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ARTICLE

Effectiveness of mass marking of juvenile sterlet (*Acipenser ruthenus*) with alizarin red S stain on field detection

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Abstract

Sterlet (*Acipenser ruthenus*), one of the last sturgeons in the Danube basin, is regularly stocked in national and international restoration programs, but the efficacy of stocking has not yet been evaluated. Effectiveness of stocking should involve marking and evaluating growth and survival of juveniles. Chemical marking with alizarin red S (ARS) was evaluated to establish optimal ARS staining approaches for juvenile sterlet. Bathing in a 100–200 mg l^{-1} ARS concentration for 12 h had no affect on fish mortality or growth. Use of a green laser on the upper five external structures (dorsal, lateral, and ventral scutes and rostral and anal plates) allowed easy detection for at least a year. Simultaneous use of multiple external structures was effective for distinguishing stocked from wild fish without harm or sacrifice. We conclude that ARS staining enabled a cost-effective, quick, and labour-nonintensive way to mark sterlet, with a high-field detectability and minimal previous experience required.

KEYWORDS
ARS staining, Danube, fish marking, management, stocking, sturgeon

1 | INTRODUCTION

Contemporary society is responsible for managing its impact on environmental health and biodiversity (Sandifer et al., 2015; WHO, 2015). Living requirements needed to provide for a growing human population have substantially altered most known ecosystems (Naiman & Dudgeon, 2011). Freshwater ecosystems are key habitats that serve as sources of water for drinking, agriculture, industry, and power plants, and as receivers of wastewater and runoff from surrounding land, often in fragmented rivers (Arthington, 2012). Consequently, freshwater fish species are under increasing pressure (Arthington et al., 2016; Reid et al., 2019). Even prehistoric fish such as sturgeon, that have lived on our planet for over a 100 million years (Vavrek et al., 2014), are not safe and today face challenges from water pollution, genetic contamination by fish from farmed stocks, habitat deterioration, and serious damage that affects accessibility and existence of suitable spawning grounds (Kubala et al., 2021; Lenhardt et al., 2006; White et al., 2023).

Sturgeon species are bioindicators of the quality of rivers and habitats and are often regarded as flagships of freshwater biodiversity conservation (Van Rees et al., 2021). Of the six native sturgeon populations that migrate (or once migrated) into the upper part of the Danube River basin (DRB), all declined dramatically during the previous century, leaving the European sturgeon (*Acipenser sturio*) extinct, four on the brink of extinction, and the sterlet (*A. ruthenus*) vulnerable (Friedrich, 2012; Friedrich et al., 2018; Gessner et al., 2009; Hensel & Holčík, 1997; Holčík et al., 2006). Previous sterlet release programs were unfortunately somewhat uncoordinated and several mistakes

occurred, including the DRB. For example, no unified methodology for trapping juveniles or older fish, such as hauling (Holčík, 2003), gill nets (Mohler et al., 2012), and trawling (Kalmykov et al., 2009), was used for recapturing and identifying stocked individuals (Ireland et al., 2002; Kapusta et al., 2015; Kubala et al., 2017; Pierre, 1999), so results of previous mark-recapture studies of stocked sturgeons were therefore inconclusive.

The Pan-European Action Plan for Sturgeon (PANEUAP), which includes sterlet, is a new management tool that clearly defines needs of sturgeon species in European waters (Friedrich et al., 2018). The PANEUAP regards marking or tagging as one of the most crucial elements to successfully assess introductions of new stocks, but provides no detailed methodological specifications. Today, many invasive or noninvasive methods are used for group or individual marking, all of which have advantages and disadvantages (Nielsen, 1992). Efficiency of a marking method is based on (i) mortality of marked individuals, (ii) cost, (iii) ease of application, (iv) retention time during marking, and (v) welfare of fish (Warren-Myers et al., 2018). Many invasive or less invasive marking methods are used, ranging from fin cutting (in the past) to fin clips, and in more recent years FLOY Tags, VIA (Visible implant alpha tags), VIE (Visible implant elastomer tags), CWT (coded wire tags) (Nielsen, 1992), and radio frequency identification systems known as PIT tags (passive integrated transponder) (Gibbons & Andrews, 2004; Hamel et al., 2012, 2013). Acoustic telemetry assists research into fish behaviour but is limited by size of young fish, time of detection (Ashton et al., 2017), and the potential for mistakes due to a need to collect data under challenging conditions (Beeman & Perry, 2012). Moreover, due to predominant use of age-0 fish in recovery programs, use of telemetry is questionable. Even when using PIT tags, size of sturgeon in the first year must be taken into account, and surgical procedures can increase retention even in the smallest age-0 fish without significant impacts on growth or swimming performance (Schumann et al., 2017). Retention and readability of visible implant tags (e.g., VIA, VIE, and CWT) depend on application location, colour, and detection time (e.g., VIE and CWT) (Kapusta et al., 2015). These methods do not affect growth or survival, but retention is not always successful (Hamel et al., 2013). Mark-detection time is also essential (Bashey, 2004; Crook et al., 2007) because detection by stocking programs must be possible for at least one or more years after marking. Detection is also complicated in fast-growing fish, such as sturgeons, especially in large rivers like the Danube, Missouri, or Hudson, where sterlet recapture efficiency was poor as a result of low (<1%) survival (Holčík et al., 2006; Mohler et al., 2012; Pekárik et al., 2017). Overall, effective marking requires nearly 100% retention of easily detectable marks, and recapture efficiency is also increased by greater size and weight of stocked sturgeons (Burtsev, 2009).

For smaller fish, chemical marking is promising, and calcein (CAL), alizarin complex one (ALC), alizarin Red S (ARS), oxytetracycline (OTC), and strontium chloride ($SrCL_2$) can all be employed. These dyes function by binding to calcified structures in the body and ALC and ARS, for example, have been used for more than two decades because they allow for fast and minimum handling time when mass-marking small sturgeon under various concentrations and protocols (Brun et al., 1999; Chang, 1999; Jatteau & Lochet, 2011; Lochet et al., 2011). Chemical marking methods (CAL, ARS, and OTC) are widely used for fish as internal markers for otolith control in short- and long-term studies (Lagardère et al., 2000; Lü et al., 2020), but are an invasive approach and fish must be sacrificed before examination.

