Serial cross-sections from the ventral to the dorsal of day 2 glochidia

DMC = Degenerating muscle cells

LP = Lateral pits

An arrowhead indicates the developing ventral plate.

(Masson' trichrome: bar = 25 μm)

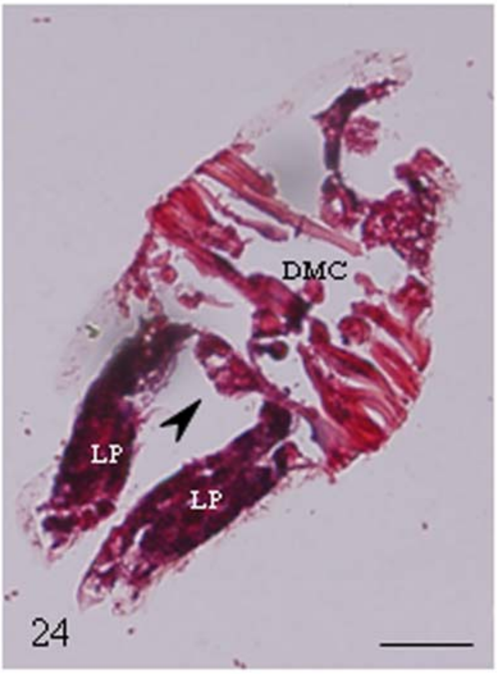
Figure 26 Serial cross-sections from the ventral to the dorsal of day 2 glochidia

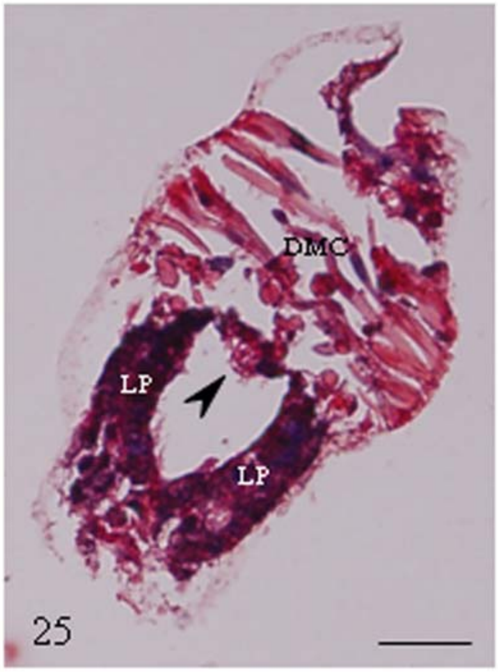
DMC = Degenerating muscle cells

LP = Lateral pits

An arrowhead indicates the developing ventral plate (early foot lobe).

(Masson' trichrome: bar = 25 μm)


DMC
LP
LP
24


DMC
LP
LP
25

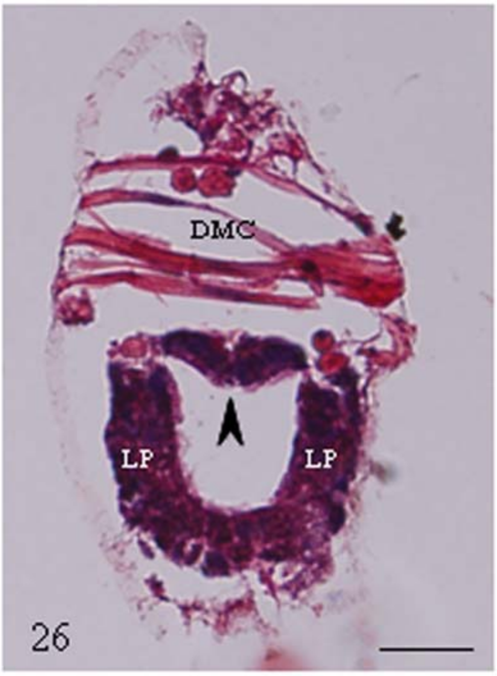

DMC
LP
LP
26

Figure 27 Cross-section of day 2 glochidia

GB	=	Gill bud
VP	=	Ventral plate

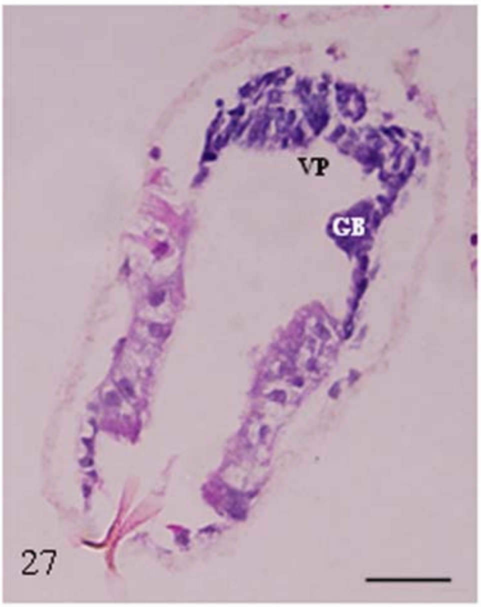
(H&E: bar = 25 μm)

Figure 28 Saggital-section of day 2 glochidia

DMC	=	Degenerating muscle cells
FL	=	Foot lobe

An arrowhead indicates the opened larval mouth.

(Masson's trichrome: bar = 25 μm)


VP
GB
27

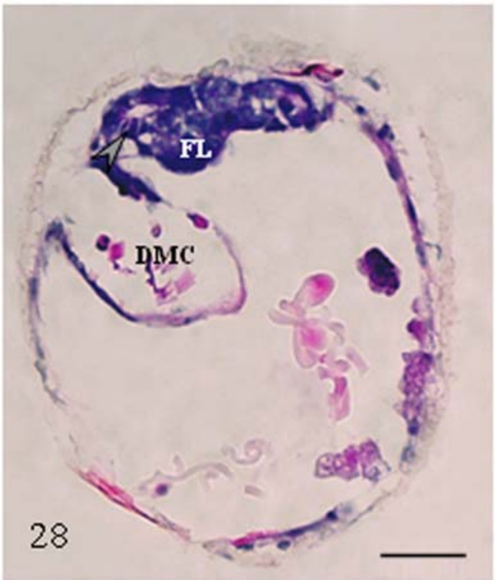

FL
DMC
28

Figure 29 Saggital-section of day 4 glochidia

FL = Foot lobe

An arrowhead indicates the degenerating larval adductor.

