

External fluorescence retention of calcein-marked juvenile brown trout *Salmo trutta* raised in natural and artificial environments

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The fluorescence retention and intensity of juvenile brown trout *Salmo trutta* marked during their first summer were monitored in a hatchery and in four natural streams. A handheld detector was used for direct examination. In the hatchery, three marking treatments (T) were compared: 3.5 min in a 0.5% calcein solution (T0.5-3.5), 7 min in a 0.5% calcein solution (T0.5-7) and 3.5 min in a 1% calcein solution (T1-3.5). The fish were raised indoors for 11 months and then outdoors until 18 months. The fluorescence retention rate was 100% in all treatments at 11 months, although T1-3.5 showed the highest mean fluorescence intensity, followed by T0.5-7 and T0.5-3.5. The fluorescence intensity was not correlated with the final total length (L_T) of the fish in two treatments, although it significantly decreased with increasing L_T in T1-3.5. At 18 months, <30% of the fish were still slightly fluorescent, suggesting a negative effect of sunlight exposure. In stream studies, the fluorescence intensity did not significantly differ according to final L_T ; an overall mean \pm s.d. retention rate of $70.7 \pm 26.6\%$ was measured at 12 months with a decrease to $48.6 \pm 24.6\%$ at 24 months. Significant differences amongst streams and within reaches of the same stream were observed. Because of a significant positive effect of the shading index on the fluorescence intensity, the use of calcein should be restricted to streams unexposed to direct sunlight. Consequently, the marking method would be reliable for 1 year monitoring studies in shaded streams.

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Key words: chemical marker; field tests; fluorescence intensity; shading.

INTRODUCTION

Stocking efficiency at early life stages is still a key concern for fishery managers, and any new mass-marking method that is cost-effective and easy to perform is of great interest. The use of fluorescent dyes such as alizarin red S, oxytetracycline or calcein allows for quick marking and produces reliable marks in otoliths (Brooks *et al.*, 1994; Baer & Rosch, 2008; Caudron & Champigneulle, 2009; Simon *et al.*, 2009), which remain throughout the fish's life. The main drawback with otolith marking is the necessity to sacrifice the recaptured fish. While collecting otoliths

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may not be a problem in lakes or large streams with recreational or commercial fishing harvest, the problem arises in small streams harbouring small or endangered fish populations.

Calcein has additional interesting properties. Larval Atlantic salmon *Salmo salar* L. 1758 (60 days post-hatch) immersed in a calcein solution showed fluorescence in the caudal fins for 234 days (Mohler, 1997). An osmotic induction before calcein immersion shortened the exposure time and enhanced calcein absorption (Mohler, 2003; Smith *et al.*, 2010). Fluorescence may be externally detected in the fin rays of live fish by the use of a handheld detector (Leips *et al.*, 2001), or in the scales using a microscope with a blue filter set (Mohler, 2003). Most studies focused on short-term monitoring in artificial environments, and tests in the field have been poorly documented (Hill & Quesada, 2010; Crook *et al.*, 2012).

Protocols for mass marking of fishes with calcein have been explored in the U.S.A. since 2003 under the sponsorship of the U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, Bozeman, MT, with the aid of the detector manufacturer (SE-MARK, Western Chemical Company; www.wchemical.com). Although many salmonid species were tested for calcein marking effects and retention, the first tests reported on brown trout *Salmo trutta* L. 1758 were performed by Stubbing & Moss (2007) in a hatchery in Dorset, U.K. The study showed no significant effect of calcein marking after an osmotic induction on the survival and growth of *S. trutta* fry in comparison with a control. Furthermore, the fluorescence was still detectable after 12 months in 100% of the marked fish reared indoors without exposure to sunlight.

Several studies have shown a sharp decrease in fluorescence intensity in the fin rays or the scales when the fishes were exposed to direct natural (Elle *et al.*, 2010; Hill & Quesada, 2010) or artificial sunlight (Honeyfield *et al.*, 2006, 2008). In the field, *S. trutta* are not continually exposed to sunlight during the day, and exposure mainly depends on the stream canopy cover and topography, the fish's behaviour and shelter availability. Therefore, it is necessary to test whether calcein could be confidently used as a long-term external marking agent in the wild.

This study combined hatchery and field surveys after calcein marking on juvenile *S. trutta* over a long period (18 and 24 months). It aimed to determine the main factors affecting mark retention in fin rays. This issue was investigated in hatchery experiments. Parallel field tests were carried out to compare responses in natural conditions and to outline a framework for calcein use in fish management.

