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Evaluation of large-scale marking with alizarin red S in different age rainbow trout fry for nonlethal field identification

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Abstract

Fry of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), was subjected to one-hour and four hours immersion in Alizarin red S (ARS) bath 150 mg·L^{-1}. The experiment involved seven age groups (40, 50, 60, 70, 80, 90, and 100 days) and was conducted to estimate the minimal age of salmonids for mass marking with ARS bath enabling subsequent effective, simple field nonlethal identification, based on fin rays checking. The fish were examined at the ages of 200 and 300 days. The results showed a high retention level of ARS traces in caudal fin rays ensured satisfactory visibility and quick detection. A success rate of marking detection was >90% at 200 and 300 days of age and the fish were immersed in ARS solution from 60 days of age (685°D). This treatment provided better results in fish bathed for four hours. Recognition of marks using a laser pointer and protective glasses was successful even in fish, with a 3.5–5.0 times length increase compared to the marking time. The results indicate a high potential for ARS marking and its field identification for juvenile salmonids, which can significantly expand the possibilities of field experiments.

Keywords

Actinopterygii, alizarin red S, chemical marking, fin rays, Salmonidae, 532 nm laser pointer detection

Introduction

Fish marking is a powerful tool in fisheries science (Thorsteinsson 2002). It is possible to obtain a wide range of information by marking fish. Methods based on the re-capturing of marked fish provide information on the composition of populations in open waters, as well as the growth and survival of stocked fish in natural conditions.

The use of farmed and restocked fish for different goals, including conservation efforts, is growing at a rapid rate. Yet, monitoring the benefits of using hatchery-raised fish for supplementation is often lacking, often due to hatcheries not marking or tagging all fish before release (Warren-Myers et al. 2018). Effective mass marking of early-age categories of fish is particularly problematic. Simultaneously, there is broad agreement among scientists that fish generally have to be stocked in their early life stages if stocking aims to support natural populations (Järvi 2002; Metcalfe et al. 2003). Typically, salmonids in captivity develop reared-related traits that diverge them from wild conspecific in feeding behavior, response to predators, and habitat use (Einum and Fleming 2001). This phenomenon substantially reduces their fitness in wild environments (Fraser et al. 2011).

Although the use of natural or chemical marks (e.g., tetracycline, calcein, and alizarin red), like exposure to stable isotopes via egg immersion or vaccination, involves no or no extra handling, subsequent analysis may require killing the fish after catch (Thorsteinsson 2002; Uglem et al. 2020). In the case of efforts to restore and strengthen weakened populations, especially of salmonid fish, the necessity of killing individuals to identify the origin is counterproductive, because it also includes the killing of wild fish offspring. Bath marking of fish in ARS uses the ability of ARS to bind to calcareous structures in organisms (bone, scale, otolith) (Puchtler et al. 1968) has been known for a long time and is described for various species of salmonid fish, published studies used identification based on otolith analysis (Baer and Rösch 2008; Caudron and Champigneulle 2009; Unfer and Pinter 2013; Lejk and Martyniak 2018; Lejk and Radtke 2021). For this identification method, it is necessary to kill the examined fish. However, several studies mention the possibility of identifying ARS markings using fin-ray analysis in cyprinids (Lü et al. 2015, 2016). For pike, *Esox lucius* Linnaeus, 1758, this procedure has been verified, including the possibility of field identification (Halačka et al. 2018a). Ossification of the fin rays is essential for the success of such marking. Therefore, it is necessary to verify the minimum age and size of individuals of various fish species for marking ARS baths, enabling the subsequent simple and effective field identification of marked fish.

The first purpose of the presently reported study was to investigate the possibility of a simple field nonlethal method of identification ARS marked salmonid fish based on fin rays checking. The second aim was a preliminary estimate of the minimal age of salmonid fish for mass marking with ARS bath enabling subsequent effective field identification with rainbow trout as model species.

