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[Article]

Safety of Fish Therapeutants to Glochidia of the Plain Pocketbook Mussel during Encystment on Largemouth Bass

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Abstract.—Mussel biologists and fisheries managers have developed propagation techniques to duplicate the natural glochidia infestation on host fish. However, in intensive culture situations, fish diseases may threaten the survival of both fish and their attached glochidia and chemical treatments may be required to control a disease epizootic. Five therapeutants were evaluated for their safety to largemouth bass *Micropterus salmoides* encysted with mussel glochidia by comparing the number of sloughed glochidia in the chemical treatment groups with that of an untreated control group. Largemouth bass were infested with glochidia from the plain pocketbook mussel *Lampsilis cardium* and treated with 20 mg chloramine-T/L, 2 mg Cutrine/L, or 200 mg formalin/L (trial 1) and 200 mg formalin/L, 100 mg hydrogen peroxide/L, or 20,000 mg sodium chloride/L (trial 2). Chemicals were applied for 60 min (15 min in the case of sodium chloride in trial 2) once every other day, for a total of three treatments (six in the case of formalin in trial 2). After the first treatment, aquaria were siphoned each weekday to determine the number of sloughed glochidia or transformed juveniles. In trial 1, the initial mean number of glochidia per fish ranged from 257 to 294, and approximately 94% of the glochidia transformed to juveniles. In trial 2, the initial mean number of glochidia per fish ranged from 97 to 115, and approximately 91% of the glochidia transformed to juveniles. The mean percent of sloughed glochidia varied by less than 2% among all test groups in each trial. There were no significant differences ($P < 0.05$) in the number of sloughed glochidia or transformed juveniles among control or treatment groups in either trial. Therapeutic treatment of diseased fish with chloramine-T, Cutrine, formalin, hydrogen peroxide, or sodium chloride at the treatment regimens evaluated are viable options for enhancing the survival of fish encysted with glochidia.

Freshwater mussel populations in the United States declined substantially in the 20th century, and many species are now classified as threatened or endangered (Williams et al. 1993). The decline in mussel diversity and numbers has been attributed to zebra mussels *Dreissena polymorpha*, decreases in suitable habitat, inadequate water quality, and human disturbances (Neves et al. 1997). Most freshwater mussel species require a fish host to complete their life cycle. Mussel larvae (glochidia) are released by the female mussel into the water column where they must come in contact with a suitable fish host to survive. Glochidia parasitize the fish host by attaching to the host's gills or fins and may remain on the host for up to 9 months (Steingraeber et al. 2005), depending on the mussel species and environmental conditions. The parasitic glochidial stage ends when they transform to free-living juveniles.

Mussel biologists have developed propagation techniques that simulate glochidial infestation on suspected fish hosts. However, cultured fish used as glochidial hosts are susceptible to pathogenic bacteria, fungus, or other opportunistic parasites. These intensively reared glochidia-infested fish may be at higher risk of disease than wild fish because of rearing at high densities that encourage disease epizootics. Chemo-

