Longevity of Oxytetracycline and Calcein in Double-marked Batches of Fry and Fingerling Largemouth Bass

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Abstract: We evaluated the ability of oxytetracycline hydrochloride (OTC) and calcein (CAL) to double-mark otoliths in largemouth bass (*Micropterus salmoides*, LMB) fry and fingerlings. To observe longevity of marks, fish were sampled at six-month intervals for two years. Marks on fry otoliths disappeared rapidly regardless of chemical used; most marks were not visible after 180 days. Marks from CAL on fingerling otoliths were short-lived; 50% were not visible after 180 days. Marks from OTC on fingerling otoliths were visible on 100% of treated fish and were retained throughout the two years of study. Immersion-marking with OTC of fingerlings is a simple, effective, and relatively long-lasting technique for mass-marking LMB for various fisheries assessments. We successfully double-marked fingerling LMB with OTC when we imposed a two-week interval between marks. Alternate marks with OTC and CAL were unsuccessful because readers were often unable to distinguish between the OTC and CAL marks.

Key words: oxytetracycline, bass, calcein, mark, fluorescence, recapture, fingerlings, fry

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Biologists frequently mark many fish to evaluate the contributions of stocked fishes to established populations (Vandergoot and Bettoli 2003, Hoffman and Bettoli 2005). Oxytetracycline hydrochloride, alizarin, and calcein, which fluoresce under ultraviolet (UV) light (Hendricks et al. 1991, Secor et al. 1991, Guy et al. 1996) and bind within bony tissues of young fish (particularly otoliths, but also bones and scales), can mark many fish quickly and inexpensively, with high survival (Wilson et al. 1987, Brooks et al.1994, Conover and Sheehan 1996, Mohler 1997). For some studies, it is necessary to differentiate treatment effects using unique marks. Multiple applications of the same chemical can produce multiple marks; however, it may become difficult to distinguish these multiple marks on older fish. Use of different chemicals may provide multiple marks that are more recognizable over time, due to different fluorescent colors. Alternating oxytetracycline hydrochloride (OTC), which fluoresces gold-brown (Wilson et al. 1987, Secor et al. 1991, Conover and Sheehan 1996, Unkenholz et al. 1997), with calcein (CAL), which fluoresces green or apple green (Wilson et al. 1987, Brooks et al. 1994, Mohler 1997, Mohler et al. 2002), may improve the ability to detect multiple marks.

This study investigated two issues: 1) mass, immersion-marking efficacy and mark retention through two years when applying double marks with OTC and CAL to largemouth bass (*Micropterus salmoides*, LMB), and 2) the interval necessary between marks before they are individually distinguishable.

Methods and Materials

Protocols for Administering Marks

Fish were randomly segregated into three groups and exposed to either OTC or CAL or were unmarked controls. To mark LMB fry (9–10 mm total length [TL]) with CAL, we used a two-step osmotic induction method (Mohler 2003). First, we immersed fish in a 20-ppt salinity solution for 3.5–4.0 minutes. Fish were then immediately immersed in a 5,000-ppm active ingredient (AI) CAL solution (3.5–4.0 minutes) in freshwater with pH matching culture water (pH was adjusted up with 6N NaOH, or down with vinegar).

To mark LMB fingerlings (30–40 mm TL) with CAL, or to mark LMB fry and fingerlings with OTC, we immersed fish in a 500-ppm AI CAL solution or 550-ppm AI OTC solution, respectively, for approximately six hours (Secor et al. 1991, Brooks et al. 1994, Conover and Sheehan 1996, Unkenholz et al. 1997). We added enough sodium phosphate dibasic buffer to maintain solution pH within 0.2 units of culture water. During immersion we allowed no freshwater inflow, but maintained at least 80% dissolved oxygen saturation. Whenever foaming occurred during the

immersion period, we added a silicon-based surfactant to break up foam (drop wise for solution volumes ≤ 20 liters and in 25-ml additions for solution volumes > 20 liters). At the end of each marking period, we added fresh water to flush the solution and temper fish to ambient water conditions.

Double-marking of Fry and Surviving Fingerlings

We marked approximately 7,000 LMB fry and marked surviving fingerlings a second time. The combinations of mark possibilities during this 730-day study were planned to be: controls (fish handled as in chemical marking, but with no chemical exposure), OTC-OTC, OTC-CAL, CAL-OTC, and CAL-CAL, where the initial mark was administered to fry and the second mark was administered later to the same fish as fingerlings. After the initial marking, we segregated fry based on the chemical that was used in marking and stocked the fry into ponds (one ~0.25 ha pond per mark). We had established zooplankton blooms in the ponds before we introduced fry (Hutson 1990). After 35–40 days, ponds were drained and fingerlings were collected to be marked again. After fingerlings were marked, they were returned to ponds with established forage for grow-out and sampled at six-month intervals via boat-electrofishing (240 volts DC at 8–10 amps collecting 20 fish per sample) to observe the quality of the mark. Fish were frozen until we could remove otoliths and measure (TL) each fish.

