

MANAGEMENT BRIEFS

Immersion of Larval Atlantic Salmon in Calcein Solutions to Induce a Non-Lethally Detectable Mark

JERRE W. MOHLER

U.S. Fish and Wildlife Service, Northeast Fishery Center
Post Office Box 75, Lamar, Pennsylvania 16848, USA

Abstract.—At 60 d posthatch, larval Atlantic salmon *Salmo salar* were immersed in water containing calcein at 125 or 250 mg/L or oxytetracycline at 250 mg/L or in untreated control water. Immersions were static, 48-h treatments. All fish immersed in calcein solutions acquired a chemical mark visible microscopically under long-wave ultraviolet light and manifested as apple-green fluorescence in calcified structures, including fin rays. No fluorescence was detected in fish immersed in oxytetracycline or untreated water when viewed in the same manner. Mortality from the time of immersion through day 10 was significantly higher ($P \leq 0.05$) in 250 mg calcein/L (10.5%) than in 125 mg calcein/L (<1.0%). At 234 d postimmersion, the calcein mark was detected non-lethally in samples of caudal fin tissue from over 93% of calcein-treated fish examined. Results suggest that calcein may be a valuable tool for mass-marking larval fish for long-term hatchery product evaluation.

Fry stocking is a primary management strategy of the U.S. Fish and Wildlife Service and cooperating state fishery agencies for restoring populations of Atlantic salmon *Salmo salar* to the New England states. In order to determine the effectiveness of this strategy in achieving management goals, identification of stocked fish is critical. Therefore, a technique is needed to mark larval (nonfeeding) Atlantic salmon with a readily recognizable tag or mark capable of being non-lethally detected in fry, parr, smolts, and returning adult fish.

Various attempts have been made to mark salmonid fry by mechanical or chemical techniques. None of these methods have been adequately refined or proven practical for marking large numbers of Atlantic salmon fry with a feature that can be non-lethally detected in subsequent life stages of the fish. Kaill et al. (1990) evaluated the use of half-length coded wire tags in emergent pink salmon *Oncorhynchus gorbuscha* and reported short-term retention rates of 93–100% and long-term retention rate estimates of 50–84%. This technique was tested on Atlantic salmon larvae in 1994 by U.S. Fish and Wildlife biologists at the Northeast Fishery Center (NEFC), Lamar, Pennsylvania, but

proved to be ineffective and impractical with this species due to the small size of fry (J. W. Fletcher, NEFC, personal communication). In general, chemical marks induced in fish from immersion in dyes or stains produce short-term marks detectable for days, rather than years. Injection of dyes and stains produce more durable marks, but fish are subjected to greater stresses during handling and marking (Muncy et al. 1990). Immersion, injection, or feeding of fluorescent chemicals, such as oxytetracycline, can produce a mark on calcified fish tissues detectable under ultraviolet (UV) light or through fluorometric techniques (Muncy et al. 1990). Oxytetracycline has been used to mark teleost otoliths for subsequent age validation, but fish must be sacrificed for mark determination. Wilson et al. (1987) reported that calcein, a compound that fluoresces green under long-wave UV light, produced a detectable mark on otoliths of three species of estuarine fish after a 2-h immersion in a calcein solution of 125 mg/L. Calcein chemically binds with calcium and shows a marked increase in fluorescence when complexed with alkaline earth metals (Wallach et al. 1959). The compound has been used as an indicator to determine calcium content of limestone and gypsum (Diehl and Elingboe, 1956), for determining submicrogram quantities of cadmium (Hefley and Jaselskis 1974), as a stain useful for angiography (Oncel et al. 1990), and for fitting soft hydrophilic contact lenses (Refojo et al. 1972). In this experiment, larval Atlantic salmon were immersed in calcein and oxytetracycline solutions to evaluate the effectiveness of the chemicals to produce a mark which could be non-lethally detected in fin tissue.