(Masson's trichrome: bar = 25 µm)

Figure 30 Frontal section of day 4 glochidia

IMC = Inner layer of larval mantle cells

OMC = Outer layer of larval mantle cells

(H&E: bar = 25 µm)

Figure 31 Cross-section of day 4 glochidia

FL = Foot lobes

(H&E: bar = 25 µm)

Figure 32 Cross-section of day 4 glochidia

FL = Foot lobes

(Masson' trichrome: bar = 25 µm)

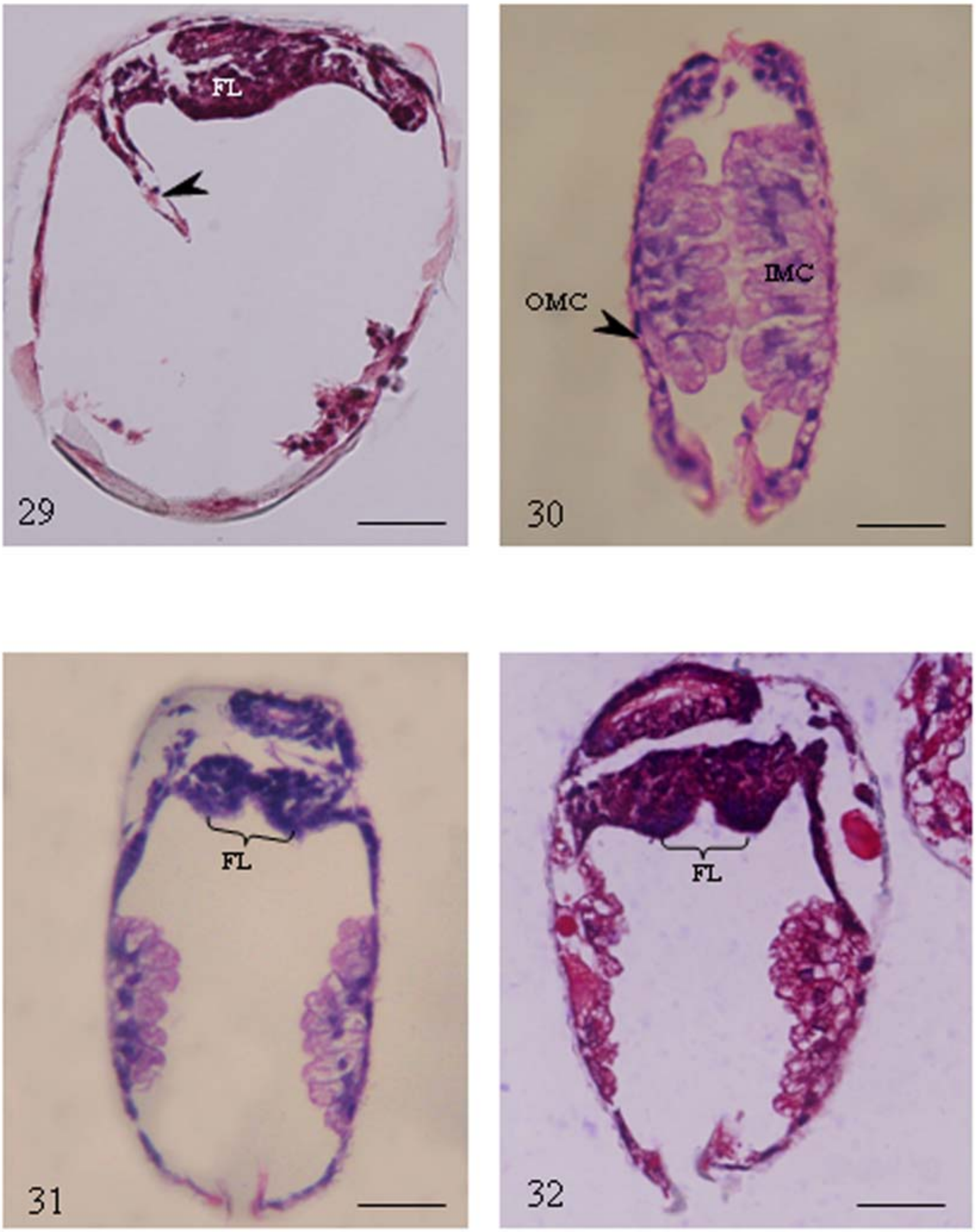

FL
29
OMC
IMC
30
FL
31
FL
32

Figure 33 Cross-section through the posterior end of day 4 glochidia

GB = Gill buds

(H&E: bar = 25 μm)

Figure 34 Cross-section through the posterior end where the proximal foot was found in day 4 glochidia

GB = Gill buds

FL = Foot Lobe

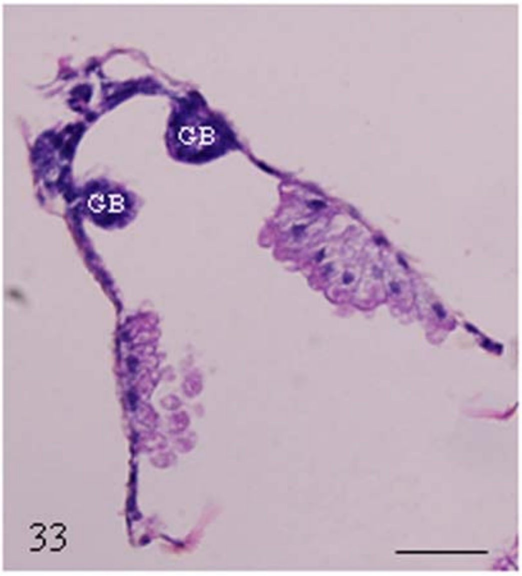
(H&E: bar = 25 μm)

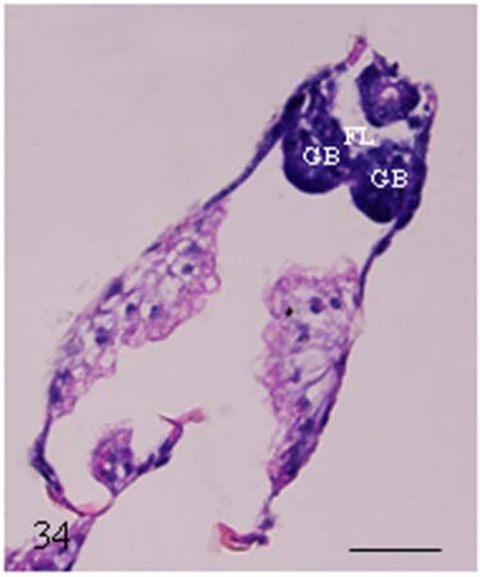
Figure 35 Cross-section through the middle part of day 4 glochidia, the developing foot was flanged by a pair of elongate gill buds

GB = Gill buds

FL = Foot Lobe

(H&E: bar = 25 μm)


GB
GB
33


FL
GB
GB
34

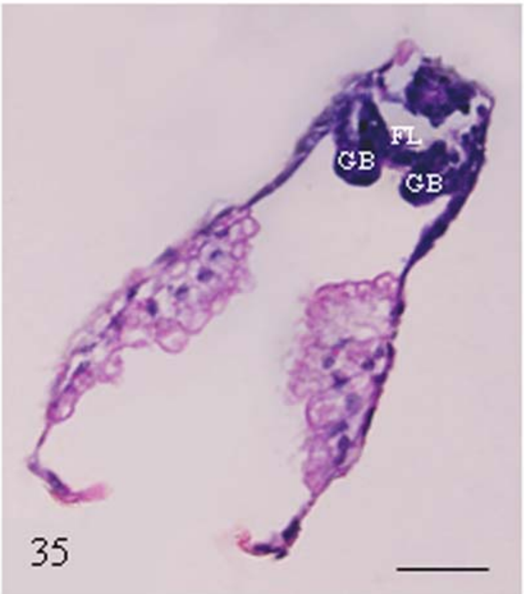

FL
GB
GB
35

Figure 36 Saggital-section of day 4 glochidia

FL	=	Foot lobe
GB	=	Gill bud

A gray arrowhead indicates the degenerating larval adductor.