For both fry and fingerling LMB, we estimated the 15-day efficacy of each treatment using a randomized block design, with aquaria (~76 liters) as blocks. We replicated the experiment three times. After marking, we immediately took approximately 20 fish from each of the three treatment batches and randomly placed each group in one of three aquaria. To keep marked fish separate, we partitioned the aquaria into three sections using saran screen. We fed fry daily, *ad libidum*, freshly-hatched, brine shrimp (*Artemia* sp.) nauplii and zooplankton harvested from ponds; we fed fingerlings live Western mosquitofish (*Gambusia affinis*) young.

Double-marking of Fingerlings

To estimate the interval necessary for readers to distinguish between two individual marks, we marked fingerlings (about 40) twice with 550-ppm OTC at 3-, 14-, or 21-d intervals between marks. We used identical procedures for marking with OTC as those we detailed above.

Evaluating Mark Efficacy and Retention

To estimate mark efficacy, both sagittal otoliths were removed from each fish and cleaned to reduce auto-fluorescence. Otoliths were dried and affixed to microscope slides sulcus-side down with cyano-acrylic cement. Otoliths were viewed for mark detection with a 100-watt ultraviolet light-source Nikon Optiphot-2 compound microscope at 100X–400X magnification, as necessary, with a 450- to 490-nm excitation filter, a 515-nm barrier filter, and a 510-nm dichromic mirror (Hendricks et al. 1991, Secor et al. 1991, Brooks et al. 1994, Conover and Sheehan 1996, Unkenholz et al. 1997). Most otoliths were viewed wet. If no marks were observed within the whole otolith, the otolith was ground with wet 600-grit sandpaper to enhance mark visibility (Maceina 1988). Many of the initially-viewed otoliths became opaque with drying and were reground to view a second time. All otoliths were treated in a way that maximized its mark's fluorescence. Many otoliths were ground further in an attempt to find the mark given to the individual as a fry. Otoliths were stored in labeled vials with distilled water to help maintain otolith transparency and stored in the dark to retain fluorescence (Harrison and Heidinger 1996).

Readers subjectively classified the quality of the mark into four brightness categories: absent (scored 0), visible but faint (scored 1), good (scored 2), or excellent (scored 3). After reviewing a set of photos in which the chemical used to produce the mark was identified, readers examined and scored all otoliths. Readers, using photographs of marked and non-marked otoliths from each collection (i.e., 6, 12, 18, and 24 months post-marking) as unknowns, were asked to determine mark presence or absence, and to identify whether marks were produced by OTC or CAL. Readers were given two photos of each otolith, one viewed under normal light conditions and the second viewed under ultraviolet light.

We tested for differences in the quality of the mark between our two treatments (i.e., OTC and CAL) using Proc MIXED (SAS 1999). The mean quality of the mark for each period and the raw *P*-values from the MIXED analysis were used by Proc MultTest to compare the appropriate pairs (SAS 1999). A stepdown Sidak test was used to control family-wise error (SAS 1999), which for all comparisons, was $P \leq 0.05$.

Modeling Mark Retention in Fingerling LMB

Using only LMB fingerling data from the double-marking experiment, we developed a model to analyze changes in the quality of the mark through time and to estimate mark retention. The model creates a transition matrix (Emlen 1984) to estimate the probability of the quality of a fish's mark changing from one brightness category to another. Transition probabilities lie within the range [0, 1], and the probabilities within a column sum to 1. The general form of the model is:

$$\begin{bmatrix} n_{3,t} \\ n_{2,t} \\ n_{1,t} \\ n_{0,t} \end{bmatrix} = \begin{bmatrix} p33 & 0 & 0 & 0 \\ p32 & p22 & 0 & 0 \\ p31 & p21 & p11 & 0 \\ p30 & p20 & p10 & p00 \end{bmatrix} * \begin{bmatrix} n_{3,(t-1)} \\ n_{2,(t-1)} \\ n_{1,(t-1)} \\ n_{0,(t-1)} \end{bmatrix}$$

where,

$n_{i,b}$ = number of otoliths observed with the quality of the mark in brightness score of "i" at time "b", and

$p_{i,j}$ = probability of an otolith having the quality of its mark changing from a brightness scored of "i" to "j" during one time step (i.e., six months).

