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Effects of rearing density and sub-sand filters on growth performance of juvenile freshwater mussels (*Chamberlainia hainesiana*) reared under recirculating system conditions

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ABSTRACT: *Chamberlainia hainesiana*, a commercially valuable bivalve, is found in Thailand. Juveniles of *C. hainesiana* have been successfully cultured in sterilized artificial media for culturing glochidia (to bypass the parasitic stage) until they develop into the juvenile stage. The survival percentage of glochidia in standard tissue culture medium (M199) supplemented with common carp plasma and antibiotics/antimycotic was $97.2 \pm 2.5\%$. All surviving larvae (100%) ultimately transformed into juveniles within 8 days. Early juveniles (0–90 days old) were reared in recirculating systems and were cultured at three density levels (500, 1500, and 3000 per culture unit) in a laboratory. The density level of 500 per culture unit resulted in the highest and most significant ($p < 0.05$) growth rate, with an average shell length and shell height; the average survival was $71.3 \pm 0.4\%$. The 90–150-day-old juveniles were reared outdoors in two different systems (with and without a filter plate). They were fed by filtering phytoplankton from the water in an earthen pond. The filter-plate system produced the highest growth rate ($p < 0.01$), with an average weight gain and shell size; the average survival was $98.7 \pm 0.6\%$. A forecasting equation was used to describe the shell length of juveniles, i.e., the relationship between shell length (L, mm) and age (t, days). The equations for 0–90-day-old early juveniles cultured in the laboratory (500 per culture unit), and for 90–150-day-old juveniles cultured using system 1 were $L = 0.5236 - 0.053\,t + 0.0023\,t^2 - 1 \times 10^{-5}\,t^3$ ($r^2 = 0.956$) and $L = -51.302 + 0.6812\,t - 5 \times 10^{-6}\,t^3$ ($r^2 = 0.940$), respectively.

KEYWORDS: stocking density, sub-sand system, culture, Unionidae

INTRODUCTION

Chamberlainia hainesiana (Lea, 1856) is the largest freshwater pearl mussel. It is endemic throughout Thailand, and thus possesses great potential as a source of production of cultured pearls[1,2]. The mussel's nacreous shell can be used for inlaying pearl furniture, ornaments, kitchen utensils, and souvenirs. These mussels are suspension feeders, and their filtration activities also contribute to maintain a clean aquatic environment and to reduce pollution. Freshwater pearl culturing techniques are generally considered to be a highly successful achievement; however the number of mussels all over the world is drastically decreasing, and some species are nearly extinct. This is due to deterioration of water resources as well as overutilization/overconsumption of mussels, as has occurred in several countries around the world[3–5], including the case of *C. hainesiana*. For these reasons, it is of utmost importance to support sustainable culture in the mussel industry, and to establish effective conservation measures for their continued future use.

The culture of freshwater pearl mussels is divided into three steps that follow the life cycle of the mussel: parasitic glochidial stage, juvenile stage, and adult. At present, juvenile freshwater mussels have been successfully cultured in the laboratory by attaching glochidia to fish (infestation) until they reach the juvenile stage[6–10]. Moreover, it is possible to use sterilized artificial media for successfully culturing glochidia bypassing the parasitic stage[11–23]. However, glochidial infestation of fish results in high juvenile mortality due to the disturbance caused by bacteria, protozoa, and contaminating fungi[13]. But using of artificial media for glochidia culture can achieve high production as well as prevent contamination[11–23]. A recent report described the use of artificial media to successfully culture glochidia of the freshwater

pearl mussel *Hyriopsis (Limnoscapha) myersiana* to the juvenile stage[17]. A sub-sand filter is commonly used to remove particulate matter, and to convert and ultimately remove nitrogenous compounds from the water in an aquaculture system by means of biological oxidation and reduction[24]. In this system, water containing high dissolved oxygen flows through the sub-sand filter; bacteria attached to the sand particles could convert ammonia nitrogen from aquatic animal excretion into nitrite and nitrate, respectively. This results in less ammonia toxicity to aquatic animals, and increases growth and survival.

Therefore, this study aimed to develop an effective culturing technique for *C. hainesiana*, with the goal of achieving high yield in to promote freshwater pearl mussel culture on a commercial scale, as well as to promote conservation by sustainable use. The growth and survival rates were compared for: 0–90-day-old juveniles reared at three different densities; and 90–150-day-old juveniles cultured with and without the use of a sub-sand filter system

MATERIALS AND METHODS

Culture of glochidia

Ten male and ten female adult freshwater mussels, *C. hainesiana*, were cultured on a raft in the *Mae Klong* Reservoir at the Kanchanaburi Inland Fisheries Research and Development Centre, Department of Fisheries, Kanchanaburi province, Thailand. These individuals had an average weight of 221 ± 64 g, length of 11.4 ± 0.3 cm, width of 3.9 ± 0.5 cm, and height of 6.2 ± 0.2 cm. Mature glochidia were aspirated from gravid mussels and transferred to artificial culture medium[15]. Approximately 5000–6000 glochidia/replication (three replicates) were placed in a culture dish (90 cm $\times$ 15 mm) containing: 10 ml of artificial medium composed of M199 (Gibco, No. 6231100-035); fish plasma (common carp, *Cyprinus carpio*); and antibiotics/antimycotic (100 µg/ml carbenicillin, 100 µg/ml gentamicin sulphate, 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.