Materials and methods

Experimental fish and marking procedure. One thousand juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), aged 30 days (after hatching), weighing 0.45 ± 0.13 g (mean weight ± standard deviation) were purchased from a local producer (Vladimír Šefl, Bušanovice, Czech Republic) where they were reared in a recirculation system (plastic trough) with water temperature 11.0 ± 0.1°C and fed commercial pellets (INICIO Plus, 0.5 mm; Biomar). Fish were randomly divided and stocked in three aquariums (volume 300 L) at the Faculty of Fisheries and Protection of Waters (FFPW) in Vodňany, Czech Republic. Aquaria were filled with tap water filtered through an active carbon filter. Each aquarium was aerated and connected to an individual external filter (Eheim professional 4+, EHEIM GmbH, Germany). Fish excrement and other sediments were drained daily at 12:00 h including 30% water change. Every 10 days, at 40, 50, 60, 70, 80, 90, and 100 days of fish age, 100 fish were randomly chosen, divided into two groups and placed in smaller aquariums with 10 L of ARS solution (alizarin red S, Carl Roth GmbH + Co. KG, Germany) in a concentration of 150 mg · L^{-1}. One group was always bathed for one hour, the other for four hours. Experimental groups were labelled 40/1, 50/1, 60/1, 70/1, 80/1, 90/1, 100/1 or 40/4, 50/4, 60/4, 70/4, 80/4, 90/4, 100/4 according to age of marking and duration of immersion. During the bath, the aquariums were aerated, and the mortality of fish was recorded. After the bath, fish were measured (standard length, SL and total length, TL; mm) and weighed individually (Table 1), and each group was stocked to separate sections of aerated aquariums (water volume 50 L) connected to an individual external filter (see above). During the experiment, fish were fed by commercial pellets of a reasonable size (INICIO Plus, 0.8 mm, and 1.1 mm; Biomar) at 2% of the stock weight daily, divided into three rations. The mortality of fish in each group was recorded daily. Every 10 days the total weight of the stock in each aquarium/section was detected due to the specification of the feed ration. During this part of the experiment (age of fish 30–100 days), the mean water temperature was 14.3 ± 1.2°C, oxygen content 10.1 ± 1.0 mg · L^{-1}, and pH 7.2 ± 0.7. Based on temperature measurements, it was possible to calculate degree-days (°D) for a certain age of fish as the multiplication product of the mean daily water temperature (°C) and the number of days after hatching.

Table 1. Biometric data for fry of rainbow trout, *Oncorhynchus mykiss* (n = 50 for each group) at times of the marking immersion.

Age [day]	Cumulative D° [°C]	SL [mm]	TL [mm]	W [g]
40	438	33.9 ± 1.2	38.8 ± 1.6	0.57 ± 0.06
50	562	37.2 ± 2.4	43.1 ± 2.9	0.81 ± 0.08
60	685	40.1 ± 3.1	48.1 ± 3.6	1.08 ± 0.12
70	833	45.1 ± 3.6	54.2 ± 4.3	1.55 ± 0.18
80	966	50.4 ± 6.5	60.3 ± 7.1	2.04 ± 0.32
90	1109	57.6 ± 8.6	67.6 ± 9.2	3.03 ± 0.53
100	1262	61.3 ± 8.9	72.1 ± 9.6	4.14 ± 0.89

D° = degree day (mean daily water temperature × number of days after hatching), SL = standard length, TL = total length, W = weight. Values of SL, TL, and W are mean ± standard deviation.

At the age of 100 days, the experimental fish groups and control group (250 ind.) were stocked separately into the flow-through circular 500-L tanks filled with river water from the Blanice (Vodňanská) River, at the experimental facility of the FFPW. Fish were reared for 100 days and fed by commercial pellets of a reasonable size (INICIO Plus, 1.5 mm, and 2.0 mm; EFICO Enviro 921, 3 mm; Biomar) at 2% of the stock weight daily. Mortality was recorded for each experimental group and control group. Based on the obtained results of the first evaluation, six groups of fish were selected (marked one and four hours at 60, 80, and 100 days of age), which were further reared to evaluate the visibility of markings at the age of 300 days.

Evaluation of marking visibility. After they were reared for another 100 days (i.e., until the age of 200 days), 20 randomly chosen fish from each group were removed for the analysis of fin rays. The marking detectability was checked and viewed using a green laser pointer (100 mW power; OEM) emitting light of wavelength 532 nm. The evaluators

were equipped with protective glasses (SOH, Prague) for working with the laser, preventing the passage of light with a wavelength of 190–540 nm but allowing to observe light with a wavelength of 580 nm, which is the emission value for the given ARS excitation. In the fin rays of the marked fish, the induced fluorescence of the fiery-red tissue can be observed (Fig. 1). During checking, the fish were placed on a damp, black plastic mat, protected from direct sunlight (the evaluation took place in a shade). The evaluation at the 300 days of fish age was provided for groups of fish marked at 60, 80, and 100 days of age. The presence of marks was verified on the fin rays of the caudal and/or anal fin. Randomly chosen marked and control fish was anesthetized with clove oil (0.03 mL · L^{-1}) and given to two trained examinators for evaluation. All fish were individually measured (SL and TL, mm) and weighted (g) before evaluation. Both examiners checked 10 fish from each marked group, randomly interspersed 80 unmarked control fish, without prior information about the belonging of a specific fish to one of the groups. Detectability of the mark was defined as either "marked" or "unmarked". After marking evaluation and recovery from anesthesia, the fish were returned to the appropriate tank.

