American Fisheries Society Symposium 5:89–95, 1988

Marking Cyprinid Larvae with Tetracycline[1]

ROBERT T. MUTH

Larval Fish Laboratory, Department of Fishery and Wildlife Biology
Colorado State University, Fort Collins, Colorado 80523, USA

THOMAS P. NESLER

Wildlife Research Unit, Colorado Division of Wildlife
Fort Collins, Colorado 80526, USA

ANTHONY F. WASOWICZ[2]

Department of Fishery and Wildlife Biology, Colorado State University
Fort Collins, Colorado 80523, USA

Abstract.—Laboratory experiments were conducted on the use of tetracycline hydrochloride (TC) for mass marking recently hatched Colorado squawfish *Ptychocheilus lucius*. Protolarvae were immersed in tris-buffered solutions of 200, 350, or 500 mg TC/L of distilled water for 4, 12, or 36 h and then reared for up to 77 d. Overall survival was lowest (0–81%) for the 36-h and 500-mg/L treatments. However, fish growth and development were not affected by treatment. Larvae preserved within 15 d of treatment exhibited externally visible fluorescence when scanned with ultraviolet (UV) light. Otoliths extracted from larvae that were preserved in 95% ethanol were in good condition, but those from larvae preserved in formalin solutions buffered to near neutral pH with phosphate degraded during storage. The otoliths of treated larvae exhibited fluorescent marks when examined by UV-light microscopy, and these marks were retained throughout the experiment with no apparent decrease in intensity. Whole-body immersion in 350 mg TC/L for 4–12 h was found to be optimal for best mark intensity and fish survival. In another experiment, protolarvae of fathead minnows *Pimephales promelas* were immersed in a tris-buffered solution of 350 mg TC/L for 4 h and then exposed to various intensities of artificial white light in the 340–650-nm range (includes near-UV spectrum) for 12 h/d for 7 d. Exposure to light had no effect on the presence or intensity of fluorescent marks in the otoliths. Our results suggest that the TC-marking technique can be used in field mark–recapture studies of Colorado squawfish larvae.

Marking individual fish is an effective means for obtaining various kinds of information on fish species and populations (Wydoski and Emery 1983). Data from mark–recapture studies often provide solutions to questions left unanswered by basic survey sampling. Tsukamoto (1985) observed that information on the early-life behavior and environmental requisites of fish is critical to understanding the dynamics of fish populations, and he noted that effective methods of marking fish early-life stages for mark–recapture studies need to be developed. Marking techniques used successfully for the early-life stages of some fish species might require modification or be unsuitable for use on other fish species.

The objective of this work was to develop or adapt a mass-marking technique for larvae of the federally endangered Colorado squawfish *Ptychocheilus lucius* for proposed mark–recapture studies in the Yampa and Green rivers in Colorado and Utah. This technique would facilitate investigation of dispersal patterns of larvae, habitat utilization by age-0 fish, relationships between larval fish abundance and recruitment, and causes of early-life mortality. For such studies, an optimal mark must be (1) applicable to late embryos or recently hatched, yolk-bearing larvae or both, (2) suitable for mass marking in the field, (3) innocuous, that is, must not affect the normal life of the fish, (4) detectable for at least 8 weeks, and (5) relatively easy and inexpensive to apply and detect.

Techniques for marking very small fish were reviewed by Laird and Scott (1978), Hettler (1984), Brothers (1985), and Tsukamoto (1985). These techniques included the use of dyes or stains, fluorescent antibiotics, trace element or nonradioactive isotopes, and growth inhibitors or environmental manipulations (e.g., of tempera-

[1]Contribution 35, Colorado State University Larval Fish Laboratory.

[2]Present address: Utah Cooperative Fish and Wildlife Unit, Utah State University, Logan, Utah 84321, USA.

ture or light) to alter growth patterns on bony structures. Many marking studies of fish early-life stages have been primarily concerned with age or growth determinations. Few successful methods for marking fish embryos or early larvae, specifically for application in field investigation, have been described (e.g., Muncy and D'Silva 1981; Brothers 1985; Tsukamoto 1985). Brothers (1985) and Tsukamoto (1985) considered otoliths ideal for marking because they are the first permanent calcified structures present in the earliest life history stages of fish and are, effectively, biological internal tags. We concluded that tetracycline marking of otoliths would most likely meet our requirements for an optimal mark. Tetracycline compounds are well-known markers for calcified structures, and they fluoresce yellow under ultraviolet (UV) light (Milch et al. 1957; Weber and Ridgway 1962, 1967; Choate 1964). They have been used to mark larvae of several fish species, for example, pinfish *Lagodon rhomboides* and spot *Leiostomus xanthurus* (Hettler 1984) and ayu *Plecoglossus altivelis* (Tsukamoto 1985), but their application to cypriniform larvae has not been reported.

