Alizarin marking of whitefish, *Coregonus lavaretus* otoliths during egg incubation

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Abstract Alizarin red S from 200 to 5000 mg L⁻¹ was used to mark the otoliths of whitefish embryos *Coregonus lavaretus* (L.) from 5 days after fertilization until shortly before hatching, to develop a method for evaluating the effect of stocking whitefish. Mark quality depended on developmental stage at the time of immersion. The best results were obtained when the otolith primordia had formed and had started aggregating to form the innermost part of the nucleus. This was verified by *in vitro* staining of otoliths where the primordia were more intensely stained than the remaining part of the nucleus. The volume ratio eggs to ARS solution, which was 1:10 in all but two treatments, must be observed when comparing fluorochrome concentrations, as mark quality decreased at higher ratios of 1:5 and 1:2. An average mortality rate of 35% in the most successful treatments was considered tolerable for a mass-marking programme being used under commercial hatchery conditions. This method for marking otoliths of whitefish embryos with ARS during egg incubation is recommended for all cases where marking of larvae is not possible.

KEYWORDS: alizarin, *Coregonus*, otolith marking, otolith primordia, whitefish.

Introduction

Labelling the otoliths of fish with fluorochrome dyes is a widespread technique used to evaluate stock enhancement programmes (Tsukamoto, Kuwada, Hirokawa, Oya, Sekiya, Fujimoto & Imaizumi 1989; Yamashita, Nagahora, Yamada & Kitagawa 1994; Nagata, Nakajima & Okada 1995; Secor & Houde 1995; Tsukamoto 1995). When hatchery-reared fish are marked at the larval or juvenile stage, they must be kept in tanks until mass marking by immersion in a fluorochrome solution can be carried out. In a situation where hatchery capacity is limited, so that all or most of the larvae must be released immediately after hatching, a different mass-marking technique is needed that can be applied at an earlier developmental stage, i.e. during egg incubation.

This situation is characteristic of many coregonid hatcheries in Europe where only a minor fraction of eleutheroembryos can be retained and reared in tanks. At Lake Constance, the second largest prealpine lake in Europe, for example, there are five hatcheries with a total incubation jar volume of around 12 000 L (Rösch 1993). With an average hatching success of 70%, approximately 375 million larvae are obtained annu-

ally, of which >95% are released immediately into the lake. Mass marking of larvae is not feasible under these conditions, but evaluating the effect of stocking is important because of ongoing re-oligotrophication of Lake Constance (Güde, Rossknecht & Wagner 1998). Fluorochrome labelling of whitefish otoliths during egg incubation was therefore evaluated. It was decided to use alizarin red S (ARS), as hatchery managers declined to use tetracycline (TC) in large-scale tagging programmes.

Earlier studies demonstrated that fish otoliths can be marked with fluorochrome dyes during embryonic development (Dabrowski & Tsukamoto 1986; Tsukamoto 1988; Blom, Nordeide, Svasand & Borge 1994; Nagata *et al.* 1995; Iglesias & Rodríguez-Ojea 1997), and that this can be carried out as early as at the time of egg fertilization (Ruhlé & Winecki-Kuehn 1992; Rojas-Beltran, Champigneulle & Vincent 1995a). In some of these studies, the transport of the fluorochrome dye across the egg envelope was promoted by osmotic shock (Ruhlé & Winecki-Kuehn 1992; Rojas-Beltran *et al.* 1995a; Rojas-Beltran, Gillet & Champigneulle 1995b), while in others the eggs were simply immersed in the labelling solution.

In preliminary trials, some of these published techniques were tested, using ARS instead of the commonly used TC, with little success. Adding ARS at the time of egg fertilization with or without osmotic shock did not mark the otoliths at all (Koechlin 1997), while immersion of eggs at different stages of development yielded inconsistent results, from no mark to easily detectable or even brilliant marks (R. Eckmann, unpublished data). It was suspected that marking success depended on the correct timing of egg immersion. Thus, the purpose of this study was to define the most appropriate phase of whitefish embryonic development for labelling otoliths with ARS.

