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Quantification of alizarin red S uptake in coregonid eggs after mass-marking

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Abstract

An easy method to measure the uptake rate of the persistent dye alizarin red S (ARS) during marking of whitefish eggs was established and used to measure the ARS content in three different whitefish species during and at the end of the marking procedure. Those values show that only 6–10% of the ARS in the marking solution will be absorbed by the eggs (0.0061–0.0119 mg per egg). Additional analyses 6, 15 and 36 months after marking showed ARS levels below the response level (<6.9 μg kg^{-1}).

KEYWORDS

C. albula, *C. macrophthalmus*, *C. wartmanni*, food security, otolith marking, residue level, stocking

Stocking is an important tool in fisheries management. For decades or even centuries in several deep, cold lakes millions of larvae of coregonids (*Coregonus* spp.) have been stocked every year (Eckmann, 2003; Rösch, 1993). To monitor stocking success, whitefish larvae were often marked prior to release during the egg stage using the persistent dye alizarin red S (ARS) (Eckmann *et al*., 2007; Martyniak *et al*., 2013). The aim of the current investigation was to evaluate how much ARS is absorbed by coregonid eggs treated in this manner. Quantifying dye uptake may facilitate more efficient use of marking solutions, for example allowing used solutions to be topped up with an appropriate amount of ARS and used again, thereby reducing costs and limiting the amount of dye that has to be disposed of. A second consideration is food safety because wild-caught whitefish is sold to consumers. Because ARS coalesces as a long-lasting nonwater-soluble complex in mineralized tissues, it tends not to be present in edible muscle tissue (Bensimon-Brito *et al*., 2016), and with marking taking place during the egg stage only otoliths will retain ARS for extended periods (Eckmann, 2003). However, since these small body parts might still be accidentally eaten, some consideration should be given to consumer safety. Although ARS is not listed as a dangerous substance (EC Regulation 1272/2008), the precautionary principle and a responsibility to inform consumers make it important to know how much ARS might accidentally be ingested in this way. The literature contains no information concerning the amount of ARS present in marked embryos or larvae and no method for measuring the ARS content of coregonid eggs has been described. To fill this gap, a method to measure the uptake of ARS during otolith marking at egg stage was established and the maximum values of ARS in marked eggs of three different whitefish species were determined. In addition, the ARS level in two whitefish species was measured 6, 15 and 36 months after marking following Kullmann *et al*. (2020).

Briefly, a marking solution was prepared in 20 l of deionized water with 1 g l^{-1} Tris buffer ($C_4H_{11}NO_3$; Sigma-Aldrich, Darmstadt, Germany), into which 1 g l^{-1} of ARS (alizarin red S monohydrate, $C_{14}H_7NaO_7S$; Waldeck Chemie, Münster, Germany) was stirred and dissolved (Eckmann, 2003). A spectral photometer (Hach Lange DR 6000, Germany) was used to measure extinction of the ARS solution over 11 dilution steps of 2.5, 5, 10, 20, 50, 80, 100, 120, 150, 170 and

200 mgARS l^{-1} in 1 g l^{-1} Tris buffer. The extinction rates for each solution were measured at a wavelength of 455 nm and a linear calibration line was established (extinction $E = 0.0108 \times$ mgARS $l^{-1} + 0.0051$; $r^2 = 0.99$). Afterwards, three aerated Zug jars (volume 1 l) (FAO, 1985) were filled with 500 ml of the marking solution and placed in a controlled temperature storage room at 5°C. According to Eckmann (2003) the volume ratio of settled whitefish eggs to labelling solution should be 1:10, so 50 ml of whitefish eggs were transferred carefully into each of the three Zug jars. During nine runs using fresh marking solutions, eggs of two whitefish species from Lake Constance, the pelagic *Coregonus wartmanni* (Bloch 1784) and the benthic *C. macrophthalmus* (Nüsslin 1882), and *C. albula*, L. from Northern Germany were exposed for 24 h. Triplicate runs were conducted for each species (for each species at three consecutive days, therefore in total 3 ×150 ml of eggs). Additionally, for each run an additional Zug jar containing marking solution with no eggs was incubated alongside the others as a control (control group, $n = 9$). All eggs were obtained from hatcheries (incubation temperature 3–5°C) at development stages 10–11 (Eckmann, 1987), around 4 weeks before hatching. Four 0.5 ml samples were taken from each of the four Zug jars at 2, 4, 6, 8 and 24 h after the start of each marking procedure. Each of the samples was diluted (1:10) with a solution of deionized water and Tris buffer (1 g l^{-1}), and extinction was measured and assessed against the calibration line. ARS uptake per egg was calculated according to the following formula:

