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RESEARCH PAPER

Low Temperature Effect on Multiple Alizarin Immersion Mass-Marking of Juvenile Sea Trout *Salmo trutta m. trutta* L. otoliths

Adam M. Lejk[1,2,*], Andrzej Martyniak[1]

[1] University of Warmia and Mazury in Olsztyn, Department of Fish Biology and Pisciculture, Oczapowskiego 5, PL 10-719 Olsztyn, Poland.
[2] National Marine Fisheries Research Institute, Department of Logistics and Monitoring, Kołłątaja 1, PL 81-332 Gdynia, Poland.

* Corresponding Author: Tel.: +48 89 523-32-90; Fax: +48 89 523-32-90;
E-mail: adam.lejk@uwm.edu.pl

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Abstract

This study presents a protocol of immersing sea trout *Salmo trutta* m. *trutta* L. larvae within an alizarin red S (ARS) solution to provide highly visible single and double fluorescent marks in their otoliths. The 14 day interval and temperature regime between the first and second batch marking sessions have been found to develop the proper distance between the ARS marks in the otoliths. The mean values of increment widths between the first and second ARS marks in the otoliths of sea trout marked twice within 14 day period ranged between 21.11–34.98 µm, depending on the year of the experiment. The distance between ARS marks corresponded to temperature during the period between first and second fish immersions which was 4.4ºC, 7.5ºC and 6.7ºC, for year 2014, 2015, and 2016 respectively. As a result, this method can be used as a routine procedure that provides clear and well-visible single and double alizarin marks in the otoliths and helps to distinguish fish originating from different stocking groups of wild-born individuals.

Keywords: salmonids, fluorochromes, alizarin red S, increment widths, Łeba River.

Introduction

Fish stocking is one of the most commonly applied methods to compensate for damages resulting from human impacts on the environment and aquatic living resources (Cowx, 1994). Stocking is also a valuable tool for sustainable fisheries management. Because of the high costs, new methods to evaluate stocking effectiveness, including fish tagging, have been developed (Nielsen, 1992).

Traditional, external tagging techniques are unsuitable for young fish, which are released at an early developmental stage. As a complementary method, otolith marking is used to compare stocking efficiency (Brothers, 1990). Mass marking of fish at early life stages by immersion in fluorochrome solutions was developed in the mid-1980s (Hettler, 1984), and this method is still widely applicable for salmonids (e.g., Caudron & Champigneulle, 2006, 2009; Champigneulle & Cachera, 2003; Dąbrowski & Tsukamoto, 1986; Eckmann, Czerkies, Helms, & Kleibst, 1998; Nagięć, Czerkies, Goryczko, Witkowski, & Murawska, 1995; Niva, Keränen, Raitaniemi, & Berger, 2005; Meerbeek & Bettoli, 2005; Van der Walt & Faragher, 2003).

Previous studies concerning the immersing of brown trout, *Salmo trutta* L., juveniles have shown the high applicability of this marking method with the use a various fluorochromes: tetracycline (Champigneulle & Cachera, 2003), oxytetracycline (Meerbeek & Bettoli, 2005), alizarin red S (Caudron & Champigneulle, 2006; Gerdeaux, Luquet, Poupart, & Tostivint, 2006) and calcein (Stubbing & Moss, 2007). In recent years, alizarin red S (ARS) has been one of the most commonly used marking substances which has been applied to *Salmo trutta* L., immersed at different life stages, including, chronologically, embryos (Unfer & Pinter, 2013), alevins (Baer & Rösch, 2008; Caudron & Champigneulle, 2006, 2009; Niva *et al*., 2005; Unfer & Pinter, 2013) and fry (Caudron & Champigneulle, 2009; Gerdeaux *et al*. 2006; Unfer & Pinter, 2013). Only two papers make references to multiple ARS marking of *Salmo trutta* L. otoliths (Caudron & Champigneulle, 2006, 2009). Accordingly, further study is necessary to develop this marking technique. Requisite data include the time and temperature needed to provide a proper distance between individual ARS rings and implementing obtained results for common use.

