Invertebrate Reproduction and Development, 50:4 (2007) 207–218
Balaban, Philadelphia/Rehovot
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Morphological development of the juvenile through to the adult in the freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana*, under artificial culture

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Received 16 July 2007; Accepted 25 September 2007

Summary

The morphological development of the freshwater mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856), was observed using light and scanning electron microscopes, from the newly transformed (0 days old) juvenile to the onset of the adult stage (360 days old). As in the glochidium, the early juvenile has a semi-oval and equivalve shell with an equilateral valve. After day 1 the shell develops a larger anterior than posterior region until day 40, after which the posterior region grows larger than the anterior region. The form of the juvenile at 260-day-old resembles that of a fully grown adult. The shell microstructure of 0–20-day-old juveniles shows two differentiated layers, the periostracum and the prismatic layer. By day 30 the prismatic layer lies under a clear columnar structure that has formed a third layer, the nacreous layer. The mantle develops incurrent and excurrent siphons when juveniles are 60 days old. The development of juvenile gills initiates from a pair of gill bars at 0 days old, and formation of the inner demibranch starts from 10 days old and the outer demibranch from 90 days old. From this stage, numerous cilia form the latero-frontal cirri of the inner demibranchs. Additionally, longitudinal and transversal interfilamentous junctions of the inner and outer demibranchs begin to develop when juveniles are 200 and 240 days old and are complete at 230 and 260 days of age, respectively. Interlamellar septa join the inner surface of descending and ascending gill filaments to form water chambers when juveniles are 250 and 280 days old, respectively, and the development of inner and outer demibranchs is complete.

Key words: Morphological structure, organogenesis, ultrastructure, bivalve, *in vitro*

Introduction

Hyriopsis (*Limnoscapha*) *myersiana* (Lea, 1856) (Order Unionoida, Family Amblemidae) is an endemic freshwater mussel in Thailand (Brandt, 1974). It is a pearl mussel (Nagachinta et al., 1986; Panha, 1990). The life cycle of freshwater mussel species, including *H.* (*L.*) *myersiana*, is atypical among bivalves since it includes a brief obligatory ecto-parasitic larval stage (glochi-

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dium) which lives on fish or amphibians prior to transformation into the free-living juvenile and subsequent development to the free-living adult phase (Seshaiya, 1941; D'Eliscu, 1972; Watters and O'Dee, 1998; Kovitvadhi et al., 2001b). The natural populations of *H.* (*L.*) *myersiana* and some other mussel species in the world have been drastically reduced to the extent that *H.* (*L.*) *myersiana* is now nearly extinct. Attempts have been made to restore their number through artificial culture (Isom and Hudson, 1982, 1984; Hudson and Isom, 1984; Keller and Zam, 1990; Kovitvadhi et al., 2001a; 2001b; 2006). Kovitvadhi et al. (2006) succeeded in culturing glochidia of *H.* (*L.*) *myersiana* to adulthood, but the percentage survival of newly-transformed (0-day-old) juveniles to 120-day-old juveniles was low. The morphological and organogenic changes during these most crucial and vulnerable stages have never been thoroughly studied.

The aim of this study is to observe the morphology and also to identify the sequence of organogenesis from juvenile to adult in the freshwater mussel, *H.* (*L.*) *myersiana*. Information about these growth stages could contribute to an understanding of organogenesis and hence assist in achieving high growth and survival rates during culture as well as increasing product potentials.

Materials and Methods

Fifty male and fifty female adult freshwater mussels, *H.* (*L.*) *myersiana*, were collected from the Mae Klong River, Kanchanaburi Province, Thailand. These individuals had an average weight of 120.95±50.7 g, length of 13.73±2.05 cm, height of 6.09±2.02 cm and width of 3.8±0.5 cm. They were introduced into an earthen pond for the production of mature glochidia. Mature glochidia were sucked from gravid mussels and transferred to culture in artificial medium according to Kovitvadhi et al. (2006) until they were 0-day-old juveniles. Newly transformed (0-day-old) juveniles to 120-day-old juveniles were cultured in the laboratory and 120–360-day-old juveniles in the earthen pond, under the conditions described by Kovitvadhi et al. (2006).

The mussels were collected in sequential developmental stages between 0 and 360 days old, distributed as follows: 1, 3, 5, 10 days old, and then at intervals of 10 days until they were 360 days old. The numbers of mussels for morphological studies were selected at each stage as follows: about 200, 100, 50, 30, 20 and 10 juveniles per sample at 1–10, 20–40, 50–70, 80–140, 150–240 and 250–360 days old, respectively.

Morphological development, namely shell form, gill, foot, mantle, and incurrent and excurrent siphons, was observed by light microscope and SEM, and shell microstructure was studied by SEM. Samples for light microscope and SEM observations were prepared in fixative solution containing 10% neutral buffered formalin for 24 h and stored in 5% neutral buffered formalin for further process. The samples for SEM were thoroughly washed under running water for 30 min and then dehydrated in a graded series of ethanols and dried to critical point. Before fixation, they were anesthetized in 2% chloral hydrate to observe the internal regions. For ultrastructural study of shell layers and microstructure of the thickened shell, the fixed samples were washed with tap water and cut with a thin diamond saw; for ultrastructural study of the thin shell, portions were simply broken off the fresh shell; for study of the thickened shell, the fixed samples were washed with tap water and cut with a thin diamond saw. All samples were mounted on SEM specimen stubs with conductive silver paint and coated with gold and observed with a Jeol Model JSM-5410LV scanning electron microscope operated at 25 KV.

