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NOTES

A Comparison of the Suitability of Alizarin Red S and Calcein for Inducing a Nonlethally Detectable Mark in Juvenile Guppies

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Abstract.—Mark–recapture studies are an important component of fisheries research. A diversity of marks is needed to meet the demands of experimental designs and to overcome species-specific variation in marking success. Suitable marks must not alter the viability of marked individuals, must be easy to detect, and must be retained for an appropriate period of time. I compared the effect of alizarin red S and calcein on the individual growth and mortality rates of guppies *Poecilia reticulata* via short-term experiments (<14 d) conducted both in environments where alizarin- and calcein-marked fish were allowed to interact with unmarked fish and in environments where fish were segregated by mark. Neither mark affected the growth or mortality of marked individuals. Both marks were easily applied, did not affect the appearance of fish, and could be detected on the skeleton of live, anesthetized fish or ethanol-preserved specimens without the additional preparation (or lethality) involved in detecting marks on otoliths. However, both marks were subject to fading with time and when fish were exposed to high water temperatures or to direct sunlight. Thus, pilot experiments should be conducted under field conditions before marks are used for long-term mark–recapture studies. I also present an inexpensive and portable technique for detecting alizarin that uses a green laser pointer as the excitation source. This alizarin detector performed as well as a portable calcein detector (Leips et al. 2001) and could easily be modified for improved performance. Alizarin red S and calcein fluoresce at different wavelengths, so both marks can be used simultaneously in studies examining multiple treatment groups or cohorts. The use of alizarin red S may be preferred to that of calcein because its red fluorescence is more easily distinguished from the autofluorescence of bone.

Mark–recapture studies are an important component of fisheries research (Hilborn et al. 1990). Mass-marking is often preferred over individual tagging methods as it is more rapidly applied, reduces handling stress, and is often the only successful way of marking eggs, larvae, or small juveniles. Several chemicals are available for mass-marking of fish by immersion: calcein, tetracycline, alizarin, strontium, and lanthanides. These chemical markers have the advantage that they provide no visible change in the appearance of the fish, so they should not affect risk of predation by visually foraging predators or influence visually dependent behaviors (e.g., Mohler et al. 2002). Strontium and lanthanides are calcium analogues readily deposited in bones and fin rays creating a traceable tag, but have the disadvantage that they require mass spectrometry or scanning electron microscopy to be detected (Ennevor and Beames 1993; Clear et al. 2000). Tetracycline fluoresces when exposed to ultraviolet or blue light (Brooks et al. 1994), but it is an antibiotic and concern has arisen over its use because of its potential to alter fish behavior, growth, and mortality (e.g., Monaghan 1993) and due to its potential environmental impacts (Blom et al. 1994). Calcein fluoresces when exposed to blue light, has proven to be an effective chemical marker in several species, and can be detected on live fish (Leips et al. 2001). Nevertheless, a diversity of chemical marking agents is often necessary to meet the demands of different experimental designs and to compensate for species-specific variability in marking success (e.g., Hernaman et al. 2000).

Alizarin compounds have also been used successfully in mass-marking experiments. Alizarin complexone has been used to estimate the survival and growth of marked fish in the wild (Tsukamoto et al. 1989; Secor and Houde 1995). Alizarin red S has also proven to be a good alternative to alizarin complexone, at only a fraction of the cost (Blom et al. 1994; Nagiec et al. 1995; Beckman and Schultz 1996; Eckmann et al. 1998; Lagardere et al. 2000). These studies have demonstrated that otolith marks can remain highly readable for long durations when fish are reared in the laboratory (up to 842 d) and have been successfully detected on fish recaptured after 128 d in the field (Nagiec et al. 1995). However, none of these studies have

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measured retention success (i.e., the percent of marked fish with readable marks) under field conditions. Additionally, data on the effect of alizarin red S on mortality and growth are sparse, only two studies to date reporting values relative to unmarked controls (Blom et al. 1994; Eckmann et al. 1998). Because key assumptions of many studies are that mark retention is near perfect and that the mark itself does not affect the growth or survival of marked fish, more studies are required before alizarin red S is accepted as a standard marking method. Furthermore, as fish growth and survival are affected by subtle environmental differences and interactions among fish, these effects are best compared when both marked and unmarked fish are able to interact in the same tank.