In this paper, we provide a methodical approach for the successful multiple immersion marking of sea trout, *Salmo trutta* m. *trutta* L., larvae with the use of alizarin red S solution, conducted in mass-marking conditions. Furthermore, the time interval and

different temperature regimes between particular marking sessions are examined to develop easily identified and well-separated double fluorescent marks.

Materials and Methods

A large-scale marking experiment was conducted at the Żelkówko Fish Farm (Northern Poland) from 2014–2016. The Żelkówko Fish Farm is a traditional, flow-through salmonid-type farm supplied by riverine and spring water. Juvenile fish dedicated to Łeba River stocking (southern Baltic Sea; 54°45'N; 17°33'E) originated from wild, native sea trout *Salmo trutta* m. *trutta* L. spawners. Adults were caught in Lake Łebsko, with the use of fyke nets, during their spawning migration in November 2013–2015. Fish were subjected to a typical controlled spawning operation, where gametes were collected manually, and the 'dry fertilization' procedure was applied. Fertilized eggs were incubated in hatching trays, placed in horizontal California-type incubators supplied by spring water. The water temperature during the eggs incubation was around 8.0 ± 0.5°C. The newly hatched larvae were kept in the same incubators for the next ten days, until the beginning of the experiment.

Marking Procedure

Ten-day-old sea trout larvae were divided into two groups: I) fish intended for stocking with alevins at the end of yolk-feeding phase and II) fish intended for stocking with fed fry 0+. Fish from both groups were kept in separate concrete tanks: raceways (with a bottom area of 4 m^2 and a depth about of 0.7 m each) at a mean density of 50,000 larvae per tank. The raceways were supplied by a mixture of riverine and spring water. Before the addition of marking solution, the inflow of the water into each tank was halted, and the water levels were lowered down to 15 cm. A concentrated solution of alizarin red S (ARS) was prepared earlier and evenly distributed in the experimental tanks. The marking procedure assumed that the fish from those two groups were simultaneously single marked by 3-h immersion in a solution of ARS (ACROS Organics™, Belgium) using a concentration of 200 mg L^{-1}. Additionally, after a 14 day interval, fish from group II were batch marked a second time using the same concentration of ARS.

Water temperature (°C), saturation (%), oxygen concentration (mg O_2 L^{-1}) and pH-levels were regularly monitored (every 30 min) during the immersion using the Hach HQ 40d portable multi-meter (HACH Corp., USA). For all years, the pH-value was relatively stable and approximately 7.81 (range 7.23–8.24). Therefore, treatment with pH-stabilizers was not necessary. As a result of the aeration, the oxygen concentration was maintained at around 10.5 mg O_2 L^{-1} and saturation at around 85.8% until the end of each immersion. The water temperature during particular marking sessions ranged from 3.1–5.8°C, 6.9–8.0°C and 6.2–6.9°C, for 2014, 2015 and 2016, respectively. The mean values of the water temperatures between the first and second fish immersions from group II varied between years and were 4.4°C, 7.5°C and 6.7°C.

Sixty days after the first (group I) and second (group II) marking sessions, 50 individuals from both experimental groups were randomly sampled for each year. Fish were anaesthetized with an overdose of 300 mg L^{-1} MS-222 (ACROS Organics™, Belgium). Each fish was measured by TL, (±0.1 mm), weighted as the BW, (±0.001 g) and otoliths (2 sagittae) were collected.

Marks Detection

Alizarin marks were detected under an epifluorescence microscope, the Nikon Eclipse 90*i* (Nikon Corp., Japan), equipped with a UV light in the G-2A filter set (EX 510-560 nm, DM 575 nm, BA 590 nm), with 40 and 100 x magnifications. The pairs of sagittae were mounted on microscope glass slides with Entellan® (Merck KGaA, Germany). To reduce the effect of reflective glare, otoliths were manually polished with 2000 grit sand paper and controlled for marks. Increment widths (± 0.01 µm) between the first and second ARS marks in the otoliths (n = 300) of sea trout from group II were measured using computer software NIS-Elements BR 3.1 (Nikon Corp., Japan) at the 100 x magnification.

