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Aquaculture 61867 (2001) xxx

Aquaculture

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Study of a suitable fish plasma for in vitro culture of glochidia *Hyriopsis myersiana*

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Received 19 December 2000; received in revised form 1 November 2001; accepted 4 November 2001

Abstract

Several organic and inorganic sources from the plasma of different fish species and horse serum were utilized as additives to the artificial culture M199 medium to improve glochidial survival and transformation of *Hyriopsis myersiana*. After 2–3 days of culturing in the medium containing plasma of Nile tilapia or hybrid catfish, striped catfish or horse serum, the glochidia presented significantly ($P < 0.05$) lower percentage survival compared to medium containing common carp plasma. The highest (93.77 ± 3.0) and lowest (32.42 ± 5.85) percentage survival rates of glochidia were found with common carp and striped catfish plasma, respectively. After 10 days, relevant signs of glochidia transformation, such as the foot and mantle edge, were observed. In all assays, the glochidia transformation reached 100% most probably due to the exchange of the medium at the fifth day and the addition of 1 ml of distilled water at the ninth day of culturing. The intense mobility of juveniles in the medium containing the common carp plasma indicated excellent culture conditions. The ideal density for this plasma corresponded to 150–200 glochidia per culture dish.

The present results suggest that M119 medium complemented with the common carp plasma and the medium exchange during culturing period may constitute a functional process to prepare an in vitro culture for freshwater mussels, particularly *H. myersiana*. The most relevant amino

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0044-8486/01/$ - see front matter
PII: S0044-8486(01)00870-5

acids for a successful development are CIT, GLX, LEU, PRO, THR and ALA particularly with the contents in the common carp plasma. © 2001 Published by Elsevier Science B.V.

Keywords: Glochidia; In vitro culture; Plasma sources; *Hyriopsis myersiana*

1. Introduction

Hyriopsis myersiana is a freshwater pearl mussel of Thailand. This mussel has a large size and a lustrous nacreous shell that can be utilized for various purposes. The native people have used it as food for domestic animals, as fish bait and in Thai cuisine such as mussel sced and thickened with coconut milk, roasted or smoke dried, fried in salt, chili and pepper, etc. The shell can be used for buttons, cooking utensils, handicraft souvenirs, ornaments for inlaying pearl furniture, and nuclei for the cultured pearls industry. Generally, the larval stage of the glochidium needs a parasitic stage (glochidiosis) on fish or some amphibians before transformation to the early juvenile stage (Lefevre and Curtis, 1910; Seshaiya, 1941; D'Eliscu, 1972; Walker, 1981; Kraemer and Swanson, 1985; Watters and O'Dee, 1998; Haag and Warren, 1999). This natural process involves many steps functioning as selective filters. In effect, a gravid female produces thousands of glochidia but only a few of them reach the adult stage. Several factors may contribute to the death of glochidia and juvenile forms, for example, the discharge process of the female and stress due to the fish infestation phenomenon. An additional factor is the vulnerability of these stages to threats by several predators, mainly fish. For these reasons, there is low juvenile production in nature, decreased still more by the infection with bacteria, protozoa and fungi in the water, mainly in the bottom mud. A further problem for the survival of some glochidia is finding of a specific host because the fish infestation requires physiological adaptations (Yeager and Saylor, 1995). The alternative, controlled mussel culture in tanks, tends to mimic the natural conditions, namely the host specificity, with the objective of increasing the juvenile production. However, many problems concerning the water quality and fish infestation still remain unsolved. In effect, even these controlled procedures are not enough to restore the mussel population in some altered ecosystems. Therefore, the use of sterilized artificial medium for culturing glochidia became pertinent to increase the mussel production. In fact, Isom and Hudson (1982, 1984a,b) and Keller and Zam (1990) developed methods for glochidia transformation in artificial medium with high percentage survival by in vitro culture. Uthaiwan et al. (2001) improved this method introducing organic and inorganic sources of fish plasma as suitable additives to artificial medium to increase the survival of glochidia and transformation to the juvenile stage.