In this study, I evaluated the effect of alizarin red S on the growth of juvenile guppies *Poecilia reticulata* by comparing alizarin-marked individuals with calcein-marked fish and unmarked controls when all three were present in the same tank. I also compared the growth of alizarin- and calcein-marked newborns when both were reared in mesocosms with unmarked guppies present at densities and size structures found in field populations. Additionally, I evaluated the potential for mark-induced mortality and examined the mark retention of alizarin and calcein under different environmental conditions. Finally, I describe a new, portable method for detecting alizarin on live fish and compare its effectiveness relative to epifluorescence microscopy and to a portable calcein detector.

Methods

Growth assessment in mixed-mark tanks.—Two experiments were conducted that assessed the growth of alizarin-marked fish when they were kept in the same environment and allowed to interact with unmarked and calcein-marked individuals. In the first experiment, three tanks were established that each contained alizarin-marked, calcein-marked, and unmarked juvenile guppies. Each tank contained 8 L of water; two tanks contained 15 fish (5 in each mark treatment), and one contained 12 fish (4 in each mark treatment). Guppies were anesthetized with MS-222 (tricaine methanesulfonate; 0.02 mg/100 mL) and measured to the nearest 0.01 mm with digital calipers under a dissecting microscope. Guppies ranged in size from 6.88–9.13 mm standard length (SL), averaging 7.84 mm. To control for effects of size on growth, fish were matched by size when assigned (otherwise randomly) to each mark treatment and tank. To apply the mark treatments, fish in each mark treatment–tank combination were held as a group in 500-mL containers for 24 h; unmarked fish were kept in water conditioned for healthy guppy maintenance (aerated and dechlorinated tap water with temperature = 23.5–24.5°C, pH = 7.2–7.8, hardness = 160–200 mg/L), while alizarin- and calcein-marked fish were held, respectively, in a 250 mg/L solution of alizarin red S (1,2-dihydroxyanthraquinone sodium sulfonate) or calcein (2,4,-bis-[N,N′-di{carbo methyl}-aminomethyl]fluorscein). Conditioned water was used to make the solutions. The pH of the calcein solution was adjusted with NaOH to match the pH of the alizarin solution and the conditioned water. After 24 h, all fish were rinsed in conditioned water and placed in their assigned tank. Each tank was given a small amount of flake food twice daily.

After 14 d, each fish was anesthetized with MS-222, measured with digital calipers under a dissecting microscope, and examined for a mark. To determine the mark, fish were placed individually on a microscope slide and placed under an epifluorescence microscope at the lowest available magnification (100–200×). An excitation filter at 495 and an emission filter at 535 was used to read the calcein mark, while an excitation filter at 545 and emission filter at 580 was used to read the alizarin mark. Instantaneous growth rate for each mark treatment-tank combination was calculated by using the mean initial and final standard length $(\log_e[\text{mean SL}_{\text{final}}] - \log_e[\text{mean SL}_{\text{initial}}])/14$. Analysis of variance (ANOVA) was used to test whether instantaneous growth rate differed due to mark treatment (alizarin, calcein, or unmarked) with tank used as a random, block effect (Potvin 1993). Planned contrasts were used to test whether either alizarin- or calcein-marked fish differed from controls. A retrospective power analysis was conducted to determine the difference in growth rate that could have been detected in this study.