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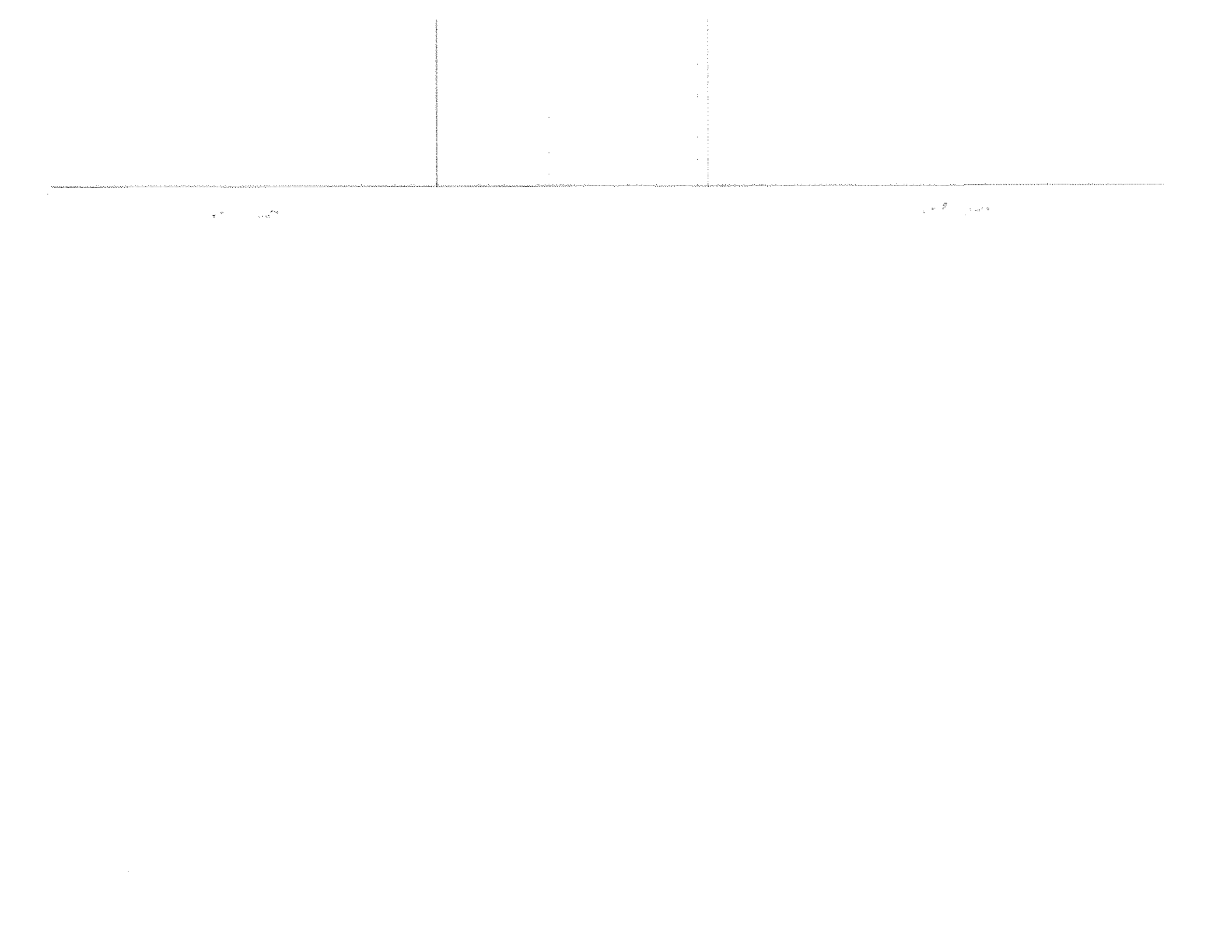

therapy may be required to control the causative fish pathogens and ensure fish and glochidia survival. However, mussel culturists are concerned that chemical treatment may be toxic to glochidia or may cause them to prematurely detach from the fish host.

Previous research has been conducted on the toxicity of many chemicals to glochidia not attached to fish, and the results have indicated various levels of toxicity for exposures of up to 72 h (Goudreau et al. 1993; Jacobson et al. 1997; Keller and Augspurger 2005; Milam et al. 2005; Valenti et al. 2005). Tests with free (unattached) glochidia have been limited to short exposures (from hours to days) because of reported declines in glochidia viability among species (Tedla and Fernando 1969; Huebner and Pynnonen 1992; Jacobson et al. 1997). Goudreau et al. (1993) ranked glochidia among the most sensitive of invertebrates with respect to chemical tolerance. However, little information exists on the safety of chemical therapeutants to encysted mussel glochidia on fish. It is therefore necessary to evaluate the safety of therapeutic fish chemicals to encysted glochidia to determine whether these chemicals can be used to treat diseased fish with attached glochidia. Mussel culturists require information to evaluate the use of therapeutants to improve fish health, without negatively affecting the attached glochidia. This study determined the safety of chloramine-T, Cutrine, formalin, hydrogen peroxide, and sodium chloride to glochidia of the plain pocketbook mussel *Lampsilis cardium* during encystment on largemouth bass *Micropterus salmoides*. A therapeutant was considered safe if statistical comparisons indicated no significant difference in the numbers of glochidia sloughed or juveniles produced between the untreated controls and chemically treated fish.