Results

Development of shell form

The early juvenile *H.* (*L.*) *myersiana* at 0 days old after transformation has semi-oval, equivalve shells with an equilateral valve, presenting the same size and shape as the glochidium and one pair of gill bars (Figs. 1, 1A). The valves are joined by a straight hinge (Figs. 1, 2). After the 1-day juvenile stage, rapid growth of the new shell is marked by the addition of co-marginal growth lines. The anterior region appears before the posterior region and grows more rapidly (Figs. 2, 3, 4, 9, 13, 14) until the juvenile is 40 days old, when the posterior region begins to increase more than the anterior (Figs. 16, 18, 20, 24, 28, 31, 32, 35). The shell of 0–20-day-old juveniles is very convex, particularly in the dorsal region, with a curve in the new growth lines (Figs. 3, 4, 9, 10). Subsequently, the convexity of the two valves decreases at successive ages until, from 40 days old, the shell even becomes distinctly laterally compressed (Figs. 18, 20, 24, 28, 31, 32, 35). The first anterior and posterior wings appear in 50-day-old juveniles (Fig. 18), with the posterior wing becoming dominant relative to the anterior from the 130-day-old stage (Figs. 28, 31, 32, 35). From this 130-day stage, the posterior region has two posterior ridges with a posterior slope between them (Figs. 28, 31, 32), and this slope is dominant from 150 days old (Fig. 31). The complete adult morphology is apparent from 160 days old (Figs. 32, 35).

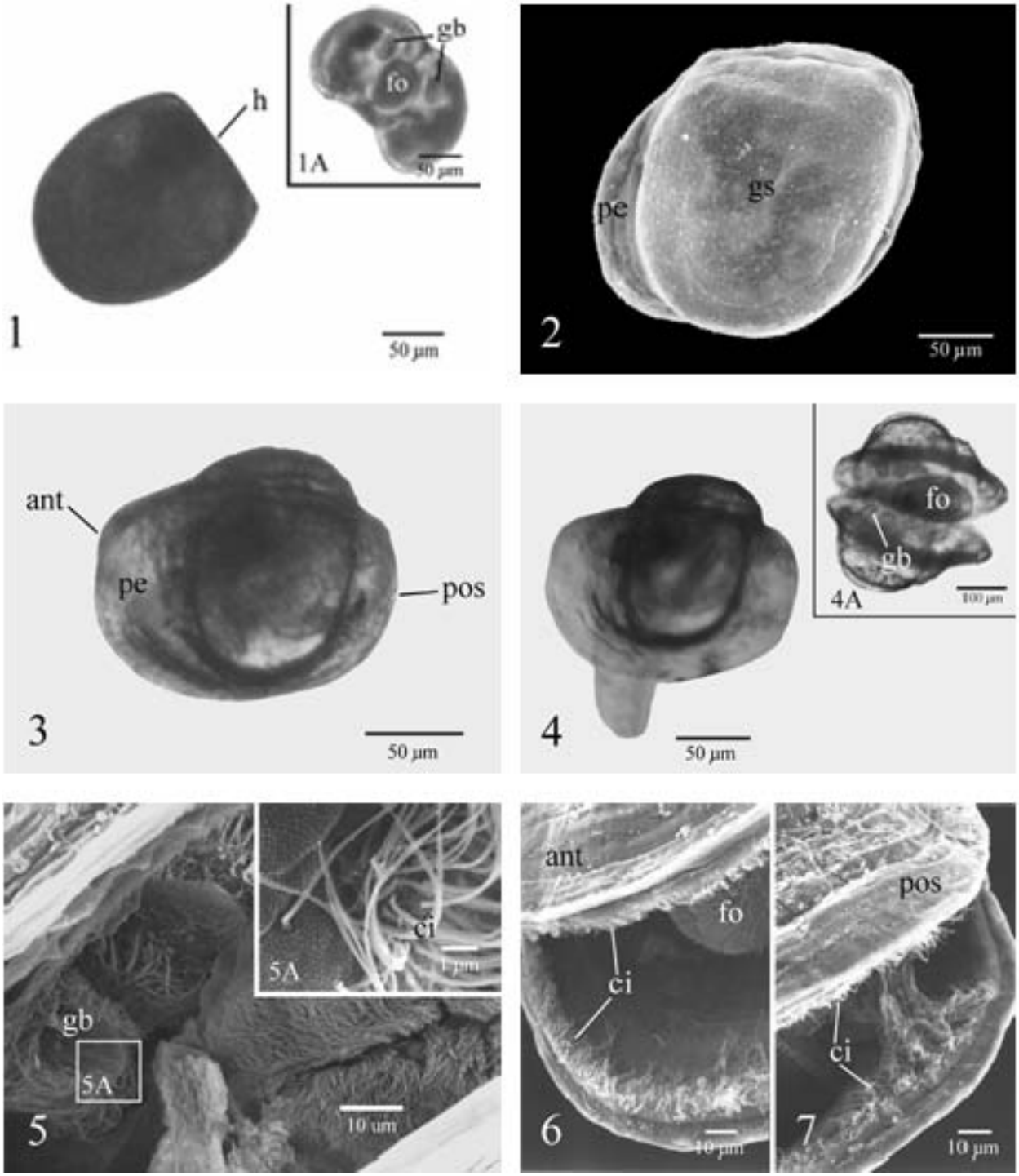

Plate 1. Fig. 1. Early juvenile (after transformation, 0-day-old) shell; note hinge (h). Fig. 1A. First gill bar (gb), on both sides close to the foot (fo). Fig. 2. SEM micrograph of 1-day-old juvenile, appearance in anterior region of new soft periostracum (pe); note glochidia shell (gs). Fig. 3. Juvenile 5 days old, anterior (ant) region appears before and grows more than the posterior (pos). Figs. 4–7. Juvenile 10 days old. Fig. 4A. Light microscopy of gill bar 10 days old. Figs. 5, 5A. The outer surface and rim of the gill bar with cilia (ci). Fig. 6. Cilia in anterior region of mantle. Fig. 7. Cilia in posterior region of mantle.

