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[Article]

Dietary Calcein Marking of Brook Trout, Atlantic Salmon, Yellow Perch, and Coho Salmon Scales

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Abstract.—Brook trout *Salvelinus fontinalis*, Atlantic salmon *Salmo salar*, coho salmon *Oncorhynchus kisutch*, and yellow perch *Perca flavescens* fed calcein for 5 d showed characteristic calcein scale marks 7–10 d postmarking. In fish fed 0.75 or 1.25 g of calcein per kilogram of feed, the percentage of fish that exhibited a calcein mark was 100% in brook trout, 93–98% in Atlantic salmon, 60% in yellow perch, and 0% in coho salmon. However, when coho salmon were fed 5.25 g calcein/kg feed, 100% marking was observed 7–10 d postmarking. Brook trout were successfully marked twice with distinct bands when fed calcein 5 months apart. Brook trout scale pixel luminosity increased as dietary calcein increased in experiment 2. For the second calcein mark, scale pixel luminosity from brook trout fed 1.25 g calcein/kg feed was numerically higher ($P < 0.08$) than scales from fish fed 0.75 g calcein/kg feed. Mean pixel luminosity of calcein-marked Atlantic salmon scales was 57.7 for fish fed 0.75 g calcein/kg feed and 55.2 for fish fed 1.25 g calcein/kg feed. Although feed acceptance presented a problem in yellow perch, these experiments provide evidence that dietary calcein is a viable tool for marking fish for stock identification.

Tagging or marking fish is an important fishery management tool employed for stock assessment, estimating recruitment or mortality, and surveying genetic contributions to a fish population. Izaak Walton in 1635 was among the first to tag Atlantic salmon *Salmo salar* with ribbon or thread inserted through the caudal fin to track the fish to their natal streams (Moring 2002). Today, fishery biologists and managers continue to rely on marked fish in their work. There are a variety of marking techniques employed; presently, these include external and internal tags, chemical marking of tissue, and the use of genetic markers. Each tag or marking process has advantages and disadvantages and has been described by Parker et al. (1990).

The chemical marking agent, calcein (2,4-bis[N,N-di(carbomethyl)-amino-methyl]fluorescein; molecular weight, 622), is currently being tested for efficacy under an investigational new animal drug (INAD) permit issued by the Food and Drug Administration (FDA) for the purpose of chemically marking fish. Calcein chemically binds with calcium phosphate, resulting in a fluorescence emission when it complexes with alkaline earth metals (Bart 2001). There is growing interest in using calcein rather than oxytetracycline (OTC). Although OTC is frequently used to mark fish, OTC is used also as an antibiotic to treat animals. Concern has been expressed regarding the potential use of antibiotics and increased incidence of antibiotic-resistant bacteria (Khachatourians 1998). Calcein produces a visual scale mark, which can readily be seen with a handheld device (Mohler et al. 2002; Mohler 2003; Negus and Tureson 2004) without sacrificing the fish. In contrast, fish are sacrificed to collect otoliths or vertebrae for evaluation of OTC-marked fish (Alcobendas et al. 1991; Gelsleichter et al. 1997).

Early work with calcein suggested that bony tissue marking efficacy was mixed (Beckman et al. 1990). The utility of calcein as a chemical agent to mark fish has been evaluated in otoliths (Wilson et al. 1987; Beckman et al. 1990; Brooks et al. 1994; Bumguardner and King 1996), fin rays, scales, and other calcified tissues (Alcobendas et al. 1992; Gelsleichter et al. 1997; Mohler 1997; Leips et al. 2001; Mohler et al. 2002). Ultrasound treatment was found to improve calcein scale marking, except in very small fish (Bart et

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al. 2001). Mohler (2003) characterized an osmotic pretreatment and calcein immersion process that is rapid and reliable and, thus, cleared the way for the FDA to issue an INAD for chemically marking fish with calcein (INAD 10–987). Under this INAD, calcein is allowed as an immersion-marking agent in fish weighing less than 2 g (U.S. Fish and Wildlife Service 2004; INAD calcein fact sheet can be accessed at http://fisheries.fws.gov/aadap/calcein.htm). Calcein has been shown to be an effective nonlethal chemical marker in several fish species, including guppies *Poecilia reticulata*, red drum *Sciaenops ocellatus*, Atlantic croakers *Micropogonias undulatus*, spot *Leiostomus xanthurus*, spotted seatrout *Cynoscion nebulosus*, silver perch *Bairdiella chrysoura*, summer flounder *Paralichthys dentatus*, and Atlantic salmon (Leips et al. 2001; Mohler et al. 2002). Immersion marking is not always practical. In addition, handling and disposal of the remaining spent calcein solution is a burden. As such, an alternative to immersion marking is desirable and would facilitate broader use of calcein.

