

Evaluation of Calcein as a Mass Mark for Rainbow Trout Raised in Outdoor Hatchery Raceways

F. STEVEN ELLE, MARTIN K. KOENIG,* AND KEVIN A. MEYER

Idaho Department Fish and Game, 1414 East Locust Lane, Nampa, Idaho 83686, USA

Abstract.—Mass marks are useful for evaluating releases of hatchery fishes for management and research purposes. Calcein is a chemical that shows potential for producing a cost-effective batch mark that can be easily applied to large numbers of small fish and can be detected externally by nonlethal means. However, calcein shows some drawbacks related to mark deterioration when exposed to direct sunlight. We evaluated calcein mark retention over time in rainbow trout *Oncorhynchus mykiss* by using both external (head, fins) and internal structures (otoliths), and we compared mark retention between fish reared in normal outdoor raceways under ambient light conditions and those reared indoors in shaded circular tanks. Calcein marks on rainbow trout that were marked as fry and reared in outdoor raceways deteriorated significantly within 8 d of marking and remained at low or undetectable levels throughout the study. Mark quality was much better for rainbow trout reared indoors but still degraded over the 7-month evaluation period. Calcein marks were visible over the entire study period for otoliths of fish reared both indoors and outdoors, with better results being obtained for fish reared indoors. While calcein shows potential for generating a successful nonlethal batch mark, it may have limited use in situations where fish are exposed to sunlight, which can degrade the external mark. Marks in otoliths were retained well but required time-consuming processing and lethal sampling. For use of otolith marks, we recommend combining calcein with some other external mark (e.g., adipose fin clip) to aid in identifying chemically marked individuals.

Mass marks are useful for evaluating releases of hatchery fishes for management and research purposes (Eldrod and Schneider 1986; Lucchesi 2002). Calcein (2,4-bis-{N,N'-di[carbomethyl]-aminomethyl}fluorescein) has been successfully used to attain a chemical batch mark in Atlantic salmon *Salmo salar* (Mohler 1997), lake trout *Salvelinus namaycush* (Honeyfield et al. 2008), walleyes *Sander vitreus* (Brooks et al. 1994), red drum *Sciaenops ocellatus*, Atlantic croakers *Micropogonias undulatus*, and spot *Leiostomus xanthurus* (Wilson et al. 1987) without adversely affecting survival or growth. Calcein chemically binds with calcium in body structures (e.g., fin rays and otoliths)

and emits green fluorescence under ultraviolet light. It can be rapidly applied to large numbers of small fish and can be detected nonlethally on external parts by using filtered ultraviolet light. One limitation is that the external mark is subject to fading over time when exposed to direct sunlight (Bashey 2004). However, Idaho Department Fish and Game (IDFG) hatcheries have limited capabilities for rearing fish under covered conditions, and therefore the options to use calcein for the mass marking of hatchery fish are reduced. Although calcein allows for rapid, nonlethal observations of marks, a second (usually lethal) alternative is to view internal bony structures (fin rays, otoliths, or other calcified tissues) using fluorescent microscopy (Wilson et al. 1987; Beckman et al. 1990). To date, evaluation of bony structures in calcein-marked fish has been limited. Our objective was to evaluate calcein mark retention over time in rainbow trout *Oncorhynchus mykiss* by examining both external and internal (otolith) structures. We compared mark retention between rainbow trout reared in outdoor raceways under ambient light conditions and those reared indoors in circular tanks.

