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MANAGEMENT BRIEF

Evaluation of Calcein and Photonic Marking for Cultured Delta Smelt

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Abstract

The Delta Smelt *Hypomesus transpacificus* is a protected osmerid endemic to the San Francisco estuary of California. We conducted laboratory tests on marked versus unmarked juvenile adult smelt to evaluate (1) calcein mark intensity and postmarking survival for juveniles and adults, (2) photonic mark retention and survival of adults, and (3) predation by juvenile Striped Bass *Morone saxatilis*. Calcein mark intensity was graded in six body sections and adults were photonically marked using four fin-color combinations. Across all immersion times (1–7 min) all fish showed 100% mark retention 7 d after exposure to calcein concentrations of 2.5 and 5.0 g/L of water. Average survival 7 d after calcein marking was 93.9% in juveniles and 98.6% in adults. After 97 d of calcein and photonic marking, adults had weaker double marking, but each type of mark still showed 100% retention. Average survival of adult fish 70 d after marking was 98.7%. Unmarked and calcein + photonically marked adult Delta Smelt exposed to juvenile Striped Bass did not experience significantly different predation rates. Calcein is both effective and practical to batch-mark juvenile and adult Delta Smelt. Combined calcein and photonic marking for adult Delta Smelt further enables identification of multiple groups while potentially improving mark detection in short-term studies.

Marking large numbers of small fish (<6.5 cm FL) is particularly challenging at the early life stages (Skalski et al. 2009; Thorrold et al. 2002). A suitable mark must be easily identified, and it should not affect capturability, health, or survivability of the marked individual (Stott 1968; Guy et al. 1996). In many cases, it may also be beneficial if marking methods can distinguish individuals, are low cost, and are quickly applied (Skalski et al. 2009).

Chemical marking by immersion can greatly reduce marking time and cost, while minimizing handling stress to fish (e.g., Hettler 1984; Tsukamoto 1985). Calcein ($C_{30}H_{26}N_2O_{13}$) is a fluorochrome marking agent that binds with alkaline earth metals (Wallach et al. 1959; Wilson et al. 1987). When excited with blue light (495 nm), calcein emits a visible bright green-yellow fluorescence (about 520 nm; Sutphin and Morinaka 2010). This chemical has been used to cryptically mark fish because such marks can be viewed only with the aid of blue light and a filter (Leips et al. 2001; Mohler 2003). Calcein has proved useful in fish for marking calcified structures such as otoliths (Yamada 1973; Wilson et al. 1987; Brooks et al. 1994) and external structures not requiring lethal detection, such as fins and scales

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(Mohler 1997; Leips et al. 2001; Sutphin and Morinaka 2010). Calcein has been applied through immersion (Brooks et al. 1994; Mohler 1997), injection (Monaghan 1993), or added to food (Honeyfield et al. 2006).

Another marking method, photonic marking, produces a subdermal mark in the fish fins through high pressure injection of rigid microspheres filled with a colored dye suspended in a biocompatible fluid (Catalano et al. 2001). Photonic marking has been evaluated in salmonids (Hayes et al. 2000), centrarchids (Catalano et al. 2001), temperate basses, and osmerids (Sutphin 2008). These studies revealed mark retention can be successful with a variety of fish species, depending on the tag location, injection pressure, and time after marking. Photonic marking of adult Delta Smelt resulted in survival rates greater than 82.5% through 28 d and retention rates >80.0% through 77–105 d postmarking (Sutphin 2008).

The Delta Smelt is considered an environmentally sensitive species because it is primarily an annual species with a relatively low fecundity, exclusively planktivorous, and endemic to the upper San Francisco estuary, California (Moyle et al. 1992; Sweetnam 1999). Historically, Delta Smelt were one of the most common pelagic fish in this system (McAllister 1963; Radtke 1966), but it was both state and federally listed as threatened in 1993 and uplisted as endangered by the state in 2009. Delta Smelt generates intense management interest because of its central role in water supply conflicts involving over 23 million people and an agricultural industry worth US$25 billion annually (Grimaldo et al. 2009; Lund et al. 2010).

Testing the effectiveness of marking and tagging tools to track the abundance, distribution, and movement of Delta Smelt is a pressing need. Despite the small adult size (approximately 5.0–9.0 cm FL), Delta Smelt sensitivity to handling stress (Swanson et al. 1996) and its relatively narrow range of tolerance to temperature and salinity (Swanson et al. 2000), both calcein marking (Sutphin and Morinaka 2010) and photonic marking (Sutphin 2008) have proven feasible for this species. However, photonic marking was deemed unfeasible for juveniles due to low mark retention and longer marking times (G. Tigan, UC Davis FCCL, personal communication) and higher postmarking mortality (Castillo et al. 2012).

