Roberts
Barnhart
1999

J. N. Am. Benthol. Soc., 1999, 18(4):477–487

Effects of temperature, pH, and CO_2 on transformation of the glochidia of *Anodonta suborbiculata* on fish hosts and in vitro

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Abstract. Low temperature is known to suppress immune function in ectothermic vertebrates, including fish. Therefore, we hypothesized that low temperature might facilitate successful encystment and transformation of the parasitic glochidia larvae of unionid mussels on fish hosts. Glochidia of the flat floater, *Anodonta suborbiculata*, are present on hosts from January through March, when water temperature is low. The % transformation success of attached glochidia in laboratory infections on fish hosts (golden shiner, *Notemigonus crysoleucas*) was significantly higher at 10°C (67%) and 15°C (62%) than at 21°C (42%). In contrast, transformation success of glochidia in vitro was significantly lower at 10°C (39%) than at 15°C (89%) or 20°C (93%). Thus, the improved survival at low temperature on fish hosts does not appear to involve direct effects of temperature on the glochidia. These results support the hypothesis that immune suppression of fish hosts by low temperature enhances transformation success of this winter-breeding species. We also tested the effects of CO_2, pH, and HEPES buffer on transformation of flat floater glochidia in vitro at 15°C. Transformation success was highest at the lowest pH tested (7.6) and the highest level of CO_2 tested (5% CO_2). This result is consistent with previous studies of other species, but is puzzling because the physiological pH of fish body fluids is higher and the level of CO_2 is much lower. Although most workers have incubated glochidia in 5% CO_2, we found that transformation success was fairly high (68%) without elevated CO_2, provided that pH was kept low. HEPES buffer reduced transformation success.

Key words: Unionidae, *Anodonta suborbiculata*, glochidia, in vitro transformation, temperature, immunity, parasitism, freshwater mussels.

The glochidium larva is a critical point in the life cycle of unionid bivalves, but little is known of the interactions between glochidia and their hosts. The need for understanding these relationships has taken on some urgency, given current concerns for threatened and endangered mussel species (Neves et al. 1997). The immune responses of fish to glochidia are particularly poorly understood. Most fish species possess natural immunity to most species of unionids. Many glochidia that attach to suitable hosts fail to transform, and suitable hosts that are exposed to successive infections may acquire immunity and show diminished tolerance (Reuling 1919, Arey 1932, Bauer 1987a, 1997b, Watters and O'Dee 1996). Therefore, it appears that unionids are under substantial selective pressure to evade the immune responses of their hosts.

One possible strategy for evasion of the host's immune response is to infect the host at low temperature. Low temperature is known to suppress immune function in ectothermic vertebrates, including fish (Corbel 1974, Avtalion and Shahrabani 1975, O'Neil 1985, Bly and Clem 1991). Therefore, we hypothesized that low temperature might facilitate the successful encystment and transformation of glochidia on fish hosts. We tested this hypothesis with the glochidia of the flat floater mussel, *Anodonta suborbiculata*. The flat floater occurs widely in the Mississippi River basin, where it is found in oxbow lakes and backwaters of large rivers (Utterback 1915, Murray and Leonard 1962, Johnson 1980). Seasonal changes in temperature are relatively marked in this shallow, lentic habitat. The release of flat floater glochidia occurs in winter, and the glochidia are present on fish hosts from January through March when water temperatures are low (Roberts 1997).

We tested the influence of temperature on survivorship, transformation success, and the duration of encystment of flat floater glochidia on fish hosts at 10, 15, and 21°C. We also examined temperature effects on the transformation of glochidia in artificial media. The latter tests were undertaken to distinguish between

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the direct effects of temperature on the glochidia and effects on the interaction with fish hosts. The in vitro tests were also extended to investigate effects of pH and CO_2 levels on transformation success.