Methods

Rainbow trout fry at the IDFG Nampa Hatchery were marked at 10–14 d post-swim-up. Our goal was to mark all fish in one entire rearing raceway. An estimated total of 48,000 fry were marked during overcast weather on February 28, 2007. Mean total length and weight at the time of marking were 25 mm and 0.2 g, respectively. Fry were marked with calcein using the osmotic induction method and high concentration–short duration method according to the protocol presented by Mohler (2003, 2004). All fish were simultaneously crowded into the upper end of the raceway and were dipnetted for transfer to the marking solution. Some fish escaped around the crowder or could not be captured with dip nets. Therefore, a small but unknown number of fry in the raceway escaped the marking process. Approximately 680 g of fish (4,400 fish) were placed in the Heath trays at a time. Each tray was submerged in a 1.5% salt bath for 3.5 min followed by immersion in a 0.5% solution of calcein (Western Chemical, Inc., Ferndale, Washington) for

* Corresponding author: martin.koenig@idfg.idaho.gov

Received January 13, 2010; accepted July 19, 2010
Published online November 24, 2010

3.5 min. Fish were rinsed before being returned to the rearing raceways. Water quality parameters during the marking process included pH of 7.8, dissolved oxygen of 8.3 mg/L, hardness (as CaCO₃) of 222 mg/L, and water temperature of 12.4°C. Sunny weather prevailed at 1 d postmarking (March 1, 2007), when a subsample of 250 fry was removed from the marked lot, transferred to indoor 1-m circular tanks at the IDFG Eagle Hatchery, and reared under shaded fluorescent light conditions. All other marked fry remained at Nampa Hatchery to rear outside in the unshaded concrete raceway.

Fry were examined for marks beginning on March 8, 2007 (8 d postmarking), and again every 2 weeks, resulting in a total of 14 samples. A sample of 10 fish was collected from each rearing hatchery through September 21, 2007 (205 d postmarking). For each sample, fish were sacrificed with an overdose of MS-222 (tricaine methanesulfonate) and were examined under dark conditions. Each fish was examined for external marks on the head and fins by using a handheld SE-MARK detector light (which uses a 495-nm excitation filter coupled with a 510-nm suppression filter). Marks were assigned an intensity score according to the criteria defined in the Investigational New Animal Drug 10-987 protocol (USFWS 2004) using a scale from 0 to 3 (3 = readily visible, bright-green mark; 2 = clearly visible green mark; 1 = dimly visible, dull-green mark; 0 = no mark). Marks on the head and on fins were ranked separately. For the head, the mark was concentrated along the lower jaw and on the ventral portion of the gill arches. For fins, the mark was most visible at the base of pectoral, pelvic, and anal fins. The detector light was battery operated. Batteries were replaced prior to each examination to guard against potential bias due to low batteries.

In addition to external marks, we also examined and ranked otoliths to evaluate deterioration of internal calcein marks. Sagittal otoliths were removed from each specimen and stored dry under dark conditions. Otoliths were mounted onto microscope slides using Crystalbond thermoplastic adhesive and were wet-sanded first with 600-grit sandpaper and then with 1,500-grit sandpaper (Brooks et al. 1994) to remove overburden and to better illuminate the mark. Prepared otoliths were examined at 40× or 100× magnification using filtered ultraviolet light. Ultraviolet filters consisted of a 450–490-nm excitation filter, a 510-nm dichromatic mirror, and a 515-nm barrier filter. Otolith marks were given an intensity score using the same scale described above. We kept track of the processing time related to evaluating the otolith marks for a typical 25-specimen batch. Processing time included extract-

ing the otoliths from the fish, mounting on a glass slide, wet-sanding, microscopic examination, and any repeated sanding (if needed).

Mean total length and weight of test fish were compared using paired *t*-tests. Mean scores of calcein marks were compared between indoor- and outdoor-reared fish by use of Wilcoxon's signed rank test. To reduce negative bias from unmarked specimens, we presumed that fish without any marks on at least one of the three structures examined (both internal and external) had escaped the marking process, and these individuals were removed from statistical comparisons. All comparisons were evaluated at a significance level of $\alpha = 0.05$.

Results

At the conclusion of the rearing period (205 d postmarking), rainbow trout reared outdoors had a mean total length of 157 mm and a mean weight of 62 g. Fish reared indoors had a mean length of 166 mm and a mean weight of 63 g. On average, rainbow trout in the outdoor raceway and indoor tanks did not differ significantly in mean length (paired *t*-test: $df = 14$, $P = 0.46$) or weight ($df = 14$, $P = 0.70$) over the duration of the experiment.