For chemical marking, sturgeon species have the advantage of external bone-like calcified structures, including plates, scutes, scales, and fin rays. However, calcification of these structures is timedependent (i.e., days post-hatching, dPH). Dye binding on bone-like structures could be limited in early life stages by a deficiency of calcium deposits (Zhang et al., 2012), and depends on ossification that begins at 10 dPH in Chinese sturgeon on dorsal scutes and runs to 25 dPH on ventral scutes (Ma et al., 2014). It follows from the above that ARS binding is best at 59 dPH, albeit with significant differences among fin rays (44–122 dPH) when calcification is visible. In addition, fish are not stained visibly, so marks are only visible using fluorescence microscopy (Lochet et al., 2011; Warren-Myers et al., 2018; Withers et al., 2019). Chemical staining is a noninvasive method for externally marking (Brun et al., 1999; Lü et al., 2015), so mass staining by ARS using proper techniques for detection (Lochet et al., 2011) could be a valuable tool for assessing efficiency of hatchery programs (Warren-Myers et al., 2018). In particular, ARS mass staining could be useful for the ongoing sterlet restoration programs based on intensive stocking of juveniles in the Danube by the Slovak Fishermen's Union (in fact the upper part of the middle Danube), and the lack of data—only a few scientific papers—on sterlet stocks since construction of the Gabcikovo dam and hydropower plant (Friedrich et al., 2016; Hensel & Holčík, 1997; Holčík, 2003; Kubala et al., 2019; Neuburg & Friedrich, 2023; Pekárik et al., 2019).

Our objectives were to: (i) assess the suitability of ARS mass marking as a simple noninvasive method for marking juvenile (0+) sterlet by nonprofessional angling associations that stock sterlet; (ii) evaluate the suitability of ARS mass marking of sterlet juveniles for long-term separation of hatchery-reared fish from natural fish to evaluate the efficiency of conservation programmes; and (iii) identify a simple method for distinguishing stocked fish by fishery managers and anglers in the field, without harming or sacrificing fish, or using complicated laboratory analyses such as fluorescence microscopy.

2 | METHODS

Sterlet were reared in facilities of Pohořelice Fisheries Plc. (Czech Republic), an entity with long experience in sterlet culture, and the only company authorised to rear and breed genetically pure sterlet for stocking in open waters of the Danube basin in the Czech Republic. The experiment was for 1 year, 28 November 2019–29 November 2020, in two phases, 1–150 days post-staining (dPS) and 151–365 dPS.

Sterlet juveniles aged 6 months ($n = 200$) were measured in individual body weight ($W = 42.35 \pm 2.07$ g; median ± 95% confidence

interval) and total length from the tip of the rostrum to the end of the longest ray of the upper lobe of the caudal fin ($TL_1 = 237.00 \pm 3.66$ mm) and from the tip of the rostrum to the end of the longest ray of the lower lobe of the caudal fin ($TL_2 = 210.50 \pm 3.33$ mm). Juvenile sterlet were randomly divided into 10 groups of 20 individuals per group for the first experimental phase. At the end of this phase (150 dPS), the four groups with the highest rate of dye detectability and visibility (N°. 5, 6, 8, and 9) were selected and used together with a control (unstained) group (N°. 10) in a second experimental phase to verify long-term durability (365 dPS) and detectability of ARS marks. Sterlet were selected based on readability of marks and total number of clearly readable structures per individual (Table 3). During the first experimental phase, fish were reared in indoor RAS conditions, with each group in a separate 3-m^3 tank, whereas all fish selected for the second experimental phase were reared together in a single 5-m^3 tank. Fish were fed ad libitum with commercially produced pellets (F-1P B40 2.5, Skretting) supplied by automatic feeders during both experimental phases. Water temperature, oxygen concentration, and pH were measured daily using a HQ 40D multimeter (Hach Lange GmbH).

A alizarin red S (ARS; VWR Chemicals Ltd.) water solution was used for mass marking of juvenile sterlet. ARS binds on calcium-rich structures to form a orange-to-red flake pigment. Use of ARS for fish staining has been confirmed safe (Beckman & Schulz, 1996; Brun et al., 1999; Lochet et al., 2011). Different amounts of ARS (50, 100, and 200 mg l^{-1}), supplied as a red powder, were diluted 50:1 in water, and fish were placed for 1-, 6-, or 12 h time intervals (Table 1). During staining, the water bath was aerated by aquarium pumps and water temperature, oxygen concentration, pH, and fish health status were checked regularly by specialist. Nine groups of fish were stained, and one group was no stained as a control. Sterlet for the second experimental phase were individually PIT-tagged (1.35-mm diameter, 7-mm long; Loligo Systems) to identify individuals from the five groups that were reared together in a single tank. PIT tags were applied behind the left-hand head plate, close to the first dorsal scale, and beneath the skin. All tagged fish were identified by scanning with a HPR Lite hand-held PIT tag reader (Biomark).