Methods

Fertilized eggs of anadromous Atlantic salmon from the Connecticut River were obtained in December 1994 and incubated at NEFC in hatchery water (mean water temperature, 7.5°C; pH, 7.5; dissolved oxygen, >11.0 mg/L). Fry began hatching in mid-December and remained in incubator

trays until February 1995. Before the onset of exogenous feeding (approximately 60 d posthatch), the experiment was initiated with four separate treatments: calcein solution at 125 mg/L (C-125), calcein solution at 250 mg/L (C-250), oxytetracycline solution at 250 mg/L (O-250), and untreated water (control). Each treatment consisted of three replicates in cylindrical 6.5-L acrylic hatching jars (USA Models, Inc., Lock Haven, Pennsylvania).

The source of oxytetracycline used to prepare immersion baths was Terramycin-343, obtained as a yellow powder (Pfizer, Inc., New York, New York). Calcein ($C_{30}H_{26}N_2O_{13}$; Chemical Abstracts Registry number 1461-15-0), was obtained as an orange powder from Sigma Chemical Co. (St. Louis, Missouri). Stock solutions were prepared by dissolving weighed quantities of each compound in 2-L graduated plastic containers with measured amounts of hatchery water. The pH of stock solutions was adjusted with sodium bicarbonate to between 7.5 and 8.0 before use in test jars. Test jars were filled with hatchery water minus 300 mL. Two hundred larvae were placed into each jar followed by 300 mL of stock solution mixed to achieve the desired chemical concentration. Single-bubble aeration was introduced via 3.2-mm-diameter plastic tubing to prevent oxygen depletion during the static, 48-h immersion. Ambient-temperature water was allowed to flow around the outside of test jars to keep water temperatures stable. At the end of 48 h, water quality characteristics (pH, temperature, and dissolved oxygen) were recorded and fresh flow-through water was introduced to all jars, thereby flushing test solutions.

At the onset of exogenous feeding, live brine shrimp *Artemia* sp., supplemented with a formulated dry diet, were introduced equitably to each replicate daily. At 7 d postimmersion, fish were anesthetized with tricaine methanesulfonate (MS 222; Argent Chemical Co., Redmond, Washington) at a concentration of 80 mg/L and viewed at 100 \times magnification with incident light fluorescent microscopy to determine mark readability. The microscope (American Optical model 120) was equipped with a vertical illuminator containing a fluor cluster exciter filter that transmitted UV light at a wavelength of 490 nm (Reichert Scientific Instruments, Buffalo, New York). After 10 d, fish were pooled by treatment group into circular culture tanks. Throughout the study, deaths were recorded on a daily basis. At 234 d, fish were judged large enough to withstand the intended sampling

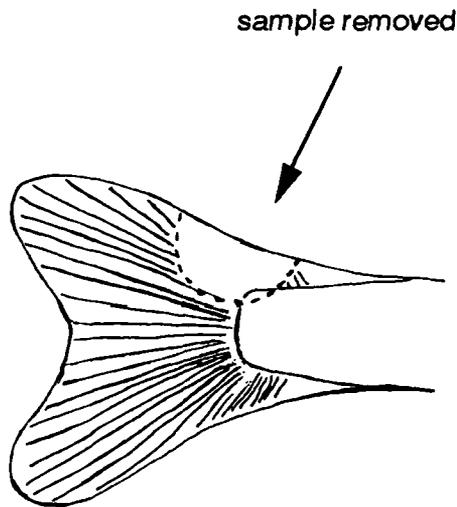


FIGURE 1.—Location of Atlantic salmon caudal fin sample at 234 d postimmersion in calcein solution.

procedure for mark evaluation. Mark readability was then determined by non-lethal removal of a portion of the caudal fin (Figure 1) from 30 fish in each calcein treatment. Fish were anesthetized as above, and fin tissue was excised with a 6-mm-diameter Keyes tissue punch (model RS-6330, Roboz Surgical Instrument Co., Rockville, Maryland). Samples were viewed under fluorescent microscopy as before and marks were scored as brilliant, medium brilliant, dim but detectable, or undetected.

Data concerning water quality and mortality were analyzed with a microcomputer software program (SAS, version 6.04; SAS Institute, Cary, North Carolina) with analysis of variance as described by Littell et al. (1991). Differences between means were compared with Tukey's honestly significant difference (HSD) test. The chosen significance level was $P \leq 0.05$ for all comparisons.

