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Chemical marking of catfishes juveniles with alizarin red S

ANA ROSA SANT´ANNA DE SÁ, SAMARA HERMES-SILVA & EVOY ZANIBONI-FILHO*
Universidade Federal de Santa Catarina, Laboratório de Biologia e Cultivo de Peixes de Água Doce, Rod. Francisco Thomaz dos Santos, 3532, 88066-260, Florianópolis, SC, Brazil.

*Corresponding author: evoy@lapad.ufsc.br

Abstract. The need for a proper technique for the mass-marking of fish of a variety of sizes and species is a constant challenge for managers who wish to evaluate fish stocking actions. The aim of this study was to define the procedures for the chemical marking of catfishes using alizarin red S (ARS). Experiments were conducted with juveniles of silver catfish *Rhamdia quelen*. Three concentrations of ARS (30, 60 and 90 mg/L) were tested in long-duration immersion baths of 18 hours divided into two groups: a) with a prior osmotic saline induction for 10 minutes and b) without osmotic induction. Additionally, three concentrations of ARS (90, 300 and 700 mg/L) were tested in short-duration immersion baths of 10 minutes, all of them with prior osmotic induction. All treatments showed the chemical marking after three months, but only treatments of 300 and 700 mg/L showed significant differences in brightness intensity. The ARS proved to be an efficient fluorochrome dye for marking catfishes.

Key words: fish stocking, fluorochrome dye, immersion bath, mark intensity, mass-marking, osmotic induction.

Resumo: Marcação química de bagres juvenis com alizarina vermelha S. A necessidade de uma técnica adequada para marcação de grandes lotes de peixes em uma variedade de tamanhos e espécies é um desafio constante para gestores que desejam avaliar ações de estocagens. O objetivo deste estudo foi definir os procedimentos de marcação química com Alizarina Vermelha para siluriformes. Os experimentos foram conduzidos com juvenis de jundiá *Rhamdia quelen*. Foram testadas três concentrações de Alizarina Vermelha (30, 60 e 90mg/L) em banhos de imersão de longa duração de 18 horas, divididos em dois grupos: a) com prévia indução osmótica salina por 10 minutos; e b) sem indução osmótica. Adicionalmente, três concentrações de Alizarina Vermelha (90, 300 e 700mg/L) foram testadas em banhos de imersão de curta duração de 10 minutos, todos com prévia indução osmótica salina. Todos os tratamentos apresentaram a marcação química após três meses dos banhos de imersão, mas apenas as concentrações de 300 e 700 mg/L apresentaram diferença significativa quanto a intensidade das marcas. A Alizarina Vermelha apresentou-se como um eficiente marcador químico para siluriformes.

Palavras-chave: banhos de imersão, corante fluorocromo, estocagem de peixes, indução osmótica, intensidade de marcação, marcação massiva.

Introduction

The need for a proper technique for the mass-marking of fish of different species, especially during the initial phases of development (at larval and juvenile sizes), is a constant challenge for managers who wish to evaluate fish stocking actions (Negus & Tureson 2004, Skalki *et al.* 2009).

Several types of internal and external marks have been used for evaluating the success of fish stocking practices, such as Passive Integrated Transponder (PIT), Visible Implant Elastomers (VIE), Coded Wire Tag (CWT), and others (Skalki *et al.* 2009). Each one of them, however, presents its limitations or problems. Despite the easy visualization of these external marks, one of their

main problems is the risk of loss, which ends up harming the proper evaluation of the results of these actions. Additionally, both external marks and some types of internal marks require individual manipulation for marking or are efficient only for a certain size of fish, making the marking process very laborious or inefficient in the case of small size fish.

Alternatively, the use of fluorochrome dyes can be a practical option for mass-marking procedures (i.e., the use of chemical compounds that bind to calcified tissues) to produce detectable marks in otoliths, scales and other calcified structures that fluoresce when exposed to ultraviolet light (Fielder 2002, Molher 2003, Liu *et al.* 2009). Many of these fluorochrome dyes have been tested on fish, such as oxytetracycline (Taylor *et al.* 2005, Abreu *et al.* 2014), alizarin (Campanella *et al.* 2013, Hermes-Silva *et al.* 2016), and calcein (Logsdon & Pittman 2012), which can be administered by injection, food supplementation or immersion (Morales-Nin *et al.* 2011). Among these methods, the use of chemical immersion is an interesting option to avoid fish stress and mortality, diminishing fish handling and permitting the marking of a large number of small fish simultaneously (Nielsen 1992).