(Masson's trichrome: bar = 25 μm)

Figure 37 Saggital-section of day 4 glochidia

FL	=	Foot lobe
H	=	Hinge
M	=	Mouth
R	=	Rectum

A gray arrowhead indicates the degenerating larval adductor.

(H&E: bar = 25 μm)


FL
GB
36

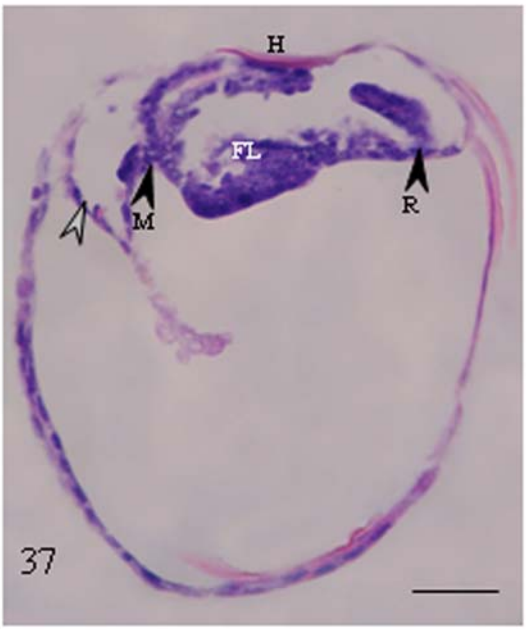

H
FL
M
R
37

Figure 38 Saggital-section of day 6 glochidia

AJA	=	Anterior juvenile adductor
F	=	Foot
GBa	=	Gill bars
PJA	=	Posterior juvenile adductor

(H&E: bar = 25 μm)

Figure 39 Frontal section of day 6 glochidia

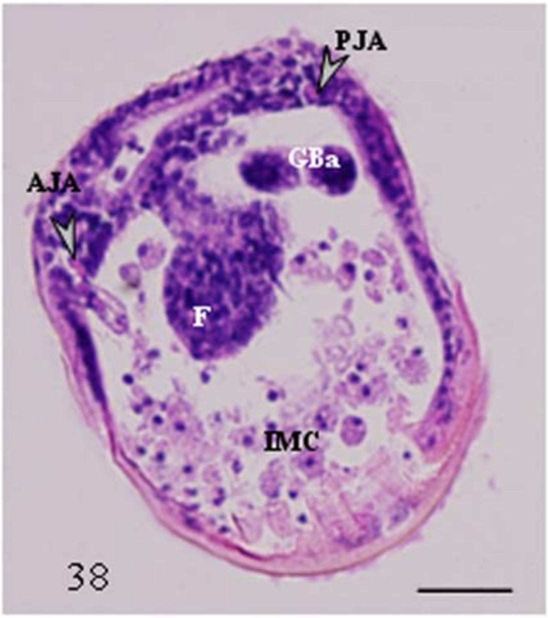
IMC	=	Inner mantle cells

(Masson's trichrome: bar = 25 μm)

Figure 40 Cross-section of day 6 glochidia

F	=	Foot
MB	=	Mushroom body

(PAS-H: bar = 25 μm)


PJA
GBa
AJA
F
IMC
38


IMC
39

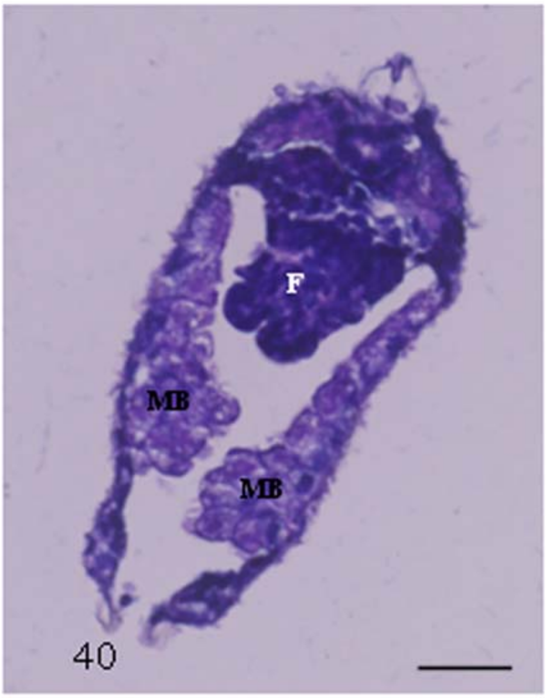

F
MB
MB
40

Figure 41 Saggital-section of day 6 glochidia

DT = Digestive tract with numerous cilia (arrowhead)

F = Foot

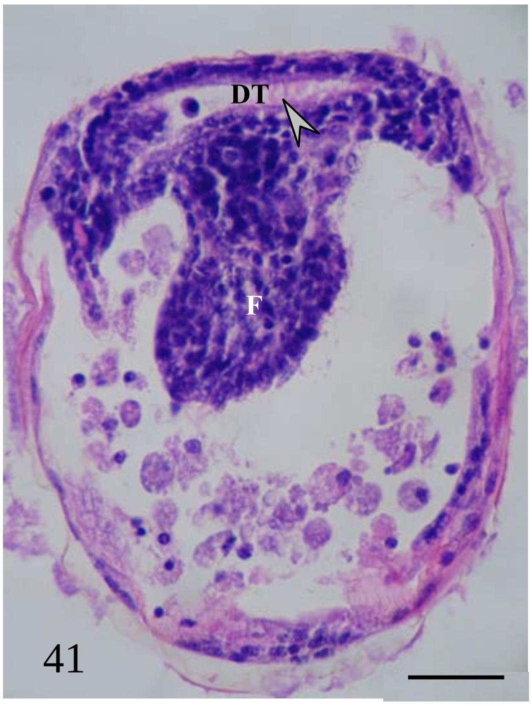
(H&E: bar = 25 μm)

Figure 42 Cross-section of day 6 glochidia

F = Foot

GBa = Gill bar

(H&E: bar = 25 μm)


DT
F
41


GBa
F
42

Figure 43 Saggital-section of day 8 glochidia

AJA	=	Anterior juvenile adductor
F	=	Foot
PJA	=	Posterior juvenile adductor

(H&E: bar = 25 μm)

Figure 44 Cross-section of day 8 glochidia

AJA	=	Anterior juvenile adductor
F	=	Foot (at the distal position)

(H&E: bar = 25 μm)

Figure 45 Cross-section through the middle foot of day 8 glochidia

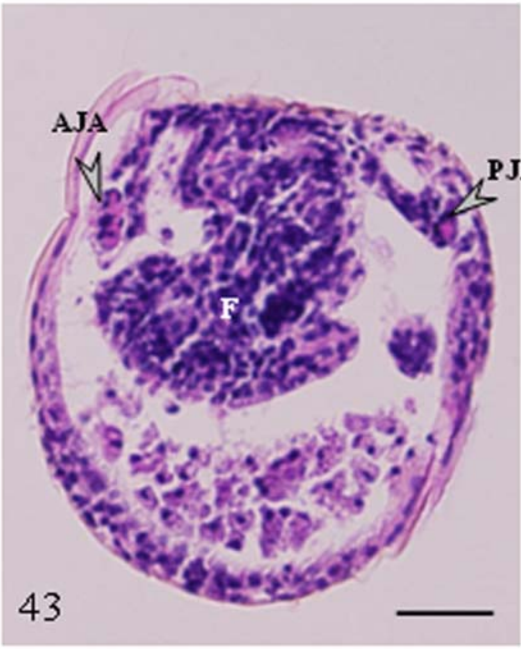
F	=	Foot
GBa	=	Gill bar
I	=	Intestine
PJA	=	Posterior juvenile adductor