External evaluation of calcein mark retention on heads and fins indicated a rapid and immediate decline in mark quality for fish reared under full sunlight compared with fish reared indoors (Figure 1). For fish reared outdoors, calcein marks on heads were faint or undetectable by 8 d postmarking; by 51 d postmarking, marks on fins were similarly degraded. The mean intensity scores for calcein marks on the head were 1.9 for fish reared indoors and 0.31 for those reared outdoors. Scores for fish reared indoors were significantly higher than scores for those reared outdoors ($P < 0.001$). Mean scores for calcein marks on the fins were 1.4 for fish reared indoors and 0.2 for fish reared outdoors, with indoor scores being significantly higher ($P < 0.001$). Fish reared indoors maintained their marks throughout the test period, although the marks showed some degradation over time, and it appeared that marks were retained better in the head region than in the fins. Low numbers of mismarked fish were removed from the comparisons of mean mark quality: only 18 fish (13%) from the outdoor-reared group and 11 fish (9%) from the indoor-reared group were removed. Fish that were excluded from analyses showed no marks on any of the structures examined, including otoliths.

For both rearing groups over the entire study period, calcein marks were retained better on otoliths than on external structures (Figure 1). However, the mean intensity score for otolith marks was signifi-

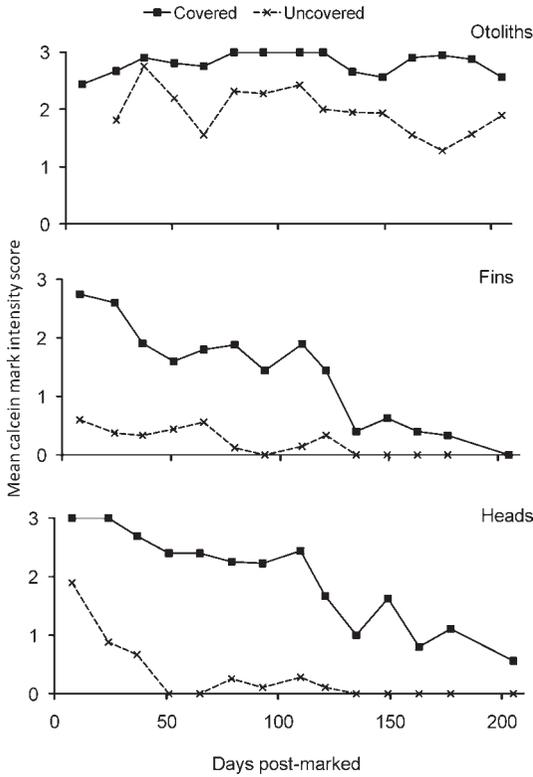


FIGURE 1.—Mean calcein mark intensity (score of 0–3, defined in Methods) on heads, fins, and otoliths in relation to days postmarking for hatchery rainbow trout reared indoors and outdoors.

cantly higher for fish reared indoors (2.6) than for fish reared in outdoor raceways (1.7; $P < 0.001$). Otoliths from fish reared indoors had little mark degradation, whereas scores for otolith marks in fish reared outdoors decreased slightly over the test period (Figure 1).

Otolith extraction, preparation, and reading to evaluate the calcein mark took a combined total of 6–10 min/fish. Since most otoliths were very small, only light sanding was necessary to enhance mark visibility. Older fish with larger otoliths could require longer processing time (i.e., more sanding) to attain clearly visible marks on otoliths.

Discussion

A mass mark that can be applied quickly at low cost presents a valuable fisheries research and management tool. Calcein is cost effective and can be quickly applied to large numbers of small fish. Our study showed that the mark was readily applied to the majority of fish, with only 9–13% having been

mismarked in the process. This likely resulted from fish escaping around raceway crowders or dip nets rather than the failure of calcein to produce a mark.