Culture of 0–90-day-old juveniles

Newly transformed juveniles were removed from the artificial medium and rinsed in dechlorinated aerated water[15]. Samples of cultured 0-day-old (newborn) juveniles were transferred to plastic culture units (width $\times$ length $\times$ height $=$ 11 cm $\times$ 20 cm $\times$ 8 cm, water level $=$ 7 cm) at three density levels (500, 1500, and 3000 juveniles per culture unit). There were three replicates of each density, and each culture unit contained 20 g of sand ($<$ 120 µm grain size) about 3 mm thick. They were reared in closed recirculating culture systems, and were fed twice daily (at 06:00 and 18:00 h) with a combination of *Chlorella* sp. and *Kirchneriella incurvata* in a ratio of 1:1 at a concentration of 1×10^5 cells/ml[17]. This system comprised three filter cabinets: a particulate filter cabinet, a macrophyte filter cabinet, and a biological filter cabinet. Water flowed through the particulate filter cabinet and then, via the second part, to the macrophyte filters cabinet. The water then flowed into the biological filter cabinet filled with BioBall (BioMérieux Industry) and then to the resting cabinet. The water from the resting cabinet was pumped at 20 ml/min into a plastic culture unit. The water circulation was turned off for 1 h during feeding. The mussels were sampled by isolating juveniles from the sand by screening (with 120 µm mesh) every 10 days for growth study during the experiment; juveniles comprised $n = 50$ from each culture unit. Growth of juveniles was assessed by recording increments of shell size (shell length and height). Juveniles were measured using a light microscope with a calibrated ocular micrometer to the nearest 0.01 mm. Growth rates were calculated as average growth rate in mm/day (average shell length or average shell height at the end of every 10-day period), and average shell length or average shell height before the initial 10-day total growth period. Survival was calculated by using the average number of living juveniles at the beginning of the experiment and at the end of every 10-day period.

Culture of 90–150-day-old juveniles

Samples of 90-day-old juveniles were reared in two systems for comparison of growth and survival. System 1 (Fig. 1a) consisted of two parts. The first part had dimensions of width $\times$ length $\times$ height $=$ 50 cm $\times$ 80 cm $\times$ 120 cm, water level $=$ 80 cm, and was overlaid with an acrylic plate (6 mm thick and with holes 3 mm in diameter throughout the plate) 10 cm above the cabinet floor. The second part, with corresponding dimensions of 50 cm $\times$ 20 cm $\times$ 120 cm, 80 cm water, was used to contain the water outflow from the rearing cabinet into an earthen pond. System 2 (Fig. 1b) had the same configuration as system 1 except for the acrylic

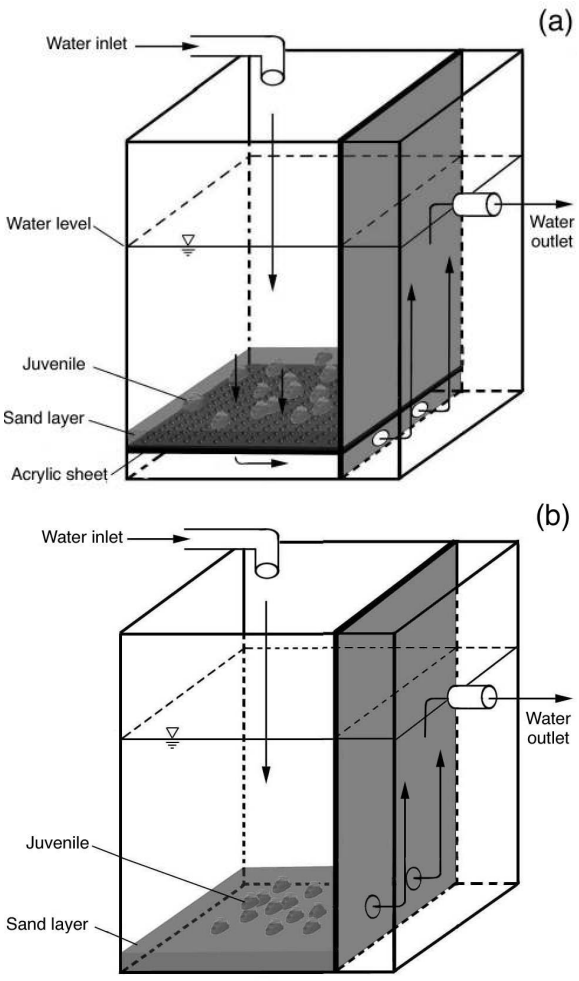

Fig. 1 Schematic diagram of the recirculating system 1 (a, with a filter plate) and 2 (b, without a filter plate) used to rear freshwater pearl mussel juveniles (90–150 days). Arrows show water current.

plate. Both systems were filled to 5 cm depth with sand ($>$ 4 mm grain size) on the plate and on the cabinet floor. A total of 2000 juveniles were cultured using both systems (0.4 juveniles/cm^2). Water for rearing juveniles in both systems was pumped from an earthen pond about 2 acres in size, at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University. The water flow rate was 3 l/min, and air was supplied to the juvenile culturing cabinets 24 h per day. There were three replicates. Fifty juveniles were randomly sampled every 20 days to measure shell length, height, and width, and to count the number of surviving juveniles.

Water analysis

Water quality analysis of cultured juveniles in the laboratory (0–90 days) was also performed in the two culture systems (90–150 days) every 10 and 20 days, respectively. Measurements were taken of water temperature (Hg thermometer), turbidity (nephelometric method), conductivity (conductivity meter), pH (pH meter), dissolved oxygen (azide modification), free CO_2 (titration), total alkalinity (phenolphthalein methyl orange indicator), total hardness (EDTA titration), total ammonia nitrogen (phenate method), nitrite (colorimetry), nitrate (cadmium reduction), phosphorus (ascorbic acid method), silica (molybdosilicate method), and calcium (EDTA titration)[25].

