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Feasibility of otolith markings in large juvenile turbot, *Scophthalmus maximus*, using immersion in alizarin-red S solutions

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The efficiency with which immersion in alizarin red S (ARS) produced marks in the otoliths of large juvenile fish was analysed. Turbots (approximately 136 mm total length and 45 g wet weight) were marked for 24 h using four treatments of seawater solutions of ARS (buffered ARS 100, 200, 400 mg l^{-1} and unbuffered ARS 400 mg l^{-1}). A single treatment with an unbuffered seawater solution of alizarin complexone (AC) (set to 120 mg l^{-1} from preliminary trials) was used as a reference marker. All fish survived to the end of the experiment (33 days after marking), and their growth rates did not differ significantly between groups. Examination of frontal sections of otoliths under UV light microscopy revealed the yellowish and pink marks induced by ARS and AC, respectively. The quality of marks was equal for both AC and ARS immersions, at a 400 mg l^{-1} concentration of ARS. ARS immersion has the double advantage of being an inexpensive and (probably) low-stress technique for marking the otoliths of large turbot juveniles. The application of ARS to large juveniles of other marine and freshwater species is suggested.

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Introduction

The capacity of bones and otoliths of fish to incorporate dyes such as fluorescent markers provides a powerful tool for understanding the formation of calcified structures (Meunier, 1974). Fluorescent marking has been applied in fisheries management (McFarlane and Beamish, 1987), in stock enhancement programmes (e.g. Tsukamoto *et al.*, 1989a; Yamashita *et al.*, 1994; Blom *et al.*, 1994; Rojas-Beltran *et al.*, 1995a, b; Secor and Houde, 1998; Eckmann *et al.*, 1998), to estimate growth rates (e.g. Campana and Neilson, 1982; Schmitt, 1984; Meunier, 1994) and to validate daily increments of otoliths (e.g. Sogard, 1991; Ahrenholz *et al.*, 1995; Hoff *et al.*, 1997; Villanueva and Molí, 1997). Various techniques for introducing the marker have been investigated: injection, dietary intake and immersion. The chosen technique depends on the life history stage and the environment (marine or fresh water). The chemical concentrations and, in the case of immersion, the duration of exposure also varied (see review by Thomas *et al.*, 1995).

Early immersions were previously restricted to eggs and larvae (Hettler, 1984; Tsukamoto, 1985, 1988) and juveniles smaller than 30 mm in length (Schmitt, 1984; Nagiec *et al.*, 1988; Tsukamoto *et al.*, 1989a; Secor *et al.*, 1991; Hoff *et al.*, 1997; Table 1). However, otolith marking using immersion also proved to be efficient in larger juveniles (Table 1), as in age-0 red drum (*Sciaenops ocellatus*) (Szedlmayer and Howe, 1995) and in hatchery-produced Japanese flounder (*Paralichthys olivaceus*) (Yamashita *et al.*, 1994). In order to minimize stress due to the low pH induced by markers, fish were usually immersed in buffered solutions (e.g. Hettler, 1984; Muth *et al.*, 1988; Tsukamoto, 1988). For marine species, salinity was maintained within a range compatible with osmoregulatory capabilities of the fish (Hettler, 1984; Szedlmayer and Howe, 1995; Thomas *et al.*, 1995). Tetracyclines were most widely used until their role was challenged by alizarin compounds following

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Table 1. Examples of otolith marking of larval and juvenile fish by immersion in AC and ARS solutions (length in total (TL) or standard length (SL) and wet weight (WW) ± standard deviations, if known). When multiple concentrations were compared, the efficient concentrations are given without parentheses.

