Larvae stage of Thai freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus* Simpson 1900, morphological development

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Abstract

Glochidia of Thai freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus* was cultured in an artificial medium consisting of M199, fish plasma (*Cyprinus carprio*), and antibiotics/antimycotic at a ratio of 2:1:0.5 (V/V). They were reared at a density of 50-100 glochidia per dish under sterile conditions until they transformed into juveniles. The culture dish was incubated at 23±2°C with a constant supply of 5% CO_2. The duration of glochidia development until the juvenile stage was 10 days. After transformation, early juvenile mussels were reared and fed with a mixture of 4 types of phytoplankton (*Chlorella* sp.2*., Kirchneriella incuvata, Navicula* sp*.* and unknown) until 15 days. The morphological study of glochidia and early juveniles was carried out using a light and scanning electron microscope. The results illustrated that glochidia and early juveniles cultured in an artificial medium exhibited normal morphological development and could undergo normal transformation. The appearance of primitive gills, foot, gastrointestinal tract, and growth lines on the shell are signs of complete metamorphosis.

Keywords: Freshwater pearl mussel, Glochidia, *Hyriopsis bialatus*, Juvenile, morphology

1. INTRODUCTION

Hyriopsis (*Hyriopsis*) *bialatus* Simpson 1900 is a Thai freshwater pearl mussel widely distributed in the bottoms of reservoirs, and rivers in central, northern, and northeastern Thailand (Brandt, 1974; Kovitvadhi & Kovitvadhi, 2002; Nuchan et al., 2022). They have been an important source of feed for different animals and economic benefit. The nacreous mussel shell can be used for inlaying pearl furniture,

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pearl button industry, ornament, kitchen utensils, and souvenirs. Besides, it can be produced freshwater pearl (Yeemin, 1997). Mussels are filter-feeders, and they siphon nutrients from the water column. It is thought that these filtering activities contribute to maintaining the river and stream ecosystem. They are also used as bioindicators of the ecological system (Hudson & Isom, 1984; Biggins et al., 1997). Moreover, mussels have antioxidant enzymes and biotransformation enzymes in their digestive gland to detoxify substances in water (Birmelin et al., 1999). However, the number of freshwater pearl mussels has been declining substantially due to over-harvesting and increased commercial use.

To increase the population of freshwater mussels, efforts have been made to use culture techniques for mass production and conservation (Isom & Hudson, 1982, 1984a; 1984b; Hudson & Isom, 1984; Keller & Zam, 1990; Uthaiwan et al., 2001; Uthaiwan et al., 2002; Kovitvadhi et al., 2003; Areekijseree et al., 2004; Areekijseree et al., 2006). This could be accomplished through understanding the general living pattern and significant changes in the developmental morphology of the animal. The life cycle of freshwater mussels is generally known that there are two critical developmental stages that make them vulnerable to changes in the environment of their habitats. First, there is a critical early stage called “glochidia” where young mussels develop within the gills of mature female mussels. When these glochidia become mature, they are released from the females and transferred to a specific host fish. They are, then, parasites living on the gills, fins, or body of the host fish. During this time transformation to the second critical stage called “juvenile” takes place and the juveniles detach themselves from their host, drop to the river bottom and live as free-living animals. These two critical stages, glochidia and juvenile are the most crucial times of their life cycle which determine the successful survival of the adult mussels. Therefore, knowing the developmental timing, as well as the morphological changes during the transformation from glochidia to juvenile would be helpful for culturing this species of mussel in an artificial medium. The aim of this investigation is to increase the population of juvenile *H. bialatus* by taking them to their habitats.

2. METHODS

2.1 Animal and rearing

Adult *H. bialatus* were obtained from the Mun River Basin in northeastern Thailand. The physio-chemical ranges of the Mun River Basin were water temperature 25.0-31.6 ^{o}C, water dept 0.8-2.0 m, transparency 10.0-170 cm, pH 6.8-7.7. The majority of soil were sand, loamy sand, sandy clay loam, clay loam and loam, respectively. They were sexually identified by needle fluid suction from gonad to observe the gametes under a microscope. Mussels were then cultured in a circle net in a pond (water dept 1.2-2.5 m, pH ranging from 7.0-7.2 and temperature ranging from 28-30^{o}C), majority of soil was clay loam and allowed to feed freely on

natural plankton in the pond *(*for example *Chlorella sp., Kirchneriella incurvate, Navicula sp.*) at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand.

