A new additive to the artificial culture medium for freshwater bivalve culture in vitro

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Received 23 September 2009; Accepted 12 May 2010

Abstract
The development of host fish techniques (in vivo) at the beginning of the 20th century, and the improvement of artificial culture media for in vitro culture of bivalves, provided an opportunity to identify the nutritional requirements needed to complete the life cycle of threatened freshwater bivalves. A freeze-dried extract of lambari, a Brazilian Tetra (Astyanax altiparanae), was used as an additive to the artificial culture medium M199 (Sigma®) to try to improve the survival of glochidia and the metamorphosis of Diplodon rotundus gratus and D. greeffeanus. We adjusted the pH of the culture medium to 6.8, the same value recorded at the collecting site of the bivalves. Glochidia were kept in an incubator at 18ºC. 40% of D. rotundus gratus glochidia survived, and 20% reached metamorphosis after 22 days of incubation. For D. greeffeanus 50% of the glochidia survived with 15% undergoing metamorphosis after 22 days of incubation. These results suggest an increase in the post-metamorphic survival with the use of the medium M199 supplemented with the fish extract, with a survival percentage of 75% after 30 days at the juvenile stage. Therefore, this constitutes an effective process in the preparation of in vitro cultures for freshwater mussels, particularly for D. rotundus gratus and D. greeffeanus.

Key words: Limnic mussel; metamorphosis; Diplodon, Hyriidae

Introduction
Limnic bivalves have declined during the last century (Watters, 1996; Cosgrove & Hastie, 2001; Villella et al., 2004; Christian et al., 2004; Bogan, 2008), including the native Brazilian fauna (Beasley et al., 2000; Beasley, 2001; Almeida, 2006). To maintain the natural stocks of bivalves the culture and management of these mollusks has been proposed (Secretariat of the Convention on Biological Diversity, 2004). The current method of propagating limnic bivalves is to infest host fishes (Zale & Neves, 1982; Neves et al., 1985; Yeager & Neves, 1986; Watters & O’Dee, 1996; Hoggarth, 1999; Haag & Warren, 2000). However, the use of artificial culture media may be the only way to avoid the extinction of some bivalves. Artificial culture techniques may produce more juveniles than the technique of infesting host fishes (Isom & Hudson, 1982; Hudson & Shelbourne, 1990). Uthaivan et al. (2001, 2002) developed a simple in vitro culture method to raise glochidia of Hyriopsis myersiana, improving those techniques proposed by Isom & Hudson (1982) and Hudson & Shelbourne (1990).
The present study aims to provide a new additive to the culture medium M199, perhaps the most adequate among the ones used so far in the artificial culture of the glochidia of freshwater bivalves, in order to obtain juvenile forms in the laboratory. In this study, we intend to improve the knowledge on the biology and conservation of two species of freshwater bivalves of the family Hyriidae, which incorporate a parasitic stage in the life-cycle involving an obligate relationship between a fish and a highly modified larva, the glochidium.

Materials and Methods

Bivalve collection

The freshwater bivalves Diplodon rotundus gratus (Wagner, 1827) (Fig. 1) and D. greefianaus (Ihering, 1893) (Fig. 2) were collected from the sediment banks of the Mogi-Guaçu river (21º50’36.10”S - 47º29’44.5”W) in Brazil, between June and August 2009, during the reproductive season (mean water temperature of 19ºC). Each specimen was placed in a 200 ml vial with water from the collecting site. Vials were then placed on ice and transported back to the Malacology Laboratory of the Biology Department, FFCLRP-USP. Bivalves, each of which was placed in a vial with a large-opening, were maintained in aquaria, with aerated spring water, at 20 ± 2ºC. The water was replaced every 2 days.

Glochidia release

Gravid females started to release their larvae up to 24 h after being collected. As the glochidia (Figs. 3, 4) were released, they were removed from the vials with a pipette and placed in a 100 ml beaker for in vitro culture. Since glochidia were released in mucus, this was removed by repeated washings with distilled water (Uthaiwan et al., 2001, Lima et al., 2006). Some glochidia as well as the parent were fixed in 70% alcohol for later correct identification. Specimens that did not liberate glochidia were maintained in the laboratory and later returned to the collecting site.

Choice of fish species

The Brazilian tetra, Astyanax altiparanae (Garutti & Britski, 2000), is a common fish of the Mogi Guacu River and its marginal lakes. This species was selected for use because field surveys had revealed that many individuals were infected with glochidia, mainly in their caudal fin. Furthermore, this species was easily captured with the aid of a sieve or net.