Reference	Species	Marker	Concentration (mg l^{-1})	Fish size at marking	Immersion time (h)
Tsukamoto *et al.* (1989a)	*Pagrus major*	AC	50–200	TL from 7 to 25 mm	24
Secor *et al.* (1991)	*Morone saxatilis*	AC	25	SL 5–7 mm	6
Blom *et al.* (1994)	*Gadus morhua*	AC	(0)–50–100–(200)	WW 1.25 ± 0.37 g	24
	(id.)	ARS	(0) 100	WW 2.24 ± 0.65 g	24
Yamashita *et al.* (1994)	*Paralichthys olivaceus*	AC	80	TL from 52 to 148 mm	24
Szedlmayer and Howe (1995)	*Sciaenops ocellatus*	AC	250	SL 67.4 ± 8.7 mm	15
Beckman and Schulz (1996)	*Catostomus commersoni*	AC	50–200–(300–400)	Larvae	12–(24)
	Catostomus commersoni	ARS	(100)–200–(400)	Larvae	12–24
	Campostoma anomalum and *Phoxinus erythrogaster*	AC	400	Postlarvae	24
	Campostoma anomalum and *Phoxinus erythrogaster*	ARS	200–300–(400)	Postlarvae	24
	Campostoma anomalum and *Phoxinus erythrogaster*	ARS	200	Juveniles SL 21.5 mm	24
Hoff *et al.* (1997)	*Deltistes luxatus* and *Chasmistes brevirostris*	AC	50	—	18

Table 2. Fish group characteristics (n, number of fish, WWi, mean initial wet weight, TLi, mean initial total length, s.d., standard deviation), concentrations and experimental conditions of treatments with ARS and AC for otolith markings of turbot juveniles.

Treatment	ARS100	Buffered ARS200	ARS400	Unbuffered ARS400unb	AC120unb
n	4	4	4	4	4
WWi ± s.d. (g)	42.8 ± 1.8	50.9 ± 10.5	45.6 ± 24.7	42.7 ± 6.9	52.8 ± 13.1
TLi ± s.d. (mm)	135.8 ± 4.8	139.3 ± 12.0	133.5 ± 23.2	135.0 ± 6.1	146.5 ± 14.3
Concentration (mg/l)	100	200	400	400	120
Immersion time (h)	24	24	24	24	24
pH	7.2	7.2	7.2	6.4	7.2

Tsukamoto's study (1988, see review by Tsukamoto *et al.*, 1989b; Blom *et al.*, 1994). Both alizarin complexone (AC) and alizarin red-S (ARS) were successful in marking the otoliths of early juvenile cod (*Gadus morhua*) (Blom *et al.*, 1994) and cyprinids (*Campostoma anomalum*, *Phoxinus erythrogaster*) (Beckman and Schulz, 1996) (Table 1). ARS, which is an inexpensive and easy-to-use chemical, has generally been chosen for mass-marking experiments requiring a large amount of marker, although its potential toxicity is still open to question (Blom *et al.*, 1994; Beckman and Schulz, 1996; Eckmann *et al.*, 1998).

Following Yamashita *et al.* (1994), who included large juvenile flatfish (up to 15 cm in total length) in AC markings, we have recently used AC immersion to induce a benchmark and validate the daily deposition of increments in 8.5 month old turbot (*Scophthalmus maximus*), and to validate the timing of the seasonal annulus in age-0, 1 and 2 sole (*Solea solea*). These previous results offer scope for new field and laboratory studies, providing that routine marking using ARS is not size limited. In addition to mass-marking programmes, individual markings of large fish could facilitate analyses of relationships between otolith and fish growth, and back-calculation procedures to estimate variations in growth. The aim of this experiment was thus to evaluate the feasibility of using ARS to mark the otoliths of large juveniles and to compare both mark quality and fish survival with those obtained using AC marking.

Material and Methods

We chose hatchery-reared turbot for their ability to tolerate handling and external tagging. Juvenile turbot (around 10 months old, N=20) were obtained from a farm (Société Aquacole de l'Ile de Ré, France). Total length (TL) ranged from 109 to 166 mm (median 136 mm) and wet weight (WW) from 22.0 to 79.7 g (median 45.2). The fish were subjected to a two-day fast before their transfer on 6 July 1998 to our field laboratory, where they were acclimatized for 10 days in a 1 m^3 tank. From the date of transfer to the end of the experiment, they were reared under semi-natural conditions (stocking density <2 kg m^{-3}, natural photoperiod, circulating seawater pumped from a nearby earthen pond, with fluctuating temperature and salinity conditions). Oxygen levels were maintained above 5–6 mg l^{-1} O_2. The fish were fed with commercial dry pellets available from self-feeders, in quantities calculated to be in excess of their daily needs.