$$\mathrm{ARS}\left(\mathrm{mg\,egg^{-1}}\right) = \left[\frac{100 - \left(\mathrm{ARS_{24h}\,mg\,l^{-1}}\,\frac{100}{\mathrm{ARS_{control}\,mg\,l^{-1}}}\right)}{\text{species-specific egg number } 100\,\mathrm{ml^{-1}}}\right],$$

where ARS_{24h} is the quantity of ARS remaining per marking solution and species after 24 h and $ARS_{control}$ is the mean ARS level after 24 h in the control solutions. Eggs of the different species vary in size, such that 100 ml amounts to around 5600 eggs of *C. macrophthalmus*, 6600 of *C. wartmanni* and 16200 of *C. albula*. Before and during each marking procedure, water temperature, pH and oxygen level were measured in the Zug jars. At the end of each marking trial, all eggs were retained in a mesh and checked for hatched larvae because it is known that stress (*i.e.*, marking procedure) can reduce incubation time (Næsje & Jonsson, 1988). According to the German Animal Welfare Act (TierSchG), approval for the present study by a review board institution or ethics committee was not necessary because only eggs were marked (and no hatched larvae or small fish).

During the marking procedure water temperature was set at 5.1 ± 0.6°C (± S.D.), oxygen levels were close to saturation and pH was stable (8.4 ± 0.2 S.D.). At the end of each trial, the egg membranes were deeply red and almost no hatched larvae were observed (0–10 individuals per Zug jar). After 24 h, the ARS level in the marking solution of *C. macrophthalmus* was 93.75% (± 1.45 S.D.), indicating an uptake by the eggs of 6.25%. The corresponding figures for *C. wartmanni* were 92.15% (±1.00 S.D.) remaining and 7.85% uptake and for *C. albula* 90.05% (±2.31 S.D.) remaining and 9.95% uptake. These proportions corresponded to a mean of 0.0111 mgARS (±0.0026 S.D.) per *C. macrophthalmus* egg, 0.0119 mgARS (±0.0015 S.D.) per *C. wartmanni* egg and 0.0061 mgARS (±0.0014 S.D.) per *C. albula* egg (Figure 1). The rate of ARS uptake followed a saturation curve, with the greatest declines in ARS in the marking solutions measured in the first 2 h of the procedure (Figure 1). After this rapid initial uptake, uptake of ARS by the eggs still occurred but at a progressively declining rate (Figure 1).

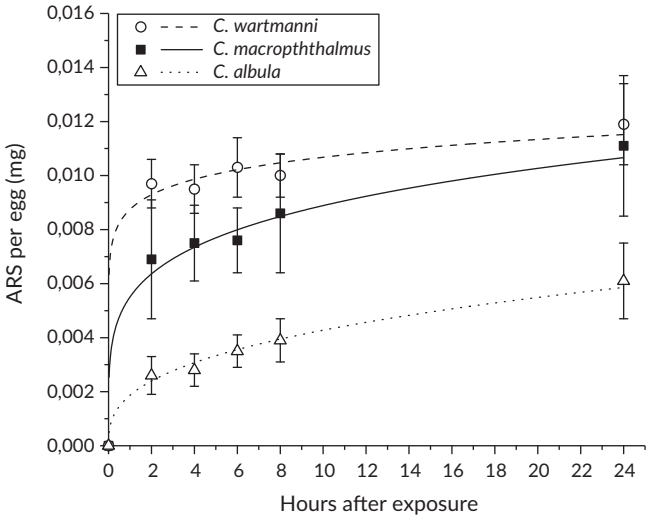

FIGURE 1 Time-dependent alizarin red S (ARS) uptake per egg of three different whitefish species 2, 4, 6, 8 and 24 h after exposure in a standard marking solution. Error bars represent standard deviation and broken, solid and dotted lines are the saturation curves for each species. ○ – – –*C. wartmanni*, ■ ——*C. macrophthalmus*, △ · · · ·*C. albula*

