North American Journal of Fisheries Management 23:1108–1113, 2003
American Fisheries Society 2003

Producing Fluorescent Marks on Atlantic Salmon Fin Rays and Scales with Calcein via Osmotic Induction

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Abstract.—Calcified tissues of Atlantic salmon in both larval and postscale developmental life stages became labeled with the fluorochrome dye known as calcein in 7 min by sequential immersion in salt and calcein solutions. I introduce the term ''osmotic induction'' to describe this process. At 47 d posttreatment, fish that had been exposed to osmotic induction of calcein as nonfeeding larvae exhibited mortality and growth equivalent to that of nonexposed fish ($P < 0.05$). Atlantic salmon parr (at 5 months posthatch) exposed to osmotic induction of calcein exhibited no mortality during a 120-d posttreatment period. In addition to inducing a calcein mark on fin rays of all larvae and parr, the procedure also produced a distinct calcein mark on scales of parr. A second exposure of parr to osmotic induction 90 d after the first produced a conspicuous 2-band pattern on scales which corresponded to the two separate marking events. Calcein marks are invisible to the unaided eye but were observed without sacrificing the experimental fish by employing a calcein detection device (patent applied for) and fluorescence microscopy. Osmotic induction of calcein marks is a potentially useful technique for batch-marking fish because it greatly reduces the amount of contact time between the fish and marking solution and produces an easily identified mark; this may enable salmon fishery managers and researchers to perform a variety of nonlethal fisheries evaluations not practical or possible using previous marking and tagging techniques.

Fisheries managers often rely on some type of fish marking or tagging to evaluate the effectiveness of strategies employed to meet their management goals. Accordingly, numerous authors have compiled and described techniques and applications for tagging and marking fish (e.g., Everhart and Youngs 1981; Wydoski and Emery 1983; Parker et al. 1990; Nielsen 1992; Guy et al. 1996). Economic considerations, concerns for fish health, and the sheer numbers to be marked dictate the development of a procedure that can mark millions of hatchery-incubated juvenile fishes simultaneously (Volk et al. 1990). One group of marking tools receiving increased attention is a group of chemical marks that rely on fluorescing compounds that label bony or calcified tissues of fish. The most commonly used fluorescent compounds are oxytetracycline and calcein (Guy et al. 1996). Although oxytetracycline is currently widely used for immersion marking, calcein has shown promise to provide an additional marking tool having capabilities of providing fisheries evaluations not possible or practical with previous marking techniques (Mohler 1997; Mohler et al. 2002). Calcein has been evaluated as a method of marking fish otoliths (Wilson et al. 1987; Beckman et al. 1990; Brooks et al. 1994; Bumguardner and King 1996), as well as fin rays, scales, and other calcified tissues (Alcobendas et al. 1991; Gelsleichter et al. 1997; Mohler 1997; Leips et al. 2001; Mohler et al. 2002).

One advantage of applying chemical marks is that large numbers of organisms can be exposed simultaneously in an immersion bath. Most studies previously performed with calcein used immersion baths that, to achieve the desired effect, were static in nature and ranged in duration from a few hours to 48 h. A technique demonstrating considerable reduction in time of contact between fishes and labeling solutions was shown by Alcobendas et al. (1991) where elvers of the European eel *Anguilla anguilla* showed rapid uptake of fluorochrome dyes when immersed in a hyperosmotic solution followed by immersion in labeling solutions. In this study I use the principle of osmotic potential to label calcified tissues of Atlantic salmon fry and parr with calcein by a process I term ''osmotic induction.'' Information on growth, survival, and potential utility of this marking technique is presented to give researchers, fisheries managers, and aquaculturists additional marking tools that may prove valuable for conducting future fishery evaluations.

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Received August 29, 2002; accepted January 13, 2003

Methods

Fry marking.—Atlantic salmon fry used in this marking study were hatched from eggs of Connecticut River domestic bloodstock obtained from White River National Fish Hatchery in Bethel, Vermont. Marking procedures took place while fry were in the yolk sac stage at a developmental index of about 85 (i.e., 85% developed to the point that exogenous feeding is initiated, as determined by tracking temperature units during incubation; P. B. Gaston, U.S. Fish and Wildlife Service, unpublished). The study design consisted of two treatments—a calcein marked group and an unmarked control group—with three replicates each. Each replicate consisted of 200 fry (mean individual weight = 0.18 g) that were batch-marked by placing fry in a plastic strainer during the osmotic induction process. The process was repeated until all replicates were complete with the same marking solutions used for the entire experiment.