Results

At the end of the 48-h immersion period, mean dissolved oxygen and temperature were similar between treatments, but mean pH of the C-250 treatment (8.12) was significantly lower ($P \leq 0.05$) than for all other treatments (range, 8.56–8.66; Table 1). Mean 10-d mortality of treatment groups ranged from a high of 21 fish/jar (10.5%) in the C-250 treatment to a low of 1.7 fish/jar (<1.0%) in C-125 treatment; mortality was significantly higher ($P \leq 0.05$) in the C-250 treatment (Figure 2). Mean 10-d mortality of control fish was cal-

TABLE 1.—Measurements of pH, temperature, and dissolved oxygen in test jars containing larval Atlantic salmon after 48 h of immersion in calcein (C-125, 125 mg/L; C-250, 250 mg/L), oxytetracycline (O-250, 250 mg/L), or control solutions. Data are means (\pm SD) of three replicate test jars in each treatment group. Within a column means without a letter in common are significantly different ($P \leq 0.05$).

Treatment	pH	Temperature (°C)	Dissolved oxygen (mg/L)
O-250	8.68 (± 0.28) z	7.8 (± 0.29) z	9.76 (± 0.25) z
Control	8.68 (± 0.25) z	7.9 (± 0.31) z	9.37 (± 0.93) z
C-125	8.56 (± 0.12) z	7.9 (± 0.06) z	10.27 (± 0.06) z
C-250	8.12 (± 0.12) y	8.2 (± 0.15) z	9.43 (± 0.42) z

culated with only two of the three replicates due to an overnight loss of 46 fish, which resulted from a water supply failure in one replicate. Mortality between day 10 and day 30 ranged from 15.4% in the C-125 group to 33.6% in the control group (Figure 3).

Gross examination at 10 d postimmersion under fluorescent microscopy revealed that all fish from both calcein treatments had acquired a mark that was detected as apple-green fluorescence in visible bony structures, including fin rays (Figure 4). No mark was detected in the fish that received the oxytetracycline treatment or in controls. At 234 d

postimmersion, 30 out of 31 (96.8%) C-250 fish sampled non-lethally had detectable marks; 28 out of 30 (93.3%) of the C-125 fish had detectable marks. The percentage of marks rated as brilliant was greater (54.8%) for C-250 fish than for C-125 fish (26.7%; Figure 5). No mortality resulted from sampling fish at 234 d postimmersion. Calcein marks in fin tissue samples were still detectable under fluorescent microscopy after 8 months of storage in 70% ethyl alcohol at ambient room temperature. However, an increase in nonspecific (yellowish) fluorescence was observed in samples stored in alcohol.

Discussion

Results of sampling for mark detection 234 d postimmersion showed that calcein marks are generally retained at the point of origin. Loss of brilliance at the distal edge of the marked area of the caudal fin suggests that some calcein may diffuse into developing fin ray tissue as growth occurs. As calcein-marked fish grow, the target area will obviously become relatively smaller and more difficult to detect. However, with the exception of one C-125 fish, results reported for the 234-d postimmersion sampling were obtained from one sample attempt per fish, suggesting that if the proper area is sampled, detection is reliable.