Phytoplankton communities

Sampling of phytoplankton in the two culture systems (90–150 days) was performed in 10 l culture cabinets. There were three replicates/cabinet. Samples were analysed for species and quantities of phytoplankton every 20 days. Samples of phytoplankton were screened through a 20 µm plankton net and preserved in a solution of 1% acidic Lugol's solution. Sampling was also conducted by counting species of phytoplankton under an inverted microscope. Species identification was based on taxonomy of phytoplankton[26–28]. All samples were examined in triplicate.

Statistical analysis

Comparison of growth rate (length and height of shell) and survival in each level of density (500, 1500, and 3000 per culture unit) in 0–90 days old juvenile using experimental design (one-way analysis) every 10 days and comparison of average values using Duncan's Multiple Range Test (DMRT) at a 0.05 significance level were implemented. For 90–150-day-old juveniles, growth rate (weight, length, height and width of shell), survival of juveniles and water quality between system 1 and 2 using experimental design (t-test) every 20 days were compared. The coefficient of correlation (r^2) of linear regression was used in relationship of juvenile during 0–90 days old which was calculated by using average of water quality characteristics with average survival or average shell length. When 90–150 days old juvenile, coefficient of correlation between average of water quality characteristics and average survival or average shell length or average weight was compared.

The relationship between ages (0–90 and 90–150 days old) with shell length was expressed by the equation: $L = b_0 + b_1 t + b_2 t^2 + b_3 t^3$, where L is the shell length (in mm), t is age (in days),

Table 1 Average growth rate and survival of 0–90 days in the laboratory.

Age (days)	Average growth rate (± SD)								Survival (%)			
	Length (mm/day)				Height (mm/day)							
	500[†]	1500	3000		500	1500	3000		500	1500	3000	
0–10	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	ns	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	ns	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	ns
10–20	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	ns	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	ns	96.3 ± 0.6^{a}	94.6 ± 0.7^{b}	91.6 ± 1.0^{c}	*
20–30	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	ns	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	ns	92.1 ± 3.8^{a}	88.8 ± 1.8^{a}	83.0 ± 1.8^{b}	**
30–40	0.05 ± 0.02^{a}	0.06 ± 0.02^{ab}	0.06 ± 0.02^{b}	*	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	ns	85.6 ± 1.3^{a}	85.6 ± 2.7^{a}	74.1 ± 2.6^{b}	**
40–50	0.07 ± 0.01	0.10 ± 0.03	0.09 ± 0.03	ns	0.06 ± 0.03	0.07 ± 0.02	0.07 ± 0.02	ns	80.5 ± 3.3^{a}	81.9 ± 6.9^{a}	73.2 ± 2.6^{b}	*
50–60	0.13 ± 0.05^{a}	0.09 ± 0.03^{b}	0.08 ± 0.02^{b}	*	0.08 ± 0.04	0.06 ± 0.02	0.05 ± 0.01	ns	76.5 ± 1.6^{a}	81.3 ± 3.5^{b}	73.2 ± 2.6^{b}	**
60–70	0.10 ± 0.04	0.07 ± 0.03	0.05 ± 0.03	ns	0.07 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	ns	72.9 ± 2.1^{a}	78.3 ± 3.7^{b}	71.1 ± 1.0^{a}	*
70–80	0.12 ± 0.06^{a}	0.03 ± 0.02^{b}	0.02 ± 0.02^{b}	*	0.08 ± 0.03^{a}	0.02 ± 0.02^{b}	0.02 ± 0.01^{b}	*	72.8 ± 2.8^{ab}	77.0 ± 2.6^{b}	68.4 ± 1.5^{a}	*
80–90	0.05 ± 0.04	0.02 ± 0.02	0.05 ± 0.05	ns	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.03	ns	72.1 ± 2.4^{a}	73.5 ± 1.2^{a}	68.1 ± 1.1^{b}	**
0–90	0.07 ± 0.00^{a}	0.05 ± 0.01^{b}	0.05 ± 0.00^{b}	*	0.05 ± 0.01^{a}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	**	71.3 ± 0.4^{a}	69.8 ± 0.3^{b}	67.8 ± 0.7^{c}	**

[†] Number of juveniles per culture unit.
Different letters within a row indicate significant difference; * = $p < 0.05$, ** = $p < 0.01$, ns = no significant difference ($p > 0.05$).

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.).

Morphological development of *C. hainesiana*

The living mussels were collected in sequential developmental stages between 0 and 150 days old. Morphological development was observed by light microscope (0–90 days old) and photography with a digital camera (110–150 days old).

RESULTS

Culture of glochidia

The glochidia of *C. hainesiana* were completely transformed within 8 days, with a survival rate of $97.2 \pm 2.5\%$. All surviving larvae transformed into the juvenile stage. The average shell length and height were 0.26 ± 0.04 mm.

Culture of 0–90-day-old juveniles

Juveniles (0–90 days old) cultured at a density of 500 juveniles/culture unit had the highest growth of shell length, with a significant difference ($p < 0.05$) compared with densities of 1500 and 3000 juveniles per culture (Fig. 2a and Table 1).

Culture of 90–150-day-old juveniles

Juveniles cultured in system 1 (with a sub-sand filter) produced greater shell length than those cultured in system 2 (without a sub-sand filter), with a significant difference ($p < 0.05$). At the termination of the experiment (Fig. 2b), both groups had the same average growth rate (90–150 days) in terms of weight, shell length, shell height, and shell width. However, there was no significant difference in the survival rates between the two systems ($p > 0.05$) (Table 2).