No studies have evaluated the use of calcein for juvenile Delta Smelt or used a combination of calcein and photonic marking. Moreover, the effect of calcein and photonic marking on the vulnerability to predation of marked fish has not been investigated for Delta Smelt. Marking-induced losses due to predation are a relevant consideration in marking studies (Catalano et al. 2001; Mohler et al. 2002; Barker and McKaye 2004; Roberts and Kilpatrick 2004).

The objectives of this Delta Smelt marking study were to evaluate (1) the effectiveness of calcein as a primary mark for batch-marking juveniles and adults, (2) the use of photonic marking as a secondary mark, and (3) predation by Striped Bass *Morone saxatilis* on marked and unmarked smelt.

METHODS

Fish Culture

All Delta Smelt used in this study were produced at the University of California Davis Fish Conservation and Culture Laboratory (FCCL) in Byron, California. Production and feeding regimes of Delta Smelt were based on methods developed at the FCCL (Baskerville-Bridges et al. 2005; Lindberg et al. 2013). Juvenile Delta Smelt ranged from 2.0 to 4.0 cm FL (mean, about 3.0 cm). Adult Delta Smelt (in which we included subadults) ranged from 4.5 to 7.7 cm FL (mean, about 6.0 cm FL).

Marking Protocols

All calcein and photonic marking was conducted at the FCCL from 2008 to 2010 (Table 1). Except for test A3, all tests were conducted indoors.

Calcein marking.—To distinguish calcein marked fish from unmarked fish, we tested SE-MARK calcein on Delta Smelt along with a SE-MARK calcein detector (Western Chemical, Ferndale, Washington). Calcein was our primary marking agent because of its feasibility for different life stages of fishes and by being a faster method for batch-marking than photonic marking. Calcein marking was conducted under the Investigative New Animal Drug permitting process (USFWS 2008). Marking included three stages: (1) pretreatment, i.e., immersing fish 3.5 min in a pretreatment static bath containing 10‰ NaCl

TABLE 1. Tests used to evaluate survival and mark grade intensity for Delta Smelt that were calcein marked at the Fish Conservation and Culture Laboratory. Calcein grading denotes the time between marking and grading. Indicated are the mean fork length and total weight ± SD.

Test	Calcein grading (d)	Fork length (cm)	Total weight[a] (g)	Calcein concentration (g/L)	Immersion time (min)	Number of fish/ treatment	Number of replicates
				Juveniles			
J1	7	3.0 ± 0.3	0.21 ± 0.08	2.5	1, 3, 7	30	2
				Adults			
A1	7	6.0 ± 0.8	1.89 ± 0.78	2.5	3, 5, 7	30	2
A2	7	6.0 ± 0.7	1.90 ± 0.69	5.0	3, 5, 7	30	2
A3	105	6.6 ± 0.7	2.44 ± 0.62	5.0	5	30	4

[a]Total weight (W) for test A3 was estimated from fork length (FL) based on tests A1 and A2: $W = 6.19 \times 10^{-3}\ \mathrm{FL}^{3.15}$ ($r = 0.96$, df = 277, $P < 0.001$).

solution to increase the uptake of calcein (Alcobendas et al. 1991; Mohler 2003) and 40 mg of MS-222/L of water (Finquel, Argent Chemical Laboratories, Redmond, Washington) to anaesthetize fish (Castillo et al. 2012), (2) calcein treatment, i.e., immersion for 1–7 min in a static bath containing calcein at 2.5–5.0 g/L of water (66 × 46 cm; depth = 16 cm), and (3) transfer, i.e., transfer of fish to a freshwater circular tanks (400 L for juveniles, 800 L for adults). For holding and transferring fish during the marking process, we used an egg tray (38 × 31 × 5 cm) with the bottom and top lids covered by plastic mesh (2 × 2 mm; Marisource, Fife, Washington). During marking, dissolved oxygen (DO) was maintained above 8.0 mg/L and the water temperature was kept similar to that in the holding tanks. After marking, water temperature was monitored daily in each posttreatment tank, and DO and pH were monitored every 3–7 d.

Control fish were immersed in treatment containers with calcein-free water (i.e., holding tank water) during each of the corresponding immersion times. In test J1, control fish exposed to different immersion times were transferred to a single posttreatment tank due to space limitations. In tests A1 and A2, however, the control for each immersion time was transferred to a separate posttreatment tank.

During indoor marking, the ambient light was reduced to about 23.0 lx (measured as sunlight) to limit stress. For the outdoor marking, light was attenuated to less than 400 lx by covering the tanks with a canopy tent. Based on preliminary tests, all pretreatments, treatments, and controls were fully covered with black plastic sheeting to reduce stress and increase survival. Post-treatment tanks also had black interiors to reduce stress in captivity (Baskerville-Bridges et al. 2005). The outdoor tanks were covered with a shade cloth to prevent avian predation. Natural daylight in the indoor tests entered through windows and a roller door. No artificial lighting was provided at night.