Methods

Study location and testing schedule.—This 2-year study, was conducted at the Upper Midwest Environmental Sciences Center (UMESC), La Crosse, Wisconsin, in 2004 and 2005. Chloramine-T, Cutrine, formalin, hydrogen peroxide, and sodium chloride were evaluated for safety to mussel glochidia during encystment on largemouth bass in trial 1 (15 March–9 April 2004) and trial 2 (25 January–19 February 2005). Each trial was terminated when 20 or fewer transformed juveniles were recovered from each aquarium for two consecutive days. On the last day of each trial, fish were euthanized with tricaine methanesulfonate (Finquel), weighed, and measured. Two fish (of 10 fish per aquarium) from each aquarium were selected without conscious bias for microscopic examination of the gills for the presence of glochidia or juveniles.

Test article.—Chloramine-T, Cutrine (chelated copper; elemental copper, 9%), formalin, hydrogen peroxide, or sodium chloride was tested in these trials. Each chemical was purchased from a commercial vendor commonly used by fish culturists. Chloramine-T (Halamid) is produced by Axcentive (Barneveld, The Netherlands), Cutrine by Applied Biochemist (Germantown, Wisconsin), formalin (Paracide-F) by Argent Chemical Laboratories (Redmond, Washington), and hydrogen peroxide (Perox-aid, 35% active ingredient) by Eka Chemicals, Inc. (Marietta, Georgia). Food-grade sodium chloride that contained no iodine was obtained from Cargill (La Crosse, Wisconsin).

Hydrogen peroxide treatment concentrations were determined by a titrimetric method (Jeffery et al. 1989) to validate the accuracy of the chemical applications. Fifteen minutes before termination of the hydrogen peroxide treatment, we removed one water sample from each aquarium; one randomly selected aquarium was sampled in triplicate.

Test organism.—Genoa National Fish Hatchery (GNFH; Genoa, Wisconsin) personnel provided largemouth bass and gravid plain pocketbook mussels for this study. Largemouth bass lengths in trial 1 ranged from 10 to 17 cm (mean, 11.6 cm), and weights ranged from 10 to 58 g (mean, 17.7 g). Largemouth bass lengths in trial 2 ranged from 9 to 17 cm (mean, 13.4 cm), and weights ranged from 8 to 49 g (mean, 27.1 g). Fish and adult mussels were maintained in UMESC well water. Largemouth bass were acclimated to 20 ± 2°C well water for 1 month before testing and fed to satiation with live rainbow trout *Oncorhynchus mykiss* fingerlings. Feeding ceased 5 d after fish were infested with glochidia to reduce fecal material and other debris in the aquariums and to improve conditions for glochidia and juvenile observations (GNFH standard operating procedures for glochidia evaluations; Tony Brady, U.S. Fish and Wildlife Service, personal communication). The first chemical treatment was applied 7 d after infestation. Before the first treatment, all aquaria were siphoned, and sloughed (dead) glochidia were discarded. Treatment was delayed to allow the removal of naturally sloughed glochidia that did not successfully encyst on the fish. Glochidia counts were initiated 24 h after the first treatment.

Glochidial infestation of fish.—One (trial 2) or two (trial 1) gravid plain pocketbook mussels were used to supply glochidia in each trial. Using a 50-mL syringe equipped with a needle, we flushed well water directly into the water tubes of the marsupial pouch to expel the glochidia used to infest fish into a glass vessel containing 500 mL of water. Glochidia flushes were pooled to provide a common source of glochidia from which we removed similar aliquots for each infestation

event. The viability and reactivity of the glochidia were checked under microscopic examination by viewing the responses of the glochidia to the addition of sodium chloride to the water. Viable glochidia responded by closing their valves.

