Satit Kovitvadhi

In vitro **Culture of Freshwater Mussel Juvenile**
Hyriopsis (*Limnoscapha*) *myersiana* **(Lea, 1856)**

Hyriopsis (Limnoscapha) myersiana
J 10 , H24   0.1 mm.

Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto
2008

Acknowledgements

Contents

ABSTRACT

Culture of the freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856) juvenile was carried out in three consecutive steps: 1) Culture of glochidia larvae in artificial medium (M199) supplement with common carp plasma. The percentage survival of glochidia was 95±2.5. All surviving larvae (100%) transformed to juvenile, the duration of transformation being 8 days. 2) Rearing the early juvenile (0-120 days old) in a laboratory. The early juvenile (0-60 days old) were fed with a mixture of four selected phytoplankton species (*Chlorella* sp., *Kirchneriella incuvata*, *Navicula* sp., and *Coccomyxa* sp.). The survival rate of juvenile was 8±0.2%. For 60-120 days old juvenile, each individual phytoplankton species was used separately as juvenile diet. The highest survival rate (65±8.32%) was obtained in the group fed with *K. incuvata*. 3) The 120-360 days old juveniles were finally cultured in an earth pond with the resultant survival rate approaching 100%.

The development and survival rate of 0-120 days old juvenile were compared when reared in two different recirculating water filtration systems. The first system consisted of a glass aquarium equipped with a filter cabinet, a UV-tube, a resting cabinet and a plastic culture unit. In the second system, the filter cabinet and a UV-tube in the first system were replaced with a particulate filter cabinet, a macrophytes filter cabinet and a biological filter cabinet. The juveniles were fed a rearing diet consisting of a mixture of micro algae, *Chlorella* sp. and *K. incurvata*, at 1:1 ratio, and density 1×10^5 cells/cm^3, given twice daily. Juveniles reared in the second system gave higher growth, average growth rate per day, and rate of survival than those cultured in the first system. The survival rate of 120 days old juvenile from the first and the second systems were 12.45±2.14 and 82.74±1.47%, respectively.

Morphometric relationships between length-height and total weight-size of the freshwater pearl mussel cultured under laboratory conditions, in acrylic containers and in pocket nets with frames in an earthen pond, were studied. Length-height in each period of culture had a linear relationship. The linear regression line actually consisted of three separate lines ($P<0.01$). The relationship between total body weight-size were seen as two different lines ($P<0.01$) for culturing in acrylic containers versus the pocket nets.

Morphological development of freshwater pearl mussel juveniles was observed, using light and scanning electron microscope, from the newly-transformed (0 day old) until the onset of adult (360 days old). The early juvenile had semi-oval and equivalve shells with an equilateral valve. From 1-day old and thereafter the mussel shell at the anterior developed better than at the posterior, until the mussel reached 40-days old, when the shell at posterior began to develop better than that at the anterior. The microstructure of the mussel shell at 0-20 days old was seen to consist of two differentiated layers, the periostracum and the prismatic layer. Thereafter the morphological changes started taking place and when the mussel reached 100-days old the shell was seen arranged as nacreous layer, with the structure of the prismatic layer resembled that of a prism. The development of the gill was initiated from a pair of gill bars. At 30-days old the inner demibranch started to develop, followed by the development of the outer demibranch which was first seen when the mussel reached 90-days old. The interfilamentous junctions were detected in 200 and 240 days old mussel and the interlamella junctions in 240 and 260 days old mussel, in the inner and the outer demibranches, respectively.

RESUMO

O cultivo dos juvenis do mexilhão de água doce, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856, foi realizada em três etapas consecutivas: 1) Cultura das larvas em meio artificial (M199) suplementado com plasma de carpa comum. A percentagem de sobrevivência dos gloquídios foi de 95±2.5. Todas as larvas sobreviventes (100%) transformaram-se em juvenis sendo a duração desta transformação 8 dias. 2) Cultivo em laboratório dos juvenis (0-120 dias de idade). Os juvenis (0-60 dias) foram inicialmente alimentados com uma mistura de quatro espécies seleccionadas de fitoplâncton (*Chlorella* sp., *Kirchneriella incuvata*, *Navicula* sp. e *Coccomyxa* sp.). A percentagem de sobrevivência dos juvenis foi de 8±0.2%. Para os juvenis com 60 a 120 dias, cada espécie individual de fitoplâncton foi usada separada como dieta juvenil. A percentagem mais elevada da sobrevivência (65±8.32%) foi obtida no grupo alimentado com *K. incuvata*. 3) Finalmente, os juvenis com 120-360 dias foram cultivados numa lagoa tendo-se obtido uma percentagem de sobrevivência que aproxima os 100%.

A percentagem de desenvolvimento e sobrevivência dos juvenis com 0 a 120 dias foi comparada para dois diferentes sistemas de filtração e recirculação da água. O primeiro sistema consistiu num aquário de vidro equipado com um compartimento de filtragem, uma lâmpada UV, um reservatório e uma unidade plástica de cultura. No segundo sistema, o módulo de filtração e a lâmpada UV do primeiro sistema foram substituídos por um filtro de partículas, um filtro de macrófitas e um filtro biológico. Os juvenis foram alimentados duas vezes por dia com uma dieta que consistia numa mistura de microalgas, *Chlorella* sp. e *K. incurvata*, numa relação de 1:1 e densidade de 1×10^5 células/cm^3. Os juvenis cultivados no segundo sistema apresentaram um crescimento mais elevado, uma percentagem de crescimento médio por dia e percentagem de sobrevivência mais elevadas do que aqueles cultivadas no primeiro sistema. A percentagem de sobrevivência dos juvenis com 120 dias do primeiro e do segundo sistema foi de 12.45±2.14 e 82.74±1.47 respectivamente.

Analisaram-se as relações morfométricas entre o comprimento-altura e o peso total - tamanho do mexilhão de água doce cultivado sob condições laboratoriais numas unidades acrílicas e em redes numa lagoa. A relação comprimento-altura teve um comportamento linear ao longo de todo o período de cultivo. A linha de regressão linear consistiu realmente em três linhas separadas ($P<0.01$). A relação entre o peso total do corpo-tamanho foi vista como duas linhas diferentes ($P<0.01$) para o cultivo nos recipientes acrílicos e para o cultivo em redes.

O desenvolvimento morfológico dos juvenis do mexilhão de água doce foi observado através de microcopia óptica e electrónica de varrimento desde os recentemente transformados (0 dias) até ao início da idade adulta (360 dias). Logo após a transformação os juvenis apresentavam conchas semi-ovais, equivales e equilateral. Desde 1 dia de idade e adiante a concha do mexilhão desenvolveu-se mais no bordo anterior do que no posterior, até o mexilhão alcançar os 40 dias de idade, quando a concha se começou a desenvolver mais no bordo posterior do que no anterior. A microestrutura da concha do mexilhão com 0-20 dias consistia em duas camadas diferenciadas, no periostraco e na camada prismática. Depois, mudanças morfológicas começaram a ocorrer e quando o mexilhão alcançou os 100 dias de idade, a concha consistia numa camada nacarada, com a estrutura da camada prismática semelhante a um prisma. O desenvolvimento das brânquias foi iniciado a partir de um par de barras branquiais. Aos 30 dias a hemibrânquia interna começou a formar-se, seguindo-se o desenvolvimento da hemibrânquia externa que era visível aos 90 dias. As junções interfilamentosas foram detectadas no mexilhão aos 200 e 240 dias e as junções interlamelares aos 240 e 260 dias, nas hemibrânquias internas e externas, respectivamente.

RÉSUMÉ

L'élevage des juvéniles de la moule perlière, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856), a suivi trois étapes successives: 1) élevage des larves en milieu artificiel (M199) supplémenté au plasma de carpe commune. Le pourcentage de survie des glochidies était de 95±2.5. Toutes les larves survenantes (100%) s'ont transformées en juvéniles, dont la durée de transformation est de 8 jours. 2) élevage en laboratoire des juvéniles (0-120 jours d'âge). Les juvéniles (0-60 jours) furent nourris d'abord avec un mélange quatre espèces sélectionnées de phytoplancton (*Chlorella* sp., *Kirchneriella incuvata*, *Navicula* sp., and *Coccomyxa* sp.). Le pourcentage de survie des juvéniles était de 8±0.2%. Pour les juvéniles de 60 à 120 jours, chaque espèce individuelle de phytoplancton était utilisée séparément comme diète juvénile. Le pourcentage de survie plus élevé (65±8.32%) fut obtenue dans le groupe nourri avec *K. incuvata*. 3) Finalement, les juvéniles de 120-360 jours étaient élevés dans un étang avec un pourcentage de survie proche des 100%.

Le pourcentage de développement et survie des juvéniles de 0 à 120 jours fut comparé en utilisant deux systèmes de filtration et de re-circulation d'eau. Le premier système comprenait un aquarium en verre équipé avec un compartiment de filtrage, une lampe UV, un réservoir et une unité plastique d'élevage. Dans le deuxième système, le module de filtration et la lampe de UV du premier système furent remplacés par un filtre de particules, un filtre de macrophytes et un filtre biologique. Les juvéniles furent nourris deux fois par jour avec une diète comprenant un mélange de micro algues *Chlorella* sp. Et *K. incurvata*, dans un rapport 1:1 et une densité de 1×10^5 cellules/cm^3. Les juvéniles élevés dans le deuxième système présentait une croissance plus élevée, un pourcentage de croissance moyenne par jour et un pourcentage de survie plus élevés que ceux élevés dans le premier système. Le pourcentage de survie des juvéniles de 120 jours du premier et du second systèmes furent de 12.45±2.14 et 82.74±1.47%, respectivement.

Les rapports morphométriques entre longueur-hauteur et pois total-taille de la moule d'eau-douce élevé dans dès conditions de laboratoire dans dès unités acryliques et dans des filets dans un étang. Le rapport longueur-hauteur s'est maintenu linéaire tout au long de la période d'élevage. Le trace de régression linéaire était composée de trois lignes séparées (*P*<0.01). Le rapport entre pois total-taille étai observe comme deux lignes séparées (*P*<0.01) pour l'élevage dans des réservoirs acryliques et dans les filets.

Le développement morphologique des juvéniles de la moule d'eau-douce fut observe par microscopie optique et électronique de balayage dès les récemment transformés (0 jours) jusqu'a l'âge adulte. Depuis leur transformation les juvéniles présentait des coquilles semi-ovales, des equivalves et valves equilaterales. Dans les juvéniles âgés d'un jour et plus la coquille s'est développé plus sur le bord antérieur que sur le postérieur, jusqu l'âge de 40 jours, moment où la coquille devient plus développée sur le bord postérieur que sur le bord antérieur. La microstructure de la coquille de la moule de 0-20 jours présentait deux couches différenciées, dans le périostraque et dans la couche prismatique. Depuis, des variations morphologiques était observées et au moment ou la moule atteint les 100 jours d'âge la coquille présentait une couche nacrée avec la structure de la couche prismatique ressemblant un prisme. Le développement des branches s'est fait à partir d'une paire de barres branchiales. A partir des 30 jours l'hémibranchie interne commence a se former, le développement de l'hémibranchie externe se faisant ensuite, visible a partir du jour 90. Les jonctions inter filamentaires furent observées aux jours 200 et 240 et les jonctions inter lamellaires aux jours 240 et 260 dans les hémibranchies internes et externes respectivement.

Status Thesis

The results of the work of this thesis have been published for publication in the following journals:

1. Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Thongpan, A., Machado, J., 2006. Optimization of diet and culture environment for larvae and juvenile freshwater pearl mussels, *Hyriopsis* (*Limnoscapha*) *myersiana* Lea, 1856. Invertebr. Reprod. Dev. 49(1-2), 61-70.

2. Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Machado, J., 2007. Morphological development of the juvenile through to the adult in the freshwater pearl mussels, *Hyriopsis* (*Limnoscapha*) *myersiana*, under artificial culture. Invertebr. Reprod. Dev. 50(4), 207-218.

3. Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Machado, J., 2008. A laboratory-scale recirculating aquaculture system for juvenile of freshwater pearl mussels *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856). Aquaculture 275, 169-177.

4. Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Trisaranuwatana, P., Machado, J., 2008. Morphometric relationship of weight and size of cultured freshwater pearl mussels, *Hyriopsis* (*Limnoscapha*) *myersiana*, under laboratory conditions and earthen pond phases. Aquaculture International (in press).

Chapter 1

General Introduction

1.1 Introduction

The phylum mollusca is one of the largest, most diverse and important groups in the animal kingdom. There are over 50,000 described species in the phylum and about 20,000 of these are found in freshwater. According the reports from several authors (Gosling, 2003; Barnes, 1987; Kaestner, 1967; Brandt, 1974; Elliott, 1952) mollusca are soft-bodied animals but most are protected by a hard exoskeleton called shell. Inside the shell is a large and fold of tissue called mantle. The mantle encloses the internal organs of the animal. Other features of the phylum are a large muscular foot that is generally used for locomotion and a specialized feeding organ called radula. There are six major classes of mollusca, being the Bivalvia a class with about 7,500 species, some arranged under very well known groups such as mussels, oysters, scallops and clams. The main feature of the Bivalvia is to possess a calcareous exoskeleton divided in two symmetrical or asymmetrical shell valves. The bivalves are in some ways highly modified from the typical mollusca, becoming over evolutionary time flattened side to side. Laterally, the body organs are covered by two mantle lobes that secrete the shell valves which are hinged dorsally. The opening and closing of the shell valves are controlled by muscles named as the anterior and posterior adductors. Lateral compression has resulted in the raising of the mouth from the substrate level; subsequently the role of catching food has shifted from radular into a ciliary system in the gills. Additionally, there is also secondary mantle ciliary system that probably helps to collect and digest food particles by acid secretions (Machado et al., 1988). Laterally to the mouth there are two labial palps that transport the food particles from the gills and mantle into the mouth. As a result, the gills have

become enormously enlarged and function as one of the most efficient systems of ciliary feeding in the animal kingdom. In the bivalvia the foot has lost its flat creeping sole, specific of the typical mollusca, becoming wedge-shaped. This organ may be extended out between the valves being more appropriate for locomotion in soft bottom habitats (Gosling, 2003).

The freshwater mussels are benthic filter feeders and occur in varied habitats such as oligotrophic streams, rivers, ponds and lakes. Ecologically, mussels are important as a food source for humans, muskrats, otters, raccoons and some fishes. They also play a significant role in filtering the water column, removing phytoplankton and particulate organic matter (Amyot and Downing, 1991; Nalepa et al., 1991). Humans have been using freshwater mussels as raw materials and food from ancient times and its use has continued into modern times. Mussel shells are used for making tools and decorative objects and mussel meat as a supplemental food source (Parmalee and Bogan, 1998; Williams et al., 1993). In the first half of the 20th century, the shells of some mussel species were harvested to manufacture several handicraft materials mainly mother-of-pearl buttons (Matteson, 1995). Presently, shell fragments of some species are used as seeds in the cultured pearl industry (Williams et al., 1993). In the far-east Asian countries freshwater pearl mussel shells are also used to make earrings, necklaces, decorations used in religious ceremonies and furniture like tables, chairs and picture frames.

Mussels are recognized for their potential in water quality biomonitoring (Matteson, 1955; Neves, 1993). In fact, as sedentary filter feeders they are exposed to large volumes of water and are unable to rapidly move away from pollution inducing a strong decline in mussel populations which may indicate water quality problems

(Matteson, 1955; Neves, 1993; Moura et al. 2000; 2001). A particular property concerns the bioaccumulation of heavy metals in their shells which added to a long life span can provide a history of metal levels in the water through the analysis of shell material (Metcalfe-Smith, 1994).

Poor water quality, habitat destruction and competition from exotic species are the main factors that have led to their decline. Direct destruction of freshwater mussel habitat can occur as a result of dredging, channelization, and the impoundment of flowing waters. The construction of dams is particularly disruptive of mussel population that not only alters the physical habitat above and below the dam, but also affects the water quality and flow characteristics. Dams result in the creation of static, deep water in the impounded area, alternations of flow, scouring of the river channel, and changes in temperature, oxygen content and substrate composition may occur (Layzer et al., 1993). In fact, mussel populations can be negatively affected by changes in water temperature, pH, depth, luminosity, and physico-chemical properties, which may result in physiological stress, with subsequent influence on growth, longevity and disruption of reproductive cycles (Matteson, 1955; Young and Williams, 1984; Gordon and Layzer, 1989; Layzer et al., 1993). Sedimentation of waterways resulting from poor land use practices, such as over-clearing in agriculture or clear cutting, also impacts mussel habitats (Layzer et al., 1993; Michaelson and Neves, 1995). The deposition of fine silt can smother mussels or impair their ability to filter feed (Imlay 1972; Box and Mossa, 1999). Muddy water is filtered less efficiently and also causes mussels to close their valves, thus reducing feeding time (Bailey, 1989; Rogers, 1999). Agricultural and mining runoff, industrial discharges and wastewater treatment effluents can both alter water chemistry and bring toxicants.

4

Inflow of these pollutions can cause, once again, alterations in dissolved oxygen content, pH, alkalinity, and hardness levels and may carry toxic substances such as zinc, copper, chlorine, cadmium, and arsenic (Michaelson and Neves, 1995; Layzer et al., 1993; Moura et al. 2000; 2001). The organic pollutants also contribute to a drastic alteration, to mention diflubenzuron, TBTO and several acid compounds (Machado et al**.,** 1988b; 1989; 1990). The above mentioned reasons pose a significant threat to mussels causing decline of mussel populations in their natural habitats almost all over the world. For example, more than 70% North American mussel species are considered to be either endangered, threatened, or of special concern (Williams et al., 1993). Similarly, Europe and Thailand mussel populations have declined tremendously as well. In particular ecosystems, some species of these mussels are nearly extinct such as *Margaritifera auricularia* in the Ebro river of Spain, *Pleurobema pyramidatum* in Mississippi (Miller and Payne, 2004), 16 species of Tennessee, North America (Parmalee and Bogan, 1998). It is recommended that such problematic should be researched and mussel populations protected jointly. Particularly, there is a high demand for culture and mass production methods to be used in the pearl industry and to be released into their natural habitats repopulating the depleted sites.

1.2 General and Specific Features in *Hyriopsis myersiana*

1.2.1 Morphology

Hyriopsis (*Limnoscapha*) *myersiana* (Lea, 1856) is a Thailand's freshwater pearl mussel classified in the family Amblemidae. It has a medium shell size with

rhomboide structure composed by two valves of equal size. The shell dorsal region has two wings with the posterior wing larger than the anterior wing. These two wings are joined by elastic ligaments. The inside face of the shell is attached by the hinge bordered by lateral teeth which form a long tapering under the hinge ligament located at posterior of the shell. Pseudocardinal teeth are much shorter than lateral teeth and are located on the anterior region of the shell (Figure 1.1). Under the interdentum is a beak cavity attached to the beak of the umbo. The umbo is located on the dorsal toward anterior of the shell with a concentric beak sculptured fashion. Next to the lateral teeth, toward posterior region, there are two muscle scars with large and round circles that are not deep into the shell. Posterior pedal retractor muscle scar is a small depression under the lateral teeth while the posterior adductor muscle scar is a larger depression under the pedal retractor muscle scar. In the anterior region of the shell there are three round or elliptical muscle scars embedded in the shell, i.e. 1) anterior adductor muscle scar located in front of pseudocardinal teeth (the largest size), 2) anterior pedal retractor muscle scar located below the pseudocardinal teeth, and 3) pedal protractor muscle scar located below the anterior pedal retractor muscle scar. Additionally, there is a characteristic curved line called "pallial line" which is located a little inside from the shell edge bordering all frontal region and which results from the rim of mantle muscle attached to the shell. On the exterior shell surface or layer, usually called "periostracum", straight or growth lines are found. These particular lines are arranged in succession from the umbo area in the lowest region of the shell. When the mussel is young the periostracum exhibit a greenish color getting brownish black with age (Brandt, 1974). The periostracum of freshwater mussels is a very thin layer but with a high resistance against pysicho-chemical agents, since it is composed

mainly by colored and hardened proteins processed by sclerotization phenomena. These proteins are similar to proteins in animal horn. In a transversal section, the middle region of shell is a denser layer than the others and is called "prismatic" since it consists of columnar structure composed by calcium carbonate crystals under a calcite or aragonite system. The inner layer is called "nacreous" layer which presents a lamellar structure composed by a thin glossy lamina of calcium carbonate crystals arranged under alternative succession with organic molecules structures. This nacreous layer exhibits features, as colour, irisdiscence and composition, similar to those of the mother-pearl. Kovitvadhi et al. (2001) and Panha and Eongprakornkeaw (1995) had studied the morphology of glochidia *H.* (*L.*) *myersiana* with a scanning electron microscope (SEM) and observed that the shell of the glochidia has a sub-oval structures with two equal hookless type shells and is composed by keratin fibers in the external shell surface.

1.2.2 Taxonomy and distribution

The classification of freshwater pearl mussel is based on significant characteristics such as hinge teeth, shell shape, muscle scar and wing. The taxonomic classification was according to Brandt (1974) as follows:-

Class Bivalvia Linnaeus, 1758
    Subclass Schizodontida Steinmann, 1888
        Order Unionoida Stoliczka, 1871
            Superfamily Unionacea Fleming, 1828
                Family Amblemidae Rafinesque, 1820
                    Subfamily Hyriopsinae Modell, 1942
                        Genus *Hyriopsis* Conrad, 1853

Subgenus *Hyriopsis* (*Limnoscapha*) Lindholm, 1932

Species *myersiana* (Lea, 1856)

This species is a common inhabitant of rivers, canal stream and reservoirs in Thailand. There have been studied on their distribution of *H.* (*L.*) *myersiana* in Table 1.1.

Table 1.1. Notable references and distribution of *Hyriopsis* (*Limnoscapha*) *myersiana.*

Notable references	Distribution
Suvatti (1950)	Maeklong, Kwae Noi River in Kanchanaburi Province, Pasak River in Ayutthaya Province and Ratchaburi Province.
Kannasut (1971)	Kwae Noi River, Kwae Yai River and Maeklong River in Kanchanaburi Province.
Brandt (1974)	Maeklong River and its tributaries Maenam Kwae Noi and Maenam Kwae Yai. Upper reaches of the Chao Praya River and its tributaries Pasak River, Ping River and Nan River, Kaek River and Kwae Noi River in Phitsanulok Province. Prachinburi River (Patrang River, Srakeo River); in South of Thailand only known from the Klong San in Phatthalung Province.
Panha (1990)	In the irrigation canals and canal near the rice fields at Chainat, Nakhonsawan, Singburi, Ayutthaya and Pathumtani Provinces.
Chaopaknam et al., (1994)	Kwae Noi River, Kanchanaburi Provinces.
Meesukko (1996)	Yom and Nan River
Kovitvadhi et al., (2000)	Maeklong River, Kanchanaburi Province.
Kovitvadhi et al., (2005)	Maeklong River in Kanchanaburi Provinces, Lopburi River in Ayutthaya Province, Chao Praya River in Angthong and Chainat Province, Klong Mone in Chainat Province, Klong Maka in Kamphaengphet Province, Ping River in Tak Province, Yom River in Phichit, Sukhothai, and Phrae Province, Nan River in Phitsanulok Province.