2.2 Glochidia and juvenile culture

Ten gravid mussels with thoroughly brown marsupia were selected for extraction of mature glochidia. They were of the average size of 108.30±6.13 cm in length, 41.33±2.40 cm in height and 22.65±2.55 cm in width. A small number of glochidia were removed using a sterilized 1 mL syringe with an 18-gauge needle and transferred to a petri dish for observation under a light microscope (x100). If their shells were periodically closed, they were considered strong and suitable for culturing in artificial medium. The remaining glochidia were removed from the marsupia and dispersed softly with sterilized distilled water. They were then collected and placed in a new petri dish to eradicate tissue residues, mucus and glochidia shell fragments by repeated changes of water. Then, they were cultured in tissue culture dishes (60x15 mm) containing 3.5 mL of artificial medium with fish plasma (*Cyprinus carprio*), antibiotic and antimycotic, at a density of 50-100 glochidia per dish under sterile conditions (Uthaiwan et al., 2001; Uthaiwan et al., 2002; Kovitvadhi et al., 2003; Polwong et al., 2003, Areekijseree et al., 2006; Nuchan et al., 2022).

The culture medium was changed on the fourth day of the culture period. The culture dish was placed in a plastic box and incubated with a constant supply of 5% CO_2 at 23±2°C (for medium pH adjustment) with room air humidity (Uthaiwan et al., 2001). They were observed daily to determine the time of glochidia transformation into a juvenile stage. The signs of transformation such as the mantle were observed, and 1 mL of sterile distilled water was added to the culture medium to synchronize transformation. After the transformation was completed, the early juveniles with the medium were transferred from the dishes to a beaker and the total medium volume was diluted with sterilized osmolarized water and aerated until the foot was observed. They were then transferred to a culture plastic box and fed initially with a mixture of 4 types of phytoplankton (*Chlorella* sp.2*., Kirchneriella incuvata, Navicula* sp.*,* and unknown) at a ratio of 1:1:1:1 (v/v), at the concentration of $1x10^3$ cell/mL twice daily (9 A.M. and 6 P.M.) for 15 days (Kovitdvadhi et al., 2003).

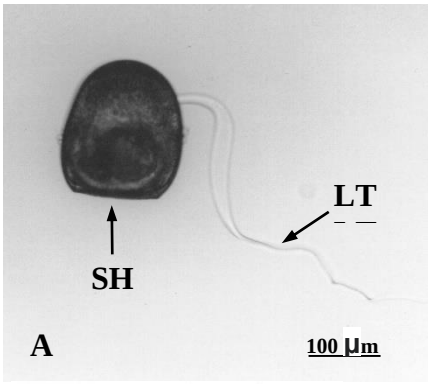
2.3 Morphological development of glochidia and juvenile

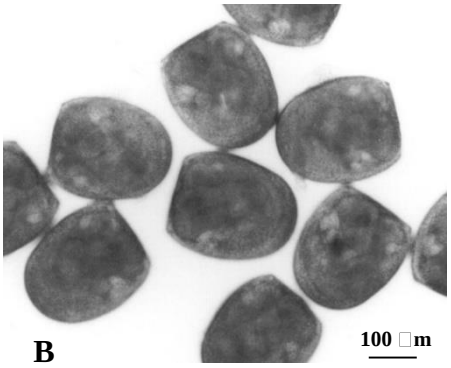
2.3.1 Preparation sample for light microscopic observation

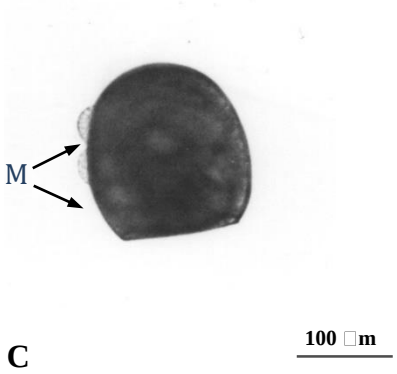
Several stages of glochidia and juveniles were anesthetized in a 2% chloral hydrate solution and observations were made to identify the mantle edge, shell growth, foot, gills, and some organs formation under the light microscope (x400) (indicate model, company, city, country).