Methods

Glochidia

Gravid flat floaters were collected from a small (0.75 km²) impoundment near the Marais des Cygnes River in Linn County, Kansas. The life history of flat floaters at this site was investigated previously (Barnhart and Roberts 1997, Roberts 1997). Mussels were transported to the lab and held at 10°C in aerated aquaria kept in a temperature-controlled incubator. Masses of glochidia were obtained by rupturing the gravid marsupial gills of the female with a sharp probe to release the gill contents. These masses were viscous and cohesive because of the extended larval threads of the glochidia. The glochidia were shaken in water to detach the larval threads and then washed several times by swirling with sterile water and decanting. After washing, subsamples of glochidia were tested for viability by exposing them to NaCl and counting the proportion that closed (LeFevre and Curtis 1912).

Transformation on fish hosts

The effect of temperature on the timing and success of transformation was tested by monitoring the drop-off of glochidia and juveniles from individual fish hosts. Golden shiners (*Notemigonus crysoleucas*) were obtained from the Hunnewell Fish Hatchery in Hannibal, Missouri. The fish were quarantined and fed daily for ~2 wk to ensure health prior to the experiment. The individuals used were between 5.0 and 9.5 cm in length. Sixty fish were artificially infected with glochidia pooled from 2 mussels. Each fish was infected by immersion in a 500-mL beaker of water containing glochidia. In this small volume, the swimming movements of the fish were sufficient to suspend the glochidia and to ensure contact with the fish. Infection intensity averaged ~90 glochidia attached per fish (see results). After infection the fish were held in aquaria at 15°C for 2 h to rinse off any unattached glochidia. Thereafter, the fish were randomly divided into 3 groups of 20 fish each that were placed at 10, 15, or 21°C in incubators with thermostats. Temperatures were checked at 1–2 day intervals. The warmest incubator was set at 20°C, but actually regulated at 21°C. One of 2 10°C incubators failed during the experiment, so the number of fishes at 10°C was reduced to 10.

The infected fish were held individually in chambers that allowed daily recovery of shed glochidia and juveniles (cf. Trdan and Hoeh 1989). Our chambers were constructed from plastic 2-L soda bottles, hung upside-down, with the base cut out to allow access. A tapered CPVC plumbing coupler was press-fit inside the mouth of the bottle and a drain tube (1.9-cm OD vinyl tubing) was attached. The neck of the bottle was partly blocked to prevent fish from entering the drain tube. Each chamber was monitored daily by draining ½ of the water through a 250-µm mesh Nitex® filter temporarily connected to the drain tube. After each sample, the filter was flushed with water to wash any recovered glochidia into individual 15-mm × 60-mm Petri dishes. The chambers were then refilled with fresh water of the same temperature.

Recovered glochidia and juveniles were examined under 10–100× magnification. Closed glochidia were presumed to be alive because the elasticity of the hinge usually opens the valves when the adductor muscle relaxes. Open glochidia were classified as dead if they were empty shells or were unresponsive to NaCl. Transformed (juvenile) mussels were distinguished from glochidia by observing at least 1 of the following characteristics: 1) valve or foot movement, 2) the presence of 2 adductor mussels, 3) heartbeat, or 4) beating cilia on the foot, gills, or mantle. Juvenile mussels were also relatively opaque compared to glochidia.

Transformation success was quantified as the % of attached glochidia that transformed on individual host fish. Treatment effects were tested by ANOVA on arc-sine transformed data, followed by Tukey paired comparisons among treatment groups.

In-vitro transformation experiments

Culture methods.—The culture medium used was similar to that of Keller and Zam (1990) and contained 4:2:1 parts by volume of Dulbecco's Modified Eagle's Medium (DME), horse serum, and antibiotic mix. The antibiotic mix followed

Isom and Hudson (1982) and contained the following (μg/mL): amphotericin B (100), gentamycin sulfate (100), carbenicillin (100), and Rifampin (50). The DME and antibiotics were combined, sterilized by vacuum filtration through a 0.22-μm Corning® filter, and stored at 0°C. Horse serum was added to the medium just before use. The final medium was distributed to the plates from a 20-mL syringe through a 0.2-μm Acrodisc® syringe filter, taking care to use good aseptic technique. Washed glochidia were distributed into plates using a sterile glass Pasteur pipette. The cultures were not axenic because the introduction of microorganisms along with the glochidia was inevitable. However, aseptic techniques were still important to minimize contamination. Glochidia that were recovered from aquaria after normal release and then cultured were quickly overrun with contaminant organisms, whereas less contamination occurred when glochidia were collected directly from the marsupia into sterile water.

