Survival of glochidia of freshwater mussels, *Pyganodon grandis* (Mollusca: Unionidae), *in vitro* in the United States

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Freshwater bivalves such *Pyganodon grandis* are found in rivers, lakes, ponds and some mud bottomed pools. They have a complex life cycle that includes a parasitic stage in which glochidia live on the gills or fins of fishes, like freshwater drum, Iowa darter and brook stickleback, from days to months depending on the host/species relationship. During this time, glochidia differentiate into free-living larvae and drop from the host. Therefore, survival and subsequent development into free-living juveniles depend upon the encounter of suitable host fish and appropriate environmental conditions for the growth of the host fish. In the face of rapidly decreasing populations of freshwater mussels (Liu *et al*. 1996), one avenue for conservation will be aquaculture of suitable host fishes on which specific mussel larvae would develop into free-living glochidia. An alternative for conservation and preservation of biodiversity of mussels is to develop *in vitro* fertilization, cultivation of embryos and larvae to free-living juveniles without the use of host fishes. Bypassing the need of host fishes will also have the advantages of yielding larger numbers of free-living mussel juveniles because there will be no predators in an *in vitro* environment. At a suitable stage of development, they will be returned to sites favorable for them to grow into reproductive adults. This approach has been successful for a number of marine invertebrates of economic value, such as *Mytilus*, oysters and shrimp. However, little or no similar work has been conducted with freshwater invertebrates, including mussels.

The difficulties involved in developing a culture system for freshwater mussels similar to those used to culture marine invertebrates include insufficient and imprecise embryological and developmental studies. Other important factors are the benthic habitat and filter feeding of freshwater mussels. These factors make it difficult to obtain clean and sterile preparations of living material for cultivation due to the presence of microbes and protozoa on and within the shells, as well as in the visceral mass. Some zooplankters and protozoa, in fact, feed on glochidia.

Figure 1. Pyganodon grandis (giant floaters)

The goal of the present investigation was to circumvent these difficulties by formulating a culture medium that discourages the growth of microbes and protozoa and favors mussel larvae. Survival of glochidia for a relatively long period of time under artificial conditions is the first step toward the establishment of a culture system that supports the transition from parasitic to free-living larval forms without host fishes.

Culture Methods

Pyganodon grandis (giant floaters, Figure 1) were collected from the marsh of the Winous Point Shooting Club, Sandusky, Ohio, USA. The animals were kept in bioboxes with about 5 cm of regular aquarium gravel with continuous aeration in running water drawn from Lake Erie at 20-22°C. The lake water intake was 300 m from shore and the water was flowed through a 200µ filter and stored in a tank. Over 90 percent survival of captive animals have been obtained in this system during an experimental period of over one year. The animals were fed a commercial fish food once a week. They were healthy and they continued to follow their natural reproductive cycle in the experimental tanks.

The chemicals used, including antibiotics and culture medium concentrates, were purchased from Fisher or Sigma, Inc. and microbiological nutrient mixes were obtained from DIFCO. De-ionized water sterilized by autoclaving was use to make up all solutions. Working solutions were Millepore-filtered through 0.22µ filters. Culture vessels and pipettes were disposable sterile Falcon or Costar tissue culture dishes. All manipulations were

conducted using aseptic techniques in a hood with no air current. All solutions were filtered and sterilized with Millepore filters.