(Masson's trichrome: bar = 25 μm)

Figure 46 Cross-section through the middle foot of day 8 glochidia

F	=	Foot
IMC	=	Inner mantle cells

(Masson's trichrome: bar = 25 μm)


AJA
PJA
F
43


AJA
F
44


PJA
GBa
I
F
45

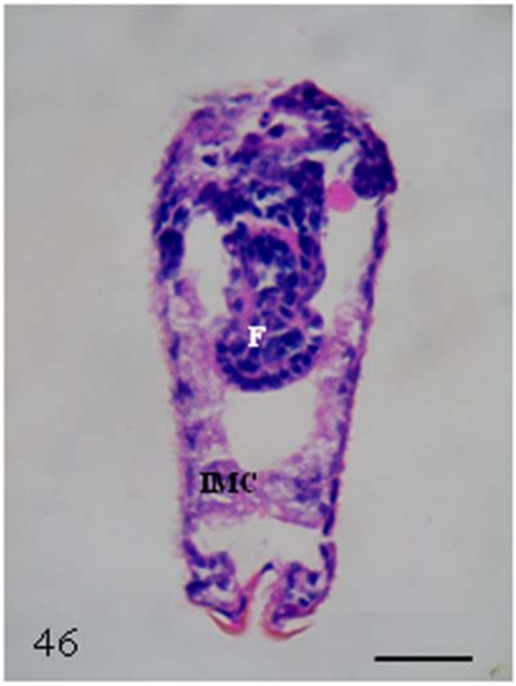

F
IMC
46

Figure 47 Cross-section of day 8 glochidia

F	=	Foot
I	=	Intestine
IMC	=	Inner layer of larval mantle cells

(H&E: bar = 25 μm)

Figure 48 Cross-section through the posterior end of day 8 glochidia

F	=	Foot (at the proximal part)
GBa	=	Gill bar

(Masson's trichrome: bar = 25 μm)

Figure 49 Cross-section through the middle part of day 8 glochidia

F	=	Foot (near middle part)
GBa	=	Gill bar

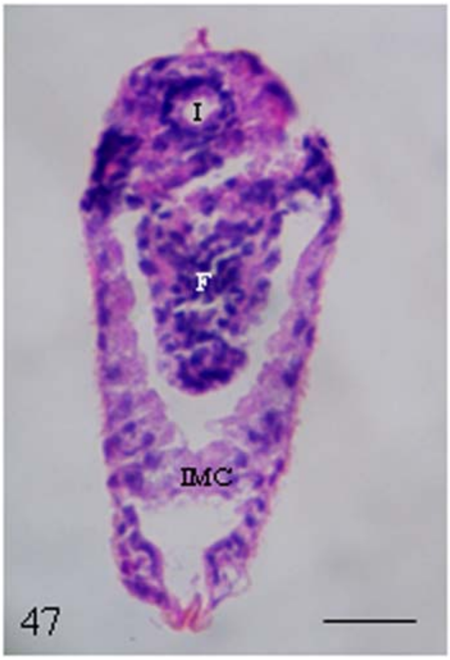
(Masson's trichrome: bar = 25 μm)

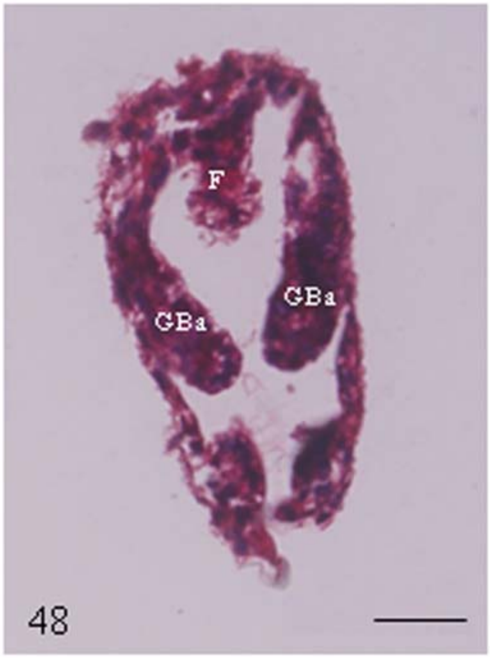
Figure 50 Saggital-section through the middle foot of day 8 glochidia

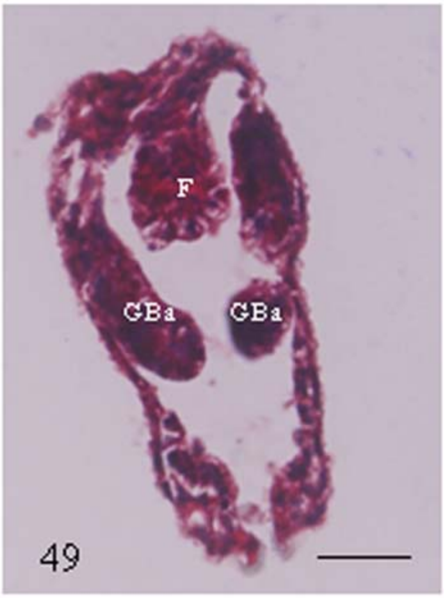
F	=	Foot (distal part)
GBa	=	Gill bar
SS	=	Style sac

An arrowhead indicates the distal part of developing foot

(H&E: bar = 25 μm)


I
F
IMC
47


F
GBa
GBa
48


F
GBa
GBa
49

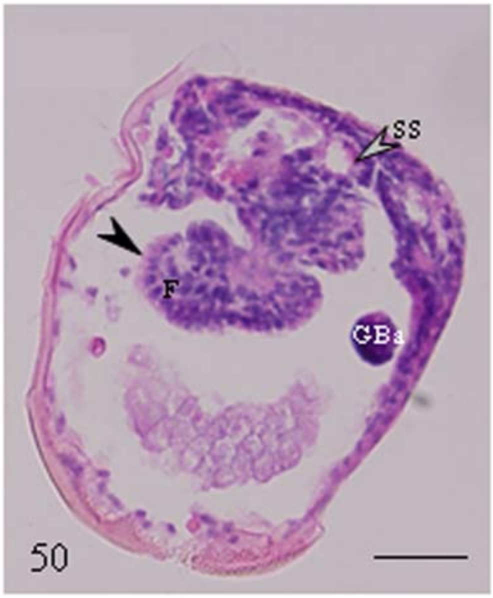

SS
F
GBa
50

Figure 51 Cross-section at the middle foot of day 8 glochidia

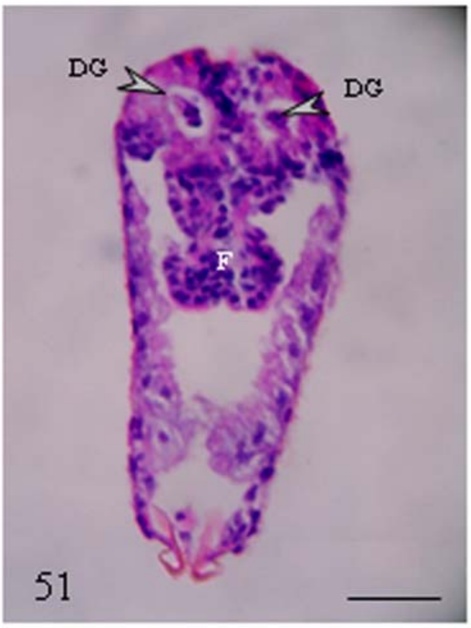
DG	=	Digestive gland (early developing stage)
F	=	Foot