Statistical analyses. A one-way analysis of variance (ANOVA) was used to assess differences in weight and length, among fish groups at both mark evaluation times. Post hoc comparisons were made by Tukey's honest significant difference test. Student's *t*-test was used to test for weight and length differences among marked fish with detected and undetected markings. Marking recognition rates were compared with the Pearson and maximum likelihood χ^2 test. Significance was accepted for values of $P < 0.05$.

Results

No effect of the marking procedure on the mortality of rainbow trout fingerlings was found. During the immersion of the fish in the alizarin solution, only two fish deaths were recorded, in groups 60/4 and 90/1. The survival rate in 14 experimental groups during subsequent rearing was 94%–100% at 200 days of age vs. 95.6% in control group. At 300 days of age, survival in the experimental 6 groups was 90%–98% vs. 93.6% in control group. No differences in weight ($F = 0.53$; $P = 0.883$), SL ($F = 0.46$; $P = 0.907$), and TL ($F = 0.37$; $P = 0.968$) were recorded between experimental and control fish at 200 days or 300 days ($F = 1.02$; $P = 0.417$ for SL; $F = 1.11$; $P = 0.356$ for TL, and $F = 2.12$; $P = 0.054$ for weight) of age (Table 2).

The detectability of marking was significantly different at both checking times (Fig. 2). At 200 days of age the mark

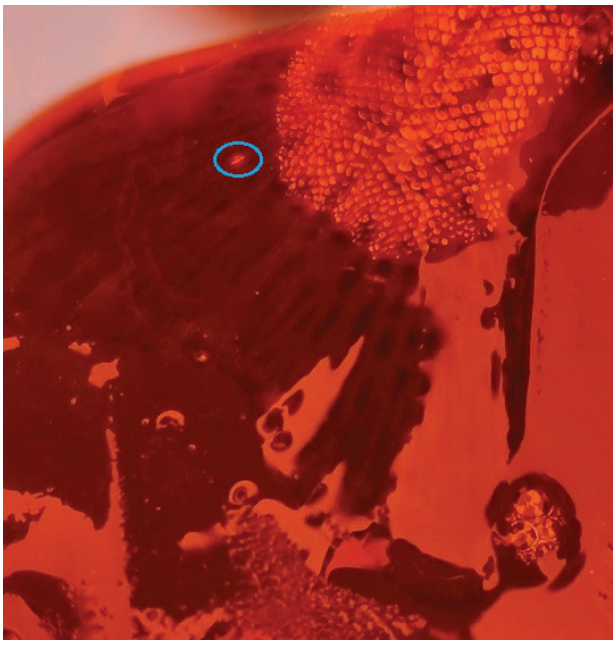

Figure 1. Visible marking in the fin ray of rainbow trout, *Oncorhynchus mykiss* stained with alizarin red S. In the blue circle, a part of the marked fin ray activated by the laser pointer is visible (photo taken through protective glasses).

Table 2. Biometric data for experimental and control (C) groups of rainbow trout, *Oncorhynchus mykiss* ($n = 20$ for marked groups, $n = 160$ for control group) in marking check times.