(A)

(B)
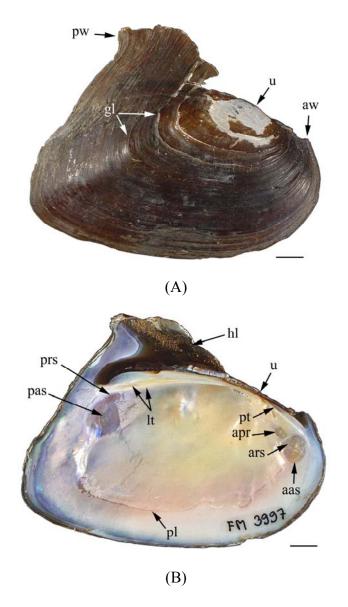
Figure 1.1. Shell morphology of freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856). The outer shell side (A) and inner shell side (B); aas, anterior adductor muscle scar; apr, anterior pedal retractor muscle scar; ars, anterior pedal protractor muscle scar; aw, anterior wing; gl, growth line; hl, hinge ligament; lt, lateral teeth; pas, posterior adductor muscle scar; pl, pallial line; prs, posterior pedal retractor muscle scar; pt, pseudocardinal teeth; pw, posterior wing; u, umbo. Scale bar = 1 cm.

9

1.2.3 Life history and habitat

Freshwater pearl mussel, *H.* (*L.*) *myersiana* is dioecious. Most varieties of freshwater mussels have separate male and female sex, but there are some species that are hermaphroditic such as *Anodonta imbecillus*, *Lasmigona compressa*, *L. subviridis*, and *Taxolasma parvus*. Ovaries and testis are yellow, located on the foot and are surrounded by intestine. They constitute much of the visceral mass and are joined with suprabranchial chamber by short ducts. *H.* (*L.*) *myersiana,* which is found in Mae Klong River, Kanchanaburi Province, has spawning seasons from October to May. One female can release glochidia larvae from 13 to 25 times (average 18.5 times) per spawning seasons. The peak of the spawning season is from December to January (Nagachinta and Meejui, 1998). Male sperm is released through a genital pore and spread into river by excurrent siphon (Figure 1.2). From there, the sperm is mixed with water and goes into the incurrent siphon of the female mussel. Then through the ostia of the gill plates will fertilize eggs in the water tubes. This results in swelling of female mussel gills to become the sac, called "marsupial or brood chamber", where the fertilized eggs develop to become zygotes. These develop in the marsupial until the first larva phase, called "glochidia" (singular, glochidium), completes with the formation of two calcareous valves and a larval thread. Many species developed a hooked valve, but *H.* (*L.*) *myersiana* is a hookless type. Glochidia emerge from the suprabranchial chamber through the excurrent siphon and float into the water. The locomotion of glochidia results from the shell opening and closing movements. Hook type glochidia will attach to a host fish on the skin, fin or gill while hookless glochidia will mainly attach under firm way to a gill of a host fish. Then the

epithelium of the host fish will cover glochidia forming a cyst. During this period the young mussel is entirely parasitic receiving nourishment from its host through absorption. After the adult organs have developed, the glochidium bursts out of the cyst and sinks to the bottom as an independent free-living animal (Elliott, 1952; Binhe, 1984). The life cycle of freshwater mussel is shown in Figure 1.3.

1.2.4 Food and feeding

The vast majority of bivalves use mainly the gills for feeding and these have become greatly enlarged to deal with their secondary derived role. This method of feeding is called suspension or filter feeding. The food of bivalves consists of minute organisms, chiefly microscopic algae and of organic matter particles or detritus suspended in the water. These are strained off by the gills, where they become entangled in mucus and swept by cilia towards the mouth. It is suggested that the mantle cilia can also retain small particles as well as secrete digestive acid mucus (Machado et al. 1988a). On each side of the mouth are two triangular and ciliated flaps, the labial palps, which sort out the food from inedible particles and convey it, also by ciliary action, into the mouth. On the contrary, rejected matter is eventually carried by cilia to the exhalent aperture and ejected (Gosling, 2003). Bivalves do not have a conventional mouth since it lack jaws and salivary glands. Instead connected with the intestine there is a gelatinous rod-like body with a crystalline style, which is continuously being used up and renewed. This mentioned structure contains a starch-digesting enzyme. A peculiar anatomic feature is that the hind part of the intestine or rectum passes through the middle of the heart (Ellis, 1978). This seems to suggest a

relevant role on the absorption of mineral and/or organic compounds contributing to the nutritional or osmoregulation mechanisms (Lopes-Lima et al., 2008).
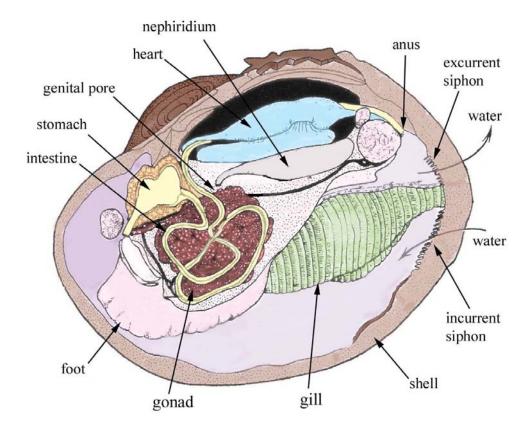
Figure 1.2. Internal structure of the freshwater mussel. (Modified from: Elliott, 1952).
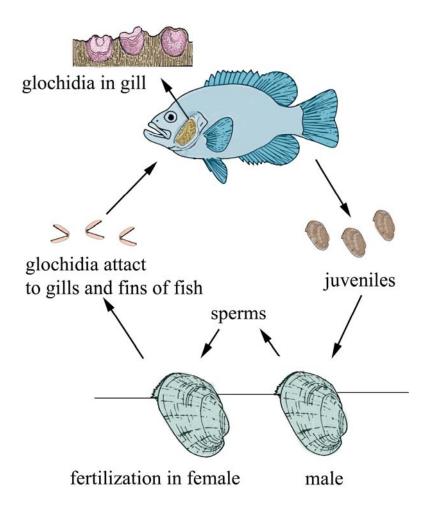
Figure 1.3. Life cycle of freshwater mussel.

Algae constitute an important component of freshwater mussels' diet. In laboratory settings, live algae are ingested and serve as a suitable diet due to specific properties for rearing juvenile mussels (Hudson and Isom, 1984; Gatenby et al., 1996; Gatenby et al., 1997; O'Beirn et al., 1998; Steg, 1998; Beck, 2001). When fed by algae, juvenile and adult bivalves exhibit selectivity correlating with particle size

(Gatenby, 1994; Beck, 2001). In fact, ingestibility is a function of size and shape where, for example, the presence of features such as elongated spines are likely to inhibit ingestion. Estimates of the cell size range that are potentially ingestible by juveniles may vary. Some studies suggest that by pedal-feeding, juveniles may take in particle sizes comparable to those ingested by adults, up to 70 μm, while other researcher suggests that smaller particle size <25 μm are more suitable (Gatenby, 1994; Beck, 2001). Sprung and Rose (1988) examined the food uptake by the freshwater mussel *Dreissena polymorpha* and found that these mussels had a maximum ingestion rate at high food concentration and could filter out from the water particles with a diameter range from 0.7 to 35 μm with a maximum retention efficiency at 5 μm. Raby et al. (1997) used epifluorescence microscopy to analyze the stomach contents of bivalve larvae collected from Western Gulf of St. Lawrence, Canada, in order to obtain information on food-particle size. They found that cyanobacteria (<2 μm) and small-sized phytoplankton (<5 μm) were major food components for all species of bivalve larvae. Gale and Lowe (1971), Huca et al. (1983) and Paterson (1986) reported that the gastrointestinal tract of adult freshwater mussels contained more phytoplankton than zooplankton. Similar results were obtained from the feeding behavior study on freshwater pearl mussel *H.* (*L.*) *myersiana* which indicated 99.99% phytoplanktons in Phylum Chlorophyta, Bacillariophyta, Cyanophyta, Phyrrophyta, Euglenophyta and Chrysophyta in the gastrointestinal tract plankton content (Kovitvadhi et al., 2000). Beside Dinoflagellate the richest phytoplankton genus found on *H.* (*L.*) *myersiana* were *Crucigenia*, *Scenedesmus, Merismopedia, Cymbella, Synedra, Coelastrum,* and *Pleodorina*

1.2.5 Environmental factor

There are many factors that affect growth in bivalves. The food supply is considered to be the most important (Seed and Suchanek, 1992) being influenced by several relevant parameters such as temperature, dissolved oxygen, alkalinity, pH, ammonia nitrogen, hardness, water depth, pollutants and population density. Additionally, some of these modulators of growth such as temperature and food may often interact synergistically. For these reasons usually it is very difficult to quantify the precise influence of a single environmental factor on the bivalves' growth in their natural habitats. Furthermore, endogenous influences that are inherent to the organism, e.g. genotype and physiological status, interact in a complex way with the environmental factors. Although the bulk of published information deals with the effect of environmental factors on absolute growth, the specific mechanisms inherent to this process should be also dealt with. Water samples from the Mae Klong River, Kanchanaburi Province, where freshwater pearl mussel *H.* (*L.*) *myersiana* lives, were analyzed in different periods obtaining quality results which are shown in Table 1.2. Ammonia occurs naturally in unpolluted surface freshwater environments with a concentration range of 0.05-0.4 mg/l (Goudreau et al., 1993). A number of harmful effects have been documented in freshwater and marine bivalves exposed to ammonia solutions such as: reduction in the amount of time valves are open for breathing and feeding (Epifanio and Srna, 1975); impaired secretion of the byssus thread (Reddy and Menon, 1979); reduction of the ciliary action (Anderson et al., 1978); depletion of the lipids, glycogen and other carbohydrate storage; metabolism alteration (Chetty and Indira, 1995) as well as acute lethal toxicity (Goudreau et al., 1993; Scheller,

1997; Summers, 1998). Dissolved oxygen levels in the flow through pond were well above 5 mg/l threshold recommended for unionids (Havlik and Marking, 1987). Estimates of potentially harmful environmental nitrate levels range from 0.8-2.13 mg/l (Lemly, 1998). Orthophosphate levels of 0.13-0.35 mg/l been reported to be problematic (Lemly, 1998). Low levels of orthophosphate are only problematic for juvenile culture if it is limiting algae production. Although initial levels of orthophosphate were relatively low, they were comparable to levels (mean 0.15 mg/l) recorded in habitats that support other freshwater mussel populations (Strayer, 1999). Binhe (1984) recommended suitable conditions for rearing freshwater pearl mussels at 1.5-3.0 m depth of running water, pH 7.0-7.5, transparency 30 cm, temperature 15-30° C and the water should be rich in diatoms, golden algae, yellow algae, green algae, rotifers and protozoa. Inorganic matter, such as calcium which is the major component of the shell, is very important for shell growth. Calcium in water should be as high as 10 mg/l while smaller contents of sodium, silica, magnesium and iron are also essential for normal growth (Moura et al., 2000).

Table 1.2. Minimum-maximum values on the water quality parameters of the Mae Klong River in Kanchanaburi Province.

Parameters	Measurement period				
	Oct.89-Mar.91[a]	Apr.-Feb.93[b]	Dec.94-Nov.95[c]	Oct.98-Apr.99[d]	Oct.02-Jan.03[e]
Transparency (cm)	23.2 - 63.7	14 - 69.9	9 - 200	6.0 - 92.8	165 - 173
Water temperature (°C)	23.8 - 26.1	24.2 - 28.4	22.0 - 32.4	22.5 - 30.5	28 - 28.5
pH	7.9 – 8.1	7.0 - 7.5	6.8 - 8.1	6.8 - 7.9	7.7 - 7.75
Dissolved oxygen (ppm)	6.2 – 8.1	5.8 - 8.1	5.1 - 9.6	3.7 - 8.9	4.9 - 5
Free CO_2 (ppm)	-	0.4 - 4.5	-	0 - 5	4 - 5
Total alkalinity (ppm)	-	80.2 - 105.5	-	69 - 115	86 - 92
Total hardness (ppm)	-	90.6 - 143	74 - 132	95 - 135	102 - 113
Ammonia nitrogen (ppm)	-	0.3 - 0.6	0.04 - 0.4	0.04 - 0.9	0.1 - 0.2
Total phosphate (ppm)	0.01 – 0.1	0.2 - 0.6	ND - 0.2	0.01 - 0.1	0.3 - 0.5
Calcium (ppm)	55.8 - 77.5	71 - 83	-	70 - 110	77 - 78
Silica (ppm)	-	0.8 - 4.04	-	0.23- 6.1	6 - 10

ND = Non – detectable, - = not measured.

a = Narksuwan (1992), b = Rakkittham (1996), c = Thientaworn (1997),

d = Kovitvadhi et. al. (1998), e = Kovitvadhi et. al. (2005).

1.2.6 Culture of glochidia and juvenile

The most common methodology to rear larvae of freshwater mussels is by mimicking the natural process based on the infestation of the host fish which are placed on the same container of the gravid mussel female. Young glochidia are parasitic and encystes in fins and gills. There, they suffer a metamorphosis process transforming into juveniles and then fall into the sediment as a free living organism. From the experiments of Uthaiwan et al. (2001) and Kovitvadhi et al. (2002) freshwater pearl mussel *H.* (*L.*) *myersiana* was successfully cultured at the glochidia phase in artificial media (M199 formula) added by common carp fish plasma under a temperature control cabinet and providing air mixed with carbon dioxide at 5%. This

17

artificial culture method is very convenient since it is time saving, practical, inexpensive and has very high survival rates up to 93%. The metamorphosis, from young glochidia to young juvenile, has a complete transformation rate up to 100% in the period of only 8-10 days. To be emphasized the fact that from a very early age is easy to culture these bivalves without disease.

It is observed that laboratory culture of larvae from the gravid female, comparatively to the natural process, offer more advantages and growth control on transformed juveniles, by artificial media. In fact, in the experiments of Hudson and Isom (1984) juveniles of *Anodonta imbecilis* were reared in a lake with natural plankton populations, i.e. *Gonium* sp., *Anabaena* sp., *Navicula* sp., *Phacus* sp., *Oscillatoria* sp. and others in residual contents. On the other hand, Uthaiwan et al. (2001) reared juvenile of *H.* (*L.*) *myersiana* with dechlorinated tap water added with *Chamydomonas* sp., *Monoraphidium* sp., and *Chlorella* sp. as food. It was found in these two experiments that juveniles reached a maximum age at 74 and 60 days with a highest length of only 5.1 and 2.4 mm, respectively. The juveniles are transparent have a small length, height and width of around 200, 150 and 75 microns, respectively. These features make these young juveniles very difficult to find in natural resources (Kovitvadhi et al., 2001). However, it was still practically impossible to maintain these juveniles from artificial culture into adult stage with a correct and a successful growth and survival in order to introduce them in their natural habitats.

18

1.2.7 Objective

Freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856) is a valuable economic mussel in Thailand, since it has a great potential for producing freshwater pearls as well as a high protein food source. The reduction of its natural stock is now an evident fact mainly due to the gradual increase of water pollution. Additional factors can limit the development of this species from the larvae (glochidia) until the adult age. In effect, the natural stress caused by glochidia discharge from the females to the water followed by bacteria, fungi and protozoa contamination as well as finally the fish encystment process are the main barriers. The posterior growing of juveniles during the first days after fish infestation constitutes a strong limitation for its survival until the adult stage.

Presently, only the *in vitro* culture of glochidia controlled by an artificial method and improved by Uthaiwan et al. (2001) is successful up to the juvenile phase. Now it is proposed to continue these studies in order to find the better conditions for juvenile development up to the adult stage based on experiments with water quality, culture system and diets such as type of phytoplankton. So, the isolation of phytoplankton from the digestive tract is here proposed to adjust a correct diet for juveniles in order to mass culture adult mussels for their introduction into the natural environment. A new laboratory-scale recirculating aquaculture system to control permanently the addition of food, the water change and the aeration will be created. Morphological studies by light microscopy and SEM observation for better understanding the organ evolution during juvenile growth will also be carried out. This study will be based on the morphometric relation of size and weight of

freshwater pearl mussel from early juvenile (0 day old) obtained in artificial culture media to adult (360 days old or more) on different environment conditions. So, these experiments under laboratory and earthen pond conditions should give morphometric data of basic importance to compare life history or morphology among freshwater pearl mussel cultured in various environments as well as for the development of culture techniques.

Chapter 2

Optimization of Diet and Culture Environment for

Larvae and Juvenile Freshwater Pearl Mussels,

Hyriopsis (*Limnoscapha*) *myersiana* (Lea, 1856)

2.1 Abstract

Culture of the freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana,* was carried out in three consecutive steps: (1) culture of glochidia larvae in artificial media, (2) rearing the early juveniles (0-120 days old) in a nursery, and (3) rearing the juveniles (120-360 days old) in an earthen pond. The percentage survival of glochidia in standard tissue culture medium (M199) supplemented with common carp plasma was 95±2.5. All surviving larvae (100%) transformed to juveniles, the duration of transformation being 8 days. The early juveniles (0-60 days old) were fed with a mixture of four selected phytoplankton species (*Chlorella* sp., *Kirchneriella incurvata*, *Navicula* sp. and *Coccomyxa* sp.). The survival rate of juveniles was 8±0.2%. The average length of these juveniles increased from 0.13±0.01 mm to 1.41±0.16 mm and the average height from 0.16±0.01 mm to 0.98±0.09 mm. Subsequently, 60-120-day juveniles were fed with one of the same four phytoplankton species or a combination of the four. Feeding the juveniles with *K. incurvata* resulted in the highest survival rate (65±8.32%), with an average length of 3.46±0.04 mm and an average height of 1.94±0.04 mm. Finally, the 120-360-day juveniles were cultured in an earthen pond. There were progressive changes in average weight (0.0037±0.002 g to 11.24±5.02 g), length (3.48±0.39 mm to 54.08±6.21 mm), height (1.97±0.24 mm to 25.09±2.48 mm) and width (0.98±0.06 mm to 12.28±3.21 mm) from 120 to 360 days. The average growth rates per day of these parameters were 0.0497±0.01 g, 0.2414±0.15 mm, 0.0975±0.08 mm and 0.0493±0.03 mm, respectively. *H.* (*L.*) *myersiana* juveniles developed the complete structural composition of the adult by 160 days, and at 360 days, gametogenesis was complete.

22

2.2 Introduction

Hyriopsis (*Limnoscapha*) *myersiana* is an endemic freshwater pearl mussel in Thailand. The nacreous mussel shell can be used for inlaying pearl furniture, ornaments, kitchen utensils and souvenirs. The mussels can also produce freshwater pearls and the meat is a source of protein for humans and animals. These mussels are suspension feeders and their filtration activities contribute to maintaining a clean environment and reducing pollution. *H*. (*L*.) *myersiana* is dioecious. The life cycle includes a unique parasitic larval stage known as the glochidium. Adults spawn from October to May and are able to do so 13-25 times a year (average 18.9 times), which is the number of times a female can release glochidia larvae in a year (Nagachinta and Meejui, 1998). The peak of the spawning season is from December to January. Glochidia of *H*. (*L*.) *myersiana* are obligate ectoparasites on a specific fish host (Arayawatanavij et al.,1992; Panha,1992; Kovitvadhi et al., 2002 and 2003). During this period, the glochidia larvae receive nourishment from their hosts through absorption. After the adult organs have developed, the glochidium bursts from the enclosing cyst and sinks to the bottom as an independent, free-living animal (Binhe, 1984).

The natural population of *H*. (*L*.) *myersiana* in Thailand has been drastically reduced to the extent that it is now nearly extinct. Thus, there is an urgent need to restore their numbers through aquaculture. Furthermore, culturing these freshwater mussels throughout their complete life cycle would lead to better understanding and contribute to the management and control of wild populations.

Freshwater mussel culture is divided into the three stages of the mussel's life cycle (i.e., the parasitic glochidia larval stage, juveniles and adults, respectively). Glochidia larvae are obligatory ectoparasites on fish hosts until they become juveniles. Uthaiwan et al. (2001) and Kovitvadhi et al. (2002) succeeded in culturing glochidia of *H*. (*L.*) *myersiana* in artificial media with a survival rate of up to 93%, and 100% of these glochidia transformed into juveniles within 10 days. Juveniles of *H*. (*L.*) *myersiana* have been cultured using dechlorinated water supplemented with *Chlamydomonas* sp., *Monoraphidium* sp. and *Chlorella* sp. for food (Uthaiwan et al., 2001). These juveniles survived to 60 days, with the average length of 2.4 mm. However, information regarding the biology and culture of juvenile *H*. (*L.*) *myersiana* is still limited. The aims of this research were to identify an appropriate diet and a suitable artificial environment for the survival of juvenile *H*. (*L.*) *myersiana* and their development to adulthood.

2.3 Materials and methods

2.3.1 Culture of glochidia

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, width of 3.8±0.5 cm, length of 13.73±2.05 cm and height of 6.09±2.02 cm. The culture method for *H*. (*L.*) *myersiana* larvae was modified from that of Uthaiwan et al. (2001) and Kovitvadhi et al. (2002): 5,000-6,000 glochidia per replicate (three replicates) were placed in a culture dish (90×15 mm) containing 10 ml of artificial medium composed of Medium199 (Gibco,

No.6231100-035), fish plasma (common carp, *Cyprinus carprio*) and antibiotics/antimycotic (100 µg/ml carbenicillin, 100 µg/ml gentamycin sulfate, 100 µg/ml rifampin, and 5 µg/ml amphotericin B) in a ratio of 2:1:0.5, respectively. The culture dishes were placed in a low temperature incubator at 25° C with 5% CO_2. The culture medium was removed and replaced with fresh medium on day 4. Finally, 4 ml of sterilized distilled water was added to the culture dish on day 7 to stimulate the transformation of glochidia into juveniles.

2.3.2 Selecting phytoplankton food species for juveniles

Phytoplankton were collected from the gastrointestinal tract of 15 mature mussels from the Mae Klong River, Kanchanaburi Province, and transferred into 1 l of f/2 media (Guillard and Ryther, 1962) and cultured under fluorescent light of 10,000 lux intensity for 18 h/day with a continuous supply of 3% CO_2. The cultured phytoplankton were sub-cultured and purified every 10 days using a streak plating technique, as described by Hoshaw and Rosowski (1973). Single colonies of these phytoplankton were then identified and mass-cultured to be used for juvenile feeding.

Phytoplankton selection was based on three criteria: (1) the ability of mussel to pass each specific type of plankton through the gastrointestinal tract, (2) digestibility of plankton, and (3) ability to mass-culture that phytoplankton species. In testing ingestion and digestibility of each phytoplankton species with juvenile, 1×10^3 cells of each species of phytoplankton were dispersed in 50 ml dechlorinated water and used to feed 50-100 early juveniles in a 100 ml beaker; three replicates per phytoplankton species were set up. After 90 min, 30 juveniles were randomly collected from each replicate to observe plankton ingestion and digestibility under the light microscope.

Ingestion was determined by the presence of phytoplankton in the gastrointestinal tract the phytoplankton being clearly visible through the transparent shells of the juveniles. Digestibility of phytoplankton was determined from the structural and color changes of the phytoplankton in the digestive gland, as well as from the feces.

From the ten different types of phytoplankton species (*Ankistrodesmus gracilis*, *Chlamydomonas* sp., *Chlorella* sp.1, *Chlorella* sp.2, *Kirchneriella incurvata*, *Monoraphidium irregulare*, *Navicula* sp., *Scenedesmus* sp., *Stichococcus* sp. and *Coccomyxa* sp.) found in the gastrointestinal tract, four species of phytoplankton (*Chlorella* sp.2, *K. incurvata*, *Navicula* sp. and *Coccomyxa* sp.) were selected as potential food sources for juveniles based on their sizes, shapes, the ability of cilia around the foot, the mantle and gill to transport them into the gastrointestinal tract, and digestibility.

