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How to pick your mussels out of a crowd: using fluorescence to mark juvenile freshwater mussels

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Abstract. Conservation plans for freshwater mussels include enhancing natural populations with artificially cultured individuals. Chemical marks on cultured juveniles would provide a way to evaluate the success of those efforts. We immersed *Lampsilis cardium* and *Actinonaias pectorosa* juveniles of various ages (1-d–210-d-old) in either oxytetracycline (OTC) or calcein (125–500 mg/L) for different lengths of time (3–48 h) and assessed growth, survival, and mark retention. There were no immediate lethal effects on 1-d-old *L. cardium* after a 24-h immersion in either chemical at a concentration of 500 mg/L. A single immersion in 250 mg/L OTC of 1-mo-old *L. cardium* inhibited growth for 2 mo post-immersion, and multiple immersions also affected survival. Neither growth nor survival were affected when older mussels (≥2-mo old) were immersed. Fluorescent marks were laid down primarily in the growing edge of the shell as a ring extending around the mussel, and marking success increased with concentration and immersion time. Immersing 7-mo-old *L. cardium* in 250 mg/L OTC for 24 h produced marks visible in all thin-sections examined 6 mo later; however, marks were not visible in whole shells. In contrast, distinctive calcein marks were visible not only in thin-sections but also on all whole 7-mo-old *L. cardium* shells 6 mo after mussels were immersed for 24 h at a concentration of 250 mg/L. A 12-h immersion at the same concentration produced inconsistent calcein marks in 5-mo-old *L. cardium* but yielded >90% correct mark identification in live 7-mo-old *A. pectorosa* 1 y after immersion. The results of our study indicate that persistent and readily identifiable chemical marks can be produced in juvenile mussels of some species ≥2-mo-old by a 24-h immersion in 250 mg/L calcein or OTC with little to no adverse effects on the mussels.

Key words: chemical marking, Unionidae, supplemental stocking, calcein, OTC, culture.

Globally, freshwater mussel populations (Unionoidea) are disappearing at an alarming rate. Drastic declines in mussel populations have received much recent attention (Bogan 1993, Williams et al. 1993, Cosgrove et al. 2000), so these declines are often thought to be a recent phenomenon; however, the decline in the eastern United States probably started with mass deforestation (Hughes and Parmalee 1999) and overharvest (Anthony and Downing 2001) in the 1800s. The stocking of artificially propagated and cultured juvenile mussels to augment existing populations is a high priority of many recovery plans established for endangered species (e.g., USFWS 1991). The national strategy for the conservation of native freshwater mussels recognizes the need to enhance existing populations through stocking, and the need to evaluate the success of those stockings (NNMCC 1998). Juveniles stocked to enhance existing populations must be recognizable at a later date to evaluate their long-term survival and growth.

Producing an identifiable mark to distinguish artificially produced mussels from wild individuals of the same species would be beneficial in assessing stocking success. Although small plastic tags (Lemarie et al. 2000), wire microtags (Heinricher 1996), and etching of shells (Layzer and Gordon 1993) have been used to successfully mark mussels, these methods are labor intensive and each mussel must be marked individually. Also, the small size (<350 μm) of newly metamorphosed juveniles precludes the use of physical tags. Tags cannot be used until the juveniles are several mm long, which may take from several mo to 2 y, depending on growth rates. If chemical marks could be produced in mussels, batch marking of a large number of small individuals could be accomplished with minimal effort.

Fluorescent chemicals (fluorochromes) are often used to mark aquatic organisms. Oxytetracycline (OTC) and calcein have been used for

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many years in marking otoliths of juvenile fish (Choate 1964, Wilson et al. 1987, Brooks et al. 1994). These techniques have also been used with marine molluscs. Nakahara (1961) used injections of tetracycline to determine growth rates of pearls inside oysters. Tetracycline has also been incorporated into the shells of abalone (*Haliotis iris*) following both injection and immersion (Pirker and Schiel 1993). Calcein has been used successfully to create a growth marker in shells of brown mussels (*Perna perna*) (Kaehler and McQuaid 1999). We wanted to determine the feasibility of using OTC and calcein to mark juvenile freshwater mussels. We also evaluated potential adverse effects of these chemicals on survival and growth of mussels. Our final objective was to determine a concentration and immersion time of OTC and calcein that would minimize toxicity and provide a distinctive and long-lasting mark on juvenile mussels.

Methods

Propagation and culture of mussels

Lampsilis cardium and *Actinonaias pectorosa* were artificially propagated in the laboratory. Glochidia were extracted from a gravid adult by pricking the water tubes with a syringe and flushing them with water (Waller and Holland-Bartels 1988). Host fish (largemouth bass, *Micropterus salmoides*, and spotted bass, *M. punctulatus*) were anesthetized using MS-222 and glochidia were directly pipetted onto their gills. These fish were maintained in aquaria until excystment of the juvenile mussels. Each aquarium was siphoned routinely through a 105-μm sieve to collect newly metamorphosed juveniles.