Incorporating calcein in feed would be an easy adaptation for most fish sizes (age) and for several species found in culture facilities. The use of dietary calcein to mark scales has not been reported. In the current studies, we report results from fish fed diets formulated with minimal calcium content. The objectives of the studies were to (1) determine whether dietary calcein would be absorbed and mark scales, (2) determine the luminosity of scales in fish fed varying concentrations of calcein in four fish species, and (3) determine whether double marks could be applied to scales.

Methods

Feed preparation.—A semipurified diet relatively low in calcium consisted of the following ingredients: casein (11%), gelatin (48%), dextrin (6%), starch (15%), fish oil (15%), sand (4%), and betaine-HCl (1%). These ingredients were mixed together with and without added calcein (Lancaster Synthesis, Inc., Peltham, New Hampshire). No vitamin or mineral supplements were included. Although this semipurified diet did not meet all known nutritional requirements, it is unlikely that nutritional deficiencies would occur within the brief 5-d feeding period in exogenous-feeding fish (NRC 1993). Feed preparation followed the method described by Honeyfield et al. (2005). The resulting dried feed was placed in a food blender and feed particle size was reduced to a size that would pass through a 10-mm sieve. With little information to use as a guide for a starting-feed calcein concentration to mark scales, a wide range was first evaluated. In experiment 1, five diets were prepared containing calcein at concentrations of 0, 1.50, 3.00, 5.25, and 50.00 g per kilogram of feed. Calcein dose may also be expressed per unit of fish by multiplying the diet concentration of calcein, the number of days fed, and the feeding rate. For example, fish fed 1.50 g calcein/kg feed at 2% of their body weight for 5 d equals 150 mg calcein/kg fish. Based on the results of experiment 1, three diets were chosen for further studies (0, 0.75, and 1.25 g calcein/kg feed). The research reported in this paper was not conducted under a specific INAD and all fish were sacrificed. The data were collected to support an application for an INAD to administer calcein in the feed.

Fish marking.—Fish were fed at 2% of their body weight per day for five consecutive days with the experimental calcein diet. Fish were held in sixty 4-L aquaria and, except for the studies with yellow perch, each aquarium was supplied with oxygenated well water (9°C) with the flow set at 1.0 L/min. For the experiment with yellow perch, well water was heated to 15°C, degassed (nitrogen saturation $< 95\%$), and the flow set at 2.10 L/min.

In experiment 1, 500 juvenile brook trout *Salvelinus fontinalis* weighing approximately 1.0 g were allocated to five aquaria (100 fish/tank) and fed one of the five experimental diets without replication. Before and after fed the experimental diets, fish were fed a standard commercial feed (Melick Aquafeed, Catawissa, Pennsylvania). In experiments 2–4, fish were fed one of three dietary calcein concentrations (0, 0.75, and 1.25 g/kg feed). In experiment 2, brook trout weighing 1.3 g/fish were allocated to three replicate tanks (100 fish/tank) per diet. For experiment 3, Atlantic salmon fingerlings (65 fish/tank) weighing 0.8 g/fish were placed in three replicate tanks per treatment. In experiment 4, three replicate groups of 48 coho salmon *Oncorhynchus kisutch* weighing 9.8 g/fish were used. In the fifth experiment, we were limited by the number of yellow perch available; therefore, only two diets were fed (0 and 1.25 g/kg feed). Yellow perch (43 fish/tank) weighing 9.8 g/fish were allocated into two replicate tanks per diet.

In the sixth experiment, we investigated the potential to double-mark brook trout scales with calcein. Brook trout that were marked in experiment 2 were allocated to 15 tanks. There were three replicate tanks of 25 fish/tank per treatment. Five treatment groups were formed: 0–0, 0.75–0.75, 0.75–1.25, 1.25–0.75, and 1.25–1.25 g/kg feed. The first number represents the calcein concentration in the fish from experiment 2 and the second number is the concentration of calcein fed 5 months after the first mark was administered.