Previous studies evaluating calcein for batch marking have demonstrated that fish may retain marks for up to 1–3 years (Mohler et al. 2002; Mohler 2004). However, whether fish are reared outdoors after marking may affect the utility of calcein as a mass mark for field evaluations. Results from our study suggest that if rainbow trout marked with calcein are reared in normal sunlight conditions, the quality of the mark is substantially degraded within weeks. Calcein marks on rainbow trout reared in outdoor raceways degraded to the point that they were unlikely to be externally visible after stocking, thus eliminating the advantage of nonlethal detection for recapture experiments. Other researchers have also reported rapid deterioration of calcein marks in fish exposed to sunlight. Bashey (2004) found that the visibility of calcein marks in guppies *Poecilia reticulata* reared with occasional sunlight was reduced in only 7 d. In another 7-d trial, Honeyfield et al. (2008) reported that lake trout reared under artificial sunlight lost 90% of calcein mark intensity on the head, body, ventral region, and pectoral fins compared with fish reared in the dark. Since marks degraded much less rapidly on fish reared indoors, calcein may retain some utility as a mass mark if fish are reared indoors prior to release. For example, Negus and Tureson (2004) found excellent long-term retention of calcein in rainbow trout and Chinook salmon *O. tshawytscha* reared in tanks. In this respect, the decline in mark visibility for rainbow trout reared indoors in our study was unexpected. As a result, we advise caution when using calcein in mass marking, even for fish reared indoors; we also suggest that the recommendation of Negus and Tureson (2004) to mark fish at the largest size possible before release should be followed. The IDFG has limited availability of indoor or shaded rearing facilities. Therefore, our potential application of calcein to achieve a nonlethal external batch mark for rainbow trout reared in traditional raceways is rather limited.

Mohler (2003) suggested the application of a rinse between the salt and calcein baths during the osmotic induction step; we omitted this rinse from our procedure. The omission of the rinse may have compromised the level of calcein mark uptake by the rainbow trout. However, at 8 d postmarking, all heads, fins, and otoliths were assigned mark intensity scores of 2–3. Although a rinse might have improved the mark quality and retention time, we argue that this does not affect the primary finding of the experiment, which is that the rate of mark decay is noticeably faster for

fish reared in sunlight and that internal structures (like otoliths) appear to retain the mark better than external structures. Even if a more intense mark had been achieved by using a rinse after the salt bath, this likely would not have changed the fact that the calcein marks showed obvious degradation in sunlight conditions and that this marking method may have limited application in typical outdoor raceway applications.

Calcein marks were retained better on otoliths than on the external structures throughout our study period. Some degradation of otolith marks occurred for fish reared under open sunlight, but the distinction in mark quality and degradation over time were much less apparent for otoliths than for external structures. These results are similar to those reported by Beckman et al. (1990) and Brooks et al. (1994). We found that mark degradation in otoliths was also more variable compared with external parts, which showed steadily decreasing mark intensity over time. This may indicate some variability in how mark intensity is scored in otoliths since otolith mark examination requires a more complex process that involves mounting, sanding, and polishing.

Use of calcein provided a batch mark on otoliths that was still visible after 6 months of full-sunlight rearing. However, in comparison with external marks, the marking of otoliths has several important drawbacks that must be considered. First, fish must be sacrificed to recover the otolith mark, presenting a drawback if a nonlethal mark is desired. Additionally, without an externally visible secondary mark, it is difficult or impossible to distinguish otolith-marked fish from unmarked fish of the same size and species. The absence of an external secondary mark (e.g., fin clip) can vastly increase the number of samples that must be prepared and examined to find marked individuals, which may offset the time and cost benefits of mass marking with calcein. For example, Koenig and Ellsworth (2008) collected 1,205 kokanee *O. nerka*, of which 305 were examined based on size criteria, and only 56 of the examined fish contained calcein-marked otoliths (2 years after release). In this case, a more traditional mark that can be detected externally (e.g., coded wire tag) may be more effective, without the drawbacks of mark degradation caused by sunlight, high water temperatures, freezing, or ethanol preservation (Bashey 2004; Negus and Tureson 2004).