Methods

Approximately 1,500 4-d-old (posthatching) Colorado squawfish protolarvae (larvae prior to development of median fin rays, sensu Snyder 1981) were obtained from the Dexter (New Mexico) National Fish Hatchery in June 1986. The larvae were placed in a flow-through holding trough that received 20°C well water; 20–22°C water was used by Hamman (1986) for hatchery culture of Colorado squawfish embryos and larvae. Most larvae were swim-up, still had 50–70% of their yolk, and measured 7.5–8.0 mm total length (TL). The sagitta and lapillus otoliths were both present. After a 24-h acclimation period, dead, damaged, or atypically behaving larvae were removed. The remaining larvae were divided into ten 140-specimen experimental groups.

Nine experimental groups were treated by placing the larvae in 1-L glass beakers containing tetracycline hydrochloride (TC) solutions of 200, 350, or 500 mg TC/L aerated distilled water. Distilled water was used as the diluent because TC binds to calcium, and hard water, which contains calcium ions, might hinder the uptake of TC by the otoliths. Each TC test solution was adjusted to pH 6.8–7.0 with tris buffer (Hettler 1984). The pH prior to buffering was about 3.6. Exposure times for each concentration were 4, 12, and 36 h. During their exposure, the treatment groups were aerated and incubated in a covered water bath at 20°C, but they were not fed. The 10th experimental group was maintained as a control.

After each prescribed exposure, the treated larvae were removed from the test solutions, and the numbers of dead larvae were recorded. Surviving larvae were placed in aerated well water in 3.8-L rearing jars and incubated in a water bath at 20°C. Control larvae were taken directly from the holding trough and placed in a rearing jar. Rearing jars were partially shaded with sheets of opaque plastic. The photoperiod was about 9-h light: 15-h dark; light intensity was 100 lx. Larvae were fed twice daily with live artemia *Artemia* sp. nauplii and TetraMin Fry Diet. After about 4 weeks, TetraMin Staple Food was included in the diet (Muth et al. 1985). Before each feeding, the rearing jars were cleaned, and the numbers of dead larvae were recorded. Larvae were reared for up to 77 d after treatment.

Ten living larvae from each experimental group were preserved and measured (TL) immediately after treatment and at weekly intervals thereafter. Two different preservation fluids were tested, and their effects on otolith structure and on TC-produced marks were compared. At each time interval, five larvae were fixed and preserved in 95% ethanol (pH = 8.0), and five were fixed and preserved in formalin solutions buffered to pH 6.8 with phosphate (Markle 1984). Larvae were fixed in 10% buffered formalin for 24 h, then transferred to 3% buffered formalin for storage. Samples were stored in the dark until they were examined 3–4 months after treatment.

Two whole larvae from each sample lot were examined with incident UV light under a dissecting microscope for the presence of external fluorescent marks. Sagittae and lapilli were extracted from larvae by procedures similar to those described by Brothers (1987) for embryonic and larval fish. Otoliths were mounted in glycerin on glass slides and examined with incident UV light under a compound microscope for the presence and intensity of fluorescent marks. The average time spent on the extraction and mounting of otoliths was about 10 min/specimen. The intensity of the mark was determined for each of the larvae that was examined, and it was ranked by a procedure similar to that used by Tsukamoto (1985). The ranking categories that we designated were absent, faint, lucid, or bright, corresponding to values of 0, 1, 2, or 3, respectively. Individual

values were summed within each sample lot, and each total was used to compare the intensities of the marks among the sample lots. For example, the maximum summed mark-intensity value for a sample lot of five specimens would be 15.