Fish were randomly assigned to five treatments groups (Table 2). Each group was identified by external tagging using laboratory-made tags with coloured pearls, which allowed us to rear all groups in the same tank and to estimate the growth rate of each fish. One group was marked without any pH adjustment in an AC sea-water solution (alizarin-3-methylamine-*N*, *N*-diacetic acid, $C_{19}H_{15}NO_8$, Sigma), at a final concentration of 120 mg l^{-1} previously tested to be effective with turbot juveniles. The AC120unb seawater working solution (pH 7.2) was used as a reference to prepare the four ARS treatments (alizarin sodium monosulfate, $C_{14}H_7O_7NaS$, Sigma): buffered solutions of 100, 200 and 400 mg l^{-1} ARS (ARS100, ARS200 and ARS400), plus a single treatment of unbuffered 400 mg l^{-1} ARS (ARS400unb). Buffered seawater solutions of ARS were prepared by adding 1–2 ml of NaOH stock solution (15 g l^{-1}) to reset the pH from 6.4 to 7.2 (Table 2). The fish were immersed in these solutions of chemical markers for 24 h on 16 July 1998 and were sampled and weighed 33 days later. The daily instantaneous growth rates per treatment were calculated from individual measurements as: $G = 100 \times (\ln W_f - \ln W_i)/(t_f - t_i)$, with W_i and W_f=initial and final weight, and $t_f - t_i$=duration of the experiment.

The sagittae were extracted under a stereomicroscope, freed from adherent tissues and rinsed in water. The antero-posterior measurements of the sagittae averaged (± s.d.) 2.84 (± 0.41) mm. Right sagittae were processed for frontal sections using 1200 grit sand paper and a polishing bar of 0.3 μm aluminium oxide, a procedure that took around 30 min. Sections were viewed with a UV light microscope fitted with block filters (band-pass

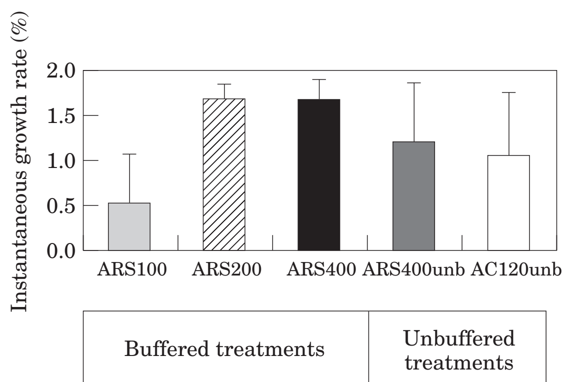

Figure 1. Variations in the growth rates of turbot juveniles subjected to five different marking treatments by immersion in buffered solutions of ARS at marker concentrations of 100, 200, 400 mg l^{-1} and unbuffered solutions of ARS and AC at marker concentrations of 400 (ARS400unb) and 120 (AC120unb) mg l^{-1}, respectively (vertical bars give the standard deviation).

filter 450–490 nm; long pass suppression filter 515 nm), to detect the incorporated AC or ARS marks under epi-illumination. Otoliths were examined by two readers, one of them operating in blind reading conditions. Mark quality was classified into four grades, absent (0), faint (1), good (2), or very good (3). From these two set of readings, grades assigned by each reader were averaged and the averaged value obtained for each otolith was kept for statistical analyses (n=20).

Differences in initial and final weights, and the growth rate between treatments were tested by the non-parametric Kruskal-Wallis method using Systat software package (1997). Kruskal-Wallis tests were also performed with the factor chemical treatment and the averaged grade values as variable.