In the second experiment, 24 trials were established that assessed the growth of marked fish in relation to three experimental factors: the size of marked fish (large versus small), population density (low versus high), and source population (two sources). In each trial, five newborn guppies were marked with alizarin and five with calcein. Newborns were measured and marked as in the first experiment. In each trial, the two groups of newborns varied in size-class, one large and one small (mean SL ± SE = 7.68 ± 0.04 mm versus 7.20 ± 0.05). Marks were assigned to groups randomly with respect to size-class. The newborns were held in a 1,000-L mesocosm with a background population of guppies. Background populations com-

prised both adult and juvenile fish in a size structure representative of size distributions of natural guppy populations (Rodd and Reznick 1997) and varied in density (14 or 56 background fish) approximating average and high field densities, respectively (Reznick et al. 2001). Additionally, newborns and background populations were derived from one of two field source populations. For the purposes of testing for an effect of mark on growth, these three experimental factors each with two levels were combined into one experimental treatment with eight levels and referred to as ''treatment.''

Trials were run for 14 d. At the end of a trial, all fish were collected from the mesocosm and were measured in standard length. Juveniles were scanned for marks using the portable alizarin detector described below and a portable calcein detector (Leips et al. 2001). If all the marked newborns were not recovered from a trial, then fish were examined on an epifluorescence scope (see the description above) to check for missed marks. Instantaneous growth rate was calculated by using the mean initial and final standard length for each mark group within a trial ($\log_e$[mean SL_{final}] − $\log_e$[mean $SL_{initial}$])/14. Growth rates were minimally affected by mortality as only 3 of 240 marked newborns in this experiment (two alizarin- and one calcein-marked fish) were not detected by either the portable detectors or the epifluorescence microscope. A mixed-model ANOVA was used to analyze the effect of mark and treatment (size-class × density × source) on instantaneous growth rate. Trial was used as a random factor, as groups of marked newborns were paired within each trial. Planned contrasts were used to examine the individual effects of size-class, density, and source on growth rate. While this analysis does not allow for testing of interactions within treatment or for interactions between these interactions and mark (i.e., three- and four-way interactions), an expanded version of this model testing for these effects found none of these interactions to be significant and for brevity will not be presented here. These analyses met the assumptions of ANOVA.

Reliability assessment in single-mark tanks.—Two experiments were conducted at the Verdant Vale Research Station (Arima, Trinidad) to determine the reliability of alizarin and calcein as marks. In each experiment, six treatments consisting of three mark types (alizarin, calcein, unmarked) crossed with two light regimes (sun versus shade) were used. I assessed reliability by testing for differential mortality or growth due to mark and by examining mark retention under each light regime. Each treatment consisted of approximately 25 juvenile guppies (6.17–9.99 mm SL) housed in a 20-L aquaria. There was only one aquarium per treatment in each experiment. The two experiments differed in that they used guppies from different source populations, were set up at different times, and were of different durations. The first experiment was run for 14 d, while the second was run for 7 d. Reliability experiment 1 experienced tank temperatures (>29°C) stressful for guppies which are found in streams with ambient temperatures of 24°C (Reznick et al. 2001). In reliability experiment 2, temperatures were on average lower than in reliability experiment 1, but still exceeded stream temperatures. In both experiments, the water temperature of tanks in the sun location were equal to or 1°C higher than the water temperature of tanks in the shade location.

To establish each experiment, juvenile guppies were measured as described above and sorted into 1-mm size-classes; then guppies from each size-class were randomly assigned to one of the six treatments. Fish were marked following the procedure described above, except that stream water (see Reznick et al. 2001 for a description of the water chemistry) was used to house fish. Tanks for each mark treatment were placed next to each other at one of two locations: a shaded site, where tanks received no direct sunlight, and a sunny site, where tanks received direct sunlight for several hours each day. At the end of each trial, guppies were checked for marks using the portable detectors (see below and Leips et al. 2001). Guppies were then measured (SL) and preserved in ethanol for rechecking using the epifluorescence setup described above. Preserved fish were stored in the dark and observed within 1 month. The visibility of the marks under the epifluorescence microscope was classified as clear, faint, or none. I used exact chi-square tests to determine whether (1) survival and (2) mark visibility were associated with mark treatment or light regime. The effect of mark treatment or light regime on growth were analyzed via ANOVA using the mean standard length for each tank at the end of the experiment.

