A Comparison of Three Techniques for Fluorochrome Marking of Juvenile Clarias gariepinus Otoliths

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Source: African Zoology, 46(1) : 72-77
Published By: Zoological Society of Southern Africa
URL: https://doi.org/10.3377/004.046.0119

A comparison of three techniques for fluorochrome marking of juvenile *Clarias gariepinus* otoliths

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Received 5 November 2010. Accepted 20 January 2011

Intramuscular injection of the antibiotic oxytetracycline (OTC) has been the only method previously employed for chemically marking *C. gariepinus* otoliths for ageing studies. This study compared intramuscular injection, immersion, and dietary incorporation methods of administering OTC to determine the most effective technique. No differences in either growth or mortality were found between experimental groups while intramuscular injection of OTC was found to be superior to either mass immersion or dietary inclusion of OTC when marking *Clarias gariepinus* otoliths.

Key words: African sharptooth catfish, oxytetracycline, fluorochrome marking.

INTRODUCTION

African sharptooth catfish, *Clarias gariepinus* (Burchell 1822), is widely distributed with a natural range that extends from southern Turkey to the Orange River, South Africa (Skelton 2001). In addition to translocations within its southerly range (Cambray 2003), Cambray (2005) noted that as a result of poor aquaculture practices and introductions from a number of unknown sources, *C. gariepinus* has now invaded South America, Europe, Asia, and Australia. Its life history characteristics include a fast growth rate to a maximum length of 1300 mm total length (TL) (Bruton 1976), a high fecundity, an omnivorous diet and the ability to breathe air (de Moor & Bruton 1988; Cambray 2003). Understanding the biology and population dynamics of this invader would assist in its management and possibly eradication.

Information on the growth, maturity, and mortality of *C. gariepinus* are all dependent on precise and accurate age estimates. As with other teleosts, age in *C. gariepinus* can be determined by counting growth increments on fish hard structures such as vertebrae (Pivnicka 1974; Willoughby & Tweddle 1978), pectoral spines (Clay 1982; Quick & Bruton 1984) and otoliths (Potts *et al.* 2008; Richardson *et al.* 2009). Determining the frequency of growth zone deposition is crucial if accurate estimates of age are to be determined (Campana 2001).

Mark–recapture of chemically tagged fishes is considered one of the most reliable methods for validating growth increment deposition rates (Campana 2001). This method relies on marking a hard structure, such as the otolith, using a calcium-chelating fluorescing chemical marker, that forms a visible mark on the otolith at the time of tagging. Later, growth zone deposition rates can be directly determined by correlating the time at liberty after tagging with the number of growth zones formed on the otolith distal to the chemical mark (Fig. 1). Choosing an appropriate marking method is therefore a crucial component of any age validation study.

Fluorescing chemical markers, known as fluorochrome markers, such as oxytetracycline (OTC) (Babaluk & Craig 1990; Simon *et al.* 2009) and alizarin red S (Blom *et al.* 1994; Beckman & Schulz 1996) have been used to mark fish hard parts not only by injection (Lang & Buxton 1983; Weyl & Booth 2008) but also by immersion (Simon *et al.* 2009; Hendricks *et al.* 1991), and dietary inclusion (Hendricks *et al.* 1991; Honeyfield *et al.* 2006).

In the past, direct injection has been the only method that has been examined to determine growth zone periodicity of *C. gariepinus* populations directly (Weyl & Booth 2008). Unfortunately this technique is costly, too time-consuming to mass mark large numbers of fish, and is often not suitable for small fishes because of the potential physical damage caused by injection and handling. As a result, only the deposition rate of growth zones

