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Pros and cons of fluorescent pigment mass marking with different colours: A 5-year long study on grayling (*Thymallus thymallus* L.)

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The ideal mark on fish would be fast to apply, of low cost, with high retention rate, no or little effect on a fish's health and behaviour, would not attract predators or alter the probability of fish being captured, and mark detection would be easy and non-lethal. None of the existing marking techniques seems to fulfil all these criteria (Lukas & Baras, 2001, McKenzie et al., 2012), but it had been argued that spray marking of juvenile fish with fluorescent pigments may fulfil most of them (Moffett et al., 1997, Friman & Leskelä, 1998, Schumann et al., 2013). We tested the suitability of the fluorescent spray marking with different pigment granules on grayling (*Thymallus thymallus* L.).

Four different groups of one-summer-old grayling that had been raised at different temperatures in the course of another study (Pompini et al., 2013) were spray-marked before release into the wild. As the different pigment granules (Swada, Chelshire, UK) seemed to vary in their physical properties, the pigment:water ratio (by weight) was adjusted. The following granules were chosen: "Lunar Yellow" (LMP27; "yellow") for group A (~3,000 fish, raised at 5°C, mean±SD length: 7.2±1.7 cm; pigment:water = 2:3), a mixture of "Lunar Yellow" and "Magenta" (LMP10; "magenta") for group B (~4,000 fish, 7°C, 9.3±1.5 cm; pigment:water = 2:4.5), a combination of "Stellar Green" (LMP8; "green") and "Laser Red" (LMP3; "red") for group C (~4,000 fish, 9°C, 9.2±1.5 cm; pigment:water = 1:1), and "Laser Red" for group D (~4,000 fish, 11°C, 9.3±1.4 cm; pigment:water = 1:1). A sandblasting gun (Asturomec, Walmec, Italy) with a 1L reservoir and a nozzle diameter of 6 mm was connected to a compressor with a valve (Airbo, Regensdorf, Switzerland) to maintain stable 6 bar. The gun was attached to the ceiling with a cord to retain a fixed distance to the freshly anesthetized fish (anesthetized with MS-222). One operator presented about 15 fish at a time in a net laid on a surface 17 cm from the nozzle. The other operator then sprayed the fish by making three passes over the net in a zig-zag manner. The spray gun was regularly shaken to maintain a good pigment-water mixture. The handling of the animals was in accordance with the Code of Ethics of the World Medical Association and with Swiss regulation.

Total mortality during marking and the following three weeks in captivity was 0.45%. After these three weeks, random samples of 40 to 66 anaesthetized fish per group were evaluated for mark retention inside a semi-closed plastic box lined with matte black plastic foil and equipped with a 15W blacklight UV-A lamp (Osram Sylvania, Wilmington, USA). In total 192 of the 198 fish (97%) showed marks, but many of these marks seemed to be in the mucus layer and hence not permanent. We therefore kept new random samples of 50 fish per treatment in a semi-natural rearing channel and released all other fish into the lake of origin (Lake Thun, Switzerland).

One year later, 68 of the fish raised in the semi-natural rearing channel could be evaluated for long-term retention of the marks with the same light box as before (Fig. 1a). Of these fish, 50 were still marked (total retention = 73.5%): 13 fish with yellow marks, 9 with yellow and magenta, 8 with green and red, and 20 with red markings only. No fish showed only magenta or only green markings (the pigments that were only used in mixtures). The retention rates per group were not significantly different from each other (likelihood ratio test: $\chi^2 = 6.8$, d.f. = 3, p = 0.08). However, the combined retention rate of only yellow and only red granules was higher than that of the combined mixed colour groups B and C ($\chi^2 = 5.2$, d.f. = 1, p = 0.02).

An ongoing monitoring program (Wedekind & Küng, 2010, Wedekind et al., 2013) allowed to sample adult grayling from the population 3.5 and 4.5 years after release of our marked fish. The fish were anesthetized (MS222) and examined under the UV-A lamp installed inside a dark tent that largely excluded ambient light and greatly improved visibility of the marks. The location of each pigment was recorded (Fig. 1b-d). In total 28 fish could be identified as marked, 5 of them as 4-years old, and 23 as 5-years old. No fish was caught twice, as concluded from the individual marking patterns. The colour of their fluorescent marks were yellow (N=10), red (N=12), or red and green (N=6). No fish was found with magenta pigments. The pigment granules were nearly exclusively (>99%) located around the base of fins, the belly, and the operculum (Fig. 1b-d). Twelve fish showed markings exclusively above the lateral line, 15 only below the lateral line, and only 1 fish showed (red) pigments both above and below the lateral line. Fish with yellow markings were more likely to be marked exclusively below the lateral line than fish with red or red and green markings ($\chi^2 = 4.1$, d.f. = 1, p = 0.04; Fig. 1d). Fish with red and green granules had on average more granules than fish with only red or only yellow marks (Kruskal Wallis, $\chi^2 = 11.3$, d.f. = 2, p = 0.004), while the number of pigment granules per fish seemed similar for only yellow or red marked (Fig. 1).