Freeze-dried fish extract

Three specimens (mean length of 6.5 cm) of A. altiparanae were macerated and then freeze-dried. The freeze-dried extract was stored in a refrigerator at 8ºC until used. For use, a 0.57 mg aliquot (0.3% of freeze-dried fish extract after filtration) was re-suspended in 1,000 ml of distilled and de-ionized water and agitated in a vortex mixer for 4 h. The suspension was filtered through 0.45 and 0.22 µm Millipore filters and then added to the medium M199. This aliquot was based on brain infusion medium.

In vitro culture

We tested the viability of the glochidia under a stereo-microscope (magnification 100×) by observing the opening and closing of the valves. About 50–100 glochidia were placed in Petri dishes (60×15 mm) under sterile conditions containing a medium consisting of M199 (Sigma®), a solution of the freeze-dried fish extract and the antibiotic/antimycotic (Invitrogen® cod. 15240062), in the proportions 2:1:0.5 ml. The pH of the culture medium was adjusted to 6.8, with 1.25 M NaOH or 1 M HCl (Keller & Zam, 1990), the same value recorded at the collecting site.

Petri dishes were kept in an incubator at 18ºC until the glochidia metamorphosed into juveniles [as indicated by the distention of the foot out of the shell (Figs. 5, 6)]. All the culture medium was carefully replaced every 6 days, and 1 ml of distilled water was added 24 h before metamorphosis, i.e., at day 21 (Uthaiwan et al., 2002, 2003; Lima et al., 2006).

In vivo culture

Astyanax altiparanae was used to create an artificial infestation. About 200 glochidia/fish were placed in contact with three fishes (mean length = 5 cm) in aquaria measuring 40×20×15 cm with 10 l of aerated spring water kept at 20±2ºC. Once fish were infested (indicated by the presence of whitish spots on the fins), they were observed daily. From the fourth day on, we siphoned the bottom of the aquarium every 2 days and observed the water from the bottom under a stereoscopic microscope in order to check whether juveniles were present. At each observation, 2/3 of the aquarium water was replaced and the fish were fed with commercial fish food.

Data analysis

The percentages of glochidia survival, and glochidia transformation to the juvenile stage after the incubation
period were determined under the light microscope (>100). In this study, experiments were done with at least three replications, and where possible 10 replications were used.

**Results and Discussion**

The development of host fish techniques (in vivo) at the beginning of the 20th century, and the recent improvements of the media for in vitro culture of bivalves, provided an opportunity to identify the nutritional requirements needed to complete the life cycle of threatened freshwater bivalves (Farris & Van Hessel, 2006).

*Diplodon rotundus gratus* and *D. greeffeanus*, with well developed glochidia, started to release their glochidia from marsupia between 12 and 48 h after they were brought back to the laboratory. The way each species released the glochidia was different. In *D. rotundus gratus*, we observed that larvae were released continuously in a mucus strand for 10 to 12 h through the exhalant aperture. Whilst *D. greeffeanus* also released their glochidia through the exhalant aperture, this occurred at regular pulses that lasted over 24 h. Glochidia release through the pedal opening as reported by Graf (2001) for species of the family Unionidae from North America, were not observed in this study. After release, the glochidia exhibited opening and closing of their valves, which indicated their viability for in vitro and in vivo culture.

The glochidia of *D. rotundus gratus* and *D. greeffeanus* cultivated in vitro at 20±2°C remained encysted for 15 days in *A. altiparanae*, with 5.3 and 1.8% undergoing metamorphosis respectively (Table 1). According to Bonetto (1954), *D. charruanus* remained encysted for 10 to 20 days in *Hoplias malabaricus*. Mansur (1999) infested specimens of *Gymnogeophagus gymnogenys* with glochidia of *D. martensi*, which remained encysted for 30 days. However, the duration of the parasitic period in freshwater bivalves may vary from several days to months, depending on biotic and abiotic factors (Zimmerman & Neves, 2002). The number of metamorphosed individuals from in vivo culture techniques is usually very low (Farris & Van Hessel, 2006). Bigham (2002) studied *Venustaconcha ellipsiformis, Ethrostoma spectabile* and *E. caeruleum* and found an average of less than 5% underwent metamorphosis. In another study with *Strophitus undulates* (Gray et al., 2002), after an incubation period of 10 days using several species of host fishes, the percentage of metamorphosis varied from 2 to 51%. Young & Williams (1984) found that in the natural environment, less than 0.01% of post-metamorphic juveniles of *Margaritifera margaritifera* survived.