2.3.3 Culture of *H.* (*L.*) *myersiana* juveniles

The culture of juvenile *H.* (*L.*) *myersiana* was undertaken in five stages, based on the passage of time: 0-60, 60-120, 120-180, 180-270 and 270-360 days. Culture conditions were as follows:

2.3.3.1 Culture of 0-60-day juveniles

About 5,000-6,000 newly transformed juveniles per replicate (three replicates) were transferred to a plastic culture unit (20×11×8 cm) containing 20 g of sand (<120 µm grain size) about 3 mm deep. This sand was collected from the natural habitat of *H.* (*L.*) *myersiana*, passed through a 120 µm mesh, washed several times with dechlorinated water and oven dried at 180° C for 24 h. Each culture unit was

maintained in a glass aquarium, the filter cabinet (dimensions, 50×46×35 cm). A combination of 5 kg of pebbles, 2 kg of ground freshwater mussel shells and 240 g of nylon fiber (~46×35×2 cm in size and ~30 g per piece) was used as filter materials. The water was then passed through a UV tube into a resting cabinet (40 l capacity) and pumped into the culture unit at the rate of 20 ml/min. (Figures 2.2 and 2.3) The culture water was subsequently recycled to the filter cabinet. Mussels were fed twice a day (06.00 h and 18.00 h) with a combination of four selected species of phytoplankton (*Chlorella* sp. 2, *K. incurvata*, *Navicula* sp. and *Coccomyxa* sp.) at a ratio of 1:1:1:1 and a concentration of $1×10^3$ cells/ml. The water circulation was turned off for 1 h during feeding. The sand and 25 % of the water in the system was replaced every other day. One hundred juveniles from each replicate were randomly harvested to measure body length every 10 days (Figure 2.1) and the survival rate was also recorded. All mussels were rinsed every 10 days.
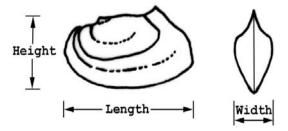
Figure 2.1. Measurements of shell size.

2.3.3.2 Culture of 60-120-day juveniles

Sixty juveniles (60-120 days old) were placed in a recirculating water system similar to that used for the previous stage, involved 20 g of coarser sand (<250 μm in size), a smaller filtration unit (40 l capacity), but no resting cabinet or UV tube (Figures 2.4 and 2.5). The flow rate of water recirculation was kept at 20 ml/min. The

sand and 25% of the water in the system was replaced every other day. All mussels from each replicate were measured both for length and height and rinsed every 10 days. The survival rate was also recorded. The 60-120-day juveniles were fed with one of the four species of phytoplankton or a combination of these species at a ratio of 1:1:1:1. All the feeds were given at the concentration of 1×10^3 cells/ml.

2.3.3.3 Culture of 120-180-day juveniles

Juveniles of 120 days old of approximately the same size were selected to rear in an earthen pond. Thirty-five juveniles were transferred to each of three culture units (each $20\times12\times72$ cm). The culture units had all four vertical sides lined with nylon net (0.42 mm mesh size) and each had a plastic lid with holes to cover the top. The lower part of the culture unit consisted of a section 2 cm in height, which fitted snugly into the culture unit, from which it could be removed (Figure 2.6A). This lower part contained 400 g of sand (<425 μm in size). The juveniles were placed directly on the sand. The culture unit was then hung in the earthen pond, located at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University. The pond was about 2 m deep with a total surface area of 0.8 ha and filled with rain water. The base of the culture unit was adjusted to a position approximately 50 cm below the water surface. The juveniles fed by filtering phytoplankton from the water in the earthen pond. All mussels from each culture unit were weighed, measured the length, height and width and rinsed every 10 days. The survival rate was recorded.

2.3.3.4 Culture of 180-270-day juveniles

Thirty-five 180-day-old juveniles per unit were placed in culture units similar to those described above, but with nylon net lining with a mesh size of 2.0 mm. Three culture units were used. These juveniles were exposed to the same conditions and feeding regime as those 120-180 days old. All mussels from each culture unit were weighed, measured (length, height and width) and rinsed every 10 days. The survival rate was recorded.

2.3.3.5 Culture of 270-360-day juveniles

Thirty-six 270-day juveniles per pocket net were transferred to three pocket nets (Figure 2.6B). Each net was equally divided into six compartments using string and six juveniles were introduced through the opening at the top of each compartment. The nets were hung 50 cm below the water surface of the earthen pond. All mussels were weighed, measured (length, height and width) and rinsed every 10 days until they reached 360 days. Their survival rate was recorded.
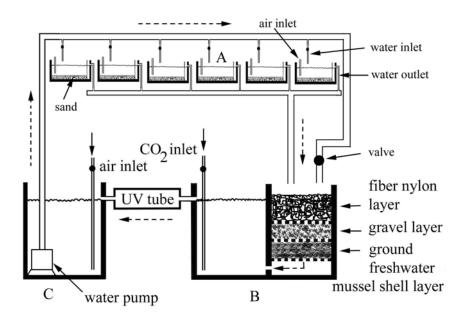
Figure 2.2. Rearing container of 0 (the early juvenile) to 60-day juveniles. The culture box (A); the filter cabinet (B); the resting cabinet (C); – – – ► = water flow.
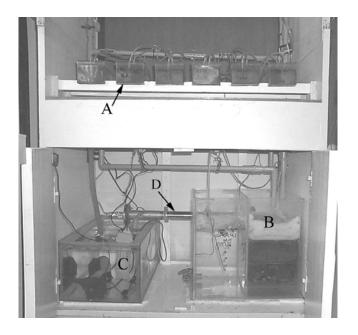
Figure 2.3. Photographic of rearing container of 0-60-day juveniles. The culture box (A); the filter cabinet (B); the resting cabinet (C); the UV-tube (D).
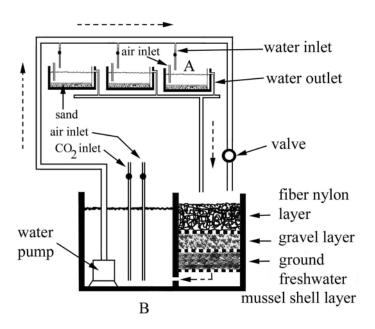
Figure 2.4. Rearing container of 60–120-day juveniles. The culture box (A); the filter cabinet (B); ‒ ‒ ‒ ► = water flow.

Figure 2.5. Photographic of rearing container of 60-120-day juveniles. The culture box (A); the filter cabinet (B); the resting cabinet (C).

A

B
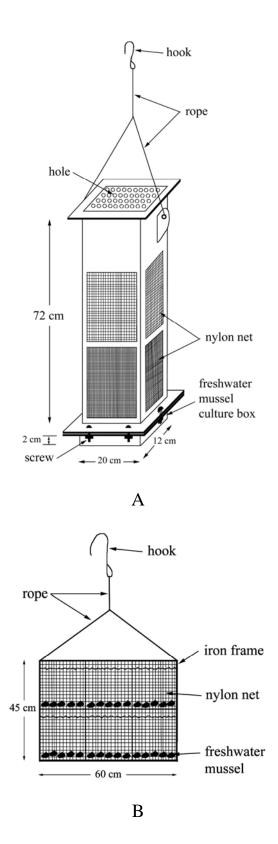
Figure 2.6. Rearing container of 120-360-day juveniles. 120-270 days old (A); 270-360-days old (B).

2.3.4 Water analysis

Water samples from laboratory culture and from the earthen pond were collected and analyzed for water temperature, pH, dissolved oxygen (azide modification), total alkalinity (phenolphthalein methyl orange indicator), free carbon dioxide (titrimetric), total hardness (EDTA titrimetric), total ammonia nitrogen (direct nesslerization), calcium (EDTA titrimetric), orthophosphate (ascorbic acid method) and silica (molybdosilicate method) (APHA, AWWA, WPCF, 1998). The analysis was carried out weekly for laboratory water samples and every other week for water in the earthen pond.

2.3.5 Phytoplankton communities

During the culture of the 120-360-day juveniles, phytoplankton in the earthen pond was collected from a depth of 50 cm using a 10 l Van Dorn sampler container every month, with two replicates. The samples were filtered through a 40 μm mesh size net, preserved with 1% acidic Lugol's solution, and counted under an inverted microscope. Species identification were based on Prescott (1951), Desikachary (1959) and Wongrat (1998, 1999). All samples were examined in triplicate.

2.4 Results

2.4.1 Culture of glochidia

The adult gravid freshwater mussels collected from the Mae Klong River and cultured in the earthen pond successfully produced glochidia during October-May, similar to those in their natural habitats. The average survival of glochidia in the artificial medium was 95±2.5%. All surviving glochidia transformed to juveniles within 8 days.

2.4.2 Selecting phytoplankton food species for juveniles

Ten species of phytoplankton (*Ankistrodesmus gracilis*, *Chlamydomonas* sp., *Chlorella* sp. 1, *Chlorella* sp. 2, *K. incurvata*, *M. irregulare*, *Navicula* sp., *Scenedesmus* sp., *Stichococcus* sp. and *Coccomyxa* sp.) were found in the gastrointestinal tracts of 15 mature *H.* (*L.*) *myersiana* and all grew well in the f/2 media. Four species, namely, *Chlorella* sp. 2, *K. incurvata*, *Navicula* sp., and *Coccomyxa* sp. (Figure 2.7) were selected as the preferred feed due to their size, shape, speed of movement into the gastrointestinal tract, and digestibility, as observed from the feeding behavior and the feces of the juveniles. During the feeding period, it was observed that phytoplankton cells needed to reach the gastrointestinal tract of the juvenile within 90 min, since any further delay caused nutritional deficiency and the juveniles became weak and finally died. As for the size and form of the phytoplankton, it was found that the small, round or oval-shaped cells could enter the juvenile mouth more readily than large and long ones (Figure 2.7). *Chlorella* sp. 2 is

round (4 μm in diameter), *K. incurvata* oval (5×6 μm), *Navicula* sp. elliptical (4×26 μm) and *Coccomyxa* sp. has a short bar shape (2×4 μm). The digestibility of the food was assessed by the changes in form of each species of phytoplankton after passing through the gastrointestinal tract to the anus. In addition, the feces were inspected for phytoplankton remains. The remains of *K. incurvata* were found to be severely transformed from the original shape and form, indicating that it had the best digestibility, followed by those of *Chlorella* sp. 2, *Navicula* sp. and *Coccomyxa* sp.

A

B

C

D
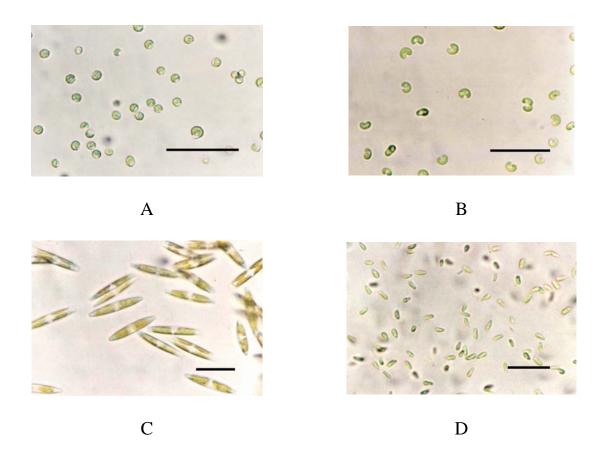
Figure 2.7. Phytoplankton species for culture *H.* (*L.*) *myersiana* juveniles. *Chlorella* sp. 2 (A); *Kirchneriella incurvata* (B); *Navicula* sp. (C); *Coccomyxa* sp. (D). Scale bar = 20 μm.

2.4.3 Culture of *H.* (*L.*) *myersiana* juveniles

2.4.3.1 Culture of 0-60-day juveniles

Having been fed with the mixture of four phytoplankton species for 60 days, the length and height of juveniles had changed from 0.13±0.01 mm to 1.41±0.16 mm and 0.16±0.01 mm to 0.98±0.09 mm, respectively (Figures 2.8A and B). The survival rate of these juveniles over the 60-day period was 8±0.2% (Table 2.1).

2.4.3.2 Culture of 60-120-day juveniles

Culture of 60-day juveniles until 120 days with individual species of phytoplankton and the mixture of four phytoplankton species, respectively, showed that mussels fed exclusively with *K. incurvata* reached the highest average body length (3.46±0.04 mm) and height (1.94±0.04 mm). These results are not significantly different ($P>0.05$) from the growth measurements of mussels fed with the mixture of four phytoplankton species, but those fed with *Chlorella* sp. 2 had significantly ($P<0.05$) lower growth rates (length 2.61±0.22 mm, height 1.53±0.08 mm) (Figures 2.8A and B). Moreover, juveniles fed only with *Navicula* sp. or *Coccomyxa* sp. live only unit 100 days and 90 days, respectively. The survival rates from 60-120 days for the juveniles fed with *K. incurvata*, *Chlorella* sp. 2 and the mixture of four phytoplankton species were 65±8.32, 61±9.43 and 27±17.63%, respectively (Table 2.1).

2.4.3.3 Culture of 120–360-day juveniles

Culture of juveniles in the earthen pond from 120-360 days resulted in changes of average weight from 0.0037±0.002 to 11.24±5.02 g, length from 3.48±0.39 to 54.08±6.21 mm, height from 1.97±0.24 to 25.09±2.48 mm, and width from 0.98±0.06 to 12.28±3.21 mm (Figures 2.9A-D). The average growth rate (weight, length, height and width) was 0.0497±0.01 g/day, 0.2414±0.15 mm/day, 0.0975±0.08 mm/day and 0.0493±0.03 mm/day, respectively (Table 2.1). The survival rate from 120-360 days approached 100%.

2.4.4 Water analysis

The range of physicochemical properties of the water in the culture system and the earthen pond during the 360 days of culturing *H*. (*L*.) *myersiana* juveniles did not differ greatly from those of the natural river water where mussels are found. The results are shown in Table 2.2.

Table 2.1. Average growth rate and survival rate of 0-60-day juveniles of *H.* (*L.*) *myersiana*. Juveniles were fed with a mixture of four phytoplankton species (*Chlorella* sp*.*, *Coccomyxa* sp*.*, *Kirchneriella incurvata,* and *Navicula* sp*.*) at the ratio of 1:1:1:1; 60-120-day juveniles were fed with four separate species of phytoplankton and a mixture of these species at the ratio of 1:1:1:1; 120-360-day juveniles were reared in the earthen pond.

Mussel age (days)	Food type	Average growth rate (±SD)				Survival (%)
		Weight (g/day)	Length (mm/day)	Height (mm/day)	Width (mm/day)	
0- 60	Mixture of four phytoplankton	-	0.021±0.01	-	-	8±0.2
60-120	*Chlorella* sp.	-	0.0207^{a}±0.004	0.0089^{a}±0.002	-	61^{a}±9.43
	Coccomyxa sp.	-	0.0078^{c}±0.004*	0.0025^{c}±0.003*	-	0*
	Kirchneriella incurvata	-	0.0364^{b}±0.002	0.0161^{b}±0.001	-	65^{a}±8.32
	Navicula sp.	-	0.0173^{a}±0.004**	0.0109^{ab}±0.004**	-	0**
	Mixture of four phytoplankton	-	0.0350^{b}±0.0002	0.0162^{b}±0.001	-	27^{b}±17.63
120-360	Food in the natural habitat	0.0497±0.01	0.2414±0.15	0.0975±0.08	0.0493±0.03	100

Remark: Groups with the same superscript letter are not significantly different (*P*>0.05).

* Juveniles fed with *Coccomyxa* sp. only survived to 90 days.

** Juveniles fed with *Navicula* sp. only survived to 100 days.

A **B**
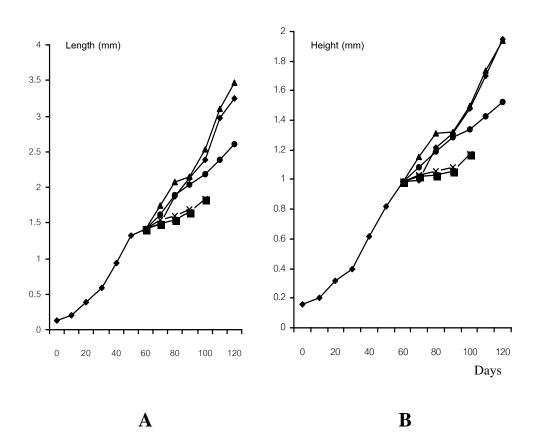
Figure 2.8. Growth of *H.* (*L.*) *myersiana* juveniles, 0-120 days. Average shell length (A) and height (B) of 0-120-day juveniles fed with different phytoplankton, ●: *Chlorella* sp.2, ×: *Coccomyxa* sp., ▲: *Kirchneriella incurvata*, ■: *Navicula* sp., ◆: mixture of four phytoplankton.
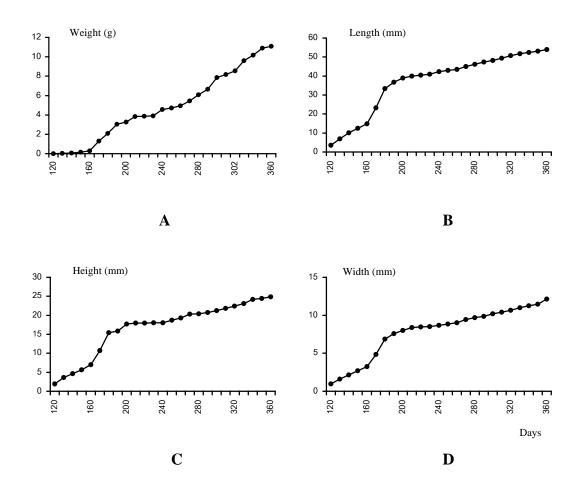
Figure 2.9. Growth of *H.* (*L.*) *myersiana* juveniles, 120-360 days. Average weight (A) and average shell size (length; B, height; C and width; D) of 120-360-day juveniles reared in the earthen pond.

2.4.5 Phytoplankton communities

Of the phytoplankton species found in the earthen pond during the culture of 120-360-day juveniles, 21 taxa were identified, including filament, colony and unicellular types. Three phytoplankton phyla are predominant, namely, Chlorophyta (92.7%), Chromophyta (6.5%) and Cyanophyta (0.8%). The most abundant were

classified as belonging to four genera, namely, *Phacus* (39.7%), *Euglena* (17.4%),

Euastrum (16.7%) and *Pediastrum* (9.1%) (Table 2.3).

Table 2.2. Water quality parameters during culturing of 0-360-day juveniles and the adult mussel habitat of *H*. (*L.*) *myersiana* in the Mae Klong River, Kanchanaburi Province.

| Parameters | Culturing of juveniles in laboratory | | Culturing of juveniles in the earthen pond | Culturing of juveniles | River water* (min. –max.) |
	0 - 60 days old (mean±SD)	60 - 120 days old (mean±SD)	120 -360 days old (mean±SD)	0 – 360 days old (min. – max.)	
Water temperature (° C)	25.0±0.74	28.0±0.54	26.5±1.1	24.5-28.5	23.8-31.6
pH	7.03±0.02	7.51±0.04	7.65±0.46	6.85-8.08	6.92-8.14
Dissolved oxygen (ppm O_2)	8.1±0.07	7.5±0.04	5.0±0.6	4.2-8.2	2.5-9.0
Alkalinity (ppm $CaCO_3$)	52.0±1.41	52.75±0.35	83.7±20.3	50.0-114.0	62.5-115.0
Free carbon dioxide (ppm CO_2)	10.0±0.04	4.25±0.35	3.95±2.7	0-10.2	0-6.0
Hardness (ppm $CaCO_3$)	154.0±2.83	123.0±9.9	196.8±12.6	121.0-222.0	90.0-133.0
Ammonia nitrogen (ppm NH_4-N)	0.42±0.02	0.28±0.01	0.44±0.19	0.20-0.82	0.22-0.88
Calcium (ppm $CaCO_3$)	139.0±9.90	89.0±4.24	101.1±5.1	86.0-142.0	65.0-105.0
Phosphate (ppm PO_4-P)	0.12±0.06	0.19±0.07	0.17±0.1	0.01-0.45	0.08-0.88
Silica (ppm SiO_2)	4.85±0.6	4.05±0.5	5.75±1.6	3.0-8.0	0.2-5.5

Remark: * = Kovitvadhi et al. (1998)

Table 2.3. List of algal taxa recorded and their abundance of the phytoplankton species (cells/l) through culturing of 120-360-day juveniles in the earthen pond.

Algal taxa	Size (µm)	Abundance of the phytoplankton species (cells/l)									%
		Aug. 02	Sep.	Oct.	Nov.	Dec.	Jan. 03	Feb.	Mar.	Apr.	
Division Cyanophyta											
Anabaena sp.	3×15	0.02	0	0.001	0	0.04	0	0.01	0.002	0.004	1.9
Oscillatoria sp.	3×25	0.01	0.02	0.03	0.02	0.02	0.01	0.02	0.03	0.01	2.9
Spirulina sp.	4×11	0.002	0.01	0.05	0	0.01	0.002	0.002	0	0.001	1. 7
Subtotal		**0.04**	**0.03**	**0.09**	**0.02**	**0.07**	**0.01**	**0.03**	**0.03**	**0.02**	**6.5**
Division Chlorophyta											
Acanthosphaera sp.	25	0.7	0.8	0.7	0	0	2.1	5.6	2.4	0.8	0.3
Closterium sp. *	11×80	0	0	0	0	0	2.1	0	0.7	0.7	0.2
Euastrum sp.*	25×52	43.1	32	273.6	234.1	252.1	37.2	16.0	45.2	112	16.7
Eudorina sp. *	12	11.8	0	14.6	2.5	0	2.8	0	1.2	4.8	1.0
Euglena sp. *	5×70	58.3	65.8	105.6	239.4	316.7	55.6	11.8	95.9	105	17.4
Oedogonium sp. *	18×40	0.7	0	0	0	0	0	0	0.7	0	0.1
Oöcystic sp. *	8×10	14.6	0	0.7	0.7	0	0	1.4	0	2	0.7
Pediastrum sp. *	20	173.6	65.4	13.9	23.1	5.95	9.1	63.9	93.8	38.7	9.1
Phacus sp. *	24×28	754.2	218.3	204.9	243.8	369.5	9	41.0	288.9	248.7	39.7
Scenedesmus sp. *	4×15	78.6	23.6	15.3	5.2	3. 5	9.1	29.9	14.6	12.5	3.2
Sphaerocystis sp. *	20	10.4	21.3	129.9	1.4	5.6	3. 5	1.4	2.1	5.6	3.1
Strombomonas sp.	22×38	25	0.7	0.7	0	2.8	0	7.0	3.5	0.7	0.9
Ulothrix sp.	5×15	5.5	0	0	0	0	0	0.7	0	0	0.5
Subtotal		**1.2**	**0.4**	**0.8**	**0.8**	**1**	**0.1**	**0.2**	**0.6**	**0.5**	**92.7**
Division Chromophyta											
Ceratium sp.	42×34	0.02	0	0.01	0	0.01	0	0.01	0	0.01	0.2
Diatoma sp. *	4×15	0	0.01	0	0	0	0	0.01	0	0.01	0.1
Fragilaria sp.	3×25	0.01	0.01	0	0	0.01	0	0	0.01	0	0.1
Gyrosigma sp.	5×25	0.01	0	0	0.01	0	0	0.01	0	0.01	0.1
Surirella sp. *	60×120	0	0.03	0	0	0	0.01	0	0.01	0.01	0.3
Subtotal		**0.05**	**0.04**	**0.01**	**0.01**	**0.01**	**0.01**	**0.02**	**0.02**	**0.04**	**0.8**
Total		**0.28**	**0.83**	**0.82**	**0.81**	**1.12**	**0.25**	**0.35**	**0.75**	**0.86**	**100**

Remark: * = the same phytoplankton species found in the gastrointestinal tract of

adult *H.* (*L.*) *myersiana* in natural habitat (Kovitvadhi et al., 2000)

2.5 Discussion

2.5.1 Culture of glochidia

Keller and Zam (1990) succeeded in culturing glochidia of the freshwater mussel, *Utterbackia imbecillis,* with a high transformation percentage (81.8%) using a complex artificial medium described by Isom and Hudson (1982), with added fish plasma. An alternative, more simple medium (M199) with horse serum supported a lower transformation percentage (65.4%) (Keller and Zam, 1990). Subsequently, Kovitvadhi et al. (2002) cultured glochidia of *H.* (*L.*) *myersiana* in M199 with fish plasma (common carp, *Cyprinus carprio*). The survival rate of 150-200 glochidia / 3.5 ml of culture medium was 93±3.0%, of which 100% transformed to juveniles within 10 days at about 23° C. With the present study, we have made an improvement by culturing a large population of 5,000-6,000 glochidia in 30 ml of artificial medium, giving a higher survival rate (95±2.5%) and an earlier transformation (8 days) at 25° C. A further modification to previous culture methods is the replacement of the culture medium with fresh medium at days 3 and 6, thus allowing the glochidia to have greater access to food. The higher culture temperature, a continuous supply of 5% CO_2 and the stimulation provided by sterilized distilled water, may also have shortened the transformation period by 2 days.