Host fishes required by many unionid species are unknown yet critical for the management and protection of this resource (Howells, 1997). This is the case of the freshwater mussel *H. myersiana* living on the muddy sand bottom at 2–5 m depth, in the slow running water of Kwae Noi River in the Center of Thailand (Nagachinta et al., 1986; Panha, 1990; Chaopaknam et al., 1994). During October–May, glochidia of *H. myersiana* are obligatory ectoparasites of different fish species: *Cyprinus carpio*,

Oreochromis niloticus, *Pangasius pangasius*, etc. (Nagachinta and Sahassanon, 1987; Arayawatanavij et al., 1992; Panha, 1992). In the present work and in general, the artificial propagation of many freshwater mussel species that utilize unknown hosts has not been possible. This means that finding a suitable host is the most problematic step in the mussel's life cycle (Jokela and Palokangas, 1993; Kirk and Layzer, 1997). In host and nonhost fish, humoral and cell-mediated responses of the immune system occur including the presence of specific antibodies to the parasites (glochidia) (Bauer and Vogel, 1987; Kirk and Layzer, 1997). Thus, host specificity of freshwater mussels is believed to have an immunological basis (Reuling, 1919; Arey, 1932; Meyers et al., 1980). It is probable that additional specific factors in the medium composition, such as the content of ion elements, free amino acids, enzymes or other organic elements, may be determinants.

In this context, it seems important to find suitable fish plasma and ideal density of glochidia per culture dish. In the present work, extracted plasma from four fish species was added to the artificial medium with antibiotic and antimycotic agents to study the effect of host specificity for improving the survival and transformation percentages of glochidia *H. myersiana*.

2. Materials and methods

This study of glochidia culture in artificial media was divided into two steps. The first step was to try and find suitable organic and inorganic sources for better survival and transformation rates. Glochidia culture method was introduced by Isom and Hudson (1982, 1984a,b) and modified by Keller and Zam (1990) and Uthaiwan et al. (2001). In the present experiment, a mixture of M199 powder (Life Technologies, No 71NO262) with different plasma sources from four fish species and horse serum and antibiotics (carbenicillin, gentamycin sulfate and rifamn)/antimycotic (amphotericin B) were used according to Keller and Zam (1990) and Uthaiwan et al. (2001). The common carp *C. carpio* (Linnaeus, 1758), Nile tilapia *O. niloticus* (Linnaeus, 1758), hybrid catfish (*Clarias macrocephalus* × *C. garienus*) and striped catfish *P. pangasius* (Hora, 1923) were chosen with total lengths of 38.6–45.9, 23.7–30.2, 35.6–54.5 and 31.0–52.0 cm, respectively. Glochidia were cultured in tissue culture dishes (60 × 15 mm). Each culture dish contained 2 ml of M199, 1 ml of horse serum or fish plasma and 0.5 ml of antibiotic/antimycotic agents as described in Isom (1987). Gravid mussels with completely brown marsua were selected for culturing. Furthermore, the test for suitability of the glochidia for culturing is the periodic opening and closing of their shells as observed under a light microscope (×400). After this, the glochidia were petted using a sterilized 1-ml syringe with 18-gauge needle and cleaned with sterilized distilled water several times to eradicate tissue residues, mucus and glochidia shell fragments. When all residues were removed, 50–100 glochidia were added to the artificial medium under sterile conditions. All glochidia culture dishes were placed in a low-temperature incubator at 23 ± 2 °C with constant supply of 5% CO_2 and room air humidity during a period of 10 days. The culture medium was exchanged at the fifth day of culturing and 1 ml sterile distilled water per culture dish was added at the ninth day to improve

transformation and survival conditions. Then, at the first step, the percentages of glochidia survival after 2–3 days and glochidia transformation to the juvenile stage after 10 days were determined under the light microscope (×400). In this study, five medium and 15 replications per medium were investigated using a completely randomized design.