Many studies have used alizarin as a fluorochrome dye in long-duration immersion baths (Ibánez *et al.* 2013, Liu *et al.* 2009, Lagardère 2000) or in short-duration immersion baths with an osmotic induction prior to the chemical treatment (Crook *et al.* 2007, Campanella *et al.* 2013, Hermes-Silva *et al.* 2016), and intense marks have been observed in otoliths, caudal fins and pectoral spines of *Oreochromis niloticus*, *Paralichthys olivaceus*, *Scophthalmus maximus*, *Leporinus obtusidens, Odontesthes bonariensis,* and *Macquaria ambigua*.

Although several studies have been carried out with chemical marking, few have been developed using the native species of the Brazilian watersheds (Campanella *et al.* 2013, Abreu *et al.* 2014, Hermes-Silva *et al.* 2016) and even fewer with Siluriformes (Stewart & Long 2011). Considering that the pattern commonly observed in neotropical watersheds is a greater abundance of the Characiformes and Siluriformes Orders (Lowe-McConnel 1999) and considering the importance of fishing management to different fish species, including Siluriformes, the aim of this study was to define the chemical marking procedures with alizarin red S (ARS) for this group of fish, offering the option of the efficient use of different concentrations and immersion times.

Methods

The Siluriformes species selected for this study was the silver catfish *Rhamdia quelen* (Quoy, Gaimard, 1824), a Heptapteridae species widely cultivated in southern Brazil, Argentina and Uruguay and thus with well-controlled juvenile production. The experiment was conducted with catfish juveniles that were hatched indoors through the artificial reproduction of wild fish from the Upper Uruguay River. Acclimatization of juveniles was done in 28 - 20-L recirculating system tanks that were lightly salinized (2.5 ppt of NaCl), and the fish were fed three times a day with *Artemia* sp. nauplii and commercial feed until they grew to approximately 4 cm in total length.

Marking procedures were performed with alizarin red S ($C_{14}H_7NaO_7S$) from Sigma-Aldrich Brazil Ltda. (São Paulo, SP, Brazil) in immersion baths with 9 g/L of fish biomass per experimental unit.

Two marking procedures were used: long-duration immersion baths of 18 hours (18H) and short-duration immersion baths of ten minutes (10 min). For the 18H baths, three ARS concentrations (30, 60 and 90 mg ARS/L) were tested, each one of them with a previous osmotic induction (OI) in a 30 ppt saline solution (NaCl) for ten minutes and without this previous osmotic induction (no OI). For the 10 min baths, other three ARS concentrations (90, 300 and 700 mg ARS/L) were tested, all of them with a previous osmotic induction. All tests were performed with three repetitions, and an extra tank with fish that have not undergone any ARS bath was maintained as a control to assess the occurrence of autofluorescence.

During treatment, fish were fasted and maintained with aeration but without water exchange, and some water quality parameters (temperature, pH and dissolved oxygen) were monitored during the initial hours to guarantee appropriate conditions for the species. To determine the saline solution for the osmotic induction, some preliminary tests were performed increasing the concentration of NaCl up to 30 g/L (30 ppt), in 10 min immersion baths, to ensure total survival of fish during treatments. After the marking procedures, fish from all treatments were released back to the 20-L recirculating system tanks and fed to satiation twice daily.

The presence of ARS marks was confirmed one week after treatment, and the permanence of marks was evaluated after three months. Nine fish per treatment were analyzed (three per tank).

Preparation of glass slides followed the protocol described by Abreu *et al.* (2014), but pieces of caudal fin rays were used instead of scales.

For mark detection, slides were examined with a fluorescence microscope LEICA DMI6000B with UV filter between 405 and 660 nm and under 50× magnification in the Central Laboratory of Electron Microscopy of the Federal University of Santa Catarina (LCME-UFSC). Images were obtained using the LEICA LAS AF software lite with the same magnification and time of exposure. The images were evaluated based on brightness intensity of the pixels within a selected area on a scale from 0 (black) to 255 (white). Selected areas of each image were the same size and were the ones with the highest fluorescence intensity.