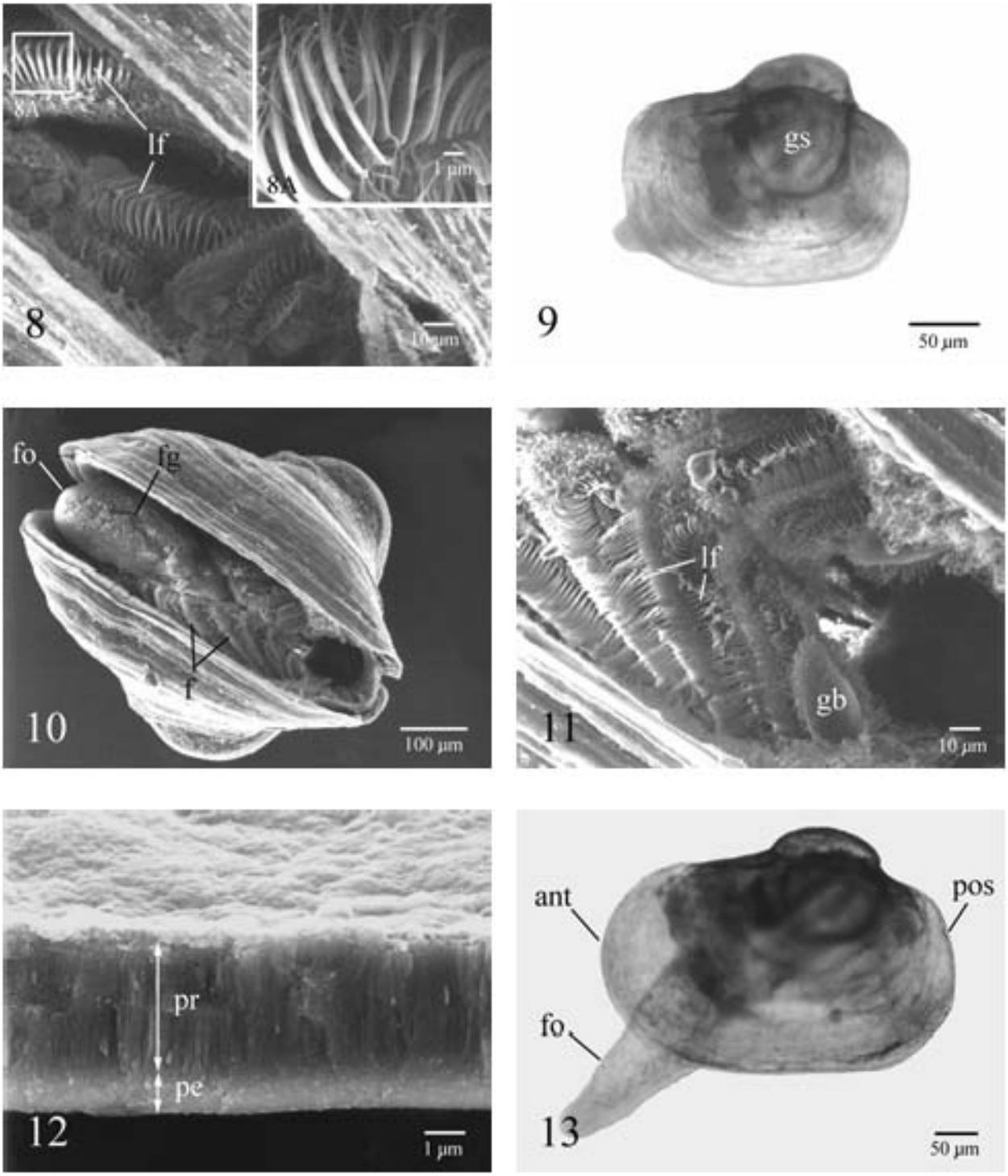

Plate 2. Fig. 8. Latero-frontal cirri (lf) on the gill filaments in 10-day-old juvenile. Fig. 8A. Higher magnification SEM micrograph of the latero-frontal cirri. Figs. 9–12. Development of gill and microstructure of shell of 20-day-old juvenile. Fig. 9. External morphology of shell; note glochidia shell (gs). Fig. 10. Position of gill filaments (f) of juvenile; note foot groove (fg); foot (fo). Fig. 11. Latero-frontal cirri at the gill filament; note gill bar (gb). Fig. 12. Microstructure of shell; note periostracum layer (pe); prismatic layer (pr). Fig. 13. External morphology of 30-day-old juvenile; note anterior (ant); posterior (pos).

