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Evaluation of Calcein Immersion for Batch-Marking Fish: Survivability and Mark Retention in Delta Smelt and Sacramento Splittail

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by

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ABSTRACT

We evaluated performance of calcein for batch-marking juvenile Sacramento splittail (*Pogonichthys macrolepidotus*) and adult delta smelt (*Hypomesus transpacificus*). We completed 3-d experiments to measure retention rates and survival of calcein marked splittail and smelt, and a 42-d experiment to measure long-term mark retention and survival of calcein marked delta smelt. Calcein marks were consistently bright and distinguishable 3 d after marking. Calcein marks on delta smelt after 42 d were less brilliant compared to marks after 3 and 21 d, but were still clearly distinguishable compared to unmarked smelt. Our results indicate calcein has no negative effect on 3-d survival of splittail or delta smelt. Mean 3-d survival of splittail marked with calcein or calcein + NaCl (at 8‰) solutions were 99%, whereas mean 3-d survival of delta smelt marked with the same solutions ranged from 84.3 to 96.7%. Mean 42-d survival of delta smelt was 80%. Our results suggest batch-marking of calcein may be a suitable technique and agent for mass marking fish.

Key words: Calcein, batch-marking, fish marking, delta smelt, Sacramento splittail

INTRODUCTION

Short-term (<2-d) mark-recapture studies are an important tool employed at California Sacramento-San Joaquin Delta (SSJD) fish collection facilities to measure effects of operational conditions on fish capture efficiency (Karp *et al*. 1995, Bowen *et al*. 1998, Sutphin and Bridges 2008). Such studies are important because species of fish endemic to SSJD, including Sacramento splittail (*Pogonichthys macrolepidotus*) and Endangered Species Act listed delta smelt (*Hypomesus transpacificus*), commonly encounter these facilities (Sweetnam and Stevens 1993, Bennett 2005). Determining an appropriate marking agent and method of application appropriate for various sizes, species, and life stages of fish prove challenging for biologists (Sutphin 2008). A suitable mark must be retained for study duration, be easily identified, and should not affect capturability, health, or survivability of the marked individual (Stott 1968, Wydoski and Emery 1983). Because of high densities of predatory fish like striped bass (*Morone saxatilis*) observed at these facilities, cryptic nature of a mark should also be considered (Liston *et al*. 1994, Portz 2007).

Marking methods that have been investigated for use at SSJD fish collection facilities include: (1) fin clipping, which requires loss of an important swimming apparatus and may result in injury, infection or death, regeneration of fins, and subsequent absence of a noticeable mark (Nicola and Cordone 1973), and (2) BMX-1000 (NEWWEST Technologies, Santa Rosa, California) injectable photonic marking solution, which has shown to be a viable marking agent for delta fish species, but has variable retention rates (Portz 2007, Sutphin 2008). Because each fish must be individually marked, each of these techniques may be inefficient for a study design that requires marking of a large number of fish. Therefore, a batch-marking agent may be more appropriate for research conducted at SSJD fish collection facilities because it can be applied rapidly to numerous individuals simultaneously, minimizing handling stress to fish and the time devoted by researchers (Bashey 2004).

We investigated use of calcein as a suitable batch-marking agent for juvenile splittail and adult delta smelt. Calcein is a fluorochrome dye that binds to calcium and brands all calcified tissues (*i.e.*, fins, jaw, otoliths, *etc.*) in fish (Mohler *et al*. 2002, Mohler 2003) and has been successfully used as a marking agent for SSJD fish species, including Chinook salmon (*Oncorhynchus tshawytscha)* and rainbow trout (*Oncorhynchus mykiss*) (Frenkel *et al*. 2002, Negus and Tureson 2004). It is typically applied through immersion techniques (bath), but can also be injected or presented orally (Monaghan 1993, Thomas *et al*. 1995). When excited with blue light (495 nm), calcein emits a bright yellow-green (~520 nm) fluorescence, producing a highly visible mark on fish. At high concentrations or long exposure time, this agent can be lethal to fish (Brooks *et al*. 1994, Bumguardner and King 1996, Mohler 1997), but at right exposures has proven to be suitable for batch-marking fish, neither affecting survivability (Beckman *et al*. 1990, Thomas *et al*. 1995, Monaghan 1993), growth (Frenkel *et al*. 2002), or predation rates (Mohler *et al*. 2002).