The second step consisted of an assay of different densities (glochidia number per culture dish) in the artificial medium. The plasma with better organic and inorganic source was selected from step 1 and added to artificial medium for the glochidia culture. The culture method was the same as in step 1 for five densities of glochidia number, namely 50–100, 150–200, 250–300, 350–400 and 450–500. Completely randomized design with five groups of different glochidia numbers and five replications per each were utilized.

The major organic and inorganic elements present in the fish plasma and horse serum were analyzed to establish the most determinant components in the glochidia culture medium. Forty-one free amino acids were analyzed by ion-exchange chromatography in a ninhydrin-based detection automatic system, using a standard five-lithium-buffer system (LMB 4151 Alpha Plus® Amino Acid Analyzer) designed for physiological fluid analysis, with L-norleucine as internal standard. The absorbances were read at 570 and 440 nm to allow hydroxyproline and proline quantification. Protein measurements were accomplished with biuret method (Henry and Berkman, 1957), triglyceride with enzymatic method (Koditscheck and Umbreit, 1969), glucose with enzymatic method (Trinder, 1969) and ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, K^{+}, S and Cl^{-}) by using a high-performance energy dispersive X-ray fluorescence spectrometer (Oxford ED^{2000} model) and also the osmolality using a freezing point osmometer (SLAMED 800cl model). Osmolality, protein, amino acid, glucose, triglyceride and ion elements were analysed in the plasma of four fish species from step 1, with three samples per species, and in six horse serum samples. The fish plasma percentage in all samples was also determined by the microhaematocrit method with three replications per sample.

A preliminary study of growing juveniles from a 10-day period to 2 months was also undertaken to test the relative survival resistance depending on different plasma treatments. Then, after complete transformation during a 10-day period at steps 1 and 2, juveniles and medium were removed from the treatment dish to a beaker and the total medium volume was diluted with sterilized dechlorinated water in a ratio of 1:1. This

Table 1
Range and average percentage plasma of *C. carpio* (Linnaeus, 1758), *O. niloticus* (Linnaeus, 1758), *C. macrocephalus* × *C. garienus* and *P. pangasius* (Hora, 1923)

Fish	Percentage plasma		
	Range	Mean	(S.D.)
Common carp	62–72	69.17[b]	(3.76)
Nile tilapia	65–73	69.83[b]	(2.93)
Hybrid catfish	75–85	79.17[a]	(4.58)
Striped catfish	64–82	72.79[b]	(5.98)

The values in the same column that have different superscripts are significantly different ($P < 0.05$).

Table 2
Range and average percentage survival after 2nd–3rd days and transformation at 10th day of *H. (Limnoscapha) myersiana* glochidia in artificial medium with five different organic and inorganic sources. Each treatment had 15 replications with a glochidia number 50–100 per tissue culture dish

Organic and inorganic sources	Percentage survival			Percentage transformation
	Range	Mean	(S.D.)	
Common carp	88.14–98.56	93.77[a]	(3.0)	100
Nile tilapia	74.21–96.79	84.47[ab]	(7.85)	100
Hybrid catfish	79.13–91.36	83.81[b]	(4.68)	100
Striped catfish	25.05–39.07	32.42[d]	(5.85)	100
Horse serum	38.03–54.84	43.16[c]	(6.83)	100

The values in the same column that have different superscripts are significantly different ($P < 0.05$).

diluted medium was changed twice during 2 days substituting half the volume with the equivalent volume of sterilized distilled water. Finally, these juveniles were fed during 2 months with a mixture of four phytoplankton species: *Chlamydomonas* sp., *Monoraphidium* sp., *Chlorella* sp., *Navicula* sp. These algae were collected from a purified stock and mixed in a beaker with sterilized distilled water until a slightly green colour was obtained. This algae mixture was added daily to the juveniles in the ratio of 1:1 after rejecting half of the medium volume. During the algae feeding period, the total volume was slowly increased to 300 ml. The main objective of this operation was to gradually adapt the juveniles from culture medium to an algae diet. The algae were selected based on the analyses of phytoplankton in the natural habitat of *H. myersiana* according to Uthaiwan et al. (submitted for publication).