The eggs of *C. albula* are much smaller than those of the other two study species, resulting in a larger total surface per volume. This is the most likely reason for the greater total uptake levels of *C. albula*, despite the lower total uptake per egg, but this assumption should be tested in future studies. However there is potential for improvement in the method for future mass-marking with ARS, as the current method utilizes only 6–10% of ARS in the marking solution. This suggests that marking solutions might be reused without affecting performance simply by replenishing the small amounts of lost ARS between procedures. Indeed, in an additional first survey at the end of the study, a used batch of marking solution was deployed a second time, resulting in almost the same uptake levels of ARS as in the main experiment (data not shown), suggesting that solutions could be used at least twice without any need for replenishment.

After the marking procedure, approximately 10 000 eggs of *C. macrophthalmus* and *C. wartmanni* were transferred to a nearby hatchery (Fischbrutanstalt Langenargen, Lake Constance). Due to the fact that *C. albula* is nonendemic in Lake Constance, this species was excluded from this step. After hatching of the larvae of *C. macrophthalmus* and *C. wartmanni*, fish were reared in circular tanks for more than 3 years. From those fish, samples were taken 6, 15 and 36 months later for measuring the ARS level according to Kullmann *et al.* (2020). All whitefish were stunned by a blow on the head and expertly killed immediately by a cardiac stab according to the German

Animal Protection Law (§ 4) and the ordinance of slaughter and killing of animals (Tierschutzschlachtverordnung § 13).

Six months after marking in collective samples of the heads of *C. wartmanni* and *C. macrophthalmus* (11–15 individuals per sample, total length of the fish between 43 and 55 mm, total sample size = 14) no ARS could be detected (below a detection limit of 6.9 μg kg^{-1}, see Kullmann *et al*., 2020). Similarly, in samples collected 15 months after marking (total length of the fish between 135 and 173 mm) from single heads (n = 10) or bodies without heads (n = 10) and 36 months after marking (heads n = 6, bodies n = 6) from both coregonid species (total length of the fish between235 and 300 mm) the ARS level was below the detection limit.

There is currently no limit set for ARS in fish products (EU, 2018) and fish products are excluded from EU Regulation 396/2005, which stipulates maximum residue levels in food products for substances without specific limits. In trials with rats (Adkins, 1965) and dogs (Rubin & Bisk, 1969) ARS was shown to have detrimental effects when administered intravenously at doses more than 100-fold higher than the maximum values recorded in the present study over a period of weeks. In Frog Embryo Teratogenicity Assay-Xenopus tests (FETAX; ASTM, 1991) using *Xenopus laevis*, very high levels (above 20 mg l^{-1}) of ARS in the culture solution lead to increasing bone deformities and higher mortality rates (Lampertsdörfer *et al*., 1991). Given that a significant proportion of ARS used in the present marking study is ligated at the surface of the eggs and does not end up in the developing larvae, the absolute ARS uptake value of 11.1 μg ARS per *C. macrophthalmus* egg and 11.9 μg per *C. wartmanni* egg measured in this study is likely to be a significant overestimate of final quantities in the fish. This statement is further corroborated by additional analyzes 6, 15 and 36 months after marking because all ARS levels were below the response level. In addition, because otoliths are the only calcium structures present in the egg stage (Eckmann, 1987) with which ARS can be bound, marking is limited almost exclusively to these structures within the head.

Whitefish is usually sold to the consumer as boneless filets (Dreßler, 2013) and even in cases where whole fish is sold (*e.g*., smoked), consumers are considered unlikely to eat the head. Taking all the facts into account, the apparent risk to the consumer from the mass-marking of whitefish eggs with ARS is negligible.

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