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Viability of glochidia of *Utterbackia imbecillis* (Bivalvia: Unionidae) following their removal from the parental mussel

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ABSTRACT: One of the critical periods in the life cycle of unionid mussels (Mollusca: Bivalvia) is after the release of the glochidia. The larvae must contact and attach to a host fish in order for successful development to the juvenile stage to occur. This study examined the length of time the glochidia of *Utterbackia imbecillis* could survive and remain developmentally competent after removal from the parental mussel. Glochidia were isolated from the adult mussel and placed individually in 96-well culture plates. Each day for 20 d, 20 glochidia were tested for valve closure when exposed to 2M KCl. Subsets of the isolated glochidia were examined for their ability to metamorphose to juveniles after removal from the parental mussel. At day 0 and every 4 d following isolation, 40 larvae were placed in a standard tissue culture protocol and later examined to determine if they had completed metamorphosis. The mean 50% survival time, as measured by closure in KCl, was 13.5 d. A small percentage of the glochidia that were isolated for 16 d underwent metamorphosis, but the maximum time at which at least 50% of the animals metamorphosed was 9 d post-isolation. A one-way ANOVA revealed a significant effect of isolation time on survival and metamorphic ability of glochidia. There was also a significant correlation between the period of responsiveness to KCl and the duration of developmental competency, suggesting that using KCl to predict the relative physiological condition of glochidia may be reliable.

Keywords: glochidia, metamorphosis, survival, transformation

Freshwater mussels of the family Unionidae (Mollusca: Bivalvia) are common inhabitants of streams and lakes of North America. Unlike marine and other freshwater bivalves, unionids have a parasitic larval stage, the glochidium. The larva typically attaches to the gills or fins of a fish and undergoes a period of development which may last from a few days to several weeks. After this period of metamorphosis, the juvenile mussel excysts and then continues its development into an adult on the substratum.

The glochidia of freshwater mussels have been well studied, with the majority of research focused on host specificity (Howard and Anson 1922, Guisti *et al.* 1975, Atkins 1979, Watters and O'Dee 1998), morphology (Kat 1984), physiology (Huebner and Pynnönen 1992, Pynnönen 1995), and sensory capabilities (Lábos and Sálanki 1963, Shadoan and Dimock 1999). An area that has received little attention is the survival time and developmental competence of glochidia after release from the parental mussel. This is a crucial time in the life cycle of freshwater mussels with mortality reported to be greater than 99.99% (Young and Williams 1984, Jansen and Hanson 1991). There are anecdotal reports that glochidia may survive for 7 - 10 d (Howard and Anson 1922, Huebner and Pynnönen 1992) or can be free in the water for 10-14 d after release from the parental mussel (Mackie 1984). Goudreau *et al.* (1993) observed that the glochidia of *Villosa iris* experienced increased mortality after 48 h of isolation from the demibranch. However, there has been no examination of the relationship between survival time post-isolation and the ability of glochidia to metamorphose.

Viability of glochidia has commonly been assessed by adding a saline solution to a sample of glochidia and measuring the number that close in response. Arey (1921) referred to the closure of glochidia in response to the addition of a few crystals of table salt as a test of their "ripeness." Since that study various concentrations of KCl and NaCl have been used to determine viability of glochidia (Huebner and Pynnönen 1992, Goudreau et al. 1993, Pynnönen 1995, Hanstén et al. 1996), however there has been no empirical evidence that this is an accurate measure of developmental competency.

This study assessed the survivorship and viability of glochidia of *Utterbackia imbecillis* (Say, 1829) following their removal from a parental mussel. This mussel is common in the Eastern U.S. (Johnson 1970), and populations in North Carolina release glochidia from early spring to late fall (personal observation). The glochidia are about 300-400 µm in length, and at 20 C complete the metamorphosis to juveniles in 7 - 14 d following attachment to a host fish or incubation in laboratory culture (Isom and Hudson 1982, and personal observation). Our primary objective was to determine the

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length of time glochidia could survive and remain developmentally competent after removal from the parental mussel. In addition, the response of glochidia to KCl as a test of "viability" was compared with the larvae's potential to complete metamorphosis in an in vitro cell culture protocol.