Materials and methods

Inshore spawning whitefish (cf. Eckmann & Roesch 1998) were sampled with bottom-set gill nets during spawning (late November/December). Egg fertilization was carried out on the fishing vessel immediately after lifting the nets. The fertilized eggs were incubated in miniature Zug jars of 300 mL capacity, which were fed with recirculated tap water. Temperature was maintained at around 4.5 °C, and about 5% of the system's 250 L total water volume was exchanged every other day.

Batches of 20 mL of eggs (corresponding to about 1300 eggs) were used for each treatment. These batches were incubated separately until hatching started. The eggs were then transferred to 14-L aquaria where hatching generally occurred within 3 days. The aquaria were fed with aerated tap water, the temperature of which was gradually increased to 12 °C within 3 days. Larvae were fed with *Artemia* nauplii until 3 weeks after hatching. Dead eggs, unhatched embryos, and dead and living larvae were counted to estimate mortality rates.

Experiment 1 was carried out in winter 1999–2000 and comprised the immersion of eggs with and without osmotic shock. All treatments with osmotic shock and one treatment without osmotic shock (at 52 days after fertilization) were replicated. In experiment 2, carried out in winter 2000–2001, eggs were only immersed without osmotic shock. In this experiment, all treatments except for one (500 mg L⁻¹ ARS at 42 days after fertilization) were replicated. There were two control groups in each experiment.

Immersion with osmotic shock

Batches of 20 mL of eggs were added to an Erlenmeyer flask containing 180 mL of ARS (Aldrich Cat. No. 11 996-2) at a concentration of 5000 mg L⁻¹ in a 5%

solution of NaCl. The pH was adjusted to around 8 with Tris buffer. The eggs were kept moving by aeration and by repeated swirling of the flask. After 15 min, the eggs were strained into a plastic sieve, thoroughly rinsed with tap water and transferred into a mini Zug jar for further incubation.

Immersion without osmotic shock

Batches of 20 mL of eggs were added to mini Zug jars that contained 180 mL of the ARS solutions at various concentrations (200 and 400 mg L⁻¹ in experiment 1, 500 and 1000 mg L⁻¹ in experiment 2). The pH was buffered as described above. The volume ratio between eggs and solution was 1:10, except for two treatments in experiment 2 where the ratio was 1:5 and 1:2, respectively. The jars were fed with air rather than water to keep the eggs constantly moving. After 24-h immersion, the air supply was shut down, the jars were supplied with water, and the ARS solution was siphoned out of the jars until the upwelling water appeared unstained. The jars were then incorporated into the recirculation system for further incubation.

Mark detection and mark quality

On the day of hatching and for up to 25 days, samples of larvae were taken and deep-frozen. Sagittae and lapilli were dissected from freshly thawed specimens, cleared from adhering tissue, dried and mounted in Entellan (Merck). Two batches of fish found to have good quality marks were reared for several months to assess mark retention. They were also used to assess the maximum fish size that allows mark detection without the otoliths having to be ground. The otoliths of these fish were embedded in epoxy resin, and they were ground with abrasive paper and polished with aluminium oxide if necessary.

Otoliths were inspected independently by two readers with an epifluorescence microscope (Zeiss filter set no. 487915, 546 nm excitation wavelength). Mark quality was scored from 0 (no mark visible at 400× magnification), through 1 (mark not visible at 100× but visible at 400× magnification), and 2 (mark easily visible at 100× magnification), to 3 (mark shining brightly at 100× magnification). It should be noted that this scoring of mark quality is conservative, as it was specifically designed to facilitate quick and reliable detection of marks in a mass-marking programme. A score of 1, therefore, can be considered as sufficient for successful mark detection, while higher scores indicate that less time is needed for otolith analysis.

Staining of ground otoliths

To check the results of the vital staining tests, otoliths of 25-day-old larvae from the control treatments were embedded in epoxy resin and ground to the midplane, so that the otolith nucleus, and particularly the primordia, became exposed at the surface. The otoliths were polished with aluminium oxide and immersed for 60 min in an ARS solution of 500 mg L⁻¹ at pH 8. After washing and drying, they were covered with a coverslip with Entellan, and the specimens were examined as described above.