Analysis of variance (one-way ANOVA) was applied to evaluate differences in brightness intensity between treatments of 18H baths, between treatments of 10 min baths, and between the two treatments using 90 mg ARS/L, followed by the Tukey test when necessary. The analyses were performed using the STATISTICA 7.0 program and considering $P<0.05$ as the significance level.

Results

No mortality was observed during marking procedures and water quality parameters were maintained at the ideal levels for the species, as observed in Table I.

After one week, the presence of ARS marks was observed in all treatments (Fig. 1). At 18H immersion baths, treatments of 90 and 60 mg/L, both with a prior osmotic induction, presented significantly brighter marks than other treatments ($P<0.05$) (Fig. 2A). At 10 min immersion baths, treatments of 300 and 700 mg/L showed significantly brighter marks than the treatment of 90 mg/L ($P<0.05$) (Fig. 2B). When comparing treatments of 90 mg/L, the use of long-duration immersion baths of 18H with a prior osmotic induction produced brighter marks than the other treatments ($P<0.05$) (Fig. 2C). No fluorescent mark was observed in fish from the control treatment.

After three months post-treatment, clear marks were still present in all treatments (Fig. 3). However, no significant difference in brightness intensity ($P>0.05$) was observed between treatments of 18H immersion baths (Fig. 4A) and between treatments of 90 mg/L (Fig. 4C). For the 10 min immersion baths, treatments of 300 and 700 mg/L continued to show significantly brighter marks than the treatment of 90 mg/L ($P<0.05$) (Fig. 4B).

Discussion

The use of ARS in immersion baths proved to be effective for the chemical marking of Siluriformes, and bright marks were produced with the use of 300 and 700 mg of ARS/L with a previous osmotic induction.

Several studies have been developed to evaluate chemical marking procedures for fish, but the vast majority of these studies have focused on the evaluation of the presence of chemical marks in otoliths and scales. While the first is considered a destructive method that requires the sacrifice of the fish, besides requiring additional time to prepare the otoliths for observation, which are factors that hinder its use and prevent the continuous revaluation of these marks; the second method cannot be used in catfishes due to the obvious absence of these structures in Siluriformes.

Stewart & Long (2011) evaluated chemical marking with oxytetracycline (OTC) in *Ictalurus punctatus* on otoliths and pectoral spines, and although they obtained visible marks with the concentration of 700 mg/L, they reported some concern due to the erosion of pectoral spine annuli, which may hinder OTC marks and, therefore, undermine an adequate assessment.

No study at the present time has reported such problems with the use of ARS. In addition, many studies mentioned the success obtained with this fluorochrome dye, observing marks in fin rays even after eight months or more post-treatment (Eckmann 2003, Crock *et al.* 2007, Campanella *et al.* 2013, Hermes-Silva *et al.* 2016). In the present study, clear and bright ARS marks were visible after three months post-treatment. Besides evaluating these

Table I. Average of water quality parameters observed during immersion baths with alizarin red S.

Immersion baths	Temperature (°C)	Salinity (ppt)	Hardness (mg $CaCO_3$/L)	pH	Dissolved Oxygen (mg/L)
Long-duration (18H)	24.8	0.24	197	7.60	6.51
Short-duration (10 min)	26.4	0.26	197	7.02	7.37

