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The use of alizarin red S and alizarin complexone for immersion marking Japanese flounder *Paralichthys olivaceus* (T.)

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ABSTRACT

Alizarin red S from 200 to 400 mg/l and alizarin complexone from 50 to 300 mg/l were used to mark Japanese flounder, *Paralichthys olivaceus*. Immersion for 24 h produced detectable marks in sagittae, ctenoid, cycloid scales, fin rays (dorsal, anal, pectoral, caudal and ventral fin rays) and parts of dissected asteriscus after 60 days in culture. All treatment concentrations had good marks on otoliths. Violet marks were visible under normal light after marking with 200–400 mg/l ARS and 300 mg/l AC. Scales and fin rays showed acceptable fluorescent marks at higher concentrations (400 mg/l ARS, 300 mg/l AC and 250–400 mg/l ARS, 300 mg/l AC, respectively). The best results were obtained after marking with ARS at 400 mg/l and AC at 300 mg/l. There was no significant difference on survival and growth of marked fish relative to controls throughout experiment ($P > 0.05$).

1. Introduction

Japanese flounder *Paralichthys olivaceus* is one of the most important commercial species along the coastal areas of China, southwest of the Korean peninsula and Japan (Zhu et al., 1963; Cheng and Zheng, 1987). Japanese flounder has declined significantly in recent years due to overexploitation. Since 1996, a hatchery-produced juvenile release program has been implemented in Shandong province. In 2007 alone, approximately, 9 million *P. olivaceus* juveniles were released into Shandong inshore waters. The success of this mass-releasing program of stock enhancement remains to be evaluated. To assess the effectiveness of the stock enhancement strategies for fisheries management (Brown et al., 2002; Taylor et al., 2005; Baer and Rösch, 2008), marking and recapture programs need to be carried out. Various methods have been developed to mark fish at different stages of the life cycle. However, fish in their early stage are too small to be marked with external marks (e.g. T-bar, plate disc and fin clipping) or internal tags (e.g. passive integrated transponder, PIT). Additionally, individual handling could involve large effort. Genetic tags are not suitable for recapture process due to high operating costs. Considering metamorphosis during the early stage of development and depressiform body structure of *P. olivaceus*, using coded wire tag seems difficult. Furthermore, thermal marking has been widely used for marking hatchery-produced salmons (e.g. Hagen et al., 1995; Volk et al., 1999) due to long period of egg incubation. However, *P. olivaceus* has relatively short incubation period when it could be thermally marked. Therefore, as far as authors know, chemical marks as internal–external marks are the most suitable tools for evaluation of large-scale stocking of small juveniles in contrast to other tagging methods (Brown et al., 2002; Simon and Dörner, 2005; Baer and Rösch, 2008). This method must be suitable for application to large numbers of small fish, handling stress should be kept at a minimum, markers should be retained for several months, marking process and recovery should be easy to carry out (Skov et al., 2001).

Fluorochrome labeling dyes can produce detectable marks in otoliths, scales and other bony structures based on differential staining of chemical dyes. Various techniques for introducing marker have been investigated both in teleosts and elasmobranchs (Gelsleichter et al., 1997). The choice of technique depends on life history stage, environment (marine or fresh water) and experiment condition (Lagardère et al., 2000), for example, injection (Monaghan, 1993), dietary intake (Honeyfield et al., 2006) and immersion.

Chemical marks mainly include: oxytetracycline (OTC) hydrochloride, calcein, alizarin red S (ARS) and alizarin complexone (AC). In recent years, alizarin red S (ARS) and alizarin complexone (AC) have been substituted for the other chemical dyes to produce clearly readable marks, causing less negative

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effect on survival and without availability limitation such as OTC in increasing salinity (Taylor et al., 2005) (e.g. Tsukamoto, 1988; Tsukamoto et al., 1989; Walt and Faragher, 2003; Baer and Rösch, 2008).

However, no controlled laboratory experiment on the mortality and mark retention induced by AC and ARS has been conducted during relatively long periods on *P. olivaceus,* although Yamashita et al. (1994) used fixed 80 mg/l AC for 24 h in field experiment. There was little detail on evaluation of AC and without reference to ARS on this species. Therefore, the aims of this study were to evaluate growth, mortality and mark quality in otoliths, scales and fin rays of *P. olivaceus* marked with AC and ARS.

2. Methods

2.1. Experimental fish

Juveniles (20–30 mm total length) were transported from a commercial hatchery company located at Wendeng City and kept in a 2 m^3 recirculatory fiberglass tank for 27 days (1.4 individuals/l). During this acclimation period, fish were fed twice daily with commercial pellets (Shengsuo, Shandong, China) to satiation. Water quality was constantly monitored throughout the holding period and maintained (temperature, $20 \pm 0.5\,^{\circ}C$; salinity, 31.0 ± 1.0; dissolved oxygen (DO), 5.26–5.32 mg/l; pH, 7.6–8.0; photoperiod, 14 L/10 D). Water was exchanged at the daily rate of 15% tank volume.