Calcein mark detection.—Fish were lightly sedated with tricaine methanesulfonate (MS-222; Western Chemical, Inc., Ferndale, Washington) before mark

inspection and scale collection. Fish were examined 7–10 d postmarking with a handheld SE-MARK calcein detector (Western Chemical) equipped with a 3× magnifying lens insert (Mohler et al. 2002). The principles of this technique have been outlined elsewhere (Brooks et al. 1994; Mohler 1997; Leips et al. 2001). Briefly, an excitation light source set at 495-nm wavelength is directed at the fish scales and fin rays. If the scale contained bound calcein, an emission of fluorescent green light will be observed when viewed through the glass filter (520-nm cutoff); no fluorescence will be observed in unmarked fish. This method is more subjective than data collected by means of a fluorescent microscope examination of the scales.

To obtain the scale pixel luminosity data reported, we recorded fluorescence luminosity in freshly removed scales (from 3 fish/tank) following the method of Frenkel et al. (2002). Scales were collected above the midline of the fish and mounted on a slide with a drop of water and viewed through a Zeiss Axioskop 2 MAT microscope with epifluorescent capabilities (Carl Zeiss MicroImaging, Inc., Thornwood, New York). Digital images were recorded at 32×magnification with phase contrast set at three. A 2-s exposure time was used with the wavelength of light set at 495 nm. In the nonfluorescent images, a 1-s exposure of white light was used. The fluorescent or white light image of the scales was digitally recorded. Fluorescence pixel luminosity (unit less number) was generated from the digital image with Adobe Photoshop 7.0 as described by Frenkel et al. (2002).

Statistics.—Analysis of variance, means, and standard error of the means of scale pixel luminosity were calculated with SAS (2003). Pixel luminosity of each band on the double-marked scales was evaluated in a 2 × 2 factorial arrangement (two levels of calcein at two marking times).

Results

The results of experiment 1 showed that all brook trout fed dietary calcein (1.50–50.00 g/kg) exhibited readily visible scale marking (Figure 1). The markings could easily be seen with the handheld SE-MARK calcein detector 7–10 d postmarking.

In experiment 2, a linear dose–response marking was observed in scale fluorescent pixel luminosity of the digital images from brook trout fed 0, 0.75, and 1.25 g calcein/kg feed (Figure 2; $P < 0.05$). All brook trout (100%) fed a diet containing calcein exhibited calcein marks (Table 1) when examined 9 d postfeeding.

In experiment 3, the percentage of Atlantic salmon fingerlings with calcein marks was 0, 93, and 98% in fish fed 0, 0.75, and 1.25 g calcein/kg feed, respectively (Table 1). Marking followed a curvilinear response in Atlantic salmon. Mean scale pixel luminosity of fish fed 0.75 or 1.25 g calcein/kg feed was 57.7 and 55.2, respectively (Figure 2). Within each treatment group (0.75 and 1.25 g/kg), fluorescent luminosity of individual fish scales was variable and suggested that fish did not uniformly consume the feed.

In experiment 4, coho salmon readily consumed the three diets, but no scale marking was evident when viewed with the handheld detector or when scales were viewed with the fluorescent microscope (Table 1; Figure 2). Clearly, coho salmon were eating the feed because the characteristic green fluorescence associated with calcein was evident in the feces and around the fish's anal orifice, but not on the scales. Five randomly selected coho salmon were immersed in calcein as described by Mohler (2003) to confirm that coho salmon scales would mark with calcein; scales were readily marked. The remaining coho salmon were then fed a diet containing 5.25 g calcein/kg for 5 d. With the handheld detector, scale marking was clearly observed in fish fed 5.25 g calcein/kg feed 7–10 d postmarking. Fluorescent scale pixel luminosity was not recorded.

In experiment 5 (yellow perch), only 11 (13%) of 85 fish exhibited scale marking (Table 1) when fed 1.25 g calcein/kg feed for 5 d without prior acclimation to the feed. Yellow perch could be seen readily refusing to eat the feed. Fish were then placed on the semipurified diet without calcein (0 g calcein/kg feed) for 14 d to acclimate the fish to the feed. Then, feed containing calcein was again offered (1.25 g calcein/kg feed) for 5 d. Although fish appeared to be actively feeding, only 60% of the yellow perch exhibited calcein marks 7–10 d later. Yellow perch scale pixel luminosity was low with an average of only 6.06. The reason for the lower marking rate and low pixel scale luminosity are not known, but food intake, poor absorption of calcein, or other factors may have been involved.