Largemouth bass were infested with glochidia in a 4-L pail containing 1 L of aerated well water. Groups of 12 fish (the number of fish equal to the number of test aquariums) were placed in the pail and then 2 mL (trial 1) or 3 mL (trial 2) of the glochidia slurry was added to the water in the pail (i.e., similar densities of glochidia in each exposure). Fish were exposed to the glochidia for approximately 5 min. Upon infestation, one fish from a group was immediately placed in an aquarium containing 16 L of water. Nine additional groups of 12 fish each were similarly exposed to provide 10 fish in each aquarium.

Chemical exposures.—The test system consisted of 12 individually plumbed aquaria (40 × 15 × 10 cm; 16 L volume) covered with Plexiglas lids. Well water was delivered by gravity flow to each aquarium at a rate of 300 ± 30 mL/min from a 100-L headbox. Water temperature was maintained at 20 ± 2°C, and water in each aquarium was aerated. The order in which fish were transferred to each test tank was randomly assigned according to a random numbers table. Assignment of treatments was likewise randomized to each aquarium. Each chemical treatment regimen was tested in triplicate ($n = 30$ fish). Fish were exposed to 20 mg chloramine-T/L, 2 mg Cutrine/L, or 200 mg formalin/L in trial 1 and to 200 mg formalin/L, 100 mg hydrogen peroxide/L, or 20,000 mg sodium chloride/L in trial 2. Each chemical was applied at a specific target concentration for 60 min (except for a 15-min exposure to sodium chloride in trial 2) once every other day for a total of three applications; in trial 2, formalin was applied once every other day for a total of six applications. The second formalin treatment regimen was added to evaluate three additional treatments to later developmental stages of glochidia. Chemical exposures were administered under static test conditions by turning the water flow off and adding to the holding water a calculated amount of chemical to achieve the target chemical concentration. The positive control aquaria received an aliquot of well water. The water level in each aquarium (including the control aquaria) was drawn down to approximately 30% of the original volume after treatment to eliminate most of the chemical that had been applied. The water flow was restored immediately after the drawdown at a rate of approximately 600 mL/min for at least 30 min to flush the remaining chemical from each aquarium. Flow to each aquarium was reset to 300 ± 30 mL/min after the flushing period.

Sloughed glochidia or transformed juveniles.—Glochidia prematurely shed from the gills or fins of a fish before transformation to the self-sustaining juvenile life stage were termed "sloughed glochidia." The day after the first chemical treatment, water from the bottom of each aquarium was siphoned through a 153-μm sieve to collect sloughed glochidia and newly transformed juveniles. The sieve was washed with well water to rinse the organisms into a petri dish divided into 12 equal-size sections. The glochidia or juveniles collected from each aquarium were counted and examined for viability by using a dissection microscope with cross-polarized illumination. Viable juvenile mussels were characterized by the presence of two adductor muscles, closed valves, and foot movement. Mussel counts continued on weekdays until trial termination.

Gill observations.—Two fish from each aquarium were examined for the presence or absence of glochidia or juveniles at study termination of each trial. Fish were euthanized with Finquel, and gill filament samples were taken from the first and second gill arches on the right side of each fish. The gill tissue was prepared as a wet mount (Lasee 1995) and microscopically examined for glochidia.

Blinding procedures.—Blinding procedures were followed for each trial to eliminate bias during data collection. For example, test concentrations of individual tanks were not known by the technician recording daily mussel counts but were known to the study director who administered the treatments. Other study procedures that were blinded included fish placement, test chemical concentration verification, gill observations, and water sampling.

