et al
2001

ELSEVIER

Aquaculture 195 (2001) 61–69

Aquaculture

www.elsevier.nl/locate/aqua-online

Culture of glochidia of the freshwater pearl mussel *Hyriopsis myersiana* (Lea, 1856) in artificial media

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Received 13 January 2000; received in revised form 14 September 2000; accepted 4 October 2000

Abstract

The freshwater pearl mussel, *Hyriopsis myersiana* (Limnoscapha) (Lea, 1856) was cultured in two artificial media at $23 \pm 2°C$. Each artificial medium contained a mixture of M199. (Life Technologies, No. 71N0262) horse serum or fish (*Oreochromis niloticus*) artificial medium plasma as a protein source, and antibiotics/antimycotics at a ratio of 2:1:0.5. Glochidia were reared until they became juveniles, i.e. until the mantle and foot could be observed under a light microscope. The duration of glochidia development until the juvenile stage was 9–10 days in both media. After 1 month of controlled feeding with phytoplankton, the juveniles showed an elongate of shell with several growth lines. The more suitable artificial culture formula for the transformation from glochidia to juvenile stage was the medium containing protein from fish plasma. Survival from glochidia to juvenile stage was up to $85.3 \pm 3.9\%$ in fish plasma, while it was equal to $46.2 \pm 12.7\%$ in horse serum. The transformation from glochidia to juvenile stage was up to $84.3 \pm 2.3\%$ in fish plasma, while it was equal to $44.3 \pm 8.9\%$ in horse serum. Percentage survival and transformation from glochidia to juvenile stage were significantly higher in fish plasma than in horse serum ($P < 0.01$). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Glochidia; Artificial culture; Freshwater mussel

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PII: S0044-8486(00)00541-X

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1. Introduction

Hyriopsis myersiana (Limnoscapha) (Lea, 1856) is one of the freshwater mussels that is economically important since its flesh is consumable with high protein content (up to 41%). The nacreous mussel shells are valuable for freshwater pearl production, as ornaments for inlaying pearl furniture, and as nuclei for the cultured pearls industry. They also play an important role in maintaining the balance of the ecological system (e.g. Dudgeon and Morton, 1984; Panha, 1992; Pennak, 1978).

At present, the water quality is deteriorating due to the accumulation of human and industrial waste as well as agricultural activities such as pesticides and herbicides (Keller and Zam, 1990). Furthermore, a massive decrease in fish populations, which are the hosts of mussels at glochidia stage, diminishes the possibility to support the environmental balance, resulting in a subsequent decrease in the mussel population. Therefore, efforts are being made to find freshwater mussel culture techniques for mass production and conservation. Generally, mussel culture tends to mimic natural culture, because in the larval stage, the glochidia need to parasitise (glochidiosis) fish or some amphibians prior to transformation into the early juvenile stage (e.g. D'Eliscu, 1972; Seshaiya, 1941; Watters and O'Dee, 1998; Kraemer and Swanson, 1985). However, high juvenile mortality in nature is evident due to the disturbance caused by bacteria, protozoa and contaminating fungi. These complex culture processes can scarcely contribute to the maintenance of the mussel population. Alternatively, it is possible to use artificial media for glochidia culture to achieve high production as well as preventing contamination. For these reasons, Isom and Hudson (1982, 1984a,b) and Keller and Zam (1990) developed simple culture techniques for glochidia in artificial media which allowed a high percentage of survival. The main goal in the present study is to improve artificial glochidia culture and juvenile production. Guidelines to increase the population of freshwater mussels for pearl culture and the development of activities in Thailand will be defined. Therefore, Isom and Hudson (1984b) and Keller and Zam (1990) culture techniques were adapted to the local species of *H. myersiana* (L.) using the plasma from the exotic fish, Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758). The most important steps in the present study concern the preparation of glochidia without contamination of specimens in order to produce strong glochidia with successful metamorphosis using an inexpensive culture system.

2. Materials and methods

2.1. Composition of the artificial media

Glochidia were cultured in two media containing different sources of protein: horse serum and fish plasma (*O. niloticus*). The composition of each formula is shown in Table 1. These artificial media were based on those improved by Keller and Zam (1990) which consisted of a modification from the formulae of Isom and Hudson (1982,

Table 1
Composition of two artificial medium for in vitro culture of *H. myersiana* (L.) glochidia

Composition of media	Concentration of each artificial medium (ml)	
	Formula 1	Formula 2
M199	2.0	2.0
Horse serum	1.0	–
Fish plasma	–	1.0
Antibiotics and antimycotic[a]	0.5	0.5

[a]Detail in Table 2.