The primary objective of this study was to determine if calcein bath immersion for juvenile splittail and adult delta smelt is a suitable batch-marking agent and technique. We tested this by evaluating fish survival for 3 d post marking and by observing mark longevity and intensity of fish marked using a calcein bath over a 42-d period. Our secondary objective was to determine whether a pre-treatment salt bath (8‰ NaCl) or increased salinity (8‰ NaCl) during immersion would have an effect on fish survival or mark quality. Exposing fish to a pre-treatment salt bath (osmotic induction) is a method recommended by Mohler (2003) to improve the diffusion of calcein into calcium structures of fish and improve mark quality.

METHODS

Fish Source and Care

Cultured adult delta smelt (52–77 mm FL) and juvenile splittail (38–55 mm FL), obtained from University of California-Davis Fish Conservation and Culture Laboratory (FCCL), were used for calcein immersion experiments. Due to facility space limitations, two research facilities, FCCL and U.S. Bureau of Reclamation (Reclamation) Tracy Aquaculture Facility (TAF), were used for fish holding and testing. Pre-test fish holding and 3-d post treatment analysis at TAF were completed using covered, outdoor, grey, 400-L cylindrical plastic tanks (55-cm x 71-cm) maintained on a flow-through system at approximately 4 L/min. Makeup water from the Delta Mendota Canal was cleansed using a Baker Hydro Filtration sand filter (Waterco Ltd., Sydney, Australia; ~80 L/min) before use. Pre-test fish holding and 3-d post treatment analysis at the FCCL was completed using indoor, black, 400-L cylindrical fiberglass tanks (114-cm × 61-cm) maintained on a recirculation system at approximately 4 L/min. Makeup water from the Clifton Court Forebay was cleansed using a settling basin for solids removal and ozone for disinfection. Recirculated water was cleansed using an ultraviolet light filter, beadfilter, and a moving bed biofilter loaded with kaldness media. Diet, feeding regime, and water quality parameters measured were the same at both facilities. Splittail were fed Silver Cup Fry Salmon Feed (Murray, Utah) and supplemented with bloodworms throughout testing. Delta smelt were fed a 2:1 mixture of Lancy (600–800 µm) and Hikari plankton (Kamihata Fish Industries, Hayward, California; 370 µm) throughout testing. Both species were fed at 4% body weight/d. Dissolved oxygen (DO) and temperature were monitored using a pre-calibrated YSI meter (Yellow Springs, Inc., Yellow Springs, Ohio), water pH was measured using an Oakton pH meter (Vernon Hills, Illinois), and total ammonia was measured using a water quality test kit (LaMotte Company, Chestertown, Maryland).

Batch-Marking of Test Fish

Immersion in a calcein solution was used to mark fish in all experiments. All calcein ($C_{30}H_{26}N_2O_{13}$) was obtained, in powder form, from Sigma-Aldrich Corporation (St. Louis, Missouri). Calcein immersion solutions were prepared at a concentration of 5 g/L. Due to acidifying effects of calcein, water pH was adjusted to a level similar to

ambient rearing waters (6.8–7.2) using sodium bicarbonate. A pre-treatment bath of 8‰ NaCl and 20 mg/L Tricaine methanesulfonate (MS-222) was prepared for use prior to all treatments to aid in the induction of calcein.

During marking, fish (9–10/treatment) were placed in a soft-meshed brine shrimp net (16-cm × 22-cm) and transferred to a black plastic tray (46-cm × 15-cm × 15-cm, 10-L) for a 4-min immersion in the pre-treatment bath. Fish and net were then briefly (<10s) submersed in a freshwater solution to remove excess salt, and then transferred to the calcein bath for another 4-min immersion. Ten and 15 repetitions were conducted for splittail and smelt, respectively, at TAF and with the following treatment conditions: (1) control: MS-222 (20 mg/L), (2) treatment A: MS-222 (20 mg/L) and calcein, and (3) treatment B: MS-222 (20 mg/L) and calcein + NaCl (8‰ uniodized NaCl). Twelve additional replicates were conducted for delta smelt at FCCL using the same treatment conditions minus treatment A. Use of MS-222, a known fish anesthetic, during batch-marking was employed because during initial pilot testing, we observed both species becoming agitated and stressed when submerged in both calcein and calcein + NaCl solutions. Delta smelt were intensely agitated and attempted jumping from the net and swam erratically into each other and the netting when not anesthetized. Use of NaCl during calcein marking provided a brief prophylactic treatment to aid in protection from pathogens and aided in osmotic balance of blood ions.