Studies using artificial media for the culture of freshwater bivalves began in the 1980s (Farris & Van Hessel, 2006), and Isom & Hudson (1982) succeeded using commercially available cellular culture media (Eagles and medium M199). These media have almost all amino acids at concentrations as high as, or even higher, than the ones found in fish plasma (Farris & Van Hessel, 2006). Glochidia in vitro cultures have proved to be very efficient when compared with in vivo cultures. In this study, *D. rotundus gratus* exhibited four times more metamorphosed juveniles and *D. greeffeanus* 7.5 times more juveniles when cultivated with the freeze-dried extract of *A. altiparanae*, compared to the in vivo culture. Similar studies produced about 10 to 20 times more juveniles from several artificial culture media (Isom & Hudson, 1982; Hudson & Shelbourne, 1990).

In this study, we sought to develop the techniques already used for obtaining juveniles, based on the results of Isom & Hudson (1982), Hudson & Isom (1984), Hudson & Shelbourne (1990), Keller & Zam (1990), Uthaiwan et al. (2001, 2002) and Lima et al. (2006). The use of freeze-dried fish extracts (*A. altiparanae*) made possible in vitro cultures in a simple and efficient way. With freeze-dried extract of *A. altiparanae* 40% of *D. rotundus gratus* glochidia were alive after 22 days and 20% reached metamorphosis (Table 1). In *D. greeffeanus* 50% of glochidia were alive after 22 days and 15% metamorphosed (Table 1). Hudson & Isom (1984) and Hudson & Shelbourne (1990) showed that the percentage

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<th><em>D. rotundus gratus</em></th>
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<td>In vitro</td>
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<td>Temperature, °C</td>
<td>18</td>
<td>20 ± 2</td>
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<td>No. of glochidia</td>
<td>50/dish</td>
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<td>Percent survival</td>
<td>40 ± 6.3</td>
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<td>50 ± 9.6</td>
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<td>Percent metamorphosis</td>
<td>20 ± 2.2</td>
<td>5.3</td>
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Table 1. Percentage of the larvae surviving and undergoing metamorphosis of *Diplodon rotundus gratus* and *Diplodon greeffeanus* glochidia raised “in vitro” and “in vivo”
of glochidia undergoing metamorphosis increased with the addition of fish plasma, reaching values between 50 and 78%. Uthaiwan et al. (2001, 2002, 2003) developed a simple technique of glochidia culture in an artificial medium resulting in 85 to 100% metamorphosis. Similar results were obtained with the European species *Anoonta cygnea*, in which 34.3% of larvae survived, whereas the proportion of larvae that metamorphosed was 60.8% (Lima et al., 2006). Although the values found in this study were smaller than the ones reported in the literature, they were far higher than the values of metamorphosis obtained through the infestation of host fishes (Table 1).

The freeze-dried extract of *A. aliparanae* used as a protein source in the culture medium provided the necessary components for the development of the glochidia of the tested species. Despite the fact that we did not have the composition of essential and non-essential amino acids of *A. aliparanae*, only its body composition (13.45% protein, 6.05% fat; Cotan et al., 2006), our results suggest an efficiency in post-metamorphic survival, 75% after 50 days (Figs. 7, 8). Some authors (e.g. Hudson & Isom, 1984) did not succeed in maintaining the post-metamorphic juveniles of *Lampsilis ovata*, *Fusconaia ebeba*, *Ligumia recta*, *Pleurobema cordatum* and *Carunculina moesta* for more than a week. Uthaiwan et al. (2001), using M199 with the plasma of *Oreochromis niloticus* and horse serum, did not succeed in maintaining the post-metamorphic juveniles of *H. myersiana* for more than 10 days. Uthaiwan et al. (2002) compared the effect of the plasma of several fish species and obtained post-metamorphic survival values of two months with common carp (*Cyprinus carpio*), one month with Nile tilapia (*O. niloticus*) and catfish hybrid, 2–3 weeks with horse serum, and 1–2 weeks with striped catfish. Recent studies by Lima et al. (2006) using *A. cygnea* cultivated in M199 with the plasma of *C. carpio* reached a post-metamorphic survival of 15 days.

The study by Tankersley (2000) showed that lipid levels in glochidia and juveniles vary according to each breeder pool and are, therefore, influenced by parental nutritional state. Moreover, he indicated that the type of culture medium used in vitro influences the lipid contents of glochidia and their subsequent juveniles. For *Utterbackia imbecillis* Fisher & Dimock (2002a, b) found that the artificially developed glochidia showed lower lipid, triglyceride, cholesterol, glycojen and protein levels than the ones developed in a host fish. Therefore, we suggest that the use of freeze-dried fish extract provides a more adequate medium for the metamorphosis of the glochidia. This study is the first account of the culture of Brazilian fresh water bivalves in an artificial medium.

**Acknowledgements**

To FAPESP for the research grant (2006/04658-7). To the PhD Program in Comparative Biology of the Faculty of Philosophy Science and Letters of Ribeirão Preto/USP. To CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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