MATERIALS AND METHODS

HATCHERY TEST

The *S. trutta* fry were procured from the fish farm of Rives (Thonon-les-Bains, France) that artificially reproduced the wild stock from the Aubonne River (Switzerland). The eggs were fertilized on 22 December and hatched 2 months later. The calcein (C₃₀H₂₆N₂O₁₃, CAS number 1461-15-0) used for the trials was sourced from Riedel-de Haën, Sigma-Aldrich (www.sigmaaldrich.com). Three distinct treatments were used to compare the effects of calcein concentration and immersion time. In the first treatment (T0.5-3.5), the marking protocol initially developed by Mohler (2003) and further adapted for *S. trutta* by Stubbing & Moss (2007) was used. After an osmotic induction in a 2.5% NaCl solution for 3.5 min, fish were bathed in a 0.5% solution of calcein for 3.5 min. The calcein solution was prepared by

dissolving calcein powder in hatchery water and readjusting the pH to 8.3 using sodium hydroxide (NaOH). The fish were first placed in a 20 cm diameter sieve and immersed in the salt solution. A quick rinse between baths removed excess salt. The sieve was then plunged into the calcein solution. A few rinses post-treatment eliminated the calcein residuals. In the second treatment (T1-3.5), the concentration of calcein was doubled to 1% (Mohler, 2003), and in the third treatment (T0.5-7), the exposure time was doubled (7 min). The other manipulations (*i.e.* salt bath and rinses) remained unchanged, and all baths were aerated with an air pump. The fish used as control (T0) were not bathed *i.e.* no osmotic induction and no calcein. The four treatments were replicated thrice, and each of the 12 batches consisted of 100 fry.

Calcein marking was performed in the early summer (6 July 2009) when fry weighed a mean \pm s.d. of 1.09 ± 0.04 g. During the first 9 months, the fish were reared in six covered rectangular tanks (215 cm \times 42 cm \times 17 cm) divided into two parts; each lot was randomly allotted in one of the 12 enclosures. The replicates were then pooled into four covered circular basins (one per treatment). After 11 months, each treatment was marked with a different visible implant elastomer (VIE) colour (Northwest Marine Technology Inc.; www.nmt.us) so that the fish could be distinguished, and all treatments were mixed until the end of the experiment (18 months) in a large concrete tank with a canvas cover [shading under cover was *c.* 70% and was calculated as the ratio of irradiance measured above and under the canvas cover using a LI-250A light meter (Li-Cor Inc.; www.licor.com)]. Every day, the fish were fed *ad libitum* with the appropriate food [Inicio plus 801, 1.5 mm, BioMar (www.biomar.com), contents = 54% protein, 18% lipids and 11% N-free extract], the tanks were cleaned and any dead fish were removed and recorded.

FIELD TESTS

In 2009 and 2010, *S. trutta* reared in a Swiss hatchery (Morrens, Switzerland) were marked and released in four Swiss streams harbouring natural *S. trutta* populations (Table I). The wetted widths of the streams ranged between 2.1 and 3.6 m (mean \pm s.d. = 2.9 ± 0.8 m). Conductivity and pH were measured at different times during the study (ranges in Table I). For each stream, hatchery fish were marked following a similar procedure: an osmotic induction in 2.5% NaCl for 3.5 min and a calcein bath thereafter. The same treatments as those applied in the hatchery tests were used (Table I). All marking campaigns were performed during the summer, between June and August, and were coupled with adipose fin clipping (double mark). The mean mass at marking was between 1.0 and 4.2 g. The fish were kept either 1 or 2 weeks in the hatchery before release. Between one and three representative reaches (inter-reach distance *c.* 1–2 km) were selected at each stream inside of the stocked section (Table I). Electrofishing was carried out 1 and 2 years post-marking during the summer period.

To test whether sunlight could affect the fluorescence intensity, a shading index was derived for each reach during the period of maximum canopy cover. For this purpose, a spherical

TABLE I. Field marking trials of *Salmo trutta* with calcein in four streams

| | Carrouge | Drize | Seigneux | Vaux |
|--|----------|---------|----------|----------|
| Year | 2009 | 2010 | 2009 | 2009 |
| Treatment | T0.5-3.5 | T0.5-7 | T1-3.5 | T0.5-3.5 |
| Number marked | 4108 | 4060 | 1120 | 1060 |
| Mean initial mass (g) | 4.2 | 2.0 | 1.0 | 1.0 |
| Stocked section (km) | 4.5 | 2.0 | 2.7 | 1.7 |
| Recapture reaches | 3 | 1 | 2 | 2 |
| Reach length (range in m) | 84–99 | 133 | 105–138 | 93–113 |
| Stream wetted width (m) | 3.6 | 3.5 | 2.3 | 2.1 |
| pH (range) | 8.1–8.5 | 8.2–8.5 | 8.5–8.7 | 8.4–8.5 |
| Conductivity (range in $\mu\text{S cm}^{-1}$) | 405–495 | 513–660 | 597–667 | 626–739 |

convex densitometer (Forestry Suppliers, Inc.; www.forestry-suppliers.com) modified according to Strickler (1959) was used to measure shading at different points, spaced 10 m apart along the stream bed median axis. Four measurements were made at every point, with each measure facing one cardinal direction (Kelley & Krueger, 2005). The shading index was the mean across all the measurements.