DO and temperature were monitored using a pre-calibrated YSI meter (Yellow Springs, Inc., Yellow Springs, Ohio), and water pH was measured using an Oakton pH meter (Vernon Hills, Illinois) during calcein immersion.

Short-Term Survival and Mark Retention

Splittail and delta smelt acute mortality and mark retention experiments were performed at TAF between November and December 2004. Water quality and survival were checked daily during testing. Fish from all treatments were culled 3 d post treatment. After culling, calcein marks were detected using a high intensity blue (495-nm) LED flashlight, and an orange acrylic sheet was used to act as a barrier filter to block out excess blue light. All mark detection and intensity verification was performed in a dark room, without influence of external light sources. Fish measurements were recorded after mark examinations. Photographs of fish, aiding in the qualitative comparison of mark retention and intensity, were taken using a Nikon Coolpix 995 digital camera (Nikon Corporation, Tokyo, Japan) fitted with an orange lens filter.

Chronic Mortality and Long-Term Mark Retention

Additional delta smelt experiments were performed at FCCL in February of 2005 to assess both short- and long-term effects of calcein and our marking methods and mark retention over 42 d. Calcein marking, mark detection, 3-d mortality, and short-term mark retention for tests completed at FCCL were the same as those conducted at TAF. However, at the end of our 3-d mortality and mark retention assessment, all calcein marked and surviving fish were transferred to a single 400-L tank. These fish were

monitored daily for mortality, and all fish were checked weekly for calcein mark retention.

Statistical Analysis

A One Way ANOVA was used to test for differences in mean fork lengths (FL) as a function of treatment condition through all experiments. Assumptions necessary to model our 3-d survival data using parametric statistics were not met. Therefore, our data were transformed by rank, and a Two Way ANOVA on ranks was used to test for differences in survival across treatment conditions. Because of temporal differences, no comparisons across holding facilities (TAF vs. FCCL) were attempted and because smelt were combined to a common tank in experiments completed at FCCL, no quantitative analysis was completed on the effects of calcein on 42-d survival and mark retention.

RESULTS

Short-Term Survival and Mark Retention

Exposure to atmosphere was minimal (<2 s) during transfer to and from immersion solutions. During calcein immersion DO levels were kept above 8.0 mg/L with the aid of pure oxygen, and frequent water changes were conducted to assure temperature did not increase 0.5°C, versus ambient, in all treatment baths. During post treatment holding at TAF, splittail and delta smelt holding tank water temperatures were maintained at 10.1 ± 0.1°C (mean ± SD) and 11.1 ± 0.4°C, respectively. Post treatment holding tank water temperatures were maintained at 10.5 ± 0.6°C and 15.9 ± 0.4°C for the delta smelt experiments conducted at FCCL between February 1–4 and February 22– 25, respectively. Total ammonia nitrogen, nitrite, pH, and DO levels were maintained at adequate levels at both facilities as recommended by Meade (1989).

Sacramento Splittail 3-Day Survival — There was no difference in splittail mean FL across all treatments (range = 43.4 – 48.8 mm, $P > 0.05$). When comparing mean (± SD) 3-d survival of splittail, there was no difference in control splittail (100.0%), splittail marked with calcein only (99.0 ± 0.03%), and splittail marked with a calcein and NaCl solution (99.0 ± 0.03%, $P > 0.05$; Figure 1).

Delta Smelt 3-Day Survival — There was no significant difference in smelt mean (± SD) fork lengths across treatments in experiments conducted at TAF (control = 62.4 ± 7.6, calcein = 63.0 ± 6.4 mm, calcein + NaCl = 63.3 ± 7.0; $P > 0.05$). Comparing data from experiments conducted at TFCF we measured no difference in mean 3-d survival of control delta smelt (mean ± SD, 91.8 ± 10.7%) compared to smelt marked with calcein (87.5 ± 13.3%) and smelt marked with a calcein and NaCl solution (84.3 ± 15.0%, $P > 0.05$; Figure 1).