One basic assumption is that the quality of the mark may attenuate through time, but not increase. We also assumed that attenuation is constant.

Results

Double-marking of Fry and Surviving Fingerlings

We recovered few fry (OTC –11.8%, Control –16.5%, and CAL –0.3%) from our ponds after six weeks. For fry LMB, marks from both CAL and OTC attenuated rapidly (Table 1), despite marks at 15 days post-marking being largely good to excellent for both CAL (mean value = 2.8) and OTC (mean value = 2.5). For fingerling LMB marked with CAL, 50% had lost their marks by 180 days, whereas 100% of fingerlings marked with OTC had visible marks throughout the 730 days of study (mean mark value ≥ 1.1). For fingerling LMB, marks on OTC-treated otoliths were significantly ($P < 0.0001$) brighter than those treated with CAL (Table 1). Because we recovered so few CAL-marked fry, only OTC-CAL and OTC-OTC double-marks were evaluated.

Double-marking of Fingerlings

We found that at least 14 days between applications of OTC were necessary before we could always observe two marks (Table 2). Readers could accurately assess whether an otolith was marked or unmarked; however, readers often had difficulty determining if the mark had been made by OTC or CAL (Table 3).

Modeling Mark Retention in Fingerling LMB

When we tried to model mark retention of CAL in fingerling LMB otoliths, we noted we had observed no excellently-marked (score "3") otoliths (Table 1) at 15 d. Under our assumption that mark intensity could not increase with time, some excellently-marked otoliths must have been initially present, for we observed excellently-marked otoliths in subsequent periods. We assumed we had missed detecting any excellently-marked otoliths at 15 d because of our small initial sample size ($N = 6$). We developed a series of four alternative scenarios (Table 4) in which data were expanded from the observed $N = 6$ fish to $N = 20$ fish, consistent with the sample sizes at other sample times. While 4845 scenarios exist for distributing 20 fish into four categories, an exploration of the likelihood surface suggested these four were most plausi-

Table 1. Quality of fluorescent marks on largemouth bass otoliths and the mean values of the brightness categories for LMB marked as fry or fingerling at various elapsed times after immersion in oxytetracycline hydrochloride (OTC) or calcein (CAL). Standard deviations are shown in parentheses.

			Number of fish in each brightness category				
Fish stage	Chemical	Elapsed time (Days)	Not visible	Visible	Good	Excellent	Mean mark value
Fry	CAL	15	0	0	2	8	2.8(0.42)
		45	0	4	1	0	1.2(0.45)
Fry	OTC	15	0	1	3	6	2.5(0.69)
		180	13	7	0	0	0.3(0.47)
		365	17	3	0	0	0.1(0.18)
		540	18	0	0	0	0.0(0)
		730	23	0	0	0	0.0(0)
Fry	Control	15	10	0	0	0	0.0(0)
		180	12	0	0	0	0.0(0)
		365	20	0	0	0	0.0(0)
		540	20	0	0	0	0.0(0)
Fingerling	CAL	15	0	4	1	0	1.3(0.42)
		180	10	7	2	1	0.7(0.82)
		365	12	6	1	1	0.6(0.71)
		540	12	6	1	1	0.6(0.70)
Fingerling	OTC	15	0	2	12	6	2.2(0.50)
		180	0	5	14	1	1.8(0.54)
		365	0	4	12	4	2.0(0.57)
		540	0	10	8	0	1.4(0.43)
		730	0	17	6	0	1.3(0.40)
Fingerling	Control	15	15	0	0	0	0.0(0)
		180	12	0	0	0	0.0(0)
		365	20	0	0	0	0.0(0)
		540	20	0	0	0	0.0(0)

Table 2. Percent of largemouth bass fingerlings with two visible oxytetracycline hydrochloride (OTC) marks 14 days after the second of two OTC immersions (550 ppm active ingredient solution for six hours) with 3, 14, or 21 days between mark applications. Tabled values are the mean size (mm TL) at the second mark application and the mean value of the brightness categories (standard deviation in parentheses).

Mark application interval (days)	*N* fish	Mean TL (mm)	% with two visible marks	Mean brightness of first mark	Mean brightness of second mark[a]
3	20	58(6.7)	40	2.8(0.44)	3.0(0.0)
14	14	78(10.7)	100	2.7(0.47)	3.0(0.0)
21	20	74(9.4)	100	1.9(0.55)	2.3(0.64)

a. Mean based on those fish with two marks.