(H&E: bar = 25 µm)

Figure 52 Cross-section at the middle foot of day 8 glochidia

F	=	Foot
S	=	Stomach

(H&E: bar = 25 µm)


DG
DG
F
51

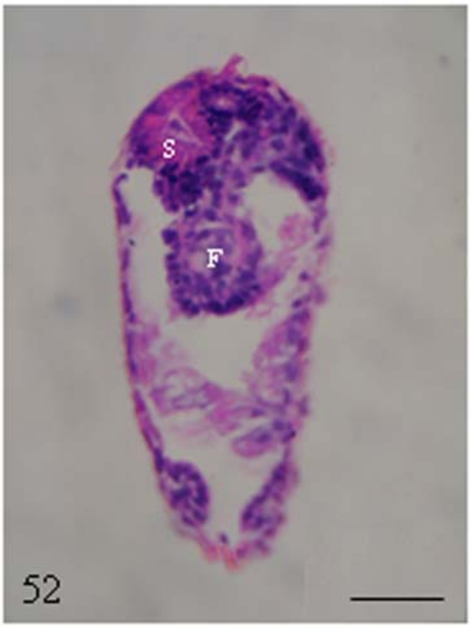

S
F
52

Figure 53 Saggital-section of day 10 glochidia

AJA	=	Anterior juvenile adductor
F	=	Foot with cilia (arrowhead)
PJA	=	Posterior juvenile adductor
SS	=	Style sac

(Masson's trichrome: bar = 25 μm)

Figure 54 Cross-section of day 10 glochidia

AJA	=	Anterior juvenile adductor muscles
F	=	Foot

An arrowhead indicates an elongate nucleus

(H&E: bar = 25 μm)

Figure 55 Cross-section through the middle foot of day 10 glochidia

FT	=	Foot
GBa	=	Gill bar
I	=	Intestine
PJA	=	Posterior juvenile adductor muscles

An arrowhead indicates an elongate nucleus

(H&E: bar = 25 μm)

Figure 56 Cross-section through the middle foot of day 10 glochidia

F	=	Foot
I	=	Intestine
GBa	=	Gill bar

(H&E: bar = 25 μm)

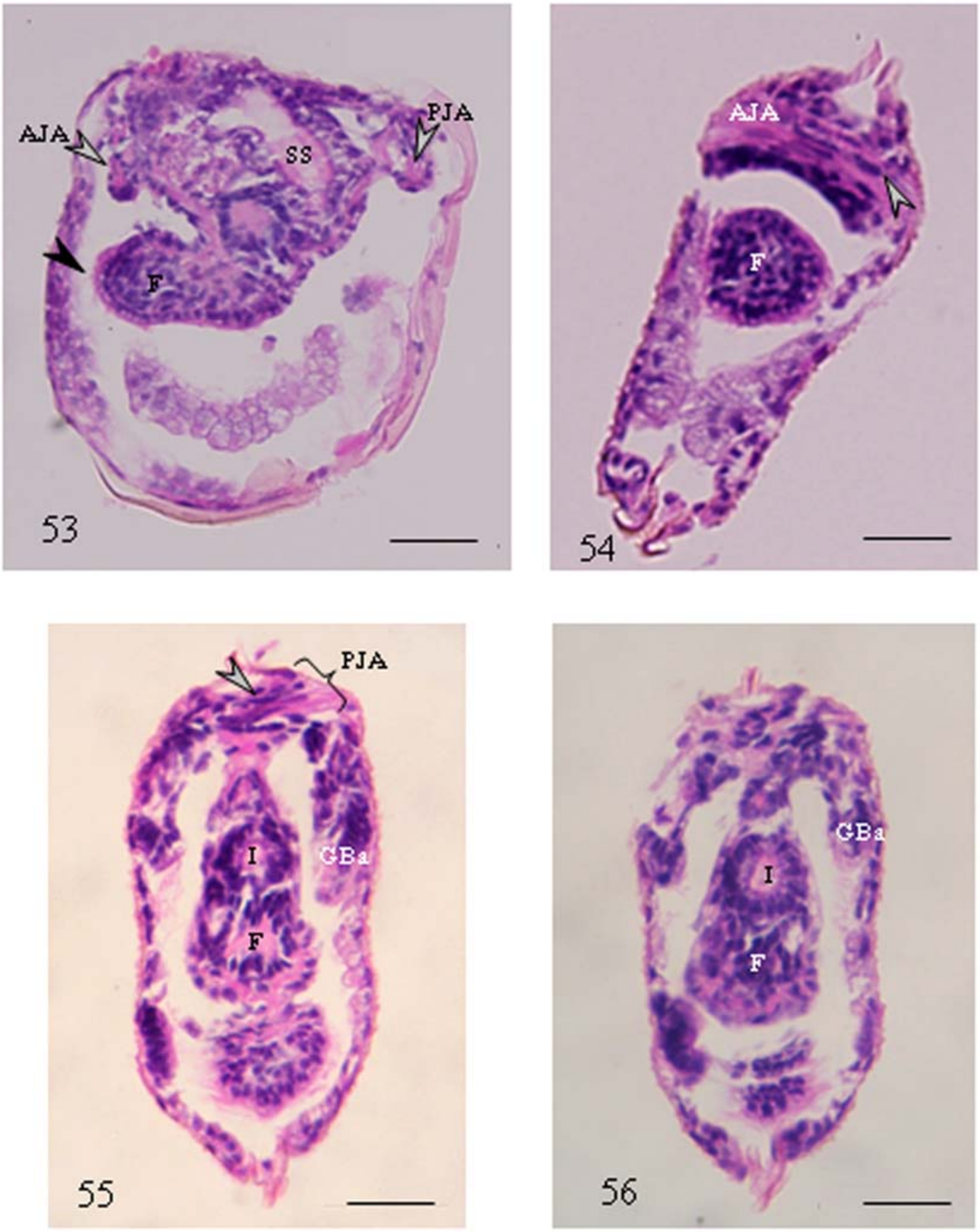

AJA
PJA
SS
F
53
AJA
F
54
PJA
I
GBa
F
55
GBa
I
F
56

Figure 57 Frontal section of day 10 glochidia

DM	=	Definitive mantle

An arrowhead indicates some mushroom body cells debris.