2.2. Immersion marking

ARS and AC were dissolved in distilled water at the concentrations of 4000 mg/l ARS and 1000 mg/l AC, respectively as stock solution with salinity adjusted to 31. Then treatment concentrations of 0, 200, 250, 300, 400 mg/l ARS were prepared and water aerated strongly in order to raise pH. The fish were starved for 24 h before immersion. About 70 sixty-day-old *P. olivaceus* was randomly allocated to each treatment at a density of 3.5 individuals/l in ARS solution (20 L volume) for 24 h. Fish immersed in seawater without ARS served as control group. They were kept covered in dark environment to avoid stress throughout the immersion period. Following completion of the immersion period, juveniles were retrieved from the dye solutions, rinsed and then transferred to separate containers with fresh seawater for 4 h to completely wash away any remnant of dye. Lastly, fish were transferred into five aquaria for 72 h to determine the degree of acute mortality caused by ARS. PH was checked regularly during the immersion period in order to maintain a tolerable condition (7.23–7.86).

The same procedure was followed in the application of AC. Fish were immersed in AC of concentrations 0, 50, 100, 150, 200 and 300 mg/l for 24 h. Fish treated in seawater without AC served as control group. For each solution, about 40 *P. olivaceus* at a density of 4 individuals/l was immersed in AC (10 L volume). Fish acute mortality due to AC was determined 72 h post-immersion.

2.3. Growth experiment

To examine the effects of ARS and AC on survival and growing condition following immersion, 60 and 33 juveniles, respectively, were randomly selected per treatment concentration. For ARS marking, each treatment consisted of three replicates of 20 individuals (0.22 individuals/l) in a recirculatory system comprising 184 L storage aquarium and 8 titanium alloy columniform tanks (D:H = 65 cm × 64 cm). The tanks were continuously aerated and constant water quality parameters maintained (DO, 5.26–5.32 mg/l; salinity, 31.0 ± 1.0; photoperiod, 14 L/10 D; ammonia-N, 0.10–0.20 mg/l; pH, 7.6–8.0; temperature, $20 \pm 0.5\,^{\circ}C$). Water was exchanged at the daily rate of 15% tank volume and recirculated with composite sand filter, foam filter, biochemical filter balls and ultraviolet sterilization.

Fish treated with AC were held in another recirculatory system, which consisted of 18 glass aquaria ($L \times W \times H$ = 35 cm × 25 cm × 33 cm) equipped with filter bed and biochemical filter balls. Water volume was about 180 L. 33 *P. olivaceus* juveniles were randomly selected from each treatment concentration. This was then replicated three times consisting of 11 individuals (0.34 individuals/l) per sub-treatment and kept in glass aquarium. Water quality was maintained daily as described for ARS treatments. Water was exchanged at the daily rate of 30% tank volume.

At the beginning of the growth experiments, all fish were starved for 24 h before total length and wet mass were measured to the nearest 0.1 mm and 0.01 g, respectively. Fish were measured from different batches of AC and ARS on 20th, 40th and 60th day to assess the extended effect of chemical marks on growth.

2.4. Sampling and mark analyses

The sagittae and some scales (ctenoid scale from backside, cycloid scale from abdomen) of all fish were removed and 2 individuals per sub-treatment (6 individuals per concentration) were taken randomly for fin rays (dorsal, anal, pectoral, caudal and ventral fin ray) dissection at the base of the pterygiophores after 60 days post-marking. Parts of asteriscus were also dissected. They were all freed from adherent tissues and rinsed with water. All samples were checked directly without resin and polishing (e.g. Champigneulle and Cachera, 2003). To prevent fading of fluorescent marks, they were stored in sample envelopes avoiding high temperature and direct sunshine (Bashey, 2004).

Marks were observed with an epifluorescence microscope (OLYMPUS BX51) equipped with a 10× objective lens and fitted with an Olympus DP70 high resolution digital camera (Table 1) and Stereoscopic Zoom Microscope (Nikon SMZ800) equipped with a 1× objective lens. Mark quality was assessed using a scale of 0–5 (0, no mark visible under fluorescence microscope; 1, poor mark under fluorescence microscope; 2, mark easily visible under fluorescence microscope; 3, mark shining brightly under fluorescence microscope; 4, mark visible in transmitted light; 5, mark distinct in transmitted light). Mark was identified by analyzing both sagittae, dissected parts of asteriscus, at least 20 cycloid and ctenoid scales of all fish and more than 4 pieces of fin rays per position of subsample fish. In general, mark quality on otoliths, scales and fin rays per specimen was analyzed separately by two researchers and where the presence of a mark was doubtful, it was determined by a third researcher. Mark quality ≥ 2 was judged an acceptable good mark as it could be readily detected in the structure (Taylor et al., 2005). Marks with high scores require less time for analyses and can be easily applied during mass-marking programs.

2.5. Data analyses

Analysis of variance (ANOVA) with Turkey's honestly significant difference (HSD) test (SPSS13.0) was used to assess the significance

Table 1
Filter wavelengths for visualizing ARS and AC marks.

Light source	Wavelength (nm)	
	Excitation filter	Barrier filter
WB	490	515
WG	545	590
UV	365	420

Both of AC and ARS marks were detected using same filters.