Mortality was monitored daily; length and weight were measured monthly during the first experimental phase and at the end of the second experimental phase, and efficacy of fish staining in different groups was individually evaluated at 1, 30, 60, 90, 120, 150, and 365 dPS. External structures (Vecsei et al., 2001) to which ARS could bind to Ca^{+2} ions included: (i) dorsal, lateral, and ventral scutes; (ii) pectoral, ventral, anal, caudal, and dorsal fin rays (visible in lateral view); and (iii) rostral protrusions and plates on the snout and preanal plates (visible in ventral view). Presence of ARS dye bound to calcified structures was determined while holding fish under a green laser module with TTL (power: 100 mV; Eclipsera Ltd.), which emitted a light ray with a 532-nm wavelength. Thermostability of the device (recommended operating temperature = 22–23°C), which was connected to a 12-V battery, was ensured with a Peltier element. While working with the laser, operators wore protective glasses with wavelength coverage of 190–540 nm. Structures rich in calcium in stained fish glowed orange-light red, whereas fish from the unstained control group emitted no radiation. Visibility and readability of ARS marks were evaluated individually in each external structure by scoring 0–2: 0 = no dye detected; 1 = weak dye detectability or visibility; and 2 = clear, easy dye detectability or visibility. Scores were assigned by two people (laser operators) simultaneously and independently of each other to avoid failures or ambiguities in scores. Each individual was rated to calculate the average rating for the whole group (total score) for each character (the average total score of all characteristics per individual). Scores were also calculated for the best-rated features for detection among all characteristics (the average total score of dorsal, lateral, and ventral scutes and rostral and anal plates for each individual). We also evaluated the share (as a proportion) of selected characters in relation to the total score, as the percentage that selected characteristics contributed to the total score. Prior to manipulation of fish, anaesthesia in clove oil essence (concentration: 0.03 ml l^{-1}, Eugenol, Sigma-Aldrich) was used to measure morphometric characteristics, and to detect ARS dye in relaxed unstressed fish.

Effects of ARS staining on survival and growth of stained fish (groups 1–9, denoted G) were compared to the control (group 10) using a *Q* coefficient:

$$Q_i(P,T,G) = \frac{M_i(P,T,G) - \mathrm{Med}\,(P,G)}{\mathrm{Med}\,(P,G)}$$

where *T* is time, *P* is a parameter (TL_1, TL_2, *W*), M_i is the measured value of a parameter *P* at time *T*, and $Med(P,G) = \sum_G M_i(P,1,G)$. The equation computes the difference in each specimen over time, compared to the median of the group at the beginning

TABLE 1 Fish staining scheme for mass marking of juvenile sterlet (*Acipenser ruthenus*) during experiment from 2019 to 2020 in Pohořelice Fisheries by alizarine red S (ARS).

ARS concentration (mg l^{-1})	50			100			200			0
Time (h)	1	6	12	1	6	12	1	6	12	0
Group number	G1	G4	G7	G2	**G5**	**G8**	G3	**G6**	**G9**	**G10**

Note: Groups of sterlet were stained using the following approaches [time of exposure (h = hours) and ARS concentrations (mg l^{-1})]: G1 = 1 h, 50 mg l^{-1}; G2 = 1 h, 100 mg l^{-1}; G3 = 1 h, 200 mg l^{-1}; G4 = 6 h, 50 mg l^{-1}; G5 = 6 h, 100 mg l^{-1}; G6 = 6 h, 200 mg l^{-1}; G7 = 12 h, 50 mg l^{-1}; G8 = 12 h, 100 mg l^{-1}; G9 = 12 h, 200 mg l^{-1}; and G10 = control/reference group with no staining. Groups in bold were also used for long-term studies.

(i.e., the change in each parameter over time independently compared to its starting statistics). A Mann–Whitney *U*-test (Mann & Whitney, 1947) was used to test the null hypothesis that TL_1, TL_2, and *W* did not differ significantly between the control group ($G = 10$) and test groups ($G = 1$–9) for short-term and long-term experiments. *U*-tests for each *P*, *G*, and *T* were compared to critical values, $U_{crit,}$ for a one-tailed distribution, $U_1 = 138$, and a two-tailed distribution, $U_2 = 127$ (Milton, 1964). The null hypothesis was not rejected if *U* was larger than U_{crit}. To select the best combination of approaches and structures for use on juvenile sterlet in the field, ARS detection and staining approaches (ARS concentrations and exposure times) were compared among structures based on scores and proportions of total scores per individual. Readability of different staining approaches (groups 1–10) was compared using Kruskal–Wallis nonparametric tests ($p < 0.05$), followed by Multiple comparisons (TIBCO Statistica, version 14.0.0.15, TIBCO Software Inc.).

3 | RESULTS

No sterlet in any experimental or control groups died during the experiment and growth in length and weight was similar among experimental and groups during both short-term and long-term studies (Figure 1). ARS marking had no significant effect on growth (TL_1, TL_2, *W*) for any staining approach or control fish over 1, 30, 60, 90, 120, 150, or 365 dPS (Table S1).

The effectivity of staining in terms of the visibility and readability of colour marks in 10 external structures in the sterlet juveniles, dependent on dye concentration and exposure time, is given