Statistical Analysis

Statistics were calculated using Statistica 12.0 (Stat Soft, Inc., USA). Data were tested for normality using a Shapiro–Wilks test, and the homogeneity of variances were tested with Levene's test. The non-parametric Kruskal-Wallis *H* test and the Pairwise Multiple Comparisons of Mean Ranks (PMCMR) test were used because data did not fulfil assumptions of normality and/or homogeneity of variances. Differences were considered statistically significant at $P<0.05$ ($\alpha = 0.05$).

Results

The effectiveness of the applied marking method was verified after 60 days and was 100% in both groups for each year. As a result, single and double fluorescent (ARS) marks were identified in the sea trout otoliths (Figure 1). Observed post-marking mortality, caused by the marking process and routine manipulations, did not exceed 5%.

The mean values of the distance (e.g., increment widths) between the first and second ARS marks in the otoliths of sea trout marked twice within 14 day period ranged between 21.11 and 34.98 µm,

depending on the year (Figure 2). The differences among years (2014, 2015 and 2016) were statistically significant (Kruskal-Wallis H test: P<0.001; Figure 2). It was due to the difference between year 2014 and 2015 (PMCMR: P<0.001), and between year 2014 and 2016 (PMCMR: P<0.001). The difference between year 2015 and 2016 was not statistically significant (PMCMR: p = 0.17; Figure 2). The distance between ARS marks in different years corresponded to temperature during the period between first and second fish immersions which was 4.4ºC, 7.5ºC and 6.7ºC, for year 2014, 2015, and 2016 respectively ($y = 4.7508x + 0.5615$, $r^2 = 0.77$, P<0.001; Figure 3).

Statistically significant differences in the body length (TL) and weight (BW) of sea trout larvae from both tested groups were observed during the following years of the experiment (Kruskal-Wallis *H* test: P<0.001; Table 1, Table 2). Fish mean TL and BW during the first marking session in 2015 did not differ significantly between either experimental group (TL: Kruskal-Wallis *H* test: p = 0.67; BW: Kruskal-Wallis *H* test: p = 0.08). For all other years, differences in initial mean TL and BW of larvae among experimental groups were statistically significant (Kruskal-Wallis *H* test: P<0.001). Observed differences were reflected in the final growth parameters of released fish (Table 3).

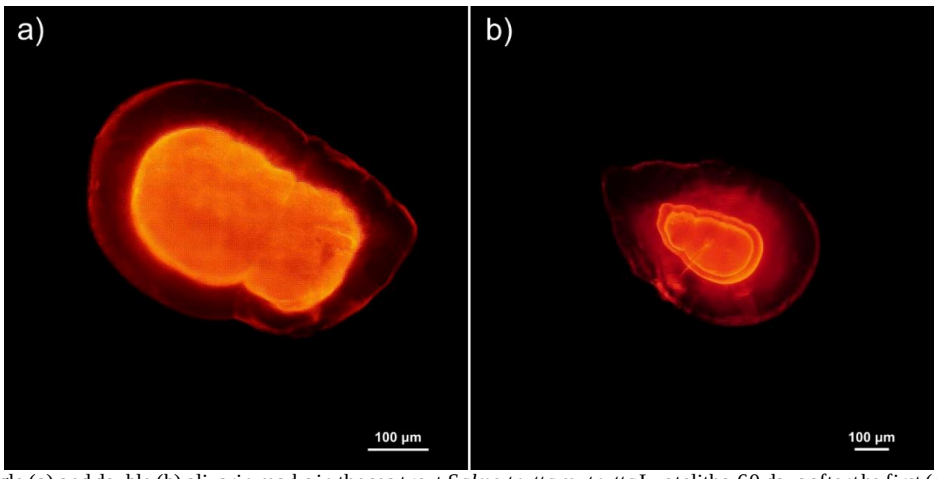

Figure 1. Single (a) and double (b) alizarin marks in the sea trout *Salmo trutta* m. *trutta* L. otoliths, 60 days after the first (group I) and second (group II) marking sessions with a 3h immersion in 200 mg L^{-1} of alizarin red S; magnification: a) 100x and b) 40x, respectively.