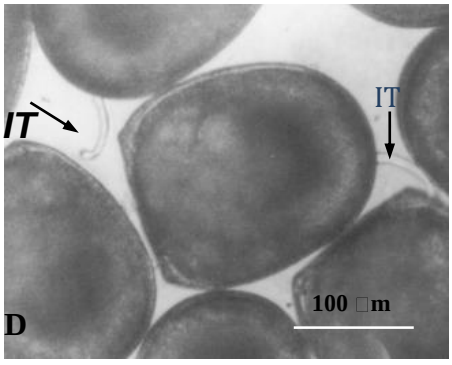
2.3.2 Preparation sample for scanning electron microscopic observation

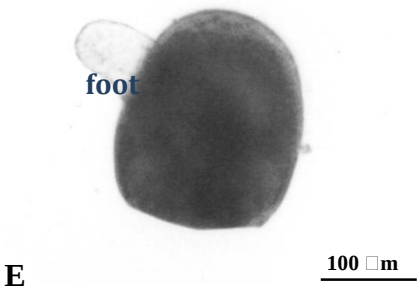
The specimens were anesthetized in a 2% chloral hydrate solution and fixed in 10% buffer formalin for 2 h and preserved in 5% buffered formalin for 24 h. They were then dehydrated in a graded series of ethanol (30%, 50%, 70% 80%, 90%, and absolute ethanol) and dried using a critical point dryer machine. All samples were mounted on SEM specimen stubs with conductive silver paint and coated with gold and observed using a Jeol JSM-35 CF and Camscan scanning electron microscope operated at 5 kV (Company, city, country).











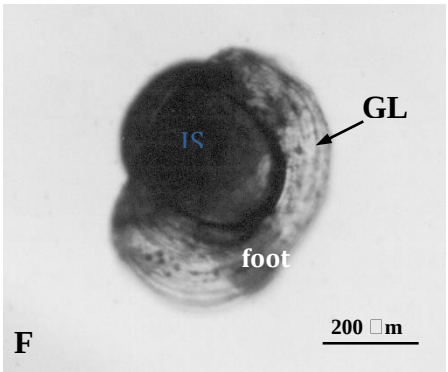
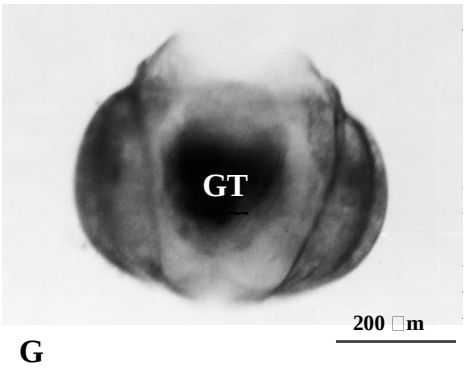


Figure 1. Features of glochidia from the gravid female gills and early juvenile *Hyriopsis bialatus* observed under light microscope (LM). A-B: mature glochidia showing larval thread (LT) and straight hinge (SH)**.** C-D: 10 days old glochidia in artificial medium showing mantle (M) and internal thread (IT). E: 1day old juvenile after transformation with a foot appearing. F: 15 days old juveniles showing the initial glochidia shell (IS); a foot; growth lines; (GL); gastrointestinal tract (GT) and primitive gills (G).



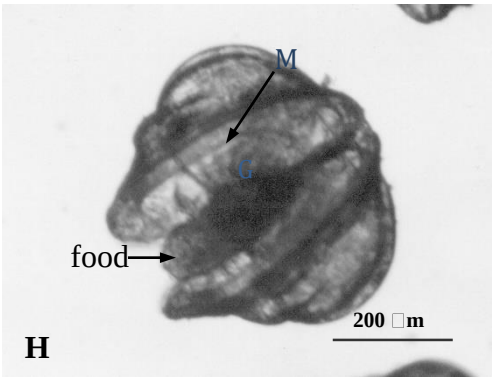


Figure 1 (Continue) Features of glochidia from the gravid female gills and early juvenile *Hyriopsis bialatus* observed under light microscope (LM). G-H: 15 days old juveniles showing the initial glochidia shell (IS); a foot; growth lines; (GL); gastrointestinal tract (GT) and primitive gills (G).