Prior to obtaining the glochidia, the mussel shells were scrubbed gently with a soft brush under cool tap water. The animals were rinsed thoroughly under de-ionized water and then bathed in de-ionized water with frequent changes until no liberation of particulate materials was visible (a process which took about an hour). The shells were opened with pliers and kept open with a cork of about 0.5 cm diameter. The visceral mass was rinsed gently 6 times with sterile mollusc Ringer's solution containing 10 µg of penicillin-streptomycin/ml. Glochidia were then flushed into Petri dishes with Ringer's with a 1 mL syringe fitted with a 26 g needle. The glochidia were collected with a Pasteur pipette and serially transferred into disposable sterile tissue culture dishes containing Ringer's solution to eliminate contaminating microbes and protozoa by dilution. Embryos were withdrawn from the gonads with a 24

Table 1. Percentage of live glochidia after various periods of time in five culture media.[a]

	Media Tested				
Days in Medium	Muller-Hinton	Nutrient Broth	Brain/Heart Infusion	Todd	Lauria
1	100	99	98	100	98
2	100	98	98	98	97
3	99	100	99	99	98
4	99	98	98	99	95
5	94	98	98	98	17
7		97	95	92	2
8		90	98	82	
12		10	84	3	
14			84	5	
19			87		
22			95		
23			79[b]		
24			86[b]		
25			57[3]		

[a]Each number is based on counting 100 glochidia.
[b]Signs of deterioration were observed though shells were closed.

Table 2. Culture media mixture compositions (ml/L except bacterial nutrient which was added at 2 ml/L as either nutrient broth [NB] or Brain/heart infusion [BH][a]).

Mixture Number	Ringer's	HEPES[b] (1 M)	Buffer-all[b] (100X)	Kanamycin (50 mg/ml)	Geneticin (50 mg/ml)	Antimycotic (100X)[c]	Bacterial Nutrient	Leibovitz[b] (ml)
1	17.80	0.5					NB	
2	17.80		0.2				NB	
3	19.45	0.5		0.05			BH	
4	17.45	0.5		0.05	0.05		NB	
5	17.45		0.2	0.05	0.05		BH	
6	17.78		0.2			0.2	NB	
7	17.30	0.7				0.2	NB	
8	15.45	0.5		0.05			NB	2
9	17.75		0.2	0.05			BH	2
10	17.45	0.5			0.05			2
11	17.78		0.2			0.2	BH	2
12	17.78		0.2	0.05			BH	2
13	17.72	0.5		0.05	0.05	0.2		2
14	15.20	0.5		0.05	0.05	0.2	NB	2
15	17.50		0.2	0.05	0.05	0.2	BH	2
16	16.50		0.2	0.05	0.05	0.2	NB	2
17	15.00	0.5	0.2	0.05	0.05	0.2	NB	2
18	20.00							

[a]NB and BH from DIFCO.
[b]Trade name of organic tissue culture buffers.
[c]The formula used was concentrated 100 times the normal concentration.

Table 3. Survival of glochidia (percent) in the 18 culture mixtures used over a period of 11 days.

Medium No.	Culture Day 1	2	4	6	7	9	11
1	88	73	54	46	49	58	23
2	100	100	62	40	40	40	31
3	100	100					
4	90	92	75	90	44	37	35
5	100	100	100	97	96	73	65
6	100	96	73	87	87	63	48
7	87	100	71	97	91	93	65
8	100	100	46	60	46	18	15
9	96	96	97	96	97	95	90
10	97	98	98	82	65	30	26
11	94	96	89	100	100	80	95
12	100	100	100	100	100	100	100
13	81	97	96	93	84	53	29
14	88	94	90	91	78	34	21
15	97	100	100	86	77	54	36
16	100	96	70	84	84	84	42
17	94	96	70	86	52	37	40
18	100	100	100	73	75	54	

g needle and were cleaned by sedimentation/re-suspension in Ringer's solution a minimum of six times.

The authors' preliminary unpublished experiments showed that mollusc Ringer's solution without nutrient mixture supplements could support the survival of glochidia for only four days. Because many cell types that are routinely used for bacterial cultures require a supplement of nutrient broth, the efficacy of different nutrient mixtures to evaluate the survival of glochidia *in vitro* were tested. Five commonly used nutrient mixtures, Muller-Hinton, Nutrient Broth, Brain/heart Infusion, Todd, and Lauria (Table 1) were selected.