An indoor recirculating system, composed of cascading series of vinyl troughs 2.7-m long by 10-cm wide, was used to culture juvenile mussels. Water was pumped from a 38-L reservoir to the top level of each series and returned by gravity flow through the troughs at a rate of 60 mL/s. Each of the 4 levels and the reservoirs were illuminated by two 40-watt, cool white, fluorescent lights set on 13:11 h light:dark cycle. City of Cookeville, Tennessee, municipal water (hardness ~70 mg/L as $CaCO_3$) filtered through a C canister filter was used for culture. Water temperature was regulated only by room temperature (15–25°C). Approximately 50% of the water in each series was changed each week, and excess algal growth in troughs was mechanically removed as needed. An algal culture (*Bracteococcus grandis*) was maintained in 96-L clear plastic tubes and fed (500–1500 mL) to each series as a food source 3 times per week. *Ankistrodesmus* sp. contaminated the culture at an unknown point during the study, and was the species used for the remainder of the experiments.

A warmwater raceway at Minor E. Clark Fish Hatchery near Morehead, Kentucky, was used for grow-out of mussels. Culture baskets ranging in mesh size from 250 μm to 4.8 mm were placed in the floating cages to hold the juvenile mussels. The baskets extended above the water surface to prevent juveniles from escaping. As mussels grew, they were moved to baskets with a larger mesh size. The mesh was cleaned periodically with a plastic brush and wash bottle to maintain water flow through the basket.

Mussel marking

Nine tests were conducted to address the study objectives. In each test, stock solutions of the fluorochromes were prepared at the highest concentration used and buffered to pH 7–8 using sodium phosphate dibasic (Na_2HPO_4). The stock solution was then diluted to achieve the desired concentrations, which covered a range previously used with fish and marine molluscs (see below). All immersions were either conducted in a dark room or covered to prevent potential breakdown of the fluorochromes by light. In tests where control groups were used, control treatments were identical to marking treatments other than a lack of exposure to a fluorochrome. Mean lengths of mussels were determined before and after each test in which growth was monitored. Before each test, all mussels being used were pooled in a single container, mixed thoroughly, and length was measured on the first 30 randomly selected individuals. After each test, all mussels from each replicate were put in a petri dish and stirred, and the first 20 individuals encountered under a dissecting microscope were measured. All juveniles or subsamples of 40 per treatment were measured at the end of nonreplicated tests, depending on the number of mussels used in the test. Juveniles <5 mm in length were measured with an ocular micrometer under a dissecting

microscope, and dial calipers were used for larger individuals. Analyses of variance (ANOVA, single and multifactor) were used to test for differences in growth and survival among treatments. A Tukey's Studentized Range Test was used for pairwise comparisons.

Juveniles cultured indoors were separated from sediment using a modified McDonald egg-hatching jar (Brady 2000). Mussels <2 mm at immersion were pipetted into a 105-μm-mesh basket and immersed; larger mussels were placed by hand into the chemical. Mussels were rinsed and transferred to fresh water at the end of the specified immersion time. The number of treatments and replicates, as well as sample sizes, varied among experiments because of the availability of juveniles of the same age (see below). Marks were viewed using a Nikon Labophot 2 microscope with a 420–490-nm excitation filter and a 520-nm barrier filter.

1-d-old L. cardium

A 3-factor design was used in Test I to determine if juvenile mussels would incorporate OTC and calcein into their shell and to address acute toxicity of those chemicals at various concentrations and immersion times. A secondary objective was to determine if the presence of sediment in immersions affected toxicity or marking success. Test I was designed primarily as a screening mechanism because there was limited information in the literature to provide guidance on suitable concentrations and immersion times to use for freshwater bivalves. We used 4 concentrations (0, 125, 250, and 500 mg/L) of each chemical and 3 immersion times (6, 12, and 24 h). Each combination of chemical, concentration, and immersion time had 2 replicates without sediment and 2 with sediment. The same control group was used for both OTC and calcein comparisons. One-d-old *L. cardium* were collected from aquaria, and 25 individuals pipetted into each of 84 mesh baskets. One-half of the baskets contained a 3 to 5 mm layer of coarse sand (0.5–1 mm); the remaining baskets contained no sediment. Baskets were then randomly assigned a treatment, and all immersions began at the same time. Survival was assessed after the 24-h immersion by rinsing the juveniles into a petri dish and examining them under a dissecting microscope. The first 10 individuals recovered from each treatment and each control group were placed on a microscope slide and examined using fluorescence microscopy.

In Test II, a total of 1080 1-d-old *L. cardium* were immersed in either 125 or 500 mg/L calcein or 250 mg/L OTC for 24 h to evaluate mark retention. Only 1 concentration of OTC could be tested because a limited number of juveniles were available. After immersion, juveniles from each treatment were divided into 5 groups ranging from 30 to 90 mussels each; more individuals were assigned to those groups assessed later in the test to increase the likelihood of some individuals surviving to the end of the test. These groups were transferred to 250-μm-mesh culture baskets, which were placed in the indoor culture system. Each basket contained a 5 to 8 mm layer of very coarse sand (1–2 mm) and fine gravel (2–4 mm). One basket from each treatment was removed at 10, 20, 35, 51, and 79 d post-marking to assess survival and mark retention. Two control groups of 90 individuals each were assessed at 35 and 79 d, respectively. Ten individuals were examined for marks using fluorescence microscopy, and the remaining individuals were placed back into the culture system.