At 7 d after marking in tests J1, A1, and A2, we determined the survival of Delta Smelt and the quality of calcein marks in six body sections (pectoral, pelvic, and caudal fins; jaw; operculum; scales). Calcein marks in test A3 were graded for adults kept alive 105 d postmarking (Table 1). Fish in test A3 were frozen 24 months before calcein grading.

Calcein grading.—A ranking scale was applied to evaluate the grade of calcein marks (USFWS 2008; Figure 1). Anaesthetized or preserved fish were individually placed in a plastic dish with water, and calcein marks were then graded with the calcein detector inside a black container used as portable dark room. The mark grade for each body section was the average of independent grades conducted by two trained individuals.

For tests J1, A1, and A2, we measured fork length and fish weight when the marks were graded to evaluate whether calcein immersion times affected growth. Tests J1 and A1 were also used to evaluate whether calcein grade varied with fish size.

Photonic marking.—We used pressurized CO_2 guns (POW'R-Ject System, model BMX2000) and BMX2000 photonic marking solutions (cobalt green, cobalt blue, and titanium white; hereafter, green, blue, and white; New West Technologies, Arcata, California). Photonically marked adult Delta Smelt were calcein marked 7 d earlier and used in replicated tests, including 770–783 fish per photonic mark (mean = 6.3 cm FL, SD = 0.8). Control fish marked with calcein at 5.0 g/L for 5 min (n = 120) were used to evaluate the survival of photonically marked fish. Marking pressures of 8.4–11.2 kg/cm^2 were applied in the dorsal, caudal, and anal fins to produce the following four marks: dorsal-green, dorsal-white, caudal-blue, and anal-blue. Before tagging, 5–10 fish were netted at a time from the holding tank and anesthetized approximately 1 min in with MS-222 at 100 mg/L of water. The fin to be marked was positioned directly against a ceramic tile to minimize fin movement due to the marking pressure. The pressurized gun was then triggered perpendicularly to the fin to maximize the amount of photonic pigment retained. Once marked, fish were placed in a circular 800-L holding tank. A 1-h prophylactic treatment of oxytetracycline at 20 mg/L of water and 5‰ NaCl was administered to the tanks for 3 d after marking. Fish mortalities were monitored daily, and survival of adults was evaluated 7 d and 70 d after photonic marking. Detection of photonic marks was conducted 97 d after marking using subsamples of 95–106 fish per photonic mark. Most photonic marks were readily visible, and no attempt was made to grade their intensity; however, a stereomicroscope was used when marking required further verification.

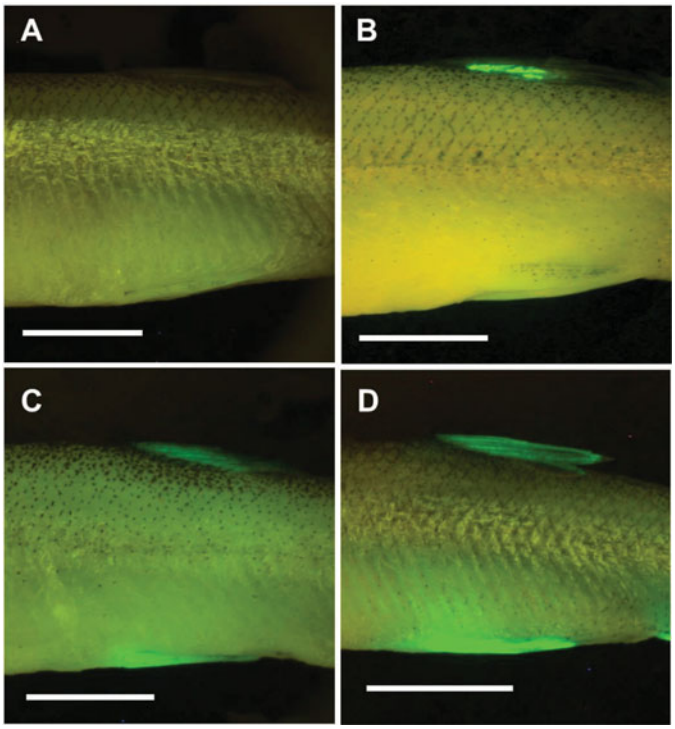

FIGURE 1. Pelvic fins of adult Delta Smelt with different grades of calcein marks: (**A**) 0 = no visible mark (control fish), (**B**) 1 = low intensity mark, (**C**) 2 = intermediate intensity mark, and (**D**) 3 = high intensity mark). Scale: 1 cm. [Figure available in color online.]