Portable alizarin detector.—A dielectric interference band-pass filter at 590 nm with a width of 35 nm (Omega Optical 590 df35-1) was placed in one ocular of a dissecting microscope to act as a barrier filter. To excite alizarin, I used a frequency doubled Nd: YAG green (532 nm) laser pointer (Power Technology LCP-GP-2). The absorption maximum for the alizarin-calcium complex is 550

nm (Connerty and Briggs 1965). This class II laser product has a maximum output power of less than 1 mW and a beam width of less than 2.5 mm and is battery powered. To read alizarin marks, live guppies were anesthetized with MS-222 and observed under the dissecting microscope individually. A dark room was required to successfully read the marks. I evaluated the effectiveness of the alizarin detector by determining the probability of the portable detector missing a mark that was visible under the standard epifluorescence setup in the second growth assessment experiment, in the reliability experiments, and in two, short-term mark–recapture studies. For comparison, I also calculated the failure rate of the portable calcein detector. The mark–recapture studies were performed in four streams on the southern slope of the Northern Range Mountains of Trinidad in March through May of 2000. Fish were released into the field for either 7 or 14 d. In the 7-d study, neonates born in the field station were marked as described above and released into their mother's stream. In the 14-d study, juveniles (6–10 mm SL) were captured from the field, measured, and marked at the field station and then returned to their stream (Bashey 2002). Upon recapture, fish were examined at the field station with the portable detectors; unmarked fish were preserved in ethanol and examined under an epifluorescence scope within 3 months of preservation.

Results

Growth Assessment in Mixed-Mark Tanks

In the first growth experiment, where alizarin-marked juveniles were present in the same tank as calcein-marked and unmarked juveniles, there was no mortality, except for one unmarked guppy that died shortly after the setup of the experiment and was not replaced. Fish grew on average 1.24 mm over the course of the experiment, which matches average field growth rates of similarly sized guppies (Bashey 2002). Instantaneous growth rates did not differ between mark treatments ($F_{2,4} = 2.05$, $P = 0.24$; Figure 1). Retrospective power analyses indicate that that this experiment had good power (>0.90) to detect a 0.2 mm difference in growth among treatments over the course of the experiment at a type I error rate of 0.05.

In the second growth experiment, newborn alizarin- and calcein-marked guppies were paired in mesocosms with background populations of guppies and mark effects were tested under a variety of experimental treatments. Newborn growth was significantly affected by treatment ($F_{7,16} = 3.45$, $P = 0.02$), with the density of the background population having the greatest effect ($F_{1,16} = 18.25$, $P = 0.0006$). However, no difference was found in growth rates of alizarin- versus calcein-marked newborns ($F_{1,16} = 0.00$, $P = 0.9861$). Additionally, there were no interactions between mark and treatment in general ($F_{7,16} = 0.76$, $P = 0.63$) or mark and density per se ($F_{1,16} = 0.23$, $P = 0.64$).

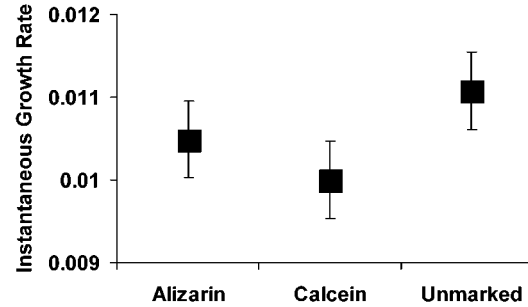

FIGURE 1.—Mean instantaneous growth rate ([$\log_e${mean SL_{final}}—$\log_e${mean $SL_{initial}$}]/14; ±SE) for each mark treatment from an analysis of variance. The growth rates do not differ significantly between the treatment groups ($F_{2,4} = 2.05$, $P = 0.24$). Planned comparisons show that the growth rates of both alizarin-marked ($F_{1,4} = 1.24$, $P = 0.33$) and calcein-marked fish ($F_{1,4} = 4.09$, $P = 0.11$) do not differ from that of unmarked controls.