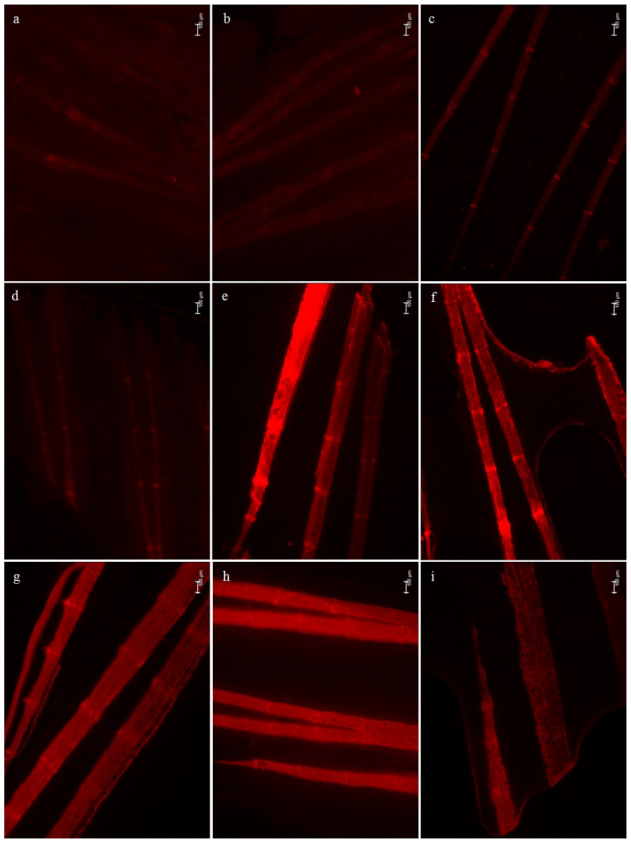

Figure 1. Images of fluorescent marks in *Rhamdia quelen* caudal fins rays one week after immersion baths in alizarin red S. Long-duration immersion bath (18H) treatments: 30 mg no-OI (a), 60 mg no-OI (b), 90 mg no-OI (c), 30 mg OI (d), 60 mg OI (e), and 90 mg OI (f); and short-duration immersion bath treatments: 300 mg OI (g), 700 mg OI (h), and 90 mg OI (i).

marks without the need for sacrificing fish, it was possible to notice that at the first evaluation (1-week post-treatment) marks were located close to the edge of the caudal fin rays; and after three months, the marks were located farther from the edge of the fins, showing that fish growth occurring during this period did not prevent the visualization of the marks.

Despite the variation observed in brightness intensity between the treatments, the presence of ARS marks was confirmed in all the treatments at both the 1-week or 3-month post-treatment evaluations. These results show that ARS can be used for the chemical marking of Siluriformes in immersion baths, as was observed for other scaled fish species (Liu *et al.* 2009, Crook *et al.* 2007, Campanella *et al.* 2013, Hermes-Silva *et al.* 2016), confirming its potential use in evaluating the success of stocking programs also for Siluriformes.

Although the use of osmotic induction prior to the ARS immersion bath resulted in brighter marks after 1-week post-treatment, this result was not