1-mo-old L. cardium

One-mo-old *L. cardium* were used to assess growth, survival, and mark retention following single and multiple immersions. A single concentration (250 mg/L) and immersion time (24 h) were used, and mussels were assigned to 1 of 5 treatments: 1) control (no immersion), 2) OTC (1 immersion), 3) OTC (4 immersions), 4) calcein (1 immersion), and 5) calcein (4 immersions). Five replicates were used for each treatment. Twenty-five groups ranging from 40 to 100 individuals were randomly assigned to 1 of the 5 treatments and placed in 105-μm mesh baskets. All groups were then immersed at the same time in their respective treatments. At the end of the 1st immersion, baskets from all treatments were rinsed, and each group was then transferred to a 250-μm mesh culture basket containing a 5 to 8 mm layer of fine gravel. All baskets were placed in the indoor culture system and randomly assigned a position in the trough. For the next 3 wk, the groups to be immersed 4 times were immersed at 1-wk intervals in their respective culture baskets and remained in the culture system between immer-

sions. Growth and survival were evaluated at monthly intervals thereafter for 4 mo, and empty shells were examined for marks. Immediately following the 4th monthly check, all juveniles were taken to the hatchery and placed in 500-μm-mesh baskets in a raceway. At the end of the growing season (7 mo post-immersion), 5 individuals from each treatment were sacrificed to evaluate mark retention. These shells were embedded in an epoxy mold, and 2 thin-sections (250 μm) were cut through the umbo of each shell using a low-speed precision saw. Sections were viewed using fluorescence microscopy.

2-mo-old L. cardium

Growth, survival, and mark retention with a lower exposure to calcein, were tested with 1 concentration (125 mg/L) and 2 immersion times (3 or 8 h). Six groups of 16 2-mo-old *L. cardium* each were randomly assigned to 1 of the 2 calcein treatments or a control group. After immersion, mussels were transferred to 250-μm-mesh culture baskets containing a 5 to 8 mm layer of very coarse sand. The baskets were placed in a trough of the indoor culture system and rinsed as needed to clean the mesh. Baskets were removed at 25 and 105 d post-marking, and surviving juveniles were counted and measured; empty shells were collected and examined for marks.

An identical design was used to test extended immersion times (24 and 48 h) at a higher concentration (250 mg/L) of calcein using 2-mo-old *L. cardium*. Twelve groups of 30 individuals were randomly assigned to 1 of the 2 treatments or a control group, and all immersions began simultaneously. Those groups immersed for 24 h were rinsed and placed in fresh water with the control for the remaining 24 h. At the end of the 48-h immersion, all groups were placed in 250-μm mesh culture baskets containing a 5 to 8 mm layer of fine gravel, and then cultured. Baskets were washed as needed with a wash bottle to remove algae from the mesh. Growth, survival, and mark retention were evaluated 30 and 90 d post-immersion.

≥*4-mo-old* L. cardium

Long-term mark retention was tested using ≥4-mo old *L. cardium*. In June 1999, 101 individuals (4-mo-old, mean length = 2.9 ± 0.2 mm) were immersed in either 250 mg/L OTC or calcein for 6 h. After immersion, mussels from each treatment were transferred to a 1-mm-mesh basket (22 × 22 cm) and placed in a floating cage in the raceway. In July 1999, 54 *L. cardium* (5-mo-old, mean length = 5.3 ± 0.3 mm) were immersed in either 250 mg/L of OTC or calcein for 12 h. After immersion, mussels from each treatment were transferred to a 1-mm-mesh basket (15 × 22 cm) and placed in a floating cage in the raceway. The sides of the basket protruded above the water surface to prevent juveniles from escaping. In September 1999, 38 *L. cardium* (7-mo-old, mean length = 13.0 ± 0.3 mm) were immersed in either 250 mg/L of OTC or calcein for 24 h. Mussels from each treatment were then transferred to a 4.8-mm-mesh basket (25 × 25 cm) and placed in a floating cage in the raceway. In November 1999, surviving *L. cardium* marked ≥4-mo old were counted and measured, and 5 individuals were sacrificed, thin-sectioned (as above), and examined for a mark. The 5 individuals were selected by blindly reaching into the mesh baskets and picking the first mussels touched. In March 2000, mussels immersed for 12 and 24 h (5- and 7-mo old at immersion, respectively) were counted, measured, sacrificed, and viewed whole using fluorescence microscopy (8 and 6 mo post-immersion, respectively). These shells were then stored in darkness, and shells of mussels immersed for 24 h were examined in September 2000 (12 mo post-immersion, 6 mo post-sacrifice) again using a blind-reading technique. Shells were randomly selected by coin toss from either the OTC or calcein treatment. The shell was then handed to the reader, who was unaware of the shell's treatment. The reader examined the shell using fluorescence microscopy and decided if the mussel had been immersed in OTC or calcein. Because OTC marks were not visible using this method, those shells served as a control with no mark.