2.5.2 Phytoplankton selection for culturing juveniles

Phytoplankton has proven to be a vital source of nutrient for several species of freshwater mussel juveniles (Hudson and Isom, 1984; Gatenby et al., 1996; Gatenby et al., 1997; O'Beirn et al., 1998; Tankersley and Butz, 2000; Henley et al., 2001; Uthaiwan et al., 2001). Similarly, Kovitvadhi et al. (2000) reported that phytoplankton contributed to 99% of the gastrointestinal tract content of the adult freshwater mussel, *H*. (*L.*) *myersiana*. This finding is consistent with gut content analyses from other bivalve species (Gale and Lowe, 1971; Huca et al*.,* 1983; Paterson, 1986; Parker et al., 1998). Consequently, phytoplankton from the gastrointestinal tract of adult *H*. (*L.*) *myersiana* were cultured and selected for juvenile feeding. Since the size and shape of phytoplankton also determine the ease of their passage into the gastrointestinal tract (Gatenby et al., 1996; Gatenby et al., 1997; O'Beirn et al., 1998; Henley et al., 2001; Uthaiwan et al., 2001), the morphological features of phytoplankton were taken into account. Selection of suitable phytoplankton food species was based on size, morphology, digestibility, and appropriate co-ordination of cilia around the foot, mantle and gill to move each type of phytoplankton. Kovitvadhi et al. (2001) found that the gills of the early juvenile *H*. (*L.*) *myersiana* (<13 days old) were not fully developed for filtering food. In the present study, it was observed that early juveniles (0-40 days) have to use cilia around the foot, the mantle and gill to move phytoplankton into the mantle cavity and ultimately to the mouth. We describe this type of food intake at the juvenile stage as "pedal-mantle-gill-feeding", rather than "pedal-feeding" as used by other research groups (Reid et al., 1992; Yeager et al., 1994; Gatenby et al., 1997). This type of feeding behavior is also different from

"suspension-feeding" at the more mature stage of mussel development where an incurrent siphon, gill and labial palp are involved in selective intake of food.

2.5.3 Culture of 0-360-day juveniles

The high survival rates (65±8.32%) and large shell size of juveniles fed with *K. incurvata* during the 60-120-day period suggests that this is the most promising phytoplankton species for use as feed for the culture of *H.* (*L.*) *myersiana*. Although the mixture of four phytoplankton species gave good results for the first 60 days of culture, the survival rate with this mixture over the 60-120-day period was markedly lower (27.5±17.63%). An attempt to use *K. incurvata* alone as feed, from day 0 to day 120, should be made to increase the survival rate after 60 days. From our observations, the first 30 days are the most sensitive of juvenile life, so that the low survival rate during this stage was expected. A similar observation was made by Hudson and Isom (1984) who observed juveniles of *U. imbecillis* in their natural habitat.

Attempts to culture juveniles of *U. imbecillis* using lake water with different species of phytoplankton were made by Hudson and Isom (1984), while Uthaiwan et al. (2001) raised *H.* (*L.*) *myersiana* juveniles using dechlorinated tap water supplemented with *Chlamydomonas* sp., *Monoraphidium* sp. and *Chlorella* sp. as food. However, these groups were only able to raise the juveniles for 74 days and 60 days, respectively, reaching maximum lengths of 5.1 mm and 2.4 mm, respectively. The 0-60-day *H.* (*L.*) *myersiana* juveniles in this study grew to the maximum length of 3.46 mm and could be reared to maturity with little or no mortality. It should be noted here that the juveniles used in the experiments of Hudson and Isom (1984), Uthaiwan et al.

(2001), and in this study, were developed from glochidia which had been cultured in artificial media, while Gatenby et al. (1997) cultured *Villosa iris* juveniles derived from parasitic glochidia on host fish. They could rear them to 60 days, to maximum lengths of 450 µm, and the survival rates at that stage was 66.5%.

Transferring 120-day juveniles to the earthen pond until they reached 360 days enabled us to observe and record in detail the development of these mussels. Although the overall weight and size of juveniles increased throughout the culture period, their sizes (length, height, width) changed most markedly during the 120-180-day period, while the weight increase was most prominent during the 280-360-day period, when it reached an average of 0.0633 g/day (Figure 2.9A and Table 2.1).

Adding sand to the culture unit of 0-270-day juveniles to a depth of 3-5 mm appears to improve survival rates. The sand may help grind the food in the gastrointestinal tract. The same effect was reported by Isom and Hudson (1982, 1984), for *U. imbecillis*, and Buddensiek (1995) for *Margaritifera margaritifera*.

2.5.4 Water analysis

Water quality of the juvenile culture medium at two different stages (0-60 days and 60-120 days) of the freshwater mussel, *H.* (*L.*) *myersiana*, was comparable to that in their natural habitat (Kovitvadhi et al., 1998), except for the values of free carbon dioxide, hardness and calcium. The high values of water hardness and calcium found in the water of the 0-60-day juvenile culture could result from the higher level of free carbon dioxide due to the recirculation of carbon dioxide under closed culture conditions depressing the pH, so that calcium from ground mussel shell in the filtration unit could readily dissolve and raise the calcium value and hence water

hardness. Water samples from the earthen pond during the culture of 120-360-day juveniles were found to have similar qualities to that of the mussels' natural habitat. However, the value of free carbon dioxide, hardness and silicate were higher.

2.5.5 Phytoplankton communities

Gale and Lowe (1971), Huca et al. (1983), Binhe (1984), Paterson (1986) and Kovitvadhi et al. (2000) found more phytoplankton than zooplankton in the gastrointestinal tract of adult freshwater mussels. They demonstrated that most phytoplankton were those of green algae, of unicellular or small colony types, long filament algae being rarely found. Kovitvadhi et al. (2000) reported that the relative abundance of phytoplankton species within the gastrointestinal tract of adult *H.* (*L.*) *myersiana* from the Mae Klong River, Kanchanaburi Province, corresponded to their prevalence in the environment. According to the present study, the main phytoplankton species in the earthen pond related closely to the species in the gastrointestinal tract contents of adult *H.* (*L.*) *myersiana*. Consequently, the 120-360-day juveniles reared in the earthen pond were able to grow vigorously and achieve survival rates approaching 100%.

Chapter 3

A Laboratory-Scale Recirculating Aquaculture System

for Juveniles of Freshwater Pearl Mussel

***Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856)**

3.1 Abstract

Growth and survival rates of juvenile freshwater pearl mussels *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856) were compared at 0-120 days when reared in two closed recirculating aquacultural systems. System I was composed of a glass aquarium with a filter cabinet (combination of pebbles, ground freshwater mussel shells and nylon fiber), a UV-tube, a resting cabinet, and a plastic culture unit. The system II was composed of 5 cabinets: a particulate filter cabinet, a macrophyte (*Limnophila heterophylla*) filter cabinet, a biological filter cabinet, a water resting cabinet and plastic culture units. Water flowed through the juvenile culture units at 20 ml/min in both systems. In each system juveniles were stocked at day 0 with sand at <120 μm and were fed twice a day on a 1:1 mixture of *Chlorella* sp. and *Kirchneriella incurvata*. Over the 120 days, average growth rate per day and rate of survival were higher in System II. Free carbon dioxide, total ammonia nitrogen, nitrate, phosphate and silica of second system were significantly lower in system II. The relationship between shell length (*L*) and age of the freshwater pearl mussels cultured in system II was $L = 0.6164 - 0.0809 \text{ Day} + 0.0032 \text{ Day}^2 - 1 \times 10^{-5} \text{ Day}^3$, $R^2 = 0.983$.

3.2 Introduction

Hyriopsis (*Limnoscapha*) *myersiana* (Lea, 1856) is a freshwater pearl mussel endemic to Thailand. At present it has a decreasing population, so culture is considered to be important in order to provide material for nacre inlays for furniture, nuclei for the cultured pearl industry and data to assist conservation measures.

Freshwater pearl mussel culture can be divided into the three stages of the life cycle: the parasitic glochidia larval stage, juveniles and adults. Only a small amount of data on freshwater juvenile biology and culture is available; juvenile survival from culture in artificial media has been assessed in only three groups of research experiments under laboratory conditions (Hudson and Isom, 1984; Uthaiwan et al., 2001; Kovitvadhi et al., 2006). Only Kovitvadhi et al., (2006) succeeded in culturing glochidia of *H.* (*L.*) *myersiana* to adulthood. However, early juveniles (0-60 days) cultured with a mixture of four phytoplankton species (*Chlorella* sp., *Kirchneriella incurvata*, *Navicula* sp. and *Coccomyxa* sp.) had a survival rate of only 8±0.2%. The cause of low survival rate is due to a higher total ammonia nitrogen of the water quality under laboratory conditions than in the mussels' natural habitat equal to 0.42; ammonia nitrogen must be removed from the culture water, as it is toxin. Glochidia and juvenile mussels are more sensitive to some chemicals such as copper, ammonia and chlorine when compared to commonly tested aquatic organisms (Wang et al., 2007).

Many species can be successfully grown and have high survival rates in recirculating (closed) aquaculture systems (RAS) due to the high-quality culture water. Therefore, RAS have been used extensively for rearing and maintaining adult and juvenile marine bivalves in captivity (Epifanio et al., 1974; Spotte, 1979; MacMillan et al., 1994), and have also been used for some juvenile and adult freshwater mussels (Coker et al., 1921; Gatenby et al., 1996; Dunn and Layzer, 1997; O'Beirn et al., 1998; Henley et al., 2001; Kovitvadhi et al., 2006). Therefore, the objective of the present study was to increase survival rate and growth of the freshwater pearl mussel at the juvenile stage (0–60 days) by a comparison of two

culture systems: system I, used by Kovitvadhi et al. (2006) and system II, using a biological filter (macrophytes and bioball) in the filter system. The water quality in these two systems was compared in order to ascertain the suitable water quality for culturing this freshwater pearl mussel.

3.3 Materials and methods

3.3.1 System design and components

Growth and survival rates of juvenile freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856), were compared in two closed recirculating systems. System I (Figures 2.2 and 2.3) was the system adopted by Kovitvadhi et al. (2006) for culturing juveniles of 0–60 days. This system consisted of a particulate filter cabinet (Length×Width×Height×Water level =50×46×35×30 cm) was divided into two equal section, resting cabinet (50×26×35×30 cm) and plastic culture unit (20×11×8×7 cm). Water from the culture unit flowed into the first section of the filter cabinet, which was composed of three layers: a nylon filter layer, a gravel layer and a layer of ground freshwater mussel shells, then flowed to the second section. Filtered water flowed through a UV tube and collected in the resting cabinet, whence it flowed into the culture unit at 20 ml per minute. The inside of the culture unit was divided into two sections. The first section (18×11×8×7 cm) was to put sand and juveniles. This sand was collected from the natural habitat of *H.* (*L.*) *myersiana*, passed through a 120-μm mesh, washed several times with tap water and oven dried at 180° C for 24 h. Then the water from the first section flowed through the screen (120-μm mesh) to the

second section (2×11×8×7 cm) which there was no sand and juveniles for trapping juveniles and at the end of the second section was an outlet for overflow water to collect into the particulate filter cabinet via a trough. The water circulation was turned off for 1 h during feeding. System II (Figures 3.1 and 3.2) comprised three filter cabinets made of 6 mm thick acrylic (particulate filter cabinet, macrophytes filter cabinet and biological filter cabinet), one water resting cabinet and nine plastic culture units. The particulate filter cabinet (46×35×51×42 cm) was divided into two equal parts, of which the first part was filled with a 30 cm thick nylon filter. Water flowed through this filter and via the second part to the macrophytes filter cabinet (80×40×51×42 cm) which was divided into four equal units. Each unit contained 57 ambulia plants, *Limnophila heterophylla* (Raxb.) Bentham; these, 228 plants in total, were introduced when they were 6 cm in height and had an average weight of 2.69±0.13 g. The plants were removed and replaced when their tips reached the water surface. The upper parts of the cabinets were equipped with three fluorescent lamps (each 20 W) 25 cm above the water surface (light intensity at the water surface, 5320 lux; 24 h). The water then flowed into the biological filter cabinet (60×34×51×42 cm) filled with BioBall to full capacity, and then to the resting cabinet (46×41×51×42 cm). In the resting cabinet there were two water pumps: the first returned water to the particulate filter cabinet at the rate of 1 l per minute continuously and the second pumped water at 20 ml per minute to nine plastic culture units (each 84×14×15×7 cm). This pump was stopped for 1 h after phytoplankton was introduced into the culture unit. The bottom of the culture unit was filled with sand at 0.27 g/cm^2. The preparation of sand was the same as for system I. The inside of the culture unit was divided into two sections, as described previously, but of different sizes (section 1-

66.1×14×15×7 cm; section 2- 17.9×14×15×7 cm). The first section in this experiment also consisted of five acrylic sheets jutting from the walls on alternate sides.

3.3.2 Preparation of juvenile mussels

Fully grown adult male and female freshwater pearl mussels, *H. (L.) myersiana*, were collected in September 2005, at Mae Klong River (13°57′30″N; 99°45′00″E), Kanchanaburi Province, and held in an earthen pond ($\approx$8,000 m^2) on Department of Aquaculture, Faculty of Fishery, Kasetsart University, culture in November 2005. Mature glochidia were sucked from gravid females to culture in artificial medium according to Kovitvadhi et al. (2006). The 0-day-old juveniles that developed were released into the two culture systems at a density of 4 juveniles per sq cm (790 and 3,700 juveniles per unit, in total 2,370 and 33,300 juveniles per system, respectively).

3.3.3 Food and feeding

Juveniles were fed *Chlorella* sp. and *Kirchneriella incurvata* over the course of the experiment. These two species were purified from the digestive tract of freshwater pearl mussels from the Mae Klong River by the streak plate technique (Hoshaw and Rosowski, 1973) on solid mixed f/2 medium (Guillard and Ryther, 1962), and placed under fluorescent lamps at 10,000 lux intensity for 18 h/day. Then the algae were separated to culture in liquid f/2 medium in pointed test tubes of 250 ml volume for 7 days under fluorescent lamps for 18 h/day and 3% carbon dioxide

53

mixed with air (24 h). They were then increased to 1 l volume by culturing in pointed test tubes under the same conditions of light and air for 5 days and then culturing for increased volume in plastic tanks (100 l) in the open air with f/2 medium and continuous aeration for 5 days.

Each species of alga was collected from the 100 l by being pumped through 0.3 μm ceramic filters and then separated from the water by centrifuging at $8,000 \times g$. The sediments of the two algal species were mixed at a ratio of 1:1 wet weight and kept in a freezer. When required, the mixture was brought to room temperature then sucked by Pasteur pipette into the all plastic culture unit in both system to an algal density of 1×10^5 cells per ml. Algae were supplied twice a day (06.00 h and 18.00 h), and the frozen stock was usually used within 7 days of collection.

3.3.4 Water analysis

The water used in culturing juveniles was free from chlorine. In both systems the following water quality parameters were analyzed every 10 days: water temperature (Hg thermometer), pH (pH meter), dissolved oxygen (azide modification), total alkalinity (phenolphthalein methyl orange indicator), free carbon dioxide (titration), total hardness (EDTA titration), total ammonia nitrogen (phenate method), calcium (EDTA titration), nitrite (colorimetry), nitrate (cadmium reduction), orthophosphate (ascorbic acid method) and silica (molybdosilicate method) (APHA, AWWA, WPCF, 1998).
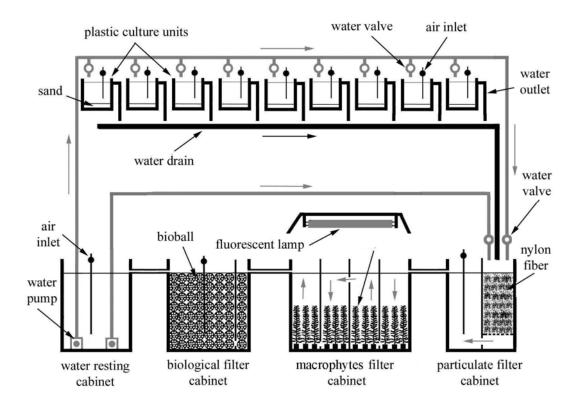
Figure 3.1. Schematic diagram of the recirculating system II used to rear freshwater pearl mussel juveniles (0–120 days).

55

Figure 3.2. Photographs of the aquaculture system II used to rearing freshwater mussel juvenile (0-120 days old). Close recirculation system (A); Particulate filter cabinet (B); Macrophytes filter cabinet (C); Biological filter cabinet (D); Reservoir tank (E); Juvenile culture box (F); Fluorescent box (G).

3.3.5 Statistical analysis

The mussels were sampled by isolate juvenile form sand with screen (120-μm mesh) every 10 days for growth during the experiment was n=50 from each culture unit. Growth of juveniles was assessed by recording increments of shell size (shell length and shell height). Juveniles were measured using a light microscope with a calibrated ocular micrometer to the nearest 0.01 mm. Growth rate were calculated as average growth rate in mm per day = (average shell length or average shell height at the end of every 10 days-average shell length or average shell height at before 10 days/total growth period in days (10 days).

Survival was calculated using the average number of living juveniles at the beginning of the experiment and at the end of every 10 days.

Group comparison (t-test) was used to compare values between two systems for growth rate, survival and water quality in different every 10 days.

The coefficient of correlation (r) of linear regression was used in relationship of water quality and survival rates or shell size (length and height) which was calculated by using averages of water quality characteristics with averages of survival rates and average shell size (shell length and shell height) throughout experiment.

The relationship between the shell size and age was expressed by the equation:
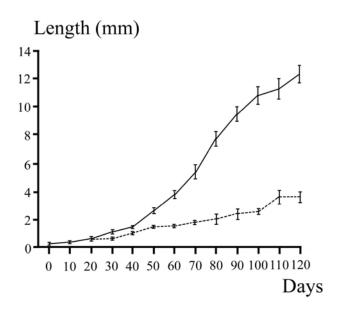
$$Y = b_0 + b_1X + b_2X^2 + b_3X^3$$

where Y is the shell size (shell length or shell height in mm), X is age (days), and b_0, b_1, b_2 and b_3 are parameters. The all group comparison and regressions analysis was used the statistical program SPSS (SPSS Inc,).

3.4 Results

3.4.1 Growth and survival of cultured juveniles

Growth and survival rate of 0–10-day-old juveniles of freshwater pearl mussels did not differ between systems I and II; however, for 10–120-day-old juveniles there were significant ($P<0.05$) and highly significantly ($P<0.01$) differences between the two recirculating systems. Shell length changed from 0.19±0.01 mm to 3.45±0.40 mm in system I and 12.17±0.68 mm in system II, and shell height changed from 0.22±0.01 mm to 1.89±0.23 mm in system I and 6.33±0.41 mm in system II (Figure 3.3). Shell length and height of juveniles cultured in each system were significantly different ($P<0.05$) from 30 to 120 days. Average growth rates calculated from shell lengths and shell heights were 0.03±0.003 mm/day and 0.01±0.002 mm/day for system I, and 0.10±0.006 mm/day and 0.05±0.003 mm/day for system II, respectively (Table 3.1). Survival rates in culture were calculated up to 120 days. Juveniles in system II had a higher survival rate (82.74±1.47%) than in system I (12.45±2.14%) (Table 3.1), with differences between the two systems being significant ($P<0.05$) and highly significantly ($P<0.01$) from 20 to 30 days and 40 days onward, respectively.

Figure 3.3. Development of *H*. (*L.*) *myersiana* juveniles. Average shell length (±SD) and height of 0-120-day-old juveniles cultured in systems I (------) and II (——).

Table 3.1. Average growth rate and survival rate of 0–120-day-old juveniles of *H.*

(*L.*) *myersiana* cultured in systems I and II.

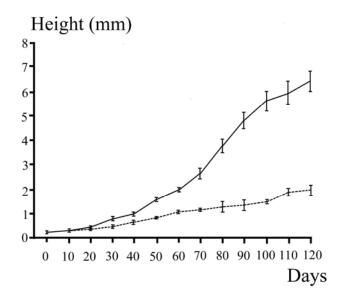| Mussel age (days) | Growth rate (average±SD) | | | | | | Survival (%) | | | |
| | Length (mm/day) | | | Height (mm/day) | | | | | | |
	System I	System II	Sig.	System I	System II	Sig.	Days	System I	System II	Sig.
0-10	0.02±0.003	0.02±0.002	*ns*	0.01±0.002a	0.01±0.002b	*ns*	10	90.57±3.25	98.54±2.29	*ns*
10-20	0.01±0.006a	0.02±0.006b	*	0.0003±0.004a	0.01±0.005b	**	20	87.42±0.27a	96.44±1.24b	*
20-30	0.01±0.007a	0.05±0.007b	**	0.01±0.005a	0.03±0.007b	**	30	77.01±0.13a	95.67±1.09b	*
30-40	0.03±0.015a	0.04±0.009b	**	0.019±0.006a	0.021±0.009b	*	40	62.42±2.04a	94.53±2.14b	**
40-50	0.04±0.009a	0.18±0.026b	**	0.02±0.006a	0.06±0.014b	**	50	39.72±3.44a	92.40±3.15b	**
50-60	0.01±0.007a	0.11±0.031b	**	0.02±0.004a	0.04±0.012b	**	60	29.55±3.56a	91.54±3.24b	**
60-70	0.02±0.009a	0.16±0.052b	**	0.01±0.007a	0.06±0.026b	**	70	25.82±2.42a	90.22±2.54b	**
70-80	0.02±0.040a	0.24±0.071b	**	0.01±0.023a	0.11±0.037b	**	80	22.42±2.61a	89.24±2.13b	**
80-90	0.04±0.031a	0.17±0.053b	**	0.01±0.016a	0.10±0.041b	**	90	19.75±3.25a	88.56±3.21b	**
90-100	0.01±0.058a	0.13±0.068b	**	0.01±0.029a	0.08±0.054b	**	100	17.84±2.44a	85.39±2.14b	**
100-110	0.04±0.084a	0.10±0.055b	**	0.03±0.059a	0.04±0.022b	**	110	15.62±1.85a	84.61±1.22b	**
110-120	0.0001±0.006a	0.10±0.068b	**	0.01±0.032a	0.05±0.057b	**	120	12.45±2.14a	82.74±1.47b	**
0-120	0.03±0.003a	0.10±0.006b	*	0.01±0.002a	0.05±0.003b	**				

Different letters at each age within each system denote significantly different values
(*P*<0.05).
* = *P*<0.05, ** = *P*<0.01, *ns* = not significant difference, *P*>0.05.