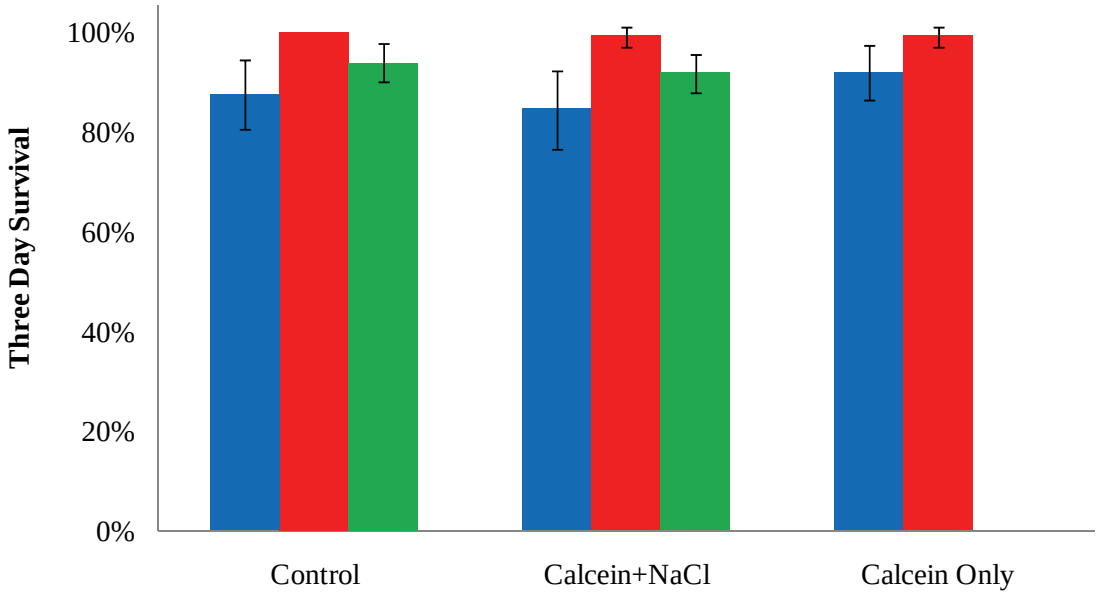

Figure 1.—Mean 3-day survival of juvenile Sacramento splittail tested at Tracy Aquaculture Facility (TAF; red), delta smelt tested at TAF (blue), and Fish Conservation and Culture Laboratory (green) batch-marked by immersion in a calcein solution or calcein + NaCl solution, and control fish. Batch-marking using calcein or calcein + NaCl had no affect on 3-day survival of either species, but when allowing for effects of differences in treatment splittail had significantly greater survival compared to delta smelt. Error bars represent ± two standard errors.

Mean FL (mm) of smelt tested at FCCL were not significantly different across treatments (control = 72.9 ± 8.6 mm, calcein + NaCl = 76.8 ± 8.5 mm; P >0.05). There were no significant differences in mean 3-d survival of control smelt (mean ± SD, 95.0 ± 6.7%) or smelt marked with a calcein and NaCl solution (96.7 ± 6.5%) tested at FCCL (P >0.05; Figure 1).

Smelt and Splittail 3-Day Mark Retention — Examination of fish, using a blue LED flashlight and filter, showed that all fish introduced to either treatment type, calcein or calcein + NaCl, displayed distinct bright green-yellow markings 3 d after marking. Marks were easily visible, in both species, on outer edges of scales and around mouth and head, but predominant on fin rays (Figure 2). Fish marked with a treatment of calcein only displayed a more intense yellow-green fluorescence compared to those marked with calcein + NaCl solution in all experiments. Marks were not visible without the aid of the blue LED flashlight and filter. Control fish did not retain the same green-yellow marks indicative of treatment fish.