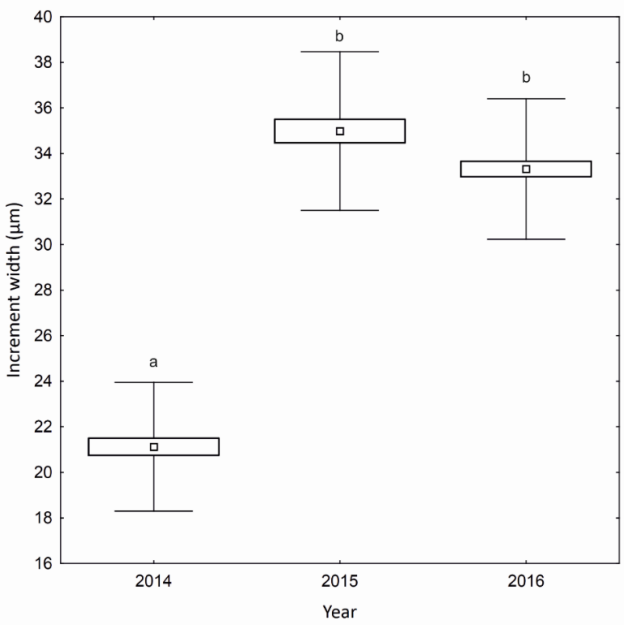

Figure 2. Increment widths (µm) in the sea trout otoliths (N = 300) between the first and second alizarin red S (ARS) marks. The centre points indicate the means, the boxes represent the standard error (*SE*) of the mean, and the whiskers indicate the standard deviation (*SD*) of the mean distance between the ARS marks. Data marked with different superscripts were significantly different (PMCMR: P < 0.001). The mean values of the water temperatures between the first and second fish ARS immersions were 4.4ºC, 7.5ºC and 6.7ºC for 2014, 2015 and 2016, respectively.

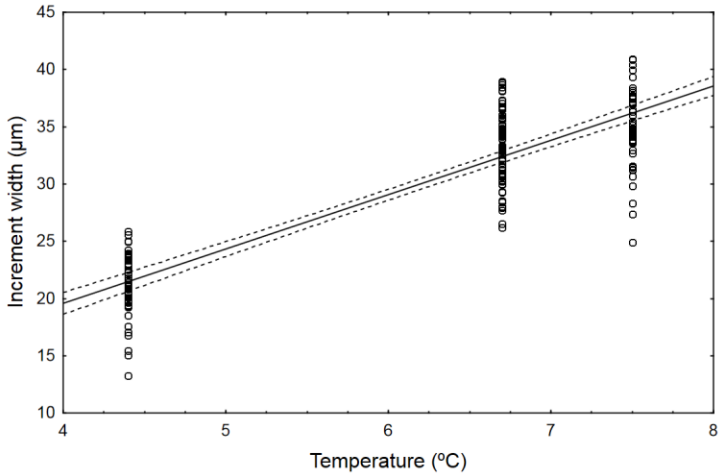

Figure 3. Relationship between water temperature (°C) and increment widths (µm) between the first and second alizarin red S (ARS) marks in the sea trout otoliths (n = 300; regression line: y = 4.7508x + 0.5615, r^2 = 0.77, P<0.001; dashed lines indicate 95% CI). The time interval between the first and second batch marking sessions was 14 days.