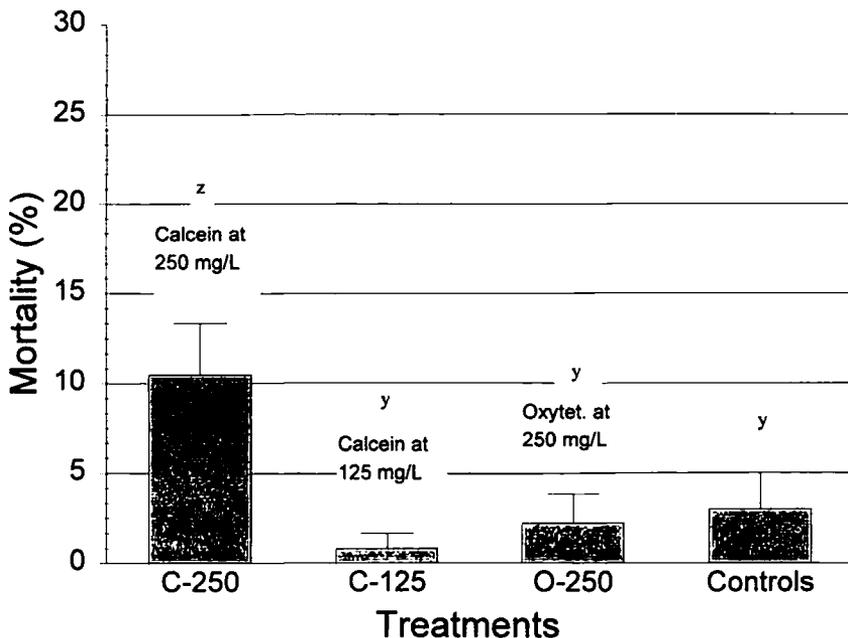


FIGURE 2.—Mean percent mortality of juvenile Atlantic salmon from day 0 to day 10 postimmersion in calcein or oxytetracycline (Oxytet.) solutions. Data are means of replicate test jars with standard deviation shown. Means with different letters are significantly different ($P \leq 0.05$).

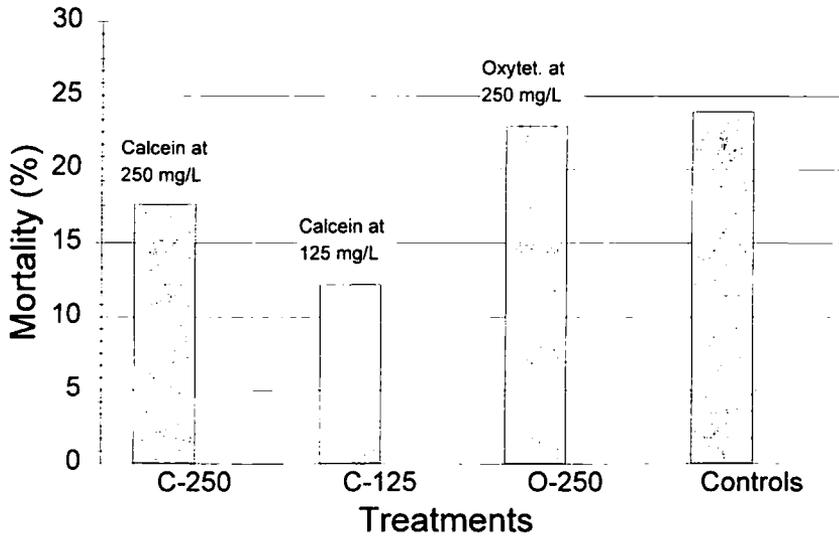


FIGURE 3.—Cumulative percent mortality of juvenile Atlantic salmon from day 10 to day 30 postimmersion in calcein or oxytetracycline (Oxytet.) solutions. Data are total percent mortality of pooled replicates. (Replicates were pooled at day 10.)



FIGURE 4.—Photomicrograph of an Atlantic salmon fry caudal fin viewed under long-wave ultraviolet light at 7 d postimmersion in a calcein solution of 250 mg/L for 48 h. The calcein-labeled fin ray tissue fluoresces a more vivid apple-green color than is shown on this reproduction. Bar = 0.5 mm