7-mo-old A. pectorosa

Actinonaias pectorosa were used to test OTC and calcein immersion on a 2nd species. Two-hundred and twenty-four *A. pectorosa* (mean length = 7.0 ± 0.2 mm) were immersed in either 250 mg/L OTC or calcein for 12 h in September 1999. After immersion, mussels were trans-

TABLE 1. Survival and growth of 1-mo-old *Lampsilis cardium* immersed in 250 mg/L oxytetracycline either 1 [OTC (1×)] or 4 [OTC (4×)] times. Mean length was 635 μm (SE = 16 μm) at the time of initial immersion. * = significantly different from controls ($p < 0.05$).

	Mean overall survival ± SE (%)			Between-month survival ± SE (%)		
Month	Control ($n = 5$)	OTC (1×) ($n = 5$)	OTC (4×) ($n = 5$)	Control ($n = 5$)	OTC (1×) ($n = 5$)	OTC (4×) ($n = 5$)
1	69.1 ± 6.9	45.0 ± 4.5	43.8 ± 6.4*	69.1 ± 6.9	45.0 ± 4.5	43.8 ± 6.4*
2	58.3 ± 5.6	37.5 ± 4.9	20.7 ± 5.9*	86.9 ± 4.5	83.4 ± 7.1	43.3 ± 11.1*
3	52.8 ± 5.3	30.2 ± 4.0	14.4 ± 4.0*	90.5 ± 1.8	81.3 ± 5.9	68.6 ± 5.5
4	47.7 ± 4.2	26.5 ± 4.6	11.4 ± 3.2*	91.0 ± 3.0	86.7 ± 8.2	78.3 ± 6.8

ferred to 500-μm-mesh baskets and placed in floating cages. In November 1999, growth and survival were evaluated, and 5 mussels were thin-sectioned and examined for marks as described above. A blind reading was conducted on live mussels in September 2000 using 40 of the largest individuals from each treatment.

Results

1-d-old L. cardium

There were no significant differences in survival among treatments (concentration, immersion times, and sediment) immediately after the 24-h immersion in Test I, nor were there any significant interactions among main effects (ANOVA, all p values > 0.05). The grand mean for survival was 83.6 ± 1.0% (±1 SE). Examination of shells under fluorescence microscopy indicated that control shells exhibited autofluorescence because of the natural structure of the shell. Controls fluoresced a yellowish color, similar to OTC, so the OTC treatment could not be distinguished from controls at this point; however, the entire shell of mussels marked in calcein fluoresced green and was distinguishable from controls. Brilliance of calcein marks increased with higher concentrations and longer immersion times. Marks on mussels immersed for 6 or 12 h in 125 mg/L calcein were very faint, but all other calcein treatments produced a distinct mark. The presence or absence of sediment did not noticeably affect the intensity of the mark.

In Test II, mussels in both calcein treatments survived only to the 51-d assessment, and marks from both the 125- and 500-mg/L concentrations were visible on all mussels evaluated to that point. Marks produced by the 500 mg/L calcein were noticeably brighter than those produced by a concentration of 125 mg/L at all evaluations to 51 d. The OTC treatment was not assessed at 51 d because of a lack of mussels available for the test, but all mussels immersed in OTC and examined at 35 d had distinct marks. The portion of the shell that had grown after immersion exhibited some autofluorescence but could be easily distinguished from both OTC and calcein marks. There were no surviving mussels in any treatment, including controls, after these evaluations.

1-mo-old L. cardium

Mean survival of *L. cardium* immersed at 1-mo old after 4 mo was 26.5% for those immersed once in 250 mg/L OTC for 24 h and 11.4% for those immersed 4 times. Survival varied significantly ($p < 0.05$) among treatments during the 4-mo period (Table 1). Survival of juveniles immersed only once in OTC did not differ significantly from controls ($p > 0.05$) for any of the evaluations. Survival between months of those immersed 4 times in OTC was significantly lower than controls after the first 2 mo ($p < 0.05$) but was similar during the 3rd and 4th mo (Table 1). Overall, mussels grew from an initial mean length of 635 ± 16 μm to mean lengths ranging from 2.0 to 2.3 mm during the 4-mo period (Table 1).

All 1-mo-old *L. cardium* immersed in 250 mg/L calcein were dead after 1 mo in the culture system. Shells appeared very thin, as if they had been dead for a considerable time, and there was no visible indication of growth after immersion. Presumably, these mussels died at the time of marking or soon thereafter.

Empty shells from both OTC treatments were examined using fluorescence microscopy, and

TABLE 1. Extended.

Mean length ± SE (μm)		
Control (n = 100)	OTC (1×) (n = 100)	OTC (4×) (n = 100)
922 ± 22	811 ± 19*	756 ± 22*
1234 ± 31	1081 ± 31*	1059 ± 78*
1692 ± 46	1557 ± 60	1599 ± 136
2322 ± 74	1991 ± 92*	2284 ± 216

100% had visible marks through the 4-mo evaluation. Marks on mussels immersed 4 times were slightly larger than on those immersed only once because individuals marked 4 times were 3 wk older and larger at the time of the final marking. Marks were not at that time, however, noticeably brighter or easier to detect than marks on mussels immersed only once. No marks could be detected on thin-sections of the shells of these juveniles using fluorescence microscopy when they were examined at 14 mm (7 mo after first immersion).