3.4.2 Water quality

Water temperature and dissolved oxygen did not significantly (*P*>0.05) differ

between the two systems, with ranges of 27.4-28° C and 7.1-7.6 ppm O_2. Total

ammonia nitrogen, free carbon dioxide, nitrate, phosphate and silica of system I

increased and were significantly (*P*<0.05) greater than in system II. Total alkalinity

and total hardness of water in system I were also higher than in system II. Free carbon

dioxide, pH, total ammonia nitrogen, phosphate, and silica were not significantly different between the two systems at an early culture stage (0–30 days) but there were significant differences ($P<0.05$) at the end of the experiment (Figure 3.4). Highly significant positive correlations with survival indicated that pH, total alkalinity, and calcium were the most consistently important water quality factors in each system. These were followed by free carbon dioxide and nitrite, which had significant negative correlations with survival in each system. Whereas total ammonia nitrogen, nitrate, phosphate and silica showed significant negative correlations with survival in system I, none of these variables was correlated with survival in system II except silica. Correlations between water quality and growth (shell length and shell height) in both systems were similar to those between water quality and culture system in shell growth. Growth was significantly negatively correlated with pH, total alkalinity, and calcium in both systems (Table 3.2). As a result of the water quality in system II suitable to cultured juveniles, they had higher survival rate and growth development than in system I. Summary of system composition, stocking density, survival rate, growth rate and water quality in system I and II shown in Table 3.3.

3.4.3 Macrophytes

In system II the average initial weight was 2.69±0.13 g/plant. When the plant tips touched the water surface, the ambulia plants were replaced; this occurred 7 times, with an average of 15.14±0.64 days per cycle and an average weight increase of 12.07±0.02 g/plant (about a 4.5 fold increase).

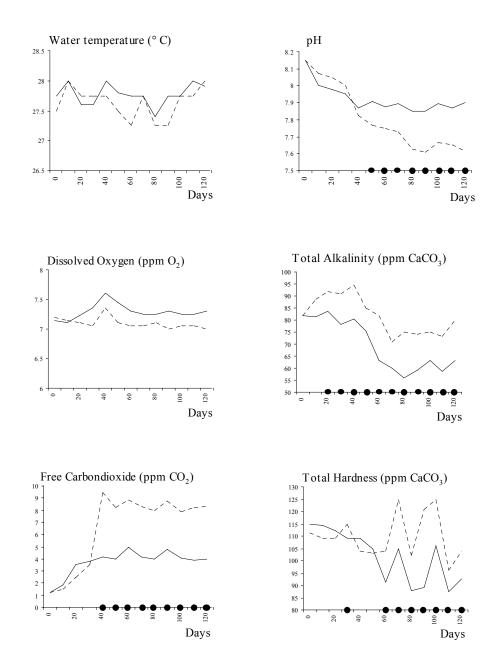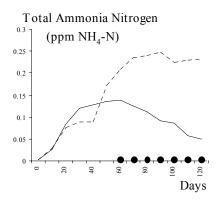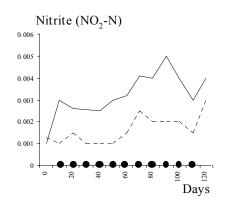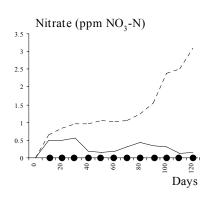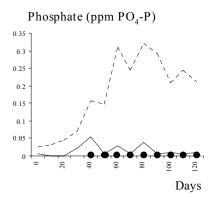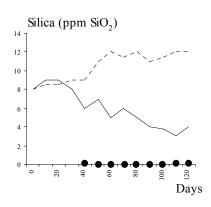**Figure 3.4.** Water quality during culture for 0–120 days of *H.* (*L.*) *myersiana* in system I (-----) and system II (——). Dots on the "Days" axes indicate that water quality in systems I and II was significantly different ($P<0.05$).
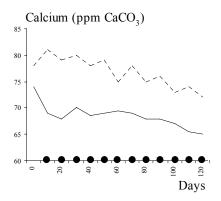
Figure 3.4. (Continued)

63

Table 3.2. Coefficient of correlation between average survival rate and water quality;
average growth rate and water quality of juvenile *H.* (*L.*) *myersiana* cultured in
systems I and II every 10 days.

Parameter	Survival		Shell length		Shell height	
	System I	System II	System I	System II	System I	System II
Water temperature	0.237^{ns}	-0.093^{ns}	0.030^{ns}	0.075^{ns}	-0.012^{ns}	0.107^{ns}
pH	0.983**	0.716**	-0.881**	-0.597*	-0.905**	-0.590*
Dissolved oxygen	0.568*	-0.118^{ns}	-0.559*	-0.055^{ns}	-0.579*	-0.035^{ns}
Total alkalinity	0.745**	0.841**	-0.685**	-0.849**	-0.726**	-0.827**
Free carbon dioxide	-0.874**	-0.634*	0.706**	0.481^{ns}	0.729**	0.476^{ns}
Total hardness	-0.001^{ns}	0.769**	-0.144^{ns}	-0.764**	-0.104^{ns}	-0.751**
Total ammonia nitrogen	-0.982**	-0.152^{ns}	0.849**	-0.051^{ns}	0.885**	-0.061^{ns}
Nitrite	-0.664**	-0.716**	0.676**	0.709**	0.722**	0.688**
Nitrate	-0.771**	0.203^{ns}	0.928**	-0.200^{ns}	0.911**	-0.218^{ns}
Phosphate	-0.903**	0.003^{ns}	0.711**	-0.085^{ns}	0.762**	-0.091^{ns}
Silica	-0.968**	0.914**	0.849**	-0.913**	0.888**	-0.091**
Calcium	0.797**	0.817**	-0.862**	-0.751**	-0.882**	-0.761**

(* = *P*<0.05, ** = *P*<0.01, *ns* = not significant difference, *P*>0.05)

Table 3.3. Summary of system composition and other in system I and II.

Item	System I	System II
Water volume (l)/each unit		
Culture unit	1.54	8.23
Number of culture unit/system	3	9
Particulate filter cabinet	69.0	67.62
UV tube	1.0	-
Macrophytes filter cabinet	-	134.4
Biological filter cabinet	-	85.68
Water resting cabinet	39.0	79.21
Total water volumes	113.62	440.98
Total mussel/system	2,370	33,300
Mussel/l	20.86	75.51
Survival rate (average±SD)	12.45±2.14	82.74±1.47
Growth rate of shell length of 0-120 day juveniles (mm/day±SD)	0.03±0.003	0.1±0.006
Water quality (average±SD)		
Water temperature (° C)	27.6±0.19	27.8±0.17
pH	7.81±0.05	7.92±0.02
Free carbon dioxide (ppm CO_2)	6.52±0.6	3.73±0.2
Dissolved oxygen (ppm O_2)	7.10±0.06	7.29±0.1
Total alkalinity (ppm $CaCO_3$)	81.79±1.1	69.5±1.2
Total hardness (ppm $CaCO_3$)	109.85±2.1	101.85±2.1
Total ammonia nitrogen (ppm NH_4-N)	0.159±0.01	0.089±0.01
Nitrite (ppm NO_2-N)	0.0016±0.001	0.0032±0.001
Nitrate (ppm NO_3-N)	1.331±0.14	0.289±0.19
Phosphate (ppm PO_4-P)	0.178±0.015	0.015±0.004
Silica (ppm SiO_2)	10.46±0.7	5.98±0.1
Calcium (ppm $CaCO_3$)	76.8±1.68	68.5±0.88

3.4.4 Length-at-age and height-at-age curves
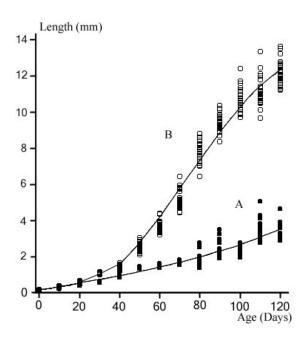
Growth was greater in system II than in system I, as a result of a more rapid increase in growth rate with increasing age (Figure 3.5). The age and size relationships of freshwater pearl mussel for both culture systems are summarized in Table 3.4.

Table 3.4. Relationships of age with shell length and age with shell height in *H.* (*L.*) *myersiana* juveniles (0-120 days) in systems I and II.

Regression	b_0	b_1	b_2	b_3	R^2
Age and Length (*n*=390)					
System I	0.1474	0.0212	-5×10^{-5}	9.3×10^{-7}	0.926
System II	0.6164	-0.0809	0.0032	-1×10^{-5}	0.983
Age and Height (*n*=390)					
System I	0.1960	0.0083	6.5×10^{-5}	-1×10^{-7}	0.935
System II	0.3954	-0.0313	0.0014	-6×10^{-6}	0.978

Regression equation : Shell size = $b_0 + b_1 \text{Day} + b_2 \text{Day}^2 + b_3 \text{Day}^3$,

R^2 = coefficient of determination.
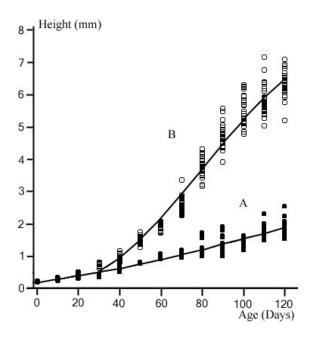
Figure 3.5. Relationship curves between age and shell size (length and height) in systems I (A) and II (B).

3.5 Discussion

Generally, the larval stage of aquatic animals is more sensitive to environmental conditions than the adult stage and this is true for the freshwater pearl mussel in its juvenile stage (Gosling, 2003). This may be because the juvenile shell is not able to close completely against unfavorable environmental conditions or predators, since the foot is of relatively larger size than in the adult. In addition, 0–50-day-old juveniles have not yet completely developed the organs necessary for ingesting food, namely the incurrent and excurrent siphon and gills with fingerlike paired lobes (Hudson and Isom, 1984; Uthaiwan et al., 2001; Kovitvadhi et al., 2006). Juveniles began to close their shells completely at 40 days, and their organs were fully developed after >50 days, in the study by Kovitvadhi et al. (2006). Therefore, the culture system and quality of water used to rear the juvenile stage must provide suitable conditions for food ingestion and digestion.

Several culture systems have been assessed, but mostly a recirculating system has been used. Water used for rearing may come from a natural water resource or from a dechlorinated supply. Phytoplankton has been a vital source of nutrients in the culture of early juvenile *H*. (*L*.) *myersiana,* (Kovitvadhi et al., 2006; Uthaiwan et al., 2001, and the present study), and other freshwater mussel species (Hudson and Isom, 1984; Gatenby et al., 1996; 1997; O'Beirn et al., 1998; Tankersley and Butz, 2000; Henley et al., 2001; Lima et al., 2006). Phytoplankton should have the size and shape to easily pass into the gastrointestinal tract so that the juveniles can digest them (Gatenby et al., 1996; 1997; O'Beirn et al., 1998; Henley et al., 2001; Uthaiwan et al., 2001; Kovitvadhi et al., 2006), and should also be appropriate for the co-

ordination of cilia around the foot, mantle and gill to move the phytoplankton into the mouth of the juveniles (Kovitvadhi et al., 2006). For the present study, culturing systems and phytoplankton for juveniles were developed from Kovitvadhi et al. (2006). It was found that growth and survival rates of the early juvenile *H*. (*L.*) *myersiana* in system II were higher than in system I. At 60 days, survival was 91.54±3.24% in system II and 29.55±3.56% in system I, and at 120 days 82.74±1.47% and 12.45±2.14%, respectively. These survival rates are higher than in Kovitvadhi et al. (2006), in which a system similar to system I but with different algal species achieved 8±0.2% survival at 60 days and 65±8.32% at 120 days. In fact, the present results suggest that the significantly higher (*P*<0.05) survival rate in system II may be due to one or both of the following reasons: (1) a specific diet more appropriate for effective digestion; (2) a better chemical water quality as a result of the recirculating system. In this study, the two algae were mixed in the ratio 1:1 (by wet weight) as diet through out the experiment. The relevant diet differences in both actual comparative experiments, *Chlorella* sp. and *Kirchneriella incurvata* were prove to be greater efficiency when there were used individually in juveniles after 60 days old (Kovitvadhi et al., 2006). Areekijseree et al. (2006) compared the protein, carbohydrate and lipid digestibility of four different phytoplanktons (*Chlorella* sp., *K. incurvata*, *Navicula* sp. and *Coccomyxa* sp.) by using crude enzyme extracts from 15 days old juvenile *Hyriopsis* (*Hyriopsis*) *bialatus*. It was indicated that a combination of *K. incurvata* and *Chlorella* sp. is a suitable food formula for juvenile culture. This aspect seems to suggest that the diet composition is very important for supporting the survival of the juvenile mussels from 60-120 days old, whereas the water quality is more crucial at 0-60 days old. In system II, sand was placed in the culturing container

since it could be a source of food such as organic matter or microorganisms (Vogel, 1981; Mann and Lazier, 1991). Juveniles can develop when sand is present, and survival rates are higher than without sand, because they can burrow into the sand as they do in nature, and this helps to prevent the attachment to the shell of feces and pseudofeces with many protozoa and later flatworms and eventual death of the juveniles (Hudson and Isom, 1984; Gatenby et al., 1996; O'Beirn et al., 1998; Kovitvadhi et al., 2006).

The difference between the two systems was the filter cabinet. System I used nylon fiber, gravel and ground freshwater mussel shells as filter materials within the same cabinet but system II used nylon fiber, macrophytes (*Limnophila heterophylla*) and BioBall and these were separated from each other. In addition, the cabinets in system II were larger. These differences resulted in different water quality. Quantities of free carbon dioxide, phosphate, total ammonia nitrogen and nitrate in system II were significantly lower than in system I from days 40, 40, 60 and 10 of rearing, respectively, because the macrophytes could tolerate the environment, and had good growth and filamentous leaves that resulted in more surface area touching the water and absorbing more substance efficiently. The introduction of macrophytes in recirculating systems for sea mussel culture is common (Neori et al., 2000) but for freshwater juvenile culture no report has been found. The pH of system II was between 7.85 to 8.15 which was close to the values measured where growth of blue mussel, *Mytilus edulis*, was slow at pH 6.7-7.1 and better at 7.4-7.6, although the growth increments were not significantly different from those at normal pH 8.1 (Berge et al., 2006). Redding et al. (1997) found that the presence of the emergent plant *Rorippa nasturtium-aquaticum*, the free-floating plant *Azolla filiculoides* or the

70

submerged plant *Elodea nuttalli* significantly reduced total ammonia nitrogen, nitrate and phosphate in comparison with systems lacking macrophytes. Ammonia is the main nitrogenous waste produced by aquatic organisms via metabolism and through the decomposition of organic wastes such as uneaten food and feces. (Goudreau et al., 1993). Sand, a nylon filter layer and BioBall stick provide a place for nitrifying bacteria that will convert toxic ammonia and nitrites into non-toxic nitrates (Al-Hafedh et al., 2003). Layzer et al. (1999) reported that total ammonia nitrogen should be lower than 0.25 ppm to be safe to freshwater unionids. For marine bivalves, MacMillan et al. (1994) reported that the highest level of nitrite and nitrate should not exceed 0.01 ppm and 19.16 ppm, respectively. In this study the total ammonia nitrogen, nitrate and nitrite of system I was higher than system II throughout the experiment but still lower than those two limits. In addition, the nylon fiber layer trapped particles from the water. Calcium is a major component of freshwater pearl mussel shells. Furthermore, silica, sodium, magnesium and iron are essential for growth (Binhe, 1984). Therefore, system II may have had less silica and calcium than system I as a result of their use for shell growth. The coefficient of correlation (*r*) between average water quality and average survival rate in system II indicated that pH, total alkalinity, total hardness, silica and calcium were important factors in juvenile survival, with a highly significant (*P*<0.01) positive correlation, while for free carbon dioxide and nitrite there was a significant negative correlation (*P*<0.05). Water temperature, dissolved oxygen, total ammonia nitrogen, nitrate and phosphate were not significantly correlated with survival rate (*P*>0.05) since those values were uniformly controlled at very low level by filter cabinets. This is in line with the report of Buddensiek (1995) who compared the coefficient of correlation between water quality

71

and survival rate and growth of the freshwater pearl mussel, *Margaritifera margaritifera* culture in four rivers; that study found that there was a different correlation, with water temperature being an important factor in development of this mussel in all water resources and there was a highly significant difference ($P<0.01$) between rivers. Hence, the present study suggests that the values of water quality parameters has mostly highly significant relationship on growth rate, survival rate or development of juveniles, whereas a gradual decrease in free carbon dioxide and nitrites is beneficial. With regard to dissolved oxygen, total ammonia nitrogen, nitrate, phosphate and temperature, it can be concluded that constant maintenance at the experimental values is very important and probably determinant for correct development.

Therefore, when macrophytes and a biological filter are used in a system developed to a large scale for culture of the freshwater juvenile phase at an industrial level, according to the water recirculating system II, it is important to control free carbon dioxide and total ammonia nitrogen. Finally, from the present study it is possible to propose a very profitable aquaculture system to maintain and control a population of *H.* (*L.*) *myersiana* under excellent conditions from the glochidia in the laboratory to the adults in the natural pond. Additionally, from this research status it should be possible to extend this aquaculture system with few modifications to other freshwater bivalve species.

Chapter 4

Morphometric Relationship of Weight and Size of

Cultured Freshwater Pearl Mussel,

Hyriopsis (*Limnoscapha*) *myersiana*

Under Laboratory Conditions and Earthen Pond Phases

4.1 Abstract

The freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856), was cultured in the laboratory (0–120 days old), in an earthen pond in acrylic containers (120–270 days old) and in pocket nets with frames (270–360 days old). Measurements were taken of length-height and total body weight-size (shell length, shell height and shell width). It was found that length-height in each period of culture showed a linear relationship and that shell height increased as shell length increased along with the same linear relationship. When statistical analyses was performed, this linear regression line consisted of three distinct lines (*P*<0.01). The general morphometric relationships between the length and height of mussels grown in the laboratory, in acrylic containers and in pocket nets were Log*H*=−0.147+0.828Log*L*, Log*H*=−0.211+0.909Log*L* and Log*H*=−0.264+0.951Log*L*, respectively. The relationship between the total body weight-size in different culture phases was a positive allometric equation and the two curved lines were not the same (*P*<0.01). The general morphometric relationships between the length and the total body weight of the mussels cultured in acrylic containers and pocket nets were Log*W* = −3.747 + 2.674 Log*L* and Log*W* = −4.149 + 2.976 Log*L*, respectively. Such measurements are useful in the management of different culture phases of *H*. (*L.*) *myersiana*.

4.2 Introduction

A general assessment of the freshwater pearl mussel often includes shell size (length, height, width) and mussel weight, combined in a calculation of the size-weight ratio. This metric has been used, for example, in analyses of the morphological

development of the shell (Anderson and Gutreuter, 1983; Gosling, 2003). It was also used by Gasper et al. (2001; 2002) in comparing the life history and morphology among populations of 25 bivalve species from different habitats or locations along the Algarve coast of southern Portugal.

In general, it is recognized that size-weight data are important basic data that explain the changes of shell proportions in bivalves found in natural water resources or from culturing in various environmental conditions, e.g. wave exposure (Akester and Martel, 2000), trophic conditions (Alunno-Bruscia et al., 2001), water depth (Claxton et al., 1998; Lajtner et al., 2004), currents (Blay, 1989; Fuiman et al., 1999), water turbulence (Hinch and Bailey, 1988), type of sediment (Newell and Hidu, 1982; Lajtner et al., 2004), type of bottom (Claxton et al., 1998) and water quality (Lajtner et al., 2004). The size-weight data are also employed in physiological investigations, and to obtain estimates of seasonal variation in growth or productivity (Gosling, 2003). However, these data have, so far, only been available for adult populations. In this study, we present size-weight measurements for pearl mussels from early juvenile (0 days old) cultured glochidia in artificial media to the adult (age 360 days) in different environments, i.e. laboratory conditions and earthen pond. The relationship between the shell length-shell height and shell length-total body weight is also examined.

4.3 Materials and methods

4.3.1 Culturing of *Hyriopsis* (*Limnoscapha*) *myersiana*

The culturing of *Hyriopsis* (*L*imnoscapha) *myersiana* was undertaken in three stages according to their growth periods: 0–120, 120–270 and 270–360 days. Culturing conditions were as follows.

4.3.1.1 Culturing *H.* (*L.*) *myersiana* (0–120 days old) in the laboratory

About 3,500 early juvenile *H.* (*L.*) *myersiana* (0 days old) were removed from the artificial medium and rinsed in dechlorinated, aerated water, as described by Uthaiwan et al. (2001), Kovitvadhi et al. (2002; 2006). They were then transferred to a plastic culture unit ($20 \times 11 \times 8$ cm^3) containing 20 g of sand about 3 mm deep (grain size <120 µm for 0–60-day-old juveniles and <250 µm for 60–120-day-old juveniles). The water was then passed through a filter cabinet ($50 \times 46 \times 35$ cm^3, glass aquarium) and pumped into the culture unit at a rate of 20 ml/min. A combination of 5 kg of pebbles, 2 kg of ground freshwater mussel shells and 240 g of nylon fiber (~$46 \times 35 \times 2$ cm^3 in size and ~30 g per piece) was used as filter material. This was a closed recirculating culture system. The mussels were fed twice daily (at 06.00 and 18.00 h) with a combination of *Chlorella* sp., and *Kirchneriella incurvata* at a ratio of 1:1 and a concentration of 1×10^5 cells/ml. The water circulation was turned off for 1 h during feeding. The sand and 25% of the water in the system were replaced every 10 days and every other day, respectively.

4.3.1.2 Culturing *H.* (*L.*) *myersiana* (120–270 days old) in acrylic containers

One hundred 120-day-old juveniles were transferred to a culture unit ($20 \times 12 \times 72$ cm^3). This unit was specifically designed to have all four vertical sides lined with a nylon net (mesh size 0.42 mm for 120–180-day-old juveniles and 2.0 mm for 180–270-day-old juveniles). A plastic lid with holes covered the top. The lower part of the culture unit contained another section 2 cm in height, which fitted snugly into the bottom part and could be removed from the main unit (Figure 2.6A). This smaller unit contained 400 g of sand of grain size <425 μm. The juveniles were placed directly on the sand. The culture unit was then hung in an earthen pond located at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The pond was about 2 m deep with a total surface area of 0.8 ha and was filled with rain water. The lower culture unit was adjusted to a position 50 cm below the water surface. The juveniles fed on phytoplankton in the earthen pond. The sand in the culture unit was replaced every 10 days.