Physical parameters.—Water quality characteristics, including temperature, pH, and dissolved oxygen, were measured daily and recorded during the study. Temperature, dissolved oxygen, and pH were measured in each aquarium on nontreatment days. During chemical treatments, the temperature, dissolved oxygen, and pH were measured approximately 45 min into the 60-min exposure (or 10 min into a 15-min treatment). Alkalinity and hardness of the test water were analyzed on the day of glochidia infestation, the first day of chemical treatment, and at trial termination.

Test water was maintained at a mean ± SD temperature of 20.0 ± 0.3°C during all trials. Mean ± SD total hardness and alkalinity (as $CaCO_3$) of the test water was 166 ± 6.3 and 126 ± 6.4 mg/L in trial 1 and 167 ± 7.2 and 117 ± 10.3 mg/L in trial 2, respectively. The pH of the test water was 7.90 ± 0.12 and 7.92 ± 0.16 during trials 1 and 2, respectively. Dissolved oxygen was 8.4 ± 0.35 and 8.2 ± 0.35 mg/L in trials 1 and 2, respectively.

Statistical analysis.—Individual glochidia experienced one of two outcomes: transformation to a juvenile or death (sloughed glochidia). Comparison of sloughed glochidia (or transformed juveniles) is therefore a binary random variable and is assumed to follow a binomial distribution. The binomial response data from the efficacy trials were the number of sloughed glochidia and the total number of glochidia initially present on the 10 exposed fish in each aquarium. The individual tank was the experimental unit in this study. Fish and attached glochidia held in a given aquarium experienced similar environmental conditions; however, those conditions may have varied randomly among aquaria. The study design for trials 1 and 2 consisted of four levels of the primary predictor (trial 1 = untreated control, chloramine-T, Cutrine, and formalin; trial 2 = untreated control, formalin, hydrogen peroxide, or sodium chloride) applied to each of three independently observed replicate tanks for a total of 12 experimental units. Glochidia survival to juvenile transformation was analyzed by logistic regression in a General Linear Mixed model (e.g., SAS PROC GLIMMIX; Wolfinger and O'Connell 1993) with treatment types as the predicator variable. Each trial was analyzed separately. The degrees of freedom were automatically adjusted by the Kenward–Roger (kr) option, making it unnecessary to drop the random term and refit the statistical model. Least squares means were determined, and results for each active treatment were compared with those for the untreated control. Treatment levels were judged statistically different if $P \leq 0.05$.

Results

The mean number ± SD of glochidia on an individual fish at the initiation of trial 1 was 294 ± 29 (control), 257 ± 29 (Cutrine), 285 ± 40 (chloramine-T), and 262 ± 56 (formalin). The largest numbers of glochidia were sloughed on day 2, then continually declined for the remainder of the study (Figure 1). The mean number of glochidia sloughed per aquarium ranged from 18 to 64 (1.8–6.4 glochidia/fish) on day 2; by day 5, approximately 75% of the glochidia were sloughed. The mean number (±SD) of sloughed glochidia per fish was 16.6 ± 2.0 (control), 17.2 ± 5.2 (Cutrine), 17.6 ± 3.4 (chloramine-T), and 16.7 ± 1.0 (formalin). There was no significant difference ($P < 0.05$) in the number of sloughed glochidia between control fish and chemically treated groups of fish, and the mean percent of sloughed glochidia varied by 1% (5.6–6.7%) among all test groups.

The mean number of juveniles ± SD produced per fish during trial 1 was 277 ± 29 (control), 238 ± 23 (Cutrine), 266 ± 38 (chloramine-T), and 243 ± 55 (formalin). More than 90% of the juveniles transformed between day 15 and day 22 (Figure 2), and the maximum number of juveniles recovered per aquarium occurred on days 17 and 18 (approximately 500–650 juveniles/aquarium or 50–65 juveniles/fish). The mean number of glochidia that transformed to juveniles ranged between 93.3% and 94.4%, and there was no significant difference ($P < 0.05$) in the number of juveniles produced by control fish versus chemically treated fish. Fewer than 1% of the juveniles were moribund at transformation. Gills from 20% of the test fish were microscopically examined for mussel presence, and no glochidia or juveniles were observed.