The morphology of glochidia transformation and the mobility of juvenile were closely observed under a light microscope to detect relevant alterations of the mantle edge, foot and gill formation and body-shell increase. The observation methodology under a light microscope was similar to that used by Uthaiwan et al. (2001).

The present data extracted from the fish plasma and horse experiments for evaluating glochidia survival and transformation percentages, the organic–inorganic content and osmolality were analyzed with ANOVA and Duncan New's Multiple Range Test.

Table 3
Range and average percentage survival after 2nd–3rd days and transformation at 10th day of *H. (Limnoscapha) myersiana* glochidia in artificial medium with different density of glochidia extracted from the same gravid female. Each treatment had five replications

Density of glochidia	Percentage survival			Percentage transformation
	Range	Mean	(S.D.)	
50–100	87.14–92.47	90.29[a]	(2.55)	100
150–200	85.29–97.96	92.39[a]	(3.78)	100
250–300	82.72–96.04	90.91[a]	(5.97)	100
350–400	75.32–95.62	88.74[a]	(7.79)	100
450–500	68.04–83.88	78.21[b]	(8.83)	100

The values in the same column that have different superscripts are significantly different ($P < 0.01$).

Table 4
The organic and inorganic elements measured in the four fishes plasma and horse serum. These compounds are organized into several groups depending on the significant and non-significant differences and according to the concentration levels. The number of samples are represented by N

Organic/Inorganic elements	Adult fishes					
	Common carp ($N=3$)	Nile tilapia ($N=3$)	Hybrid catfish ($N=3$)	Striped catfish ($N=3$)	Horse serum ($N=6$)	Significance
Amino acids (μmol/l)						
The first group						
ANS	0^{b}	0^{b}	0^{b}	0^{b}	5.0 ± 7.1^{a}	*
CAR	0^{b}	0^{b}	0^{b}	0^{b}	9.0 ± 12.7^{a}	*
PEA	1.0 ± 2.0^{b}	1.67 ± 2.9^{ab}	1.33 ± 2.3^{b}	0^{b}	5.0 ± 2.8^{a}	*
CYS2	9.5 ± 5.5^{ab}	1.33 ± 1.2^{b}	14.7 ± 6.5^{a}	6.4 ± 3.7^{b}	3.0 ± 1.4^{b}	*
ABU	10.0 ± 2.2^{ab}	5.33 ± 2.1^{b}	9.67 ± 2.1^{ab}	16.6 ± 7.1^{a}	11.5 ± 3.5^{ab}	*
HYP	14.5 ± 10.7^{ab}	13.33 ± 11.9^{ab}	35.0 ± 9.5^{a}	39.2 ± 21.4^{a}	0^{b}	*
CYSTA	1.0 ± 2.0^{b}	0^{b}	44.67 ± 20.8^{a}	3.8 ± 3.8^{b}	3.5 ± 4.9^{b}	* *
The second group						
ORN	74.5 ± 26.2^{a}	21.0 ± 9.9^{b}	40.67 ± 16.1^{b}	80.4 ± 15.3^{a}	78.0 ± 8.5^{a}	* *
MET	82.25 ± 29.1^{a}	27.67 ± 14.7^{b}	80.33 ± 18.9^{a}	34.6 ± 6.1^{b}	58.5 ± 4.9^{ab}	* *
TYR	72.0 ± 18.1^{bc}	55.33 ± 9.9^{c}	100.0 ± 6.1^{a}	57.8 ± 13.2^{c}	93.0 ± 2.8^{ab}	* *
ILE	120.25 ± 26.3^{a}	54.67 ± 4.6^{b}	97.3 ± 16.2^{a}	101.6 ± 12.4^{a}	125.0 ± 4.24^{a}	* *
SER	131.75 ± 37.0^{ab}	78.67 ± 48.05^{b}	168.0 ± 35.5^{a}	74.6 ± 18.9^{b}	170.5 ± 12.0^{a}	* *
AAD	5.5 ± 6.8^{b}	1.33 ± 2.3^{b}	257.33 ± 136.65^{a}	5.2 ± 7.7^{b}	33.0 ± 26.9^{b}	* *
VAL	234.5 ± 38.2^{a}	131.0 ± 13.9^{b}	147.67 ± 31.5^{b}	145.8 ± 22.1^{b}	271.5 ± 10.6^{a}	* *
TAU	320.0 ± 78.2^{ab}	240.0 ± 266.9^{ab}	428.0 ± 137.9^{a}	246.4 ± 69.3^{ab}	102.0 ± 25.5^{b}	*
GLY	338.0 ± 32.1^{b}	223.67 ± 109.7^{b}	717.33 ± 198.1^{a}	358.8 ± 117.3^{b}	737.0 ± 203.7^{a}	* *
The third group						
ßALA	0	0	6.67 ± 11.6	0	0	ns
HYL	0	1.33 ± 2.3	0.67 ± 1.2	0	3.0 ± 4.2	ns
HCY2/GABA	2.0 ± 4.0	3.3 ± 5.8	0	2.2 ± 2.5	0	ns
PPS	3.5 ± 2.9	5.33 ± 9.2	13.0 ± 5.6	6.6 ± 3.2	9.0 ± 2.8	ns
3MHIS	9.25 ± 5.6	9.0 ± 4.4	4.0 ± 5.3	9.0 ± 15.2	16.5 ± 4.9	ns
1MHIS	17.25 ± 23.8	3.67 ± 1.2	2.0 ± 1.7	5.4 ± 0.6	3.5 ± 4.95	ns