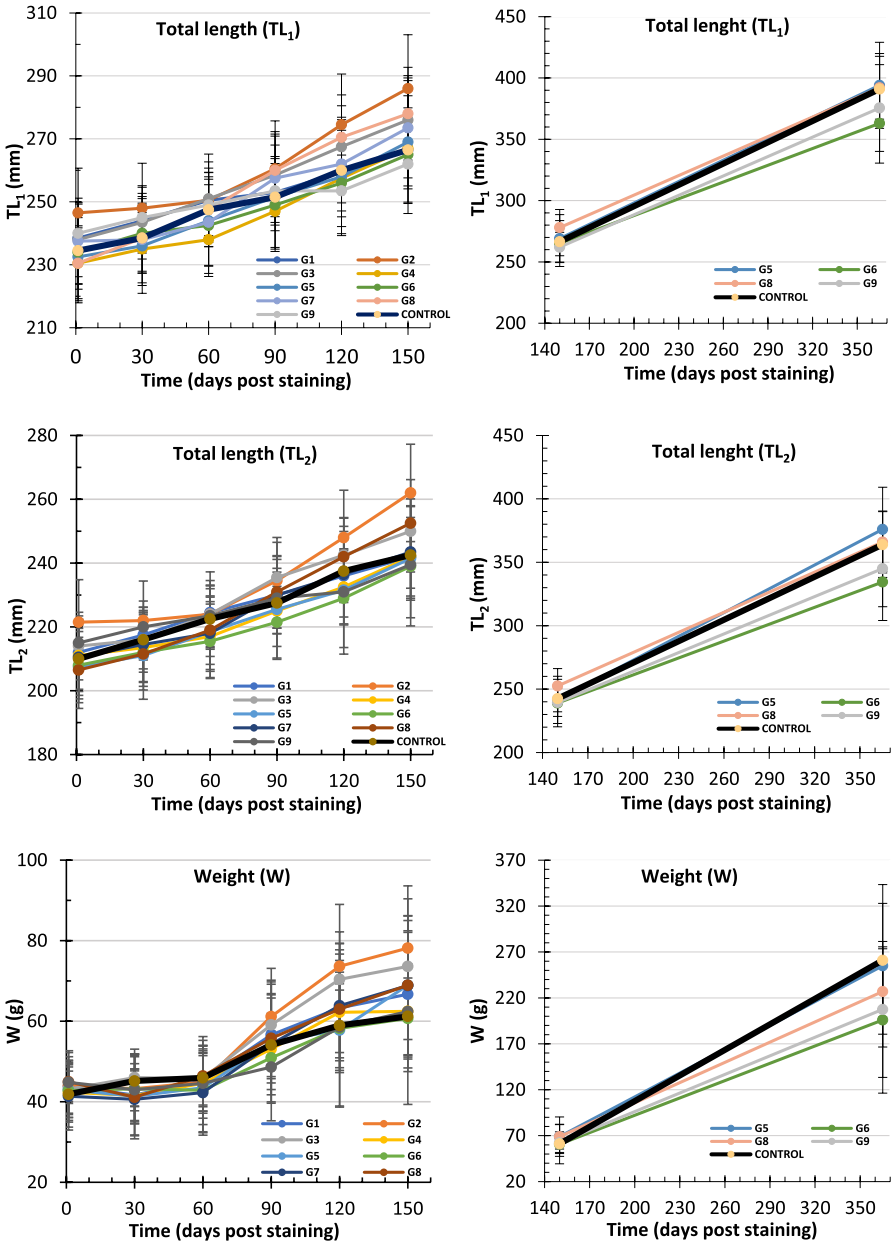

FIGURE 1 Growth in total length (TL_1, TL_2; mm) and weight (W; g) of juvenile sterlet (*Acipenser ruthenus*) marked by staining with alizarin red S (ARS) during the first experimental phase (left-hand column) and second experimental phase (right-hand column) at 1, 30, 60, 90, 120, 150, and 365 days post staining (median ± 95% CI). The groups of sterlet were stained using the following approaches (time of exposure and ARS concentrations): G1 = 1 h, 50 mg l^{-1}; G2 = 1 h, 100 mg l^{-1}; G3 = 1 h, 200 mg l^{-1}; G4 = 6 h, 50 mg l^{-1}; G5 = 6 h, 100 mg l^{-1}; G6 = 6 h, 200 mg l^{-1}; G7 = 12 h, 50 mg l^{-1}; G8 = 12 h, 100 mg l^{-1}; G9 = 12 h, 200 mg l^{-1}; and G10 = control/reference group without staining.

for three crucial time points: 1 dPS, at beginning of the first phase of the experiment (Table 2); 150 dPS, at the end of the first experimental phase (Table 3); and 365 dPS, at the end of the second experimental phase (Table 4). Readability and visibility of ARS marks on all scutes and plates did not change significantly over the entire study period (1–365 dPS) for groups 6, 8, and 9. For other external structures in selected groups and all structures in other experimental groups, visibility of ARS marks decreased 1 month after staining. Moreover, marks were often too weak to be readable or were completely invisible 5 months after staining (150 dPS), especially for fins. Among groups selected for the second experimental phase due to high mark visibility at the end of the first experimental phase, mark readability for group 5 was 20–30% poorer than for other groups at the end of second experimental phase (365 dPS).

Scutes and plates (dorsal, lateral, and ventral scutes and rostral and anal plates) were the most suitable, easily detected, and usable structures for ARS detection, and together were more than 82% (group 9) or 90% (groups 5, 6, and 8) of the total score for all structures at 365 dPS (Table 5). In contrast, the proportion of fins to total individual scores declined shortly after staining and was negligible by the end of the experiment (365 dPS), except for the pectoral fin, which was 6–13% of the total score.

4 | DISCUSSION

Conserving the sterlet and other sturgeon species in the Danube river catchment [i.e., LIFE projects Nos.: LIFE Sterlet (Boku, 2015); LIFE Living Rivers (Vuvh., 2023); LIFE-Boat-4-Sturgeon (Boku, 2022)] requires robust assessment of success. Stocking of juvenile sterlet requires effective marking without causing excessive harm or stress and high mark retention of released fish for future recapture. For such small fish, fluorochrome markers are very promising (Lochet et al., 2011; Warren-Myers et al., 2018; Withers et al., 2019). Our study sought to refine current knowledge of effective marking approaches for sterlet and how best to detect released fish in the field (search for the best body parts or structures for reading marks). Our results could be crucial for recovery programs and vital for current projects, such as PANEUAP (Friedrich et al., 2018) operating in countries of the Danube basin.

Retention and readability of fluorochrome are related to many factors, including exposure time (time of staining and age of stained fish), application site (Crook et al., 2007; Kapusta et al., 2015; Kozłowski et al., 2017), fish size in relation to calcification (Ma et al., 2014; Zhang et al., 2012), and whether fish are ray-finned (Baer & Rosch, 2008; Caraguel et al., 2015; Warren-Myers et al., 2018) or cartilaginous (Jatteau & Lochet, 2011; Lochet et al., 2011; Withers et al., 2019). Fish density, temperature, bath exposure time, and dye quality, combined with a saline bath (1%), increases efficiency and cuts marking cost (Simon, 2024). Mass marking with alizarin is also limited by UV radiation, which reduced and shortened marking detection in rainbow trout (Elle et al., 2010). For rheophilic species like sterlet, which

TABLE 2 Readability of alizarin red S (ARS) marks on juvenile sterlet (*Acipenser ruthenus*) 1 day after staining (mean ± SD).