2-mo-old L. cardium

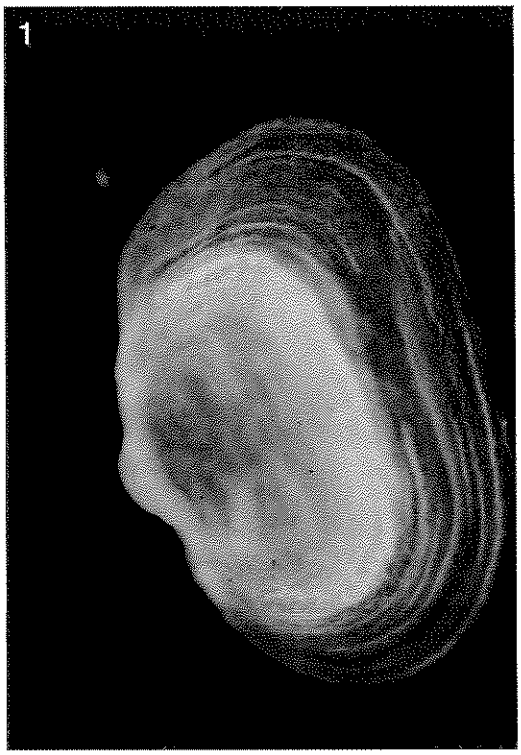
Survival and length of 2-mo-old *L. cardium* immersed in 125 mg/L calcein for either 3 or 8 h did not differ significantly ($p > 0.05$) from controls at 25 or 105 d. Mean survival ranged from 51.8 to 56.6% and mean length ranged from 2.0 to 2.1 mm across all treatments after 105 d (Table 2). Shells examined under fluorescence microscopy exhibited marks that were distinct, but somewhat faint. Survival and length of 2-mo-old *L. cardium* immersed in 250 mg/L calcein for either 24 or 48 h also did not differ significantly ($p > 0.05$) from controls at 30 or 90 d. Mean survival ranged from 65.0 to 72.5% and mean length ranged from 1.63 to 1.68 mm across all treatments after 90 d (Table 2). Marks from this higher concentration and longer immersion times were very distinct (Fig. 1) on all shells examined during the 3-mo period.

≥*4-mo-old* L. cardium

Most 4-mo-old *L. cardium* immersed for 6 h in 250 mg/L OTC or calcein in June 1999 survived and grew from an initial length of 3 mm to 22 mm by November 1999 (Table 3). No OTC marks and only 1 calcein mark were found in the 5 individuals from each treatment that were thin-sectioned and examined. Nearly all 5-mo-old juveniles immersed for 12 h in 250 mg/L OTC or calcein in July 1999 survived and grew from an initial length of 5 mm to 21 mm by November 1999 (Table 3). Out of 5 mussels sectioned that were immersed in OTC, 2 thin sections had recognizable marks. Marks were found on 3 of the 5 mussels immersed in calcein that were thin-sectioned. Both OTC and calcein

TABLE 2. Survival and length-at-age of 2-mo-old *Lampsilis cardium* immersed in calcein at varying concentrations and immersion times. Separate control groups were used for each immersion concentration because dates of immersion differed for each concentration.

Calcein concentration (mg/L)	Days after immersion	Immersion time (h)	Survival		Length	
			n	Mean ± SE (%)	n	Mean ± SE (%)
125	25	0	2	71.9 ± 13.3	23	1252 ± 73
		3	2	68.8 ± 12.5	22	1222 ± 83
		8	2	72.4 ± 9.9	24	1144 ± 70
	105	0	2	56.3 ± 0.0	18	2036 ± 140
		3	2	56.6 ± 6.3	18	2047 ± 119
		8	2	51.8 ± 10.7	17	2143 ± 112
250	30	0	4	85.0 ± 1.7	80	1591 ± 45
		24	4	80.8 ± 5.8	79	1558 ± 42
		48	4	78.3 ± 2.9	80	1515 ± 37
	90	0	4	72.5 ± 6.3	76	1674 ± 44
		24	4	67.5 ± 8.6	72	1686 ± 41
		48	4	65.0 ± 4.4	73	1630 ± 36



marks were readily identified. Calcein marks were visible on portions of 20 of the 21 mussels when the remaining mussels from the July immersion were harvested (March 2000) and whole shells were examined for fluorescence. Marks from OTC, however, could not be seen on whole shells.

All 7-mo-old juvenile *L. cardium* immersed for 24 h in 250 mg/L OTC or calcein in September 1999 survived and grew from a mean length of 13 mm to ~17 mm in 2 mo (Table 3). Distinct marks of both chemicals were visible on 100% of shells that were thin-sectioned. Very distinct calcein marks were visible as a continuous ring around the mussel when the remaining mussels were harvested (March 2000) and whole shells were examined for fluorescence. Marks produced by OTC could not be seen on whole shells. During the blind-reading 6 mo later, 100% of the whole shells examined were identified correctly; calcein marks were unmistakable (Fig. 2), whereas OTC marks were not visible and served as a control.