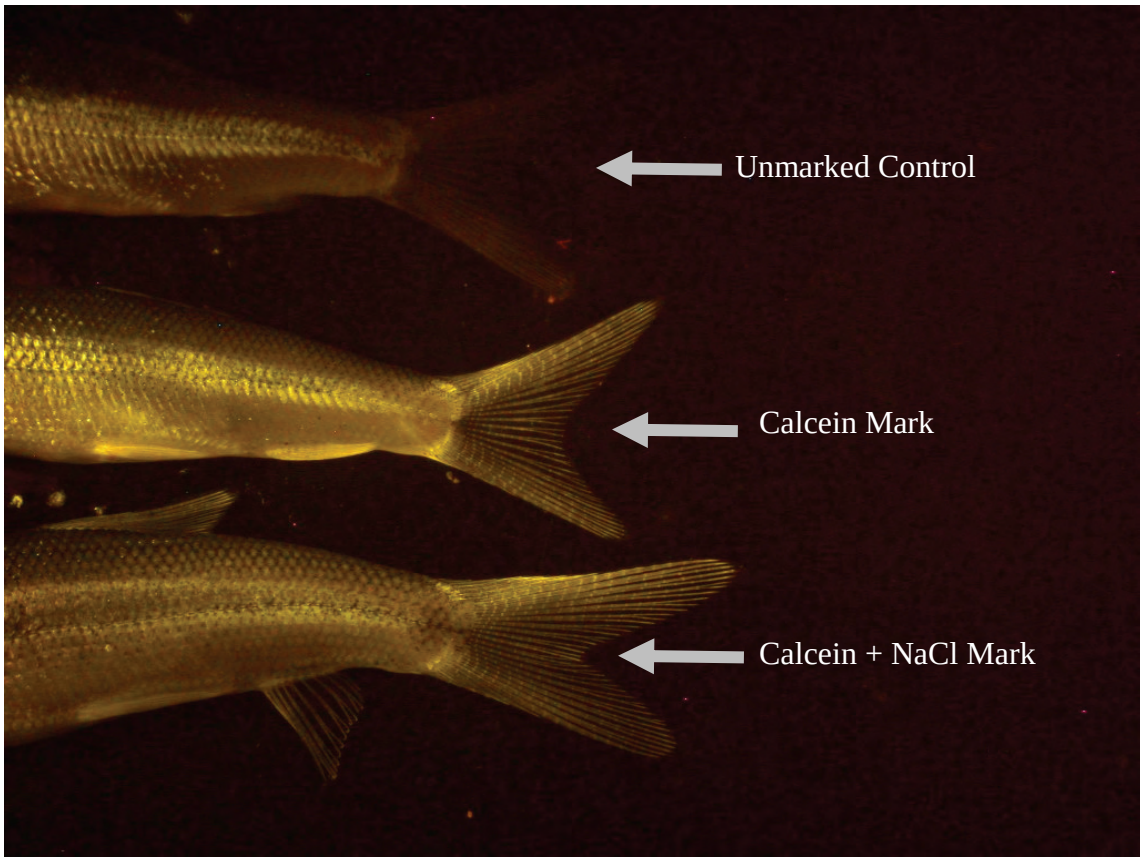

Figure 2.—Sacramento splittail not marked (top), and 3 days after being marked by immersion in a calcein solution (5 g/L; middle), or marked with a calcein + NaCl solution (8‰; bottom) viewed under filtered (via orange acrylic) high intensity blue light (495 nm).

Chronic Mortality and Long-Term Mark Retention

Delta Smelt 42-Day Mark Retention — Delta smelt marked with calcein had a 42-d group survival rate of 80.0%, respectively. Under filtered blue light, calcein marks were visible in head area (jaw and opercle), dorsal fin, pectoral fins, pelvic fins, anal fin, and caudal fin of all smelt surviving 42 d. A visual comparison of images taken of delta smelt 3, 21, and 42 d post marking suggests marks faded slightly over 42 d, but were still easily distinguishable when compared to unmarked fish (Figure 3).

Species Comparison

Allowing for effects of differences in treatment, splittail survival rates were greater than those for delta smelt ($P < 0.05$). Only delta smelt were batch-marked in the third experiment. Though delta smelt survival rates appeared similar to those conducted in experiments completed at TAF, no cross species comparisons were attempted using