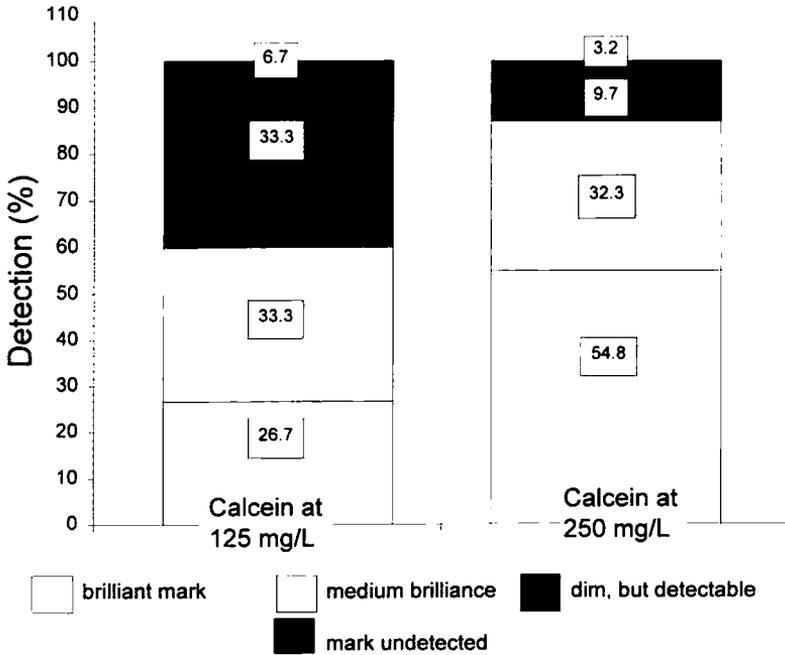


FIGURE 5.—Non-lethal detection of a calcein mark in caudal fin tissue of Atlantic salmon at 234 d postimmersion in calcein at two concentrations.

The significantly higher level of 10-d mortality (10.5%) in C-250 fish may be a reflection of higher toxicity of calcein at that concentration than the other solutions tested. Even though mean pH was also significantly lower in that treatment (Table 1), it was still within the range acceptable for Atlantic salmon culture. Although anecdotal, fish in C-250 treatments were also observed to be less active than those in other treatments during the 48-h immersion. Wilson et al. (1987) reported no mortality in red drum *Sciaenops ocellatus*, Atlantic croaker *Micropogonias undulatus*, or spot *Leiostomus xanthurus* when immersed as juveniles in a solution of estuarine water containing calcein at 125 mg/L. Likewise, in this study, low mortality (<1%) occurred in fish immersed in the 125 mg/L calcein solution.

Use of calcein for mass-marking larval Atlantic salmon before release appears to be a valuable tool for fishery managers, at least during the first year of liberation. By using the calcein-marking techniques described in this paper, fish population studies could be performed non-lethally by sampling the caudal fin tissue and preserving the sample for analysis at a later date. Use of alcohol for sample storage may prove to be unwise due to increased background fluorescence observed in fish tissue stored during this study. A high level

of background fluorescence could add uncertainty to calcein mark determination in samples where quality of the original mark was medium or dim but detectable. Koenings et al. (1986) found natural background fluorescence highly problematic when attempting qualitative determinations of oxytetracycline marks in young sockeye salmon *Oncorhynchus nerka*. This problem was overcome by using a fluorometric method that employed chemical extraction of the oxytetracycline and subsequent measurement with a spectrofluorometer. Most likely, a similar detection technique could be formulated for calcein-marked fish tissue.

Fish immersed in the 250 mg/L calcein solution had a higher percentage of brilliant marks than those immersed in the 125-mg/L solution. This is consistent with observations of Beckman et al. (1990) that the highest calcein bath concentration yielded the brightest calcein marks in otoliths of spotted seatrout *Cynoscion nebulosus*. More study is needed to determine the immersion conditions that will yield the brighter calcein marks most consistently in fin tissue. Additional study is also needed to determine whether chemical composition of source water for immersion affects mortality or mark uptake in larval fish. Ultimately, long-term (2–5-year) analyses of calcein-marked fish is necessary to determine whether the mark

can be detected in presmolt and adult Atlantic salmon.

Even though evaluation of the performance of hatchery-produced Atlantic salmon fry and other fish species may be improved as a result of calcein-marking techniques, U.S. Food and Drug Administration (FDA) approval for use of this compound on potential food fish is required before calcein-exposed fish may be released into the wild. Before the compound could be considered for approval by the FDA, it must go through the Investigative New Animal Drug (INAD) approval process. The first step in the INAD process is to have the compound included on a prioritized list with other compounds awaiting consideration for approval. This can only be initiated by increasing awareness among fishery managers through published information which demonstrates that calcein could be an additional tool for evaluation of stocked fish (D. Erdahl, U.S. Fish and Wildlife Service, personal communication). Regardless of its approval status for use on potential food fish, calcein may have immediate utility for researchers who do not intend to release fish but who need an easily administered batch mark to facilitate comparisons between marked and unmarked groups of fish combined as fry. In summary, data collected in this study showed that, at 250 mg/L, calcein caused higher mortality of larval Atlantic salmon than other treatments. However, subsequent (10–30-d-postimmersion) mortality of pooled replicates was similar between all treatment groups, indicating that calcein-treated fish were able to commence exogenous feeding and survive at rates comparable with those of their study counterparts.

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