7-mo old A. pectorosa

Juvenile *A. pectorosa* immersed for 12 h in 250 mg/L OTC or calcein in September 1999 (Table 3) grew from a mean length of 7.0 mm to >8.5 mm in 2 mo with no significant differences ($p > 0.05$) between treatments. There was 98.2% survival in the OTC treatment and 96.4% survival in the calcein treatment. Distinct OTC marks were seen in 3 of 10 mussels thin-sectioned. In contrast, all 10 thin-sections examined from the calcein treatment contained visible marks. Marks were identified correctly 93.8% of the time when the blind reading was conducted on live *A. pectorosa* 1 y post-immersion (mean length = 23.2 ± 0.4 mm).

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FIG. 1. Shell of *Lampsilis cardium* immersed in 250 mg/L calcein for 48 h: green fluorescent area indicates size at immersion (~1 mm long); nonfluorescing area reflects growth during 3 mo following immersion.

FIG. 2. Portion of whole shell (17 mm long) of *Lampsilis cardium* immersed at a length of ~13 mm in 250 mg/L calcein for 24 h. Green fluorescent band indicates shell margin at time of immersion.

TABLE 3. Survival and length of *Lampsilis cardium* (Lc) and *Actinonaias pectorosa* (Ap) immersed in 250 mg/L oxytetracycline (OTC) or calcein at various ages and immersion times. Immersions began in June 1999 and the test ended in November 1999. Months post-immersion indicates the length of time from immersion to evaluation of growth and survival at the end of the test.

Month (1999)	Age at immersion (mo)	Chemical	Immersion time (h)	Species	*n*	Mean length ± SE (mm)		Months post-immersion	Survival (%)
						At immersion	End of test		
June	4	OTC	6	Lc	50	2.9 ± 0.8	22.5 ± 2.4	5	90
		Calcein	6		51	2.9 ± 0.8	22.0 ± 2.0	5	100
July	5	OTC	12	Lc	27	4.9 ± 1.6	21.3 ± 2.5	4	100
		Calcein	12		27	5.6 ± 1.5	20.7 ± 1.9	4	96
September	7	OTC	24	Lc	19	12.8 ± 2.0	16.5 ± 2.1	2	100
		Calcein	24		19	13.3 ± 2.3	17.4 ± 2.6	2	100
September	7	OTC	12	Ap	112	7.0 ± 0.2	8.5 ± 0.2	2	98.2
		Calcein	12		112	7.0 ± 0.2	8.8 ± 0.2	2	96.4

Discussion

Survival and growth

The adverse effects of fluorochromes on *L. cardium* and *A. pectorosa* seem to depend on age at immersion. Many details about mussel life history are unknown, but high mortality of juveniles during the first 2 mo of culture (Buddensiek 1995, O'Beirn et al. 1998, Westbrook 1999) indicates they are most sensitive at this life stage. If this assumption is true, immersions done on juveniles <2-mo old would likely yield the most conservative results regarding exposure tolerance to chemical marking. Our study indicates that OTC and calcein are likely most toxic when mussels are <2-mo old, but are safer for older mussels. In Test I, there were no immediate lethal effects to 1-d-old *L. cardium* after an exposure in either OTC or calcein at concentrations up to 500 mg/L for 24 h. A 24-h immersion in 250 mg/L OTC had toxic effects on 1-mo-old *L. cardium*, but there were no apparent effects when ≥2-mo-old mussels were immersed in either chemical. We believe that the death of all 1-mo-old mussels immersed in 250 mg/L calcein for 24 h may have resulted from experimental error or an anomaly. Shells examined 1 mo after immersion had not grown beyond the size when the juveniles were immersed, which suggests all individuals died at the time of immersion or soon thereafter. No other test resulted in immediate mortality to mussels immersed in calcein. In fact, both survival and growth of mussels immersed in calcein were similar to controls in all other trials.

Multiple immersions within a 1-mo period had adverse effects compared to a single immersion. Growth was affected when 1-mo-old *L. cardium* were immersed once in 250 mg/L OTC for 24 h, but both survival and growth were lower after 4 immersions in a 1-mo period. Results of monitoring these juveniles over 4 mo indicated that toxic effects were short-lived (1–2 mo post-immersion). Mussels in both the single and multiple immersion groups were significantly smaller than controls after 4 mo, but they grew more slowly during the first 2 mo (Table 1). Mussels immersed 4 times also grew slower during the first 2 mo but were similar in length to controls after 4 mo. Their faster growth rate during the last 2 mo indicated a recovery from marking stress, but it may also reflect density dependent growth because high mortality greatly reduced densities in the culture baskets. Similarity of between-month survival rates in the 4 immersion groups after 2 mo also indicated that toxic effects were short-lived. Multiple marks could be useful to distinguish between different groups of mussels, but immersions should be carried out at least 1 mo apart to allow for recovery from stress and growth between markings.