Results

Otoliths of whitefish embryos could be marked with ARS during embryonic development, but mark quality depended on the age of the embryos at the time of immersion and on ARS concentration. The uptake of ARS through the egg chorion was not enhanced by hyperosmotic conditions (Table 1), and this technique was therefore abandoned in the second experiment.

In the first experiment with 400 mg ARS L⁻¹, immersion of young embryos, i.e. 5 days after fertilization, did not mark the otoliths at all, whereas immersion of advanced embryos, i.e. 38 days after fertilization, provided better mark quality (Table 1). The mark qualities observed with the most advanced embryos, i.e. 52 days after fertilization and older, however, varied considerably between individuals from the same treatment, and even among the four otoliths of one fish: while some otoliths were scored 1 or 1.5, others had no mark at all. In general, mark quality in the first experiment was inferior to that in the second experiment where higher ARS concentrations were applied.

In the second experiment, the best results were obtained with embryos treated with 1000 mg ARS L⁻¹. Mark quality increased significantly with the age of the embryos at the time of immersion (Kruskal–Wallis test, $P < 0.05$). In embryos treated 28 days after fertilization or later, fluorescent marks were easily visible at 100× magnification, and mark quality was uniform within and between replicates: all otoliths were scored 1 or better. Higher egg:solution ratios (cf. Table 1) resulted in significantly inferior mark quality (Kruskal–Wallis test, $P < 0.05$).

Mortality rates varied among treatments and between replicates (Table 2). In the first experiment, mortality rates in the treatments surpassed those in the controls in all but one case. Mortality depended significantly on both the age of the embryos at the time of immersion and on ARS concentration (two-way ANOVA, $P < 0.05$). Mortality of eggs treated with osmotic shock did not differ from those treated with 400 mg ARS L⁻¹ but was significantly higher compared with eggs treated with 200 mg ARS L⁻¹ (Tukey test, $P < 0.05$). In the second experiment, mortality rates in the treatments were similar to those in the controls (except for 14 days old embryos treated with 1000 mg ARS L⁻¹). There was no significant relationship between either age of the embryos at the time of immersion or ARS concentration and mortality (two-way ANOVA, $P < 0.05$). Mortality among the controls (29.5% on average) was higher than in the first series of experiments, but it was within a range that is commonly observed in commercial whitefish hatcheries. An average mortality rate of 35% in the most successful treatments of this experiment, i.e. in embryos immersed 28 days after fertilization or later in 1000 mg ARS L⁻¹, was considered tolerable, even for a mass-marking programme.

Table 1. Mark quality (sagittae and lapilli combined) of whitefish larvae immersed in various concentrations of alizarin red S (ARS) at different times of embryonic development

Days after fertilization	ARS concentration (mg L ⁻¹)			Days after fertilization	ARS concentration (mg L ⁻¹)	
	200	400	5000 (in 5% NaCl)		500	1000
5	0*	0*	0	14	0	0.6†
18	0	0.1	0†	21	0.1	1.0†
38	0.1	0.4	0.1†	28	0.3	1.3† 0.2§ 0¶
52	0	0.2‡	0.1†	35	0	1.6†
66	0	0.1‡	0†	42	0.2	2.5†

Ten larvae were analysed per treatment (*5 larvae, †20 larvae). Experiment 1 (left data block): ‡mark quality highly variable both between and within individual larvae. Experiment 2 (right data block): the ratio between eggs and ARS solution was 1:10 in all but two treatments (§eggs:solution = 1:5, ¶eggs:solution = 1:2). Hatching occurred 68 days after fertilization in both experiments.

The best marks were always characterized by intensely stained otolith primordia (Fig. 1a). These spherical structures started to appear at around

28 days after fertilization, and they started to fuse and form the precursor of the otolith nucleus some 4–6 days later. The otolith nucleus had formed by

Table 2. Total mortality (%) of whitefish embryos, immersed in various concentrations of alizarin red S (ARS) at different times of embryonic development, from the time of immersion marking until three weeks after hatching. Experiment 1: left data block, experiment 2: right data block. Results for replicate treatments are separated by a slash