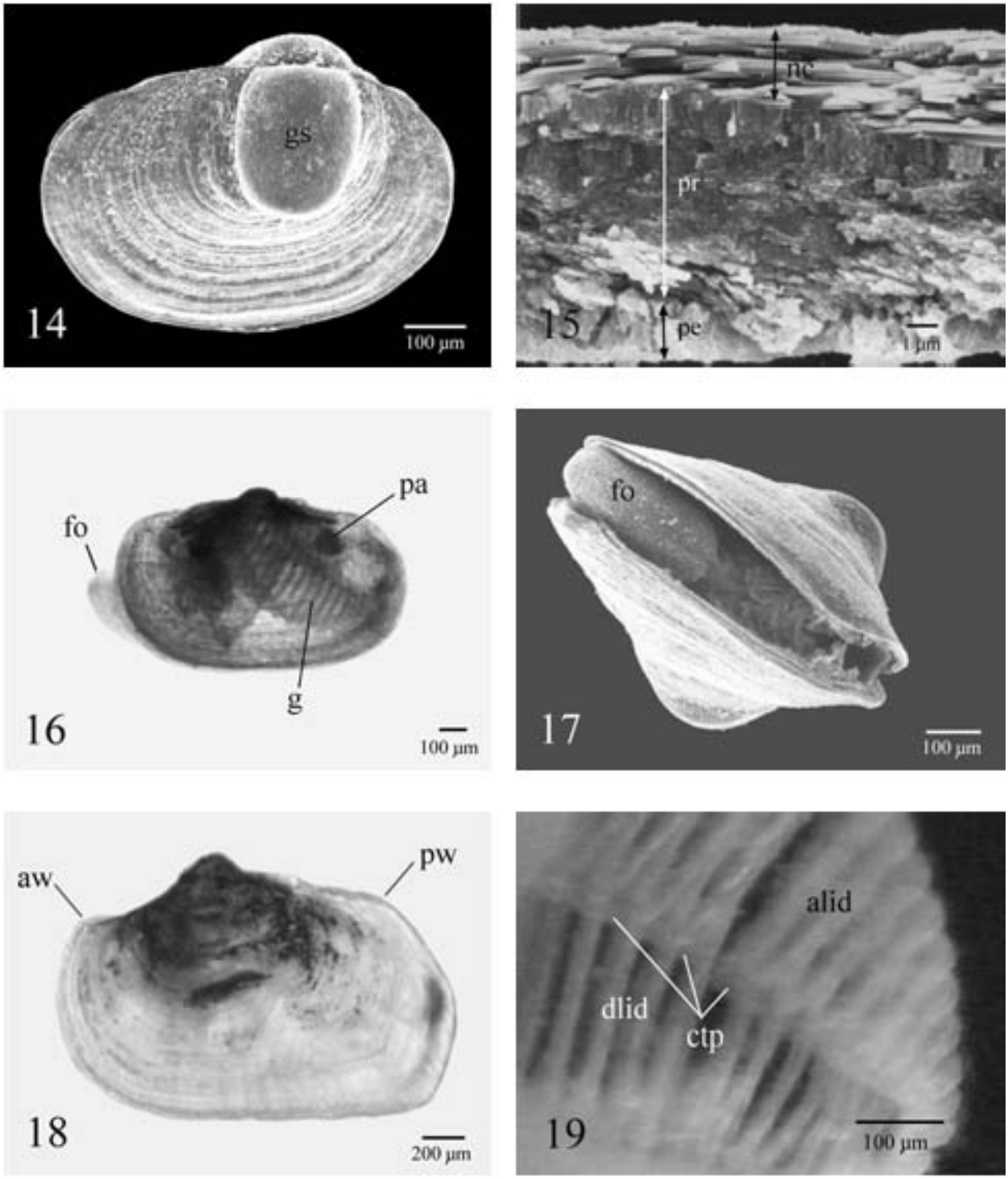

Plate 3. Figs. 14, 15. Shell form and microstructure of 30-day-old juvenile. Fig. 14. SEM micrograph of shell surface; note glochidia shell (gs). Fig. 15. Microstructure of shell; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). Figs. 16, 17. Light microscopy and SEM of development of shell, 40-day-old juvenile. Fig. 16. External morphology; note foot (fo); gill (g); posterior adductor muscle (pa). Fig. 17. Ventral side of juvenile. Figs. 18, 19. Light microscopy of shell form and gill development, 50-day-old juvenile. Fig. 18. External morphology of shell; note anterior wing (aw); posterior wing (pw). Fig. 19. Tip of ascending lamella (alid) as a connecting plate by connective tissue (ctp); note descending lamella of inner demibranch (dlid).