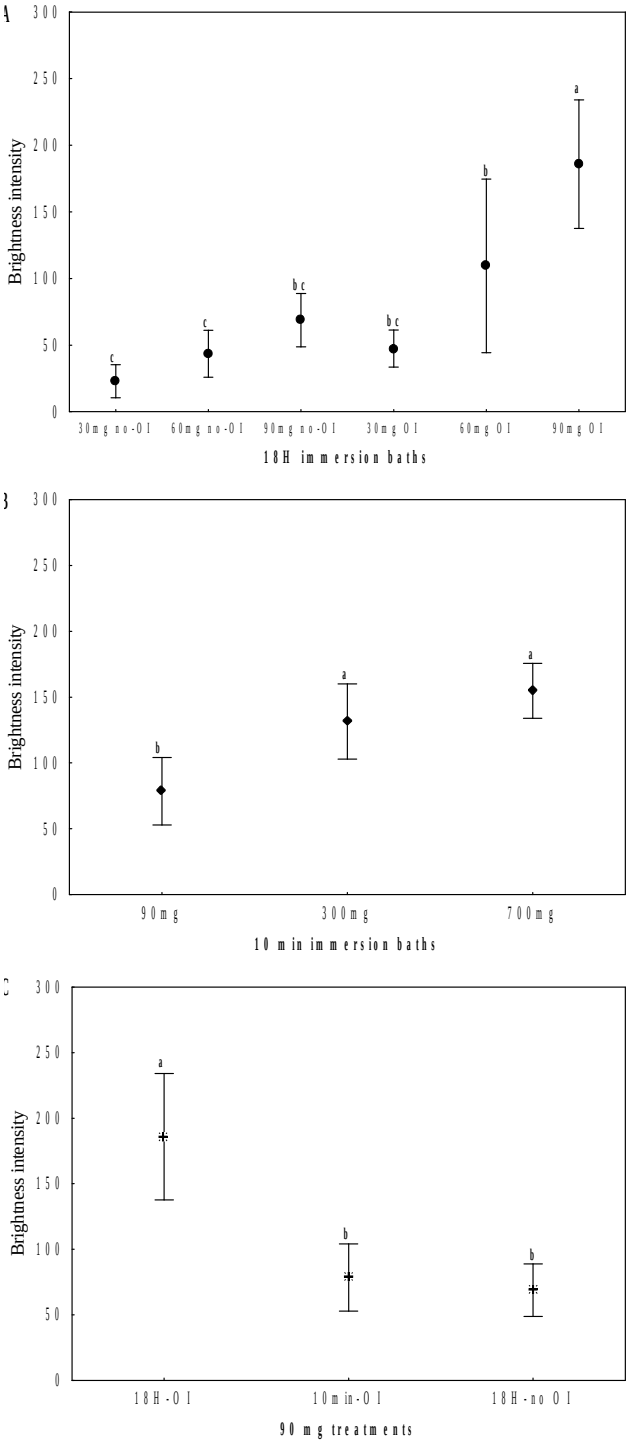

Figure 2. Fluorescent brightness of *Rhamdia quelen* caudal fins rays after one week of immersion baths in alizarin red S: (A) Long-duration immersion baths (18H), (B) Short-duration immersion baths (10 min), and (C) Treatments of 90 mg/L. Different letters indicate significant differences (P<0.05).

confirmed after 3 months. Mohler (2003) suggested that submitting fish to short immersion baths in a saline solution before immersion in the

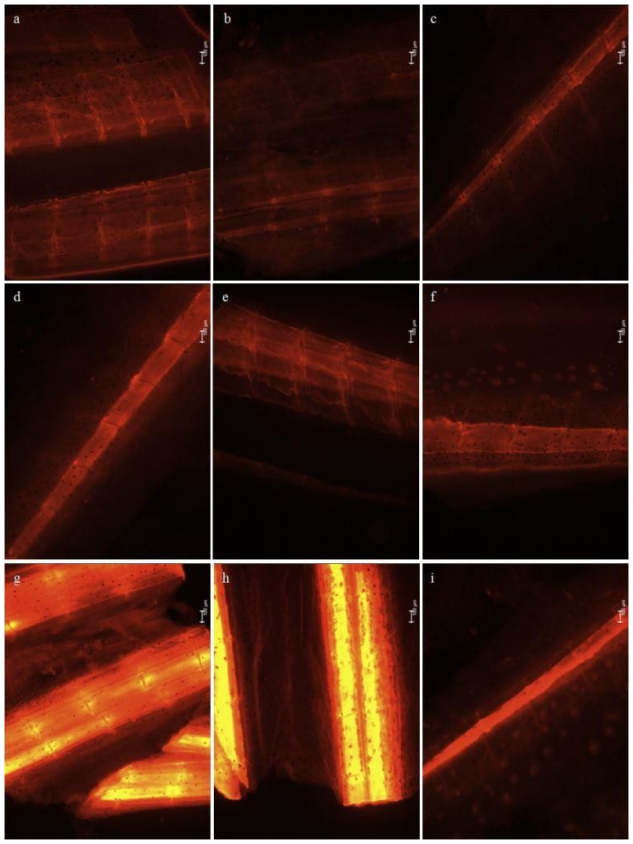

Figure 3. Images of fluorescent marks in *Rhamdia quelen* caudal fins rays three months after immersion baths in alizarin red S. Long-duration immersion bath (18H) treatments: 30 mg no-OI (a), 60 mg no-OI (b), 90 mg no-OI (c), 30 mg OI (d), 60 mg OI (e), and 90 mg OI (f); and short-duration immersion bath treatments: 300 mg OI (g), 700 mg OI (h), and 90 mg OI (i).

fluorochrome solution would decrease absorption time of the dye, but this may not be true for longer immersion baths such as the 18H treatment used in this study. Even if the absorption of the dye is increased in the first minutes of contact, as the fish continues to be in contact with the dye for a longer time it may produce similar marks after the total period of the treatment.