Days after fertilization	ARS concentration (mg L ⁻¹)			Days after fertilization	ARS concentration (mg L ⁻¹)	
	200	400	5000 (in 5% NaCl)		500	1000
5	23	40	58/78	14	20/16	57/52
18	28	32	46/46	21	28/22	26/33
38	19	26	39/36	28	25/42	26/34
52	7/21	14/12	18/9	35	29/33	35/53
66	9	11	17/20	42	35	35/27
Control			5/13	Control		27/32

Figure 1. (a) Sagittae from whitefish larvae, which were immersed during egg incubation at the age of 42 days after fertilization in a solution of 1000 mg L⁻¹ alizarin red S for 24 h. The primordia are much more intensely stained than the remaining part of the nucleus. Epifluorescent and transmitted light were applied simultaneously. Scale bar 100 µm. (b) Ground and polished sagitta from a control group whitefish under transmitted and epifluorescent light. The preparation was immersed in a solution of 500 mg L⁻¹ alizarin red S for 60 min. The primordia are more intensely stained than other parts of the nucleus and better than daily increments. Scale bar 100 µm.

approximately 50 days after fertilization. It consisted of a variable number of primordia, which were surrounded by a layer of lower optical density. This layer continued to grow until the larvae hatched and a hatch-check was formed. Thereafter, daily increments were deposited (cf. Fig. 1b).

When ground and polished otoliths from unmarked larvae were immersed in an ARS solution, the primordia were much more intensely stained than the remaining part of the nucleus (Fig. 1b). This result was further supported by the observation that primordia were less intensely stained as long as they were not exposed at the polished otolith surface but were still covered by the material that surrounds the primordia.

The ARS marks were retained for at least 8 months but the sagittae from fish larger than 45 mm total length had to be ground to reveal the mark. As whitefish lapilli are smaller than the sagittae, ARS marks could in many cases still be detected in unground lapilli. In the largest fish examined (90–100 mm total length), marks were still recognizable in the unground lapilli, but their detection was much improved by grinding. With both lapilli and sagittae, it was not necessary to grind the otoliths close to the nucleus to enhance mark detection, so that the risk of overgrinding can easily be avoided.

Discussion

This study shows that the otolith primordia of coregonids are stained more intensely with ARS than the rest of the otolith nucleus. This observation is particularly relevant for the correct timing of ARS marking of whitefish embryos. At an advanced stage of embryonic development, i.e. when the primordia were already surrounded by material that was less intensely stained than the primordia, mark quality was low. When the eggs were immersed shortly before hatching, a fluorescent mark was deposited which was closely associated with the hatch-check but mark quality was very variable, and larvae started to hatch during immersion, which is not practical for large-scale marking in hatcheries. Thus, the most appropriate time for mass marking of whitefish embryos with ARS is from the appearance of the first primordia until shortly after the primordia have fused to form the innermost part of the otolith nucleus. At an average incubation temperature of 4.5 °C, this occurs after approximately 50–60% of embryonic development, i.e. 34–42 days after fertilization.

A similar result was reported by Dabrowski & Tsukamoto (1986) who immersed eyed whitefish eggs in TC, while other authors did not specify which part

of the otolith was stained by their particular treatment. Nagata *et al.* (1995) and Tsukamoto (1995) successfully marked *Oncorhynchus masou* Brevoort embryos as eyed eggs, by immersion in 200 mg L⁻¹ alizarin complexone (AC) for 24 h. Rojas-Beltran *et al.* (1995b) marked *Salvelinus alpinus* (L.) with TC either at the time of fertilization or as eyed eggs, and it appears that the otolith primordia were stained in both treatments. Immersion of embryos shortly before hatching was successful with *Scophthalmus maximus* (L.) (Iglesias & Rodríguez-Ojea 1997) and *Plecoglossus altivelis* Temminck & Schlegel (Tsukamoto 1988). In the latter study, the AC mark was reported to form a ring, so the outer region of the otolith nucleus was stained but not the primordia.