Methods

Adult *Utterbackia imbecillis*, a simultaneous hermaphrodite unionid (Hoeh 1990) were obtained from Davis' Pond (Davidson, North Carolina) and maintained in the laboratory until mature glochidia were present in the marsupia. Glochidia were removed from the outer demibranchs of 2 mussels, washed in sterile moderately hard EPA water (Lewis et al. 1994) and pooled before use. Individual glochidia were then placed into wells of a 96-well microtiter plate together with 380 µl of EPA water. Microtiter plates were placed in an incubator at 21 C and 125 µl of water was changed every other day for 20 d. Each day 20 animals from consecutive wells were tested for their response to the addition of a drop of 2M KCl added to their well. The number of glochidia that closed in response to the KCl was recorded, and the percent "survival" calculated. At day 0 and every 4 d following isolation, 40 animals were removed from the microtiter plates (10 animals from each of 4 plates) and placed into 4 - 60 x 15mm culture dishes in 3 ml of a standard tissue culture medium (Isom and Hudson 1982, Dimock and Wright 1993). The dishes were maintained in a 5% CO_2 incubator at 21 °C for 7 d and then the mussels were examined to determine the number that had undergone metamorphosis to juveniles. Individuals were considered to have metamorphosed if they possessed two adductor muscles, a juvenile foot and a visible heartbeat. A one-way ANOVA was conducted for each measure of viability to determine if survival or metamorphic ability decreased significantly over time. Correlation analysis was performed to determine if the two measures of viability are related.

Results

The response of glochidia to KCl decreased significantly with the duration of isolation (Table 1; Fig. 1). More than 50% of the larvae failed to close when exposed to 2 M KCl about 13.5 d after removal from the parental mussel. The percentage of glochidia that completed metamorphosis in tissue culture showed an initial increase between days 0 and 4, and then decreased with the time of isolation (Table 1; Fig.1). Significant differences occurred among all time periods except days 12 and 16 (Tukey's HSD test). Approximately 5% of the glochidia that had been isolated for 16 d were able to complete metamorphosis, but the maximum time at which at least 50% of the animals metamorphosed was 9 d post-isolation. There was a significant correlation between KCl response and ability to metamorphose, ($r = 0.88$, $df = 4$, $P < 0.05$).

Discussion

The period between release from the parental mussel and attachment to a host fish is a time of high mortality in the life cycle of freshwater mussels (Young and Williams 1984, Jansen and Hanson 1990). Although some mussels have modified mantle structures or conglutinates (Kraemer 1970, Kat 1984, Haag and Warren 1999) that mimic prey items, and thus may improve the probability of contact between a larva and an appropriate host, *Utterbackia imbecillis* apparently has no special adaptations for synchronizing the release of larvae with the presence of a fish host. It is unclear if the glochidia of *U. imbecillis* can contact a host fish once they have settled out of the water column; however, prolonged viability following release may increase the probability of attachment.

Our data indicate that glochidia of *U. imbecillis* may survive as long as 19 d following their release from a parental mussel, but they suffer 50% mortality within 13.5 d. These values are consistent with the few data on longevity that have been reported for other unionids. Howard and Anson (1922) observed glochidia that survived for 1 wk post-isolation, and Mackie (1984) reported that some glochidia can remain free in the water column for 10-14 d after release. However, survival may not be indicative of developmental competency. Glochidia must retain the ability to attach to the fish and undergo metamorphosis for development to continue. Our data indicate that a small percentage of glochidia remain developmentally competent for up to 16 d after isolation from the parent, but by 9 d post-isolation only 50% were capable of metamorphosis. Thus glochidia are able to survive longer than they remain developmentally competent.