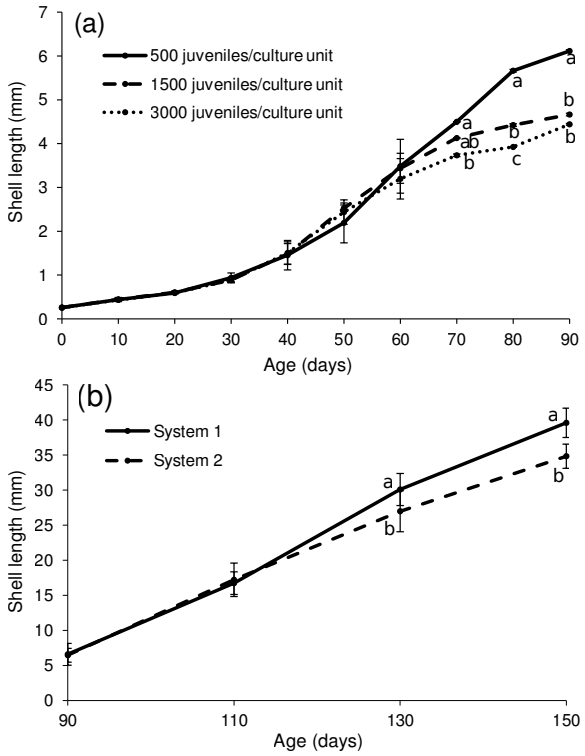

Fig. 2 Development of *C. hainesiana* juveniles. Average shell length (± SD) of (a) 0–90-day-old juveniles cultured at different densities and (b) 90–150-day-old juveniles cultured in system 1 (with a filter plate) and 2 (without a filter plate). Different letters at each age within each density and system denote significantly different value ($p < 0.05$).

Water quality

Average water quality throughout the culture of 0–150-day-old mussels is shown in Table 3. In a comparison of water quality between systems 1 and

Table 2 Average growth rate and survival of 90–150 days cultured in system 1 and 2.

Age (days)	Average growth rate (± SD)								Survival (%)	
	Weight (g/day)		Length (mm/day)		Height (mm/day)		Width (mm/day)			
	System 1	System 2	System 1	System 2	System 1	System 2	System 1	System 2	System 1	System 2
90–110	0.01 ± 0.00	0.02 ± 0.00^{ns}	0.50 ± 0.00	0.53 ± 0.08^{ns}	0.30 ± 0.01	0.39 ± 0.06^{ns}	0.12 ± 0.00	0.15 ± 0.02^{ns}	100.0 ± 0.0	100.0 ± 0.0^{ns}
110–130	0.11 ± 0.02	0.12 ± 0.02^{ns}	0.66 ± 0.03	$0.48 \pm 0.03^{**}$	0.53 ± 0.01	$0.43 \pm 0.03^{*}$	0.21 ± 0.01	$0.15 \pm 0.01^{**}$	99.4 ± 0.3	98.5 ± 0.9^{ns}
130–150	0.19 ± 0.02	$0.13 \pm 0.00^{**}$	0.47 ± 0.03	0.39 ± 0.06^{ns}	0.38 ± 0.03	0.34 ± 0.04^{ns}	0.19 ± 0.01	$0.15 \pm 0.02^{**}$	99.0 ± 0.6	97.7 ± 0.8^{ns}
90–150	0.10 ± 0.01	$0.08 \pm 0.01^{**}$	0.55 ± 0.01	$0.47 \pm 0.01^{**}$	0.44 ± 0.00	$0.39 \pm 0.17^{*}$	0.17 ± 0.00	$0.15 \pm 0.00^{*}$	98.7 ± 0.6	96.8 ± 0.8^{ns}

$* = p < 0.05$, $** = p < 0.01$, ns = not significant difference ($p > 0.05$) between systems 1 and 2.

Table 3 Average ± SD of water quality parameters during culture over 0–150 days.

Parameters	0–90 days old	90–150 days old		
		System 1	System 2	
Water temp. (°C)	25.0 ± 0.0	30 ± 10	30.1 ± 1.1	ns
Turbidity (NTU)	–	9.8 ± 1.1	9.8 ± 2.4	ns
Conductivity (µS)	269.7 ± 3.2	355 ± 21	357 ± 18	ns
pH	7.86 ± 0.12	7.63 ± 0.16	7.42 ± 0.25	ns
Dissolved oxygen (ppm O_2)	7.3 ± 0.4	6.52 ± 0.20	6.39 ± 0.31	ns
Free CO_2 (ppm CO_2)	3.5 ± 0.8	10.2 ± 1.2	10.9 ± 2.1	ns
Total alkalinity (ppm $CaCO_3$)	74 ± 8	51.5 ± 3.0	53.1 ± 3.2	ns
Total hardness (ppm $CaCO_3$)	124 ± 9	246 ± 15	236 ± 7	*
Total ammonia N (ppm NH_3-N)	0.07 ± 0.03	0.18 ± 0.06	0.24 ± 0.08	**
Nitrite (ppm NO_2^--N)	0.007 ± 0.004	0.013 ± 0.006	0.014 ± 0.011	ns
Nitrate (ppm NO_3^--N)	0.22 ± 0.09	0.024 ± 0.018	0.017 ± 0.017	*
Phosphorus (ppm P)	0.005 ± 0.016	0.004 ± 0.002	0.004 ± 0.002	ns
Silica (ppm SiO_2)	4.9 ± 0.9	18.3 ± 2.1	18.0 ± 2.1	ns
Calcium (ppm $CaCO_3$)	74 ± 4	104 ± 7	104 ± 7	ns

$* = p < 0.05$, $** = p < 0.01$, ns = not significant difference ($p > 0.05$) between system 1 and 2.