	Scutes/plates					Fins			
Group	Dorsal	Lateral	Ventral	Rostral	Anal	Pectoral	Ventral	Anal	Caudal	Dorsal
G1	1.90 ± 0.45^{a}	0.95 ± 0.22^{a}	0.95 ± 0.22^{a}	1.00 ± 0.00^{a}	0.95 ± 0.22^{a}	0.95 ± 0.22^{a}	0.95 ± 0.22^{a}	0.95 ± 0.22^{a}	0.95 ± 0.22^{a}	0.95 ± 0.22^{a}
G2	2.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	2.00 ± 0.00^{b}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
G3	2.00 ± 0.00^{a}	1.00 ± 0.00^{a}	2.00 ± 0.00^{b}	2.00 ± 0.00^{b}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
G4	2.00 ± 0.00^{a}	1.00 ± 0.00^{a}	2.00 ± 0.00^{b}	2.00 ± 0.00^{b}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
G5	$\mathbf{2.00 \pm 0.00^{a}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{1.15 \pm 0.37^{ab}}$	$\mathbf{1.15 \pm 0.37^{a}}$	$\mathbf{1.15 \pm 0.37^{a}}$	$\mathbf{1.15 \pm 0.37^{ab}}$	$\mathbf{1.15 \pm 0.37^{a}}$
G6	$\mathbf{2.00 \pm 0.00^{a}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{1.20 \pm 0.41^{ab}}$	$\mathbf{1.20 \pm 0.41^{a}}$	$\mathbf{1.20 \pm 0.41^{a}}$	$\mathbf{1.20 \pm 0.41^{ab}}$	$\mathbf{1.20 \pm 0.41^{a}}$
G7	1.80 ± 0.41^{a}	1.80 ± 0.41^{b}	1.80 ± 0.41^{b}	2.00 ± 0.00^{b}	2.00 ± 0.00^{b}	1.80 ± 0.41^{bc}	1.10 ± 0.31^{a}	1.10 ± 0.31^{a}	1.80 ± 0.41^{bc}	1.80 ± 0.41^{b}
G8	$\mathbf{2.00 \pm 0.00^{a}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{2.00 \pm 0.00^{bc}}$
G9	$\mathbf{2.00 \pm 0.00^{a}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{2.00 \pm 0.00^{bc}}$
G10	$\mathbf{0.00 \pm 0.00^{b}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{d}}$

Note: Groups of sterlet were stained using the following approaches (time of exposure and ARS concentrations): G1 = 1 h, 50 mg l^{-1}; G2 = 1 h, 100 mg l^{-1}; G3 = 1 h, 200 mg l^{-1}; G4 = 6 h, 50 mg l^{-1}; G5 = 6 h, 100 mg l^{-1}; G6 = 6 h, 200 mg l^{-1}; G7 = 12 h, 50 mg l^{-1}; G8 = 12 h, 100 mg l^{-1}; G9 = 12 h, 200 mg l^{-1}; and G10 = control/reference group with no staining. Ten external structures (scutes, plates, and fins) were evaluated by scoring in range 0–2 points where 0 = undetectable; 1 = poor readability; and 2 = clear/easy readability. Experimental groups chosen for the second experimental phase are highlighted in bold text. Groups with the same superscript letter do not significantly differ from each other ($p = 0.05$).

TABLE 3 Readability of alizarin red S (ARS) marks on juvenile sterlet (*Acipenser ruthenus*) 150 days after staining (mean ± SD).

	Scutes/plates					Fins				
Group	Dorsal	Lateral	Ventral	Rostral	Anal	Pectoral	Ventral	Anal	Caudal	Dorsal
G1	0.15 ± 0.37^{ae}	0.20 ± 0.41^{af}	0.35 ± 0.49^{ad}	0.95 ± 0.22^{a}	0.05 ± 0.22^{ae}	1.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.05 ± 0.22^{a}	0.95 ± 0.22^{a}	1.00 ± 0.00^{a}
G2	0.35 ± 0.49^{ae}	0.80 ± 0.41^{ab}	0.75 ± 0.44^{ab}	1.30 ± 0.47^{ab}	0.65 ± 0.49^{a}	0.20 ± 0.41^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.95 ± 0.22^{a}	1.00 ± 0.00^{a}
G3	1.10 ± 0.31^{ab}	1.25 ± 0.44^{bc}	1.20 ± 0.41^{b}	1.80 ± 0.41^{bc}	1.05 ± 0.51^{ab}	1.00 ± 0.00^{a}	0.65 ± 0.49^{bc}	0.35 ± 0.49^{b}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
G4	1.15 ± 0.37^{ab}	1.40 ± 0.50^{bc}	1.35 ± 0.49^{bc}	1.70 ± 0.57^{bc}	1.35 ± 0.49^{bc}	1.05 ± 0.22^{a}	0.25 ± 0.44^{b}	0.55 ± 0.51^{bc}	0.95 ± 0.22^{a}	1.00 ± 0.00^{a}
G5	$\mathbf{1.70 \pm 0.47^{bc}}$	$\mathbf{1.85 \pm 0.37^{cd}}$	$\mathbf{1.85 \pm 0.37^{bc}}$	$\mathbf{1.85 \pm 0.37^{bcd}}$	$\mathbf{1.30 \pm 0.47^{bc}}$	$\mathbf{1.00 \pm 0.00^{a}}$	$\mathbf{0.85 \pm 0.37^{bc}}$	$\mathbf{0.65 \pm 0.49^{bc}}$	$\mathbf{1.00 \pm 0.00^{a}}$	$\mathbf{1.00 \pm 0.00^{a}}$
G6	$\mathbf{2.00 \pm 0.00^{cd}}$	$\mathbf{2.00 \pm 0.00^{de}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{1.95 \pm 0.22^{cd}}$	$\mathbf{1.90 \pm 0.31^{d}}$	$\mathbf{1.25 \pm 0.44^{a}}$	$\mathbf{0.65 \pm 0.49^{bc}}$	$\mathbf{0.70 \pm 0.47^{bc}}$	$\mathbf{1.00 \pm 0.00^{a}}$	$\mathbf{1.00 \pm 0.00^{a}}$
G7	1.85 ± 0.37^{bcd}	1.95 ± 0.22^{cde}	1.85 ± 0.37^{bc}	1.65 ± 0.49^{bc}	1.80 ± 0.41^{d}	1.25 ± 0.44^{a}	0.50 ± 0.51^{bc}	0.30 ± 0.47^{b}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
G8	$\mathbf{2.00 \pm 0.00^{cd}}$	$\mathbf{2.00 \pm 0.00^{de}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{2.00 \pm 0.00^{cd}}$	$\mathbf{2.00 \pm 0.00^{d}}$	$\mathbf{1.40 \pm 0.50^{a}}$	$\mathbf{0.75 \pm 0.44^{bc}}$	$\mathbf{0.20 \pm 0.41^{b}}$	$\mathbf{1.00 \pm 0.00^{a}}$	$\mathbf{1.00 \pm 0.00^{a}}$
G9	$\mathbf{2.00 \pm 0.00^{cd}}$	$\mathbf{2.00 \pm 0.00^{de}}$	$\mathbf{2.00 \pm 0.00^{c}}$	$\mathbf{2.00 \pm 0.00^{cd}}$	$\mathbf{2.00 \pm 0.00^{d}}$	$\mathbf{1.50 \pm 0.51^{a}}$	$\mathbf{0.55 \pm 0.60^{bc}}$	$\mathbf{0.65 \pm 0.75^{bc}}$	$\mathbf{1.25 \pm 0.44^{a}}$	$\mathbf{1.2 \pm 0.41^{a}}$
G10	$\mathbf{0.00 \pm 0.00^{e}}$	$\mathbf{0.00 \pm 0.00^{f}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{e}}$	$\mathbf{0.00 \pm 0.00^{e}}$	$\mathbf{0.00 \pm 0.00^{b}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{b}}$	$\mathbf{0.00 \pm 0.00^{b}}$