Petri plates (15 × 60 mm) were prepared with 3.5 mL of medium and stocked with ~300 glochidia each. A few glochidia (<3%) failed to close in response to the medium. These glochidia were considered dead and were not included in subsequent calculation of transformation success. It was important not to overstock culture plates with excessive numbers of glochidia. Two plates were intentionally overloaded with glochidia (~600/plate). Glochidia in these plates showed some development (the beginning of a foot and gill buds), but all died before development was complete. Nutrient depletion or waste accumulation may limit the number of glochidia able to develop in a plate.

The plates were incubated within sealed gas chambers rather than a CO_2 incubator. These chambers were 4-L wide-mouth glass jars with metal screw caps. Small stopcock valves were soldered into the caps to permit flushing with gas mixtures, and the caps were fitted with rubber gaskets. The seals were tested before use by filling the chambers with 5% CO_2. The internal CO_2 was tested with an infrared gas analyzer and was unchanged after 2 d, showing that the vessels were gas-tight. In use, each chamber was gassed every other day with an analyzed gas mixture prepared by a commercial supplier (5% CO_2: 95% air) or with gas mixtures prepared using a Matheson® gas mixing system and mass flow controllers. The gas was humidified by bubbling through water to avoid desiccating the culture plates.

Temperature effects.—Three groups of 8 plates each were incubated in 5% CO_2 at 10, 15, and 20°C. Temperatures were checked at 1–2 day intervals. The length of time the glochidia were left in the culture medium was based on the mean transformation times observed in vivo on golden shiners and on observations of the progress of transformation. All glochidia in a group were transferred to water simultaneously. Immediately afterward, the % of transformed individuals was determined for each plate and the pH of the medium was measured. Dead glochidia and juveniles were counted again after the first 24 h in fresh water to determine the % of juveniles surviving the transfer from the medium to fresh water.

Transformation success was quantified as the % of inoculated glochidia that transformed in individual culture plates. Treatment effects were tested by ANOVA on arc-sine transformed data, followed by Tukey paired comparisons among treatment groups.

Acid–base effects.—We tested the effects of pH, CO_2, and HEPES organic buffer (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) on transformation success of flat floater glochidia in a separate in vitro experiment. The experiment was run at 15°C only, in gas chambers to control CO_2 as described above. Eight combinations of variables were tested. Media were titrated to pH 7.6, 7.9, and 8.2 at each of 3 levels of CO_2: 0.04% (air), 2%, and 5%. Five similar series were prepared with and without HEPES (0.2% w/v). The pH was measured with an Accumet® pH meter and Ross electrode calibrated at 2 points with fresh buffers. CO_2 in the media was controlled during titration by bubbling the media with these gas mixtures in a 30-mL vessel, with a water-jacket for temperature control. Five plates of glochidia (90 plates total) were tested in each treatment, with ~100 glochidia per plate. Transformation success was quantified as described above. Following the experiment, the medium from each plate was equilibrated with the appropriate gas mixture and pH was remeasured to detect any change in pH resulting from glochidial metabolism.