In the present experiments with ARS, it was not possible to mark the otoliths of whitefish when eggs were immersed at the time of, or shortly after, fertilization. With TC, however, it was possible to mark whitefish (Ruhlé & Winecki-Kuehn 1992) and Arctic charr (Rojas-Beltran *et al.* 1995b) otoliths by osmotic incorporation at the time of fertilization. The TC that was taken up during egg hardening must have been deposited within the chorion and then became incorporated into the otolith primordia some weeks later. The ARS, although it forms complexes with calcium in a similar way to TC, apparently has different characteristics, which do not permit its storage within the chorion for a prolonged time. The present study shows that ARS can be transported across the chorion, even without osmotic shock, but ARS must be complexed with calcium and deposited soon thereafter, otherwise it will be leached out of the egg.

Mark quality depended on ARS concentration and on the amount of eggs per unit of ARS solution. Thus, when an ARS solution of 1000 mg L⁻¹ produced good marks, this cannot be considered in absolute terms but must be related to the amount of eggs per unit of solution. The recommended concentration of 1000 mg ARS L⁻¹ will produce good quality marks only when the egg:solution ratio does not increase above 1:10. Some authors do not report precisely the volume ratio of eggs to fluorescent solution or the number of eggs per unit of solution. Consequently, it is difficult to use these results in mass-marking programmes without additional tests.

In an earlier study, Eckmann, Czerkies, Helms & Kleibs (1998) observed that pH values of ARS solutions are critical for the survival of coregonid larvae, and recommended adjusting pH in the range of 7.5–8. Therefore, in the present study pH of the ARS solutions was always adjusted to around 8. It is

conceivable though, that embryos within the chorion are less affected by low pH values compared with eleutheroembryos, as pH of the perivitellin fluid is buffered against fluctuations of ambient pH values (Kuegel & Peterson 1989). While this problem has not been analysed in detail, it is recommended to use ARS solutions with pH values around 8.

Mark retention was verified in this study over at least 8 months, while much longer retention times of fluorescent marks have already been reported (Rojas-Beltran *et al.* 1995a; Unkenholz, Brown & Pope 1997; Eckmann *et al.* 1998). Thus, it appears safe to assume that mark retention will not be a problem with ARS-marked otolith primordia of whitefish. For reliable detection of marks in larger juveniles, i.e. to avoid false negative results, otoliths should be ground. The preparation of lapilli in addition to the sagittae may help to reduce the need for grinding, because marks are more easily visible in unground lapilli compared with the larger and thicker sagittae.

Marking whitefish larvae with ARS is easier than marking embryos, because a lower ARS concentration is applied during a shorter time and the developmental stage of the fish is not critical (Eckmann *et al.* 1998). Nevertheless, immersion marking of eggs does provide some advantages for mass marking under hatchery conditions compared with the marking of larvae. (1) There is a time window of about 2 weeks, from the appearance of the first primordia until shortly after the primordia have fused when immersion marking can be carried out. The entire egg stock of a hatchery can thus be treated in small batches within this time window. (2) For the immersion of larvae, a much larger volume of solution is needed compared with when eggs are immersed. In the present study, around 1350 eggs were immersed in 180 mL of solution, while a density of 1250 larvae L⁻¹ was used by Eckmann *et al.* (1998). Most published reports on immersion marking of larvae with fluorescent dyes used even lower densities (e.g. Hettler 1984; Blom *et al.* 1994; Brooks, Heidinger & Kohler 1994; Nagiec, Czerkies, Goryczko, Witkowski & Murawska 1995; Iglesias & Rodríguez-Ojea 1997). Therefore, when eggs instead of larvae are immersed in a fluorescent solution, it is necessary to handle a much smaller volume of solution in accordance with local safety and water protection standards. (3) Handling of eggs, e.g. retrieving them from the fluorescent solution, washing, transferring between jars or tanks, is much easier than with larvae.

In conclusion, a method is now available for marking the otoliths of whitefish embryos with ARS during egg incubation, whereby mortality rates because of the marking process are tolerable. This

technique can be used in mass marking operations under hatchery conditions, and it is recommended for all those cases where marking of larvae is not possible.

Acknowledgments

Many thanks to Myriam Schmid for her invaluable assistance during all laboratory work, especially for her skilful preparation of otoliths. Antje Zahn collected whitefish spawners with gill nets. The members of my group helped with the regular control of egg incubation. Mary Morris corrected the English. This study was supported by Deutsche Forschungsgemeinschaft within the collaborative research center SFB 454, Littoral of Lake Constance.

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