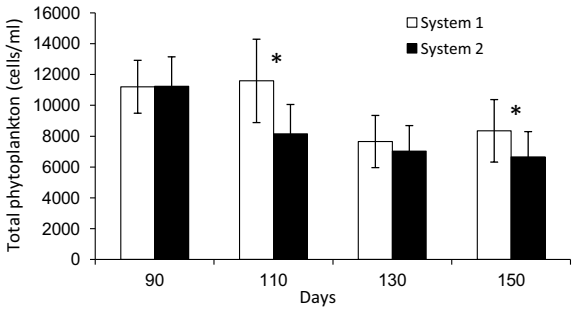

Fig. 3 Comparison of total of phytoplankton (cells/ml) between system 1 and 2 during 90–150-day-old cultures. (*) Indicated total of phytoplankton of system 1 which was different from system 2 of the same day ($* = p < 0.05$).

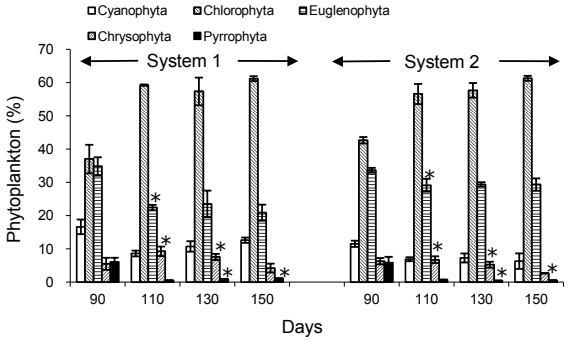

Fig. 4 Percentage of phytoplankton in each division between 90–150-day-old cultures in system 1 and 2. (*) Indicated percentage of phytoplankton in the same division of system 1 which was different from system 2 of the same day ($* = p < 0.05$).

2 (90–150 days), it was found that water quality mostly exhibited no significant difference ($p > 0.05$), except that total hardness and nitrate had significant difference ($p < 0.05$), total ammonia nitrogen had a highly significant difference ($p < 0.01$). Culture of mussels in the laboratory (0–90 days) revealed that both survival and shell length had highly significant relationships ($p < 0.01$) with ammonia nitrogen, nitrate, silica, and calcium (Table 4). In comparing the culture of mussels between the two systems (90–150 days), it was found that survival, total weight and shell length had an inverse relationship with total hardness and silica, respectively.

Phytoplankton community

Based on average total phytoplankton quantities in the two systems, it was found that there were greater quantities of phytoplankton in system 1 than in system 2, with a significant difference ($p < 0.05$) on days 110 and 150 (Fig. 3). The percentages of types of phytoplankton that were found between the two culture systems determined that the division Chlorophyta was most prevalent, followed by Euglenophyta, Cyanophyta, Chrysophyta, and Pyrrophyta, respectively (Fig. 4).

Table 4 Coefficients of correlation between average survival and water quality parameters, and average growth and water quality parameters of juveniles over 0–90 and 90–150 days.

	Water temp.	Turbidity	Conductivity	pH	DO	Alkalinity	CO_2	Total hardness	Ammonia nitrogen	Nitrate	Nitrite	Phosphorus	Silica	Calcium
0–90 days														
Survival														
500†	0.03	–	0.28	−0.15	0.58	−0.00	−0.37	0.63*	0.88**	0.93**	−0.23	0.39	−0.79**	0.94**
1500	0.30	–	0.26	−0.09	0.63*	0.03	−0.37	0.66*	0.81**	0.89**	−0.29	0.27	−0.8**	0.92**
3000	0.50	–	0.16	−0.00	0.62*	0.15	−0.35	0.53	0.76**	0.80**	−0.21	0.25	−0.68**	0.91**
Length														
500	0.04	–	−0.46	0.38	−0.56	0.14	0.23	−0.9**	−0.83**	−0.94**	0.33	−0.35	0.93**	−0.76**
1500	0.20	–	−0.42	0.38	−0.59	0.18	0.26	−0.84**	−0.89**	−0.98**	0.35	−0.38	0.92**	−0.81**
3000	0.24	–	−0.40	0.35	−0.55	0.14	0.28	−0.83**	−0.89**	−0.98**	0.29	−0.39	0.92**	−0.82**
90–150 days														
Survival														
System 1	−0.83	0.89	−0.74	−0.11	−0.95*	0.55	0.83	−0.97*	−0.03	−0.67	0.70	−0.61	0.93	−0.98*
System 2	−0.84	0.67	−0.76	0.95*	−0.80	−0.20	0.78	−0.99**	−0.01	−0.68	0.63	−0.61	0.92	−0.83
Weight														
System 1	0.75	−0.66	0.97*	−0.42	0.66	−0.85	−0.48	0.72	−0.43	0.93	−0.58	0.90	−0.99**	0.94*
System 2	0.84	−0.38	0.98**	−0.83	0.32	−0.03	−0.33	0.83	−0.48	0.51	−0.90	0.94*	−0.98*	0.91
Length														
System 1	0.81	−0.82	0.85	−0.09	0.88	−0.67	−0.71	0.91	−0.16	0.78	−0.70	0.73	−0.98*	0.99**
System 2	0.82	−0.49	0.87	−0.97*	0.60	0.21	−0.57	0.97*	−0.27	0.71	−0.83	0.77	−0.99**	0.86

† Number of juveniles per culture unit.
* $= p < 0.05$, ** $= p < 0.01$, no asterisk = no correlation ($p > 0.05$).