In experiment 6, brook trout fed calcein 5 months after the first marking showed a distinct second or double-mark pattern. Mean scale luminosity (Figure 3) was higher from fish fed 1.25 g calcein/kg feed than observed in scales from brook trout fed 0.75 g calcein/kg feed in the inner band or first marking ($P < 0.0013$). Although scale pixel luminosity tended to be higher in fish fed 1.25 g calcein/kg feed in the outer band or second mark, this difference was not significant ($P < 0.08$). No interaction was detected between the luminosity of the first and second mark ($P < 0.82$). A high degree of variability was noted in scale luminosity within each treatment group as illustrated in Figure 4.

Discussion

The primary purpose of this work was to determine the feasibility of marking fish scales with dietary calcein. Feeding fish calcein resulted in the scales of

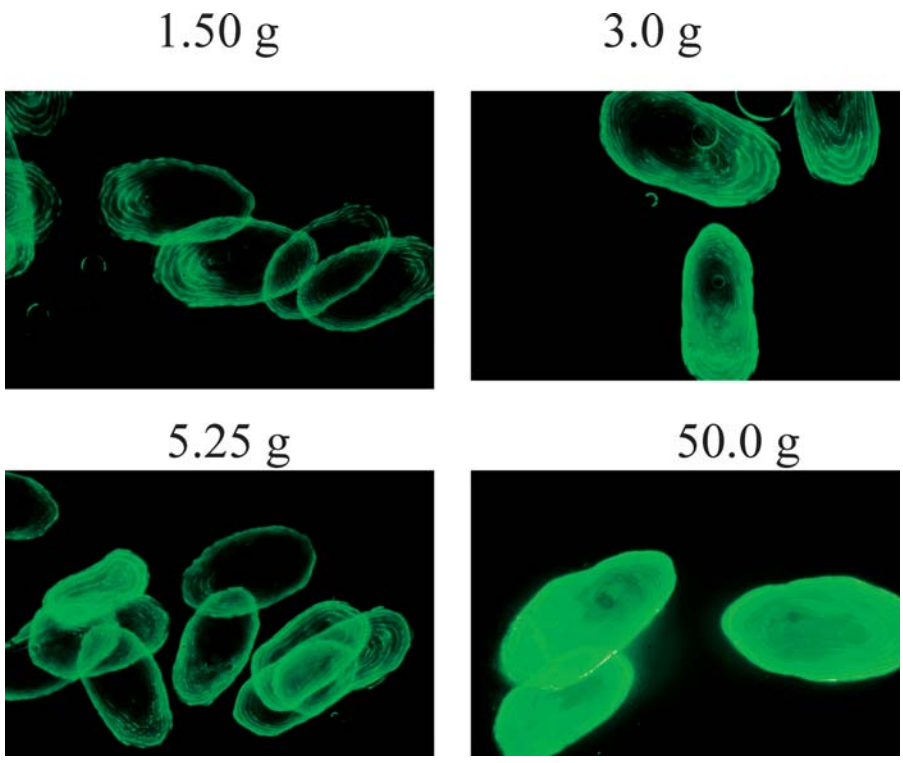

FIGURE 1.—Brook trout scales from fish fed calcein at four concentrations (1.50, 3.0, 5.25, and 50.0 g calcein/kg feed) for five consecutive days. Only a black background was visible in the fluorescent image of scales of fish fed 0 g calcein/kg feed (not shown).

brook trout (100%), Atlantic salmon (93–98%), and coho salmon (100% at 5.25 g/kg) being chemically marked when fish were fed at 2% body weight for five consecutive days. The scale markings could be observed with the handheld detection device on brook trout scales and fin rays as early as the third day of feeding calcein. Visually, the fish were similar to published images of calcein-immersed trout (Negus and Tureson 2004). Thomas et al. (1995) reported marked otoliths after 1 d in red drum fed 25 or 50 g calcein/kg feed fed at 7% body weight. Red drum were fed 20–66 times (7% body weight) the concentration of calcein used in our experiments (2–6). The authors did not investigate the marking of red drum scales. To have confidence in correctly identifying calcein-marked fish, using the handheld detector we conducted a blind study (data not shown) with three individuals unfamiliar with calcein-marked fish. There was 100% correct identification of unmarked fish in experiment 1 and all the examiners tended to rate fish as having been fed a higher concentration of calcein than was actually fed. Thereafter (experiments 2–6), fish were evaluated by only one person with the handheld detector.