The osmotic induction marking procedure required preparation of two solutions: a salt bath and a calcein bath. The salt bath was prepared by dissolving 50 g of noniodized NaCl in 1 L of ambient hatchery water resulting in a 5% salt solution. The calcein bath was prepared by dissolving 10 g of calcein powder (Sigma Chemical Company, St. Louis, Missouri) in 1 L of ambient hatchery water, resulting in a 1% calcein solution. The pH of the solution was adjusted with sodium bicarbonate to a level of 7.2, which was similar to that of the ambient hatchery water at 7.1. Both solutions were poured into stainless steel bowls and acclimated to ambient hatchery water temperature (8.1°C). Osmotic induction marking procedures began by immersing the strainer containing each batch of fry for 3.5 min in the salt solution followed by a momentary immersion in freshwater to rinse off excess salt. The strainer was then momentarily placed on paper towels to absorb excess water. Fry were then immersed for 3.5 min in the calcein solution. Control replicates were exposed to the same salt bath process as the treatment group, but they were not exposed to the calcein bath. Another set of controls to evaluate the effects of the salt bath on fry were not deemed necessary due to previous observational studies at the Northeast Fishery Center (NEFC) that showed no adverse effect on survival of Atlantic salmon fry exposed to similar salt treatments. In addition, the salt bath pretreatment is essential for rapid induction of a consistent, high quality calcein mark when utilizing concentrated calcein solutions (J. W. Mohler, unpublished 1999 Biological Activities Report).

Once processed as described above, each batch was placed into a separate 9-L rectangular plastic tank supplied with ambient (temperature) flow-through water. After a 24-h acclimation period, 1 g of commercially available dry starter feed was introduced to each replicate daily along with 2 mL of decapsulated cysts of brine shrimp *Artemia* sp. in a salt solution. Fish were maintained indoors under the natural photoperiod supplemented with overhead fluorescent lighting from 0800 to 1600 hours. After 22 d of this feeding regimen, brine shrimp cysts were no longer offered and only commercially available dry diet was supplied at a rate of 3 g per replicate daily. At 47 d postimmersion, biomass of each replicate was determined, and fry were consolidated into larger tanks for long-term rearing. Mortality and biomass data from this inventory were compared between treatments using *t*-tests at $\alpha = 0.05$. Subsamples of about 20 fish from each replicate were evaluated for a calcein marks using a hand-held calcein mark detector (SE-MARK; patent pending, Western Chemical Company, Ferndale, Washington), which allows the operator to immediately discern between marked and nonmarked fish by the presence or absence of a visible green fluorescence in the fin rays and other calcified structures.

Parr marking.—At the age of 5 months posthatch (August 14, 2000), Atlantic salmon that were previously unmarked were viewed microscopically to verify scale development. Twenty-five fish (mean weight and length 0.85 g and 46.6 mm) were randomly selected and immersed for 3.5 min in a 1.5% salt solution followed by a 3.5-min immersion in a 1% calcein solution to induce a calcein mark on the scales. No replicated treatments were established with this observational study. At 24 h posttreatment, all fish were examined with the calcein detector. Scale samples were taken from a few fish and examined via a compound microscope outfitted with a blue filter set (model Axioskop with filter set #487909; Carl Zeiss Inc., Thornwood, New York). At 90 d after the first induction (8 months posthatch) two of the previously marked parr were randomly selected from the rearing unit and exposed to a second induction episode, as described above except that the calcein bath was mixed at a lower concentration of 0.5%. At 24 h posttreatment, scale samples were taken and again evaluated microscopically for a calcein mark. In January 2002 (17 months postimmersion), scale-marked parr were reevaluated for mark retention

TABLE 1.—Mean 10-d mortality, 47-d mortality, and final biomass of Atlantic salmon fry over a 47-d period after exposure to calcein (treatment) or hatchery water (controls) using an osmotic induction technique. Within columns, means with different letters are significantly different ($P \leq 0.05$).

Replicate	Mortality (%) at 10 d	Mortality (%) at 47 d	Final biomass (g)
Calcein-treated			
1	0	13	44.0
2	0	17	43.3
3	0	19	43.6
Mean (SD)	0.0 (0.0) z	16.3 (3.1) z	43.6 (0.4) y
Control			
1	1	28	39.9
2	0	24	40.5
3	0	11	42.6
Mean (SD)	0.3 (0.5) z	21.0 (8.9) z	41.0 (1.4) z

and readability using the previously mentioned calcein detection device, as well as fluorescence microscopy. Photos of calcein-marked scales were made using a digital camera (model Fine-Pix S1 Pro; Fuji Photo Film, Inc., Elmsford, New York) mounted on the microscope previously described.