FLUORESCENCE SCORING

The fluorescence was observed using a SE-MARK detector under a dark opaque curtain. The detector uses a 495 nm excitation filter and a 510 nm filter for fluorescence observation. A handheld detector was adapted to an external power source to provide constant power, which optimized the detection of the mark. The fish were anaesthetized with 3 ml of 10% clove oil in 10 l of water and were individually weighed and measured (total length, L_T). Each fish was then observed with the detector on six different body parts: head (including jaws and visible gill arches), pectoral fin, pelvic fin, anal fin, caudal fin and dorsal fin. These tissues were graded as 0 (no fluorescence), 1 (faint) or 2 (bright). The fluorescence intensity (fluosum) was calculated as the sum of the six scores of each individual. Fluosum values ranged from 0 to 12 and were unit-less. This semi-quantitative score is a better integrator of the fish's overall fluorescence than single control points, especially because the decrease in fluorescence varies greatly with time among fins and head (unpubl. data) and between individuals. The retention rate was calculated as the percent of fluorescent fish among marked fish. All the observations were made by a single operator who scored each fish without any information about its initial treatment.

In the hatchery tests, the evaluation of fluorescence was performed at 3, 9, 11, 12, 15 and 18 months post-marking. A sub-sample of 30 individuals per treatment was observed, except at 11 months, when all fish were checked for fluorescence before VIE marking. In the field, captured fish were observed with the detector, retention rates were calculated at each river reach and fluosum was assessed.

DATA ANALYSIS

All statistical tests were performed using R software version 2.15.0 (R Development Core Team; www.r-project.org). The retention rates were compared between treatments (hatchery) and between rivers (field experiment) using either Fisher's exact tests (small sample sizes) or Pearson's χ^2 tests and the false discovery rate method to adjust the P -value for multiple comparisons (Benjamini & Hochberg, 1995). The 95% c.i. was computed for retention rates using the Wilson procedure without any correction for continuity (Newcombe, 1998). Fluosum was compared between treatments, L_T classes or river reaches with non-parametric Kruskal–Wallis (KW) H -tests and *post hoc* multiple comparison tests using the *pgirmess* package (Giraudoux, 2012). The fluorescence intensity in the field experiments was also analysed using an ordered regression (logit link) mixed model implemented by means of the ordinal package in R (Christensen, 2012). Final L_T of fish and shading index were ln-transformed and used as independent explanatory variables. Fluosum was the dependent ordinal variable (13 levels from 0 to 12). A random river effect was added in the model and accounted for differences between marking treatments and river characteristics. The significance level was set at 0.05.

RESULTS

HATCHERY TESTS

No mortality was observed 1 week after marking. Thereafter, a *Flavobacterium* sp. epizooty caused a general mortality in all basins and treatments (30.4% at 30 days). Because of this high mortality value, which significantly affected fish densities, growth analysis was not carried out.

The retention rates and fluosum remained high and stable over the first 9 months (Fig. 1). At 11 months, fluosum started to decline, but not the retention rate. The mark intensity significantly differed between treatments ($\chi^2 = 47.36$, $P < 0.001$). Treatment T0.5-3-5 displayed lower mark intensity than treatment T0.5-7 (*post hoc* KW test, $P < 0.05$), which was less fluorescent than treatment T1-3-5 ($P < 0.001$). At 15 months, the retention rates dropped and significantly differed between treatments ($\chi^2 = 7.62$, d.f. = 2, $P < 0.05$), with a sharper decrease for treatment T0.5-3-5. All the marked fish displayed very low fluosum (≤ 4). At 18 months, the retention rates were $\leq 30\%$ and did not differ between the three treatments ($\chi^2 = 3.08$, d.f. = 2, $P > 0.05$). The control fish scored zero throughout the experiment, except two fish (one at 11 months and one at 12 months) that were misread because of autofluorescence.

To test any effect of L_T on fluorescence intensity, all the 320 observed fish at 11 months were assigned to a size class (Fig. 2). Calcein intensity differed according to L_T in treatment T1-3-5 (KW test, $H = 9.56$, $P < 0.01$), with fish ≥ 175 mm being less fluorescent than fish < 150 mm (*post hoc* KW test, $P < 0.05$). In contrast, fluosum did not significantly differ between L_T classes in the other two treatments ($H = 4.17$, $P > 0.05$ and $H = 2.12$, $P > 0.05$ for T0.5-3-5 and T0.5-7, respectively).