The mean number ± SD of glochidia on individual

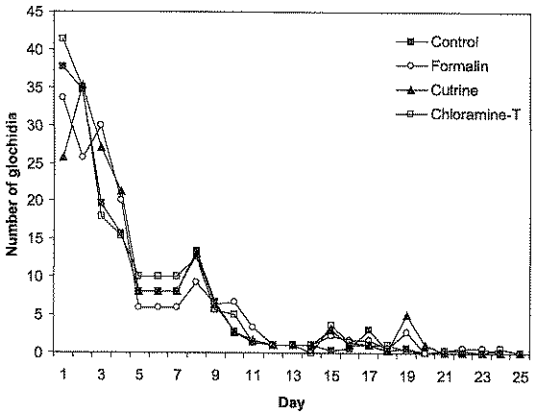

FIGURE 1.—Daily mean number of glochidia sloughed from largemouth bass treated with 20 mg chloramine-T/L, 2 mg Cutrine/L, or 200 mg formalin/L (trial 1) for 60 min once every other day, for a total of three treatments.

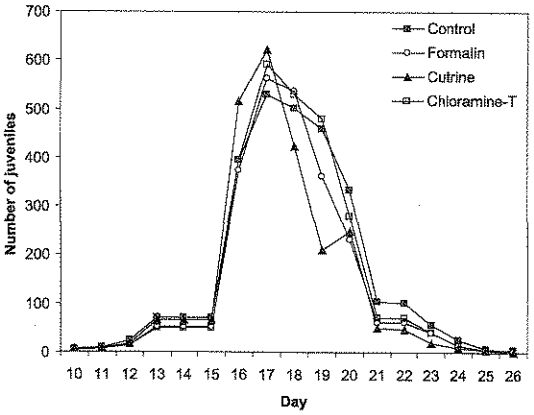

FIGURE 2.—Daily mean number of *L. cardium* juveniles produced from glochidia-infested largemouth bass treated with 20 mg chloramine-T/L, 2 mg Cutrine/L, or 200 mg formalin/L (trial 1) for 60 min once every other day, for a total of three treatments.

fish at the initiation of trial 2 was 97 ± 16 (control), 109 ± 10 (formalin), 107 ± 5.6 (hydrogen peroxide), and 115 ± 13 (sodium chloride). The greatest number of glochidia was sloughed on day 2 and declined for the remainder of the trial (Figure 3). The mean number of glochidia sloughed per aquarium ranged from 17 to 32 (1.7–3.2 glochidia/fish) on day 2, and approximately 70% of the glochidia were sloughed by day 5. The mean number ± SD of sloughed glochidia per fish was 9.9 ± 3.1 (control), 9.1 ± 1.3 (formalin), 8.9 ± 1.8 (hydrogen peroxide), and 10.0 ± 1.3 (sodium chloride). There was no significant difference ($P < 0.05$) in the number of sloughed glochidia between control fish and chemically treated groups of test fish, and the mean percent of sloughed glochidia varied by less than 2% (8.3–10.2%; control 10.2%) among all test groups.

The mean number ± SD of juveniles produced per fish in trial 2 was 87 ± 14 (control), 98 ± 10 (formalin), 97 ± 6 (hydrogen peroxide), and 103 ± 14 (sodium chloride). More than 90% of the juveniles transformed between day 15 and day 28 (Figure 4), and the maximum number of juveniles were recovered on days 18–20 (approximately 94–164 juveniles/aquarium each day; 9.4–16.4 juveniles/fish). The mean percentage of glochidia that transformed to juveniles ranged from 89.8% to 91.7% (control 89.8%), and there was no significant difference ($P < 0.05$) in the number of juveniles produced by the control fish and chemically treated fish. Fewer than 2% of the juveniles were moribund at transformation (chemical treatment groups had a 1% greater mortality than the controls). Gills from 20% of the test fish were microscopically examined for mussel presence, and no glochidia or juveniles were observed.