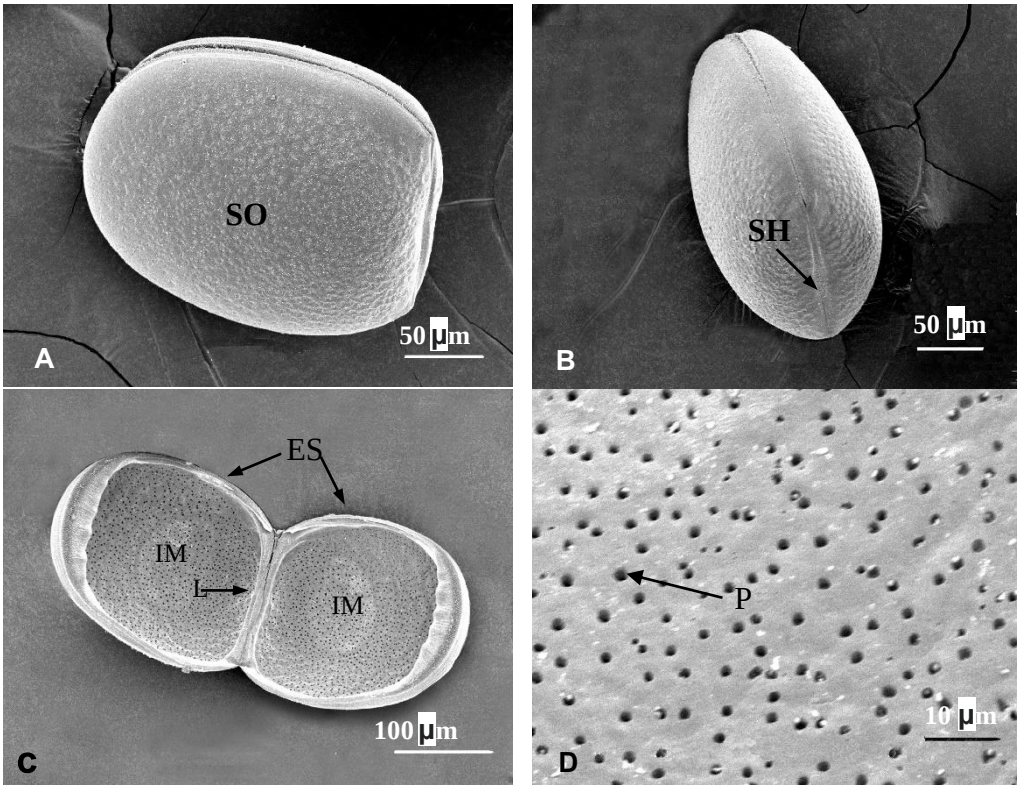


Figure 2. Several ultrastructural aspects observed in mature glochidia *Hyriopsis bialatus* using scanning electron microscopy (SEM): A-B: mature glochidia showing semi-oval valves (SO); straight hinge (SH). C-D: the internal surface of glochidia valves showing hookless equivalve shell (ES); shell ligament (L); insertion area of adductor muscle (IM) and pores in the calcareous layer (PO)