Predation on Marked versus Unmarked Fish

To investigate potential mark related predation, cultured adult Delta Smelt (mean = 7.3 cm FL, SD = 0.8) were provided as prey to wild juvenile Striped Bass (mean = 37.5 cm FL, SD = 3.0). Predation was compared between a double-marked fish group (calcein + photonically marked fish) and an unmarked fish group. In addition, predation was compared among three types of photonically marked fish (blue, green, and white) included in the previously referred double-marked fish group. Calcein immersion (5.0 g/L) was applied for 5 min to 150 fish and an additional 150 unmarked fish were used as a control. After 7 d, all calcein marked fish were divided into three groups (about 50 fish/group), and each group was photonically marked in the anal fin with one color.

Striped Bass were selected as the test predator because this species is highly abundant in the Delta (Feyrer and Healey 2003) and considered to be a key piscivore (Nobriga and Feyrer 2007). Wild juvenile Striped Bass (28–40 cm FL) were collected in the South Delta from late winter to early spring; then, they were acclimated in outdoor tanks 14–30 d before predation tests. Based on preliminary tests, Striped Bass were starved 7 d before predation tests to enhance the feeding response.

Four Striped Bass were used in each of the four predation tests and were introduced in a circular test tank (4,000 L) 1 d before each test. Each predator and prey was only used in one test. In three of these tests, 60 Delta Smelt were introduced in the tank (10 fish per photonic mark group × 3 photonic colors + 30 unmarked control fish). An additional test included 30 Delta Smelt (5 fish per photonic mark × 3 photonic colors + 15 control fish). To initiate feeding tests, equal numbers of marked and unmarked prey were transferred into two 19-L buckets and released simultaneously from opposite sides of the test tank.

To facilitate enumeration of uneaten prey and termination of predation tests, a 2.43-m circular net (5 mm mesh) was inserted in the test tank before introducing predators and prey. A hoist system attached to the net was used to quickly terminate each test by lifting all predators and prey out of the water so the remaining prey could be counted after the feeding period. Each test was terminated when about half of the fish had been eaten (within 1–2 h). Predators were then immersed in a lethal solution of MS-222 (200 mg/L) and dissected to remove any eaten Delta Smelt.

Statistical Analyses

The F-test was used in one-way and two-way ANOVAs to compare differences in survival for different calcein immersion times and concentrations. Two-way ANOVA was also used to compare differences in calcein mark grade among factors (body sections and immersion times). The Ryan-Einot-Gabriel-Welsch multiple range test ($P = 0.05$) was further used in these analyses to evaluate significant differences among means. The previous test was also used in test A3 for evaluating differences in calcein grade among body sections and photonic marks. Simple linear regression analysis was used to evaluate possible relations between calcein immersion time and fish size or weight, and between marking grade and fish size. Differences in Striped Bass predation between calcein + photonically marked fish and unmarked groups were evaluated using a paired t-test. The Friedman's test S-statistics (adjusted for ties) was used to compare how many Delta Smelt in each photonically marked group were eaten.

RESULTS

Calcein Marking Survival

Survival of juvenile Delta Smelt immersed in 2.5 g/L calcein ranged from 88.3% to 100% at 7 d after marking (Figure 2A) and immersion time did not affect survival ($F_{3,8} = 1.11$, $P = 0.40$). Water temperature range was 14.8–16.2°C, DO was 8.4–13.8 mg/L, and pH was 7.6–8.3 during the test.