Hermes-Silva *et al.* (2016), without evaluating brightness intensity, observed that low concentrations of alizarin resulted in lighter and more unequal marks, especially in treatments without previous osmotic induction. This irregularity in fish marking was also mentioned in other chemical marking studies (Campanella *et al.* 2013, Ibáñez *et al.* 2013), even when using more concentrated dosages such as 1 g/L (Campanella *et al.* 2013). As a piece of the caudal fin is removed from each fish for evaluation, and in this study, marks were not visible with the naked-eye, we suspected that the same problem of unequal marks

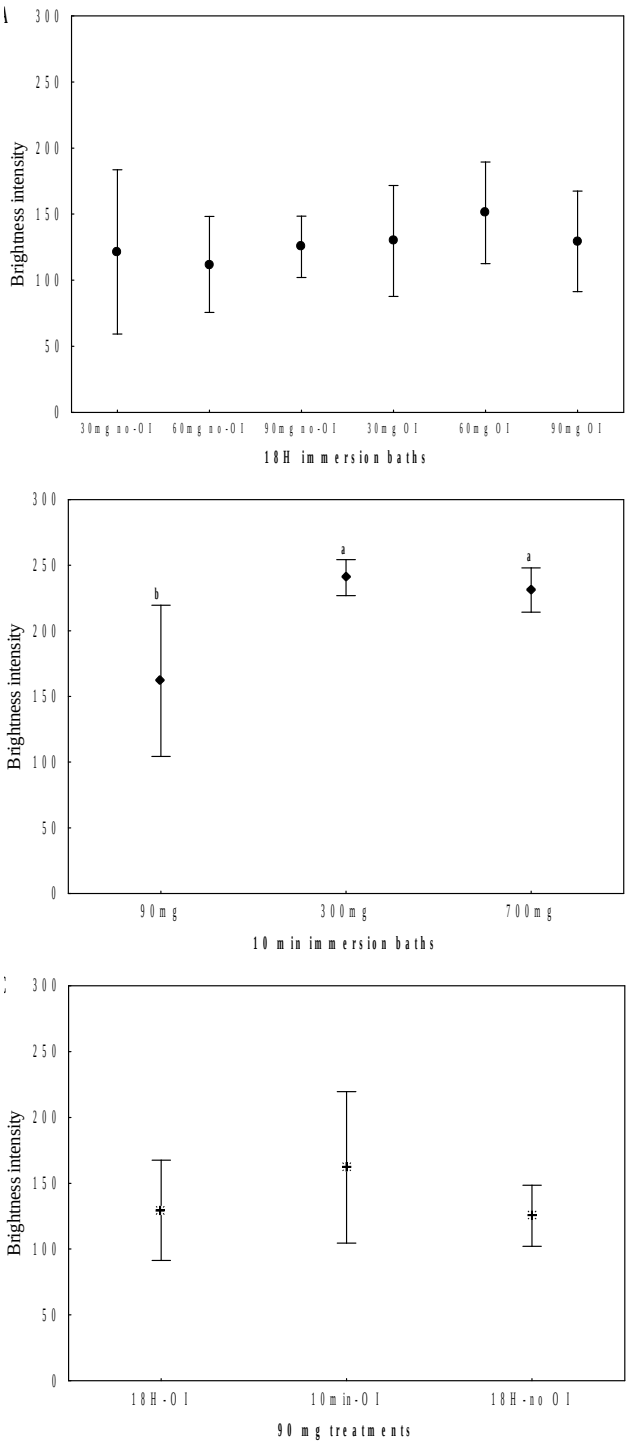

Figure 4. Fluorescent brightness of *Rhamdia quelen* caudal fins rays three months after immersion baths in alizarin red S: (A) Long-duration immersion baths (18H), (B) Short-duration immersion baths (10 min), and (C) Treatments of 90 mg/L. Different letters indicate significant differences ($P<0.05$).

that was observed by Hermes-Silva *et al.* (2016) with *Leporinus obtusidens* also occurred at the low concentration treatments with *R. quelen*. In addition,

because of this, only treatments using 300 and 700 mg/L resulted in significant differences in brightness intensity.

The potential use of ARS as a chemical marker in immersion baths for Siluriformes was presented in this study. However, future research is needed to evaluate the quality of these marks in fish exposed to sunlight and the homogeneity of the marks produced by different marking methods. In this way, the results observed in this study with *R. quelen* suggest the use of short-duration immersion baths of 10 minutes in an ARS solution with a previous osmotic induction of 10 minutes in a 30 ppt saline solution.

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