ETN	16.25 ± 11.7	9.67 ± 16.7	9.67 ± 2.1	31.6 ± 34.4	8.0 ± 2.8	ns
PHE	87.75 ± 13.2	76.0 ± 16.4	71.33 ± 4.9	69.6 ± 23.2	95.0 ± 5.7	ns
HIS	83.0 ± 61.4	65.3 ± 24.0	47.33 ± 9.3	96.4 ± 29.5	74.0 ± 14.1	ns
ARG	182.0 ± 49.1	170.67 ± 130.8	162.0 ± 26.23	99.80 ± 24.1	119.0 ± 2.8	ns
ASX	192.25 ± 166.3	119.33 ± 70.8	145.0 ± 7.0	99.0 ± 16.0	65.5 ± 10.6	ns
LYS	201.0 ± 147.3	232.0 ± 152.6	265.33 ± 37.3	204.4 ± 43.7	132.5 ± 7.8	ns
The fourth group						
CIT	36.75 ± 9.6^{b}	8.67 ± 2.5^{c}	8.33 ± 5.8^{c}	7.0 ± 4.0^{c}	75.0 ± 4.2^{a}	* *
LEU	253.25 ± 47.2^{a}	118.33 ± 17.62^{c}	181.0 ± 26.5^{b}	179.6 ± 24.2^{b}	197.0 ± 14.1^{b}	* *
GLX	197.25 ± 43.0^{b}	61.0 ± 25.9^{d}	122.0 ± 17.5^{c}	57.6 ± 23.6^{d}	271.5 ± 14.9^{a}	* *
PRO	415.25 ± 94.6^{a}	73.67 ± 42.1^{b}	57.67 ± 23.5^{b}	56.4 ± 22.9^{b}	95.0 ± 4.2^{b}	* *
THR	331.0 ± 148.3^{a}	68.67 ± 13.6^{b}	136.67 ± 29.8^{b}	69.8 ± 17.1^{b}	185.5 ± 13.4^{b}	* *
ALA	537.75 ± 54.6^{a}	224.67 ± 62.1^{b}	306.67 ± 13.3^{b}	238.6 ± 58.5^{b}	314.5 ± 34.7^{b}	* *
Protein (g/dl)	3.93 ± 1.1^{b}	3.86 ± 0.8^{b}	3.77 ± 0.3^{b}	3.67 ± 0.9^{b}	7.93 ± 1.2^{a}	* *
Glucose (mg%)	248.17 ± 83.7^{a}	257.07 ± 107.4^{a}	252.70 ± 113.6^{a}	222.27 ± 65.1^{a}	36.93 ± 19.3^{b}	*
Triglyceride (mg/dl)	347.59 ± 120.6^{a}	286.7 ± 33.9^{a}	106.60 ± 8.9^{b}	381.16 ± 38.1^{a}	173.03 ± 39.16^{b}	* *
Inorganic elements (mg/g)						
Cu^{2+}	0.33 ± 0.49	0.27 ± 0.46	0	0.43 ± 0.8	0.33 ± 0.58	ns
Mn^{2+}	3.63 ± 4.0	7.23 ± 4.4	3.73 ± 4.3	2.27 ± 3.2	0.83 ± 1.44	ns
Ca^{2+}	271.73 ± 129.7	173.97 ± 21.9	182.07 ± 5.7	161.07 ± 23.4	177.76 ± 19.9	ns
Mg^{2+}	254.17 ± 102.1	191.50 ± 35.1	256.0 ± 60.9	246.57 ± 37.9	210.13 ± 169.7	ns
Na^{+}	2713.57 ± 479.8	654.93 ± 378.12	2403.2 ± 475.3	1564.03 ± 1118.3	2641.43 ± 234.4	ns
K^{+}	166.27 ± 33.8^{b}	183.80 ± 70.82^{ab}	264.77 ± 50.5^{a}	273.45 ± 39.4^{a}	218.27 ± 26.1^{ab}	*
S	632.30 ± 179.3^{b}	531.60 ± 191.4^{bc}	400.07 ± 25.5^{c}	346.97 ± 16.6^{c}	977.07 ± 49.2^{a}	* *
Cl^{-}	3804.23 ± 125.0^{a}	3962.13 ± 484.1^{a}	3486.93 ± 200.7^{ab}	3172.10 ± 137.5^{b}	3556.20 ± 120.7^{ab}	*
Osmolality (mosM)	335.33 ± 67.3	365.0 ± 71.2	332.67 ± 50.6	303.67 ± 11.71	324.33 ± 60.4	ns