Length at age relationship curves

Shell growth had different density culture (0–90 days) and different culture system (90–150 days), as a result of more rapid increase in shell length during an increase of age (Fig. 5). The relationship between shell length and each culturing duration was highly significant ($p < 0.01$). Various values of equations are shown in Table 5.

Table 5 Length-age relationships for freshwater pearl mussels (*C. hainesiana*) cultured in a laboratory (0–90 days) and in outdoors tanks (90–150 days).

	b_0	b_1	b_2	b_3	r^2
0–90 days ($n = 500$)					
500†	0.5236	−0.0530	0.0023	-1×10^{-5}	0.956
1500	0.4694	−0.0532	0.0029	-2×10^{-5}	0.967
3000	0.4414	−0.0494	0.0027	-2×10^{-5}	0.960
90–150 days ($n = 1200$)					
System 1	−51.302	0.6812	0	-5×10^{-6}	0.940
System 2	−47.447	0.6071	0	-1×10^{-6}	0.948

† Number of juveniles per culture unit.
Regression equation: $L = b_0 + b_1 t + b_2 t^2 + b_3 t^3$.
L = shell length in mm. t = age in days. n = number of mussels. r^2 = coefficient of determination.

Morphological development of *C. hainesiana*

The morphological development of *C. hainesiana* juveniles in culture (0–150 days old) is shown in Fig. 6. The early juvenile (0 days old) after transformation has equal length and height: i.e., 0.26 ± 0.04 mm, subrotund, equivalve shells with an equilateral valve, presenting the same size and shape as the glochidium. The anterior region appeared before the posterior region, and grew more rapidly until the juvenile was 90 days old, when the posterior region began to increase more than the anterior. The shell began to completely close at 20 days. The first anterior and posterior wings appear at 50 days, with the posterior wing becoming dominant relative to the anterior after 90 days. The shell was so thin during 0–90 days of age that the internal organs could be seen clearly under a microscope: e.g., the foot, gill, intestine, stomach, heart, and bundle of muscle. The first incurrent siphon and excurrent siphon appeared at 50 days. The complete adult morphology was apparent in 90-day-old mussels.

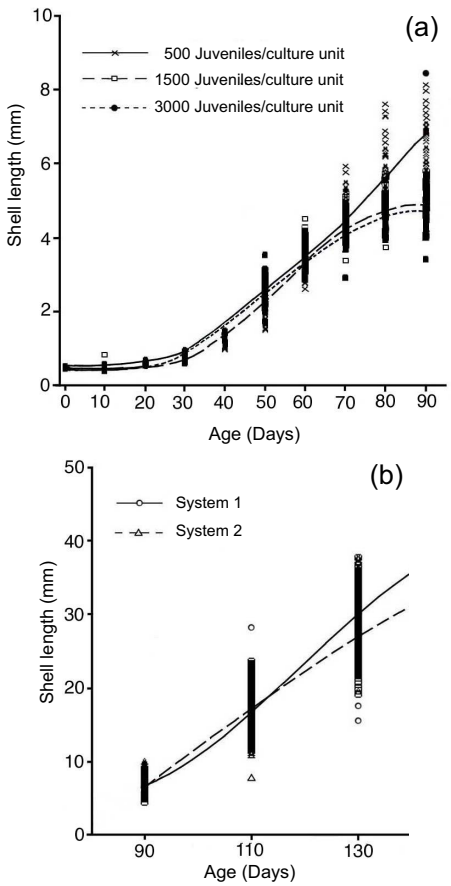

Fig. 5 Relationship curves between age and shell length in (a) different density culture and (b) different culture system.

DISCUSSION

Culture of glochidia

This study demonstrated that glochidia of *C. hainesiana* could successfully develop into a juvenile stage when cultured in artificial media and demonstrated a high rate of survival of up to $97.2 \pm 2.5\%$, with 100% of all surviving larvae transformed into juveniles; duration of transformation was 8 days. As with cultured glochidia of the freshwater pearl mussel *H. (L.) myersiana* in the same artificial media [14, 15], the temperature of the incubator was different (23 °C). The percentage of survival of glochidia was 93 ± 3–$95 \pm 2\%$. In addition, other freshwater pearl mussels were cultured in artificial media: *Hyriopsis (Hyriopsis) bialatus* [21, 23], *Anodonta cygnea* [20], *Ligumia recta* [11], and *Anodonta imbecillis* [12]. They were transformed into juveniles with survival rates of 100, 60.8, 48.8, and 65.4%, respectively. The important factors for transformation of glochidia into juveniles could be, successively: glochidia maturity, suitable medium (particularly fish plasma) as a growth factor for glochidia development, incubator temperature, and contamination.