Acknowledgments

We would like to thank the staff at Eagle Fish Hatchery—including Dan Baker and Dan Green—and the staff at Nampa Fish Hatchery—including Rick Alsager, Jamie Mitchell, Bob Becker, Bob Turik, Gary

Ady, and Dick Bittick—for rearing our test fish prior to and during the study. Joe Kozfkay provided guidance on working with calcein. Liz Mamer, Roberta Scott, and Sharon Landin provided valuable assistance with microscope setup and fluoroscopy techniques. This manuscript also benefited from the input of two anonymous reviewers. Funding for this project was provided in part by the Federal Aid in Sport Fish Restoration program.

References

- Bashey, F. 2004. A comparison of the suitability of alizarin red and calcein for inducing a non-lethally detectable mark in juvenile guppies. *Transactions of the American Fisheries Society* 133:1516–1523.
- Beckman, D. W., C. A. Wilson, F. Loricca, and J. M. Dean. 1990. Variability in incorporation of calcein as a fluorescent marker in fish otoliths. Pages 547–549 in N. C. Zarker, A. E. Giorgi, R. C. Heidinger, D. B. Jester Jr., E. D. Prince, and G. A. Winans, editors. *Fish-marking techniques*. American Fisheries Society, Symposium 7, Bethesda, Maryland.
- Brooks, R. C., R. C. Heidinger, and C. C. Kohler. 1994. Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. *North American Journal of Fisheries Management* 14:143–150.
- Eldrod, J. H., and C. P. Schneider. 1986. Evaluation of coded wire tags for marking lake trout. *North American Journal of Fisheries Management* 6:264–271.
- Honeyfield, D. S., T. Kehler, J. W. Fletcher, and J. W. Mohler. 2008. Effects of artificial sunlight on the retention of external calcein marks on lake trout. *North American Journal of Fisheries Management* 28:1243–1248.
- Koenig, M., and K. Ellsworth. 2008. Hatchery trout evaluations. Idaho Department of Fish and Game, Job Performance Report 08-10, Project F-73-R-30, Boise.
- Lucchesi, D. O. 2002. Evaluating the contribution of stocked walleye fry and fingerlings to South Dakota walleye populations through mass marking with oxytetracycline. *North American Journal of Fisheries Management* 22:985–994.
- Mohler, J. W. 1997. Immersion of larval Atlantic salmon in calcein solutions to induce a non-lethally detectable mark. *North American Journal of Fisheries Management* 17:751–756.
- Mohler, J. W. 2003. Producing fluorescent marks on Atlantic salmon fin rays and scales with calcein via osmotic induction. *North American Journal of Fisheries Management* 23:1108–1113.
- Mohler, J. W. 2004. Evaluation of calcein-marked and unmarked Atlantic salmon fry stocked into the West Branch Sheepscot River, Maine. U.S. Fish and Wildlife Service, Lamar Information Leaflet LM-04-01, Lamar, Pennsylvania.
- Mohler, J. W., M. J. Millard, and J. W. Fletcher. 2002. Predation by captive wild brook trout on calcein-marked versus nonmarked Atlantic salmon fry. *North American Journal of Fisheries Management* 22:223–228.

- Negus, M. T., and F. T. Tureson. 2004. Retention and nonlethal external detection of calcein marks in rainbow trout and Chinook salmon. *North American Journal of Fisheries Management* 24:741–747.
- USFWS (U.S. Fish and Wildlife Service). 2004. Fact sheet: calcein INAD 10-987. USFWS, Aquatic Animal Drug Approval Partnership Program, Bozeman, Montana.
- Wilson, C. A., D. W. Beckman, and J. M. Dean. 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. *Transaction of the American Fisheries Society* 116:668–670.