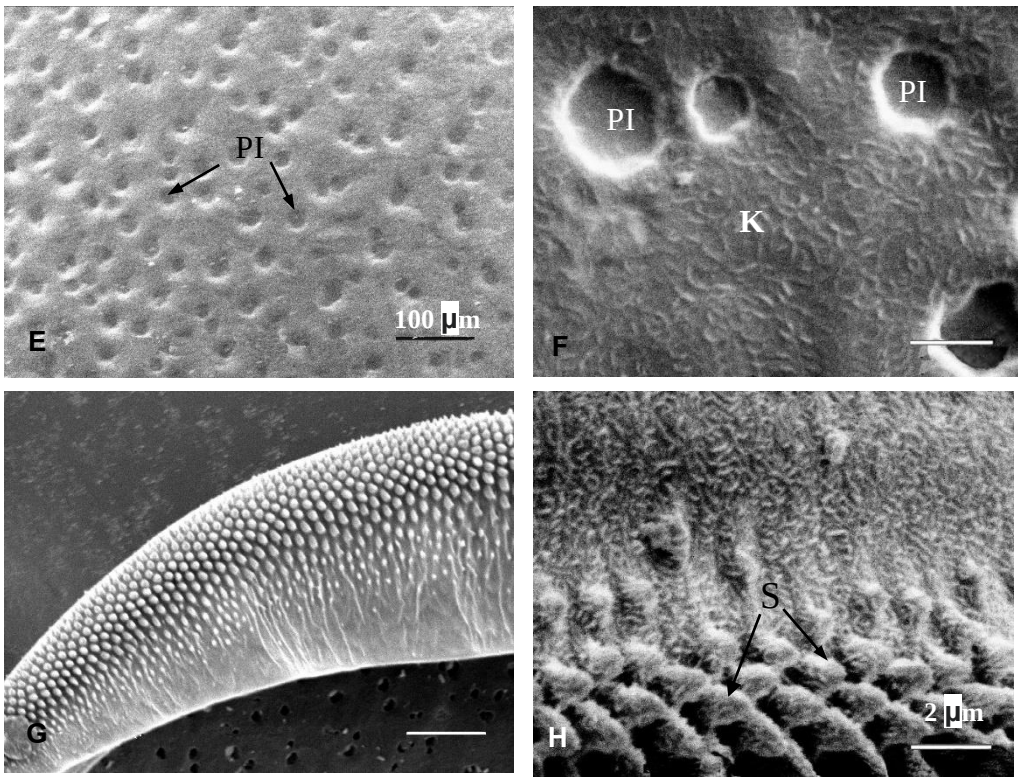


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Figure 2 (Continue) Several ultrastructural aspects obse ed in mature glochidia *Hyriopsis bialatus* using scanning electron microscopy (SEM): E-F: the external surface of glochidia valves showing pits (PI) and keratin fibers (K). G-H: the terminal surface of glochidia shell showing numerous spines (S).

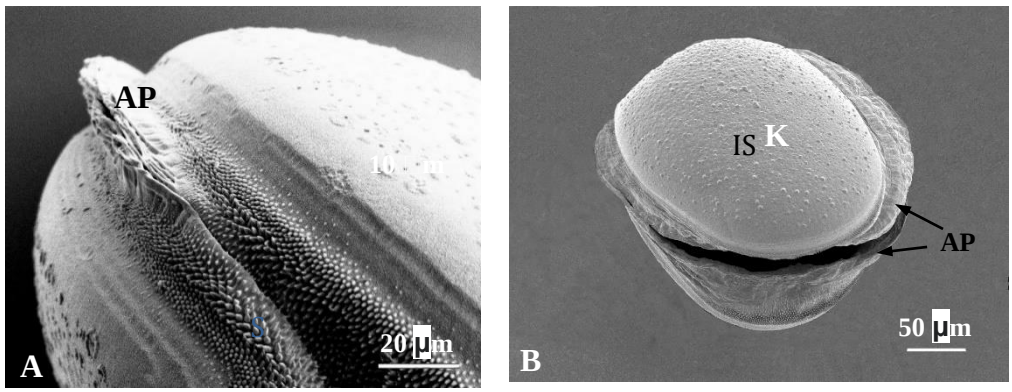

Figure 3. Several ultrastructural aspects observed in juvenile *Hyriopsis bialatus* using scanning electron microscopy (SEM): A: 2 days old juveniles, B: 7 days old juveniles