Culture of juveniles

From the culture of 0–90-day-old mussels in a laboratory-scale recirculating aquaculture system, the density of cultured mussels, under otherwise similar conditions, had an effect on the rates of development and survival [17]. Densities of 500 mussels/culture unit had the highest value, and were (highly) significantly different from other densities ($p < 0.01$) in terms of height and survival, as opposed to a significant difference ($p < 0.05$) of the same length. The mussel diet played an important role in this experiment, which used *Chlorella* sp. and *K. incurvata* [15, 17]. From observation of algae colour under a microscope after feeding for 30 min, it was found that the colour of algae existing in the digestive gland, stomach, and intestine had changed from green to yellow, or orange to brown, and that such colours indicated high digestibility of algae and changing algae morphology from normal shape to debris; this resulted in increased growth of mussels and consequently high survival rates. For the study in vitro digestibility of phytoplankton a crude enzyme extract of *H. (H.) bialatus* juveniles was used. Based on the digestion of carbohydrate, protein and lipid content, it was found that *Chlorella* sp. 2 and *K. incurvata* are the most efficiently digested by juveniles [19, 21]. When comparing the growth rate in length of *H. myersiana* in a report [17] where they cultured with the same system in this experiment, it was found that the growth of *C. hainesiana* was closely related to the growth rate in the previous study. Cultures of 0–90-day-old *C. hainesiana* had values between 0.05–0.07 mm/d, as compared to growth rates of *H. myersiana* of: 0–120 days old, 0.03–0.1 mm/d [17]; 0–60 days old, 0.021 mm/d; and 60–120 days old, 0.007–0.036 mm/d [15]. Rearing of juvenile freshwater mussels, *A. imbecillis*, which were cultured from an artificial medium, with river water containing a diversity of plankton: namely, the genera *Gonium*, *Anabaena*, *Achnanthes*, *Navicula*, *Oscillatoria*, *Bodo*, *Fragilaria*, *Eudorina*, *Stentor*, *Vorticella*, *Scenedesmus*, *Trachelomonas*, *Crucigenia*, *Phacus*, *Stephanodiscus* and *Chlorococcales*. The oldest was 74 days, and was more than 5.1 mm in length (original length was 0.28 mm) [29]. As with culture of juvenile unionids: they were four species of *Lampsilis* spp. and *Ligumia recta*. It was found that the maximum cultured age at 12 weeks showed a growth rate in length between 0.005–0.012 mm/d; growth rates depended upon several factors, such as culturing methods and diet, as well as mussel species [30].

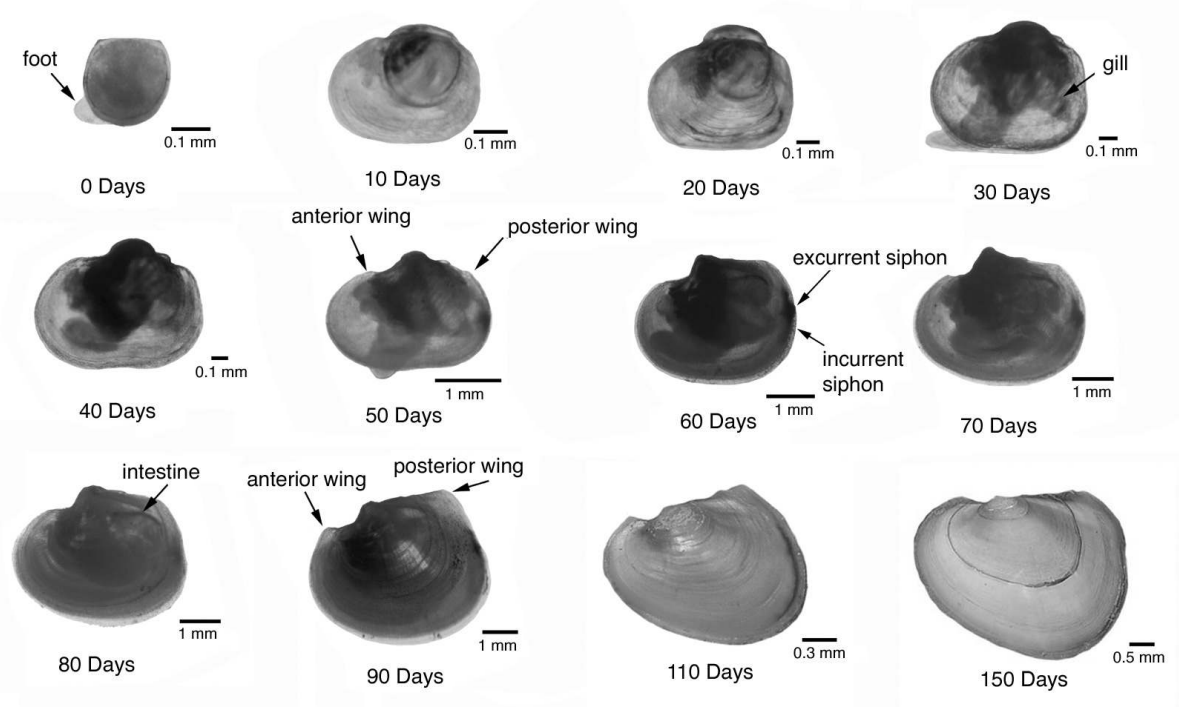

Fig. 6 Morphological development of 0–150-day-old juveniles of *C. hainesiana*.

When juvenile freshwater pearl mussels *H. (L.) myersiana* reared in the laboratory were transferred outdoors[15], it was found that the suitable juvenile stage for outdoor culture required fully developed organs, particularly the organs for ingesting food (namely the incurrent and excurrent siphons and gills) and that their shells had closed completely. These are factors which will support increased survival and growth of juveniles. Juveniles of *C. hainesiana* began to close their shells completely when they were about 20 days old, and their organs were fully developed at 90 days; hence, this age was chosen for outdoor culture. Culture of 90–150-day-old juveniles by system 1 had higher growth rates of weight, width, height and length of shell, with a significant difference from system 2; this might be due to the culturing condition of system 1 being closer to nature, as was indicated in the above studies[31–33]. Biofiltration using a sub-sand filter is probably the most popular ammonia removal method, with the ammonia being oxidized to nitrite and then to nitrate in the nitrification process[34]. As with the findings of a previous study, the ammonia content in system 1 was significantly less ($p < 0.01$) than in the system 2. This is because in system 1, dissolved oxygen in water could flow through a sand stratum, causing continuing oxidation of ammonia nitrogen and therefore resulting in less ammonia in the water. In system 2, however, dissolved oxygen in water could not flow through the sub-sand filter, which resulted in slower oxidation of ammonia nitrogen; this caused increased accumulation of ammonia nitrogen deeper down into the sand stratum. Therefore, a rearing system with a sub-sand filter could assist in lowering ammonia nitrogen content. Also, culturing mussels without a substrate, which resulted in very low survival[35], had some important effects on pedal feeding behaviour, proper orientation of the mussels for filtering efficiency, and stability from physical disturbances.