Note: Groups of sterlet were stained using the following approaches (time of exposure and ARS concentrations): G1 = 1 h, 50 mg l^{-1}; G2 = 1 h, 100 mg l^{-1}; G3 = 1 h, 200 mg l^{-1}; G4 = 6 h, 50 mg l^{-1}; G5 = 6 h, 100 mg l^{-1}; G6 = 6 h, 200 mg l^{-1}; G7 = 12 h, 50 mg l^{-1}; G8 = 12 h, 100 mg l^{-1}; G9 = 12 h, 200 mg l^{-1}; and G10 = control/reference group with no staining. Ten external structures (scutes, plates, and fins) were evaluated by scoring in range 0–2 points where 0 = undetectable; 1 = poor readability; and 2 = clear/easy readability. Experimental groups chosen for the second experimental phase are highlighted in bold text. Groups with the same superscript letter do not significantly differ from each other ($p = 0.05$).

TABLE 4 Readability of alizarin red S (ARS) marks on juvenile sterlet (*Acipenser ruthenus*) 365 days after staining (mean ± SD).

	Scutes/plates					Fins				
Group	Dorsal	Lateral	Ventral	Rostral	Anal	Pectoral	Ventral	Anal	Caudal	Dorsal
G5	$\mathbf{1.55 \pm 0.51^{a}}$	$\mathbf{1.63 \pm 0.48^{a}}$	$\mathbf{1.28 \pm 0.44^{a}}$	$\mathbf{1.18 \pm 0.59^{a}}$	$\mathbf{1.00 \pm 0.65^{a}}$	$\mathbf{0.45 \pm 0.60^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$
G6	$\mathbf{1.90 \pm 0.31^{b}}$	$\mathbf{1.90 \pm 0.31^{a}}$	$\mathbf{1.90 \pm 0.31^{b}}$	$\mathbf{1.90 \pm 0.31^{b}}$	$\mathbf{1.90 \pm 0.31^{b}}$	$\mathbf{1.00 \pm 0.86^{ab}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$
G8	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{a}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{1.00 \pm 0.73^{ab}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$
G9	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{a}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{2.00 \pm 0.00^{b}}$	$\mathbf{1.83 \pm 0.37^{b}}$	$\mathbf{1.65 \pm 0.67^{bc}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.15 \pm 0.37^{b}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.30 \pm 0.47^{b}}$
G10	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{b}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$	$\mathbf{0.00 \pm 0.00^{a}}$

Note: Groups of sterlet were stained using the following approaches (time of exposure and ARS concentrations): G5 = 6 h, 100 mg l^{-1}; G6 = 6 h, 200 mg l^{-1}; G8 = 12 h, 100 mg l^{-1}; G9 = 12 h, 200 mg l^{-1}; and G10 = control/reference group with no staining. Ten external structures (scutes, plates, and fins) were evaluated by scoring in range 0–2 points where 0 = undetectable; 1 = poor readability; and 2 = clear/easy readability. Experimental groups chosen for the second experimental phase are highlighted in bold text. Groups with the same superscript letter do not significantly differ from each other ($p = 0.05$).

TABLE 5 Individual scores (sum of points per individual) of alizarin red S stained groups and control juvenile sterlet (*Acipenser ruthenus*) 1 day after staining (dPS), 150 dPS (end of the first experimental phase), and 365 dPS (end of the second experimental period).