Results

The differences in initial weights between groups were not significant (KW=3.4, df 4, n=20, p=0.49), neither were the final weights (KW=5.2, df 4, n=20, p=0.27). Post-marking survival was 100% after 33 days for all treatments. Mean instantaneous growth rates (±s.d.) varied between treatments (Fig. 1), increasing from 0.53 (±0.54)% in the ARS100 treatment, to 1.03 (±0.70)% and 1.19 (±0.62)%, in the AC120unb and ARS400unb treatments, reaching 1.66 (±0.23) and 1.68 (±0.17) in the ARS400 and ARS200 groups. These differences were not significant (KW=8.2, df 4, n=20, p=0.08) although the ARS100 groups had the lowest rank sum (RS=19), in comparison with the ARS400 and ARS200 groups (RS=58 and 59, respectively).

Microscopic examination of otolith sections through transmitted light indicated changes in microstructures, with two checks (i.e. stress-induced discontinuities, Kalish *et al.*, 1995) separating the inner increments from wider outermost increments (Fig. 2a–b and 2d–e). The position of the incorporated mark, as revealed by UV light, allowed us to ascribe the stronger check (C_t) to events occurring before marking, presumably during the two-day pre-transfer fast and the transfer itself, and the second check (C_m) to the marking. Beyond this C_m check, increments were deposited in a more regular pattern. When viewed under UV light, AC produced a pink-orange ring (Fig. 2c) and ARS a yellowish ring (Fig. 2f), whereas the otolith edge (O) was greenish due to its autofluorescent properties.

All AC marks were classified as good or very good (Fig. 3). Marks were also seen on all otoliths tagged with ARS, but the quality of the mark varied significantly according to the concentrations (KW=8.6, df 3, n=16; p=0.03). The mark was poorly visible in most otoliths of the ARS100 group, which had the lowest rank sum (RS=13), whereas the ARS400 groups had the highest rank sum (RS=48). Marks resulting from the ARS200 treatment ranged from faint to very good, in contrast with the good and very good marks obtained with both ARS400 and ARS400unb treatments. The ARS100 group was excluded from statistics and there was no differences between quality of marks for the three remaining ARS treatments (KW=2.2, df 2, n=12, p=0.33). When the AC120unb group with the three efficient ARS treatments was added to the test, there were still no differences in the quality of marks between alizarin compounds and unbuffered or buffered treatments (KW=2.3, df 3, n=16, p=0.51). Nevertheless, rank sums indicated a decreasing efficiency from the ARS400 treatment (RS=42.5), compared with ARS400unb (RS=37.5), AC120unb (RS=32) and ARS200 (RS=24).

Discussion

Following Tsukamoto *et al.* (1989a), the use of alizarin to mark otoliths of juvenile marine fish has increased (Table 1), compared with tetracycline, which has the disadvantge of producing an antibiotic effect (Szedlmayer and Howe, 1995). ARS immersion appears to be a promising technique for marking the otoliths of small juveniles (Blom *et al.*, 1994; Beckman and Schulz, 1996). However, questions remain regarding the upper size limitation (Yamashita *et al.*, 1994; Beckman and Schulz, 1996), toxicity of AC *versus* ARS, and the most efficient concentrations and durations of exposure (Tsukamoto, 1988; Blom *et al.*, 1994; Beckman and Schulz, 1996).

The size limitation and toxicity of ARS appeared to be less drastic than previously thought, since we obtained ARS markings in turbot juveniles as large as the Japanese flounder marked by Yamashita *et al.* (1994)