Reliability Assessment in Single-Mark Tanks

There was considerable mortality (54%) in the first reliability experiment; however, there was no differential mortality due to mark ($\chi^2 = 0.39$, df = 2, $P = 0.82$) or light regime ($\chi^2 = 1.05$, df = 1, $P = 0.31$). Mortality in the second reliability experiment was much lower (<3%), and no mortality occurred in the alizarin treatments. In both reliability experiments, there was no significant effect of mark (experiment 1: $F_{2,2} = 0.08$, $P = 0.93$, experiment 2: $F_{2,2} = 0.77$, $P = 0.57$) or light regime (experiment 1: $F_{2,2} = 0.24$, $P = 0.67$, experiment 2: $F_{2,2} = 2.44$, $P = 0.26$) on final standard length.

In reliability experiment 1, both marks were prone to fading (Table 1). Fading appeared more pronounced in the sun location, but this effect was not significant ($\chi^2 = 1.23$, df = 2, $P = 0.54$). In reliability experiment 2, there was significant reduction in the visibility of the marks in the sun location ($\chi^2 = 19.56$, df = 2, $P < 0.0001$) due exclusively to the fading of calcein. Using a standard epifluorescence setup, alizarin marks appeared more clear than calcein marks (Table 1;

TABLE 1.—Quality of alizarin and calcein marks under different light treatments. Marks were detected on whole, ethanol-preserved fish with an epifluorescence microscope (see Methods for a description). Marked fish (*N*) were preserved for 14 or 7 d postmarking (reliability experiments 1 and 2, respectively).

Treatment	*N*	Visibility of mark (% of *N*)		
		Clear	Faint	None
Reliability experiment 1				
Alizarin, shade	7	85.7	0.0	14.3
Alizarin, sun	13	76.9	15.4	7.7
Calcein, shade	11	45.4	36.4	18.2
Calcein, sun	11	9.1	63.6	27.3
Reliability experiment 2				
Alizarin, shade	25	100.0	0.0	0.0
Alizarin, sun	25	100.0	0.0	0.0
Calcein, shade	24	100.0	0.0	0.0
Calcein, sun	23	30.4	52.2	17.4

experiment 1: $\chi^2 = 11.99$, df = 2, $P = 0.0015$, experiment 2: $\chi^2 = 20.38$, df = 2, $P < 0.0001$). The lower visibility of calcein was largely due to the difficulty in distinguishing marks from the autofluorescence of bone in ethanol-preserved specimens (Figure 2).

Portable Alizarin Detector

The portable alizarin detector performed comparably to the portable calcein detector describe by Leips et al. (2001; Table 2). Overall, the alizarin detector had a failure rate of 12.6%, while the calcein detector had a failure rate of 15.6%. The failure rate of both portable detectors was correlated with the strength of the fluorescent marks. In laboratory-reared fish (second growth assessment experiment), the marks were the strongest