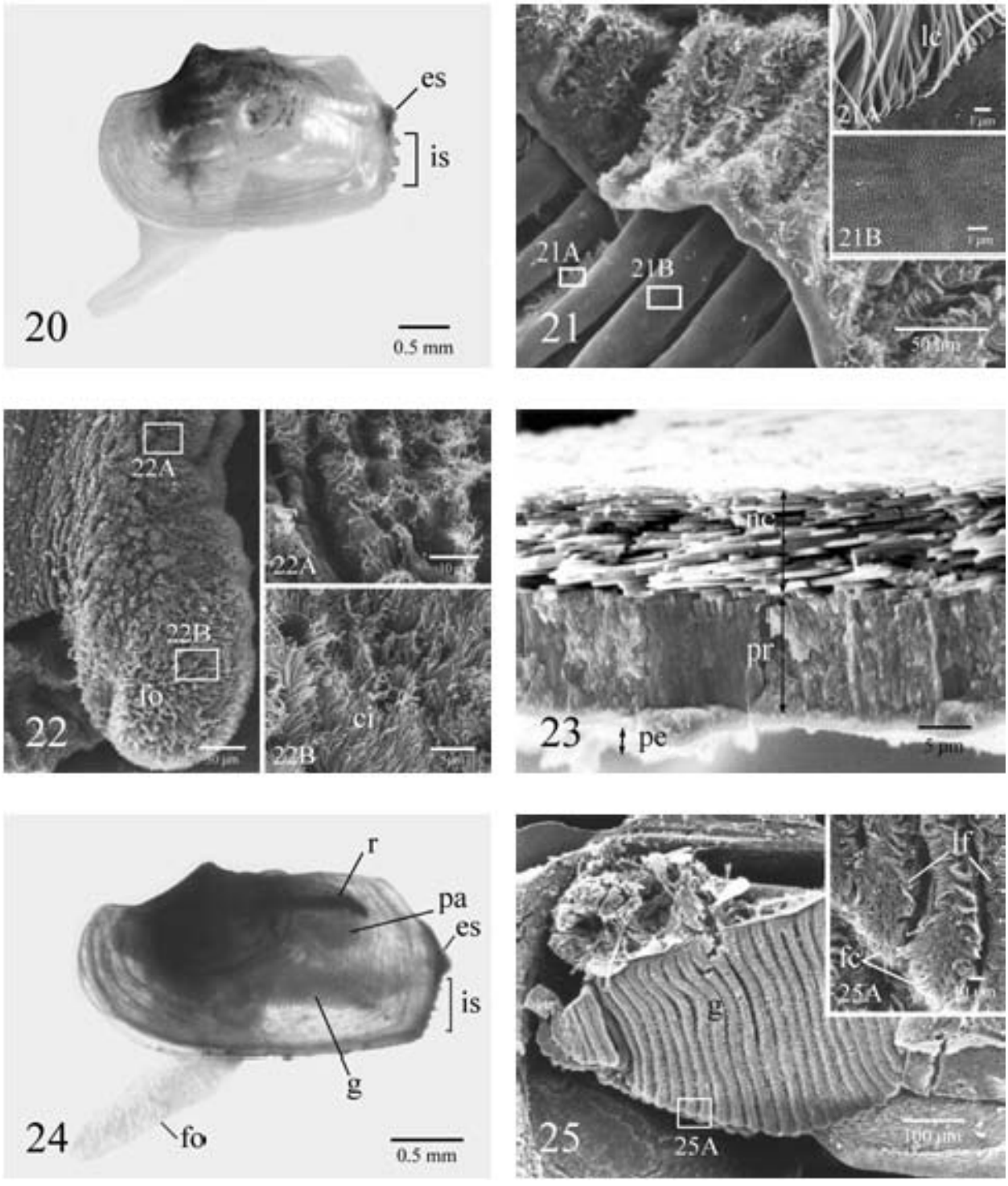

Plate 4. Figs. 20–23. Development of shell form, siphon, gill, foot and shell microstructure, 60 days-old juvenile. Fig. 20. External morphology of shell; note excurrent siphon (es); incurrent siphon (is). Fig. 21. SEM, inside and outside of inner demibranch surface. Fig. 21A. Rim of inside surface; note lateral cilia (lc). Fig. 21B. Inside surface of inner demibranch. Fig. 22. SEM, cilia on the foot (fo) surface. Fig. 22A. Cilia at the base region of foot. Fig. 22B. Cilia (ci) at the terminal of foot surface. Fig. 23. Shell microstructure of juvenile; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). Fig. 24. External morphology of 100-day-old juvenile; note gill (g); posterior adductor muscle (pa); rectum (r). Fig. 25. SEM, cilia on the 100-day-old gill. Fig. 25A. Latero-frontal cirri (lf) and frontal cilia (fc) on gill filaments.

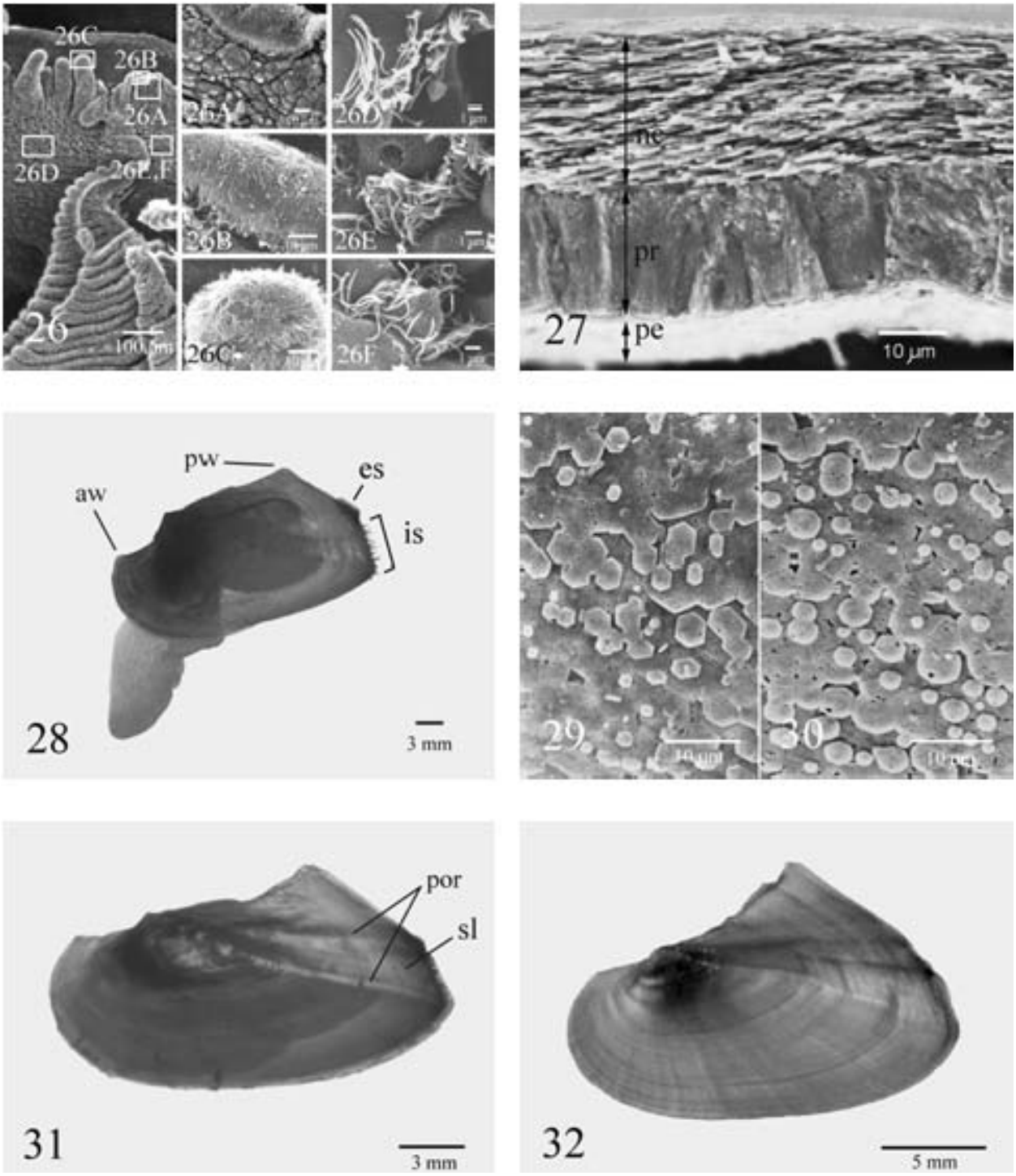

Plate 5. Figs. 26, 27. SEM, incurrent and excurrent siphon, and shell microstructure, 100 days old. Figs. 26A, 26B, 26E, 26F. Surface of excurrent siphon area. Fig. 26C. Cilia on the surface of papilla. Fig. 26D. Surface of incurrent siphon area. Fig. 27. Shell microstructure; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). Fig. 28. Shell morphology of 130-day-old juvenile; note anterior wing (aw); excurrent siphon (es); incurrent siphon (is); posterior wing (pw). Fig. 29. SEM, nacreous layer of the shell with polygonal. Fig. 30. The nacreous layer of the shell with round tablets. Figs. 31,32. Juveniles 150 and 160 days old; note posterior ridge (por); posterior slope (sl).