(H&E: bar = 25 µm)

Figure 58 Cross-section at the proximal foot of day 10 glochidia

F	=	Foot
GBa	=	Gill bar

(H&E: bar = 25 µm)

Figure 59 Cross-section at the distal foot of day 10 glochidia

DM	=	Definitive mantle
F	=	Foot

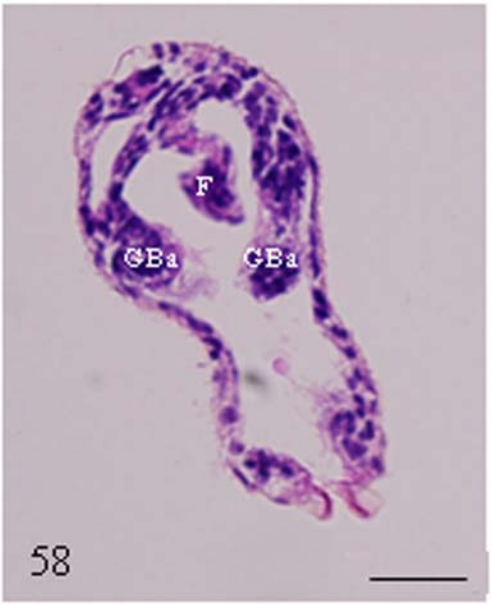
(H&E: bar = 25 µm)

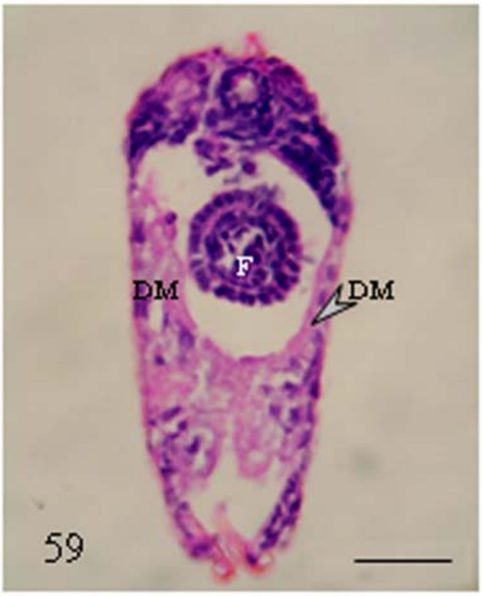
Figure 60 Cross-section at the distal foot of day 10 glochidia

DM	=	Definitive mantle
F	=	Foot

(H&E: bar = 25 µm)


DM
DM
57


F
GBa
GBa
58


F
DM
DM
59


DM
DM
F
60

Figure 61 Cross-section at the distal foot of day 8 glochidia

DF = Distal foot with cilia (arrowhead)

(H&E: bar = 25 μm)

Figure 62 Cross-section at the posterior side of day 8 glochidia

DM = Definitive mantle

GBa = Gill bar

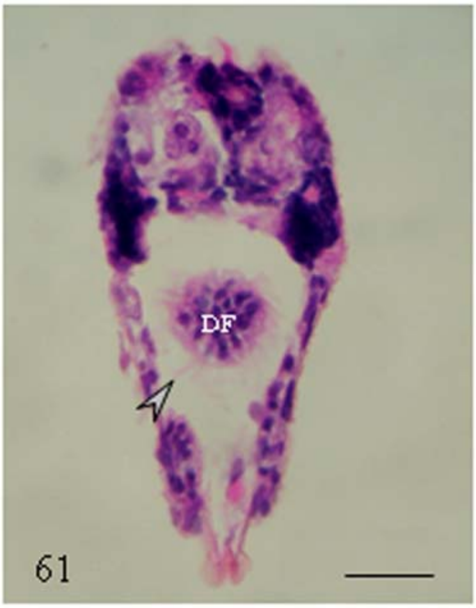
(H&E: bar = 25 μm)

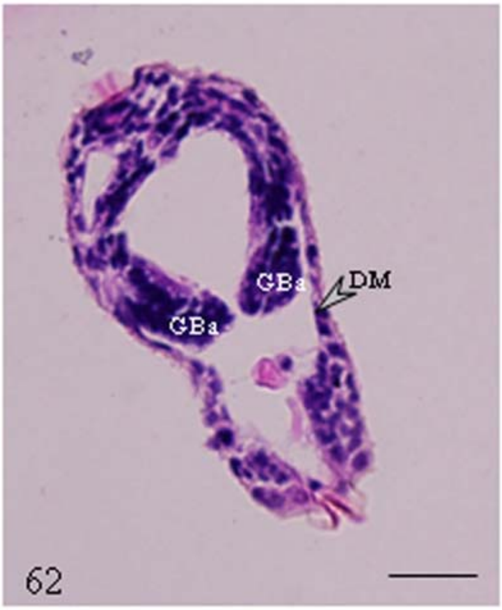
Figure 63 Cross-section at the posterior side of day 8 glochidia

GBa = Gill bar

PJA = Posterior juvenile adductor

(H&E: bar = 25 μm)


DF
61


GBa
DM
GBa
62


PJA
GBa
63

<u>Table 1</u>. Morphological development of *H. (H.) bialatus* glochidia cultured in artificial media.

<table>
<tr><th colspan="3" rowspan="2">Morphological development</th><th colspan="6">Duration of glochidia cultured in artificial media (day)</th></tr>
<tr><th>0 (Mature glochidia)</th><th>2</th><th>4</th><th>6</th><th>8</th><th>10 (Early juvenile)</th></tr>
<tr><td colspan="3">Shape and size</td><td colspan="6" rowspan="2">NC</td></tr>
<tr><td colspan="3">Spines</td></tr>
<tr><td colspan="3">Sensory hair cells</td><td rowspan="4">+++++</td><td rowspan="4">D</td><td rowspan="4">D</td><td rowspan="4">D</td><td rowspan="4">D</td><td rowspan="4">D</td></tr>
<tr><td colspan="3">Larval thread</td></tr>
<tr><td colspan="3">Thread gland</td></tr>
<tr><td colspan="3">Larval adductor</td></tr>
<tr><td rowspan="2">Juvenile adductors</td><td colspan="2">anterior</td><td rowspan="2">ND</td><td rowspan="2">ND</td><td rowspan="2">ND</td><td rowspan="2">+</td><td rowspan="2">+++</td><td rowspan="2">+++++</td></tr>
<tr><td colspan="2">posterior</td></tr>
<tr><td colspan="3">Foot</td><td rowspan="2">ND</td><td rowspan="2">+</td><td rowspan="2">++</td><td rowspan="2">+++</td><td rowspan="2">++++</td><td rowspan="2">+++++</td></tr>
<tr><td colspan="3">Gill bar</td></tr>
<tr><td rowspan="6">Digestive tract</td><td colspan="2">Mouth</td><td>ND</td><td>+</td><td>++</td><td>+++</td><td>+++</td><td>+++</td></tr>
<tr><td colspan="2">Esophagus</td><td>ND</td><td>ND</td><td>+</td><td>++</td><td>++</td><td>+++</td></tr>
<tr><td colspan="2">Stomach</td><td>ND</td><td>ND</td><td>ND</td><td>+</td><td>++</td><td>+++</td></tr>
<tr><td colspan="2">Digestive glands</td><td>ND</td><td>ND</td><td>ND</td><td>+</td><td>+</td><td>++</td></tr>
<tr><td colspan="2">Intestine</td><td>ND</td><td>ND</td><td>+</td><td>+++</td><td>++++</td><td>+++++</td></tr>
<tr><td colspan="2">Rectum</td><td>ND</td><td>ND</td><td>+</td><td>+</td><td>+</td><td>+++</td></tr>
</table>