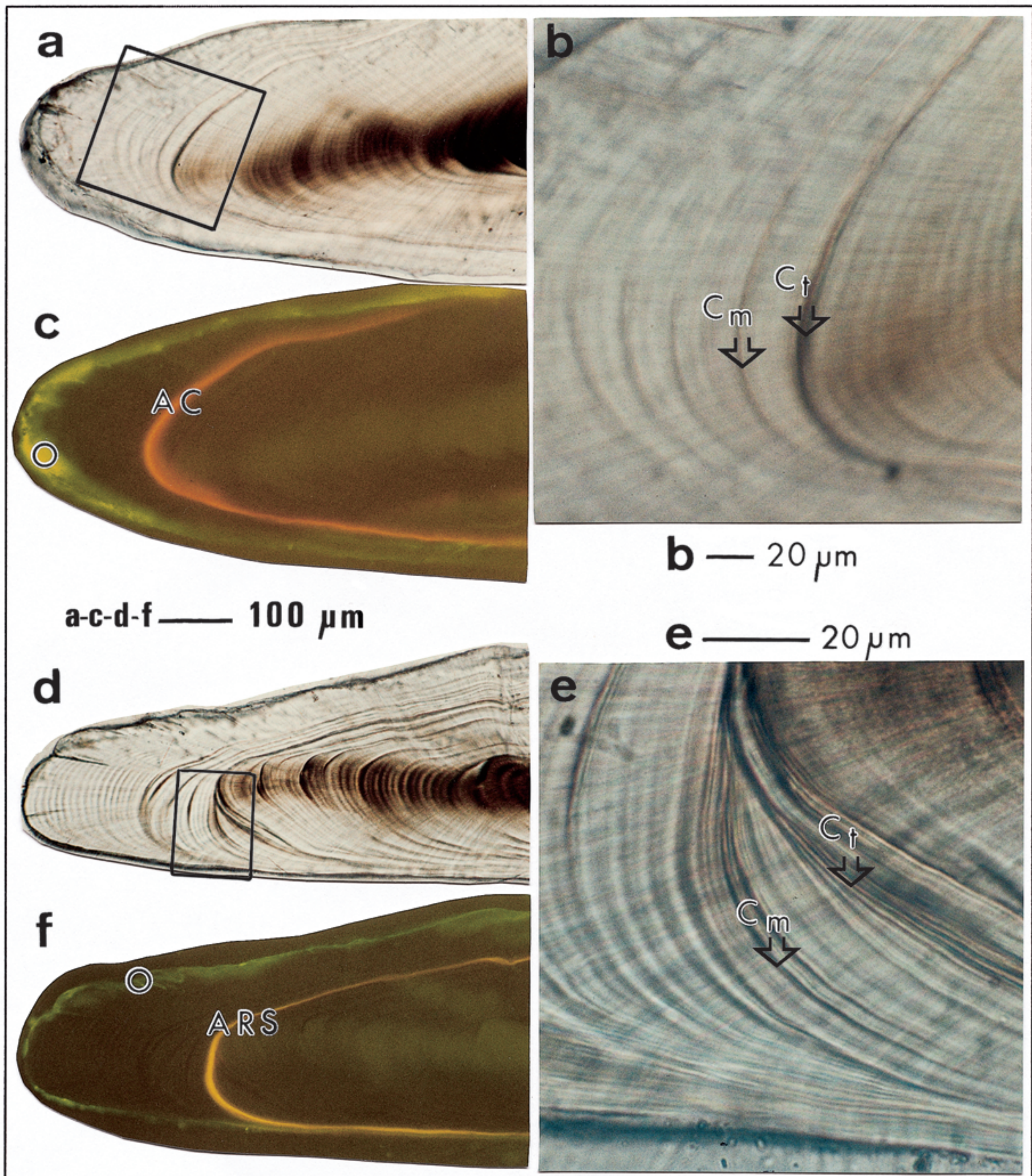

Figure 2. Frontal sections of otoliths of turbot juveniles marked with AC (a, b and c) and ARS (d, e and f). (a and b, inset of a) and (d and e, inset of d) Anterior tips viewed through transmitted light showing two checks, C_t ascribed to handlings due to transfer and C_m, which corresponded to the alizarin marks; (c and f) same sections as (a) and (d) viewed under UV epi-illumination (AC, alizarin-complexone mark; ARS, alizarin-red S mark, O autofluorescence of the otolith edge).

with AC immersion. Using both ARS and AC, we obtained 100% marking success, no mortality and no cessation of growth. Although obtained from small-scale experiments, our results confirm that post-marking mortality could be restricted to a 0–10% range (e.g. Tsukamoto *et al.*, 1989a, b; Peterson and Carline, 1996). Depending on the treatments, the averaged growth rates varied from 0.5 to 1.7% (Fig. 1). From standard deviations of initial sizes (Table 2), one can infer that there was no relationship between these growth variations and