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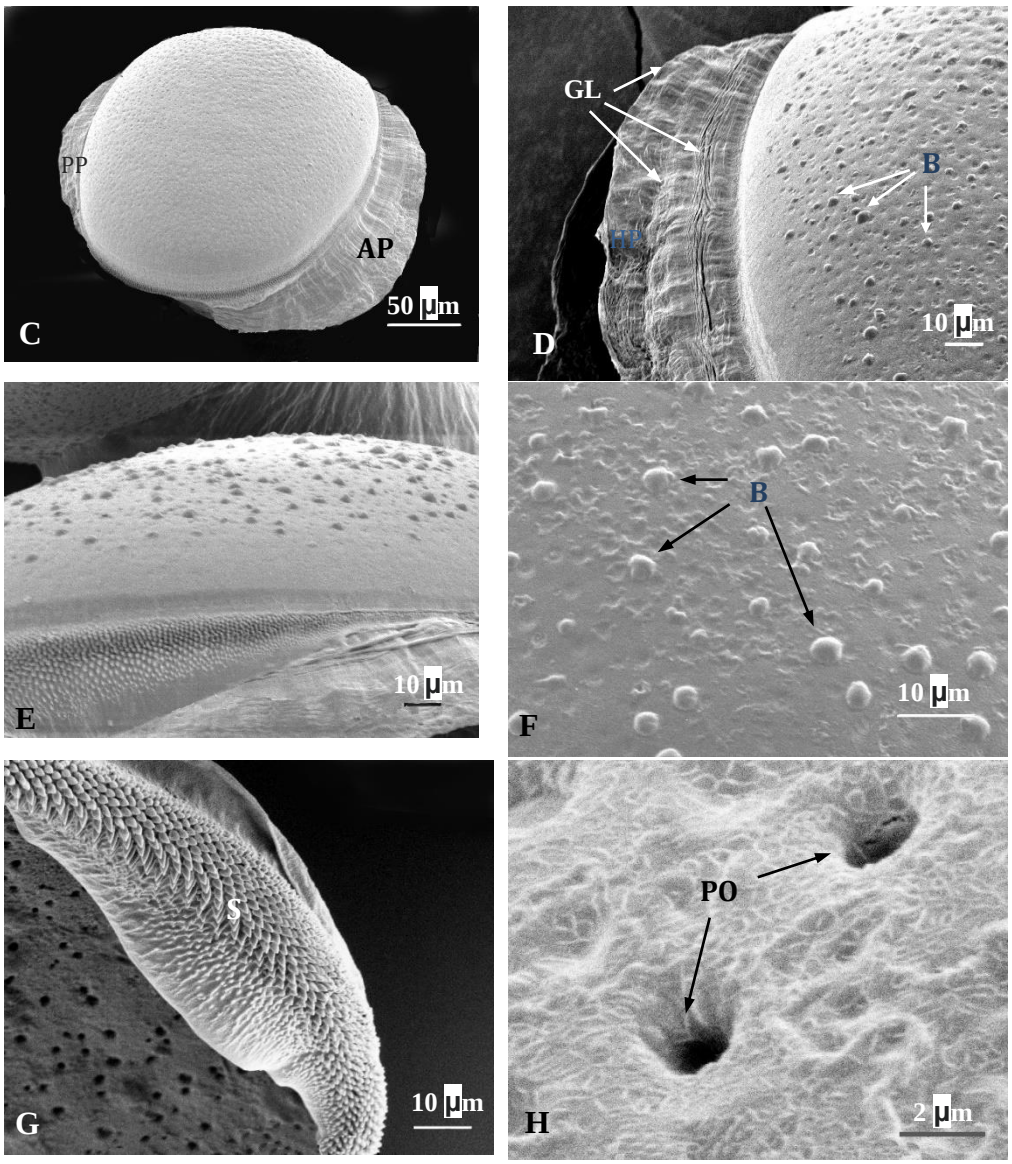

Figure 3. Several ultrastructural aspects observed in juvenile *Hyriopsis bialatus* using scanning electron microscopy (SEM): C-H: 10 days old juveniles showing anterior periostracum (AP); initial glochidia shell (IS); posterior periostracum (PP); growth lines of periostracum (GL); hard periostracum (HP); cuticle buds (B); pores in the internal calcareous layer (PO); spines (S) and keratin fibers (K).