Group	Age 200 days			Age 300 days		
	SL [mm]	TL [mm]	*W* [g]	SL [mm]	TL [mm]	*W* [g]
40/1	165.7 ± 12.6	187.7 ± 13.0	73.3 ± 12.5	—	—	—
40/4	163.3 ± 18.0	183.3 ± 18.8	74.3 ± 16.1	—	—	—
50/1	161.0 ± 20.2	182.9 ± 22.1	71.5 ± 21.6	—	—	—
50/4	167.3 ± 14.2	188.5 ± 15.6	74.8 ± 16.2	—	—	—
60/1	163.6 ± 18.1	184.8 ± 19.4	69.4 ± 17.7	248.7 ± 20.2	277.7 ± 21.0	249.5 ± 55.5
60/4	166.8 ± 18.5	189.0 ± 19.6	77.0 ± 21.1	249.9 ± 19.6	279.0 ± 20.5	263.3 ± 50.4
70/1	164.6 ± 16.2	186.3 ± 16.5	77.6 ± 17.4	—	—	—
70/4	175.1 ± 14.2	196.9 ± 15.4	88.7 ± 17.3	—	—	—
80/1	158.7 ± 15.9	180.3 ± 17.6	69.8 ± 20.6	235.8 ± 27.4	264.5 ± 30.2	229.0 ± 71.5
80/4	164.0 ± 15.4	185.4 ± 17.3	75.6 ± 18.1	251.2 ± 22.7	279.9 ± 26.1	286.6 ± 53.7
90/1	164.4 ± 15.8	184.7 ± 16.2	73.7 ± 19.1	—	—	—
90/4	168.6 ± 17.0	189.1 ± 15.7	79.9 ± 17.0	—	—	—
100/1	163.1 ± 20.6	183.8 ± 22.6	78.3 ± 24.0	250.8 ± 21.4	279.5 ± 24.3	272.9 ± 62.9
100/4	165.7 ± 12.5	187.2 ± 14.6	74.5 ± 21.3	251.5 ± 36.5	281.2 ± 39.1	290.4 ± 99.6
Control	162.8 ± 15.3	182.1 ± 16.5	71.5 ± 19.2	244.9 ± 26.5	271.5 ± 27.7	254.5 ± 68.7

Values are mean ± standard deviation, *n* = number of fish, SL = standard length, TL = total length, *W* = weight. In SL, TL, and *W*, no significant ($P < 0.05$) differences between groups according to ANOVA in both checking times were found.

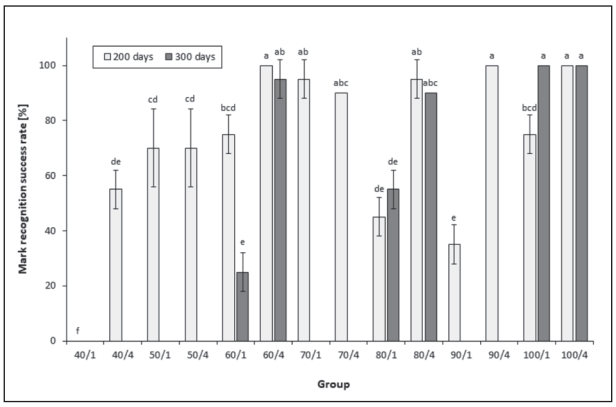

Figure 2. Alizarin red S marking recognition rate in fin rays of experimental groups of rainbow trout, *Oncorhynchus mykiss*, at 200 and 300 days of age. Different letters indicate significant differences ($P < 0.05$) between groups according to Pearson and maximum likelihood χ^2 test.

recognition success rate was from 0% (group 40/1) to 100% (groups 60/4, 90/4, and 100/4). Except for fish tagged at 50 and 70 days of age, the mark recognition was always higher in the four-hour immersion group than the one-hour immersion one, with the difference being statistically significant in some groups. When evaluated at 300 days of age, there was a significant decrease in the detectability of marking in the 60/1 group and an increase in the 100/1 group compared to the evaluation at 200 days. For the other groups, the detectability of marking changed but not statistically significantly. At both evaluation times, no significant differences in weight ($F = 1.35$; $P = 0.657$ for 200 days evaluation; $F = 2.55$; $P = 0.244$ for 300 days evaluation), SL ($F = 1.45$; $P = 0.905$ for 200 days evaluation; $F = 2.18$; $P = 0.982$ for 300 days evaluation), and TL ($F = 1.45$; $P = 0.995$ for 200 days evaluation; $F = 2.52$; $P = 0.275$ for 300 days evaluation), were recorded between the fish of the experimental groups with successful and unsuccessful marking detection (Table 3).

Discussion

During the experiment, there was no negative effect of immersion in 150 mg · L^{-1}ARS solution on the survival and growth of marked fish in both duration time one and four hours. The observed death of two fish during the immersion in our experiment can probably be attributed to stress or injury during handling. Concentration ARS immersion up to 300 mg · L^{-1} is published as safe for different fish species by many studies (Lü et al. 2015; Lü et al. 2016), although Unfer and Pinter (2013) recorded increased mortality in brown trout fry and alevin at three-hour duration immersion in ARS concentrations of 150 and 300 mg · L^{-1} compared to control fish. Jurgelėnė et al. (2022) point out the possibility of different toxicity of alizarin red S produced by different manufacturers. Baer and Rösch (2008) recorded more than 95% mortality of 20-day-old brown trout (TL 25–29 mm) immersed for three hours in ARS 300 mg · L^{-1} bath with added 10 g · L^{-1} sodium chloride

Table 3. Biometric data for marked rainbow trout, *Oncorhynchus mykiss*, with detected and undetected marks in marking check times.