The effects of acid-base variables on in vitro transformation were tested with binary logistic regression. All statistical analyses were carried

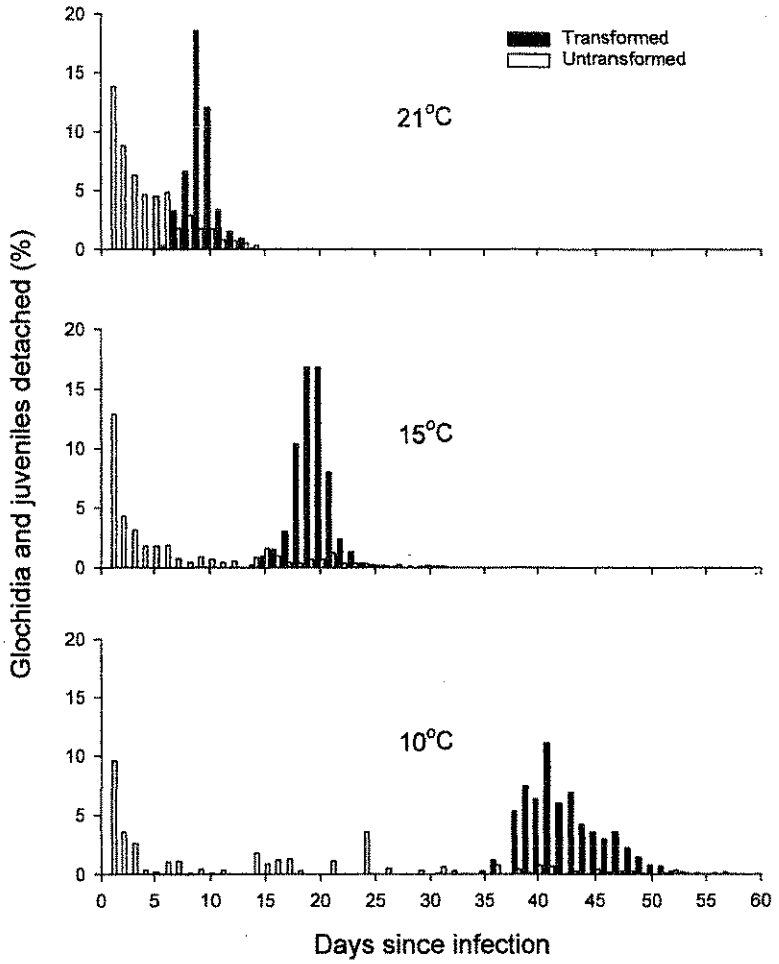

FIG. 1. Temperature effects on the detachment of glochidia and juvenile *Anodonta suborbiculata* from host fish.

out using Minitab® software (release 12, Minitab Inc., State College, Pennsylvania).

Results

Transformation on fish hosts

Infection intensity varied from 2 to 200 glochidia per fish (mean ± 1 SD = 88 ± 54, n = 50). The total number of glochidia attached per individual fish in artificial infections was significantly correlated with length of the fish (number attached = −76.1 + 22.1 * length, R^2 = 33.6%, p = 0.041). Fish length ranged from 5 to 9.5 cm. No significant correlation was found between the infection intensity and the % of transformed glochidia at any temperature.

Transformation success (% of glochidia that transformed) on fish hosts decreased significantly at higher temperature (ANOVA, p < 0.001) (Table 1, Figs 1, 2). Paired comparisons indicated that transformation success on fish at 21°C was lower (p < 0.001) than at 10°C or at 15°C. Transformation success varied among individual fish, particularly at 21°C (Fig. 2). Larger proportions of both live and dead glochidia detached from the hosts without transformation at 21°C than at the lower temperatures (Table 1). Most detachment of glochidia, particularly live glochidia, occurred within the first 3 d of the infection, but loss continued throughout the duration of the infection and overlapped the period when live juveniles were detaching (Fig. 1, Table 1).

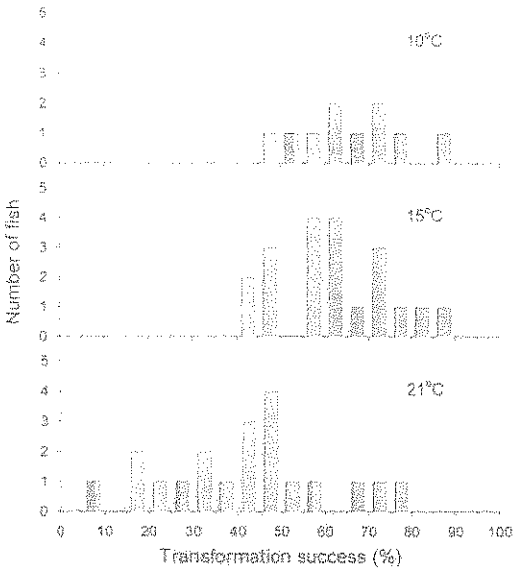

Fig. 2. Transformation success of glochidia on individual fish hosts at 3 temperatures. n = 20 fish at 15 and 21°C and 10 fish at 10°C. Transformation success was significantly lower at 21°C than at 15 or 10°C.