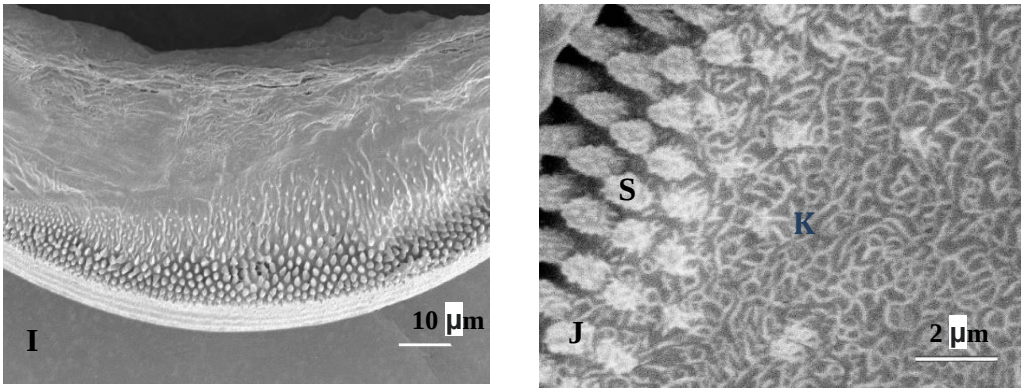

Figure 3. Several ultrastructural aspects observed in juvenile *Hyriopsis bialatus* using scanning electron microscopy (SEM): I-J: 10 days old juveniles showing anterior periostracum (AP); initial glochidia shell (IS); posterior periostracum (PP); growth lines of periostracum (GL); hard periostracum (HP); cuticle buds (B); pores in the internal calcareous layer (PO); spines (S) and keratin fibers (K).

3. RESULTS AND DISCUSSION

Glochidia *H. bialatus* could survive and successfully transformed into juvenile stage in the artificial medium containing fish plasma (*Cyprinus carprio*) as a food additive. The transformation occurred within 10 days after culturing in the artificial medium. The average percentage survival and average percentage transform were 94.45±2.37 and 97.36±2.2.7.

The form of mature glochidia *H. bialatus* observed under a light microscope was semi-oval with an equivalve shell and equilateral valve shape where the hook formation in the ventral region was absent in this species (Fig. 1A, 2A, and 2C). The mature glochidia shell was 186.51±2.18 mm in length, 216.25±3.69 mm in height, and 60.63±0.86 mm in width. There was one larval thread for host attachment while shell valves were held together by a straight hinge (Fig. 1A and 2B). The mature glochidia shells periodically closed and rapidly closed the shell after being transferred to the artificial medium (Fig. 1B). Either the edge of the mantle border or an internal thread was observed outside the shell on day 9 before transformation occurred (Fig. 1C and 1D). During the transformation process, the early juvenile (1 day old) remains of the same size and shape until the foot appears (Fig. 1E). In fact, the foot of the early juvenile was fully formed, showing its quick movement when stimulated by distilled water and aeration. This organ showed intense activity by shortening and enlarging until the size of the shell was doubled. The early juvenile in this study (0-15 days old) kept the valves always open to facilitate food movement.

The growth of the juvenile shell was relatively rapid. Calcium was deposited on the remnant of the juvenile shell, making a newly formed line of the growing shell (Fig. 1F). The gastrointestinal tract contained organic matter derived from phytoplankton feed (Fig. 1G). The

gills of the 0–15-day old juvenile had not been fully developed to filter food. The effective use of cilia around the foot and mantle was considered essential for phytoplankton movement and, thus, food selection. The expanded foot, primitive gill, and fully developed shell (Fig. 1H) were clearly seen 15 days after transformation.

Observation by scanning electron microscope (SEM), the glochidia *H. bialatus* was seen on each valve in its internal surface with an imprint corresponding to adductor muscle attachment (Fig. 2C). The inner surface of glochidia valves contained numerous open pores (Fig. 2D), while the external surface was covered with keratin fibers and numerous pits (Fig. 2E and 2F). The rim of glochidia valves, particularly in the ventral region, showed different arrangement patterns with numerous large spines (Fig. 2G and 2H).

Within 2 days after transformation, the early juvenile stage was observed with a soft periostracum formation in the anterior region as the first step towards new shell formation under the glochidia shell (Fig. 3A, 3B, and 3C). The morphological changes, which characterized the juvenile shape from the first day, were well observed within 7 days of the juvenile life (Fig. 3B). In seven-day-old juveniles, the anterior region was seen with only a slight growth that increased continuously, possibly due to the hardening by periostracum's sclerotization, which was associated with the initial calcium precipitation. In 7 to 10 days old juveniles, parallel and slender lines were seen in the newly formed shells predominantly in the anterior region (Fig. 3C and 3D). The external surface of juvenile valves was covered with numerous cuticle buds instead of the pits seen in glochidia (Fig. 3E and 3F). The rim of the shells was lined with rows of tiny and large spines (Fig. 3G). The internal surface of the shell was covered with numerous pores (Fig. 3G and 3H). Numerous spines with keratin fibers were also observed at the terminal surface of the shells (Fig. 3I and 3J).