FIELD TESTS

One year after marking, the percent of fluorescent fish ranged from 32% for the Flon de Carrouge stream to 91% for the Drize stream (Fig. 3) and strongly varied among the four streams ($\chi^2 = 48.7$, d.f. = 3, $P < 0.001$). The fish from the Flon de Carrouge stream displayed a lower retention rate than those living in the other three streams (multiple χ^2 tests, $P < 0.05$). The overall mean \pm s.d. was $70.7 \pm 26.6\%$, but it increased to $83.6 \pm 7.3\%$ without including the Flon de Carrouge fish. Within-stream analyses of fluosum showed significant differences between reaches in the Flon de Carrouge ($H = 7.26$, d.f. = 2, $P < 0.05$) and the Seigneux streams ($H = 8.84$, d.f. = 1, $P < 0.01$), where shading rates were contrasted between different reaches (Table II). Conversely, fluosum did not differ between the two reaches in the Vaux stream ($H = 0.01$, d.f. = 1, $P > 0.05$), where shading rates were very close (0.85 and 0.89). Ordinal logistic regression (Table III) showed an overall positive effect of the shading index on fluosum [likelihood ratio (LR) $\chi^2 = 16.50$, $P < 0.001$]. This indicates that higher fluorescence intensity was observed on fish living in heavily shaded river reaches. The final L_T of fish was not correlated to fluosum (LR $\chi^2 = 2.69$, $P > 0.05$), but the (random) river effect was highly significant ($P < 0.001$).

Two years after marking, an overall decrease in the mean retention rate ($48.6 \pm 24.6\%$) was observed despite the low number of recaptures (Fig. 3). The highest retention rate was observed in the Vaux stream (71%, $n = 7$). Conversely, the retention rate in the Seigneux stream dropped significantly (Fisher's exact test, $P < 0.05$) as only one of five of the marked fish was still fluorescent.

DISCUSSION

In this study, fluorescence retention and intensity of calcein-marked juvenile *S. trutta* were assessed. The respective effects of final L_T of fish and shading were

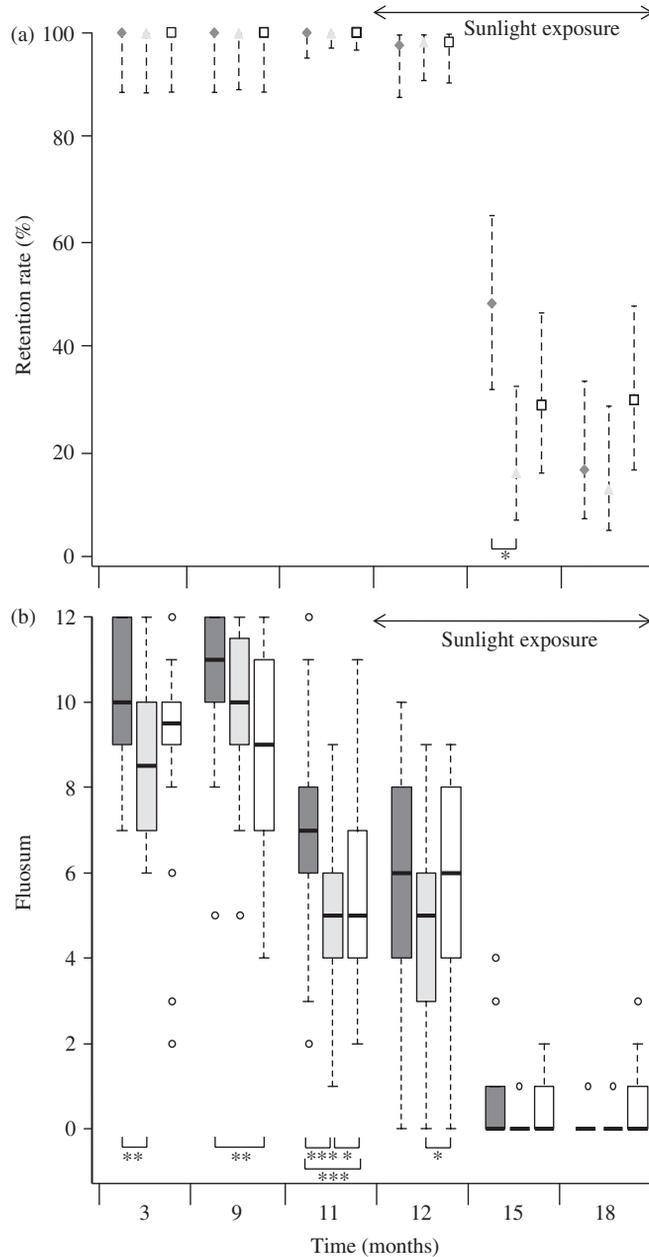


FIG. 1. (a) Variation of retention rate \pm 95% C.I. over time per treatment: 3.5 min in a 1% calcein solution (T1-3.5; ◆), 3.5 min in a 0.5% calcein solution (T0.5-3.5; ▲) and 7 min in a 0.5% calcein solution (T0.5-7; □), and (b) cumulative head and fin fluorescence intensity (fluosum) over time per treatment: 3.5 min in a 1% calcein solution (T1-3.5; ■), 3.5 min in a 0.5% calcein solution (T0.5-3.5; □) and 7 min in a 0.5% calcein solution (T0.5-7; □) in hatchery *Salmo trutta*. All the fish were raised in the absence of light until 11 months and were then exposed to sunlight under canvas. Significance of *post hoc* Kruskal–Wallis tests is indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). The bottom and top of the box are the first and third quartiles, and the band inside the box is the median. The whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box. ○, outliers.