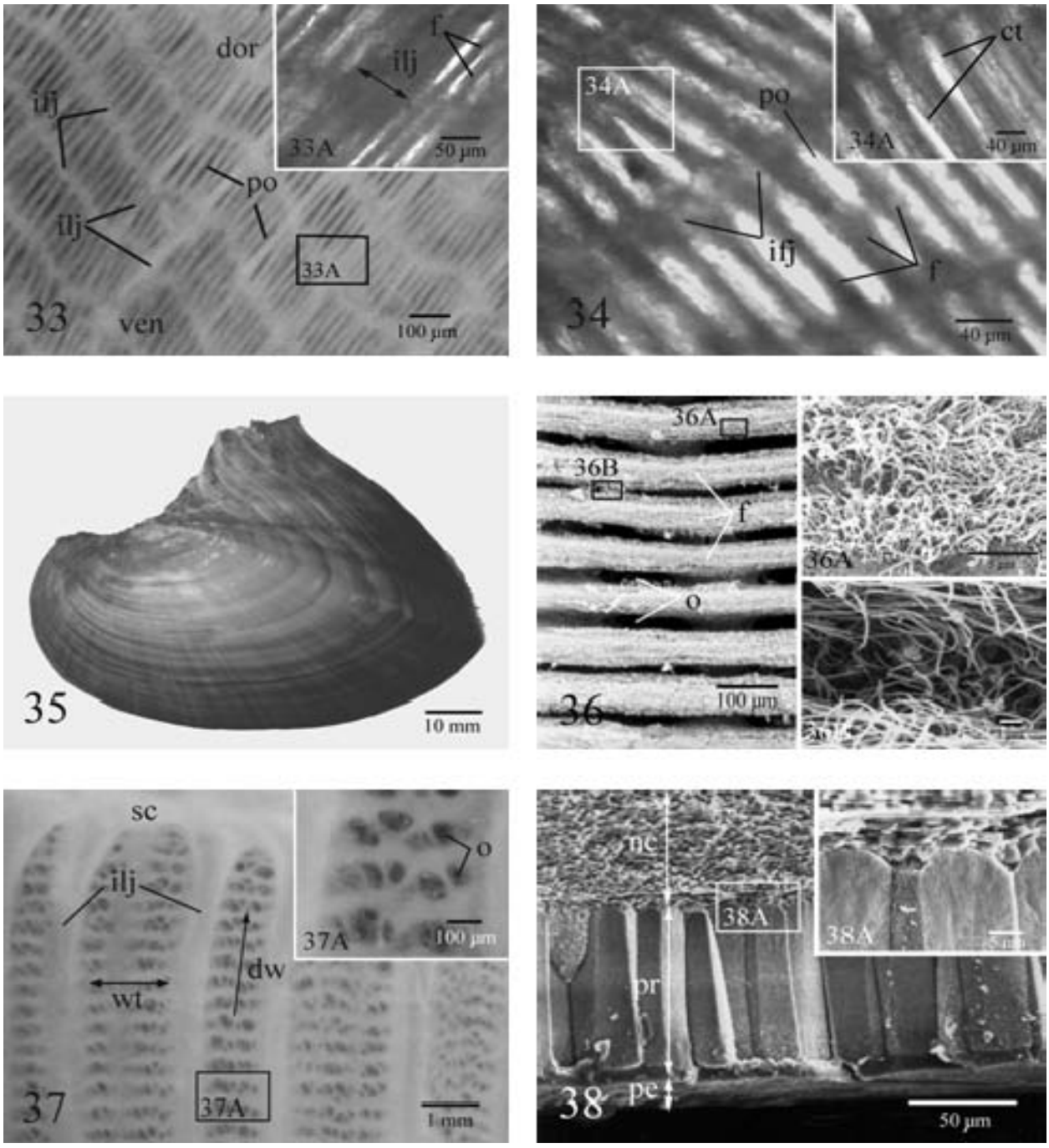

Plate 6. Figs. 33,34. Development of gill, 200-day-old juvenile. Fig. 33. Inside surface of inner demibracnhs; note dorsal (dor); transversal interfilamentous junction (ifj); longitudinal interfilamentous junctions (ilj); ventral (ven); gill pore (po). Fig. 33A. 2–3 gill filaments (f) fused by connective tissue throughout longitudinal demibranch. Fig. 34. Inner surface of the gill filament connected by connective tissue at greater intervals occurred throughout transverse demibranch. Fig. 34A. Gill pore surrounded with connective tissue (ct). Figs. 35–38. Shell form, development of water tube and shell microstructure, 360-day-old juvenile. Fig. 35. External shell morphology. Fig. 36. Outer surface of gill filaments; note ostia (o). Fig. 36A. Cilia on gill filaments. Fig. 36B. Cilia between the gill filaments. Fig. 37. Light microscopy of inner demibranch, each divided by longitudinal interfilamentous junctions; note direction of water current (dw); suprabranchial chamber (sc); water tube (wt). Fig. 37A. Inner site of the ostia. Fig. 38. Shell microstructure, the prismatic structure well-defined thicker columnar structure; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). Fig. 38A. Higher magnification between the nacreous layer and prismatic layer.