Note:

NC	=	no change	+	=	start to develop
ND	=	not developed	++	=	early developed
D	=	degenerated	+++	=	moderately developed
			++++	=	clearly developed
			+++++	=	fully developed

DISCUSSION

Reports on glochidial development, especially histological observation from glochidia to juvenile stage are scarcely found. Although several groups have tried to culture glochidia by setting a mimic condition and using host fish, it is difficult to collect adequate samples and observe their morphological development until *in vitro* culture methods by Keller and Zam (1990) and later on modified by Kovitvadhi *et al*. (2001) have led to substantial improvement of glochidial survival and development. In addition, all glochidia in each culture plate could be manipulated to develop at the same transforming stage. Therefore, a controlled process involving the artificial culture of glochidia could provide enough samples to follow the transformation of glochidia into juveniles.

The transformation period of Uninacean glochidia varied depending on freshwater mussel species. Under the same condition of 25 ^{o}C with 5% CO_2, *H. (H.) bialatus* glochidia could transform into juvenile within 10 days while *H. myersiana* needs only 7-8 days in culture medium (Kovitvadhi, 2001).

Shape and size of mature glochidia

Two groups of *Unionacean* glochidia, one hookless and another with hooked shells, have been distinguished (Lefevre and Curtis, 1910). The general appearance of the glochidium of *Hyriopsis (Hyriopsis) bialatus* resembled other unionid larvae, but more closely to the glochidium of *Hyriopsis myersiana* (Kovitvadhi *et al*., 2001). Both species had semi-oval and equilateral-valved shells. During transformation process, these two species remained in a semi-oval shaped until the last day of transformation. The size of *H. (H.) bialatus* was somewhat smaller than *H. myersiana* but quite different comparing to other unionoids (Appendix Table 1; Arajo and Ramos 1998).

Attachment organs

Two types of attachment organs were found in *H. (H.) bialatus* glochidia, i.e, numerous spines (or microscopic teeth) and larval threads. Like *H. myersiana*, the rim of glochidial valves of *H. (H.) bialatus* was lined by numerous spines (Kovitvadhi *et al*., 2001). During transformation, these small spines still covered the ventral of the larval shell margin until they reached early juvenile stage. The microscopic teeth of shell margin, on the other hand, were covered by a rim of the periostracum in the glochidium of *H. depressa* and *M. margaritifera*. Only Harms (1907, 1909) has reported the presence of six or seven microscopic teeth in *M. auricularia*, while Jupiter and Byrne (1997) found just one microscopic blunt tooth at the ventral margin of each valve of the glochidia of *H. depressa*. The presence of microscopic teeth or numerous spines which acted as attachment organs in the glochidia may be unique characters to identify the taxonomic differences among genus of freshwater mussels (Davis and Fuller 1981; Smith and Wall 1984).

There were two types of larval thread of the mature glochidia of *H. (H.) bialstus*, internal and external larval threads. The external larval thread rising from the thread gland was long (up to 2 mm externally), sticky, and pliable, and could attach itself to rough surface organs such as the fin or gill of host fish. The internal larval thread, on the other hand, was located around the larval adductor muscle. These threads consisted of neutral mucopolysaccharide or mucin as seen from the positive staining with Masson’s trichrome and PAS, supporting the work of Wood (1974) as seen on the glochidia of *Anodonta cygnea* staining with only PAS. The TEM observation of *A. arcaeformis* as also reported by Jeong *et al*. (1992), confirmed that the larval thread was non-cellular and not bound by a membrane, while the matrix of the larval thread did not contain any organelles other than numerous microtubule-like structures. It was speculated that these structures were related to the movement of larval thread (Lodish *et al.,* 1995). Although there was no larval thread or thread gland observed in the glochidia of *M. auricularia* but the thread fragments was found in the culture media as reported by Araujo and Ramos (1998).

Organogenesis

To prepare themselves for the free-living condition at juvenile stage, glochidia began to form essential structures such as mantle, foot, gill bar and digestive tract for independent use. In the glochidium of *H. (H.) bialatus*, all cell masses were temporarily aggregated as rudiment organs of juvenile. The adductor muscle and the ciliated organs (ventral plate and lateral pits) were the most conspicuous cell masses (Araujo and Ramos, 1998). Other rudiment organs such as pericardium, kidney, heart or gonad were not observed in the glochidia or at early juvenile stage of *H. (H.) bialatus, H. myersiana, U. imbecillis, M. margaritifera* and *M. auricularia* (Harms, 1907; 1909, Araujo and Ramos, 1998; Kovitvadhi *et al*., 2001; Fisher and Dimock, 2002).

Like the glochidia of *M. auricularia*, no respiratory organs have developed in *H. (H.) bialatus* at this stage, so the cilia of the lateral pits and ventral plate were probably used to aerate the larva (Araujo and Ramos, 1998). The ventral plate and lateral pits were the origins of foot and gills as also described by Lillie (1895). Autoradiographic data of *U. imbecillis* also indicated that these structures were the areas of high rate RNA and DNA synthesis during the early stage of transformation, suggesting that the gene expression (both transcription and translation) were confined primarily to the lateral pits and the ventral plate that later formed several juvenile structures (Fisher and Dimock, 2002). These active cell masses of *H. (H.) bialatus* were darkly basophilic stained because they contained numerous basophilic molecules such as DNA, RNA, and other basic proteins.

Gill formation

There were two gill buds (left and right) in the developing glochidia of *H. (H.) bialatus* similar to those of *H. myersiana* as reported by Kovitvadhi *et al*. (2001). These two gill buds found in *H. (H.) bialatus* completely turned into gill bars within 8-10 days in artificial media but they could not fully function for filter feeding at this stage. As pedal-mantle feeder, these glochidia have numerous cilia covering the junenile foot, gill bars, as well as mantle, and moved themselves very quickly and vigorously to transfer the food into the mantle cavity (Kovitvadhi *et al*., 2001; Fisher and Dimock, 2002).

Foot formation

Foot formation required different period of time depending on freshwater mussel species. Both *H. (H.) bialatus* and *H. myersiana* took 8-10 days (Kovitvadhi *et al*., 2001), while *U. imbecillis* took only 6-7 days to form the juvenile foot (Fisher and Dimock, 2002). It is interesting to see that the foot lobes, formed within 4 days in artificial medium, were originally seen as two lobes. They were probably separately formed and later fused to become a single foot. This interesting aspect of foot organogenesis was also observed under SEM in the glochidia of *A. cygnea* showing the foot formation involved several growing stages from the two small symmetric and curved lobes on the base of future foot. These two distinct lobes eventually fused to form a bifurcated structure until completely joined as a definitive foot (Lima *et al*., 2006).