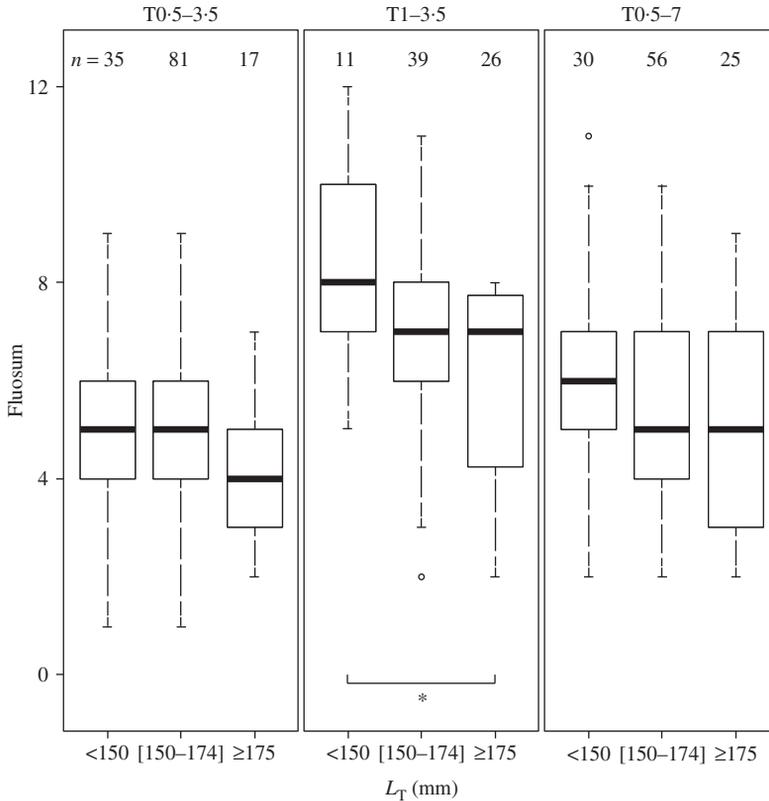


FIG. 2. Effect of final total length (L_T) on fluorescence intensity (fluosum) (see Fig. 1) of hatchery-reared *Salmo trutta* at 11 months post-marking. Fish were sorted into three L_T classes: <150, between 150 and 174 and ≥ 175 mm. Significance of *post hoc* Kruskal-Wallis tests is indicated (* $P < 0.05$). The bottom and top of the box are the first and third quartiles, and the band inside the box is the median. The whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box. \circ , outliers.

investigated both in a hatchery and in a natural environment. In the hatchery, all the marked fish showed recognizable fluorescent marks in their fins after being reared indoors for 11 months, whatever the calcein treatment. These results are in accordance with Stubbing & Moss (2007), who noted 100% mark retention until 12 months post-marking when the *S. trutta* were kept in a shaded environment and a decrease to 32% at 19 months after moving them to a shaded outdoor raceway. In this experiment, fish were also moved outdoors at 11 months; fluorescence retention and intensity rapidly decreased only after 4 months of partial exposure to sunlight (70% shading). Less than 30% of the marked fish still displayed fluorescence at very low intensity at 18 months.

Some differences were apparent between initial marking treatments. The fish marked with a lower calcein concentration combined with a shorter immersion time (T0.5-3.5) showed the sharpest decline in fluorescence intensity among the three treatments. By investigating the respective influence of immersion time and calcein concentration on mark intensity on golden perch *Macquaria ambigua* (Richardson