1984a,b). The main difference concerns the composition of the commercial media M199. Furthermore, while the protein source was exclusively horse serum in Keller and Zam (1990), in the present work, fish plasma was used as an alternative support to the medium.

2.2. Preparation of glochidia media

2.2.1. M199 preparation

Distilled water was poured into 1-l beakers, which contained one packet of M199 powder (Life Technologies, No. 71N0262) to make 1 l of solution, then stirred. Two grams of $NaHCO_3$ were added and pH adjusted to 7.3–7.4 using 1.25 M NaOH or 1 M HCl (Keller and Zam, 1990). Finally, the whole solution was filtered through 0.45- and 0.20-μm filter paper, respectively.

2.2.2. Fish plasma preparation

O. niloticus was anesthetized with 50–1000 mg/l of quinaldine (Sado, 1985). Fish blood was collected from the caudal vein, in the tail area, using a syringe needle no. 18 (1.2 mm in diameter and 40 mm in length). The syringe was coated with sodium heparin at 1000 unit/ml. Then, the blood was centrifuged at 1000 and 3000 rpm for 10 min each.

The blood samples were placed in test tubes and centrifuged again at 3000 rpm for 10 min. Plasma portion (clear yellow in colour) was separated and filtered through 0.45- and 0.20-μm filter paper, respectively.

2.2.3. Preparation of glochidia

Soil and algae were thoroughly removed from the outer shells of gravid mussels. Tongs were used to open the shell slightly in order to observe marsupia colour, which indicates the development of the embryonic stage. Yellow colour indicated glochidia still in the immature stage, while partially brown colour means the beginning of maturity. Only gravid mussels with completely brown marsupia were selected in order to extract glochidia. After a preliminary inspection of the gravid mussel marsupia, the glochidia activity was tested by using a sterilized 1-ml syringe with an 18-gauge needle.

For inspection, the glochidia were sucked and discharged into a 100-ml beaker with sterilized distilled water and aeration. Then, the glochidia were examined under a light microscope (×400). If their shells periodically closed, they were considered strong and suitable for culturing in artificial media. After this, the gravid mussels were forced open with tongs and the glochidia were sucked out by inserting the needles into the marsupia. Since glochidia are usually found in groups, attached to each other, they had to be separated by placing them in a petri dish with sterilized distilled water and using a pipette to spray water onto them. Water spraying for glochidia separation had to be carefully performed to avoid shell breaking and dying. Then, they were collected and placed in a 100-ml beaker with sterilized distilled water and aeration. Thereafter, the water was changed slowly several times to eradicate tissue residues, mucus and glochidia shell fragments. The cleaning was necessary because the strong glochidia initially fall to the beaker bottom. Once there were no more residue, the cleaning was considered complete and the stronger glochidia were used for culture. It was found that glochidia from gravid mussels should be placed in artificial media no later than 5 h after harvesting since mortality then rapidly increases.

2.2.4. Glochidia culture

Glochidia were cultured in tissue culture dishes (60 × 15 mm). Each culture dish contained 3.5 ml of artificial media (Table 1) with fish plasma or horse serum. In these media, there were incubated 50–100 glochidia/dish. The natural supply of nutrients, growth factors, oxygen and pH maintenance were simulated by the artificial conditions. The culture media were not circulated or even changed during culture to the juvenile stages. In fact, disturbance caused by some manipulation or medium flux caused a stress effect during the larval stage. To prevent contamination from pathogenic organisms, antibiotic and antimycotic compounds were added to the media according Tables 1 and 2.

All culture dishes were placed in plastic boxes and then inside an incubator with a constant supply of 5% CO_2 and room humidity. The internal temperature in the box was kept at 23 ± 2°C until glochidia transformed into an active juvenile stage. The foot extended outside the shell could be observed as an indicator for this transformation. Glochidia mortality counts under a light microscope (×400) were then performed in

Table 2
Combination of antibiotics and antimycotic for in vitro culture of *H. myersiana* (L.) glochidia

Compound	Concentration (μg/ml)
Antibiotics	
Carbenicillin	100
Gentamycin sulfate	100
Rifampin	100
Antimycotic	
Amphotericin B	5

order to estimate the glochidia transformation success into juvenile stage. A completely randomized design with two treatments and 30 replications was utilized. Data analysis was done using ANOVA.

2.2.5. *Juveniles culture*

After complete transformation, which took 9–10 days, early juveniles were removed from the culture medium, rinsed in a mixture of sterilized water and M199, and placed in a beaker containing 200–300 ml of dechlorinated and aerated water. The juveniles were fed initially with a mixture of 3 phytoplankton species (*Chlamydomonas* sp.; *Monoraphidium* sp.; *Chlorella* sp.), collected from a purified stock and added to the water until it gave a slightly green colour. Half this culture water was changed daily. The juveniles were fed for 2 months.