A second experiment was conducted to examine the stability of TC deposited in otoliths of fish larvae that were then exposed to white light. About 300 7-d-old (posthatching) fathead minnow *Pimephales promelas* protolarvae were immersed in an aerated, tris-buffered solution of 350 mg TC/L for 4 h. Larvae were 5.5–6.5 mm TL, and lapilli and sagittae were present. After immersion, five larvae were preserved in 95% ethanol. The remaining larvae were divided equally into six experimental groups and placed in 38-L aquaria containing aerated, dechlorinated tap water at 22°C. Then, the larvae were exposed to white light at replicated treatment intensities of 10, 300, or 1,000 lx (measured at the water surface) for 12 h/d for 7 d. The two lower light intensities were achieved by covering the aquaria with sheets of opaque plastic or fine-mesh screening. Illumination was provided by paired General Electric cool white 40-W fluorescent tubes positioned 20 cm above the aquaria. According to the manufacturer, these tubes emit light in the 340–650-nm range. Weber and Ridgway (1967) stated that tetracycline molecules that were deposited in fish bone were excited to a fluorescent state by absorbing UV energy at 360 nm. Larvae were fed twice daily with live artemia nauplii. Larvae in each light-intensity treatment were sampled once daily and preserved in 95% ethanol. Then, otoliths were extracted, mounted, and examined in the same manner as for Colorado squawfish larvae.

Results

Mortality of Colorado squawfish larvae was high for all 36-h-exposure treatments; 71–100% of the fish in each group died. In the 500-mg/L treatment for 4- and 12-h, mortality was moderate (16 and 24% of each group, respectively), then it stabilized nearly to zero during rearing. For the remaining treatments, survival was high during exposure to TC and during rearing. Overall survival (90–92%) of each treatment group was higher than that of the control group (79%). Fish growth, measured in increments of total length, was similar for all experimental groups. Exposure to TC had no obvious effect on fish development or behavior.

Fluorescence was visible externally when whole treated Colorado squawfish larvae were scanned with UV light. The entire bodies of larvae

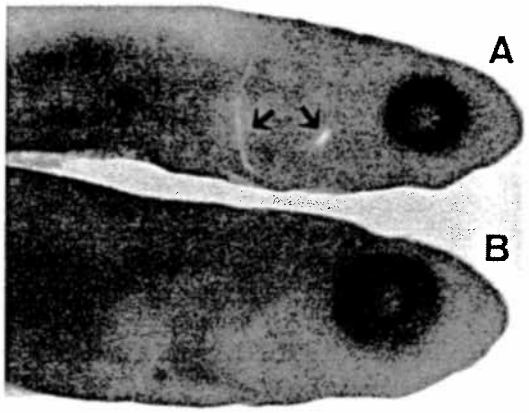

FIGURE 1.—A. Externally visible fluorescent marks (arrows) on preopercal and subopercal bones of a Colorado squawfish larva (10.7 mm total length, TL) scanned with ultraviolet (UV) light 14 d after whole-body immersion in a 350-mg tetracycline hydrochloride/L solution for 4 h. B. Untreated control larva (10.5 mm TL) scanned with UV light.

from all treatments that had been preserved in ethanol or buffered formalin within 15 d of exposure to TC fluoresced pale yellow. Fluorescence was most evident in the yolk and gut regions. Larvae that had been preserved in ethanol revealed distinct yellow fluorescent marks on the developing dorsoanterior tip of the preopercal bone and posterior edge of the subopercal bone (Figure 1). Fluorescent marks on these bones were not present in larvae that had been preserved in buffered formalin. Larvae that had been preserved 2 weeks or more after treatment revealed externally visible fluorescence that was indistinguishable from reflections caused by incident illumination.

The otoliths extracted from larvae that had been preserved in ethanol appeared structurally intact. However, the otoliths of larvae that had been preserved in buffered formalin had either disintegrated during storage or degenerated to thin, transparent disks that crumbled upon handling. These otoliths were unsuitable for microscopic examination. Asteriscus otoliths were formed in larval Colorado squawfish during the third week of rearing, and they were not examined for fluorescent marks.

Readily detectable marks were visible in otoliths of all TC-treated Colorado squawfish larvae examined by UV-light microscopy (Table 1 and

TABLE 1.—Relative intensity of fluorescent marks in otoliths from Colorado squawfish larvae that were immersed in tetracycline hydrochloride (TC) solutions. Mark intensities were determined for five larvae from each treatment and sampling time (days after treatment) and were ranked 0–3 (absent–bright). Values were summed within each sample lot to produce a batch mark-intensity value. All fish immersed in TC solutions of 350 or 500 mg/L for 36 h died during treatment.