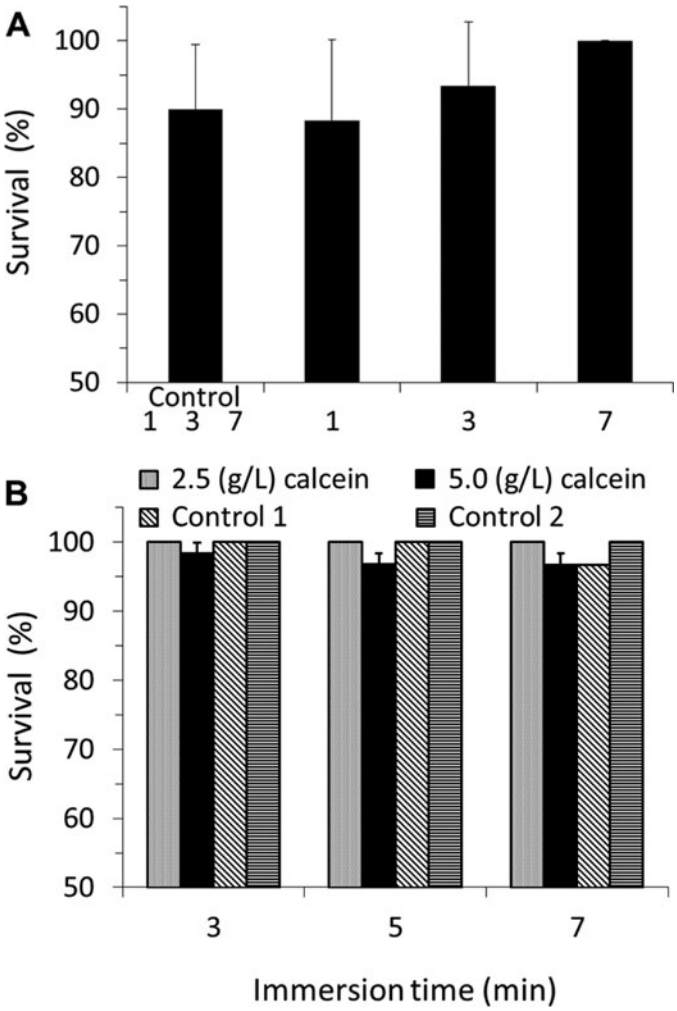

FIGURE 2. Mean (error bars = SDs) survival of Delta Smelt 7 d after marking in relation to calcein immersion times: (**A**) juveniles marked with calcein at 2.5 g/L of water (1–7 min) versus unmarked fish (control), and (**B**) adults marked with calcein at 2.5–5.0 g/L of water (3–7 min) versus unmarked fish (unreplicated controls 1, 2).

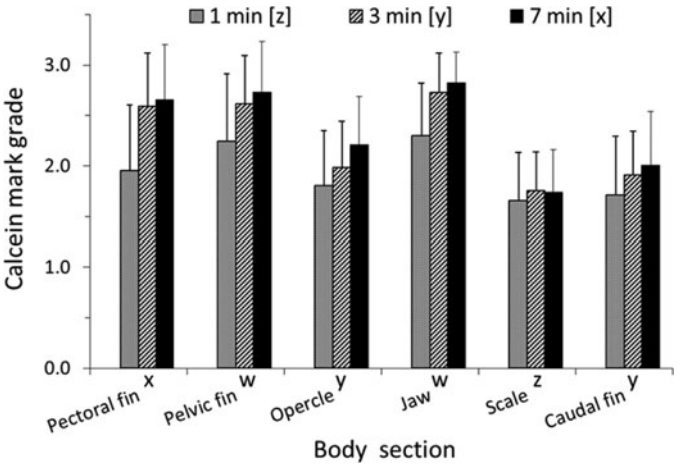

FIGURE 3. Mean (error bars = SDs) calcein mark grades for juvenile Delta Smelt 7 d after being marked with calcein at 2.5 g/L of water (test J1). Significant differences in grade among body sections or among immersion times are indicated by the lack of a lowercase letter in common.

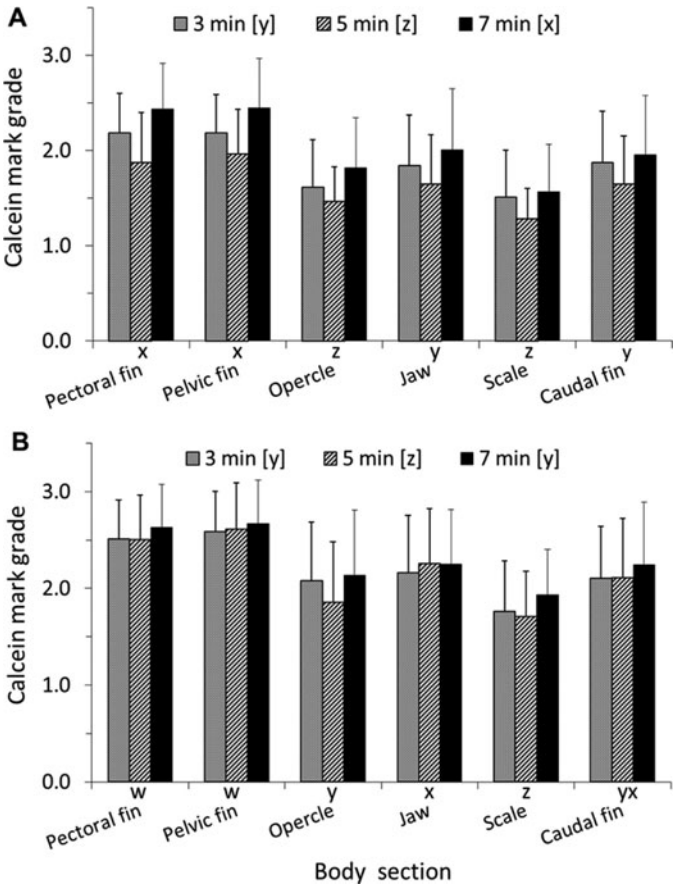

FIGURE 4. Mean (error bars = SDs) calcein mark grade for adult Delta Smelt 7 d after being marked with calcein at **(A)** 2.5 g/L of water (test A1) and **(B)** 5.0 g/L of water (test A2). Significant differences in grade among body sections or immersion times are indicated by the lack of a lowercase letter in common.