Results

Fry Marking Experiment

Comparison of data from the replicated treatments showed that 47-d mean mortality of fry was not different ($P = 0.438$) between calcein-marked and control fish at 16.3 and 21.0 individuals, respectively (Table 1). Mean tank biomass of calcein-marked fish at 43.6 g was slightly higher ($P = 0.035$) than controls at 41.0 g (Table 1). Visual examination of all fish with the calcein detector showed that fin rays of fish from the calcein treatment were labeled with calcein while those from the control group were not (Figure 1).

Observations of Parr Marking

Visual examination of all fish with the calcein detector showed that fin rays and scales of parr that received a single osmotic induction treatment were labeled with calcein. No mortality occurred in these 25 fish during the first 120 d postimmersion. Scale samples from the 2 individuals that received a second osmotic induction treatment 90 d after the first one were examined microscopically and found to have two highly-distinguishable fluorescent bands (Figure 2).

Fish were re-evaluated at 17 months after the first immersion at which time their mean length was 23.2 cm and weight was 125.8 g. Three mortalities occurred over the 17 months, one of which was a double-marked individual. Survivors evaluated with the calcein detector had easily discernible calcein marks on their scales. At 17 months after the first osmotic induction treatment, microscopic evaluation of scale samples from both single-treated and the double-treated fish revealed obvious calcein marks consistent with the number of immersion episodes (Figure 2).

Discussion

The osmotic induction method I tested considerably reduced contact time between marking solution and fish compared with the first calcein-marking study of early life-stage Atlantic salmon as reported by Mohler (1997). Mean 47-d mortality of control fish appeared to be higher (21.0 individuals, SD 8.9) than that of calcein-treated fish (16.3 individuals, SD 3.1; Table 1). However, due to the high variability, the *t*-test was unable to detect a statistical difference in observed mortality using data from only 3 replicates per treatment. Under normal circumstances, it is not likely that treating fish with calcein increases early survival; however, the accompanying salt bath conceivably affords fish some prophylactic protection from pathogens, which could enhance early survival. Based on observed mortality, we saw no evidence of calcein toxicity to fish in our study.

Mean final biomass of calcein-marked fish (43.6 g, SD 0.4) was slightly higher than controls (41.0 g, SD 1.4; Table 1), but this was probably due to the fact that more control fish died during the study and feed rates were not adjusted to reflect the resulting change in tank biomass. The relatively small difference in mean biomass between treatments accompanied by low variability resulted in the ability of the *t*-test to show a statistical difference between means using data from only three replicates per treatment (Table 1).

By exposing fish to water of relatively high salinity, we created a condition where the external environment was hyperosmotic to body fluids of the fish. Yolk sac larvae of many teleost species can survive in a very wide range of salinities, and their ability to tolerate changes in salinity depends upon (1) the ability of body fluids to function, at least for a short time, in an abnormal range of internal osmotic and ionic concentrations, and (2) the ability of the larvae to regulate the body fluids to restore levels of osmotic pressure to near normal. Furthermore, osmotic and ionic movement takes place through the skin of the larvae to an extent that depends on the salinity of the water

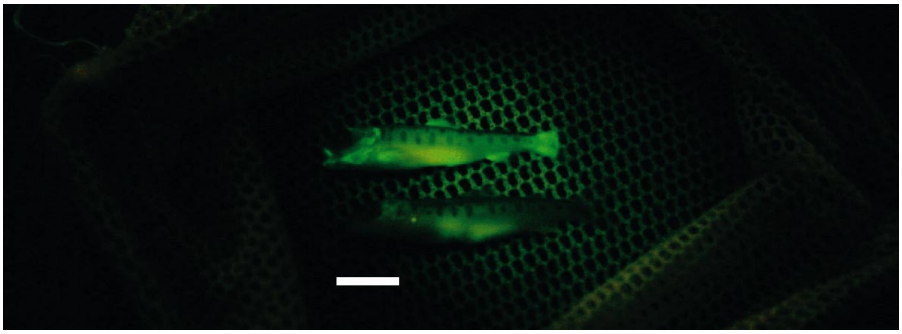

FIGURE 1.—Photograph of a calcein-marked (top) and a control (bottom) Atlantic salmon fry 47 d after the osmotic induction of the fluorochrome calcein. Fin rays and other bony structures fluoresce bright green on the calcein-treated individual. Bar = about 1 cm. Green fluorescence of calcified structures is revealed on the photograph by utilizing calcein detection equipment during photography.