2.2.6. *Glochidia and juvenile morphology*

After incubation, morphological transformation was carefully observed using light microscopy until the distinct signs of early juveniles appeared. These signs were found to consist mainly of the mantle edge and the foot formation. After this metamorphosis, shell growth as well as the appearance of gills and the accumulation of some organic matter inside the gastrointestinal tract were also observed.

3. Results

The blood collection from caudal vein of *O. niloticus* was satisfactory since we obtained an average of 10 ml blood/kg of fish and at the same time the fish could be kept alive for further use.

The microscopic observations showed glochidia with semi-oval shapes 1–2 weeks after fertilization (Fig. 1). Some successful transformations occurred within 7–8 days in culture medium. The edge of the mantle bordered outside the shell (Fig. 2). During the transformation process, the larvae remained in a semi-oval shape until the foot appeared (Fig. 3). The foot was totally formed within 9–10 days resulting in quick movements when stimulated by addition of distilled water and aeration. The juvenile shell increased rapidly during the first month with the glochidia shell remaining near the hinge (Fig. 4), and the gastrointestinal tract contained organic matter (Fig. 5). Growth lines on the juvenile shell were evident (Fig. 5). The expanded foot and shell growth lines (Fig. 6) and the gills (Fig. 7) were clearly evident for 2 months, as well as other important details of the juvenile formation.

We found that glochidia could transform into the juvenile stage in the media containing either fish plasma or horse serum as protein sources. the transformation period was 9–10 days for both media. The average percent survival in fish plasma and in horse serum from glochidia to early juvenile stage was 85.3 ± 3.9 and 46.2 ± 12.7, respectively, and the percent transformation was equal to 84.3 ± 2.3 and 44.3 ± 8.9, respectively (Table 3). Metamorphosis was observed until the appearance of the foot, which is a clear indication of transformation.

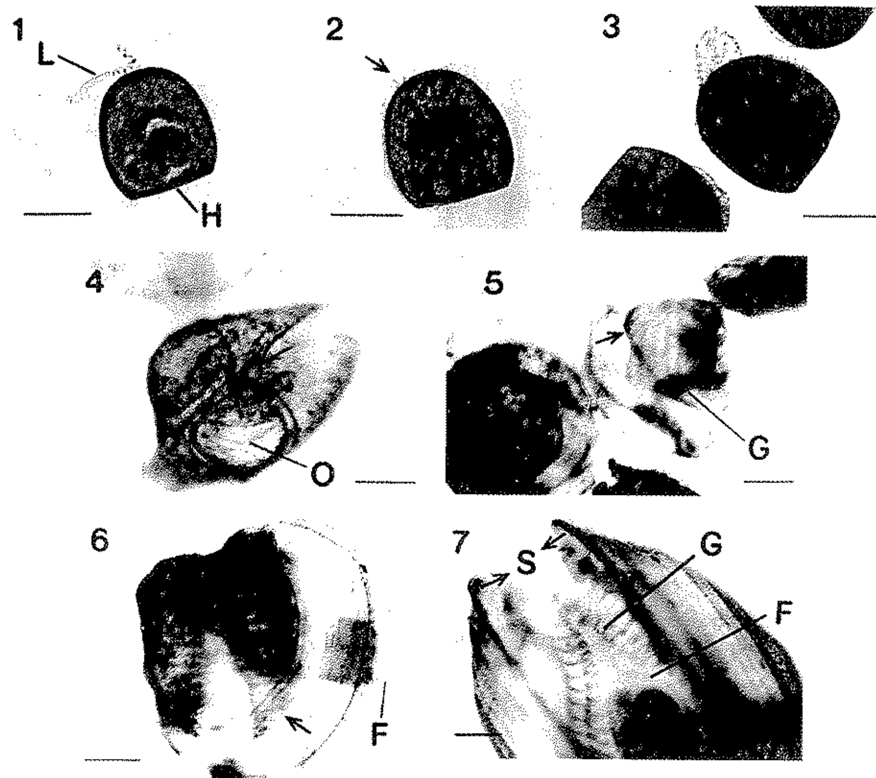

Fig. 1. *H. myersiana* glochidia 1–2 weeks after fertilization. H: hinge; L: larva thread. Bar = 100 μm.

Fig. 2. *H. myersiana* glochidia with a bordering mantle edge (arrow) bordering shell outside. Bar = 100 μm.