The 7-d postmarking survival of adult Delta Smelt in tests A1 and A2 ranged from 97.3% to 100% and was not influenced by immersion time ($F_{2,3} = 0.88$, $P = 0.50$) or calcein concentration ($F_{2,3} = 3.59$, $P = 0.16$; Figure 2B). Water temperature range was 8.0–13.5°C, DO was 8.4–13.5 mg/L, and pH was 7.6–9.3 during these tests.

Calcein Mark Intensity

Calcein marks for juvenile Delta Smelt at 7 d after marking showed highest intensity in the jaw and the pelvic fin ($F_{5,846} = 105.2$, $P < 0.001$), higher calcein grades resulting from longer immersion times ($F_{2,846} = 101.7$, $P < 0.001$) (Figure 3).

No differences (regression analyses) were observed across calcein treatments in test J1 for juvenile Delta Smelt size ($r = 0.11$, $F_{1,221} = 3.85$, $P > 0.05$) and weight ($r = 0.10$, $F_{1,221} = 3.26$, $P > 0.05$).

In adult Delta Smelt, calcein grade was positively related to the calcein concentration at marking (Figure 4A, B; $F_{1,1,042} = 183$, $P < 0.001$). Calcein grade for adult Delta Smelt tended to be higher with longer immersion time under both calcein concentrations, i.e., 2.5 g/L ($F_{2,504} = 132.5$, $P < 0.001$) and 5.0 g/L ($F_{2,504} = 11.4$, $P < 0.001$). Moreover, calcein intensity was consistently higher in the pelvic and pectoral fins than in other body sections under both calcein concentrations: 2.5 g/L ($F_{5,504} = 45.4$, $P < 0.001$) and 5.0 g/L ($F_{5,504} = 31.6$, $P < 0.001$).

Regression analyses suggested no differences in adult Delta Smelt size and weight across calcein treatments under both calcein concentrations: 2.5 g/L (size: $r = -0.08$, $F_{1,360} = 0.49$, $P > 0.05$); weight: $r = -0.02$, $F_{1,360} = 0.19$, $P > 0.05$) and 5.0 g/L (size: $r = -0.03$, $F_{1,355} = 0.24$, $P > 0.05$; weight: $r = 0.04$, $F_{1,355} = 0.62$, $P > 0.05$). However, over the size range (2.2–6.9 cm FL), Delta Smelt exposed 7 min to 2.5 g/L calcein tests (J1 and A1) showed an inverse relation between average mark grade and fish size ($r = -0.41$, $F_{1,118} = 24.48$, $P < 0.001$).

Adult Delta Smelt in the outdoor test A3 showed 100% mark retention 105 d after calcein marking. Relative to the 7-d tests, these fish had consistently lower mark grade in all body sections, pelvic fins showing the highest calcein intensity ($F_{5,696} = 180.6$, $P < 0.001$; Figure 5). Calcein mark grade in test A3 also differed among photonic groups, but to a lower extent than body sections ($F_{3,696} = 14.5$, $P < 0.001$; Figure 5). In test A3, the water temperature range was 7.1–20.0°C, DO was 6.6–15.2 mg/L, and pH was 7.1–8.4.

Photonic Marking Survival

The four groups of photonically marked adult Delta Smelt had high survival, both at 7 d postmarking (mean = 99.3%, SD = 0.9) and at 70 d postmarking (mean = 98.7%, SD = 1.2). The 7 d and 70 d postmarking survival of calcein-only controls was 100%; thus, there was no significant effect of photonic marking on survival of adult Delta Smelt.

Detection of photonic marks for adult Delta Smelt was 100% in the four marked groups, at least over a period of 97 d. The

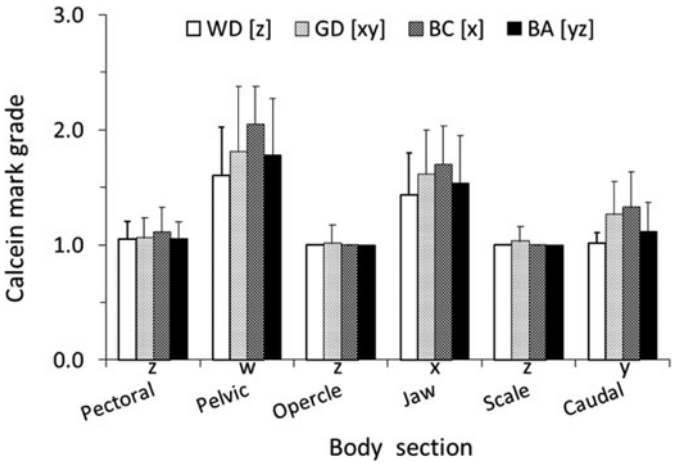

FIGURE 5. Mean (error bars = SDs) calcein mark grade for adult Delta Smelt that also were photonically marked (WD = white-dorsal, GD = green-dorsal, BC = blue-caudal, and BA = blue-anal) and maintained alive 105 d after marking. Significant differences in grade among body sections or photonic mark groups are indicated by the lack of a lowercase letter in common.

photonic marks were readily visible to the naked eye in the great majority of fish: green-dorsal (97%), white-dorsal (90%), blue-caudal (99%), and blue-anal (96%). Photonic marks were detected in all remaining fish under dissecting scope magnification.