4. DISCUSSION

Although, the study of fish specificity for the infestation of glochidia *H. bialatus* had not been reported, the plasma from common carp was selected as the food additive to culture glochidia *H. bialatus* not only because it is the host fish of other glochidia *H.* (*L.*) *myersiana* as reported by Arayawatanavij (1992) but also because it has been used as a successful feed for other species of mussel, i.e., *H.* (*L.*) *myersiana, Chamberlainia hainesiana, Scabies scobinata, S. crispata, S. phaselus, Pilsbryoconcha exilis exilis, P. exilis compressa* and *Ensidens ingallsianus ingallsianus.* (Uthaiwan et al., 2001; Uthaiwan et al., 2002; Kovitvadhi et al., 2003; Polwong et al., 2003; Sangsawang et al., 2019). Having achieved transformation for culturing *H. bialatus* in an artificial medium, the study is further aimed at maintaining juveniles until they reach the mature stage so that mass production for conservation and propagation would be possible. Like all other species of freshwater mussels, *H. bialatus* is at risk of becoming an endangered species. Nevertheless, the species possesses an advantageous property of fast

reproducibility and can spawn all year round (Jindamonkol et al., 2003), while other species such as *H*. (*L*.) *myersiana* and *Chamberlainia hainesiana* have only a limited short period of spawning (Nagachinta & Meejui, 1998). Moreover, the species is widely distributed in large areas of Thailand, such as the Mekong River, the Mun River, the Chi River, the Choen River, the Songkram River, the canal in the Garden Palace of Bang Pa-In; Bung Boraphet; Ban La Po, Pisanulok Province and the Pong River, Kon Khaen Province (Brandt, 1974; Kovitvadhi & Kovitvadhi, 2002). Therefore, increasing the mussel population from artificial culturing of glochidia and juveniles to replenish the number of *H. bialatus* in nature is quite promising.

One important point from this study is that the combination of artificial medium (M199) with common carp plasma provides good transformation and survival. Moreover, this culture method is straightforward, noninvasive, and practical for *in vitro* culture of glochidia using an inexpensive incubator and available commercialized medium. It is also reasonable to point out that one of the most critical factors for a successful culture is the use of plasma from fishes cohabiting with the same local mussel species.

Selection of phytoplankton as feed for juvenile was based on several criteria, i.e., the size and morphology of phytoplankton, digestibility, as well as the co-ordination of cilia and mantle to ingest each type of phytoplankton. Observation of feeding behavior of juvenile under light microscope indicated that 4 species of phytoplankton (*Chlorella* sp.2*, Kirchneriella incurvata, Navicula* sp.*,* and an unknown) were chosen as preferred feeding for juvenile as considered from their size, shape and fast movement of cilia to transport them into the gastrointestinal tract. A combination of 4 species of phytoplankton (*Chlorella* sp.2*, Kirchneriella incurvata, Navicula* sp.*,* and an unknown) at the ratio of 1:1:1:1 (v/v) was fed to juvenile *H. bialatus* during the first 15 days after transformation gave a good development of juvenile mussel. Moreover, these 4 species of phytoplankton are commonly found in the natural habitat of freshwater mussels as well as in the gastrointestinal tract of adult freshwater mussel *H. myersiana.* The growth and proliferation of these phytoplankton in the laboratory have successfully been established and the species could be mass-produced for artificial culture (Kovitvadhi et al., 2003). They are convenient to prepare and could be used effectively as mussel feeding formula for juvenile at different stages of development.

Morphological development of glochidia and juvenile *H. bialatus* from *in vitro* culture observed under light, and scanning electron microscope exhibited normal morphological development and could undergo normal transformation in 15 days. The glochidia shell of *H. bialatus* has hookless, semi-oval valves. The morphology of the shell of glochidia was close to *H.* (*L*.) *myersiana*y. The duration of glochidia development until the juvenile stage of *H.* (*L*.) *myersiana* was 9–10 days. The glochidia of the

two species differed in the timing of transformation. The primitive gills, foot, gastrointestinal tract, growth lines on the shell, and rapid movements are signs of complete metamorphosis (Kovitvadhi et al., 2001). The culture of glochidia in an artificial medium could observe morphological changes during transformation, which otherwise would not be possible since juveniles in nature were very difficult to obtain.

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