In these preliminary experiments, complete M199 and F12 media with bicarbonate buffer in both air and five percent CO_2 or in air, were used. A mollusc Ringer's mixture was selected as the basic solution and was supplemented with the mammalian cell culture mixture, Leibotviz medium. The

Table 4. Survival of glochidia (percentage) over up to 49 days with respect to duplicate cultures (a and b) using six culture media mixtures.

Days in Culture	Mixture 1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b
1	97	100	100	99	100	99	93	97	100	97	91	98
3	90	96	97	98	97	99	90	99	99	93	95	96
5	98	98	100	--	100	99	92	--	100	98	100	97
7[a]	98	95	97	--	97	--	97	--	--	98	--	91
9	89	96	96	--	96	89	97	--	--	94	--	91
12[a]	81	91	74	--	77	--	80	--	--	84	--	--
13	90	80	79	--	79	--	--	--	--	81	--	--
15[a]	79	78	61	--	79	--	--	--	--	59	--	--
16	65	--	50	--	74	--	--	--	--	--	--	--
17	62	--	64	--	53	--	--	--	--	--	--	--
20[a]	66	--	42	--	80	--						
24	54	--	47	--	76	--						
26	49	--	55	--	60	--						
30	55	--	55	--	51	--						
33[a]	61	--	52	--	56	--						
35	46	--	44	--	53	--						
36	61	--	55	--	50	--						
37[a]	61	--	56	--	55	--						
40	55	--	59	--	52	--						
42[a]	51	--	54	--	52	--						
45	58	--	51	--	52	--						
47	39	--	41	--	42	--						
49	38	--	29	--	36	--						

[a]Days on which media were changed.

rationale to choose the Leibotviz formula was that it can be used with organic buffer as well as with bicarbonate in air or a in CO_2 atmosphere. Although the Leibotviz mixture was selected to supply amino acids and vitamins, nutrient broth was also needed for survival. The two most efficient mixtures were Brain/Heart Infusion and Nutrient Broth from DIFCO.

Subsequently, to test the efficacy for glochidia growth *in vitro*, 18 different working mixtures or culture media were tested for a period of 11 days. The different concentrations of the constituent ingredients in the culture media used are shown in Table 2. The compounds were chosen using the following information: 1) antibiotics were of the broad spectrum type, and 2) Leibotviz culture media can be buffered with organic buffer, HEPE, and does not require a CO_2 atmosphere. Most importantly, in order to discourage contamination, diluted culture media in mollusc Ringer's was used. The survival rates by percentage during the 11 day period were calculated (Table 3). Mixtures 9, 11, and 12 were chosen as the growth media for the next experiments (Table 4). Media 13, 14, and 15 were arbitrarily chosen as controls. Media were changed at intervals as indicated.

Cultures were monitored daily except Sundays. An inverted microscope with phase optics was used to ascertain larval vitality. Living cells and dead cells were different in their refraction. About 100 larvae were counted in random fields to calculate survival rates. Those larvae with open shells were considered dead.

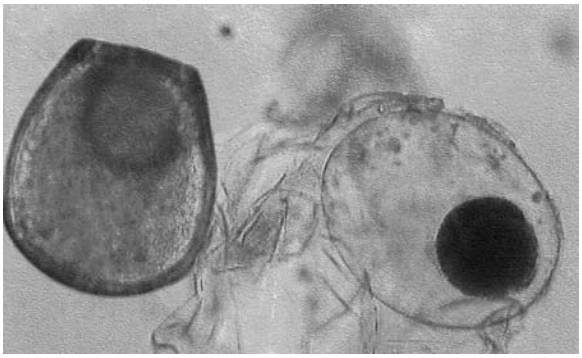

Figure 2. Embryos with and without fertilization membranes along with the membranes.