The time that successfully transformed individuals (juveniles) remained attached to the fish host depended upon temperature. The mean time from attachment to detachment of juveniles increased from 9.3 d at 21°C to 42.6 d at 10°C (Table 2). The interval during which the transformed juveniles were detaching from the fish also increased, from 9 to 26 d (Fig. 1).

In-vitro transformation experiments

Temperature effects.—Flat floater glochidia transformed readily in cell culture medium. Transformation success in culture medium was high at 20°C (93%) and 15°C (89%), but dropped to 39% at 10°C (Table 3). Transformation times were similar on fish hosts and in vitro at 20–21°C (9 d) and 15°C (20 d). However, transformation time was longer in vitro (52 d) than in vivo (43 d) at 10°C. From 1/3 to 1/2 of in vitro transformed juveniles died within 24 h after transfer from the culture medium to water (Table 3). Survival after 24 h was highest at 15°C and was significantly lower at 10 and 20°C, where survival was similar. The pH of the culture media increased by ~0.5 units during incubation at each temperature (Table 3).

Acid–base effects.—Each of the test variables (pH, CO_2, and HEPES buffer) significantly affected transformation success ($p < 0.01$ in each case). Transformation success was higher at lower pH and at higher CO_2 (Table 4). The interaction term between pH and CO_2 was also significant; effects of pH were enhanced at low CO_2 and effects of CO_2 were enhanced at high pH. Although elevated CO_2 generally enhanced transformation success, reasonably high transformation success was observed among glochidia incubated in air, particularly at the lowest pH tested (7.6). The pH of the medium generally increased during the incubation period, except for a slight decrease in the plates that were incubated in air (Table 4). The presence of HEPES did not appear to stabilize pH, and transformation success was lower with HEPES present.

Discussion

Loss of attached glochidia from hosts

More than ½ of the glochidia that initially attached to the fish host detached without transforming. Live glochidia accounted for ~28% of the total that failed to transform (calculated from Table 1). Most of these live glochidia detached during the first 3 d. Heavy losses of glochidia from suitable hosts in the first few days after infection have been previously reported in *A. woodiana* attached to *Gambusia affinis* (Dudgeon and Morton 1984) and in *Margaritifera margaritifera* on trout (Fustish and Millemann 1978, Meyers et al. 1980, Bauer and Vogel 1987). In those studies, the initial loss of glochidia was attributed to an acute, nonselective tissue response of the host, similar to the responses that lead to sloughing of the glochidia from non-host species. However, it is possible that some glochidia may simply fail to attach firmly or may attach to surfaces that are unsuitable for encystment. Hooked glochidia frequently attach to areas other than gills and fins, including scales, fin spines, mouth, throat, and gill rakers (Dartnall and Walkey 1979, Dudgeon and Morton 1984, Waller and Mitchell 1989, Jansen 1991).

In addition to the early loss of live glochidia, loss of dead glochidia continued throughout the duration of the infection and accounted for ~72% of those that failed to transform. Presumably these glochidia died within the cysts. Loss of dead glochidia was also reported in *M. margaritifera* and was attributed to a humoral (an-

TABLE 1. Fate of glochidia on fish hosts. Figures are means ±1 SD of % of attached glochidia. n = 20 fish at 15 and 21°C and 10 fish at 10°C. Closed glochidia were classified as alive, and open glochidia were classified as dead (see text).