Predation Tests

No difference in Striped Bass predation between unmarked and marked Delta Smelt (calcein + photonically marked) was evident ($t = -0.08$, df = 30, $P = 0.93$; Figure 6). The numbers of Delta Smelt eaten by Striped Bass were not significantly different among the three photonic colors (S-statistics = 1.73, df = 2, $P = 0.42$; Figure 6).

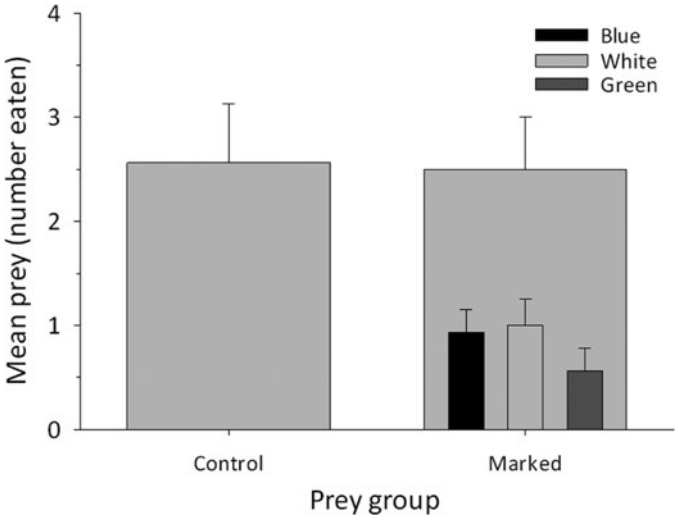

FIGURE 6. Mean (error bars = SDs) numbers of unmarked (control) and calcein + photonically marked (blue, green, white) adult Delta Smelt preyed upon by juvenile Striped Bass in four predation tests.

DISCUSSION

Delta Smelt can be readily batch-marked using calcein, with high survival and mark retention over a period of at least 7 d (juveniles) and 100 d (adults). The combined use of calcein and photonic marking for adult Delta Smelt resulted in high postmarking survival and 100% retention of both marks, of at least 97 d, enabling multiple groups of fish to be distinguished from each other. Photonic marking (Sutphin 2008) and calcein marking (Sutphin and Morinaka 2010) also have been used to double-mark adult Delta Smelt (Castillo et al. 2012), enabling instant and noninvasive mark detection and minimizing processing time.

Calcein mark intensity has been shown to decline over time in several fish species (e.g., Negus and Tureson 2004; Elle et al. 2010). In addition to differences in calcein concentration, immersion time, and postmarking time, the differences in marking intensity among body sections, within, and between life stages is attributed to the degree of calcified structures to which calcein binds (Wilson et al. 1987), and to their proximity to the epidermis and dermis through which calcein must first penetrate and then fluoresce in response to blue light. Hence, both the degree of calcification and skin structure may have influenced the inverse relation between calcein grade and Delta Smelt size reported here. Nevertheless, Negus and Tureson (2004) showed no differences in initial calcein intensity for Rainbow Trout and Chinook Salmon marked at different ages, and longer mark retention in fish marked at older life stages.

Calcein mark retention can also decrease with the duration of light exposure (Honeyfield et al. 2008; Hill and Quesada 2010). For example, calcein marks on Rainbow Trout fry reared in outdoor raceways deteriorated significantly within 8 d of marking (Elle et al. 2010). However, Mohler (2003) detected calcein marks 17 months after marking in all juvenile Atlantic Salmon *Salmo salar* reared indoors and after marking Atlantic Salmon residing 12 months in the wild. In the case of Delta Smelt, calcein mark intensity was lower but mark retention was still 100% for fish maintained in partially shaded outdoor tanks for up to 30 d (juveniles) and 90 d (adults; Castillo et al. 2012). Sutphin and Morinaka (2010) reported calcein marks on adult Delta Smelt after 42 d were less brilliant than marks after 3 and 21 d but were still clearly distinguishable. Mohler (1997) reported nearly 240 d of calcein mark retention in caudal fin tissue from over 93% of marked larval Atlantic Salmon held in captivity. Thus, both age at marking (Frenkel et al. 2002) and sunlight exposure (Leips et al. 2001; Logsdon and Pittman 2012) may play a role in the attenuation of calcein marks over time. Moreover, sunlight exposure may be influenced by factors such as water turbidity, fish distribution in the water column, and available cover.