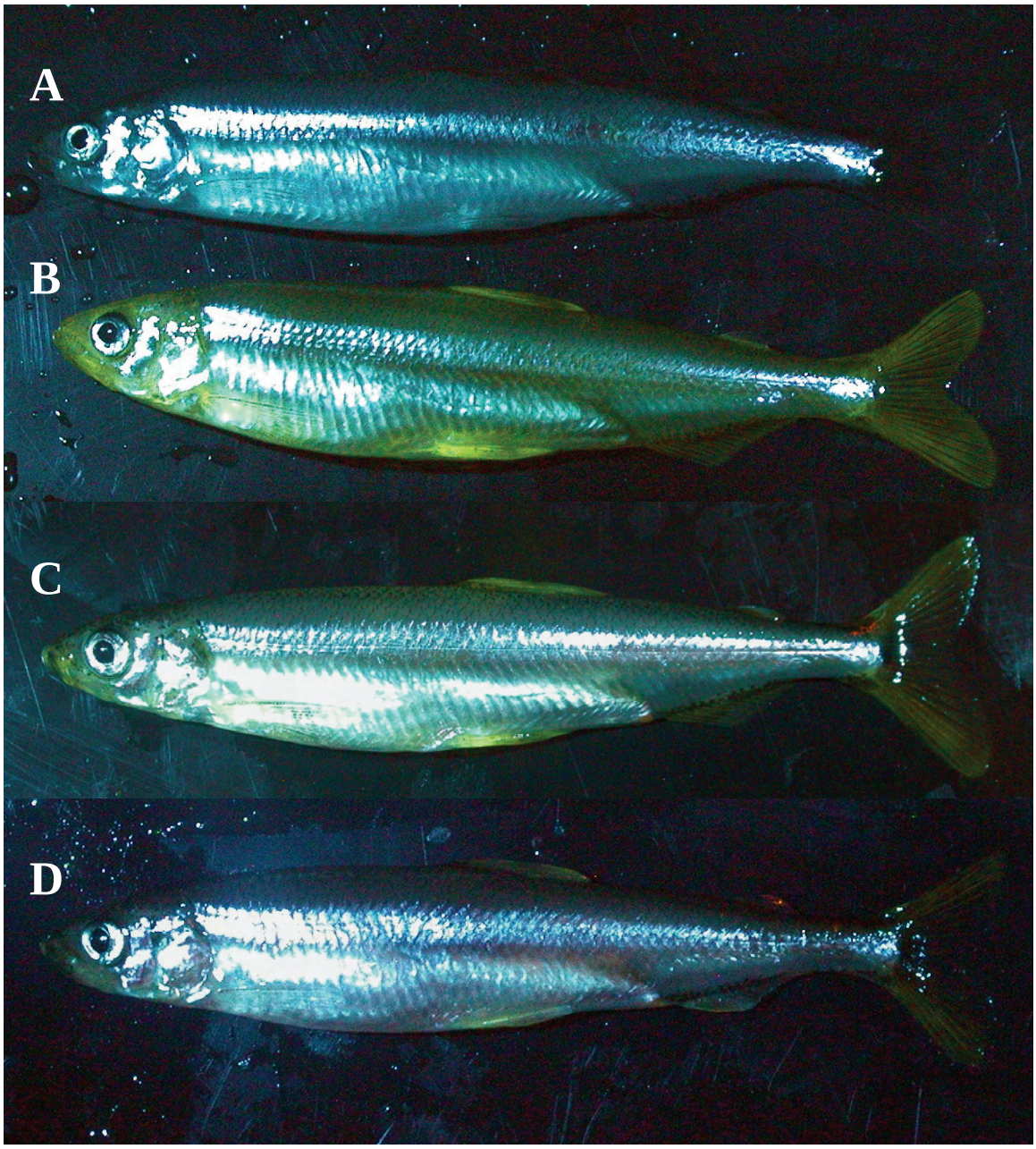

Figure 3.— Delta smelt not marked (A), and 3 (B), 21 (C), and 42 days (D) after being marked by immersion in a calcein (5 g/L) + NaCl (8‰) solution and viewed under filtered (via orange acrylic) high intensity blue light (495 nm).

fish from experiments conducted at FCCL, against all other experiments, due to temporal differences and differences in post treatment holding environments.

DISCUSSION

Immersion in a calcein bath, with and without the addition of NaCl, was effective as a batch-marking agent for juvenile splittail and adult delta smelt. Calcein marks were easily distinguishable compared to unmarked splittail through 3 d and for delta smelt through 42 d. Both species tolerated, with no initial mortality, both calcein treatments. The calcein concentration (5 g/L) we used for batch-marking is well below the 96-h lethal levels (96-h LC_{10} = 160 mg/L) to juvenile striped bass (Bumguardner and King 1996). Also, calcein exposure time during marking was only 4 min, and we detected no difference in mortality of control and marked fish. We attribute the decline in survival rates of delta smelt compared to splittail in our experiments, in part, to the age and life stage, as well as the sensitivity of the species. The delta smelt used in our experiments were age-2 fish and some female fish from the same stock, not used in experimentation, were noticeably gravid and began projecting eggs shortly after we initiated experiments. Wild delta smelt are generally annual fish, and fish older than 2 years in age are rare because of high mortality rates associated with elevated stress and energetic demands of spawning (Moyle 2002). Cultured fish are more likely to live past age-2, but also see increases in mortality after first and second spawning seasons (Bradd Bridges 2004, personal communication). Also, delta smelt are notoriously sensitive to handling stress (Swanson *et al*. 1996, Swanson *et al*. 1998). So it is possible handling stress and prolonged confinement in dip nets resulted in mortality of some smelt.