	Age 200 days		Age 300 days	
	Detected	Undetected	Detected	Undetected
n	197	83	93	27
SL [mm]	165.2 ± 17.4	164.9 ± 14.5	246.5 ± 27.3	253.0 ± 17.1
TL [mm]	186.4 ± 18.6	186.4 ± 15.4	275.5 ± 29.5	282.0 ± 18.6
W [g]	75.9 ± 19.7	74.9 ± 17.1	265.0 ± 74.3	266.2 ± 51.2

Values are mean ± standard deviation, *n* = number of fish, SL = standard length, TL = total length, *W* = weight. In SL, TL, and *W*, no significant ($P < 0.05$) differences between groups according to Student's *t*-test in both checking times were found.

(NaCl) for the possibility to increase the marking efficiency. Immersion in 50 and 150 mg · L^{-1} ARS was founded to be safe, with or without NaCl added. Halačka et al. (2018b) found 100% mortality in a 72-h toxicity test on zebra fish, *Danio rerio* (Hamilton, 1822), in 150, 300, and 600 mg · L^{-1} ARS immersions if NaCl (10 g · L^{-1}) was added compared to zero mortality in the same without NaCl. Due to these inconsistent results, NaCl was not used in the marking immersion in the presently reported experiment to reduce the risk of experimental fish mortality, which could compromise the evaluation of the experiment.

Our experiment demonstrated the possibility of using ARS immersion for mass marking of young age categories of salmonid fish with minimal losses of fish. When followed by simple non-lethal mark detection in the field this approach is a suitable choice for monitoring the stocking programs with young salmonids. The majority of the study results published so far, which have focused on the possibility of non-lethal detection of chemical marking of fish, state the necessity of tissue sampling and detection of the marking in the laboratory using a special microscope (Lü et al. 2015; Lü et al. 2016), although Mohler (2003) describes the possibility of using a hand-held calcein mark detector to immediately discern between marked and non-marked fish by the presence or absence of a visible green fluorescence in the fin rays and other calcified structures. The possibility of field identification of fluorescent markers is also mentioned by Uglem et al. (2020). The method of detection of ARS marking, used in the presently reported study, was described in the methodology of Halačka et al. (2018a) for pike. Its main advantage is the possibility of identifying the marked fish directly in the field, which minimizes the stress of the researched fish and the time and personnel requirement of the research.

The second important goal of the study was to determine the minimum age (size) of the fish, necessary for the formation of a sufficiently distinct mark. The principle of the method namely requires the calcification of the fin rays; they enable the establishment of ARS. To achieve high mark detection rate (90% +) in rainbow trout in the presently reported study, the minimum age of tagged fish for mark application was 60 days (685°D, SL 48.1 ± 3.6 mm), while better results were achieved in fish with immersion duration of four hours compared to those im-

mersed for one hour. In contrast, Halačka et al. (2018a) did not find a significant difference in the identifiability of tags between fish immersed for one or three hours in 150 mg · L^{-1} ARS solution for pike fry.

The results of our study also demonstrate a good detectability of marking even in the case of significant length growth of marked fish. Deterioration of marking identifiability at 300 days of age compared to 200 days was noted only in the 60/1 group, which proves the already mentioned advantage of a longer duration of the marking bath. Fish checked at 300 days of age had 3.7–5.0 times longer body length than at the time of marking. It is the growth of the fish and possible changes in the color and physiological changes of the fish tissues that are mentioned as a frequent reason for the deterioration of the detectability of internal tags (e.g., Halačka et al. 2018b; Thorsteinsson 2002). Thus, it seems that the increase in length of the marked fish does not significantly reduce the identifiability of the ARS markings in rainbow trout, and thus in salmonid fish in general. The final size of the fish in the presently reported experiment corresponded to the size of wild adult brown trout found by Turek et al. (2010) during local mark–recapture experiments in the Blanice (Vodňanská) River. Considering the lower growth rate of stocked salmonid fish in nature, it can be assumed that the presented method of marking and identification will make it possible to recognize fish stocked as fed fry even several years after stocking.

In conclusion, a four-hour duration ARS immersion (150 mg · L^{-1}) is suitable for marking the juveniles salmonids from 60 days of age (685°D) for later nonlethal field identification, based on fin rays checking by 532 nm laser pointer using protective glasses preventing the passage of light with a wavelength of 190–540 nm. The use of the presented marking and identification methods can significantly improve and simplify the implementation of mark-recapture experiments in salmonids in open waters.

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