Results

Preliminary experiments. During the manipulation of embryos and the glochidia, we found that the freshwater mussel embryos of *P. grandis* were much more fragile than those of marine invertebrates such as sea urchins and starfish. Even mild centrifugation using a hand centrifuge would rupture the membranes and fragment the embryos. Washing, therefore, must be done gently by hand. Embryos recovered from the gonad consisted of different stages of development. Embryos with and without fertilization membranes along with the membranes are shown in Figure 2. In that Figure, we placed a glochidium and an embryo on the same slide to show their equivalency in overall size. The thin and trans-

parent shells of glochidia flushed from the marsupia stayed open for a short duration, about 5-10 sec, then closed under the present culture conditions.

The Brain/Heart Infusion supported survival for the longest duration, up to at least 22 days, with more than 85 percent survival. Brain/heart Infusion broth therefore constituted part of the growth media for freshwater mussel glochidia in subsequent experiments.

Based on the preliminary results, another series of experiments were developed in order to test larval survival efficacy of new mixtures presented in Table 3. Mixtures 9, 11, and 12, all containing Brain/heart Infusion, were the best media with survival rates of 90, 95 and 100 percent over the 11 day experimental period. The same mixtures supported survival of the larvae for as long as 49 days in those experiments. Glochidia in the controls did not survive longer than 15 days. The duplicates were highly contaminated rendering them unable to support the growth of the glochidia.

Discussion

This study represents one of the few investigations using defined culture media in an attempt to bypass the parasitic stages of developing freshwater mollusc glochidia. Isom and Hudson (1982) reported that a defined medium supplemented with fish serum would support the transformation of glochidia obtained from the gills of mussels. However, unlike serum from large vertebrates like goats or horses, fish serum in quantity and of high quality is difficult to obtain and thus not is practical for a large-scale cultivation.

Hudson and Shelbourne (1990) had some success in the use of a serum-free commercially available medium to culture free-living juveniles. Juveniles could survive up to 96 hours in studies conducted by Dimock and Wright (1993). These sporadic reports did indicate the possibility of adopting mammalian tissue culture techniques for the survival and subsequent transformation *in vitro* free-living stages without host fishes.

One of the difficulties in glochidia cultivation *in vitro* is the continuous presence of protozoa and other microbes in even the viscera of the mussels resulting in contamination of the cultures. Some protozoa will feed on the glochidia as well as competing for nutrients. The reasons for high mortality rates in the controls during the last series of experiments are unclear even though steps of aseptic techniques were strictly followed. The importance of aseptic techniques in the cultivation of freshwater mussel cells is considered crucial. Although antibiotics are necessary, our experiments showed that none of the ones used was superior over the others. It is more important to start with a population of glochidia with the least amount of contaminating organisms. Bathing the mussels in sterile water until no visible particulate matter is released is the minimum requirement to obtain a clean culture. Knowing this and other difficulties in establishing a successful culture system, we formulated a medium that discouraged the growth of microbes and protozoa. The reasons for choosing DIFCO products for the preliminary experiments were based on availability. Products of other companies will most likely be just as effective.

We have accomplished the first goal which was to obtain high survival of glochidia *in vitro*_for a relatively long period of time without an expensive CO_2 incubator. The next step will be to find a suitable nutrient mixture that can support metamorphosis and differentiation to free-living juveniles without the host fish. Because various species parasitize host fish for different durations, requirement for survival through metamorphosis into free-living juveniles will differ, hypothetically, *in vitro*.

To reach the level of efficacy as exists in mammalian cell culture, there remains much work to be accomplished. The concept of adopting mammalian cell culture technology presented here represents an alternative and practical approach to finding specific host fishes for the rescue and replenishment of populations of freshwater mussels, especially the endangered species. To that end, we have accomplished the first step, survival in an *in vitro* environment that can be refined for the transformation and metamorphosis of freshwater mussel larvae.

Our studies presented here have demonstrated the feasibility of the use of diluted amino acid mixtures designed for cell cultures, broad spectrum antibiotics and the appropriate use of aseptic techniques. The aseptic techniques are the most important step in establishing a culture condition suitable for survival first and subsequent development.

Notes

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