Shell microstructure

At each stage of development of *H.* (*L.*) *myersiana*, there are differences in shell microstructure. The 0–20-day-old juveniles have two differentiated layers: a thin outer organic layer, the periostracum, about 1 µm in thickness, and an inner prismatic layer about 4 µm in thickness adhering to it (Fig. 12). The prismatic layer is a single layer of elongated calcium carbonate crystals oriented at 90° to the periostracum. From 20 days old and through the juvenile and adult stages, this prismatic layer always forms a thinner structure perpendicular to the periostracum sheet (Figs. 27, 38). The prismatic structure, about 100 µm in thickness, is clearly observed under the well-defined thicker columnar structure when the mussels are 360 days old (Figs. 38, 38A). A third layer in the shell, the nacreous layer, develops from the 30-day-old juvenile onwards (Fig. 15) and consists of successive thin lamellae of small $CaCO_3$ crystals parallel to the plane of the shell (Figs. 15, 23, 27, 38) and, like the periostracum, perpendicular to the prismatic layer. The thickness of the nacreous layer is proportional to the number of lamelle, and this depends on age. The upper lamella of the nacreous layer is composed of polygonal and round tablets of $CaCO_3$ crystals which later merge into a nacre sheet. This can be seen clearly when the lamella crystals are just forming (Figs. 29, 30).

Development of incurrent and excurrent siphon

In the developing *H.* (*L.*) *myersiana,* the two lobes of the mantle extend down on either side of the body. These thin membranes are attached to the two valves of the shell. The mantle bordering the new shell is covered with numerous cilia (Figs. 6, 7). The mantle lobes of 0–50-day-old juveniles are joined dorsally and are free ventrally (Figs. 10, 11, 17). The siphons appear after 60 days. The posterior mantle margins fuse to form the incurrent and excurrent siphons and are seen clearly from 100 days old (Figs. 20, 24, 28). The ventral incurrent siphon is surrounded by sensory papillae covered by microvillae, and the internal walls of the incurrent chamber are covered by cilia. In contrast, the dorsal excurrent siphon is only bordered by numerous cilia and its surface has a smooth aspect (Figs. 26, 26A–26F).

Gill development

The newly transformed juvenile of *H.* (*L.*) *myersiana* has one gill bar on each side, close to the mantle in the region of the posterior adductor muscle. These will develop into the inner demibranch (Fig. 1A). The edges of the gill bars are covered by numerous cilia during the transformation period (Figs. 5, 5A, 11). In several 10-day-old juveniles the gill bars had already formed two branches per bar and which constitute gill filaments (Figs. 4A, 5). From this stage, numerous cilia on the filaments project along the lateral and frontal edges and which will form the cirri (Figs. 8, 8A, 11). Each filament bears two rows of fused cilia, joined at this base along the lateral-frontal edges and which form the latero-frontal cirri (Figs. 8, 8A, 11, 21, 21A, 25, 25A). Each cirrus is already well-developed at 20 days-old (Figs. 8, 8A, 11). From day 30, each gill filament extended ventrally and recurved inwards towards its origin (descending lamellae) until 50 days old, and the connective tissue developed along the tip of the filaments as a connecting plate that will attach the ascending lamellae of the gill filaments connecting to the visceral mass wall (Fig. 19). When the juveniles have reached 360 days old, the basal portions of the lateral-frontal cilia are seen not to be fused (Figs. 36, 36A, 36B).

At 60 days old, the internal surface of the inner demibranch has no cilia but numerous buds (Fig. 21B). The development of cilia and cirri on the outer demibranch is similar to that on the inner demibranch, although the timing is different (see below). The foot is entirely covered with cilia (Fig. 22), these being long in the terminal region (Fig. 22B) and shorter in the basal region (Fig. 22A).

At 200 days old, in 2–3 gill filaments, the inner surface of ascending and descending lamellae of the gill filaments of the inner demibranchs are fused by connective tissue. These connections, the longitudinal interfilamentous junctions, run from the dorsal to the ventral region after 230 days to form a thin network. Alternating with this thin tissue, connections at larger intervals of about 4–5 gill filaments occur throughout the inner demibranch (Figs. 33, 33A). These form a larger net of transversal interfilamentous junctions formed by the connective tissue. When juveniles have reached 200–230 days old, they join the gill filaments at regular intervals. In a similar way, at 240–260 days old, longitudinal and transverse interfilamentous junctions connect the gill filaments of the outer demibranch. The lateral alignment of transversal connections in inner and outer demibranch filaments forms the lateral pores (Figs. 33A, 34, 34A). Later (360-day-old, i.e., adult), the pores are narrowed by development of connective tissue around the aperture, thereby forming the ostia (Figs. 37, 37A). Within the thin network, an interlamellar septum formed by fused connective tissue was observed in the inner demibranch at 230–250 days old and in the outer demibranch at 260–280 days old. These interlamellar septa form water channels in the ridge of each demibranch (Fig. 37) which open into the suprabranchial chamber.

Discussion

The early life history, mainly of the juvenile stage, has been studied in several bivalve groups such as *Unio*, *Potomida*, *Anodonta* and *Margaritifera* (Harms, 1907; 1909; Herbers, 1914; Giusti, 1973; Castilho et al., 1989; Araújo and Ramos, 1998). Culture *in vitro* was difficult to accomplish until the methods of Keller and Zam (1990) and Kovitvadhi et al. (2001b, 2006) substantially improved glochidial survival and development. Use of these methods in the present study has allowed successful development to the juvenile and adult stages and thereby permitted detailed morphological and ultrastructural descriptions. This work contributes new knowledge regarding the early ontogeny of *H.* (*L.*) *myersiana*.