There was a positive relationship between dietary level of calcein and scale pixel luminosity or the amount of fluorescent light observed from marked brook trout scales. These results suggest that it may be possible to designate groups of fish by varying dietary calcein with different scale luminosity. In our studies,

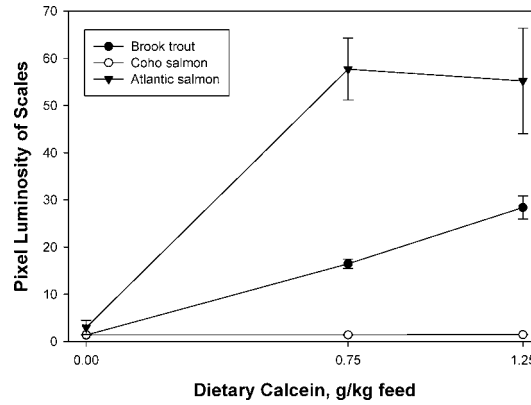

FIGURE 2.—Average luminosity and standard error of the means of scales from brook trout, coho salmon, and Atlantic salmon fed 0, 0.75, and 1.25 g calcein/kg feed for 5 d.

TABLE 1.—Number (*N*) and percentage of fish exhibiting calcein marks 7–10 d postmarking when fed dietary calcein in a semipurified diet for five consecutive days at 2% of body weight.

Species	Calcein (g/kg feed)	Fish (*N*)	Fish marked (*N*)	Percent marked
Brook trout	0	100	0	0
	1.5	100	100	100
	3.0	100	100	100
	5.25	100	100	100
	50.0	100	100	100
	0	300	0	0
	0.75	300	300	100
	1.25	300	300	100
Atlantic salmon	0	195	0	0
	0.75	195	181	93
	1.25	195	191	98
Coho salmon	0	140	0	0
	0.75	139	0	0
	1.25	141	0	0
	5.25[a]	25	25	100
Yellow perch	0	86	0	0
	1.25	85	11	13
	1.25[b]	85	51	60

[a]Second attempt to mark fish by increasing the concentration to 5.25 g calcein/kg feed.
[b]Yellow perch fed 1.25 g calcein/kg diet for 5 d then 14-d diet adaptation to the semipurified diet with 0 g calcein. This was followed by a second 5-d feeding of the 1.25 g calcein/kg diet.

the two calcein-marked groups of brook trout were identifiable at 10 d and after 5 months (Figures 2 and 3). Longer-term calcein mark retention has been reported in fish injected or immersed in calcein. In two reports, rainbow trout *Oncorhynchus mykiss* retained their external calcein marks for at least 12 months in young fish (Negus and Tureson 2004; Frenkel et al. 2002), and older rainbow trout (3.5 months) kept identifiable calcein marks into adulthood (Negus and Tureson 2004). Calcein-marked otoliths from summer flounder were readable 194 d later (Monaghan 1993). With immersion treatment, calcein-marked Atlantic salmon had readable marks at 234 d (Mohler 1997). More importantly, calcein-marked Atlantic salmon have been recovered from the West Branch of the Sheepscot River in Maine after 16 months (Mohler 2004).

Criticism of the relatively short duration of the present studies is somewhat blunted for two reasons: (1) once calcein is bound to bony fish tissue (regardless of its mode of administration), its chemistry is likely to be the same; and (2) as noted in the previous paragraph, calcein-marked fish have been captured 16 months after being released. Because calcein is a relatively new chemical marking agent, confidence in its utility will be enhanced with additional studies and with several fish species.

Our third objective was to investigate the possibility of marking scales twice. In this study, feeding two concentrations of calcein resulted in the double-band pattern. Differences in scale luminosity as a result of dietary calcein concentration of the outer band or second mark were not as prominent as that observed with the inner band or first mark. Variability of calcein mark luminosity within treatment groups was a contributing factor and an example can be seen in Figure 4. Beckman et al. (1990) reported similar variability in calcein-marked otoliths with immersion application. The potential benefit of intentionally varying luminosity in both the first and second marks would be to increase the number of possible mark combinations. Additional studies will be needed to refine the use of