Remark: The values in the same row that have different superscripts are significantly different.

ns = Non-significant difference ($P > 0.05$).

* = Significant difference ($P < 0.05$).

* * = Highly significant difference ($P < 0.01$).

3. Results

The fish plasma for different culture medium experiments was extracted from the adult stage. The minimum percentage average of 69.17 ± 3.76 was determined in common carp plasma and did not differ significantly from that of tilapia and striped catfish (Table 1). On the other hand, the maximum average of the plasma percentage collected from hybrid catfish was significantly higher ($P < 0.05$) and equal to 79.17 ± 4.58.

The glochidia *H. myersiana* presented the highest significant survival percentage (93.77 ± 3.0) after 2–3 days of culturing in artificial medium with common carp plasma. An exception, with non-significant difference, occurred in culture medium with Nile tilapia plasma (Table 2). The transformation with 100% success was observed in juvenile stage after 10 days for all treatments.

The glochidia culture of *H. myersiana* in different density levels of artificial medium was tested with common carp plasma as its survival percentage showed the highest values. The density level of 150–200 glochidia per 3.5 ml of artificial medium after 2–3 days presented the significant ($P < 0.05$) highest percentage (92.39 ± 3.78) (Table 3). The transformation percentage from glochidia to juvenile stage after 10 days was 100% in all density levels.