Because post batch-marking analyses, for both species, were of a relative short duration (3 d) and the intended use of this agent at SSJD fish collection facilities is for short-term mark and recapture studies, we did not quantify effects of calcein on growth, long-term health or long-term mark retention rates. Other studies have determined immersion in calcein can provide a detectible mark for a relatively long period and has no negative effect on growth of fish (Leips 2001, Frenkel *et al*. 2002, Mohler 2003). Mohler (1997) observed 234-d retention of calcein marks in larval Atlantic salmon (*Salmo salar*). In a later study Mohler (2003) observed obvious calcein markings in all juvenile Atlantic salmon at 17 mo (months) post marking. However, mark retention time is likely size dependent (Frenkel *et al*. 2002, Negus and Tureson 2004), and calcein marks may begin to deteriorate rapidly in some species as early as 6 mo post marking (Negus and Tureson 2004).

Aside from low mortality and mark retention rates, there are a number of other benefits to using calcein for marking fish. Calcein batch-marking can be performed in a time and cost-efficient manner. With two employees and our described methods, fish can be marked at an approximate rate of 1,000 fish/h. Since calcein, appropriately stored in a refrigerated environment, can be re-used multiple times, we approximate cost/fish at $0.01. However, with a larger volume, calcein immersion bath, and larger nets, it would be simple to batch-mark >10 fish/immersion. In comparison, high pressure injection of fluorescent pigments, the most common marking agent and method used to mark delta smelt and splittail at SSJD fish collection facilities, can be accomplished at a rate of

~300 fish/h and at a cost of ~$0.10/fish (Sutphin 2008). As mentioned previously, the cryptic nature of marks used at SSJD fish collection facilities is an important concern. Twenty-four hours after initial immersion, calcein tags were not visible to the human naked eye. Though some fish can reportedly detect different wavelengths compared to humans (Neumeyer 1986), data published by Mohler (2002) suggest calcein provides a camouflaged mark that does not result in differential predation mortality. In contrast, Catalano *et al*. (2001) indicated bluegill (*Lepomis macrochirus*) marked with bright colored injectable fluorescent tags were preyed upon at a higher rate by largemouth bass (*Micropterus salmoides*).

There are a number of factors that may make batch-marking fish with calcein inappropriate for mark and recapture research. Even though it can be used to mark individual fins (Frankel *et al*. 2002), calcein omits a single color mark thereby limiting the number of individual marks to the number of fins present on a particular species. Injectable fluorescent pigments can also be applied to individual fins, and are also available in multiple colors which permit a large number of fins plus color marking combinations (Sutphin 2008). Also, the process of individually marking fins using calcein immersion negates reduced time and cost benefits of batch-marking. Detection of calcein, using our methods, may also be problematical when performing field research. Our experiences indicated calcein marks became much harder to perceive when marked specimens were identified under normal outdoor light conditions. However, there are commercial portable hand-held devices (*e.g*., SE-MARK Detector, Western Chemical, Ferndale, Washington) that may improve calcein mark detection in the field.

In conclusion, our research shows immersion in calcein is appropriate as a batch-marking agent for delta smelt and splittail, and considering the sensitive nature of delta smelt, is likely appropriate for most species of fish resident to SSJD. However, a number of research endeavors should be undertaken to investigate improving suitability of calcein as a batch-marking agent. We recommend measuring long-term growth, health (fungal development), and mortality of delta smelt marked by calcein immersion. Higher concentrations of calcein during marking tend to produce more brilliant marks (Beckman *et al*. 1990, Mohler 1997), but calcein levels as low as 125 mg/L produce adequate marks (Wilson *et al*. 1987, Mohler 1997). Therefore, we recommend testing lower doses and reduced immersion times to reduce exposure time to calcein and also minimize handling and confinement stress during marking. Also, Negus and Tureson (2004) indicated higher salt concentrations during pre-treatment baths resulted in stronger marks. Evaluating effects of higher concentration pre-treatment salt baths may permit use of a lower calcein dosage or shorter duration immersion, possibly improving cost and fish survival.

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