Although the viability of glochidia is commonly tested by closure in response to KCl (Arey 1921, Huebner and Pynnönen 1992, Goudreau et al. 1993, Pynnönen 1995, Hanstén *et al.* 1996), there has been no empirical test of this method. The data reported herein indicate that this technique may adequately assess developmental competency, especially shortly after larvae are released from the brood chambers. The proportion of glochidia that closed in response to KCl was significantly correlated with the proportion able to complete metamorphosis, following the same duration of isolation.

Table 1. Results of a one-way ANOVA showing the effect of time on survival (A) and metamorphosis (B) of glochidia.

Source of Variation	df	SS	MS	F	P
A: Survival					
Among days	20	49140.8	2457.0	20.9	< 0.001
Within days	42	4932.9	117.4		
B: Metamorphosis					
Among days	4	5115.4	1278.9	16.7	< 0.001
Within days	10	765	76.6		

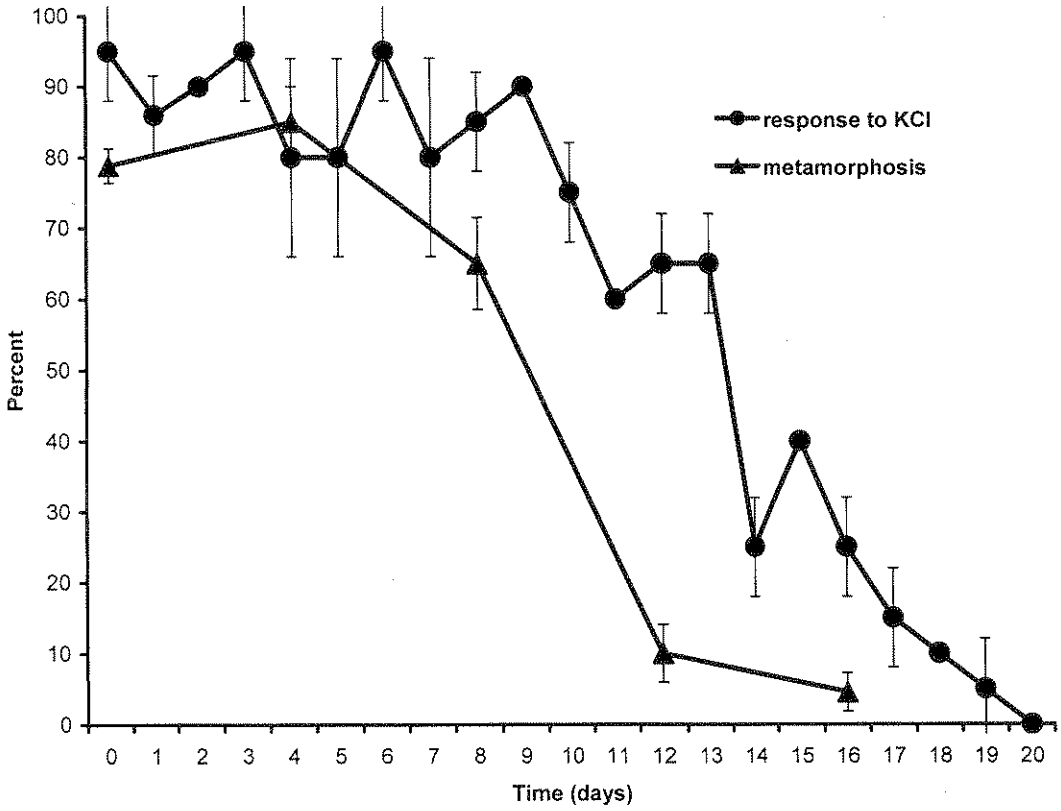

Figure 1. The effect of isolation from a parental mussel on the percentage of glochidia that close in response to 2 M KCl and that undergo metamorphosis. Points are the $\bar{x} \pm$ SD. The effect of isolation time on metamorphosis was significant for all comparisons except between days 12 and 16 (one-way ANOVA, $F_{(4,10)} = 16.7$, $P < 0.001$; Tukey's HSD).

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