		Untransformed detached in first 3 d (%)		
Temperature (°C)	Transformed (%)	Live	Dead	Total
10	66.5 ± 11.52	5.2 ± 3.08	9.3 ± 5.01	14.5 ± 6.97
15	61.9 ± 13.10	7.5 ± 5.49	14.9 ± 9.74	22.4 ± 14.35
21	42.2 ± 18.39	14.6 ± 8.35	16.6 ± 8.61	31.3 ± 13.82

tibody-mediated) response (Bauer 1987a, Bauer and Vogel 1987). Antibody production is a delayed process in fish (Corbel 1974). These glochidia may have been encysted and begun development, but were destroyed within the cyst before development was complete.

Transformation success varied considerably among individual fish hosts (Fig. 2). Few other studies have documented variation in glochidia transformation success among individual fish hosts (Kirk and Layzer 1997, Riusech and Barnhart, in press). If these differences are genetically based, natural or artificial selection could readily alter the ability of a host population to support glochidia. Alternatively, the differences could be physiologically based and reflect differences in condition of individual fish. Genetically based individual differences in host tolerance of parasites have been documented in other organisms (Snyder et al. 1996).

Temperature effects on transformation success

The higher transformation success observed on fish hosts at low temperature may be a result of immunosuppression of the host. Detachment of both live and dead glochidia was higher at 21°C than at 10 or 15°C (Table 1), suggesting that both tissue and humoral responses to glochidiosis may be affected by temperature. An alternative explanation for temperature effects on transformation success is that lower temperatures may be more suitable for glochidial development. However, the results from the in vitro experiments do not support the latter hypothesis. Transformation success in vitro was 100% in several plates at 20°C and was not significantly different from 15°C (Table 3). Therefore, it appears that the higher temperature did not directly inhibit the glochidia. A 3rd possible temperature effect on transformation success is through effect on fish activity. Fish may be more active at warmer temperatures, which may cause increased mechanical detachment of glochidia. This possibility seems plausible, but was not tested in our study.

Temperature effects on transformation time

Temperature effects on rate processes can be quantified by Q_{10}, the factor by which rate changes with a 10°C increase in temperature, calculated according to the equation:

$$Q_{10} = (k_2/k_1)^{10/(t_2-t_1)}. \quad [1]$$

The terms k_1 and k_2 are rates at temperatures t_1 and t_2, respectively. Q_{10} for most chemical reactions and many physiological processes is ~2–3 (Schmidt-Nielsen 1990). Substantially lower or higher values of Q_{10} may indicate adaptations to reduce or to enhance temperature-induced changes, respectively.

We calculated Q_{10} for unionid development, using the inverse of transformation time to represent rate of development. Temperature effects on transformation time have been reported by LeFevre and Curtis (1912), Howard and Anson (1922), Dudgeon and Morton (1984), Parker et al. (1984), Weaver et al. (1991), Hruska (1992), and our study. Q_{10} for transformation time exceeds 4 in 4 of 5 species tested (Table 2). The high values of Q_{10} suggest that developmental rate of these species is more strongly affected by temperature than expected from thermodynamic considerations alone. The slowing of development at low temperatures might serve to switch off development during the winter months and prolong the encystment period at a time when conditions are not suitable for feeding and growth. Conversely, the acceleration of development at higher temperatures should result in rapid development as the water warms in the spring. It is interesting in this regard that temperature quotients are higher in the winter-

TABLE 1. Continued.

Untransformed detached after first 3 d (%)			Total untransformed (%)		
Live	Dead	Total	Live	Dead	Total
3.3 ± 2.42	16.6 ± 15.26	19.9 ± 15.76	8.7 ± 12.15	25.6 ± 12.15	34.3 ± 21.74
2.0 ± 8.48	13.7 ± 5.61	15.7 ± 6.83	9.5 ± 5.92	28.6 ± 8.75	37.8 ± 13.13
4.1 ± 4.86	22.3 ± 11.06	26.4 ± 11.44	18.8 ± 9.40	38.9 ± 13.98	57.7 ± 18.34

breeding *A. suborbiculata* than in the summer-breeding *A. woodiana* (Table 2).