Time (dPS)	1			150			365		
Group	**TS**	**SSS**	***P* (%)**	**TS**	**SSS**	***P* (%)**	**TS**	**SSS**	***P* (%)**
G1	10.50 ± 2.24^{a}	5.75 ± 1.12^{a}	56.82 ± 10.16^{ab}	4.70 ± 1.03^{a}	1.70 ± 0.98^{ad}	34.02 ± 11.86^{a}	n.e.	n.e.	n.e.
G2	12.00 ± 0.00^{a}	7.00 ± 0.00^{a}	58.33 ± 0.00^{ab}	6.00 ± 1.26^{a}	3.85 ± 1.27^{ab}	62.67 ± 11.68^{b}	n.e.	n.e.	n.e.
G3	13.00 ± 0.00^{ab}	8.00 ± 0.00^{ab}	61.54 ± 0.00^{bc}	10.40 ± 1.67^{b}	6.40 ± 1.39^{ab}	61.26 ± 6.53^{b}	n.e.	n.e.	n.e.
G4	13.00 ± 0.00^{ab}	8.00 ± 0.00^{ab}	61.54 ± 0.00^{bc}	10.75 ± 2.24^{b}	6.95 ± 1.85^{ab}	64.22 ± 8.22^{b}	n.e.	n.e.	n.e.
G5	$\mathbf{15.75 \pm 1.83^{bc}}$	$\mathbf{10.00 \pm 0.00^{c}}$	$\mathbf{64.17 \pm 6.11^{bcd}}$	$\mathbf{13.05 \pm 2.14^{bc}}$	$\mathbf{8.55 \pm 1.57^{bc}}$	$\mathbf{65.32 \pm 4.30^{b}}$	$\mathbf{7.08 \pm 1.81^{a}}$	$\mathbf{6.63 \pm 1.51^{a}}$	$\mathbf{94.52 \pm 7.05^{ab}}$
G6	$\mathbf{16.00 \pm 2.05^{bc}}$	$\mathbf{10.00 \pm 0.00^{c}}$	$\mathbf{63.33 \pm 6.84^{bcd}}$	$\mathbf{14.45 \pm 1.36^{c}}$	$\mathbf{9.85 \pm 0.49^{c}}$	$\mathbf{68.61 \pm 5.73^{b}}$	$\mathbf{10.50 \pm 1.91^{ab}}$	$\mathbf{9.50 \pm 1.54^{b}}$	$\mathbf{91.06 \pm 7.39^{ab}}$
G7	17.00 ± 2.64^{bc}	9.40 ± 1.23^{bc}	55.56 ± 2.21^{abc}	13.15 ± 1.90^{bc}	9.10 ± 1.21^{c}	69.43 ± 4.86^{b}	n.e.	n.e.	n.e.
G8	$\mathbf{20.00 \pm 0.00^{d}}$	$\mathbf{10.00 \pm 0.00^{c}}$	$\mathbf{50.00 \pm 0.00^{a}}$	$\mathbf{14.35 \pm 0.81^{c}}$	$\mathbf{10.00 \pm 0.00^{c}}$	$\mathbf{69.90 \pm 4.01^{b}}$	$\mathbf{11.00 \pm 0.73^{ab}}$	$\mathbf{10.00 \pm 0.00^{b}}$	$\mathbf{91.29 \pm 6.06^{ab}}$
G9	$\mathbf{20.00 \pm 0.00^{d}}$	$\mathbf{10.00 \pm 0.00^{c}}$	$\mathbf{50.00 \pm 0.00^{a}}$	$\mathbf{15.15 \pm 2.37^{c}}$	$\mathbf{10.00 \pm 0.00^{c}}$	$\mathbf{67.46 \pm 9.84^{b}}$	$\mathbf{11.93 \pm 1.05^{ab}}$	$\mathbf{9.83 \pm 0.37^{b}}$	$\mathbf{82.86 \pm 6.27^{b}}$
G10	$\mathbf{0.00 \pm 0.00^{e}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{e}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{d}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$	$\mathbf{0.00 \pm 0.00^{c}}$

Note: The total score (TS; total score of all investigated external structures per individual), score of selected structures (SSS; total score of dorsal, lateral, and ventral scutes and rostral and preanal plates per individual), and the proportion of the score of selected structures of the total score (*P*; in %) are given in the table. n.e. = not evaluated (groups not used in the second experimental phase). Data are shown as mean ± SD. The maximum possible value of the total score is 20 points. The experimental groups chosen for the second experimental period are highlighted in bold text. Groups of sterlet were stained using different approaches (time of exposure and ARS concentrations): G1 = 1 h, 50 mg l^{-1}; G2 = 1 h, 100 mg l^{-1}; G3 = 1 h, 200 mg l^{-1}; G4 = 6 h, 50 mg l^{-1}; G5 = 6 h, 100 mg l^{-1}; G6 = 6 h, 200 mg l^{-1}; G7 = 12 h, 50 mg l^{-1}; G8 = 12 h, 100 mg l^{-1}; G9 = 12 h, 200 mg l^{-1}; and G10 = control/reference group with no staining. Groups with the same superscript letter do not significantly differ from each other ($p = 0.05$).

typically live in running waters at depths of 4–10 m (Holčík, 1998), exposure to UV radiation is limited, but should be verified.

Identifying of the best approach for staining and detection is crucial for stocking programmes in large rivers, where recapture of marked fish is complex, due to lower survival, limited capture methods, or large size of habitats, and generally low recapture rates for estimating survival (Holčík et al., 2006; Mohler et al., 2012; Pekárik et al., 2017; Puzzi et al., 2009; Steffensen et al., 2010). Survival can be increased by appropriate breeding and nursery care (Cámara-Ruiz et al., 2018; Chebanov & Billard, 2001) or by ensuring that time, release method, and size and combination of individuals of different ages are correct (Burtsev, 2009; Ireland et al., 2002). Nevertheless, poor marking can lead to few recaptured fish. Increased size can increase success to allow for a wider range of marking methods, but can also reduce survival of released fish through loss of feeding, hiding, or anti-predatory behaviours (Brown & Laland, 2001; Johnsson et al., 2014). Larger fish are also more expensive to rear.