4.3.1.3 Culturing *H.* (*L.*) *myersiana* (270–360 days old) in pocket nets with frames

Thirty-six 270-day-old juveniles were transferred to a pocket net (10 pocket nets = 360 juveniles). The net frame was 60×45 cm^2 fitted with a nylon net (0.5 mm mesh size) (Figure 2.6B). The pocket net itself was equally divided into six compartments using string, and six juveniles were introduced through the opening at the top of each compartment. The containers were then hung 50 cm below the water

surface of the earthen pond. All mussels were rinsed every 10 days until they were 360 days old.

4.3.2 Data sampling

Shell measurements included: length (the maximum distance between the anterior and posterior margins of the shell, *L*) and height (the maximum distance between the umbo in 0–60-day-old juveniles or the posterior wing in 60–360-day-old juveniles and the ventral shell margin, *H*). For 0–120-day-old juveniles, these dimensions were measured to the nearest 0.01 mm using a micrometer eyepiece. For 120–360-day-old juveniles, the measurements also included shell width (at the thickest part of the two shell valves, *Wi*), length and height. All dimensions were measured to the nearest 0.01 mm with a digital caliper. The total body weight (*W*) was measured using an analytical balance with precision of 0.001 g. The mussels were blotted dry in absorbent paper prior to weighing every 10 days (120–360 days old). The mussel was measured for shell length, height and width and then weighed.

4.3.3 Morphometric relationships

The estimation of the morphometric relationships between these variables was made by the adjustment of a linear function to the data (Ricker 1973):

$$\log Y = \log a + b \log X$$

where $\log Y$ and $\log X$ are the log shell size (height and width) or total body weight and log shell size (length, height and width), respectively, while $\log a$ is the intercept

and *b* is the slope. The association degree between variables was calculated by the determination coefficient (r^2). The values of *b* obtained in the linear regression were significantly different from the isometric value ($b = 1$) or allometric range (negative allometry: $b<1$ or positive allometry: $b>1$) when a *t*-test (H_0: $b=1$) with a confidence level of 95% was applied, expressed by the following equation (Sokal and Rohlf 1995; Lleonart et al. 2000):

$$t_s = \frac{b - 1}{S_b}$$

where t_s is the *t*-test value; *b* is the slope (relative growth rates of variables) and S_b is the standard error of the slope (*b*).

4.3.4 Statistical analysis

The morphometric data of length-height, length-width, length-weight, height-weight and width-weight (log-transformed) were determined by regression analysis. Significant differences between regression lines (0–120, 120–270 and 270–360 days old) were analyzed by hypothesis testing in normal linear models (Dobson 2002) using indicator variables for culture phases.

4.4 Results

The shell size (length, height, and width) and total body weight relationships of cultured *H.* (*L.*) *myersiana* for each culture phase (0–120, 120–270 and 270–360 days old) are summarized in Tables 4.1 and 4.2. The coefficient of determination (r^2)

of the shell length and total body weight during the period in acrylic containers (120–270 days old) and in pocket net with frames (270–360 days old) was greater than the shell size (height and width) and total body weight, suggesting that application of the morphometric relationship between the shell length and total body weight is the most suitable for cultured *H.* (*L.*) *myersiana*.

The morphometric relationships between the length and height of the shell were analyzed during culture in the laboratory (0–120 days old) and in the earthen pond (120–270 and 270–360 days old). It was found that, with each age phase, the shell length and height increased in a linear relationship. Regression lines from the three periods do not exactly coincide ($P<0.01$) (Figure 4.1). The morphometric relationships between the length and width of the shell were analyzed during culture in the earthen pond (120-270 and 270-360 days old) (Figure 4.2).

The relationship between the total body weight and the shell length, height and width in each phase (120–270 and 270–360 days old) was found to be a positive allometric equation (Figures 4.3, 4.4 and 4.5, respectively). It was found that the two regression lines were not the same ($P <0.01$).

During this study, it was found that a general morphometric relationship of the freshwater pearl mussel at each phase could be described using separate regression lines.

Table 4.1. Descriptive statistics parameters of the cultured freshwater pearl mussel *Hyriopsis* (*Limnoscapha*) *myersiana* during culture in the laboratory (0-120 days old), acrylic containers in an earthen pond (120-360 days old) and in pocket nets with frames (270-360 days old).

Parameters	During culture (days old)		
	0-120	120-270	270-360
Number of individuals	3,044	1,076	696
Shell length (mm)			
Mean±S.D.	1.82±0.74	27.88±16.08	49.63±6.16
Min-Max	0.36-5.85	1.60-58.63	39.62-78.60
Shell height (mm)			
Mean±S.D.	1.16±0.37	12.52±6.95	22.28±2.76
Min-Max	0.30-2.78	1.07-23.95	17.11-33.44
Shell width (mm)			
Mean±S.D.	-	5.82±3.28	10.51±1.43
Min-Max	-	0.33-11.46	7.02-17.72
Total body weight (g)			
Mean±S.D.	-	2.41±2.10	8.30±3.59
Min-Max	-	0.002-9.58	3.77-31.97

Table 4.2. Morphometric relationship parameters of the cultured freshwater pearl mussel *Hyriopsis* (*Limnoscapha*) *myersiana* during culture in the laboratory (0-120 days old), acrylic containers in an earthen pond (120-360 days old) and in pocket nets with frames (270-360 days old)

During culture		Allometric equation	Determination coefficient (r^2)	S.E. of b (95% C.I. of b)	Relationship (t-test)
H and *L* relationship					
0–120	A	Log$H$=–0.147+0.828Log$L$	0.969**	0.003(0.823–0.833)	- Allometry
120–270	B	Log$H$=–0.211+0.909Log$L$	0.996**	0.002(0.906–0.913)	- Allometry
270–360	C	Log$H$=–0.264+0.951Log$L$	0.843**	0.016(0.920–0.981)	- Allometry
Wi and *L* relationship					
120–270	A	LogWi=–0.591+0.941Log$L$	0.995**	0.002(0.937–0.945)	- Allometry
270–360	B	LogWi=–0.747+1.043Log$L$	0.879**	0.015(1.014–1.072)	+ Allometry
W and *L* relationship					
120–270	A	Log$W$=–3.747+2.674Log$L$	0.993**	0.011(2.653–2.695)	+ Allometry
270–360	B	Log$W$=–4.149+2.976Log$L$	0.938**	0.029(2.919–3.033)	+ Allometry
W and *H* relationship					
120–270	A	Log$W$=–3.124+2.936Log$H$	0.983**	0.011(2.915–2.958)	+ Allometry
270–360	B	Log$W$=–2.856+2.785Log$H$	0.880**	0.039(2.708–2.862)	+ Allometry
W and *Wi* relationship					
120–270	A	Log$W$=–2.062+2.832LogWi	0.982**	0.012(2.809–2.855)	+ Allometry
270–360	B	Log$W$=–1.808+2.649LogWi	0.920**	0.030(2.591–2.707)	+ Allometry

H = shell height (mm); *L* = shell length (mm); *Wi* = shell width (mm); *W* = total body weight (g); S.E. = standard error; C.I. = confidence interval; ** = *P*<0.01.

Different capital letters indicate significant differences (*P*<0.05) among regression models for the same morphometric relationship.
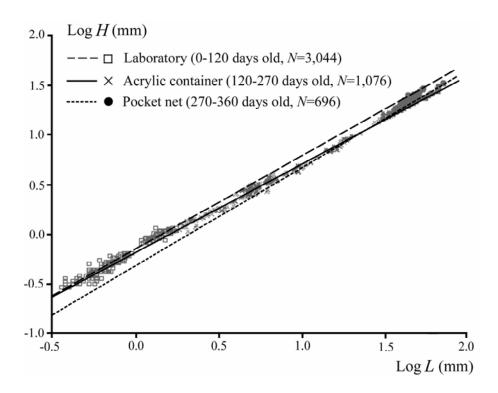
Figure 4.1. Morphometric relationship between log shell length (Log *L*) and log shell height (Log *H*) of the cultured freshwater pearl mussel, *H.* (*L.*) *myersiana* during culture in the laboratory (0-120 days old) and in the earthen pond (120-360 days old). The extent of the regression line encompasses the age range studied.

Figure 4.2. Morphometric relationship between log shell length (Log *L*) and log shell width (Log *Wi*) of the cultured freshwater pearl mussel, *H.* (*L.*) *myersiana* during culture in the earthen pond (120-360 days old). The extent of the regression line encompasses the age range studied.
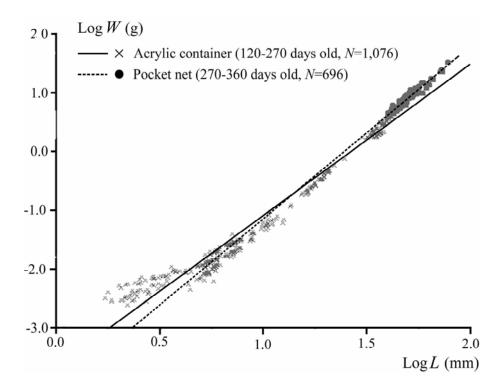
Figure 4.3. Morphometric relationship between log shell length (Log *L*) and log total body weight (Log *W*) of the cultured freshwater pearl mussel, *H.* (*L.*) *myersiana* during culture in the earthen pond (120-360 days old). The extent of the regression line encompasses the age range studied.

Figure 4.4. Morphometric relationship between log shell height (Log *H*) and log total body weight (Log *W*) of the cultured freshwater pearl mussel, *H.* (*L.*) *myersiana* during culture in the earthen pond (120-360 days old). The extent of the regression line encompasses the age range studied.
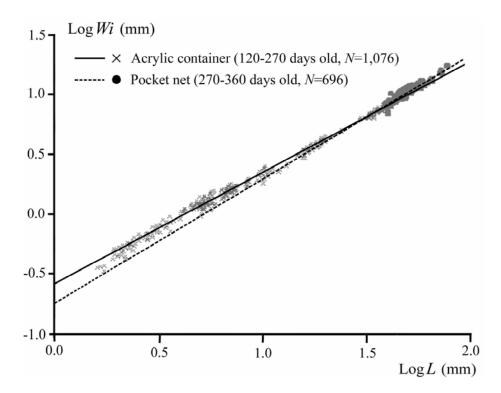
Figure 4.5. Morphometric relationship between log shell width (Log *Wi*) and log total body weight (Log *W*) of the cultured freshwater pearl mussel, *H.* (*L.*) *myersiana* during culture in the earthen pond (120-360 days old). The extent of the regression line encompasses the age range studied.
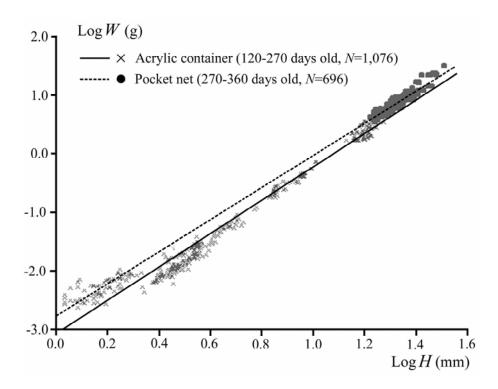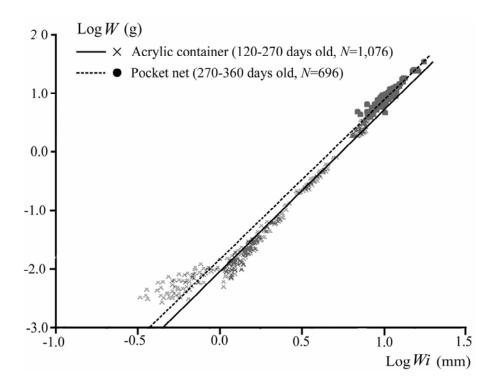
4.5 Discussion

Among the few known studies examining size-weight relationships in freshwater mussels, nearly all deal with fully grown adults living in various natural environments. Blay (1989) examined the relationship between the whole wet weight (live weight) and shell length of *Aspatharia sinuate* from five natural water resources (two reservoirs and three rivers). Even among mussels from the same site, the variation in the ratio was highly significant ($P<0.01$). Significant differences ($P<0.05$) were also found among mussels from the five different sites. Similarly, Bailey and Green (1988) sampled the freshwater mussel, *Lampsilis radiata siliquoidea* from the Inner Long Point Bay, Lake Erie, Canada, and found that the differences were highly significant ($P<0.0001$).

Our results reveal significant differences in the mussel shell size-weight ratio according to the various culture conditions. Analysis shows that the exponents of the weight-size ratio (*b*) of the freshwater pearl mussel cultured in the experiment were between 2.649 and 2.976 which was significantly higher than 1.0 ($P<0.01$). This result is in accordance with Hanson et al. (1988) who studied population size, growth and production of a unionid clam, *Anodonta grandis simpsoniana*, in a small, deep boreal forest lake in central Alberta. The *b* value of the live weight to length ratio was 2.95 and the value of the shell weight to length ratio was 2.70.

In the present study, the exponent of the length-height and length-width relationships (*b*) increases in value as the pearl mussels become older (Table 4.2). The shell length is seen to increase with age. Bivalve shell growth and shape are influenced by biotic (endogenous/physiological) and abiotic (exogenous/environmental) factors. A variety

of environmental factors are known to influence shell morphology and the relative proportions of many bivalve species (Gasper et al. 2002). For examples, the type and quality of phytoplankton as a food source of the mussels (Kovitvadhi et al. 2006; Alunno-Bruscia et al. 2001), water quality (Lajtner et al. 2004; Buddensiek, 1995), water depth (Claxton et al. 1998; Karayücel and Karayücel 2000, Lajtner et al. 2004), currents (Blay 1989; Fuiman et al. 1999), water turbulence (Hinch and Bailey 1988), type of sediment (Newell and Hidu 1982; Lajtner et al. 2004), type of bottom (Claxton et al. 1998) and wave exposure (Akester and Martel 2000). Buddensiek (1995) described the culture of juvenile *Margaritifera margaritifera* in cages as a useful method of raising the early post-parasitic stages in four suitable rivers. Analyzing the effect of 12 water quality variables, he found a negative relationship between growth and water temperature. Claudi and Mackie (1994) reported that the most important environmental factors for the survival and growth of zebra mussels were temperature, calcium levels and pH. In another study on the zebra mussel (*Dreissena polymorpha*), Lajtner et al. (2004) examined the shell morphometrics in three sites of different sediment types, depth, and physical and chemical conditions. Their analysis showed that the shells differed significantly between all three sites. Alunno-Bruscia et al. (2001) identified food availability and population density as important determinants of shell morphometry and shell length-body mass ratio in *Mytilus edulis*.

Similarly, under our culture conditions, the differences in morphometrics among culture phases may reflect variability in the environmental parameters (e.g. water quality, water depth, water current), feeding and rearing conditions (e.g. phytoplankton, container, stocking density) employed for each culture phase.

The results from the current study lead to four insights into the application of morphometric data in cultured mussels. Firstly, measurements of shell length rather than shell height and shell width were consistently proportional to the total body weight during the period 120–360 days old. Secondly, the application of the morphometric relationship between the shell length and total body weight is the most suitable for cultured *H.* (*L.*) *myersiana*. Thirdly, the shell length might be employed in evaluating total body weight of the freshwater pearl mussel during the period 120–360 days without use of an analytical balance. Finally, an analysis to compare the relationship between length-height, length-width and total body weight-size of this freshwater pearl mussel showed different regression equations under different conditions, suggesting the likely effects of different culturing environments.

Chapter 5

**Morphological Development of the Juvenile
Through to the Adult in the Freshwater Pearl Mussel,
Hyriopsis (*Limnoscapha*) *myersiana*,
Under Artificial Culture**

5.1 Abstract

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.

5.2 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 (glochidium) 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; Uthaiwan et al., 2001). 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; Uthaiwan et al., 2001; Kovitvadhi et al., 2001; 2006). Kovitvadhi et al. (2006) succeeded in culturing glochidia of *H.* (*L.*) *myersiana* to adulthood, but the survival percentage 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.

5.3 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 ethanol 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.

5.4 Results

5.4.1 Development of shell form

The early juvenile *H*. (*L.*) *myersiana* at 0 day 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 (Figures 5.1, 5.1A). The valves are joined by a straight hinge (Figures 5.1, 5.2). 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 (Figures 5.2, 5.3, 5.4, 5.9, 5.13, 5.14) until the juvenile is 40 days old, when the posterior region begins to increase more than the anterior (Figures 5.16, 5.18, 5.20, 5.24, 5.28, 5.31-5.32, 5.35). The shell of 0-20-day-old juveniles is very convex, particularly in the dorsal region, with a curve in the new growth lines (Figures 5.3, 5.4, 5.9, 5.9A, 5.10). Subsequently, the convexity of the two valves decreases at successive ages until, from 40 days old, the shell even becomes distinctly laterally compressed (Figures 5.18, 5.20, 5.24, 5.28, 5.31-5.32, 5.35). The first anterior and posterior wings

appear in 50-day-old juveniles (Figure 5.18), with the posterior wing becoming dominant relative to the anterior from the 130-day-old stage (Figures 5.28, 5.31, 5.32, 5.35). From this 130-day stage, the posterior region has two posterior ridges with a posterior slope between them (Figures 5.28, 5.31, 5.32), and this slope is dominant from 150 days old (Figure 5.31). The complete adult morphology is apparent from 160 days old (Figures 5.32, 5.35).

5.4.2 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 (Figure 5.12). 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 (Figures 5.27, 5.38). 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 (Figure 5.38). A third layer in the shell, the nacreous layer, develops from the 30-day-old juvenile onward (Fig. 5.15) and consists of successive thin lamellae of small $CaCO_3$ crystals parallel to the plane of the shell (Figures 5.15, 5.23, 5.27, 5.38) and, like the periostracum, perpendicular to the prismatic layer. The thickness of the nacreous layer is proportional to the number of lamella, 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 (Figures 5.29, 5.30).

5.4.3 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 (Figures 5.6, 5.7). The mantle lobes of 0-50-day-old juveniles are joined dorsally and are free ventrally (Figures 5.10, 5.11, 5.17). 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 (Figures 5.20, 5.24, 5.28, 5.28A). 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 (Figures 5.26, 5.26A-5.26F).

5.4.4 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 (Figure 5.1A). The edges of the gill bars are covered by numerous cilia during the transformation period (Figures 5.5, 5.5A, 5.11). In several 10-day-old juveniles the gill bars had already formed two branches per bar

and which constitute gill filaments (Figures 5.4B, 5.5). From this stage, numerous cilia on the filaments project along the lateral and frontal edges and which will form the cirri (Figures 5.8, 5.8A, 5.8B, 5.11). Each filament bears two rows of fused cilia, joined at this base along the lateral-frontal edges and which form the latero-frontal cirri (Figures 5.8, 5.8A, 5.8B, 5.11, 5.21, 5.25, 5.25A-B). Each cirrus is already well-developed at 20 days old (Figures 5.8, 5.8A, 5.8B, 5.11). 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 (Figure 5.19). When the juveniles have reached 360 days old, the basal portions of the lateral-frontal cilia are seen not to be fused (Figures 5.36, 5.36A-5.36B).

At 60 days old, the internal surface of the inner demibranch has no cilia but numerous buds (Figure 5.21B). 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 (Figure 5.22), these being long in the terminal region (Figure 5.22B) and shorter in the basal region (Figure 5.22A).