Marking success

Marking success tended to increase with increasing concentration and immersion time. This trend was expected because it has been documented previously in fish (Weber and Ridgway 1962, Hettler 1984, Brooks et al. 1994)

and marine molluscs (Pirker and Schiel 1993, Day et al. 1995, Kaehler and McQuaid 1999). Very faint marks were produced in Test I when 1-d-old *L. cardium* were immersed in the lowest concentration for 6 and 12 h in comparison to higher concentrations and longer immersion times. In Test II, brighter calcein marks were produced by immersion in 500-mg/L calcein compared to a concentration of 125 mg/L. Longer immersion times also produced more consistent results with mussels ≥4 mo old. The longest immersion time (24 h) produced the most reliable marks with 100% recognition in OTC and calcein thin-sections and whole shells from the calcein treatment.

Marking success may also be influenced by factors other than chemical concentration and immersion time. Marking success probably depends on mussel growth, as marks were most visible at the growing edge of the shell. Day et al. (1995) found marks only at the growing edge in abalone (*Haliotis rubra*); temperature, feeding, and animal stress affected incorporation of fluorescence into shells. Consequently, freshwater mussels should be marked when they are in an active growth phase. Persistence of marks is also a major concern in long-term studies. Direct and prolonged exposure to light deactivates the fluorescence of tetracycline (Choate 1964); however, we found that fluorescent marks persisted for at least 1 y in live mussels. The burrowing behavior of juvenile mussels (Yeager et al. 1994) probably diminishes the light that actually reaches the fluorochrome in the shell. We found no reports of fluorochrome marks fading in fish or marine molluscs, so we believe that recognizable marks may persist in freshwater mussels for several years. Mark retention will likely vary among streams because of water-quality variables that could affect the rate of shell dissolution. The size at which a mussel is marked in relation to shell erosion rates and its size as an adult will also dictate how long a mark remains. Brilliant marks were detected when mussels were at a length of ~2 mm (4 mo post-immersion) when 1-mo-old *L. cardium* (mean length = 630 ± 16 μm) were immersed; however, marks had either eroded away or were too small to find by thin-sectioning when the mussels were re-examined at a length >14 mm (7 mo post-immersion).

Calcein was more effective than OTC for marking juvenile *L. cardium* and *A. pectorosa* for the exposures we tested. Calcein is more expensive, but it produced brighter marks and better mark recognition when used at identical concentrations and immersion times to OTC. We immersed *A. pectorosa* into calcein at a mean length of 7 mm with excellent survival, and recognized >90% of the marks 1 y later on live mussels when they had grown to a mean length of 23 mm. Higher concentrations or longer immersion times of OTC would be necessary to produce visible marks on live mussels, which would risk adverse effects. Also, the fluorescence of OTC was similar to the natural fluorescence in mussel shells (Day et al. 1995), and some care must be taken to distinguish the two.

Interspecific variability

The most appropriate marking protocol to achieve quality marks without toxic effects may vary between species. Calcein is more toxic than OTC to some fish species (Brooks et al. 1994, Bumguardner and King 1996) and less toxic to other species (Wilson et al. 1987, Monaghan 1993). If the toxicity of these chemicals varies between fish species, it is reasonable to assume that species-specific toxicity also occurs in freshwater mussels. For this reason, we strongly recommend caution when immersing rare or threatened mussels. Marking success also may vary between species. Hettler (1984) found that spot (*Leiostomus xanthurus*) more readily incorporated tetracycline into their otoliths than pinfish (*Lagodon rhomboides*). We found that marking success varied between species; a 12-h immersion in 250 mg/L calcein was more effective in marking *A. pectorosa* than marking *Lampsilis cardium*. Brilliant marks were detected in all 10 thin-sections of *A. pectorosa* compared to only 3 of 5 sections of *L. cardium*. In addition, marks on *A. pectorosa* consisted of very distinct rings around the whole shell, whereas marks on shells of *L. cardium* were patchy.

Recommendation

Based on the species marked so far, we recommend a single immersion of mussels >2-mo old for 24 h in 250 mg/L calcein. Chemical marking of juveniles <2-mo old may be useful for some laboratory experiments, but it is unlikely that juveniles marked at <2-mo old and released into the wild would retain marks for

very long because of umbo dissolution. Marking larger mussels (>5 mm) should increase mark detection over time.

In conclusion, our study demonstrated that OTC and calcein immersions are effective for marking juvenile freshwater mussels. Immersions can be done that produce reliable marks and minimize toxicity. The ability to identify fluorescent marks on live mussels broadens the use of chemical marking to include endangered species because it eliminates the need to sacrifice mussels, or to be limited to assessing stocking success only from dead shells. The successful application of this technique for marking juvenile freshwater mussels opens the door for evaluating supplemental stocking of mussels because artificially cultured individuals can be readily identified.

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Literature Cited

ANTHONY, J. L., AND J. A. DOWNING. 2001. Exploitation trajectory of a declining fauna: a century of freshwater mussel fisheries in North America. Canadian Journal of Fisheries and Aquatic Sciences 58:2071–2090.

BOGAN, A. 1993. Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. American Zoologist 33:599–609.

BRADY, T. R. 2000. Effects of substrate characteristics on growth and survival of juvenile freshwater mussels. MS Thesis, Tennessee Technological University, Cookeville, Tennessee.

BROOKS, R. C., R. C. HEIDINGER, AND C. C. KOHLER. 1994. Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. North American Journal of Fisheries Management 14:143–150.