Fig. 3. *H. myersiana* early juveniles with a foot. Bar = 100 μm.

Fig. 4. Dorsal view of juvenile *H. myersiana* shell showing the original glochidia shell (O).

Fig. 5. View from side of juvenile *H. myersiana* shell showing the gastrointestinal tract with organic matter (G) and growth lines (arrow). Bar = 400 μm.

Fig. 6. View from side of juvenile *H. myersiana* shell, showing the expanded foot outside the shell (F) and shell growth lines under elongated shape (arrow).

Fig. 7. Inside view of juvenile *H. myersiana* with grills (G), foot (F) and open shell (S). Bar = 400 μm.

The results of range and average percent survival and percent transformation of glochidia into juvenile stage are shown in Table 3. It is clear that the SD value of horse serum is higher than that of fish plasma, showing the variation of media treatments. The lower variation associated with high survival and significant average percentage of transformation reflects more consistent and superior environmental conditions in the fish plasma culture medium.

Table 3
Range and average percent survival and transformation of *H. myersiana* (L.) glochidia in artificial media with two different protein sources

Media	Percent survival			Percent transformation		
	Range	Mean	(SD)	Range	Mean	(SD)
M199 + horse serum	38.5–60.8	46.2^a	(12.7)	34.3–51.3	44.3^a	(8.9)
M199 + fish plasma	81.8–89.5	85.3^b	(3.9)	80.60–85.40	84.3^b	(2.3)

Each treatment had 30 replicates with 50–100 glochidia number.
Values in the same column with a different superscript are significantly different. ($P < 0.01$).

Percent transformation from glochidia to juvenile stage and percent survival in fish plasma were significantly higher than in the horse serum situation ($P < 0.01$).

4. Discussion and conclusion

O. niloticus, also known by Nile Tilapia, was introduced to Thailand about 1950 and showed high tolerance to the local environmental conditions (Noakes and Balon, 1982). The water temperatures of 20–22°C in the south of this country were favorable for the growth and reproduction of Nile Tilapia (Dan and Little, 2000). Thus, this species was soon used as a natural resource in Thailand (e.g. Little et al., 1995; Bhujel, 2000). *O. niloticus* was also selected as the fish host of *H. myersiana* (L.) because of their successful parasitism, as reported by Arayawatanavij et al. (1992): the percentage of infested fish was 100, 100, 90 and 10 for *O. niloticus*, *Labeo rohit* (Hamilton, 1822), *Cyprinus carpio* (Linnaeus, 1758) and *Puntius gonionotus* (Bleeker, 1850), respectively. All species of experimental fishes, except *P. gonionotus*, produced mussel juveniles. when considering the percentage of survival of the infested fishes as well as the incidence and intensity of the infestation. *O. niloticus* was the most appropriate host for glochidia of *H. myersiana* (L.) (Arayawatanavij et al., 1992). Thus, it was selected to supply plasma as a protein source for the artificial culture in the present work.

The medium formulae of Isom and Hudson (1982, 1984b) were modified by Keller and Zam (1990) for artificial production of mussel *Utterbackia imbecileis* (Howells et al., 1996) and replaced with standard tissue culture media (M199 and DME). No significant difference of juvenile production from DME and M119 media with horse serum was found by Keller and Zam (1990), being 65.8 ± 16.7 and $65.4 \pm 12.9\%$, respectively. However, the juvenile production with the medium of Isom and Hudson (1982, 1984b) was equal to 51.2 ± 11.6, significantly lower than values with M199 and DME media. Since M199 medium is efficient and cheap, it is the preferred for culturing *H. myersiana* (L.).

Our results showed that the percentage of survival and transformation in M199 medium were significantly higher with fish plasma than with horse serum as the protein source. The percent transformation in fish plasma (84.3 ± 2.3) was comparable with similar values ($81.8 \pm 7.5\%$) in the Isom and Hudson medium, as found Keller and Zam (1990). However, our results are better, since the period of glochidia transformation

cultured in fish plasma showed less variation in a more available medium (M199). Our research suggests that the exotic fish, *O. niloticus*, may present an effective adaptation close to the specific host properties. This eases either the successful natural enclosure of mussel *H. myersiana* (L.) or the artificial culture. This is according to Yeager and Saylor (1995), who pointed out that some freshwater fishes show high host specificity for glochidia due to their physiological adaptations.

Concerning other parameters, it is very important to keep the pH at 7.3–7.4 by adding $NaHCO_3$ and using 5% CO_2 as done by Keller and Zam (1990). However, the humidity does not constitute any problem for culture.