At specific calcein concentrations, doses, and immersion times for other species no effect on postmarking survival (Beckman et al. 1990; Frenkel et al. 2002), growth (Leips et al. 2001; Mohler 2003; Negus and Tureson 2004), and vulnerability to predation (Mohler et al. 2002) have been reported. Likewise,

over the range of calcein concentrations and immersion times we report here, calcein marking did not significantly influence the survival or growth of juvenile and adult Delta Smelt.

Sutphin and Morinaka (2010) reported the 3-d mean survival of age-2 Delta Smelt marked with calcein or calcein in 8‰ NaCl solutions ranged between 84.3% and 96.7%, and the 42-d mean survival was 80.0%. Possible reasons for the higher survival of Delta Smelt in our study could be the use of age-1 fish, as opposed to older and mature fish, and the use of SE-MARK—calcein manufactured specifically for use on fish and sold as a liquid buffered to a pH of 7.0—instead of preparing a marking solution from an alternative solid calcein brand; which could have resulted in a less soluble and less pH buffered marking agent.

The observed Delta Smelt survival in our calcein marking tests may not apply outside the range of water quality variables and light conditions tested. Juvenile Delta Smelt in our study had slightly lower calcein postmarking survival than adults, implying higher physiological sensitivity, including light exposure (Lindberg et al. 2013). Although the 90% average control survival of juvenile Delta Smelt in test J1 tentatively suggests immersion time did not greatly influence survival (Figure 2A), such interpretation would not be possible if the combined control survival for different immersion times in holding tank water had been lower or if the exposure of control fish to ambient light had not been greatly minimized. That emphasizes the need for appropriate consideration of controls in the experimental design.

Comparing calcein intensity by photonic groups was deemed important to verify whether the calcein grade differed among photonic mark groups (Figure 5). Calcein grade could be inadvertently influenced by grading biases due to double-marking in the same body section. In the case of test A3, one of the calcein-marked body sections (caudal fin) coincided with the location of one of the photonic marks (blue caudal). Yet, we found no evidence that photonic marks in the caudal fin influenced the assigned calcein mark intensity relative to nondouble-marked fins. Besides, calcein grade could be influenced by differences in light exposure among the holding tanks containing each double-marked group. In this case, however, the overall differences in calcein grade among photonically marked groups after 105 d of calcein marking were less apparent than the differences among body structures (Figure 5).

Equal vulnerability to predation between marked and unmarked fish is a relevant assumption in mark–recapture studies (Guy et al. 1996). Our predation tests suggested no differences in predation induced mortality between marked (calcein + photonic) and unmarked Delta Smelt and no differences in mortality among the three photonic marked groups. Although we did not evaluate size-selective predation as part of this study, mark–recapture experiments for Delta Smelt conducted in a reservoir populated by Striped Bass and other predators suggested size-selective predation for juveniles but not adults (Castillo et al. 2012).

Though predator satiation can influence selectivity of different prey species (Ivlev 1961), the results of our laboratory tests are consistent with field mark–recapture tests that considered the same photonic colors used in our predation tests (Castillo et al. 2012). In the referred field study, fish were released to a reservoir where fish losses are all attributed to predation by Striped Bass and other piscivores (Kano 1990; Clark et al. 2009). Based on the February 2009 experiments (Table 3 in Castillo et al. 2012), no significant differences between the observed and expected numbers of four photonically marked groups of Delta Smelt released and lost in the referred reservoir were detected ($\chi^2 = 0.55$, df = 3, $P > 0.90$).

Although photonic marking enables researchers to recognize multiple groups of fish based on different combinations of fins and marking colors (Sutphin 2008; Castillo et al. 2012), the advantages of calcein over photonic marking include quick batch marking that negates the need to handle the fish individually, higher survival, and effective application to very small fish (Negus and Tureson 2004). Excluding labor cost, calcein marking is estimated at US$0.01/fish (Sutphin and Morinaka 2010) and photonic marking at $0.10/fish (Sutphin 2008). Labor costs per fish decrease with the number of calcein-marked fish and can be substantially lower than photonic marking when marking thousands of fish. If calcein marking involves fish for human consumption or fish for release in the USA; then, guidelines for Investigational New Animal Drug may apply (USFWS 2008).

Our study supports a range of applications for calcein and photonic marking methods in short-term mark–recapture studies, including entrainment at water diversions, survival under different hydrological conditions, and fish migration between rearing and spawning areas. We recommend further evaluations of calcein and photonic mark retention, growth, and survival for mark–recapture studies intended to last over 30 d for juveniles and 90 d for adults and for marking effectiveness where Delta Smelt are maintained under different conditions before release. We also suggest further evaluations of alternative marking methods for juvenile and adult Delta Smelt, including fluorescent elastomer (e.g., Bonneau et al.1995), visible implant alpha–numeric tag (e.g., Lindberg et al. 2013), natural marks (e.g., Van Tienhoven et al. 2007), and transgenerational marking (Hobbs et al. 2012) for longer-term studies.

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