Digestive tract formation

Like other Protostome embryos, development of digestive tract of *H. (H.) bialatus* began with the mouth formation by invagination of the oral plate or endodermic sac which turned into estomodeum and the mid-gut (Araujo and Ramos, 1998). As the larval adductor muscle was sliding into the anterior side, the continuous tube of digestive tract was formed underneath the growing foot where numerous eosinophilic granules were found. These yolk granules might be the source of nutrition for the glochidial development especially at the foot and digestive tract. The complete digestive system of *H. (H.) bialatus* was formed within 10 days while the glochidial digestive tracts of *U. imbecillis* were completely formed within only 6-7 days in the culture medium (Fisher and Dimock, 2002). The digestive system developed for free-living at juvenile stage consisted of mouth, esophagus, stomach, style sac, digestive glands, and intestine which terminated in the rectum. However, there was no crystalline style present in the style sac of *H. (H.) bialatus* glochidia as seen in later stages of juvenile. It probably has not been formed at this stage or the concentration of protein component might be too low to be detected by this technique.

Mantle development

There were two layers of mantle cells, outer and inner layer, found in mature glochidia of *H. (H.) bialatus*. The outer layer was very thin and inconspicuous. It directly adhered to the shell by a connective tissue. The inner layer, on the other hand, comprised of low columnar cells containing concentric nuclei and numerous eosinophilic granules in the cytoplasm. The presence of two layers of mantle cells has been reported in several species including *A. corpulenta*, *L. luteola* (Blystad, 1923; Arey, 1932b), *M. auricularia* (Araujo and Ramos, 1998), *U. imbecillis* (Fisher and Dimock, 2002) and *M. margaritifera* (Limnaeus) (Karna and Millemann, 1978).

It is interesting to see a mucoid structure which located between the outer layer of larval mantle and larval shell of each valve. This structure was found to be neutral mucopolysaccharide or neutral mucin from the positive staining with both Masson's trichrome and PAS. Although its function is still unknown, it probably acts as skeletal-like structure of glochidia. Therefore, it was named "supporting band". It should be noted that this supporting structure has never been observed in other freshwater mussel species before.

In *H. (H.) bialatus*, the larval mantle cells were found to form the mushroom body as also seen in the glochidia of *U. imbecillis* and persisted until the end of metamorphosis (Fisher and Dimock, 2002). This mushroom body was reported to be processed by the enlargement of larval mantle cavity (Braun, 1978). Arey (1932b) also described this structure in both *L. luteola* and *A. corpulenta* suggesting that the mushroom body persisted after the end of parasitism in the hookless glochidia, whereas it disappeared towards the end of parasitism in the hooked glochidia.

On the final day of transformation, the mushroom body was clearly seen, but disappeared on the following day. This structure was not necessary for free-living juvenile because it only facilitated the glochidial acquisition of nutrients from the mussels' host (Blystad, 1923; Arey, 1932b). However, the fate of larval mantle cells in freshwater mussels during metamorphosis is still inconclusive. It was not clear whether the cells were digested by the animal itself or they were sloughed off during the final transition to juvenile. Our results, however, showed that some parts on the free end of the mushroom body of *H. (H.) bialatus* were found as debris sloughing off in the mantle cavity (Figure 57). Arey (1932b) suggested that the decrease in height of larval mantle of *L. luteola* and *A. corpulenta* during metamorphosis was possibly caused by the pressure from the growing foot and independent shortening of the mantle itself. It seemed that the larval mantle decreasing processes in *H. (H.) bialatus* followed the same pattern as described by Arey (1932b) as well as from the pressure of gill bars exerted onto it.

Adductor muscle development

The adductor of *H. (H.) bialatus* glochidia in artificial culture medium and in the host fish was found to consistently contract while the valves remained closed until the juvenile was completely formed as also seen in *U. imbecillis* (Fisher, 2001). During this time, the larval adductor muscle became degenerate and the two juvenile adductor muscles (anterior and posterior) developed *de novo*. Histochemical and actin-specific staining of *U. imbecillis* showed the complete degradation of the larval adductor muscle during the first few days of metamorphosis. This phenomenon was also observed by Zn.Nagy and Lábos (1969) indicating that the larval and adult adductor muscles of *Anodonta cygney* were two distinctively different structures. The adult adductor muscles were initially quite small and presumably enlarged as development continues. In contrast, Fukuhara *et al*. (1990) suggested that the adult anterior adductor muscle was derived in part from the larval muscle in *Anodonta woodiana* which was quite different from other research groups.

Nervous ganglia and sensory hair cells

Like glochidia of *M. auricularia* and *M. margaritifera*, the nervous ganglia in the glochidia of *H. (H.) bialatus* were not present. So, the contractile response was transmitted by the sensory hair tufts or hair cells (Pekkarinen and Valovirta, 1996). Ultrastructure of *A. arcaeformis*, showed two types of sensory hair cells in the larval mantle (Jeong *et al*., 1992), one type consisted of three solitarily positioned and highly specialized cells in the mantle. Each cell possessed a bunch of protruding hair. This type presumably perceived chemical stimuli (Wood, 1974). The function of the other type of hair cellslocated in the posterior margin near the lateral pits is still unknown. This second type of hair cells was found to be variable in numbers and have been used for taxonomic classification in some species. For example, there were two pairs in both *M. margaritifera* and *M. auricularia* instead of the four pairs found in most of other Unionoid glochidia (Lefevre and Curtis, 1912; Pekkarinen and Englund, 1995; Pekkarinen & Valovirta, 1996) Unfortunately, in genus *Hyriopsis* the number and distribute of this type of hair cells in the individual specimen of *H. (H.) bialatus* and *H. myersiana* was quite varied (Kovitvadhi *et al*., 2002) and therefore is unlikely a good indicator for taxonomic classification of this genus.

CONCLUSIONS

The successful transformation of the glochidia in *Hyriopsis (Hyriopsis) bialatus* occurred within 10 days in artificial culture media. During transformation, the larvae retained their semi-oval shape and did not significantly change their size. Numerous spines still covered the ventral of the larval shell margin until early juvenile stage while other structures such as sensory hair cells, larval thread, thread gland, and larval adductor were degenerated on day 2. The two juvenile adductor muscles, anterior and posterior, began to develop *de novo* on day 6.

The larval mantle of mature glochidia could be divided into two layers; outer and inner layer. A supporting band located between the larval mantle and the inner shell surfaces probably acted as a skeletal-like structure. During transformation, the mantle cells formed the mushroom body on day 4 and turned into the definitive mantle on the last day of transformation by sloughing off their free ends.

There were three cell masses developed in this stage; ventral plate (the foot rudiment), lateral pits (the gill rudiment), and oral plate or endodermic sac (origin of the digestive tract). Both foot and gill formation began on day 2 and completed within 10 days. The digestive tract began with the mouth formation on day 2. Then, the continuous tube of digestive tract with numerous cilia became an esophagus and the distal part of digestive tract formed an intestine and a rectum on day 4. A stomach with a pair of digestive glands began to develop on day 6.

This work clearly illustrated the sequential development of *H. (H.) bialatus* from glochidia to juvenile stage. Several controversial aspects of organogenesis have been inferred, i.e. de novo formation of anterior and posterior juvenile adductor, fate of mushroom body structure, and a foot lobe formation from 2 separate precursor lobes. More over, the evidence of supporting band (mucoid structure) in the mature glochidia of *H. (H.) bialatus* has never been reported in other freshwater mussel species and needs to be further investigated on its function and composition.

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