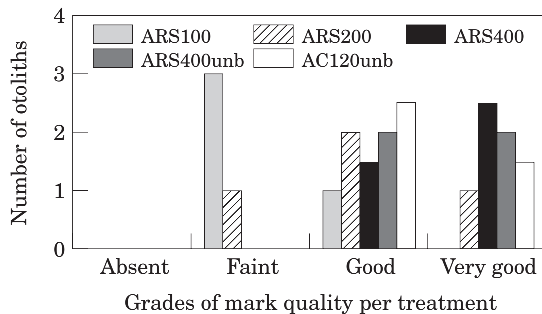

Figure 3. Distribution of otolith grades according to mark qualities per treatments. Grades (n=20) were averaged between the two readers' assessments (ARS100, 200, 400: concentration in mg l^{-1} of buffered ARS solutions; ARS400unb and AC120unb: concentration in mg l^{-1} of unbuffered treatments of ARS and AC).

the random assignation of individuals to a group: the lightest (22 g, G=1.35%) and the heaviest fish (79.7 g, G=1.69%) belonged to the ARS400 group. However, these differences in growth rate between treatments were not statistically significant. Furthermore, these values were in the range of those achieved by 6–12 month old turbot juveniles of similar initial weight under various experimental conditions (e.g. Burel *et al.*, 1996). Other evidence that stress was limited relies on examination of otolith patterns (Fig. 2). Restriction of fish growth usually produces otolith stress indices, which result from changes in the process of increment deposition (e.g. Mugiya & Muramatsu, 1982; Burke *et al.*, 1993). In our experiment, these changes were limited to the two checks induced by transfer and handling preliminary to marking. Following these checks, the regular deposition of increments indicated that most of the fish had resumed normal growth.

Alizarin dyeing was faint when viewed under a bright-field light source, and its detection with UV light required otolith preparation. This could be explained by our initial larger fish sizes, in contrast to results obtained from markings of smaller fish (e.g. up to 60 mm, see Eckmann *et al.*, 1998). By using sections, the marks could be easily detected with a UV light microscope and readers' assessments of the quality of marks varied between "good" and "very good". Depending on the species and its size at immersion, recommended ARS concentrations ranged from 100 mg l^{-1} (Blom *et al.*, 1994) to 200 mg l^{-1} (Beckman and Schulz, 1996), but the latter authors suggested that larger fish could require higher concentrations. We obtained significantly lower grades of mark quality for an ARS concentration of 100 mg l^{-1} (Fig. 3). Unexpectedly, the fish submitted to this less stressful treatment had also lower growth rates (g=0.53 ± 0.54), which could explain fainter marks. At concentrations of ARS ≥ 200 mg l^{-1}, differences in grades were not statistically different between groups, albeit the "good" and "very good" marks were only achieved at an ARS concentration of 400 mg l^{-1}, and the AC treatment (120 mg l^{-1}). Buffered treatments did not improve the quality of marks at 400 mg l^{-1}, neither did they affect growth rates or survival, which could be due to the buffering properties of seawater solutions (Secor *et al.*, 1991). Nevertheless, the use of buffers increased the solubility of alizarin compounds (Tsukamoto, 1988; Blom *et al.*, 1994; this study), which probably contributed to their marking efficiency.

In conclusion, a small-scale experiment with turbot juveniles has demonstrated the feasibility of otolith markings by immersion of large fish in ARS seawater solution, at a final concentration of 400 mg l^{-1}, thus offering an alternative to techniques such as injection. Detection of ARS marks requires the same microscope block-filter combinations as AC marking. If large otoliths require sectioning, standardized sections permit precise observations and standard measurements. Application of ARS immersion should be feasible with other marine fish species, either hatchery-produced or taken from the wild, and probably freshwater species, providing marker concentrations are adjusted both to fish sizes and the number of individuals stocked during immersion.

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