Water quality

Water quality during each period of mussel culturing is shown in Table 3. Water quality in the laboratory culture during 0–90 days was mostly close to the value in a previous study[17], which used the same system for culturing juveniles except that the temperature used in this study was 25 °C. Most of the water quality values that were used in culturing juveniles during 90–150 days had higher values than in culture (0–90 days), since cultured water from the resting pond was only derived from rainwater. This was accomplished by rotating some portion of water to be used by other aquatic animals and then returning the water to the original pond (closed system), which resulted in the accumulation of a high mineral content

as seen from the high values of conductivity and total hardness. When water quality was compared between system 1 and 2, it was found that water quality was not significantly different, except total hardness and nitrate ($p < 0.05$) and total ammonia nitrogen ($p < 0.01$) indicating that the culture system of freshwater pearl mussel through water sub-sand filtered (system 1) could reduce the total ammonia nitrogen content. Bacteria attached to surface area of sand particles may act as a biological filter altering ammonia nitrogen into nitrite and nitrate, respectively, which resulted in increasing higher nitrate content than in system 1 (with sub-sand filter). This is in agreement with a previous study[36] in which biological filtration was used in freshwater mussel culturing system with ammonia nitrogen and nitrate values ranging from 0.001–019 and 0.35–1.9 ppm, respectively.

Culture of freshwater mussel *H. (L.) myersiana* (0–120 days old) by the same culturing system and culturing method in 0–90 days old[17] of *C. hainesiana* except culturing temperature equal to 25 °C, it was found that shell length had correlation to total hardness, nitrite, silica, and calcium like in this study. When 90–150-day-old juveniles were brought for outdoor rearing, it was found that pH, dissolved oxygen, total hardness, and calcium had correlation to survival and conductivity, pH, total hardness, phosphorus, silica, and calcium. In a previous study[8] where juveniles of *Margaritifera margaritifera* were cultured from four rivers, it was found that water temperature, dissolved oxygen, conductivity, pH, ammonia, nitrite, nitrate, phosphorus, sodium, potassium, magnesium, and calcium had correlation to growth. Moreover, it was found that pH, alkalinity, total hardness and calcium had a significant relationship to the survival and growth rate of zebra mussel (*Dreissena polymorpha*) adults[37].

Phytoplankton communities

In the study of 90–150-day-old cultured juveniles, it was found that system 1 had increased numbers of phytoplankton as opposed to system 2 during days 110–150, with a significant difference ($p < 0.05$). Of the percentages of phytoplankton found in systems 1 and 2, the most was Chlorophyta (equal to 54 ± 11, and $55 \pm 8\%$, respectively). This was in accordance with a study[31] that found Chlorophyta to be the most prevalent in the gut contents, similar to the previous studies[38–42] that found more phytoplankton than zooplankton in the gastrointestinal tracts of adult freshwater mussels. This includes a study[15] using collected and cultured phytoplankton from the gastrointestinal tracts of mature *H. myersiana* from the river. Two species (*Chlorella* sp. and *Kirchneriella incurvata*) from ten species of phytoplankton were used for juvenile feeding, which conformed to the study[19] in vitro digestibility of the four species of phytoplankton (*Chlorella* sp. 2, *K. incurvata*, *Navicula* sp., and *Coccomyxa* sp.) using juvenile crude enzyme extract, resulting in data on digestion of carbohydrate, protein and lipid content. The results indicated that a combination of *Chlorella* sp. and *K. incurvata* would be a suitable food formula for culture of juveniles.

Length at age relationship curves

Correlation between age and shell length when cultured in laboratory and outdoor, it was found that there was in correlation form of cubic equation and there was high correlation ($p < 0.01$) (Table 5) with coefficient of determination (r^2) between 0.940–0.967. In the study[43] which they cultured juvenile rainbow mussels (*Villosa iris*) with natural river water flow-through culture system until 90 days. Then they were brought to culture in natural water source for 3 years including from the study[30] in culturing 8 species of freshwater mussel juvenile 8 species with the bucket rearing system for 44–72 days. In these two studies, correlation form between age and shell length was a simple linear equation but also from the study[17] relating juvenile of freshwater pearl mussel, *H. (L.) myersiana* (0–120 days old) reared in the laboratory that had given correlation equation between shell length and age in cubic equation form as same as those previous study.

Morphological development of *C. hainesiana*

Morphology of shell at the beginning from 10–150 days old will be the same to full-grown adult that the shell shape is inflated since possibly during initial stage to form a curve containing new increments co-marginal and shell border. Twenty days onward certain organs slowly come to be laterally compressed, distinctly true foot that at the initial stage looks like club, the anterior portion begins to grow rapidly than the posterior portion. This is an advantage to the juvenile because the large foot is the main organ in the anterior portion and requires protection from predators and physical agents to fulfil the important function of finding food.

CONCLUSIONS

The results of this study indicate that glochidia of *C. hainesiana* could be cultured in artificial media containing mixtures of M199 and common carp plasma, and were able to develop into juveniles. The best growth and survival was produced by culturing

0–90-day-old juveniles in a recirculating system in the laboratory at a density of 500 juveniles/culture unit, and by culturing 90–150-day-old juveniles in a sub-sand filter system.

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