Table 1. Mean body length ± *SD* (range), *mm*, and mean body weight ± *SD* (range), *g*, of sea trout *Salmo trutta* m. *trutta* L. larvae from group I during marking with a 3h immersion in 200 mg L^{-1} of alizarin red S. Data in columns marked with different superscripts were significantly different (PMCMR: P<0.05)

Year	*N sample*	Length ± *SD* (range), *mm*	Weight ± *SD* (range), *g*
2014	50	19.5 ± 1.3^{a} (16.0–22.3)	0.084 ± 0.015^{a} (0.046–0.117)
2015	50	20.0 ± 1.0^{a} (16.9–21.3)	0.106 ± 0.016^{b} (0.076–0.132)
2016	50	20.8 ± 0.7^{b} (19.1–22.8)	0.118 ± 0.014^{c} (0.092–0.152)

Table 2. Mean body length ± *SD* (range), *mm*, and mean body weight ± *SD* (range), *g*, of sea trout *Salmo trutta* m. *trutta* L. larvae from group II during the first and second markings with a 3h immersion in 200 mg L^{-1} of alizarin red S. Data in columns marked with different superscripts were significantly different (PMCMR: P<0.05)

Year	*N sample*	1st batch marking		2nd batch marking	
		Length ± SD (range), *mm*	Weight ± *SD* (range), *g*	Length ± *SD* (range), *mm*	Weight ± *SD* (range), *g*
2014	50	20.6 ± 0.9^{a} (17.7–22.1)	0.097 ± 0.014^{a} (0.073–0.119)	21.5 ± 1.0^{a} (17.9–22.7)	0.101 ± 0.017^{a} (0.075–0.135)
2015	50	20.0 ± 1.1^{b} (15.9–22.1)	0.111 ± 0.015^{b} (0.080–0.137)	23.0 ± 1.3^{b} (18.9–25.1)	0.110 ± 0.019^{a} (0.070–0.148)
2016	50	21.3 ± 0.7^{c} (19.5–22.6)	0.139 ± 0.013^{c} (0.108–0.165)	24.4 ± 0.8^{c} (22.6–26.1)	0.156 ± 0.020^{b} (0.116–0.195)

Table 3. Mean body length ± *SD* (range), *mm*, mean body weight ± *SD* (range), *g*, and number of sea trout originating from groups I and II released into the Łeba River system, in 2014–2016. Data in columns marked with different superscripts were significantly different (PMCMR: P<0.05)

Year	*N sample*	Alevins			Fry		
		Length ± *SD* (range), *mm*	Weight ± *SD* (range), *g*	*N* of released fish	Length ± *SD* (range), *mm*	Weight ± *SD* (range), *g*	*N* of released fish
2014	50	$27.0 \pm 2.0^{a,b}$ (24.0–30.8)	0.145 ± 0.045^{a} (0.085–0.261)	33,000	40.0 ± 4.2^{a} (31.3–55.8)	0.623 ± 0.244^{a} (0.276–1.832)	94,444
2015	50	26.6 ± 1.5^{a} (22.7–29.0)	0.155 ± 0.025^{a} (0.102–0.202)	71,230	38.0 ± 2.2^{b} (34.1–43.7)	0.502 ± 0.104^{b} (0.333–0.821)	170,160
2016	50	27.4 ± 0.9^{b} (25.0–29.4)	0.156 ± 0.022^{a} (0.111–0.223)	100,600	41.3 ± 2.4^{c} (34.2–45.5)	0.741 ± 0.134^{c} (0.379–0.995)	188,200