Additional data from microscopic observations gave us morphological information on the normal development of early juveniles. The foot, gills, gastrointestinal tract, growth lines on the shell and movements are signs of complete metamorphosis.

In summary, the percentage of transformation found by Keller and Zam (1990) was high, but with inactive and lethargic juveniles. Artificial media (M199; DME) with horse serum produced a significantly lower percentage but more healthy individual (Keller and Zam, 1990). Our study showed that the combination of artificial media (M119) and fish plasma gives a high percentage of transformation and good survival during juvenile stages.

Acknowledgements

We are very grateful to Mr. Rungsan Suangchompun, Director of the Kanchanaburi Inland Fisheries Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives, Kanchanaburi Province, Thailand, and also to Mrs. Aurapa Nagachinta and Mrs. Oodium Meejui, whose supply of mussels was greatly appreciated. Our special thanks to Dr. Amara Thongpan and Dr. Martin Shwartz for reviewing and correcting the manuscript. We also would like to thank Prof. Dr. Anake Kijjoa for his valuable suggestion and kind assistance throughout this study.

References

Arayawatanavij, P., Nagachintam, 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), 32 pp.

Bhujel, R.C., 2000. A review of strategies for the management of Nile tilapia (*Oreochromis niloticus*) broodfish in seed production systems, especially hapa-based systems. Aquaculture 181, 37–59.

Dan, N.C., Little, D.C., 2000. The culture performance of monosex and mixed-sex new-season and overwintered fry in three strains of Nile tilapia (*Oreochromis niloticus*) in northern Vietnam. Aquaculture, 221–231.

D'Eliscu, P.D., 1972. Observation of the glochidium, metamorphosis, and juvenile of *Anodonta californiensis* Lea, 1857. The Veliger 15 (1), 57–59.

Dudgeon, D., Morton, B., 1984. Site selection and attachment duration of *Anodonta woodiana* (Lea, 1834) (Bivalvia: Unionacea) glochidia on fish hosts. Journal of Zoology London 204, 355–362.

Howells, R.G., Neck, R.W., Murray, H.D., 1996. Freshwater Mussels of Texas. Texas Parks and Wildlife Press, Austin, 218 pp.

Isom, B.G., Hudson, R.G., 1982. In vitro culture of parasitic freshwater mussel glochidia. The Nautilus 96 (4), 147–151.
Isom, B.G., Hudson, R.G., 1984a. Freshwater mussels and their fish hosts: physiological aspects. Journal of Parasitology 70 (2), 318–319.
Isom, B.G., Hudson, R.G., 1984b. Culture of freshwater mussel glochidia in an artificial habitat utilizing complex liquid growth media. U.S. patent 4,449,480, 18 pp.
Keller, A.E., Zam, S.G., 1990. Simplification of in vitro culture techniques for freshwater mussels. Environmental Toxicology and Chemistry 9, 1291–1296.
Kraemer, L.R., Swanson, C.M., 1985. Functional morphology of eyespots of mantle flaps of Lampsilis: evidence for their role as effectors, and basic for hypothesis regarding pigment distribution in bivalve mantle tissues. Malacologia 26 (1–2), 241–251.
Little, D.C., Lin, C.K., Turner, W.A., 1995. Commercial scale tilapia fry production in Thailand. World Aquaculture 26 (4), 20–24.
Noakes, D.L.G., Balon, E.K., 1982. Life histories of tilapias: an evolutionary perspective. In: Pullin, R.S.V., McConnell, R.H.L. (Eds.), The Biology and Culture of Tilapias. ICLARM Conference Proceedings, International Center for Living Aquatic Resources Management, Manila, Phillippines. pp. 61–82.
Panha, S., 1992. Infection experiment of glochidium of a freshwater pearl mussel, *Hyriopsis (Limnoscapha) myersiana* (Lea, 1856). Venus 51 (4), 303–314.
Pennak, R.W., 1978. Freshwater Invertebrates of the United States. 2nd edn. Wiley, London, 628 pp.
Sado. E.K., 1985. Influence of the anaesthetic quinaldine on some Tilapias. Aquaculture 10, 55–62.
Seshaiya, R.V., 1941. Tadpoles as hosts for the glochidia of the freshwater mussel. Current Science 10, 535–536.
Watters, G.T., O'Dee, S., 1998. Metamorphosis of freshwater mussel glochidia (Bivalvia: Unionidae) on amphibians and exotic fishes. American Midland Naturalist 139, 49–57.
Yeager, B., Saylor, C.F., 1995. Fish hosts for four species of freshwater Mussels (Pelecypoda: Unionidae) in the upper Tennessee River Drainage. American Midland Naturalist 133, 1–6.