Table 4 shows analyses of the 37 free amino acids of horse serum and fish plasma from four species. These amino acids were distributed among four distinct groups based on contents and significant differences ($P < 0.05$ or $P < 0.01$) of common carp plasma when compared to the other sources. The first group consisted of those that had the lowest concentrations of amino acids between 0 and 44.67 μmol/l (ANS–CYSTA) with significant random difference. The second group represents higher concentrations, from 50 to 737 μmol/l (ORN–GLY) also with significant random difference. The third group comprises the lowest and highest concentrations between 0 and 497 μmol/l (ßALA–LYS) with non-significant differences. The fourth group concerns the most relevant meaning amino acids with the significant ($P < 0.01$) lowest and highest concentrations between 7.0 and 537.75 μmol/l (CIT–ALA).

The protein, triglyceride and glucose elements in four distinct plasma showed a significant difference ($P < 0.05$ or $P < 0.01$) when compared to the horse serum, except for the triglyceride content in hybrid catfish plasma. The inorganic element analyses showed non-significant difference ($P > 0.05$) for the osmolality and Cu^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} and Na^{+} concentrations. On the contrary, the K^{+}, S and Cl^{-} in common carp plasma were significantly different ($P < 0.05$) when compared to those in other treatments, except Cl^{-} in Nile tilapia.

4. Discussion and conclusion

Several studies on host specificity for the glochidia were accomplished for different species of glochidia showing some relevant dependence on their fish species hosts (Fuller, 1974; Meyers and Millemann, 1977; Waller et al., 1985; Weaver et al., 1991; Gordon et al., 1994; Yeager and Saylor, 1995). The intensity of infestation (number of

glochidia per infested fish) with *Lampsilis radiata* glochidia showed the highest values for the small sizes of yellow perch (Tedla and Fernando, 1969). Experimental results from Arayawatanavij et al. (1992) indicated that the percentage of fish infested with glochidia of *H. myersiana* was 100, 100, 90 and 10 for Nile tilapia, striped catfish, common carp and common silver barb, respectively. These results suggested that in the natural process of glochidia development, the nutrient uptake from the fish blood might represent a determinant factor besides the immunological reactions.

Keller and Zam (1990) cultured glochidia *Anodonta imbecillis* with a high transformation percentage (81.8%), but in a complex artificial medium from Isom and Hudson (1982) mixed with fish plasma. Alternatively, Keller and Zam (1990) improved the glochidia culturing by another simple medium (M199) mixed with horse serum, although decreasing the transformation percentage to 65.4%. Further work was carried out by Uthaiwan et al. (2001) with glochidia *H. myersiana* culture in a simple M199 medium mixed with fish plasma. This represented a more simple and efficient methodology, since it could obtain a higher percentage survival (85.32%) and transformation (84.28%).

The present investigation aimed to study specific effects caused by the plasma of different fish species using the method by Uthaiwan et al. (2001). As a general observation, we found that the critical period for glochidia survival occurred during a 2- to 3-day period after incubation. Thus, the survival percentage under different conditions refers specifically to this period. The present results with Nile tilapia and horse serum had similar survival percentages when compared to those by Uthaiwan et al. (2001). However, the survival percentage was now higher (93.77 ± 3.0) with common carp plasma than with other treatments. It is relevant to note that the juvenile from common carp plasma incubation exhibited intense movements by foot. This suggests a great improvement when compared with the Nile tilapia assay by Uthaiwan et al. (2001). The percentage transformation of 100%, in all treatments after the 10th day, was also significantly higher than in the experiments of Keller and Zam (1990) and Uthaiwan et al. (2001). In the present assays, the change of the culture medium, at the fifth and ninth day of incubation, may constitute an important factor for inducing the high transformation of glochidia. Perhaps, this is the reason why the glochidia transformation reached 100% in this experiment with Nile tilapia plasma, whereas it was only 84.28% in the work by Uthaiwan et al. (2001). Thus, the present study suggests that the survival of glochidia depended mainly on the plasma specificity, whereas the transformation to juvenile on better sterile conditions. Supporting the plasma specificity are still the preliminary observations with the juvenile rearing with algae diet during 2 months. In fact, the juveniles cultured with common carp plasma could survive 2 months, whereas those with Nile tilapia and hybrid catfish plasma yielded only 1 month, horse serum 2–3 weeks and striped catfish plasma 1–2 weeks.