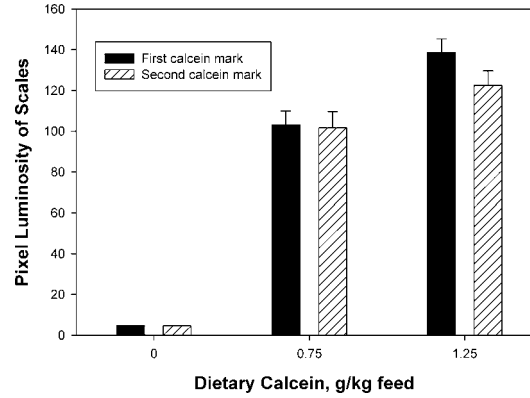

FIGURE 3.—Mean brook trout scale luminosity of two calcein marks applied 5 months apart. Data were collected 10 d after application of the second mark. The probability (*P*-values) of differences in mean scale luminosity between fish fed 0.75 and 1.25 g calcein/kg feed was less than 0.0013 for the first or inner marks and less than 0.08 for the second or outer marks. Error bars are standard errors of the means.

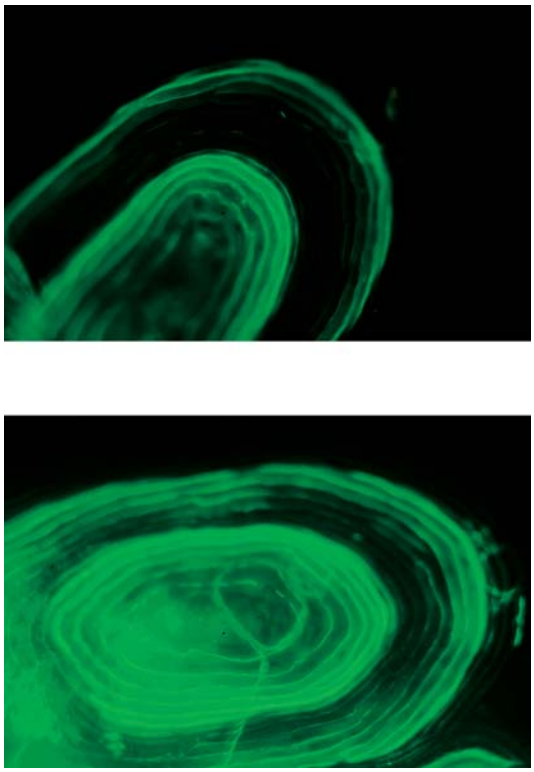

FIGURE 4.—Variability in scale luminosity observed in scales from two brook trout fed 1.25 and 0.75 g calcein/kg feed; the upper panel shows the inner mark for a fish fed 1.25 g in experiment 2, and the lower panel shows the outer mark for a fish fed 0.75 g in experiment 6.

different luminosities as a means of differentiating groups of fish.

No mortality was observed in the four species fed calcein in these studies. Monaghan (1993) and Gelsleichter et al. (1997) reported no mortality in fish injected with calcein. However, a potential problem with feed consumption was identified in yellow perch. These fish did not aggressively feed when the diet was abruptly changed to the semipurified diet or after fish were acclimated to the diet for 14 d. Palatability of the semipurified diet appeared to be low and the yellow perch may have detected the presence of calcein and, thus, further avoided their feed. Calcein readily dissolves in water and has an acidic pH of approximately 3 in solution. We did not measure the pH of the feed and made no attempt to adjust the acidity of the feed containing calcein during the feeding of yellow perch. Atlantic salmon also showed a lack of aggressive feeding on the semipurified diets. As most fish culturists are aware, acceptance of feed can vary for several reasons (Weber and Ridgeway 1962; Lorson and Mudrak 1987). Besides acclimating the fish to their feed, another approach to overcome the problem of feed palatability might be to add calcein to standard fish meal-based diets. Bioavailability of calcein is not known in the presence of bone and divalent cations found in a fish meal-based feed.

Based on results with coho salmon, brook trout, and Atlantic salmon, there appear to be differences in pixel luminosity of scale marks among fish species fed the same concentration of calcein. Dietary concentration rather than total amount of calcein fed appears to be important. In our work with coho salmon, extending the feeding period of fish fed 1.25 g calcein/kg feed for an additional 5 d (10 d total) was ineffective in marking coho salmon scales. This suggests there may be something influencing intestinal uptake of calcein that is species specific. Therefore, dietary concentration of calcein may need to be determined on a species-by-species basis. In summary, these experiments demonstrate that feeding calcein is a feasible delivery method. For calcein in general, which appears to be a good candidate chemical marking agent, studies are needed to determine the duration of scale mark retention in order for this compound to become a standard fishery management tool.

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