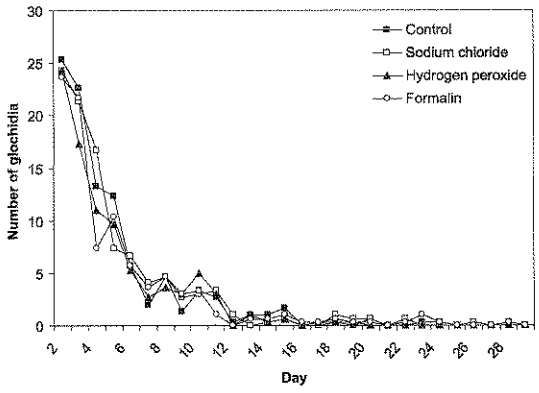

FIGURE 3.—Daily mean number of glochidia sloughed from largemouth bass treated with 200 mg formalin/L, 100 mg hydrogen peroxide/L, or 20.0 g sodium chloride/L (trial 2) for 60 min once every other day, for a total of three treatments (sodium chloride was administered for 15 min, and formalin was applied on six occasions).

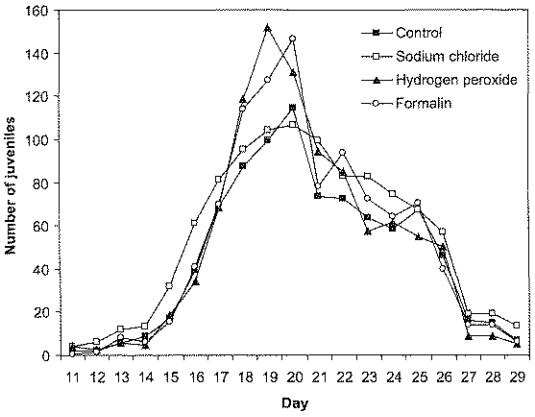

FIGURE 4.—Daily mean number of *L. cardium* juveniles produced from glochidia-infested largemouth bass treated with 200 mg formalin/L, 100 mg hydrogen peroxide/L, or 20.0 g sodium chloride/L (trial 2) for 60 min once every other day, for a total of three treatments (sodium chloride was administered for 15 min, and formalin was applied on six occasions).

Discussion

Induced infestation of fish with mussel glochidia is a procedure with multiple variables that can affect the success of juvenile mussel propagation and the ability to conduct valid, reproducible research. Variables that can directly affect success of glochidial infestations on fish include size of fish (gill or fin surface area), mussel fecundity, fish susceptibility to infestation, and maturity of glochidia to infest fish (Rogers and Dimock 2003). These variables may be influenced by physical factors, especially water temperature. The infestation of all test fish from commonly pooled glochidia on the same date, and the randomization of fish placement (10 separate infestations to achieve the desired number of fish) in each aquarium, appeared to reduce the effect of variable fish sizes, individual fish susceptibility to infestation, and maturity of glochidia at release from marsupial gill pouch. The effect of variables encountered in this study was further reduced by selecting individual aquariums (not individual fish) as the test unit. Even though this study was conducted with fish of various sizes (weight ranged between 8 and 58 g) and different mussel infestation dates (infested on 15 March 2004 and 25 January 2005), the infestation and chemical treatment procedures of this study provided statistically valid data for evaluating the safety of chemical therapeutants to mussel glochidia infested on largemouth bass.