Advantages of winter glochidia release

The glochidia of flat floaters are present on fish from mid-January until mid-April (Roberts 1997). *Anodonta couperiana*, which is closely related to *A. suborbiculata* (Hoeh 1990), also releases larvae in the winter (Heard 1975), as do many (but not all) other species in the genus *Anodonta* (Dartnall and Walkey 1979, Dudgeon and Morton 1983, Jansen 1991, Jokela et al. 1991). Although most unionids release glochidia primarily in the spring and summer, glochidia of lampsiline species are frequently present on hosts during the cold months as well (Neves and Widlak 1988, Weiss and Layzer 1993, Wat-

TABLE 2. Temperature effects on encystment period of glochidia. Figures from this study are mean ±1 SD. n = 20 fish at 15 and 21°C and 10 fish at 10°C. Other figures are from the literature cited. Q_{10} values were calculated according to equation 1 (see discussion). * = studies measured average encystment duration among glochidia; other studies reported only a range or a single value for transformation time.

Mussel species	Fish species	Temperature (°C)	Encystment period (d)	Q_{10}	Reference
Anodonta suborbiculata	*Notemigonus crysoleucas*	10	42.6 ± 3.63		This study*
				4.71	
		15	19.6 ± 1.12		
				4.44	
		21	9.3 ± 1.34		
Anodonta woodiana	*Cambusia affinis*	15	14.4		Dudgeon and Morton 1984*
				2.52	
		22	7.5		
				1.59	
		27	6.0		
				2.45	
		33	3.5		
Anodonta piscinalis	No reported	9	80		Harms 1907
				5.22	
		17	21		
				6.46	
		20	12		
Glebula rotundata	*Lepomis cyanellus* and *L. macrochirus*	22	16		Parker et al. 1984
				4.28	
		30	5		
Pleurobema oviforme	*Campostoma anomalum*	22	14.5		Weaver et al. 1991
				4.35	
		24.5	10.5		

TABLE 3. Temperature effect on in vitro transformation success and subsequent survival. Figures are mean ±1 SD. n = 8 plates with ~100 g glochidia each.

Temperature (°C)	Transformation success (%)	Average pH change (pH units)	24-h survival in water (%)
10	39.0 ± 11.03	0.59 ± 0.05	46.6 ± 23.18
15	89.1 ± 2.53	0.40 ± 0.36	72.8 ± 2.74
20	93.1 ± 5.97	0.46 ± 0.08	49.9 ± 14.07

ters and O'Dee, in press). Thus, it is possible that low water temperature may be relevant to the transformation success of other unionids in addition to *Anodonta*. This possibility should be taken into account when testing host relationships. Lower water temperatures might allow unionids to use some species of hosts that reject infections at higher temperatures.

High transformation success, perhaps because of immunosuppression of the host by low temperature, may be only one of several advantages to the winter release of glochidia (Roberts 1997, Watters and O'Dee 1998). Low temperature may also prolong the survival of glochidia after release from the female, thus increasing the odds of contacting fish. Prolonged residence on fish hosts may also be an advantage. Movement of *A. suborbiculata* among oxbow lakes on floodplains probably depends primarily upon the dispersal of fish hosts carrying glochidia. The increased length of time spent encysted on the host at winter temperatures, and the likelihood of early spring floods, probably enhance the chance of movement between these relatively ephemeral habitats. Another advantage of releasing glochidia during winter months may be a longer growing season in the 1st year because of transformation in very early spring.

In-vitro transformation

Transformation success was higher in cell culture media than on fish hosts at 15 and 20°C. However, transformation was slower at 10°C and transformation success was lower in vitro than on fish hosts. The prolonged incubation time at 10°C may have affected survival if 1 or more ingredients in the media did not maintain their integrity. In future studies, we suggest that the media should be changed at least once during the incubation period, to ensure stable conditions.