Table 3. Accuracy of readers to differentiate unidentified marked (with oxytetracycline hydrochloride (OTC) or calcein (CAL)) and unmarked largemouth bass otoliths at six-month intervals following marking.

Months since mark	*N* fish unmarked	Accuracy of identifying unmarked fish (%)	*N* fish marked	Accuracy of finding any mark	*N* marked with OTC	Accuracy of correctly identifying mark as OTC mark (%)	*N* marked with CAL	Accuracy of correctly identifying mark as CAL mark (%)
6	5	80	11	91	7	100	4	25
12	10	100	10	100	4	100	6	33
18	8	100	17	100	11	91	6	67
24	4	100	12	100	12	75	0	NA

ble based on the assumption that the observed distribution was drawn from a multinomial distribution. In each alternate scenario, the number of marked fish was increased to be consistent with the sample that we had observed under a sample size of $N = 6$, while also allowing for the idea that some random variation would occur under the expanded sample. Residual sums of squares following model fit was similar for all four scenarios, suggesting all scenarios are equally plausible given the data. We found the probabilities for transitions $p33$ and $p32$ were strongly related to the initial conditions of the scenario, but other transition probabilities were more robust. When there was one initial excellently-marked otolith, then $p33 = 1.00$, because one "3" is observed in each subsequent sample throughout the 730 days; when more than one excellently-marked otolith was in the initial sample, $p33 < 1.00$. The pattern for the rest of the transition matrix suggests substantial decay of marks each 180 days. Because we noted substantial and consistent decay in all but scenario "D," where we estimated $p33 = 1.00$, the resultant transition matrix gives the average values for all parameters over the other three alternative scenarios:

$$\begin{bmatrix} 0.66 & 0 & 0 & 0 \\ 0.34 & 0.41 & 0 & 0 \\ 0.00 & 0.59 & 0.43 & 0 \\ 0.00 & 0.00 & 0.57 & 1.00 \end{bmatrix}$$

With OTC, every otolith examined had a visible mark (Table 1). We estimated a transition matrix of:

$$\begin{bmatrix} 0.31 & 0 & 0 & 0 \\ 0.69 & 0.75 & 0 & 0 \\ 0.00 & 0.25 & 1.00 & 0 \\ 0.00 & 0.00 & 0.00 & 1.00 \end{bmatrix}$$

Abnormal otoliths were encountered in <1% of LMB in this study. Abnormal otoliths were more difficult to score, although marks could be seen in all cases.

Table 4. Likely alternative scenarios when the observed data ($N = 6$) were expanded to $N = 20$. The table shows the number of largemouth bass otoliths and the quality of their calcein marks: not visible (0), visible (1), good (2), and excellent (3). Also included is the resulting residual sum of squares (Residual SS) following the fitting of the transition-matrix model.

	N fish in brightness category of				
Scenario	Not visible	Visible	Good	Excellent	Residual SS
A	0	11	5	4	30.1
B	0	12	5	3	27.8
C	0	12	6	2	29.2
D	0	13	6	1	28.4

Discussion

This study builds on previous studies, extending the utility of fluorescent-chemical marks. While results associated with fingerling LMB are quite promising, results associated with LMB fry were somewhat disappointing due to low recovery rates. Low recovery rates of LMB fry are likely due to handling stress, which has been noted as the key factor in survival of fish (especially fry) during immersion marking (Kayle 1992, Brooks et al. 1994, Peterson and Carline 1996, Unkenholz et al. 1997, Lucchesi 2002, Logsdon et al. 2004).

We, like others (Choate 1964, Lorson and Mudrak 1987, Beckman et al. 1990, Kayle 1992, Brooks et al. 1994, Brown et al. 2002, Jenkins et al. 2002, Lucchesi 2002), were unable to attain excellent marks in all fish, even at the earliest observations (Table 1). Assuming the quality of the mark at day 15 is approximately that observed on day 0, the model for CAL suggests we would expect approximately 60% of the otoliths to have "visible marks," 30% to have "good marks," and 10% to have "excellent marks" (Fig. 1), but for OTC, we would expect approximately 10% of the otoliths to have "visible marks," 60% to have "good marks," and 30% to have "excellent marks" (Fig. 2). By the end of the first year (Fig. 1), we would expect about 60% of the otoliths in fish marked with CAL to have no visible mark, illustrating the short life of CAL marks in this study. Calcein has faded rapidly in other fishes, which has necessitated remarking for long-term studies (Leips et al. 2001).