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 (Figures 5.33, 5.33A). These form a larger a 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 (Figures 5.33A, 5.34, 5.34A). Later (360-day-old, i.e. adult), the pores are narrowed by development of connective tissue around the aperture, thereby forming the ostia (Figures 5.37, 5.37A). Within the thin network, an interlamellar septum forms 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 (Figure 5.37) which open into the suprabranchial chamber.
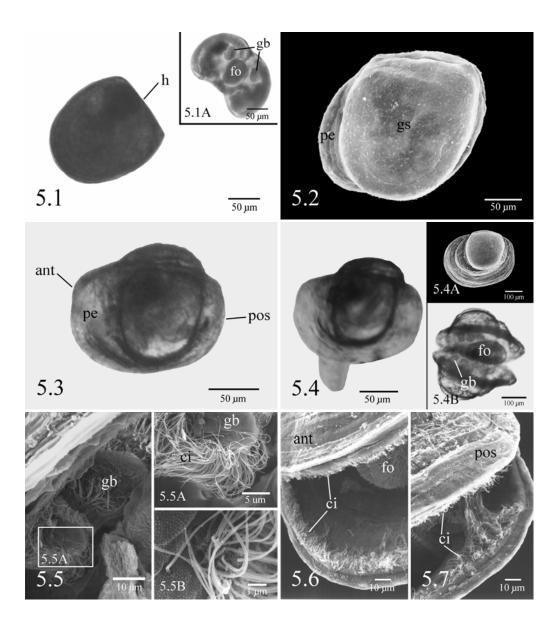
Figure 5.1. Early juvenile (after transformation, 0-day-old) shell; note hinge (h). Figure 5.1A. The first gill bar (gb), in both sides close to the foot (fo). **Figure 5.2.** SEM micrograph of juvenile 1-day-old, appearance in anterior region of new soft periostracum (pe); note glochidia shell (gs). **Figure 5.3.** Juvenile 5 days old, anterior (ant) region appears before and grows more than the posterior (pos). **Figures 5.4-5.7.** Juvenile 10 days old, Figure 5.4A. Light microscopy of gill bar 10 days old. **Figures 5.5, 5.5A.** The outer surface and rim of the gill bar with cilia (ci). **Figure 5.6.** Cilia at anterior region of mantle. **Figure 5.7.** Cilia at posterior region of mantle.
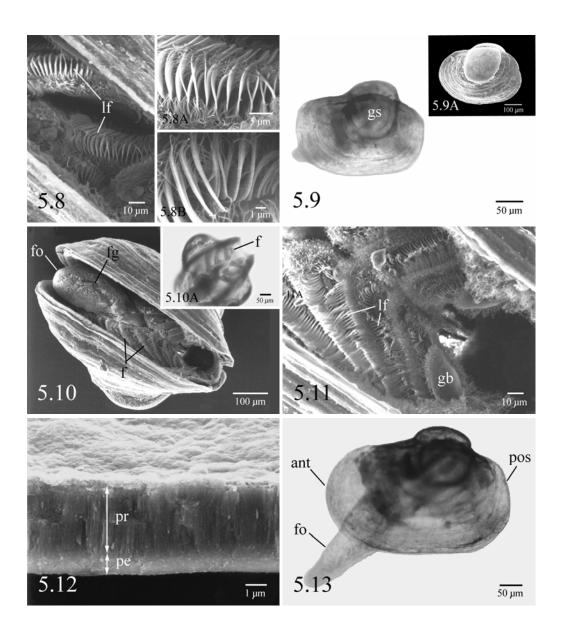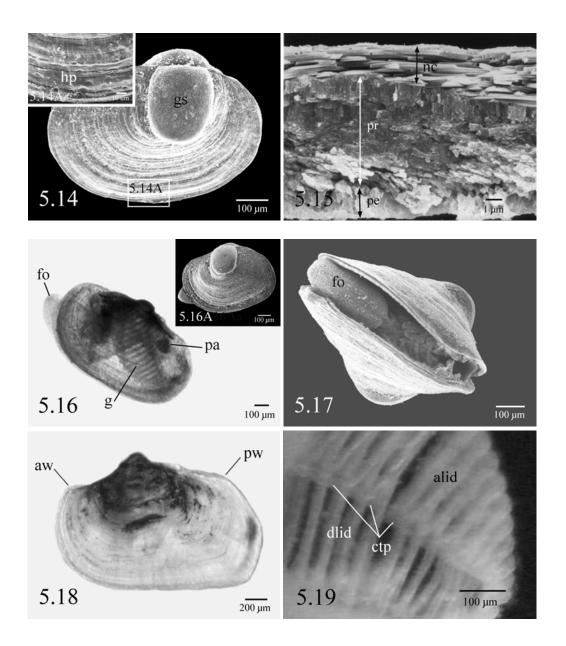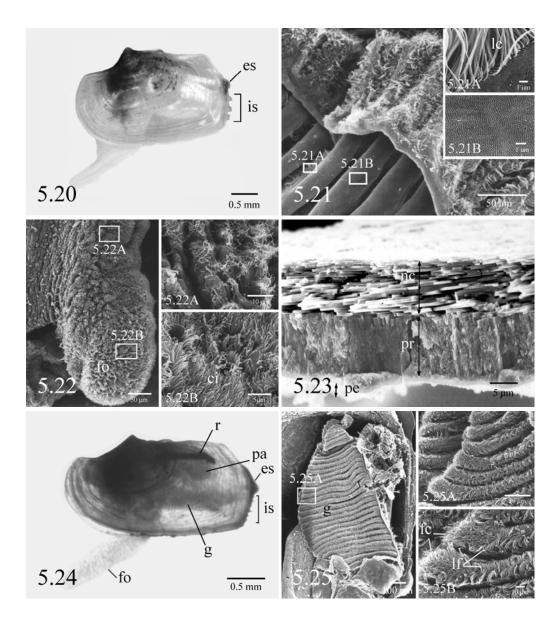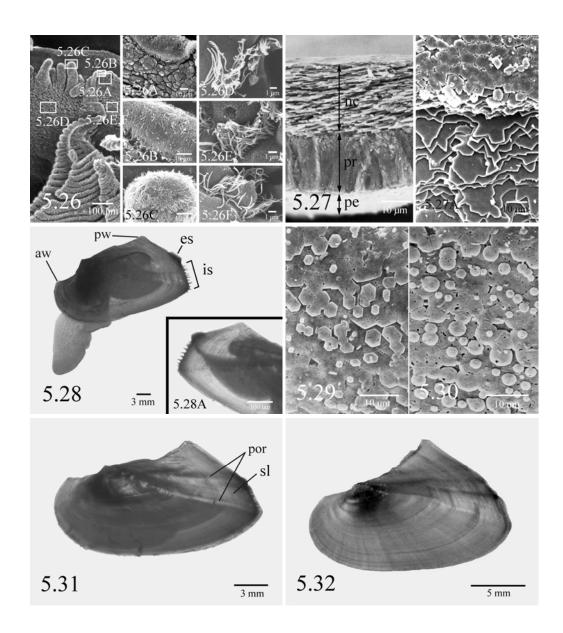
Figure 5.8. Latero-frontal cirri (lf) on the gill filaments in 10-day-old juvenile. Figure 5.8A. Higher magnification SEM micrograph of the latero-frontal cirri. **Figures 5.9-5.12.** Development of gill and microstructure of shell of 20-day-old juvenile. **Figure 5.9.** External morphology of shell; note glochidia shell (gs). **Figure 5.10.** Position of gill filaments (f) of juvenile; note foot groove (fg); foot (fo). **Figure 5.11.** Latero-frontal cirri at the gill filament; note gill bar (gb). **Figure 5.12.** Microstructure of shell; note periostracum layer (pe); prismatic layer (pr). **Figure 5.13.** External morphology of 30-day-old juvenile; note anterior (ant); posterior (pos).
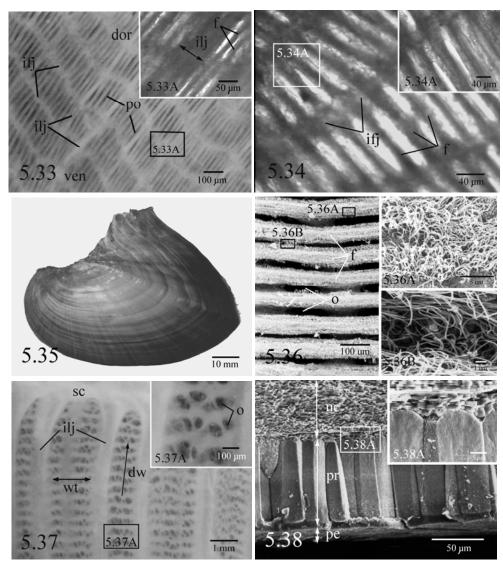
Figures 5.14-5.15. Shell form and microstructure of 30-days-old juvenile. **Figure 5.14.** SEM micrograph of shell surface; note glochidia shell (gs). **Figure 5.15.** Microstructure of shell; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). **Figures 5.16-5.17.** Light microscopy and SEM of development of shell, 40-day-old juvenile, **Figure 5.16.** External morphology; note foot (fo); gill (g); posterior adductor muscle (pa). **Figure 5.17.** Vental side of juvenile. **Figures 5.18-5.19.** Light microscopy of shell form and gill development, 50-day-old juvenile. **Figure 5.18.** External morphology of shell; note anterior wing (aw); posterior wing (pw). **Figure 5.19.** Tip of ascending lamella (alid) as a connecting plate by connective tissue (ctp); note descending lamella of inner demibranch (dlid).

Figures. 5.20-5.23. Development of shell form, siphon, gill, foot and shell microstructure, 60-day-old juvenile. **Figure 5.20.** External morphology of shell; note excurrent siphon (es); incurrent siphon (is). **Figure 5.21.** SEM, inside and outside of inner demibranch surface. Figure 5.21A. Rim of inside surface; note lateral cilia (lc). Figure 5.21B. Inside surface of inner demibranch. **Figure 5.22.** SEM, cilia on the foot (fo) surface. Figure 5.22A. Cilia at the base region of foot. Figure 5.22B. Cilia (ci) at the terminal of foot surface. **Figure 5.23.** Shell microstructure of juvenile; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). **Figure 5.24.** External morphology of 100-day-old juvenile; note gill (g); posterior adductor muscle (pa); rectum (r). **Figure 5.25.** SEM, cilia on the 100-day-old gill. Figure 5.25A. Latero-forntal cirri (lf) and fontal cilia (fc) on gill filaments.

Figures. 5.26-5.27. SEM, incurrent and excurrent siphon, and shell microstructure, 100 days old. Figures 5.26A, 5.26B, 5.26E, 5.26F. Surface of excurrent siphon area. Figure 5.26C. Cilia on the surface of papilla. Figure 5.26D. Surface of incurrent siphon area. **Figure 5.27.** Shell microstructure; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). **Figure 5.28.** Shell morphology of 130-day-old juvenile; note anterior wing (aw); excurrent siphon (es); incurrent siphon (is); posterior wing (pw). **Figure 5.29.** SEM, nacreous layer of the shell with polygonal. **Figure 5.30.** The nacreous layer of the shell with round tablets. **Figures 5.31-5.32.** Juveniles 150 and 160 days old; note posterior ridge (por); posterior slope (sl).

Figures 5.33-5.34. Development of gill, 200-day-old juvenile. **Figure 5.33.** Inside surface of inner demibracnhs; note dorsal (dor); transversal interfilamentous junction (ifj); longitudinal interfilamentous junctions (ilj); ventral (ven); gill pore (po). Figure 5.33.A. 2-3 gill filaments (f) fused by connective tissue throughout longitudinal demibranch. **Figure 5.34.** Inner surface of the gill filament connected by connective tissue at greater intervals occurred throughout transverse demibranch. Figure 5.34A. Gill pore surrounded with connective tissue (ct). **Figures 5.35-5.38.** Shell form, development of water tube and shell microstructure, 360-day-old juvenile. **Figure 5.35.** External shell morphology. **Figure 5.36.** Outer surface of gill filaments; note ostia (o). Figure 5.36A. Cilia on gill filaments. Figure 5.36B. Cilia between the gill filaments. **Figure 5.37.** The light microscopy of inner demibranch, each divided by longitudinal interfilamentous junctions; note direction of water current (dw); suprabranchial chamber (sc); water tube (wt). Figure 5.37A. Inner site of the ostia. **Figure 5.38.** Shell microstructure, the prismatic structure well-defined thicker columnar structure; note periostracum layer (pe); prismatic layer (pr); nacreous layer (nc). Figure 5.38A. Higher magnification between the nacreous layer and prismatic layer.

5.5 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 Uthaiwan et al. (2001) and Kovitvadhi et al. (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*.

5.5.1 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; Uthaiwan et al., 2001). 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.

5.5.2 Development of incurrent and excurrent siphon

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; Uthaiwan et al., 2001). Kovitvadhi et al. (2001) 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.

5.5.3 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 this also occurs 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 gill. 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.

Chapter 6

Conclusion

The natural populations of *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856) in Thailand have been drastically reduced to the extent that it is now nearly extinct. Thus, there is an urgent need to restore their population through controlled aquaculture. Furthermore, culturing these freshwater mussels throughout their complete life cycle would lead to a better understanding and may contribute to the correct management and control of wild populations.

The most current methodology consisting of infestation is inconvenient relative to a very low transformation and survival percentage, the requirement of large technical apparatus, a large number of fishes, and culturing area for juvenile development under semi-natural conditions.

From the experiment of Uthaiwan et al. (2001) and Kovitvadhi (2002) it was possible to obtain high glochidia transformation (84.28%) and survival (85.32%) percentages based on a more simple and efficient methodology. They could successfully culture freshwater pearl mussel at the glochidia phase in artificial media (M199 formula) by having common carp fish plasma as an important composition factor, controlled temperature and air mixed with carbon dioxide at 5%. However, the juveniles could only survive for around 13 days, which becomes totally unpractical for a good maintenance of any natural mussel population.

The present research could completely improve all artificial culture conditions by changing greatly the process mainly in the juvenile culture stage in order to obtain a very reproducible and efficient method. Not less than 5,000-10,000 adults per female could be produced from cultured juveniles up to 3 years and ready to be reintroduced in the wild. From this success range it is possible to select the best

strains as the broodstock for future reproduction. In fact, a complete culture success was carried out in juveniles of freshwater pearl mussel, *H.* (*L.*) *myersiana* when the method was supported on two basic and relevant parameters: the algae composition and aquaculture systems. These experiments were accomplished under similar conditions described by Kovitvadhi (2002) for glochidia culture, i.e., when reared in artificial media (M199) with common carp plasma in an incubator at controlled 25° C and carbon dioxide 5%. The glochidia transformed to juvenile with a better percentage, around 95%, due to earlier media change (at 4 days) and the use of a larger water volume (4 ml) in the media from 7 days.
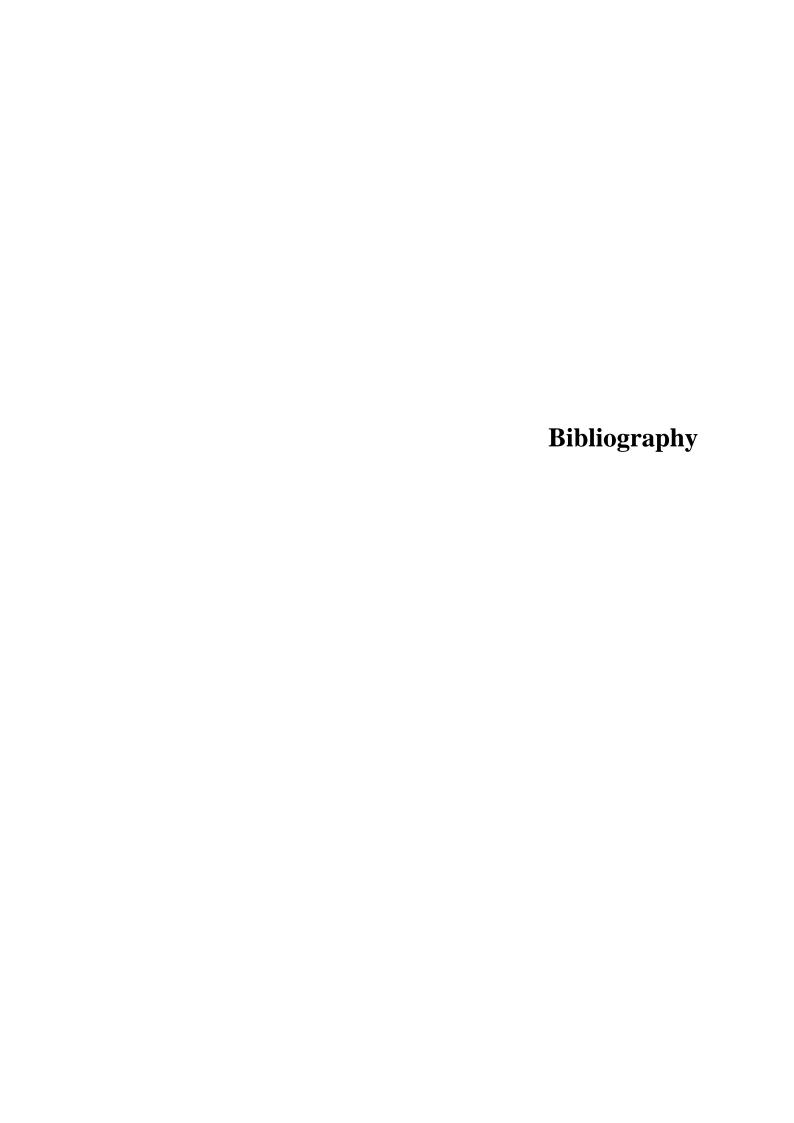
Actually, the survival rates of juvenile freshwater pearl mussel, *H.* (*L.*) *myersiana* (0-120 days old) was greatly improved with the recirculating system II composed of 3 filter cabinets (particulate filter cabinet, macrophytes filter cabinet and biological filter cabinet), 1 water resting cabinet and 9 plastic culture units. When they were reared up to 120 days it was found that they had an average length of 12.17 mm, height of 6.33 mm, and a survival rate equalled to 82.74%. The juveniles 120-180 and 180-270 days old were reared in the pond inside of plastic boxes (20×12×71 cm) which were covered with nylon net (0.42 mm and 2.0 mm size, respectively). Then all boxes were hung in the earthen pond at a depth of about 50-cm from water surface, when they were 270 days old. Their average length, height and percent survival were equal to 45.06 mm, 20.33 mm and 100%, respectively. Juveniles during 270-360 days old were reared inside a frame pocket net with 1 cm nylon net. When they were 360 days old, it was found that their average length, height, width, and weight measurements and percent survival were equal to 54.08 mm, 25.09 mm, 12.28 mm, 11.24 g and 100%, respectively.

The study of morphometric relationships were carried out between length-height and total weight-size of the shell in freshwater pearl mussel, *H.* (*L.*) *myersiana* cultured in a laboratory (0-120 days old), in acrylic containers in the earthen ponds (120-270 days old) and in pocket net with frame (270-360 days old). It was found that their length-height in each period of culture had a linear relationship and the linear regression line consisted of three lines ($P<0.01$). The relationship between total body weight-size (length, height and width) would be an allometric equation and those two curved lines were not the same line ($P<0.01$). From this study, it was found that a general morphometric relationship of the freshwater pearl mussel at each phase could be explained by using separate regression line.

The morphological development of freshwater pearl mussel was also observed by light and scanning electron microscope from the newly-transformed juvenile (0 day old) until the onset of the adult (360 days old). The early juvenile shell had semi-oval and equivalve shells. After mussel 1-day-old shell at anterior better developed than posterior until mussel 40 days old. Microstructures shells of mussel at 0-20 days old showed that there were 2 differentiated layers (periostracum and prismatic layer) after that shell has arranged as nacreous layer, prismatic layer structure similar to prism, when it was 140 days old. The development of gills initiated from a pair of gill bars. When 30 days old, the juveniles started the inner demibranch development, then the outer demibrandh began when 90 days old. Interfilamentous junctions developed from mussel when 200 to 240 days old and interlamellar junctions from mussel 240 to 260 days old for inner and outer demibranches respectively.

Finally, from the present study it is possible to propose a very profitable aquaculture system to maintain and control a population of *H.* (*L.*) *myersiana* under

excellent conditions and success from the laboratory to the natural pond period during glochidia and adult stages, respectively. In the past there was no report on culturing juvenile from artificial diet until adulthood, 360 days old. Now, supported by this system is possible to obtain them and based on the morphometric correlations and morphological aspects, we can predict the main characteristics of a freshwater mussel population, such as the number of adults, size and life quality by adapting the culture systems at all time. From this research, it is also proposed to extent this aquaculture system to other freshwater bivalve species with few eventual modifications in order to establish the equilibrium for other species in the nature. Actually, we understand that the main conditions are created in order to support and promote the diverse activities such as decorative handicrafts, animal foods, pearl cultures and even natural pharmacy products. However, for a mass production of freshwater bivalves it is pertinent to observe additional details such as: to evaluate the natural conditions in different geographic ecosystems; to collaborate with official and local services in order to adjust the balance and the water quality; finally to train technicians in this methodology relating to complete cycle culture of freshwater pearl mussel. So, the perfect accomplishment of this artificial culture on freshwater bivalves associated with the correct fulfillment of the above mentioned requirements will offer excellent conditions to attain future objectives. Of course, that additional technique adaptations and improvements for culturing other different species of freshwater bivalves is necessary to carry out, mainly those required by the specificities relative to the ecological parameters.

Bibliography

Bibliography

Akester, R.J. and Martel, A.L., 2000. Shell shape, dysodont tooth morphology, and hinge-ligament thickness in the bay mussel *Mytilus trossulus* correlate with wave exposure. Can. J. Zool. 78, 240-253.

Al-Hafedh, Y.S., Alam, A., Alam, M.A., 2003. Performance of plastic biofilter media with different configuration in a water recirculation system for the culture of Nile tilapia (*Oreochromis niloticus*). Aquac. Eng. 29, 139-154.

Alunno-Bruscia, M., Bourget, E., Frechette, M., 2001. Shell allometry and length-mass-density relationship for *Mytilus edulis* in an experimental food-regulated situation. Mar. Ecol. Prog. Ser. 219, 177-188.

Amyot, J.P., Downing, J.A., 1991. Endo-and epibenthic distribution of the unionid mollusc *Elliptio complanata*. J. North Am. Benthol. Soc. 10, 280-285.

Anderson, K.B., Sparks, R.E., Paparo, A.A., 1978. Rapid assessment of water quality, using the fingernail clam, *Musculium transversum*. Water Resources Center Research Report No. 133. Water Resources Center, University of Illionois, Urbana, Illinois, pp. 1-20.

Anderson, R.O., Gutreuter, S.J., 1983. Length, Weight, and Associated Structural Indices. In: Nielsen, L.A., Johnson, D.L. (Eds.), Fisheries Techniques. American Fisheries Society, USA, 300 pp.

APHA, AWWA, WPCF, 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed. American Public Health Association, American Water Works Association, Water Pollution Control Federation, Washington, DC, pp. 2-24 – 4-159.

Araújo, R., Ramos, M.A., 1998. Description of the glochidium of *Margaritifera auricularia* (Bivalvia, Unionoidea). Phil. Trans. R. Soc. Lond. 353B, 1553-1559.

Arayawatanavij, P., Nagachinta, A., Tispijit, N., 1992. Site selection and attachment duration of freshwater pearl mussel glochidia on 4 species of freshwater fishes. Technical paper, No.5. Inland Fisheries Division, Department of Fisheries, Ministry of Agriculture, Bangkok (in Thai), pp. 1-32.

Areekijseree, M., Engkagul, A., Kovitvadhi, S., Kovitvadhi, U., Thongpan, A., Rungruangsak-Torrissen, K., 2006. Development of digestive enzymes and *in vitro* digestibility of different species of phytoplankton for culture of early juvenile of the freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus* Simpson, 1990. Invertebr. Reprod. Dev. 49, 255-262.

Bailey, R.C., 1989. Habitat selection by a freshwater mussel: an experimental test. Malacologia 31, 205-210.

Bailey, R.C., Green, R.H., 1988. Within-basin variation in the shell morphology and growth rate of a freshwater mussel. Can. J. Zool. 66, 1704-1708.

Barrington, E.J.W., 1979. Invertebrate Structure and Function, 2nd ed. Thomas Nelson and Sons Ltd, Great Britain, 425 pp.

Barnes, R.D., 1987. Invertebrate Zoology. Saunders Collage Publishing, Fort Worth, USA, pp. 342-419.

Beck, K.M., 2001. Development of an algal diet for rearing juvenile freshwater mussels (Unionidae). Master's Thesis, Virginia Polytechnic Institute and Stage University, Blacksburg, Virginia.

Berge, J.A., Bjerkeng, B., Pettersen, O., Schaanning, M.T., Øxnevad, S., 2006. Effects of increased sea water concentrations of CO_2 on growth of the bivalve *Mytilus edulis* L. Chemosphere 62, 681-687.

Binhe, G., 1984 Freshwater Pearl Culture. Fourth Training Course for Senior Aquaculturists in Asia and the Pacific Region Tigbauan, Iloilo, Philippines, 20 pp.

Blay, J., 1989. Morphometric length-weight relationships and length distributions of five populations of the freshwater bivalve *Aspatharia sinuate* (Unionacea, Mutelidae) in Nigeria. Malacologia 30, 365-372.

Bottjer, D.J., Carter, J.F., 1980. Functional and phylogenetic significance of projecting periostracal structures in the Bivalvia (Mollusca). J. Paleontol. 54, 200-216.

Box, J.B., Mossa, J., 1999. Sediment, land use, and freshwater mussels: prospects and problems. J. North Am. Benthol. Soc. 18, 99-117.

Brandt, R.A.M., 1974. The non-marine aquatic mollusca of Thailand. Archiv fur Molluskenkunde 105, 1-423.

Buddensiek, V., 1995. The culture of juvenile freshwater pearl mussels *Margaritifera margaritifera* L. in cages: A contribution to conservation programs and the knowledge of habitat requirements. Biol. Conserv. 74, 33-40.

Castilho, E., Machado, J., Reis, M.L., 1989. Utrastructure study of the embryonic and larval shell of *Anodonta cygnea*. Can. J. Zool. 67, 1659-1664.

Chaopaknam, B., Toopbucha, K., Nagachinta, A., 1994. Study on some biological and ecological aspects of freshwater pearl mussel in Kwae Noi River, Kanchanaburi, Thailand. Technical paper No. 5/1994. Inland Fisheries Division, Department of Fisheries, Ministry of Agriculture, Bangkok (in Thai), 24 pp.

Chetty, A.N., Indira, K., 1995. Adaptive changes in glucose metabolism of a bivalve to ambient ammonia stress. Arch. Environ. Contam. Toxic. 54, 83-89.

Claudi, R., Mackie, G.L., 1994. Practical Manual for Zebra Mussel Monitoring and Control. Lewis Publishers, Boca Raton, 227 pp.

Claxton, W.T., Wilson, A.B., Mackie, G.L., Boulding, E.G., 1998. A genetic and morphological comparison of shallow- and deep-water populations of the introduced dreissenid bivalve *Dreissena bugensis*. Can. J. Zool. 76, 1269-1276.