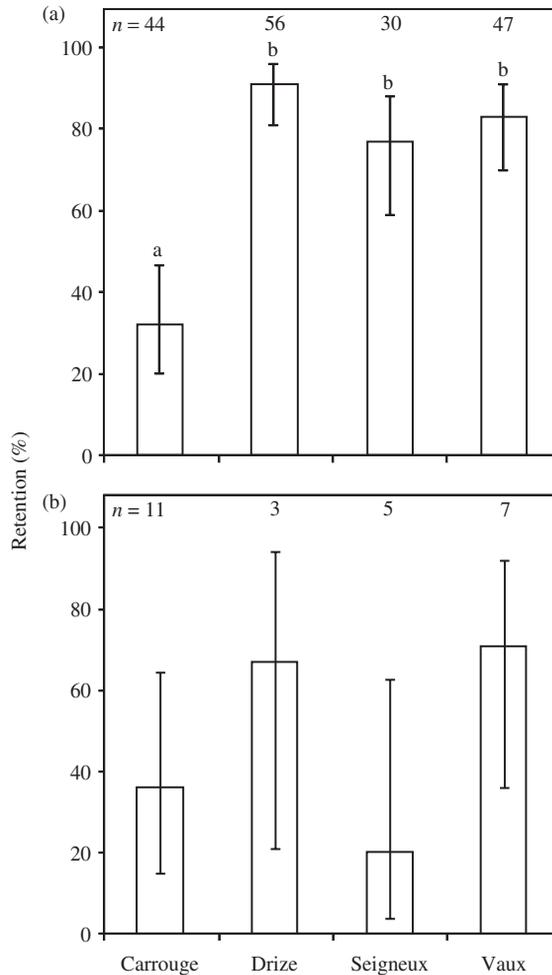


FIG. 3. Retention rate of fluorescence in marked *Salmo trutta* ($\pm 95\%$ c.i.) at (a) 1 and (b) 2 years post-marking in four streams. Fluorescence retention was compared between streams at each sampling occasion. Values sharing no common lower case letter are different at $P < 0.05$.

1845), Crook *et al.* (2009) concluded that calcein concentration was the prime influence. They showed that a 1% calcein concentration led to higher mark intensity than a 0.5% concentration, whereas increasing the exposure time in the 0.5% solution only slightly increased the mark intensity, but did not compensate for the difference due to concentration. In this study, the 1% solution induced brighter marks than the 0.5% solution with twice the exposure time until 11 months. After 11 months, these two treatments did not differ in mark intensity.

The hatchery tests showed evidence of a negative relationship between the final L_T of the fish at 11 months and the fluorescence intensity in one of the three treatments, although a negative trend appeared in the other two treatments. In the wild, L_T was not significantly correlated with the fluorescence intensity. The growth of tissue (skin and calcified tissues) over the marks was shown, however, to cause fading of the

TABLE II. Within-stream comparison of fluorescence intensity (fluosum) of calcein-marked *Salmo trutta* observed 12 months after marking in four streams. Shading index, mean final total length (L_T) of fish and retention rate were also compiled. Fluosum differed in the two streams displaying contrasted shading index between reaches

| Stream | Reach | Shading index | n | Mean \pm s.d. final L_T (mm) | Retention rate (%) | Median fluosum (minimum–maximum) | H test | P |
|----------|-------|---------------|-----|----------------------------------|--------------------|----------------------------------|----------|-------|
| Carrouge | 1 | 0.71 | 9 | 137.4 \pm 8.9 | 0.0 | 0 (0–0) | 7.26 | <0.05 |
| | 2 | 0.90 | 20 | 137.8 \pm 14.1 | 50.0 | 0.5 (0–3) | | |
| | 3 | 0.89 | 15 | 122.6 \pm 14.5 | 26.7 | 0 (0–2) | | |
| Drize | 1 | 0.81 | 56 | 151.4 \pm 15.2 | 91.1 | 3.5 (0–10) | – | – |
| Seigneux | 1 | 0.65 | 26 | 155.8 \pm 12.7 | 73.1 | 1.5 (0–6) | 8.84 | <0.05 |
| | 2 | 0.87 | 4 | 146.5 \pm 11.0 | 100 | 7 (4–9) | | |
| Vaux | 1 | 0.89 | 25 | 123.3 \pm 13.3 | 84.0 | 1 (0–6) | 0.01 | >0.05 |
| | 2 | 0.85 | 22 | 128.9 \pm 12.6 | 81.8 | 1.5 (0–10) | | |

external mark intensity with time (Frenkel *et al.*, 2002). Stubbing & Moss (2007) hypothesized that the loss of identifiable marks on *S. trutta* after 12 months was linked to size, not age. The present results did not corroborate this suggestion and factors other than fish size appear to affect fluorescence intensity.

In the hatchery, all fish were moved outdoors at 11 months. The subsequent sharp decreases in retention and intensity can best be related to a change in environmental conditions, mainly solar radiation exposure. No control fish in this study could corroborate this effect; previous studies, however, explained the loss of fluorescence intensity on calcein-marked salmonids due to sunlight exposure. Hill & Quesada (2010) observed a rapid decrease in fluorescence retention in Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) and rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) after only 2 weeks in tanks exposed to direct sunlight and after 8–9 weeks with intermittent sunlight exposure. Similarly, in a long-term monitoring of the fluorescence intensity in *O. mykiss* fry raised indoors or outdoors, Elle *et al.* (2010) showed that the external evaluation of calcein mark retention had already dropped 8 days post-marking in fish reared under full sunlight and that the mark was almost undetectable after 50 days. In contrast, fish reared under cover retained marks in their fins and heads for 205 days post-marking. Therefore, the main limitation of calcein use for external fish marking appears to be the fading of the fluorescence under sunlight.