Development of shell form and microstructure

The marked convexity of the mussel form from 0 to 20 days old may be due to the tendency of the initial shell shape to form a curve with the new increments co-marginal with the shell border. After day 20, the shell shape and also some organs gradually became laterally compressed. This is particularly true of the foot, which is initially like a club. Initially, the anterior region grows more rapidly than the posterior region. This offers an advantage to the juvenile, since the large foot is the main organ in the anterior region and needs to be protected against predators and physical agents so that it can fulfill the important function of seeking food. As in other bivalve shells, from the 30-day-old juvenile through to the adult, the shell microstructure of *H.* (*L.*) *myersiana* consists of three layers, namely, periostracum, prismatic layer and nacreous layer (Hedegaard and Wenk, 1998). The periostracum layer of *H.* (*L.*) *myersiana* is thin and this may offer little resistance to aggressive agents and organisms in the environment (Bottjer and Carter, 1980; Kovitvadhi et al., 2001a). Our observations have shown that, in the juvenile shell, the well-defined prismatic layer of calcium carbonate deposited in a proteinaceous matrix presents a columnar structure in a young adult of 360 days-old and is thinner than the nacreous layer. The nacreous layer has polygonal and round tablets of calcium carbonate crystals, joined and arranged under thin lamellae as flat sheets. In their morphology, these prismatic and nacreous layers resemble those of other species, e.g. *Anodonta cygnea* (Machado et al., 1991), in which aragonite crystals form the prismatic and nacreous layers under columnar and rhombohedral structures, respectively.

Incurrent and excurrent siphon development

An important source of nutrients for juveniles and adults of species of freshwater mussel is phytoplankton (Gale and Lowe, 1971; Hudson and Isom, 1984; Paterson, 1986; Gatenby et al., 1997; Kovitvadhi et al., 2000, 2001b). Kovitvadhi et al. (2001a) found that the gills of the early juvenile *H.* (*L.*) *myersiana* (<13 days old) are not fully developed for filtering food. Furthermore, Kovitvadhi et al. (2006) observed that early juveniles (0–40 days) use cilia around the foot, the mantle and gill in order to move phytoplankton into the mantle cavity and ultimately to the mouth. However, possibly the ciliary mechanisms are not sufficient for nutrient uptake, so survival rate is low during this stage. This feeding behavior of early juveniles differs from suspension feeding at the more mature stage, in which an incurrent siphon together with cilia of the mantle, gill and labial palp are involved in selective intake of food. In the present study, formation of organs related with water movement start at around 40 days, with the incurrent siphon completely developed in 80-day-old juveniles. This is the main organ for intake of food. The many cilia on the papillae around the opening of the incurrent siphon increase the efficiency of water movement into the mantle cavity. A ciliated excurrent siphon was present by day 80 and water passing through the gills is expelled through it. The degree to which the siphons protrude from the shell depends on the habitat, according Gale (1976), Yonge (1982) and Morton et al. (1998). *H.* (*L.*) *myersiana* has a short siphon that protrudes only little, which relates to the animal being buried in the thin layer of sediment.

Gill development

The development of the demibranch from the early juvenile phase (after transformation) to the adult stage in the freshwater mussel has been little studied. Kaestner (1967) reported that *Anodonta* sp. develops eight papillae between the mantle and the body wall at the age of six weeks (shell length, 0.7 mm) which grow to form simple filaments and become descending limbs. When 18 filaments have formed, they recurve and grow dorsally, producing ascending limbs, the tips of which fuse to the foot–visceral mass to yield an inner demibranch. The outer demibranch develops later, when the shell length reaches 3–5.7 mm. Although this was similarly observed in the present study of *H.* (*L.*) *myersiana*, the timing differed, with juveniles 10, 20, 30, 40, and 50 days old, having 4, 6, 10, 16 and 18 gill filaments, respectively. Indeed, 1–2 gill bars were seen in the inner demibranch when *H.* (*L.*) *myersiana* juveniles were only 0–10 days old, and the ascending lamellae of the inner demibranch formed at 30 days. The whole development process of inner and outer gill demibranchs occurs much earlier that in *Anodonta* sp.

The suprabranchial cavity develops when the

connective tissue of distal ends of the inner and outer demibranch filaments attach to the visceral mass and mantle wall, respectively (Barnes, 1987; McMahon and Bogan, 2001). This is seen in *H.* (*L.*) *myersiana*, in which the suprabranchial cavity of inner and outer demibranchs appears when the juvenile is 200 and 240 days-old, respectively.

The formation of new connective tissues at various points within the gill and their respective extensions vary structurally and chronologically among groups of lamellibranchs with their various types of gills. Barnes (1987) reported that, in general in the mussel, three junctions in inner and outer demibranchs occur: (1) interlamellar junction between the reflected lamellae and which are present in *H.* (*L.*) *myersiana* as interlamellar septa; (2) interfilamentar junctions between adjacent filaments, identified here in *H.* (*L.*) *myersiana* as longitudinal and transverse interfilamentar junctions; and (3) connective tissues attaching the tips of ascending inner and outer demibranchs to the visceral mass and the mantle wall, and these also occur in *H.* (*L.*) *myersiana*.

In this study of *H.* (*L.*) *myersiana*, the interlamellar septa dividing the cavity in the gills are formed by fusion of 2–3 adjacent gills. This contrasts to the situation depicted in four diagrams of a demibranch by Pierce and Maugel (1987), Kays et al. (1990), Pechenik (1996) and Barrington (1979), which depict individual gill filaments in the interlamellar septa.

In conclusion, all aspects of morphology and ultrastructure observed in *H.* (*L.*) *myersiana* were similar in sequence to the ontogenic development of other species, although the timing differs. In juveniles of *H.* (*L.*) *myersiana* the organs are formed much earlier and reach the adult stage more quickly than in other freshwater bivalves studied, even other Thai species.

Acknowledgements

The authors are very grateful to Assoc. Prof. Yaowaluk Chitramvong from the Biology Department, Faculty of Science, Mahidol University, for discussions and advice. We are also grateful to Mr. Ben Parslew from the Graduate School, Kasetsart University, for his revision of our manuscript. Finally, we thank the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, for providing a pond for culturing the mussels.

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