Coker, R.E., Shira, A.F., Clark, H.W., Howard, A.D., 1921. Natural history and propagation of freshwater mussels. Bull. U.S. Bur. Fish. 37, 76-181.

D'Eliscu, P.D., 1972. Observation of the glochidium, metamorphosis, and juvenile of *Anodontacali forniensis* Lea, 1857. Veliger 15, 57-59.

Desikachary, T.V., 1959. Cyanophyta. Indian Council of Agricultural Research, New Delhi, pp. 77-430.

Dobson, A.J., 2002. An Introduction to Generalized Linear Models. Chapman & Hall, New York, 225 pp.

Dunn, C.S., Layzer, J.B., 1997. Evaluation of various holding facilities for maintaining freshwater mussels in captivity. In: Cummings, K.S., Buchanan, A.C., Mayer, C.A., Naimo, T.J. (Eds.), Conservation and Management of Freshwater Mussels II: Initiatives for the Future. Proceedings of a UMRCC (the Upper Mississippi River Conservation Committee) symposium, 16-18 October 1977, St. Louis, Missouri, pp. 205-213.

Elliott, A.M., 1952. Zoology. New York Appleton Century Crofts, INC. USA, 703 pp.

Ellis, A.E., 1978. British Freshwater Bivalve Mollusca. Academic Press, New York, 109 pp.

Epifanio, C.E., Pruder, G., Hartman, M., Srna, R., 1974. An interdisciplinary study on the feasibility of recirculating systems in mariculture. Proc. World Maric. Soc. 4, 37-52.

Epifanio, C.E., Srna, R.F., 1975. Toxicity of ammonia, nitrite ion, nitrate ion, and orthophosphate to *Mercenaria mercenaria* and *Crassostrea virginica*. Mar. Biol. 33, 241-246.

Fuiman, L.A., Gage, J.D., Lamont, P.A., 1999. Shell morphometry of the deep sea protobranch bivalve *Ledella pustulosa* in the Rockall Trough, north-east Atlantic. J. Mar. Biol. Ass. UK. 79, 661-671.

Gale, W.F., 1976. Vertical distribution and burrowing behavior of the fingernail clam, *Sphaerium transversum*. Malacologia, 15, 401-409.

Gale, W.F., Lowe, R.L., 1971. Phytoplankton ingestion by the fingernail clam, *Sphaerium transersum* (Say), in pool 19 , Mississippi river. Ecol. 52, 507-512.

Gasper, M.B., Santos, M.N., Vasconcelos, P., 2001. Weight-length relationships of 25 bivalve species (Mollusca: Bivalvia) from the Algarve coast (southern Portugal). J. Mar. Biol. Assoc. UK. 81, 805-807.

Gasper, M.B., Santos, M.N., Vasconcelos, P., Monteiro, C.C., 2002. Shell morphometric relationships of the most common bivalve species (Mollusca: Bivalvia) of the Algarve coast (southern Portugal). Hydrobiologia 477, 73-80.

Gatenby, C.M., 1994. Development of a diet for rearing juvenile freshwater mussels. Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Gatenby, C.M., Neves, R.J., Parker, B.C., 1996. Influence of sediment and algae food on cultured juvenile freshwater mussels. J. North Am. Benthol. Soc. 15, 597-609.

Gatenby, C.M., Parker, B.C., Neves, R.J., 1997. Growth and survival of juvenile rainbow mussels *Villosa iris* (Lea, 1829) (Bivalvia: Unionidae), reared on algal diets and sediment. Am. Malacol. Bull. 14, 57-66.

Giusti, F., 1973. The minute shell structure of the glochidium of some species of the genera *Unio*, *Potomida* and *Anodonta* (Bivalvia, Unionacea). Malacologia 14, 291-301.

Gordon, M.E., Layzer, J.B., 1989. Mussels (Bivalvia: Unionidae) of the Cumberland River: a review of life histories and ecological relationships. US Department of Interior, Fish and Wildlife Service, Biological Report 89 (15). Washington, DC, pp. 1-35.

Gosling, E., 2003. Bivalve Molluscs: Biology, Ecology and Culture. Fishing News Books, UK, 443 pp.

Goudreau, S.E., Neves, R.J., Sheehan, R.J., 1993. Effects of wastewater treatment plant effluents on freshwater mollusks in the upper Clinch river, Virginia, USA. Hydrobiologia 252, 211-230.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve). Gran. Can. J. Microbiol. 8, 229-239.

Hanson, J.M., Mackay, W.C., Prepas, E.E., 1988. Population size, growth, and production of a unionid clam, *Anodonta grandis simpsoniana*, in a small, deep Boreal Forest lake in central Alberta. Can. J. Zool. 66, 247-253.

Harms, W., 1907. Uber die postembryonale Entwicklung von *Anodonta piscinalis*. Zool. Anz. 31, 801-807.

Harms, W., 1909. Postembryonable Entwicklungsgeschichte der Unioniden. Zool. Jahrb. Abt. Anatomie Ontog. Tiere 28, 326-386.

Havlik, M.E., Marking, L.L., 1987. Effect of contaminants on naiad mollusks (Unionidae). US Department of Interior, Fish and Wildlife Service: Resource Publication 164. Washington, DC, pp. 1-45.

Hedegaard, C., Wenk, H-R., 1998. Microstructure and texture patterns of mollusk shells. J. Mollus. Stud. 64, 133-136.

Henley, W.F., Zimmerman, L.L., Neves, R.J., Kidd, M.R., 2001. Design and evaluation of recirculating water systems for maintenance and propagation of freshwater mussels. N. Am. J. Aquac. 63, 144-155.

Herbers, K., 1914. Entwicklungsgeschichte von *Anodonta cellensis* Schröt. Zeitschr. Wiss. Zool. 108, 174-182.

Hinch, S.G., Bailey, R.C., 1988. Within- and among-lake variation in shell morphology of the freshwater clam *Elliptio complanata* (Bivalvia: Unionidae) from south-central Ontario lakes. Hydrobiologia 157, 27-32.

Hoshaw, R.W., Rosowski, J.R., 1973. Methods for microscopic algae. In: Stein, J.R. (Ed.), Handbook of Phycological Methods, Culture Methods and Growth Measurements. Cambridge University Press, USA, pp. 68-73.

Huca, G.A., Brennerand, R.R., Niveiro, M.H., 1983. A study of the biology of *Diplodon delodontus* (Lamarck,1819). Veliger 25, 51-58.

Hudson, R.G., Isom, B.G., 1984. Rearing juveniles of the freshwater mussels (Unionidae) in a laboratory setting. Nautilus 98, 129-135.

Imlay, M. J., 1972. Greater adaptability of freshwater mussels to natural rather than to artificial displacement. Nautilus 86, 76-79.

Isom, B.G., Hudson, R.G., 1982. *In vitro* culture of parasitic freshwater mussels glochidia. Nautilus 96, 147-151.

Isom, B.G., Hudson, R.G., 1984. Freshwater mussels and their fish hosts; physiological aspects. J. Parasitol. 70, 318-319.

Kaestner, A., 1967. Zoology. John Wiley and Son Inc., USA, pp. 352-393.

Kays, W.T., Silverman, H., Dietz, T.H., 1990. Water channels and water canals in the gill of the freshwater mussel, *Ligumia subrostrata*: ultrastructure and histochemistry. J. Exper. Zool. 254, 256-269.

Karayücel, S., Karayücel, İ., 2000. The effect of environmental factors, depth and position on growth and mortality of raft-cultured blue mussels, (*Mytilus edulis* L.). Aquac. Res. 31, 893-899.

Kannasut, C., 1971. On the fauna of fresh-water clams of Thailand. Annual Report 1971, Department of Fishery, Ministry of Agriculture and Cooperation, Bangkok, pp. 59-138.

Keller, A.E., Zam, S.G., 1990. Simplification of *in vitro* culture techniques for freshwater mussels. Environ. Toxicol. Chem. 9, 1291-1296.

Kovitvadhi, S., Kovitvadhi, U., Phasugdee, S., Nagachinta, A., Meejui, O., Chatchavalvanich, K., Siripaisarnpipat, S., Wongchaisuwat, A., Pitiyon, B., Boonyaprapat, N., Chongchaithet, N., 2005. Development of freshwater pearl culture in Thailand. Scientific Council of Thailand, Bangkok, 344 pp.

Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Thongpan, A., Machado, J., 2006. Optimization of diet and culture environment for larvae and juvenile freshwater pearl mussels, *Hyriopsis* (*Limnoscahpa*) *myersiana* Lea, 1856. Invertebr. Reprod. Dev. 49, 61-70.

Kovitvadhi, U., Chaopaknam, B., Nagachinta, A., Jongrungwit, K., Kulayanamit, S., 1998. Ecology of freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856) in the Mae Klong River, Kanchanaburi Province. Kasetsart J. (Nat. Sci.) 32, 1-12.

Kovitvadhi, U., Chatchavalvanich, K., Noparatnaraporn, N., Machado, J., 2001. Scanning electron microscopy of glochidia and juveniles of the freshwater mussel, *Hyriopsis myersiana*. Invertebr. Reprod. Dev. 40, 143-151.

Kovitvadhi, U., Nagachinta, A., Aungsirirut, K., 2000. Species composition and abundance of plankton in the gut contents of freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana*. J. Med. Appl. Malacol. 10, 203-209.

Kovitvadhi, U., Pakkong, P., Noparatnaraporn, N., Vilarinho, L., Machado, J., 2002. Study of a suitable fish plasma for *in vitro* culture of glochidia *Hyriopsis myersiana* (Lea, 1856). Aquaculture 209, 197-208.

Kovitvadhi, U., Pakkong, P., Noparatnaraporn, N., Vilarinho, L., Machado, J., 2003. Studies on the plasma composition of fish hosts of the freshwater mussel, *Hyriopsis myersiana*, with implications for improvement of the medium for culture of glochidia. Invertebr. Reprod. Dev. 44, 53-61.

Lajtner, J., Marušić, Z., Klobučar, G.I.V., Maguire, I., Erben, R. 2004. Comparative shell morphology of the zebra mussel, *Dreissena polymorpha* in the Drava river (Croatia). Biologia 59, 595-600.

Layzer, J.B., Gordon, M.E., Andrson, R.M., 1993. Mussels: the forgotten fauna of regulated rivers. A case study of the Caney Fork River. Regulated Rivers: Res. Manag. 8, 63-71.

Layzer, J.B., Madison, L.M., Khym, J.R., Quinn, R.D., 1999. Developing technology for long-term holding of mussels in captivity. Annual Report to the U.S. Fish and Wildlife Service. Biol. Rep. 89(15), 98.

Lemly, D.A., 1998. Bacterial growth on stream insects: potential for use in bioassessment. J. North Am. Benthol. Soc. 17, 228-238.

Lima, P., Kovitvadhi, U., Kovitvadhi, S., Machado, J., 2006. *In vitro* culture of glochidia from the freshwater mussel *Anodonta cygnea*. Invert. Biol. 125, 34-44.

Lleonart, J., Salat, J., Torres, G.J., 2000. Removing allometric effects of body size in morphological analysis. J. Theor. Biol. 205, 85-93.

Lopes-Lima, M., Pereira, L., Freitas, S., Gouveia, E., Andrade, J., Checa, A., Machado, J., 2008. Effects of Heavy Metals on the Ionic Regulation and Shell Mineralization of bivalve *Anodonta cygnea*. Ecotoxicology (submitted).

Machado, J., Castilho, F., Coimbra, J., Sá, C., Monteiro, E., 1988a. Ultrastructural and cytochemical studies in the mantle of *Anodonta cygnea*. Tissue and Cell 20 (5), 797-807.

Machado J., Coimbra J., Castilho F., Sá C., 1990. The effects of diflubenzuron on the shell formation of the freshwater clam, *Anodonta cygnea*. Arch. Environ. Contam. Toxic. 19, 35-39.

Machado J., Coimbra J., Sá C., 1989. Shell thickening in *Anodonta cygnea* by TBTO treatments. Comp. Biochem. and Physiol. 92C (1), 77-80.

Machado J., Coimbra J., Sá C., Cardoso I., 1988b. Shell thickening in *Anodonta cygnea* by induced acidosis. Comp. Biochem. Physiol. 91 A (4), 645-651.

Machado J., Reis, M.L., Coimbra, J., Sá C., 1991. Studies on chitin and calcification in the inner layers of the shell of *Anodonta cygnea*. J. Comp. Physiol. 161, 413-418.

MacMillan, R.J., Cawthron, R.J., Whyte, S.K., Lyon, P.R., 1994. Design ad maintenance of a closed artificial seawater system for long-term holding of bivalve shellfish. Aquac. Eng. 13, 241-250.

Mann, K.H., Lazier, J.R.N., 1991. Dynamics of Marine Ecosystems: Biological-Physical Interactions in the Ocean. Blackwell Scientific Publications, Cambridge, 466 pp.

Matteson, M.R., 1955. Studies on the natural history of the Unionidae. Am. Midl. Nat. 53, 126-145.

McMahon, R.F., Bogan, A.E., 2001. Mollusca: Bivalvia. In: Ecology and Classification of North American Freshwater Invertebrates, 2nd ed., J.H. Thorp and A.P. Covich (eds.), Academic Press, San Diego, pp. 315-374.

Meesukko, C., 1996. Karyotype of freshwater Amblemid mussels in Yom and Nan Watersheds. Master's Thesis, Chulolongkorn University, Bangkok, Thailand.

Metcalfe-Smith, J.L., 1994. Influence of species and sex on metal residues in freshwater mussels (family Unionidae) from the St. Lawrence River, with implication for biomonitoring progeams. Environ. Toxicol. Chem. 13, 1433-1443.

Michaelson, D.L., Neves, R.J., 1995. Life history and habitat of the endangered dwarf wedgemussel *Alasmidonta heterodon* (Bivalvia: Unionidae). J. North Am. Benthol. Soc. 14, 324-340.

Miller, A. C., Payne, B. S., 2004. Reducing risks of maintenance dredging on freshwater mussels (Unionidae) in the Big Sunflower River, Mississippi. J. Environ. Manag. 73, 147-154.

Morton, B., Prezant, R.S., Wilson, B., 1998. Class Bivalvia. In: Mollusca: The Southern Synthesis, Part A Fauna of Australia Vol. 5, P.L. Beesley, G.J.B. Ross and A. Wells (eds.), CSIRO Publishing, Melbourne, pp. 195-234.

Moura, G., Almeida, M.J., Machado, M.J.C., Machado, J., 2001. Effects of heavy metal exposure on ionic composition in the fluids and nacre of *Anodonta cygnea* (Unionidae). Haliotis 30, 33-44.

Moura, G., Vilarinho, L., Guedes, R., Machado, J., 2000. The action of some heavey metals on the calcification process of *Anodonta cygnea* (Unionidae): nacre morphology and composition changes. Haliotis 29, 43-53.

Nagachinta, A., Meejui, O., 1998. Reproductive biology of Thai freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856). Phuket Mar. Biol. Cent. Special Publication 18, 103-106.

Nagachinta, A., Sanassanonta, N., Tispijit, K., Tispijit, N., 1986. Preliminary study on pearl culture in two species of freshwater mussel. Proceeding of the Annual Meeting Seminar, Kasetsart University, Fisheries Section, Bangkok (in Thai), pp. 395-401.

Nalepa, T.F., Gardener, W.S., Malcyk, J.M., 1991. Phosphorus cycling by mussels (Unionidae: Bivalvia) in Lake St. Clair. Hydrobiologia 219, 239-250.

Narksuwan, S., 1992. Species composition and abundance of phytoplankton various the watershed classes in the Maeklong River basin. Master's Thesis, Kasetsart University, Bangkok, Thailand.

Neori, A., Shpigel, M., Ben-Ezra, D., 2000. A sustainable integrated system for culture of fish seaweed and abalone. Aquaculture 186, 279-291.

Neves, R.J., 1993. A State-of-the-Unionids Address. In: Cummins, K.S., Buchanan, A.C., Koch, L.M. (Eds.), Conservation and Management of Freshwater Mussels. Proceedings of a UMRCC symposium. Upper Mississippi River Conservation Committee, Rock Island, Illinois, pp. 1-10.

Newell, C.R., Hidu, H., 1982. The effects of sediment type on growth rate and shell allometry in the soft shelled clam *Mya arenaria* (L.). J. Exp. Mar. Biol. Ecol. 65, 285-295.

O'Beirn, F.X., Neves, R.J., Steg, M.B., 1998. Survival and growth of juvenile freshwater mussels (Unionidae) in a recirculating aquaculture system. Am. Malacol. Bull. 14, 165-171.

Panha, S., 1990. The site survey and the study on reproduction cycles of freshwater pearl mussels in the central part of Thailand. Venus 49, 240-257.

Panha, S., 1992. Infection experiment of glochidium of a freshwater pearl mussel, *Hyriopsis* (*Limnoscapha*) *myersiana* (Lea, 1856). Venus 51, 303-314.

Panha, S., Eongprakornkeaw, A., 1995. Glochidium shell morphology of Thai amblemid mussels. Venus 54 (3), 225-236.

Parker, B.C., Patterson, M.A., Neves, R.J., 1998. Feeding interactions between native freshwater mussels (Bivalvia: Unionidae) and zebra mussels (*Dreissena polymorpha*) in the Ohio River. Am. Malacol. Bull. 14, 173-179.

Parmalee, P.W., Bogan, A.E., 1998. The Freshwater Mussels of Tennessee. University of Temmessee Press, Knoxville, 328 pp.

Paterson, C.G., 1986. Particle-size selectivity. I The freshwater bivalve *Elliptio complanata* (Lightfoot). Veliger 29, 235-237.

Pechenik, J.A., 1996. Biology of the Invertebrate, 3rd ed. Times Mirror Higher Education Group, Inc., USA, 475 pp.

Pierce, S.K., Maugel, T.K., 1987. Illustrated Invertebrate Anatomy: A Laboratory Guide. Oxford University Press, New York, 357 pp.

Prescott, G.W., 1951. Algae of the Western Great Lakes Area Exclusive of Desmids and Diatoms. Cranbrook Institute of Science, Michigan, pp. 17-267.

Raby, D., Mingelbier, M., Dodson, J.J., Klein, B., Lagaduce, Y., Legendre, L., 1997. Food-particle size and selection by bivalve larvae in a temperate embayment. Mar. Biol. 127, 665-672.

Rakkittham, C., 1996. Investigation on species composition and abundance of plankton in the Maeklong River, Kanchanaburi Province. Master's Thesis, Kasetsart University, Bangkok, Thailand.

Redding, T., Todd, S., Midlen, A., 1997. The treatment of aquaculture wastewaters: A botanical approach. J. Environ. Manag. 50, 283-299.

Reddy, N.A., Menon, N.R., 1979. Effects of ammonia and ammonium on tolerance and byssogenesis in *Perna viridis*. Marine Ecology-progerss Series 1, 315-322.

Reid, R.G.B., McMahon, R.F., Foighil, D.Ó., Finnigan, R., 1992. Anterior inhalant currents and pedal feeding in bivalves. Veliger 35, 93-104.

Ricker, W.E., 1973. Linear regressions in fishery research. J. Fish. Res. Bd. Can. 30, 409-434.

Rogers, S.O., 1999. Population biology of the Tan Riffleshell (*Epioblasma florentina walkeri*) and the effects of substratum and light on juvenile mussel propagation. Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Scheller, J.L. 1997. The effect of die-offs of asian clams (*Corbicula fluminea*) on native freshwater mussels (Unionidae). Master's Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Seed, S., Suchanek, T.H., 1992. Population and community ecology of Mytilus. In: Gosling, E. M. (Ed.), The Mussel Mytilus: Ecology, Physiology, Genetics and Culture. Elsevier Science Publishers B.V., Amsterdam, pp. 87-169.

Seshaiya, R.V., 1941. Tadpoles as host for the glochidia of the freshwater mussel. Curr. Sci. 10, 535-536.

Sokal, R R., Rohlf, F.J., 1995. Biometry, 3rd Edition. Freeman, New York, 887 pp.

Spotte, S., 1979. Fish and Invertebrate Culture: Water Management in Closed Systems. John Wiley and Sons, New York, 179 pp.

Sprung, M., Rose, U., 1988. Influence of food size and food quantity in the feeding of the mussel *Dreissena polymorpha*. Oecologia 77, 526-532.

Steg, M.L., 1998. Identification of host fishes and experimental culture of juveniles for selected freshwater mussel species in Virginia. Master's Thesis, Virgenia Polytechnic Institute and State University, Blacksburg, Virginia.

Strayer, D.L., 1999. Use of flow refuges by unionid mussels in rivers. J. North Am. Benthol. Soc. 18, 468-476.

Summers, J.M., 1998. Response of artificially and naturally transformed juvenile Mussels, *Utterbackia imbecillis*, to environmental contaminants. Master's Thesis, Clemson Univresity, Clemson, South Carolina.

Suvatti, C., 1950. The Fauna of Thailand. Department of Fisheries, Bangkok,1100 pp.

Tankersley, R.A., Butz, S.W., 2000. Design and evaluation of a laboratory-scale recirculating aquaculture system for the captive care of freshwater mussels. Proceedings of the Conservation, Captive Care, and Propagation of Freshwater Mussels Symposium, pp. 127-134.

132

Thientaworn, P., 1997. Relationships between phytoplankton and water quality in the Maeklong River. Master's Thesis, Kasetsart University, Bangkok, Thailand.

Uthaiwan, K., Noparatnaraporn, N., Machado, J., 2001. Culture of glochidia of the freshwater pearl mussel *Hyriopsis myersiana* (Lea, 1856) in artificial media. Aquaculture 195, 61-69.

Vogel, S., 1981. Life in Moving Fluids: The Physical Biology of Flow. Princeton University Press, Princeton, New Jersey, 352 pp.

Wang, N., Christopher, G.I., Hardesty, D.K., Ivey, C.D., Kunz, J.L., May, T.W., Dwyer, F.J., Roberts, A.D., Augspurger, T., Kane, C.M., Neves, R.J., Barnhart, M.C., 2007. Acute toxicity of copper, ammonia, and chlorine to glochidia and juveniles of freshwater mussels (Unionidae). Environ. Toxicol. Chem. 26, 2036-2047.

Watters, G.T., O'Dee, S., 1998. Metamorphosis of freshwater mussel glochidia (Bivalvia: Unionidae) on amphibians and exotic fishes. Am. Midl. Nat. 139, 49-57.

Williams, J.D., Warren, M.L., Cummings, K.S., Harris, J.L., Neves, R.J., 1993. Conservation status of freshwater mussels of the United States and Canada. Fisheries 18, 6-22.

Wongrat, L., 1998. Zooplankton. Faculty of Fisheries, Kasetsart University, Bangkok (in Thai), 787 pp.

Wongrat, L., 1999. Phytoplankton. Faculty of Fisheries, Kasetsart University, Bangkok (in Thai), 851 pp.

Yeager, M.M., Cherry, D.S., Neves, R.J., 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, *Villosa iris* (Bivalvia: Unionidae). J. North Am. Benthol. Soc. 13, 217-222.

Yonge, C.M., 1982. Mantle margins with a revision of siphonal types in the Bivalvia. J. Mollus. Stud., 48, 102-103.

Young, M., Williams, J., 1984. The reproductive biology of the freshwater pearl mussel *Margaritifera magaritifera* L. in Scotland I. Field Studies. Archiv fur Hydrobiologie 99, 405-422.