Trials 1 and 2 were conducted during different months of the year and in different years (2004 and 2005); this probably resulted in glochidia of differing maturity upon release from the females. Also, different female mussels provided pooled glochidia for infestation (trial 1). These factors could have explained the variation in the number of glochidia on each fish at a trial initiation; therefore, we attempted no direct comparison between the two trials. However, the mean percent of glochidia sloughed and juveniles transformed were similar between trials (within approximately 3%), and the number of glochidia sloughed and juveniles transformed followed a similar trend over time (Figures 1–4). The initial number of glochidia on a fish and the number of glochidia sloughed were higher for trial 1; however, the overall trend of glochidia sloughed in trials 1 and 2 (Figures 1 and 3) was similar. The majority (70%) of glochidia were sloughed within the first 5 d of the trial; from day 12 through the conclusion of each trial, the number of sloughed glochidia was minimal. The date of peak juvenile transformation (day 17, trial 1; day 18, trial 2) was similar in both trials (Figures 2 and 4). In trial 1, the majority (90%) of juveniles transformed within a week, whereas in trial 2, juvenile transformation was slower and continued over a 2-week period. The extended period of juvenile transformation may be attributed to the parent mussel used in trial 2. The adult mussel may have had the glochidia in the marsupial pouch for a shorter period (in trial 1, glochidia were removed on 15 March 2004; in trial 2, glochidia were removed on 19 February 2005), and the glochidia may not have been as mature as those used in trial 1. This explanation seems plausible, as Khym and Layzer (2000) reported that the amount of time black sandshell *Ligumia recta* glochidia were in the marsupial gills was more important than temperature in regulating the length of time to metamorphosis on sauger *Sander canadensis*.

There were no significant differences in the number of sloughed glochidia or transformed juveniles between the control and treatment groups in either trial. Further, the mortality of juvenile mussels at transformation was less than 2% for each group tested. In each of the trials, the formalin and control groups exhibited no significant increase in the number of sloughed glochidia (three applications: 16.6 control versus 16.7 formalin; six applications: 9.9 control versus 9.1 formalin). Extended formalin applications (5 d versus 11 d) did not appear to increase toxicity to the glochidia or affect juvenile transformation. There was no evidence of toxicity from chloramine-T, Cutrine, formalin, hydrogen peroxide, or sodium chloride to glochidia, when the chemicals were applied at commonly used therapeutic treatment levels. The use of these therapeutants to control disease epizootics on fish infested with glochidia appears to be a viable tool for intensive mussel culture.

Researchers have reported that ammonia, copper, mercury, fluoride, and numerous other compounds (exposures up to 72 h) are highly toxic to unattached glochidia during acute tests (Goudreau et al. 1993; Jacobson et al. 1997; Keller and Augspurger 2005; Milam et al. 2005; Valenti et al. 2005). In this study, glochidia encysted on largemouth bass were exposed to each chemical for 60 min (15 min for sodium chloride; trial 2), once every other day on three occasions (six occasions for formalin; trial 2), after which no significant mussel toxicity was detected. Jacobson et al. (1997) reported similar findings in *Actinonaias pectorosa*, *Villosa iris*, and *Pyganodon grandis* exposed to aqueous copper (up to 200 μg) at 19–21°C for 12–20 d. Unattached glochidia seem to be the life stage most sensitive (Goudreau et al. 1993) to chemical toxicity because they experience direct chemical exposure through a thin permeable shell enclosing undifferentiated tissue (rudimentary organs). However, attached glochidia can be rapidly encapsulated (depending on water temperature) within 2–6 h (Arey 1932; Waller and Mitchell 1989; Jacobson et al. 1997) within layers of fish tissue (cyst formation) that isolates the permeable-shelled glochidium from direct exposure to aquatic chemicals (Jacobson et al. 1997). Also, the encysted glochidia are not filter feeders (as are all other mussel life stages), receiving their nutrients directly from the fish host. This direct uptake of nutrients by the glochidia reduces or eliminates their susceptibility or exposure to water-borne chemicals, unless the chemicals are readily absorbed into the bloodstream or tissue of a fish host.

The results from this study indicate encysted glochidia will not be harmed from the chloramine-T, Cutrine, formalin, hydrogen peroxide, or sodium chloride applied in typical hatchery treatment regimens. This conclusion is further supported from longer (12–20 d) continuous exposures of aqueous copper to encysted glochidia reported by Jacobson et al. (1997). The chemical treatment of glochidia-infested fish may be used as a tool to improve fish host survival and enhance mussel propagation efforts.

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