The low mortalities during marking and the three weeks afterwards confirm previous observations (Moffett et al., 1997, Friman & Leskelä, 1998, Gaines & Martin, 2004) and suggest that the marking is benign to the fish. Red and yellow granules showed better retention than green and magenta granules, and their retention rate one year after marking were comparable to the ones reported from other studies on salmonids (Moffett et al., 1997, Friman & Leskelä, 1998, Gaines & Martin, 2004). There are three possible explanations for the low retention rates of green and magenta. First, these pigments granules could be more likely to get lost during later life-history stages than the other granule types. Second, magenta could potentially have been misinterpreted as red on the adult fish, i.e. the colour differences of these marks on adult fish could have been too small for reliable calling under field conditions (Friman and Leskelä (1998) recommend to use frozen marked fish as a reference to avoid misinterpretations). Third, being successfully marked with magenta could have reduced survival under natural conditions, regardless of whether the marking was dorsal or ventral. We would then expect the respective sensibility for magenta in at

least one of the predators of grayling. The preferred whereabouts of lake-dwelling grayling is at depths where the water column has mostly absorbed the longer wavelengths of the sunlight. It has long been assumed that red or magenta colors are then of no importance for fish. However, some fish are capable of seeing red luminescence at depths were red light is virtually absent (Michiels et al., 2008).

The patterns of marks on adults suggest selection against yellow marking: while red and green markings were commonly observed dorsally, only two adults showed yellow dorsal marking, with only one single pigment granule each that were both located at the base of the posterior half of the dorsal fin, i.e. at a location that may often be covered by the extra-ordinary large dorsal fin of grayling. These observations suggest that yellow fluorescent pigments attract predators that typically spot their prey from above, i.e. possibly birds.

Differences in pigment retention or differences in pigment-induced mortality can be problematic for mixed colour markings. As a green-red pigment mixture was used to mark group C and red pigments for group D, and as red pigments had a better retention than the green ones, it is possible that some group C fish were wrongly identified as members of group D. This may explain the rather large fraction of group D representatives among the recaptured fish. Analogously, if the yellow-magenta mix lead to fish with both colour pigments or only one of each, and if magenta indeed increases mortality by predation, the higher survival of only yellow-marked fish of the group B would make them more likely to be wrongly interpreted as belonging to group A. In our case, the groups A and B and the groups C and D would therefore each have to be summarized, respectively.

To conclude, spray marking with fluorescent pigments allows for efficient, cost-effective, and benign mass marking of grayling at fingerling size. It is, in principle, possible to use different colours or mixes of colours to mark different groups of fish. However, retention rates of individual pigments should be determined from captive samples to improve interpretations of recapture rates in the wild. Moreover, certain types of quantitative comparisons between differently marked groups can be problematic, not only because the different pigments seem to have different retention rates and may, in some cases, be difficult to distinguish under field conditions, but also because some pigments may increase predation risks. Our first results suggest that magenta pigments and dorsally located yellow pigments increase predation on grayling.

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Figure 1 Fluorescent pigment granules found on successfully marked fish. (A) Example of a mark (here one green granule on the body side) as observed after one year in captivity. The marks brightly glow under long-wave ultraviolet light. (B-D) Locations where fluorescent pigment granules could be found on 4- or 5-year old fish that had been released into the wild, with (B) red marks only, (C) a mixture of red and green marks, and (D) yellow marks only. The white areas indicate the regions where marks were usually found (in total > 500 pigment granules), the white arrows indicate the location of the only 4 pigment granules that were found outside these regions. N gives the number of fish marked with a given type of pigment at the indicated area, the number of individual pigments per fish is given in parentheses (numbers > 25 were estimates in order to minimize handling time). No magenta pigments could be identified.