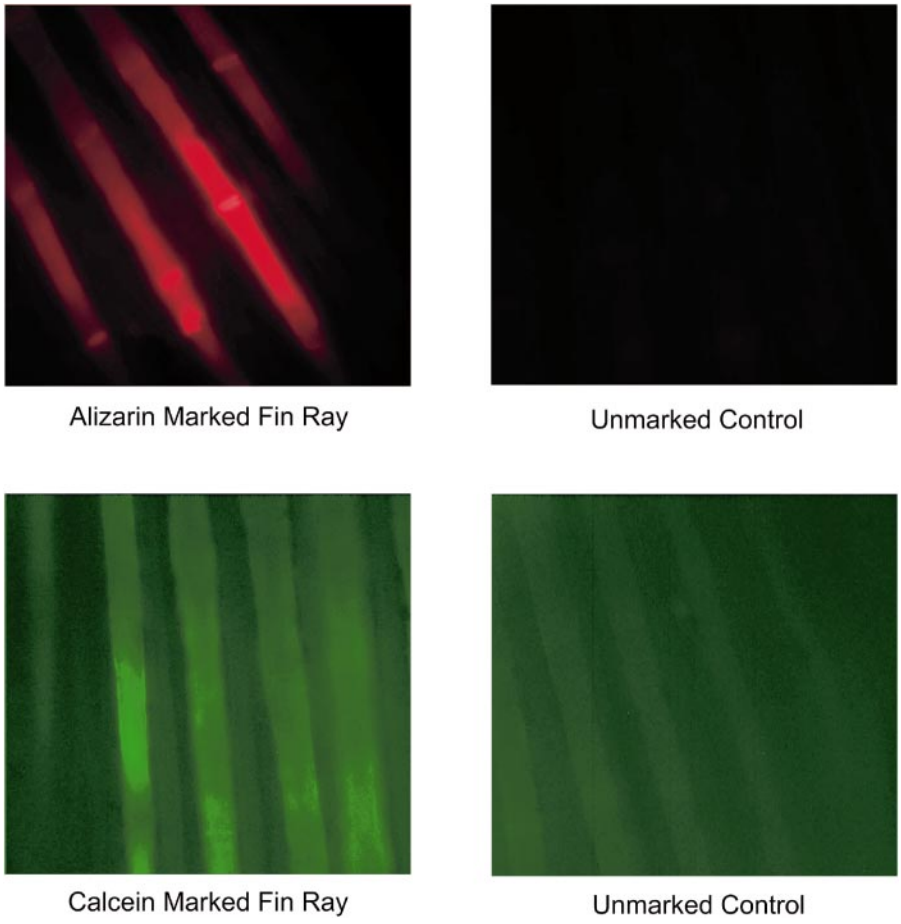

FIGURE 2.—Photomicrographs of the tail fins of alizarin- and calcein-marked fish along with that of an unmarked control. The fish were from the second reliability experiment (shade treatment) and were preserved in ethanol 7 d postimmersion. These images were taken with the epifluorescence microscope setup described in Methods. Notice that the autofluorescence in the unmarked control is absent when viewed under the filter setup for alizarin.

TABLE 2.—Failure rates of portable alizarin and calcein detectors. The letter *N* represents the number of marked fish detected by using either of the detectors or an epifluorescence microscope to examine whole fish. The failure rate is the percentage of these fish whose marks were not detected by the portable detectors.

	Alizarin detector		Calcein detector	
Experimental study	*N*	Failure rate (%)	*N*	Failure rate (%)
Second growth assessment	118	2.5	119	0.0
Reliability experiments				
Experiment 1, shade	6	66.7	9	44.4
Experiment 1, sun	12	75.0	8	87.5
Experiment 2, shade	25	20.0	24	0.0
Experiment 2, sun	25	16.0	19	63.2
Field mark–recapture study				
7 d	97	3.1	104	5.8
14 d	263	16.3	371	19.7

and the detectors performed the best. In the reliability experiments where fish were subject to heat stress or bright sunlight, the portable detectors performed the worst; in the field mark–recapture studies, the detectors performed intermediately (Table 2). Interestingly, all of the missed calcein marks from the reliability experiments were classified as faint when read with the epifluorescence scope, while only two of the missed alizarin marks were classified as faint. This was because even a small amount of alizarin mark was easily distinguished on ethanol-preserved specimens as a mark. In contrast, small amounts of calcein are less distinguishable from autofluorescence. The main difficulty in using the alizarin detector arose from the narrow diameter of its excitation light ($<$2.5 mm) which required the user to scan only part of a fish at a time. As a result, it was possible for the region with the mark to be missed with the portable detector used on an anesthetized fish, yet picked up with a more leisurely examination under the epifluorescence scope used on a preserved specimen. The calcein detector has a wider excitation light (25 mm), which made it easier to use. Attempts to diffuse the excitation beam of the alizarin detector with a convex lens to make a larger beam resulted in an emission response that was too faint to be detected.