Up to ½ of the in vitro juveniles died within 24 h after transfer to water. These individuals may have died because development was not complete. Individual variability in the timing of drop-off from the fish host may reflect variation among glochidia in transformation time. How-

TABLE 4. Effects of pH, CO_2, and HEPES buffer on transformation success (mean ±1 SE). The pH change of the culture medium during incubation is also indicated. n = 5 plates per group and ~100 glochidia per plate. Effects of CO_2, pH, and HEPES were all statistically significant (binary logistic regression, $p < 0.01$).

		Without HEPES		With HEPES	
CO_2 (%)	Initial pH	pH change	Transformation success (%)	pH change	Transformation success (%)
5	7.6	0.03 ± 0.015	77.3 ± 3.26	0.14 ± 0.032	52.6 ± 7.44
5	7.9	0.37 ± 0.053	68.7 ± 7.54	0.19 ± 0.050	40.9 ± 1.41
5	8.2	0.64 ± 0.025	73.6 ± 5.19	0.38 ± 0.025	24.3 ± 6.47
2	7.6	0.04 ± 0.019	66.2 ± 5.02	0.22 ± 0.049	55.0 ± 3.76
2	7.9	0.17 ± 0.025	64.3 ± 3.58	0.47 ± 0.016	48.9 ± 4.99
2	8.2	0.36 ± 0.017	56.4 ± 5.50	0.66 ± 0.0260	45.2 ± 4.35
0	7.6	0.19 ± 0.342	67.5 ± 8.17	0.01 ± 0.029	0
0	7.9	−0.12 ± 0.355	45.1 ± 19.91	0.08 ± 0.122	0
0	8.2	−0.01 ± 0.204	37.4 ± 9.02	0.19 ± 0.106	0

ever, in vitro juveniles were all transferred to water at once because it was not practical to monitor glochidia individually. Individuals that were relatively advanced or delayed in development may have suffered increased mortality.

Transformation success was highest at the lowest pH tested (7.6) and the highest CO_2 (5%) (Table 4). These results are consistent with previous work (Isom and Hudson 1982). The results are puzzling, however, because the pH of fish body fluids in vivo is considerably higher (~8.0), and PCO_2 is much lower (Heisler 1984). The PCO_2 within the cysts surrounding the glochidium is not known and may be higher than that of the fish's body fluids, but it is unlikely to exceed 1%.

The use of non-bicarbonate buffers such as HEPES would simplify in vitro culture of mussels if it eliminated the need to regulate high CO_2 in the culture medium. However, mussels failed to transform in the HEPES medium in the absence of CO_2 (Table 4). Keller and Zam (1990) reported a similar result. In our study, HEPES did not improve stability of pH. Change in the pH of the media during incubation varied, but the magnitude of the change was not consistently less when HEPES was present. In any event, successful transformation was observed in cultures incubated without added CO_2, although transformation success was somewhat reduced (Table 4). The optimum pH for in vitro culture may be lower than 7.6. Isom and Hudson (1982) recommended pH = 7.2. High transformation success might be achieved without added CO_2 if the pH of the medium is sufficiently low. In any event, a CO_2 incubator is not necessary for in vitro transformation if the chambers described herein are used.

In summary, the transformation success of the glochidia of *A. suborbiculata* was higher at low temperatures on fish hosts, but not in vitro. We suggest that immunosuppression of the host by low temperature may explain these results. *Anodonta suborbiculata* and other unionids that parasitize their hosts at low temperature during the winter months might benefit from enhanced transformation success and also from enhanced opportunities for dispersal during the prolonged period of attachment. Low pH and high CO_2 enhanced transformation success of glochidia in vitro. A simple closed system is described for maintaining high CO_2 during in vitro culture.

Acknowledgements

We thank Frank Riusech, Karen Couch, Shannon Fennel, Ashley Farnsworth, Don George, Wyatt Hoback, Ed Miller, and Carl Wakefield for help with various portions of this study. Funding was provided by the Kansas Department of Wildlife and Parks, the Missouri Department of Conservation, and the US Fish and Wildlife Service.

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Received: 30 July 1998
Accepted: 28 September 1999