Concerning the composition of organic sources added to the medium, the present results suggest that amino acids may be very determinant elements for high survival rates of glochidia. In effect, the fourth group in Table 4 has LEU, PRO, THR, ALA added by CIT, GLX with higher significant concentration in common carp if compared to other all or only fish treatments, respectively. Significant differences also occurred for the inorganic elements namely K^+, S and Cl^-. Perhaps, the content of these amino acids

and ions with common carp plasma incubation are close to the optimum values if it is considered that maximum percentage survival was almost reached (93.77 ± 3.0). On the contrary, the plasma from striped catfish induced low percentage survival possibly due to its lowest organic content in the fourth group. Curiously, the amino acids mentioned are well represented in the composition of organic fluids of *Anodonta cygnea* and *Unio ctorum* (Moura et al., 1995, 2000). Thus, the results indicated that the high specificity for the plasma of the common carp might be related to the requirement of these amino acids for the development of glochidia. The non-significant difference in the contents of 13 free amino acids in the third group (ßALA-LYS) measured in all organic sources (Table 4) suggested that its average content is essential to support the common basic conditions for the glochidia survival. It is also possible that the osmolality and inorganic components such as Cu^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} and Na^{2+} may play the same role. The free amino acids shown in the first group lowest (ANS-CYSTA) and second group highest (ORN-GLY) concentrations as well as proteins, glucose and triglyceride at the significant random contents (Table 4) seem to indicate no plasma specificity. In fact, it is relevant to note that some organic elements in the plasma from common carp (first and second groups) are not significantly different from those of striped catfish. Both groups of free amino acids may only express a range of low and high values within survival is possible. The presence of some free amino acids (first, second and third groups) in organic fluids of *A. cygnea* and *U. ctorum* measured by Moura et al. (1995, 2000) support their relative importance for glochidia culture.

The experiments on the glochidia density in the artificial medium with common carp plasma showed the highest survival percentage of glochidia after 2–3 days, with a non-significant difference ($P > 0.05$), among 50–100, 150–200, 250–300 and 350–400 glochidia/3.5 ml densities. Thus, to make the glochidia culture of *H. myersiana* more profitable, it is possible to use a density around 400 glochidia/3.5 ml, lower than in the assay by Keller and Zam (1990) (500–1000 glochidia/3.5 ml) and higher than in experiment by Uthaiwan et al. (2001) (50–100 glochidia).

The present investigation about plasma volume percentages of four fish species showed that hybrid catfish had the highest significant value (79.17 ± 4.58), even higher than that of common carp. However, it is possible to use bigger common carp to compensate its smaller plasma volume and thus maintain the same profitability.

On the basis of these results, we propose the addition of common carp plasma to artificial medium M199 as a standard source of organic and inorganic components for in vitro culture of glochidia *H. myersiana*. However, depending on glochidia species, its successful culture may require plasma of different fish species.

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 whose supply of mussels was greatly appreciated. Also to Dr. Dulce Quelhas for helping with amino acid measurements at the Instituto de Genética Médica Jacinto

Magalhães. Special thanks to Dr. John Bulger, Dr. Kovit Kovitvadhi, Dr. Reiner Bleher and Dr. Anna Olsson for reviewing the manuscript.

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