Sterlet are usually stocked as age-0+ juveniles (LIFE Sterlet; LIFE Living Rivers; regular stocking activities of the Slovak Angling Union), so noninvasive marking is the most practical marking method. Fluorochrome dyes, like ARS in our study, bind to calcium ions, so are feasible for sturgeon species at some ages (Jatteau & Lochet, 2011) because of the way in which calcified body parts form, including fins and scutes (Ma et al., 2014; Zhang et al., 2012). For sterlet, we recommend a minimum staining age of 59–122 days after hatching, as our study in which sterlet were 6 months post-hatching, as a prerequisite for proper development of external calcified structures such as scutes, plates, and fin rays.We found no effects of ARS marking on growth of sterlet in our study, unlike another study (Jurgelėnė et al., 2022), so we recommend using a verified ARS brand.

Other studies that found similar results about staining different body structures of sturgeons (fin rays and scutes) mostly focused on otoliths, an invasive method requiring sacrifice of fish (Warren-Myers et al., 2018). The best treatment in our study was a concentration of 100–200 mg l^{-1} for 12 h, similar to other teleost species like brown trout (*Salmo trutta*) at 150 mg l^{-1} for <3 h (Baer & Rosch, 2008) and cartilaginous species like Siberian sturgeon (*Acipenser baerii*) at 100–200 mg l^{-1} for 12–24 h (Lochet et al., 2011). In contrast, a concentration of 300 mg l^{-1} caused 95% mortality in brown trout (Baer & Rosch, 2008). Marking success was >90% on dorsal scutes of Siberian sturgeon, but only after sacrificing or injuring fish to remove scutes for analysis (Lochet et al., 2011). Control of otoliths resulted in 100% retention 1 month after staining, retention of external fluorochrome marks was lower (<90%) on fast-growing external parts of pectoral fins (first ray) and was influenced by age (days post-hatching in young individuals) (Lochet et al., 2011). Marks were detected on European sturgeon (*A. sturio*) on otoliths and the first ray of pectoral fins after 14 months (Brun et al., 1999). However, earlier studies failed to evaluate mark detection over longer time periods without sacrificing fish. Based on our experience working with sturgeons and our results, the most likely retention time was 14 months because marks were highly detectable by noninvasive methods, even on scutes and plates, after 1 year.

A major limitation of ARS marking is complex manipulation of a fluorescent microscope in the field, together with inherently complicated handling of live fish (Crook et al., 2009). For easy field detection of external marks from ARS staining, we obtained highly promising results without sacrificing marked fish, using small mobile lasers, and focusing on the most visible external marks. The best structures for detection of ARS stain in our study were dorsal, ventral, and lateral scutes and rostral and anal plates, because stain was detected 1 year after staining, similar to otolith spines and dorsal scutes of lake sturgeon (*A. fulvescens*) that were detected by an ultraviolet light source for 72–436 days (Withers et al., 2019). Similarly, ARS-marked golden perch (*Macquaria ambigua*) were externally detectable even after 2 years (Crook et al., 2007). In our study, readability changed rapidly through time for some structures, and fins were mostly undetectable or very poorly detectable, as reported elsewhere (Jatteau & Lochet, 2011). We assume that readability is affected by rapid growth as freshly ossified layers gradually cover stained layers (the first thick ray grows in length and there is also a periosteal reaction), so the stained structure sinks inward (deep) into the ray to become less visible over time. In our study, ARS marks on fins were difficult to detect, although fins may be useful for other purposes (Jatteau & Lochet, 2011; Lochet et al., 2011). We found that scutes and plates were more appropriate for external identification of ARS-marked sterlet in the field than fin rays, which were nearly undetectable unless fish were injured or sacrificed. Dorsal, lateral, ventral, rostral, and anal scutes and plates are easily accessible sites for reading marks and mark retention is high, a combination that can increase the probability of identifying fish correctly. The only possible factor that may negatively influence readability of marks on scutes and plates is abrasion, especially on ventral fins of bottom-dwelling species like sturgeon (Holčík et al., 1989).

We found that ARS staining was effective for mass marking of sterlet (and likely other sturgeon species) in stocking programmes, and its use makes field mark-recapture approaches and identification of stocked fish inexpensive, easy, and effective, with no evident stress-related effects on growth or survival. We also found that monitoring can be done by workers with limited experience and minimal equipment, with no need to sacrifice fish or use complicated laboratory procedures. Above all for sturgeons, this method is valuable for use with external bone-like structures and for identifying the origin of fish (natural or stocked), and may have an increasing range of applications in fish and sturgeon stocking programs.

5 | CONCLUSIONS

Ensuring maximum mark retention when studying wild populations is essential, especially in large river basins where recapture is difficult and juvenile survival is low. Correct rearing, marking, and release of fish, with appropriate and accurate detection methods that minimally impact fish fitness (breeding, marking, and mark detection) can help to accurately estimate stock size, and the proportion of fish released by a recovery program. Collected data can thus enhance recovery

programs through availability of more useful data. We found that ARS staining of sterlet was relatively inexpensive, quick, and not overly labourintensive. Similarly, little experience and equipment were needed for detecting fish in the field. Simultaneous use of a combination of more external structures and one ARS staining were effective for identifying stocked fish over the long term. Nevertheless, the greatest advantage of ARS staining is no need to damage or sacrifice living organisms, which is especially crucial for management of endangered or vulnerable species. In conclusion, a combination of ARS staining, with age estimation from pectoral fins (Collins & Smith, 1996), sex determination (Barulin, 2018, 2019), and population analyses through mark-recapture (Amstrup et al., 2010), is a valuable tools for effective fishery management. Such a comprehensive approach would be particularly beneficial for restoration of endangered sturgeon populations, such as the sterlet in the Danube River. Longer-term ARS mark detection will need to be further investigated by focusing on the best-detected characters in our study (scutes and plates).

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CONFLICT OF INTEREST STATEMENT

All authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All activities were performed according to the principles of the Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Vodňany, based on the EU harmonised animal welfare act of Czech Republic. The above mentioned Ethical Committee (IACUC) specifically approved this study. The principles of laboratory animal care and the national laws 246/1992 and regulations on animal welfare were followed (Ref. number 22761/2009–17210).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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