TABLE III. Ordered regression mixed model on fluorescence intensity (fluosum) performed on field recaptures of *Salmo trutta* at 1 year post-marking in the four studied streams

| Model | n | Parameter | Estimate | LR χ^2 | d.f. | P -value |
|---------|-----|----------------|----------|-------------|------|------------|
| Fluosum | 177 | Shading index | 25.81 | 16.50 | 1 | <0.001 |
| | | Final L_T | –2.31 | 2.69 | 1 | >0.05 |
| | | River (random) | – | 67.66 | 1 | <0.001 |

L_T , total length; LR, likelihood ratio.

In field experiments, small streams with significant woody cover were selected for this study. Overall, the fluorescence retention after 1 year was acceptable in three of four streams (range = 77–91%), although some within-stream differences were apparent. The fish from highly shaded stations (coverage > 80%) showed higher fluorescence intensity than those from more open areas (coverage = 60–70%). This effect of shading was observed even though fish could move during the study and might have experienced various shading conditions before recapture. To date, despite some studies that have addressed fluorescence retention or intensity in hatchery conditions, very few marking experiments have been carried out in the field. Hill & Quesada (2010) showed a significant loss of fluorescence at 133 days post-marking in *O. tshawytscha* fry released into the Metolious River, but no information was provided about the river bank canopy. Sunlight exposure on calcein-marked fish in the wild may be mitigated either by (natural or artificial) shadow over the stream bed or by water turbidity (Crook *et al.*, 2012) and colour. This latter factor is of major importance in streams or lakes with high levels of suspended organic matter or in deep large rivers where sunlight does not reach the pelagic and benthic zones. In a lowland turbid river, calcein marks were detected up to 583 days after release in *M. ambigua* (Crook *et al.*, 2012). In most rivers harbouring *S. trutta*, the main factor limiting sunlight exposure is the shade provided by riparian cover. Experimental releases of marked fish in streams along wide gradients of shading indexes might help in understanding the effect of solar radiation, and help with the identification of the types of rivers in which this marking method could reliably be employed.

Factors other than sunlight may also influence calcein marks. Fluorescence retention and intensity decreased faster in the Flon de Carrouge than in the other streams although shading rate was high at some sites. Several hypotheses could explain this result. First, the larger size of fish at marking in this river (late summer) could have been the reason for such contrasting values. If it is acknowledged that marking too early should be avoided because of the low calcification of the fin rays just after hatching (Frenkel *et al.*, 2002), there is no available information that indicates that a later marking would negatively affect the calcein retention. On the contrary, Negus & Tureson (2004) found that *O. mykiss* marked as swim-up fry (mean mass < 1 g) lost their fluorescence at 22 months, whereas fish marked 3.5 months post-hatch (mean mass = 8 g) showed evident calcein marks until 35 months. In addition, they concluded that larger fins at the time of marking would ensure longer mark retention. The low retention rate in this stream could also be explained by the re-use of the marking solution and a reduced calcein concentration during successive marking. In this study, a 2 l solution was used to mark *c.* 2000 fish. Larger fish (mean mass > 3 g) could not be marked more than 100 at a time, whereas up to 200 smaller fish could be bathed in one sieve lot. Thus, the number of baths was increased and calcein concentration might have been reduced near the end of the marking process. Lastly, the chemical properties of the stream water may also be a possible cause for this variation in fluorescence retention. In this study, the pH values measured in the Flon de Carrouge were similar to those of other streams, but the conductivity levels were slightly lower. Nevertheless, it is unclear whether water chemical properties (*e.g.* concentration of calcium or other metal ions) could induce calcein-bound calcium to be mobilized from the tissues and thus affect fluorescence intensity.

The portable SE-MARK detector is a good device to rapidly detect fluorescence. Because this method is subjective, operators should have sufficient experience with

the detector to distinguish autofluorescence (noise) from real fluorescence. This method remains cheaper and more effective than scale or fin sampling and later laboratory analyses under an epifluorescence microscope. Fluorescence is less detectable in frozen (Negus & Tureson, 2004) or ethanol-preserved tissues (Bashey, 2004) because of autofluorescence. Moreover, Negus & Tureson (2004) showed that marks in the scales of *O. tshawytscha* and *O. mykiss* recorded as parr (mean mass = 2 and 8 g) faded faster than those in the other tissues. They also noticed that the marks are generally more detectable at the base of the fin. Thus, the direct examination of the fin rays and the head (including jaws and gill arches) is the best way of detection.

Calcein can be used as an external marking tool to assess the efficiency of *S. trutta* stocking in the wild for up to 1 year. Practitioners should restrict the use to studies in shaded streams or in turbid or deep water bodies, where fish are not exposed to natural sunlight. Using the handheld SE-MARK detector to directly assess the fluorescence intensity in the wild proved to be efficient.