(Holliday 1969). Conte (1969) states that the marine environment causes an obligatory water loss from body fluids (through the skin, gills, and kidney to a minor extent) because of the differential osmotic concentrations. Using this as a model of the water budget in our fish during calcein-marking, we can predict that the salmon will lose a certain amount of water to their environment when placed in the salt bath for 3.5 min. Subsequently, when the fish are abruptly moved into a calcein solution, the resulting osmotic difference results in rapid uptake of the calcein solution as water is replaced via osmosis through the skin and gills. Calcein binds calcium and other metal ions by chelation (Wallach et al. 1959; Hefley and Jaselskis 1974); therefore, once it is introduced to the internal fluids of the fish, it can bind with and label calcified tissues that are present. In the fry-marking study a 1% calcein bath was used, but subsequent observations showed that a 0.5% solution is equally effective in producing calcein marks in Atlantic salmon.

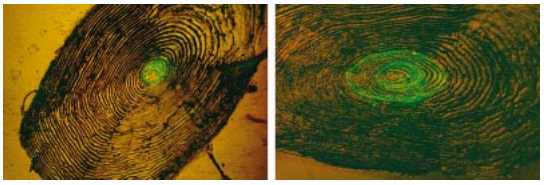

FIGURE 2.—Photomicrograph of Atlantic salmon scales marked via osmotic induction of calcein marks. The scale on the left shows a fluorescent calcein mark at the center, resulting from a single mark induction at about 5 months posthatch. The scale on the right shows an additional calcein band resulting from a second induction at about 8 months posthatch. Scales were removed from the fish and photographed at 17 months (left) and 14 months (right) after their last exposure to calcein, as evidenced by presence of many additional circuli. The photo was taken using fluorescence microscopy at a magnification of 40×.

Calcein mark longevity, as it relates to nonlethal detection in fish, has not been fully investigated, but at NEFC in 2001 it was found that 85% of Atlantic salmon ($N = 26$) immersed as sac fry and reared indoors retained fin ray marks that were immediately recognizable on intact fins and detectable with the nonlethal calcein detection device, even after 3 years of growth (average size 37.8 cm and 607 g). However, marks were not visible in fish with 5 years of postimmersion growth (Mohler, unpublished 2000 Biological Activities Report). Sunlight can negatively affect the fluorescent properties of tetracycline (Choate 1964; Trojnar 1973), but the effect of sunlight on fluorescent properties of calcein has not been investigated. Longevity of calcein marks on fish released into the wild is unknown at this time, but an experimental release and recapture using endangered stocks of calcein-marked Atlantic salmon fry was performed in the West Branch of the Sheepscot River in Maine in 2001 and 2002. Recapture of stocked fish 1 year after release as nonfeeding fry showed that calcein-marked fish were still identifiable via the hand-held calcein detection device (Mohler, unpublished 2002 Biological Activities Report).

Application of the calcein mark via osmotic induction procedures, as compared with a simple long-term immersion, requires a salt bath pretreatment, which could present a problem for large-scale marking programs. However, a pilot study for large-scale marking of salmonids with calcein via osmotic induction was performed at the Craig Brook National Fish Hatchery in Maine in 2001

and 2002. In each year of the pilot study, 30,000 nonfeeding Atlantic salmon fry were marked by allowing them to remain in their respective incubation tray inserts and subsequently transferring each insert into a salt solution and then into a calcein solution. The entire procedure took less than 4 h, minimized handling of fish, and required preparation of only 6 L of calcein solution (M. Millard, U.S. Fish and Wildlife Service, personal communication).

In conclusion, my study showed that the osmotic induction method for applying calcein marks to fin rays and scales of Atlantic salmon is not harmful to the fish and results in saving a considerable amount of contact-time between fish and marking solution. Moreover, these calcein marking techniques will provide an additional tool for researchers and fishery managers to use in performing numerous evaluations previously not practical or possible with existing marking or tagging techniques because the mark can be instantly and nonlethally detected.

In the United States, calcein has not been approved drug by the U.S. Food and Drug Administration (FDA) for use on potential food fish. However, the first step in gaining approval for its use has been initiated through the FDA via an Investigative New Animal Drug (INAD) permitting process. In addition, the U.S. Fish and Wildlife Service has entered into a licensing agreement with Western Chemical, Inc. of Ferndale, Washington, which will result in commercial availability of the calcein detection device.

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