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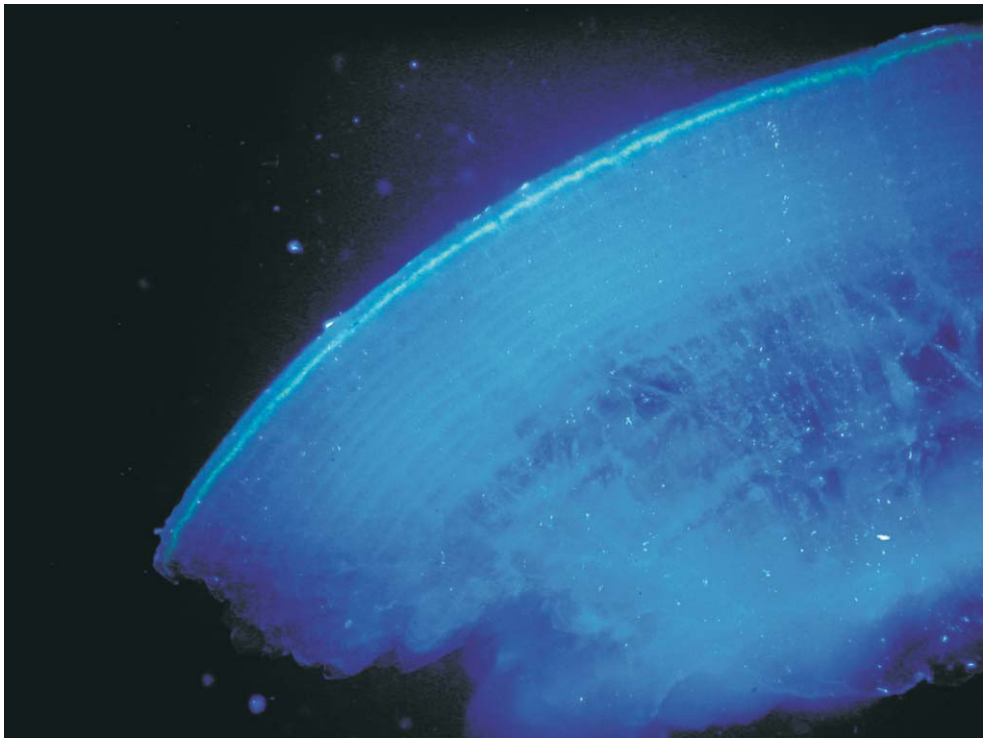

Fig. 1. Photomicrograph of a sectioned adult *Clarias gariepinus* sagittal otolith injected with oxytetracycline (OTC) prior to recapture and viewed under transmitted fluorescent light. The annulli are visible as alternating optically translucent and opaque zones. The chelated OTC is clearly visible as fluorescent mark offset from the margin of the otolith.

in otoliths of adult *C. gariepinus* have been validated.

For *C. gariepinus* it has not yet been determined whether mass immersion or dietary inclusion are, perhaps, as effective, or even more efficient, methods of marking *C. gariepinus* hard structures. Immersion or dietary inclusion could provide for a straightforward application of fluorochrome markers, particularly in smaller juveniles where injection methods can be tedious, for absolute age validation and determination of the first growth increment via mark recapture of young-of-the-year fish. This study aims to determine the most practical technique for marking the otoliths of juvenile *C. gariepinus* with OTC by comparing injection, immersion and dietary inclusion methods. Three treatment groups were compared to a control group in terms of growth, mortality, and the proportion of otoliths exhibiting an OTC mark.

MATERIALS & METHODS

A total of 108 artificially-spawned and hatchery-reared young-of-the-year juvenile *C. gariepinus* (180–250 mm TL) were acclimatized for two months in a 1.35 m^3 flow-through outdoor pond system. Water was filtered in an adjacent 5 m^3 gravel filter. Throughout the study fish were fed twice daily to satiation on 3-mm trout pellets (Nutri-Science®). Water temperature fluctuated with ambient air temperatures and ranged between 16 and 21°C.

At the start of the experiment, fish (215 mm–304 mm TL) were randomly sorted into four treatments each comprising 27 fish. The experimental treatments included an injection treatment, a feeding treatment, and immersion treatment and a control treatment. Prior to the administration of the treatment, each fish was anaesthetized in 0.2 ml/l 2-phenoxyethanol, measured, and tagged using Hallprint® (Victor Harbour, South Australia) T-bar anchor tags (model TBA-2). All fish were treated on the same day to ensure that all fish were exposed to the same environmental conditions. Water temperature over this period was 19°C. All fish in the injection treatment were injected with commercially available veterinary-grade OTC (HiTet; Bayer, Leverkusen, Germany). A 0.15 ml

Table 1. Number of fish used per treatment, the number that survived the experiment, the number of surviving fish with otoliths that displayed a fluorescent oxytetracycline (OTC) mark, and the number of surviving fish with otoliths that exhibited autofluorescence on the margin.

Treatment group	Initial	Survived	OTC mark visible	Autofluorescent margin
Control	27	20	0	10
Injection	27	22	19	9
Feeding	27	19	1	10
Immersion	27	25	1	14
Total	108	86	21	43

OTC solution (0.075 ml HiTet diluted in 0.075 ml distilled water) based on Weyl & Booth's (2008) recommended dosage of 60 mg/kg, was injected into the dorsal musculature using an insulin syringe. Immersion treatment fish were immersed in 500 mg/l OTC for six hours, as advocated by Jenkins *et al.* (2002), in 50-l tanks within a darkened room. Fish of the feeding treatment were placed into an adjacent outdoor housing pond where they were fed OTC-impregnated 3 mm commercially manufactured trout pellets (150 mg/kg fish at 3% mean body weight) during a single treatment event. Feeding was carried out at dusk to prevent the degeneration of the OTC due to direct sunlight exposure. Trout pellets were formulated by mixing the water-soluble OTC hydrochloride powder into 10 ml of sunflower oil (Flora®), which was then thoroughly mixed into the pellets. The control treatment fish underwent no further treatment after anaesthesia and tagging. After anaesthesia, tagging and the administration of the respective experimental treatment, all fish were returned to the outdoor housing pond. A rapid drop in ambient air temperature and concomitant decrease in water temperature from 16°C to 11°C over two days caused considerable mortality and resulted in the cessation of the experiment on day 42.