BUDDENSIEK, V. 1995. The culture of juvenile freshwater pearl mussels *Margaritifera margaritifera* L. in cages: a contribution to conservation programmes and the knowledge of habitat requirements. Biological Conservation 74:33–40.

BUMGUARDNER, B. W., AND T. L. KING. 1996. Toxicity of oxytetracycline and calcein to juvenile striped bass. Transactions of the American Fisheries Society 125:143–145.

CHOATE, J. 1964. Use of tetracycline drugs to mark advanced fry and fingerling brook trout (*Salvelinus fontinalis*). Transactions of the American Fisheries Society 93:309–311.

COSGROVE, P. J., M. R. YOUNG, L. C. HASTIE, M. GAYOOD, AND P. J. BOON. 2000. The status of the freshwater pearl mussel *Margaritifera margaritifera* Linn. in Scotland. Aquatic Conservation: Marine and Freshwater Ecosystems 10:197–208.

DAY, R. W., M. C. WILLIAMS, AND G. P. HAWKES. 1995. A comparison of fluorochromes for marking abalone shells. Marine and Freshwater Research 46: 599–605.

HEINRICHER, J. R. 1996. Reproductive ecology of two species of freshwater mussels in Tennessee. MS Thesis, Tennessee Technological University, Cookeville, Tennessee.

HETTLER, W. F. 1984. Marking otoliths by immersion of marine fish larvae in tetracycline. Transactions of the American Fisheries Society 113:370–373.

HUGHES, M. H., AND P. W. PARMALEE. 1999. Prehistoric and modern freshwater mussel (Mollusca: Bivalvia: Unionoidea) faunas of the Tennessee River: Alabama, Kentucky, and Tennessee. Regulated Rivers: Research and Management 15:25–42.

KAEHLER, S., AND C. D. MCQUAID. 1999. Use of the fluorochrome calcein as an in situ growth marker in the brown mussel *Perna perna*. Marine Biology 133:455–460.

LAYZER, J. B., AND M. E. GORDON. 1993. Reintroduction of mussels into the upper Duck River, Tennessee. Pages 89–92 *in* K. S. Cummings, A. C. Buchanan, and L. M. Koch (editors). Conservation and management of freshwater mussels. Proceedings of an Upper Mississippi River Conservation Committee Symposium, 12–14 October 1992, St. Louis, Missouri. Upper Mississippi River Conservation Committee, Rock Island, Illinois.

LEMARIE, D. P., D. R. SMITH, R. F. VILLELLA, AND D. A. WELLER. 2000. Evaluation of tag types and adhesives for marking freshwater mussels (Mollusca: Unionidae). Journal of Shellfish Research 19:247–250.

MONAGHAN, J. P. 1993. Comparison of calcein and tetracycline as chemical markers in summer flounder. Transactions of the American Fisheries Society 122:298–301.

NAKAHARA, H. 1961. Determination of growth rates of nacreous layer by the administration of tetra-

cycline. Bulletin of the National Pearl Research Laboratory 6:607–614.

NNMCC (National Native Mussel Conservation Commission). 1998. National strategy for the conservation of native freshwater mussels. Journal of Shellfish Research 17:1419–1428.

O'BEIRN, F. X., R. J. NEVES, AND M. B. STEG. 1998. Survival and growth of juvenile freshwater mussels (Unionidae) in a recirculating aquaculture system. American Malacological Bulletin 14:165–171.

PIRKER, J. G., AND D. R. SCHIEL. 1993. Tetracycline as a fluorescent shell-marker in the abalone *Haliotis iris*. Marine Biology 116:81–86.

USFWS (US Fish and Wildlife Service). 1991. Fanshell (*Cyprogenia stegaria* (= *C. irrorata*)) recovery plan. Southeast Regional Office, US Fish and Wildlife Service, Atlanta, Georgia. (Available from: Fish and Wildlife Reference Service, 4th Floor, 1776 Jefferson Street, Rockville, Maryland 20852 USA.)

WALLER, D. L., AND L. E. HOLLAND-BARTELS. 1988. Fish hosts for glochidia of the endangered freshwater mussel *Lampsilis higginsi* Lea (Bivalvia: Unionidae). Malacological Review 21:119–122.

WEBER, D. D., AND G. J. RIDGWAY. 1962. The deposition of tetracycline drugs in bones and scales of fish and its possible use for marking. Progressive Fish-Culturist 24:150–155.

WESTBROOK, O. J. 1999. Production of juvenile mussels in fish hatchery raceways. MS Thesis, Tennessee Technological University, Cookeville, Tennessee.

WILLIAMS, J. D., M. L. WARREN, K. S. CUMMINGS, J. L. HARRIS, AND R. J. NEVES. 1993. Conservation status of freshwater mussels of the United States and Canada. Fisheries 18(9):6–22.

WILSON, C. A., D. W. BECKMAN, AND J. M. DEAN. 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. Transactions of the American Fisheries Society 116:668–670.

YEAGER, M. M., D. S. CHERRY, AND R. J. NEVES. 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, *Villosa iris* (Bivalvia:Unionidae). Journal of the North American Benthological Society 13:217–222.

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