Discussions

The applied protocol of immersing sea trout larvae in conditions of a traditional, open flow salmonid-type farm with alizarin red S solution produced highly visible single and double fluorescent marks. The present study approves the method of multiple marking of sea trout larvae with a 3h immersion in a 200 mg ARS L^{-1} solution without requiring prior osmotic induction. This corresponds with the results presented in previous studies where 100% effective ARS concentrations for batch marking of *Salmo trutta* L. larvae ranged between 100–150 mg ARS L^{-1}, which also kept fish mortality below 5% (Baer & Rösch, 2008; Caudron & Champigneulle, 2006, 2009; Niva *et al*., 2005; Unfer & Pinter, 2013). At higher alizarin concentrations, up to 300 mg ARS L^{-1}, high fish mortality (about 95%) was observed immediately after a 3h immersion (Baer & Rösch, 2008). Different results were reported by Unfer & Pinter (2013), who recorded a post-marking mortality rate of brown trout larvae of 0.8 Ind./day during 38 days, after immersion in 300 mg ARS L^{-1} for 3h. Gerdeaux *et al*. (2006) and Unfer & Pinter (2013), noted immediate post-marking mortality of brown trout 'swimming fry' ranging between 1–2% following immersion in 300 mg ARS L^{-1} for 2.5 h and 3 h. Due to these discrepancies, it is recommended to conduct a test-marking at least 24 hours before each marking session regardless of ARS concentration. Application of high, but acceptable ARS concentrations, could allow a reduction of the immersion time, which is important in terms of mass-marking.

The time interval of 14 days between each batch marking and thermal regime has been found to provide the proper distance between the first and second ARS marks in the otoliths so that, they can be observed as two well-separated rings. Furthermore, observed differences of mean water temperature between the first and second marking sessions in fish from group II among particular years did not affect the readability or visibility of the double marks. Low mean water temperatures between marking sessions in 2014 resulted in smaller increment widths in the otoliths than the widths seen in 2015 and 2016 (Figure 2). Nonetheless, the 2014 ARS rings were still easily identified. These findings show the high potential and prospective possibilities in controlling the distance between ARS marks by simultaneous manipulation of temperature and time intervals applied between the subsequent marking sessions. Within the range of water temperatures noted throughout the experimental procedures, it is possible to effectively immerse the sea trout larvae in alizarin red S solution twice within 24 days after hatching (~ 190ºD). Nevertheless, previous studies showed that this period may be longer for *Salmo trutta*, exceeding 230ºD (Caudron & Champigneulle, 2006, 2009). This provides an adequate safety margin of time to guarantee at least two successful batch marking sessions of sea trout at an early developmental stage and makes the methods presented widely applicable for commercial mass-marking.

Observed significant differences of initial body length and weight of juvenile sea trout (Table 1, Table 2) could be caused by the difference in the size of the females stripped in consecutive spawning seasons. The total length and body weight subsequently increased from year to year, with the following mean values of TL and BW, respectively: 60.5 cm and 2360 g in 2013, 63.7 cm and 2919 g in 2014, 69.3 cm and 3790 g in 2015 (A.M. Lejk, unpublished data). As a consequence, large females produced bigger eggs (Brooks, Tyler, & Sumpter, 1997; Jonsson & Jonsson, 1999) and thus produced larger larvae (Kamler, 1992). However, this fact did not influence the final quality of single and double fluorescent marks or the efficiency of the marking procedure.

In the experiment, a total of 204,830 fish from group I and 452,804 fish from group II (Table 3) were marked and released into the Łeba River system (Northern Poland). Stocking was funded under the National Programme "Stocking of Polish Marine Areas". Steps towards defining long-term retention and quality of single and multiple ARS marks in sea trout otoliths are currently being conducted. This is an indispensable requirement for the use of the presented marking method for monitoring or recovery programmes of long-living fish species (Martyniak, Stańczak, Kozłowski, Mierzejewska, Wziątek, Lejk, & Hliwa, 2013).

Thus, the method described in this study can be advised as a routine procedure that provides clear and well-visible single and double ARS marks in otoliths and helps distinguish fish originating from different stocking groups of wild-born individuals. Moreover, it fulfils the five key criteria required for marking methods used to evaluate fish stocking effectiveness (Brown & Harris, 1995). This work is a good example of the successful implementation of laboratory-scale results to the mass-scale production of marked sea trout juveniles.

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