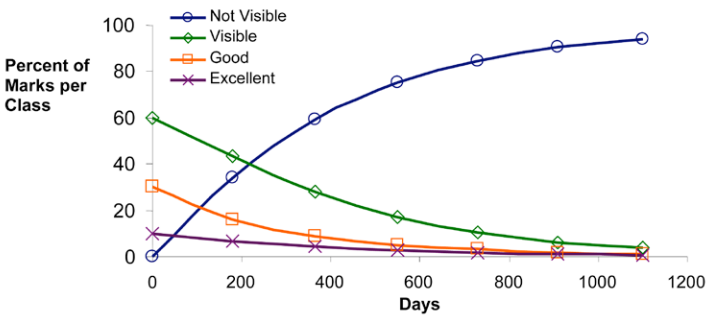

Figure 1. Estimated percent of largemouth bass otoliths with calcein (CAL) marks over elapsed days from mark application, where the quality of the marks were not visible (0), visible (1), good (2), and excellent (3).

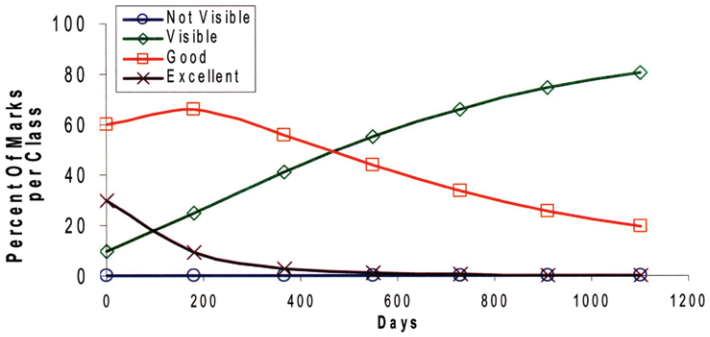

Figure 2. Estimated percent of largemouth bass otoliths with oxytetracycline hydrochloride (OTC) marks over elapsed days from mark application, where the quality of the marks were not visible (0), visible (1), good (2), and excellent (3).

Conversely, the data and the model suggest a multiple-year life expectancy of OTC marks. Variability in mark-quality among fish has been related to water hardness, which causes differential solution of the marking chemical (Brown et al. 2002), and fish size, growth rate, or metabolism, which causes varying chemical uptake (Beckman et al. 1990, Conover and Sheehan 1996, Mohler 1997, Isermann et al. 1999, Jenkins et al. 2002). Harrison and Heidinger (1996) found that walleye fed to satiation throughout treatments had brighter marks than similarly-treated fish fed ≤1% of their body weight/day. While luminescence was sufficient for this study, with more study, the conditions that will yield the brightest, most consistent, and longest-lasting marks for LMB may be better understood.

In some studies, it is necessary to differentiate treatments using multiple batch marks. Secor et al. (1991) proposed multiple marks would be useful in study of striped bass (*Morone saxatilis*) although no time interval was tested. Hendricks et al. (1991) found a 10-day interval, and to some extent a 5-day interval, provided good separation of multiple marks with American shad (*Alosa sapidissima*). Our results show that multiple marks can be successfully used with LMB when at least 14 days between applications of OTC is allowed between marks.

Readers could accurately assess whether an otolith was marked or unmarked; however, readers often had difficulty determining if the mark had been made by OTC or CAL (Table 3). This result is contrary to findings of Wilson et al. (1987) and Brooks et al. (1994). This was especially true in those otoliths with faint marks, as marks were very similar in color. We found it best to view otoliths either freshly removed from fish or stored in darkness in distilled water. Currently, typical protocols use subjective scoring by readers. Subjective scoring is easy to implement, requires less equipment, and can be consistent when readers are well-trained. To reduce the potential for bias, we used a single-blind design and trained the readers before they were allowed to score the otoliths. In the future, others might investigate whether it may be possible to eliminate this subjectivity by using some type of UV light meter.

Abnormal otoliths would hinder the utility of this approach. We were pleased to find that no fish had two abnormal otoliths, similar to findings of Conover and Sheehan (1996). Occurrence of abnormal otoliths suggests scientists should collect and use both sagittae from each fish.

This work demonstrates that proper application of OTC provides the ability to create multiple, long-lasting marks on LMB otoliths. If CAL must be used, the study should be carried out for only a short time, or fish should be remarked. Alternating OTC and CAL to provide different colored batch-marks was unsuccessful, as readers had difficulty distinguishing between the two colors.

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