Discussion

This study further supports the suitability of using alizarin red S as a mark in fisheries research and suggests that it provides a good alternative to calcein. Both marks have no visible effect on the appearance of marked fish under normal light, yet can be readily detected on the calcified structures of marked fish when observed under the proper optical conditions. The experiments presented here demonstrate that alizarin does not affect growth rate when marked fish are raised in either single-mark groups or in mixed-mark tanks. Moreover, alizarin red S and calcein appear to be equally suitable marks as they have no distinguishable effects on the growth rates of marked juveniles or on the growth rates of marked newborns when both are raised with unmarked juveniles and adults. Furthermore, neither mark affected survival. Despite the suitability of alizarin and calcein, this study also documents that these marks can fade within 14 d.

Marks were retained at a high rate ($>$98%) when fish were raised under appropriate temperature and lighting conditions as in the growth experiments. However, when fish were exposed to high temperatures or sunlight as in the reliability experiments, 18% of marks were difficult to read and 8% were undetectable. Intense light is known to bleach fluorescence (Hoyland 1999); however, sunlight alone is not sufficient to explain the loss of marks, as fish kept in the shade also lost marks. Shade-reared fish were exposed to higher-than-optimal temperatures, which perhaps resulted in a loss of marks due to a higher turnover of skeletal calcium. While both marks were subject to fading and loss, calcein had lower retention than alizarin. Despite the poor retention of skeletal marks in this study and in another poeciliid (Leips et al. 2001), alizarin has been detected in the otoliths of field-released grayling 4 months postimmersion, and calcein has been detected nonlethally in the skeleton of field-released Atlantic salmon *Salmo salar* after 1 year (Mohler 2003). The difference in mark retention among studies may be due to different methods of mark detection or related to differences among taxa or environments (e.g., poeciliids typically experience higher water temperatures than

salmonids). Because marking success and fitness consequences vary among species, it is recommended that the experimental evaluation of these factors, relative to controls, be conducted before any major marking effort is undertaken.

This study (along with Leips et al. 2001) also demonstrates that both marks can be read on live, anesthetized fish or ethanol-preserved specimens with no additional preparation. This feature is especially useful when the goal of the study is not to validate otolith increment formation, but rather to follow marked fish. To detect marks, either an epifluorescent microscope with appropriate filter sets must be used or a specialized detector must be obtained. In this paper, I describe a low-cost (<US$500) portable alizarin detector fashioned from a red filter mounted on a dissecting microscope and a green laser pointer. This detector was highly successful on laboratory-marked fish, although it had a high failure rate under field conditions. The performance of the alizarin detector was comparable to the calcein detector described by Leips et al. (2001); however, unlike the calcein detector, failure of the alizarin detector was not directly associated with poor mark retention. The most likely cause for the failure to detect an alizarin mark was the narrow width of the excitation light of the alizarin detector (the green laser pointer) which made it difficult to quickly scan the whole fish for a mark. Currently, more powerful green laser pointers (5–15 mW, class III a–b) are available which, although they should not be used directly on live fish, could be diffused to create a wider beam which would improve the ease of use and the success rate of the detector. Additionally, an alternative calcein detector, SE-MARK, is now commercially available (Western Chemical, Ferndale, Washington) and may be adaptable for use on alizarin.

Because alizarin has different spectral properties than calcein, both can be used in studies in which more than one mark is needed. Additionally, while multiple marking of individual fish may be used to create additional, unique marks (Tsukamoto 1988), the effects of multiple marking on the growth and survival of fish should be tested experimentally. If only one mark is needed, alizarin may be preferred to calcein because its emission is more easily distinguishable from the autofluorescence of bone and thus even small amounts of mark can be clearly identified. Also, alizarin marks on otoliths, unlike calcein marks, can be read without fluorescence microscopy (Beckman and Schultz 1996). Both calcein and alizarin red S are easy and inexpensive to apply, although at the time of this study, the per gram cost of calcein was 10 times that of alizarin red S ($15.78/g versus $1.56/g; Sigma-Aldrich).

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