At the termination of the experiment all fish were measured (237–308 mm TL) and sacrificed by pithing. Otoliths were removed and stored dry in a darkened room. Otoliths were then embedded in clear casting resin, sectioned transversely through the nucleus at a thickness of 0.2 mm and mounted on glass microscope slides using DPX mountant. Each section was assigned a positive integer, and a random number generator was used to determine the order of examination. All sections were examined without reference to fish size or treatment type. To test whether each treatment resulted in a visible fluorescent mark, the sections were examined for fluorescent marks with a compound microscope under blue fluorescent light (460–490 nm).

Differences in mortality and the presence of fluorochrome marks between treatments was assessed using χ^2 tests, and an ANOVA was used to test for differences in specific-growth rate between treatments. Specific growth rate (SGR) for each individual fish measured at the start, TL_S, and end, TL_E, of the experiment was calculated as $SGR = \ln(TL_E) - \ln(TL_S))/42$

RESULTS & DISCUSSION

Of the 108 initial fish treated, 80% survived to day 42 (Table 1). There was no significant difference in mortality between treatments ($\chi^2 = 3.31$, d.f. = 3, $P > 0.05$) that resulted from the onset of a secondary fungal infection initiating at the tag insertion point ($n = 18$) and cannibalism ($n = 4$). The otoliths from all surviving fish ($n = 86$) removed for later analysis.

Mean change in length for each of the experimental groups was 3.85 ± 3.03 mm TL for the control, 4.14 ± 3.29 mm TL for the injection treatment, 6.05 ± 3.50 mm TL for the feeding treatment, and 5.44 ± 3.91 mm TL for the immersion treatment. There was no significant difference in specific-growth rate (ANOVA: $F = 1.71$, d.f. = 3.82, $P > 0.05$) between experimental groups.

Fluorescent marks were distinguishable in the otoliths as strongly fluorescing blue bands distal from the margin on 21 of 86 otoliths examined (Fig. 2). The injection treatment was shown to mark a significantly greater number of otoliths when compared to the other treatments ($\chi^2 = 61.63$, d.f. = 3, $P < 0.01$). The immersion and feeding treatments had one fluorescing otolith each. No otoliths were fluorescently marked in the control treatment (Table 1).

Autofluorescence was noted and positively identified on 43 of the sectioned otoliths (Fig. 2). Jenkins *et al.* (2002) also identified autofluorescence

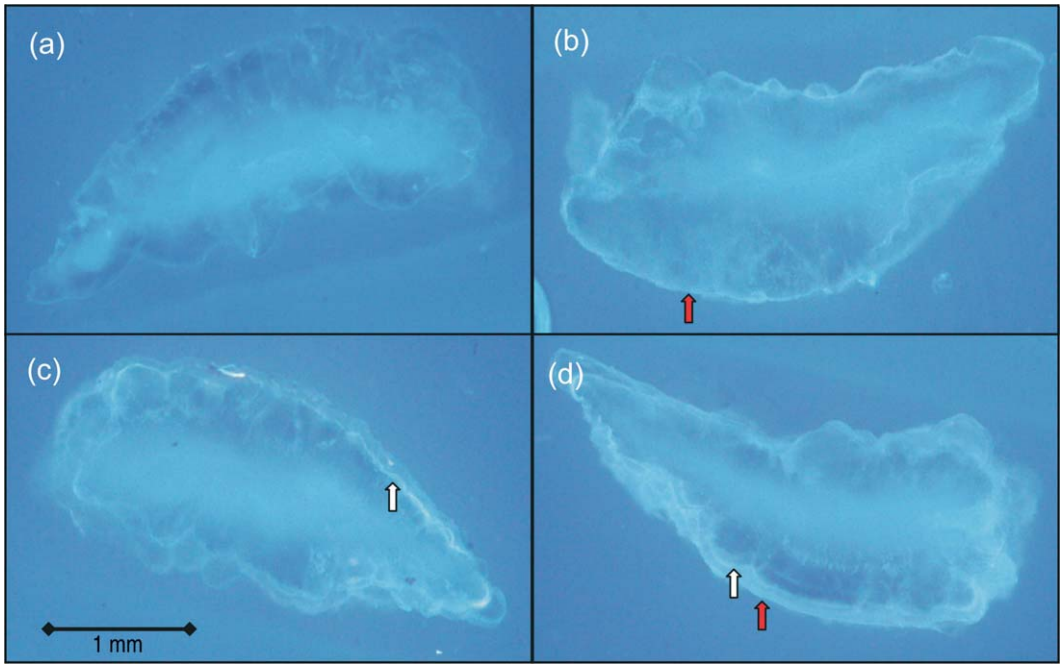

Fig. 2. Composite photomicrograph of juvenile *Claria gariepinus* sagittal otoliths viewed under fluorescent light. **a**, Control group displaying no autofluorescence and no oxytetracycline (OTC) fluorescence. **b**, Control group displaying autofluorescence (red arrow) and no OTC fluorescence. **c**, Injection treatment displaying no autofluorescence and OTC fluorescence (white arrow). **d**, Injection treatment displaying both autofluorescence (red arrow) and OTC fluorescence (white arrow).