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References

- Baer, J. & Rosch, R. (2008). Mass-marking of brown trout (*Salmo trutta* L.) larvae by alizarin: method and evaluation of stocking. *Journal of Applied Ichthyology* **24**, 44–49.
- Bashey, F. (2004). A comparison of the suitability of alizarin red s and calcein for inducing a nonlethally detectable mark in juvenile guppies. *Transactions of the American Fisheries Society* **133**, 1516–1523.
- Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B* **57**, 289–300.
- Brooks, R. C., Heidinger, R. C. & Kohler, C. C. (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.
- Caudron, A. & Champigneulle, A. (2009). Multiple marking of otoliths of brown trout, *Salmo trutta* L., with alizarin redS to compare efficiency of stocking of three early life stages. *Fisheries Management and Ecology* **16**, 219–224.
- Crook, D. A., O'Mahony, D. J., Sanger, A. C., Munro, A. R., Gillanders, B. M. & Thurstan, S. (2009). Development and evaluation of methods for osmotic induction marking of golden perch *Macquaria ambigua* with calcein and alizarin red S. *North American Journal of Fisheries Management* **29**, 279–287.
- Crook, D. A., O'Mahony, D. J., Gillanders, B. M., Munro, A. R. & Sanger, A. C. (2012). Quantitative measurement of calcein fluorescence for non-lethal, field based discrimination of hatchery and wild fish. *American Fisheries Society Symposium* **76**, 389–396.
- Elle, F. S., Koenig, M. K. & Meyer, K. A. (2010). Evaluation of calcein as a mass mark for rainbow trout raised in outdoor hatchery raceways. *North American Journal of Fisheries Management* **30**, 1408–1412.

- Frenkel, V., Kindschi, G. & Zohar, Y. (2002). Noninvasive, mass marking of fish by immersion in calcein: evaluation of fish size and ultrasound exposure on mark endurance. *Aquaculture* **214**, 169–183.
- Hill, M. S. & Quesada, C. J. (2010). Calcein mark retention in chinook salmon and steelhead fry in artificial and natural rearing environments. *North American Journal of Fisheries Management* **30**, 1370–1375.
- Honeyfield, D. C., Ostrowski, C. S., Fletcher, J. W. & Mohler, J. W. (2006). Dietary calcein marking of brook trout, Atlantic salmon, yellow perch, and coho salmon scales. *North American Journal of Fisheries Management* **26**, 431–437.
- Honeyfield, D. C., Kehler, T., Fletcher, J. W. & Mohler, J. W. (2008). Effect of artificial sunlight on the retention of external calcein marks on lake trout. *North American Journal of Fisheries Management* **28**, 1243–1248.
- Kelley, C. E. & Krueger, W. C. (2005). Canopy cover and shade determinations in riparian zones. *Journal of the American Water Resources Association* **41**, 37–46.
- Leips, J., Baril, C. T., Rodd, F. H., Reznick, D. N., Bashey, F., Visser, G. J. & Travis, J. (2001). The suitability of calcein to mark poeciliid fish and a new method of detection. *Transactions of the American Fisheries Society* **130**, 501–507.
- 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.
- Negus, M. T. & Tureson, F. T. (2004). Retention and nonlethal external detection of calcein marks in rainbow trout and Chinook salmon. *North American Journal of Fisheries Management* **24**, 741–747.
- Newcombe, R. G. (1998). Two-sided confidence intervals for the single proportion: comparison of seven methods. *Statistics in Medicine* **17**, 857–872.
- Simon, J., Dörner, H. & Richter, C. (2009). Growth and mortality of European glass eel *Anguilla anguilla* marked with oxytetracycline and alizarin red. *Journal of Fish Biology* **74**, 289–295.
- Smith, J. E., Macreadie, P. I. & Swearer, S. E. (2010). An osmotic induction method for externally marking saltwater fishes, *Stigmatopora argus* and *Stigmatopora nigra*, with calcein. *Journal of Fish Biology* **76**, 1055–1060.
- Strickler, G. S. (1959). Use of the densitometer to estimate density of forest canopy on permanent sample plots. *USDA Forest Service Research Note* **180**.
- Stubbing, D. N. & Moss, R. D. (2007). Success of calcein marking via osmotic induction in brown trout fry, *Salmo trutta*. *Fisheries Management and Ecology* **14**, 231–233.

Electronic References

- Christensen, R. H. B. (2012). *ordinal: Regression Models for Ordinal Data. R Package Version 2012.09-11*. Available at <http://www.cran.r-project.org/package=ordinal/>
- Giraudeau, P. (2012). *pgirmess: Data Analysis in Ecology. R Package Version 1.5.4*. Available at <http://CRAN.R-project.org/package=pgirmess/>