in red drum 56 days after immersion and considered it to be troublesome when attempting to identify fluorochrome marks. Autofluorescence can be described as a fluorochrome-like fluorescent band occurring on the margin of the otolith when the otolith is viewed under fluorescent light. This mark is caused by the optical properties of the margin of the otolith when the otolith is mounted on a plane that is at a slightly different angle to that of the slide on which it is mounted. Studies where insufficient otolith growth has occurred to enable a distinction between OTC-fluorescence and marginal-autofluorescence will likely be confounded. Results of the present study however indicate that, for juvenile *C. gariepinus*, 42 days was adequate to allow for the distinction between OTC fluorescence and marginal autofluorescence. There was no significant difference in the occurrence of autofluorescence between treatments ($\chi^2 = 1.14$, d.f. = 3, $P > 0.1$). This random occurrence of autofluorescence, and its independence from the marking technique employed, suggests that this phenomenon should not affect studies validating age by fluorochrome marking of otoliths in juvenile *C. gariepinus* at least 42 days after marking.

The results show that intramuscular injection is the only effective method of incorporating an OTC fluorochrome marker into *C. gariepinus* otoliths. While studies conducted on other species have successfully incorporated OTC using both immersion (Lorson & Mudrak 1987; Simon *et al.* 2009) and dietary inclusion (Hendricks *et al.* 1991; Honeyfield *et al.* 2006), these techniques proved unsuccessful in this experiment.

The reason for only one specimen exhibiting a positively identifiable OTC mark in the mass immersion treatment is likely due to the obligatory air breathing nature of *C. gariepinus.* During the six-hour immersion period, fish were observed gulping air at a much higher frequency than usual, despite adequate aeration being supplied to the treatment tanks. Kaiser (Rhodes University, pers. comm., 2010) noted that when anaesthetizing air-breathing fishes (such as anabantids) by immersion, the time until adequate anaesthetization takes substantially longer than in non air-breathing fish. It is hypothesized that obligatory air breathers will gulp air more frequently when placed in a solution that is perhaps unpleasant to the fish being immersed, thereby avoiding uptake of the anaes-

thetic solution, or, as may have been the case in this study, the OTC solution. Mass immersion in a fluorochrome marker solution is therefore likely not to be a suitable method of age validation for obligatory air breathers such as *C. gariepinus.* Future studies should investigate whether anaesthetized fish can indeed absorb these chemicals.

The lack of a positively identifiable fluorochrome mark in all but one of the dietary inclusion treatment fish could be a result of food palatability. Upon being presented the OTC-impregnated food fish were observed to feed less actively than usual and it is therefore possible that they did not ingest the full 3% of their body weight. Hustvedt *et al.* (1991) noted that including OTC into the diet of rainbow trout (*Oncorhynchus mykiss* (Walbaum 1792)) resulted in a 61% reduction in feed ingestion and it is therefore also possible that the OTC altered the taste of the food, resulting in *C. gariepinus* rejecting any food they may have initially ingested. Pond turbidity (with a water transparency of less than 20 cm) was high such that it was not possible to observe any uneaten food. Alternatively, fish within this treatment were fed at dusk which could have resulted in less active feeding due to the immediate change in feeding time.

CONCLUSION

Despite investigating intramuscular injection, immersion and dietary incorporation as possible methods for administering OTC to juvenile *C. gariepinus,* only intramuscular injection showed successful chelation of a fluorescent mark in the otoliths of juvenile *C. gariepinus.* It is recommended that further research into the optimization of all three methods in terms of OTC concentration, water temperature and the duration and frequency of treatments, be conducted. Given that there was no difference between the injection experimental group and the control group in either mortality or growth in the present study, intramuscular injection is recommended as the most suitable administration method based on these preliminary results.

ACKNOWLEDGEMENTS

This article is based on work supported by the National Research Foundation of South Africa (FA2007043000021). Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto. The authors would like to thank the Eastern Cape Department of Environmental Affairs and Tourism for the research permits (WRO-40/05WR; WRO-13/05WR). The Electron Microscopy Unit, Rhodes University is thanked for use of equipment.

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Responsible Editor: J.H. van Wyk