Days after treatment	Mark intensity by TC concentration and exposure time						
	200 mg/L			350 mg/L		500 mg/L	
	4 h	12 h	36 h	4 h	12 h	4 h	12 h
0	5	9	10	10	14	10	14
7	5	10	12	12	15	13	14
28	5	8	12	15	14	14	15
56	5	10		13	13	15	14
77	5	9		13	14	14	14

Figure 2). Marks appeared as yellow fluorescent bands against a dark-green background and were retained throughout the 11-week experiment with no apparent decrease in intensity over time. Fluorescent marks in sagittae became slightly more diffuse as otoliths changed shape with growth; however, mark intensity was not affected (Figure 2). Overall, mark intensity was higher for the 350- and 500-mg/L treatments than for the 200-mg/L treatments, but mark intensity increased with exposure time at all treatment concentrations. In the second experiment, otoliths of all fathead minnow larvae examined by UV-light microscopy had fluorescent marks similar to those observed in otoliths of Colorado squawfish larvae. Mark intensity was high for all three light-intensity exposures throughout the 7-d experiment.

Discussion

Our results demonstrated that otoliths of protolarval Colorado squawfish can be successfully marked by whole-body immersion in TC solutions. Tetracycline deposited in otoliths of larval fish has been successfully used as a mark for several species (e.g., Hettler 1984; Tsukamoto 1985), but, to our knowledge, this demonstration is the first documented use for marking the early larvae of cyprinids. The technique is relatively easy to use, reasonable in cost, and suitable for the mass marking of fish in the field; it offers 100% marking success and at lower concentrations and exposure times has no adverse effect on fish mortality, growth, or development. For the best survival and mark intensity, larvae should be immersed in a solution of 350 mg TC/L for 4–12 h. Hettler (1984) and Tsukamoto (1985) reported similar results. Tsukamoto (1985) observed that larval ayu survival decreased as exposure time in 500 mg TC/L increased. This relationship was not noted at lower TC concentrations. The lowest ayu survival occurred in TC concentrations of 500 mg/L or greater for all exposure times tested. Tsukamoto also reported that TC treatment had no affect on ayu growth, and he recommended whole-body immersion in 200–300 mg TC/L for 3–24 h for otolith marking. Hettler (1984) found that the percentage of larval spots with otoliths that were successfully marked by oxytetracycline hydrochloride (OTC, a compound closely related to TC) increased as exposure time was increased, and it reached 100% in OTC concentrations of 250 and 500 mg/L at exposures of 120 and 60 min, respectively.

Mark Retention

The knowledge of mark-retention time is necessary for mark–recapture studies. In our laboratory experiments, TC deposits in otoliths of larval Colorado squawfish were retained in detectable amounts at least 77 d after treatment in all of the fish that were examined. Larval ayu that were treated with 100 mg TC/L for 7 h and then reared in a greenhouse retained fluorescent marks in their otoliths for at least 164 d (Tsukamoto 1985). Several authors (Choate 1964; Trojnar 1973; Laird and Scott 1978; Hettler 1984; Brothers 1985) reported that tetracycline-produced marks are labile in light because sunlight deactivates the fluorescent properties of tetracycline compounds. However, they provided no confirmatory evidence that marks in internal bony structures would be lost or degraded under natural conditions. Deactivation of tetracycline is presumably caused by molecular excitation and subsequent decay through absorption of solar energy in the UV spectrum (particularly at 360 nm, Weber and Ridgway 1967). Excited molecules of fluorescent substances are usually unstable and have a mean lifetime of only about 10^{-8}–10^{-9} s (Lehninger 1975). If this were true, the reliability of tetracycline marking of larval fish for use in field mark–recapture studies would be suspect. However, this presumption is questionable, particularly for TC deposited in the otoliths of fish released into natural waters. In our experiment using fathead minnow larvae treated with TC, exposure of larvae to artificial white light (in the 340–650 nm range) at surface light intensities as high as 1,000 lx during rearing in pure tap water had no effect on the presence or intensity of fluorescent marks in otoliths. Water absorbs UV

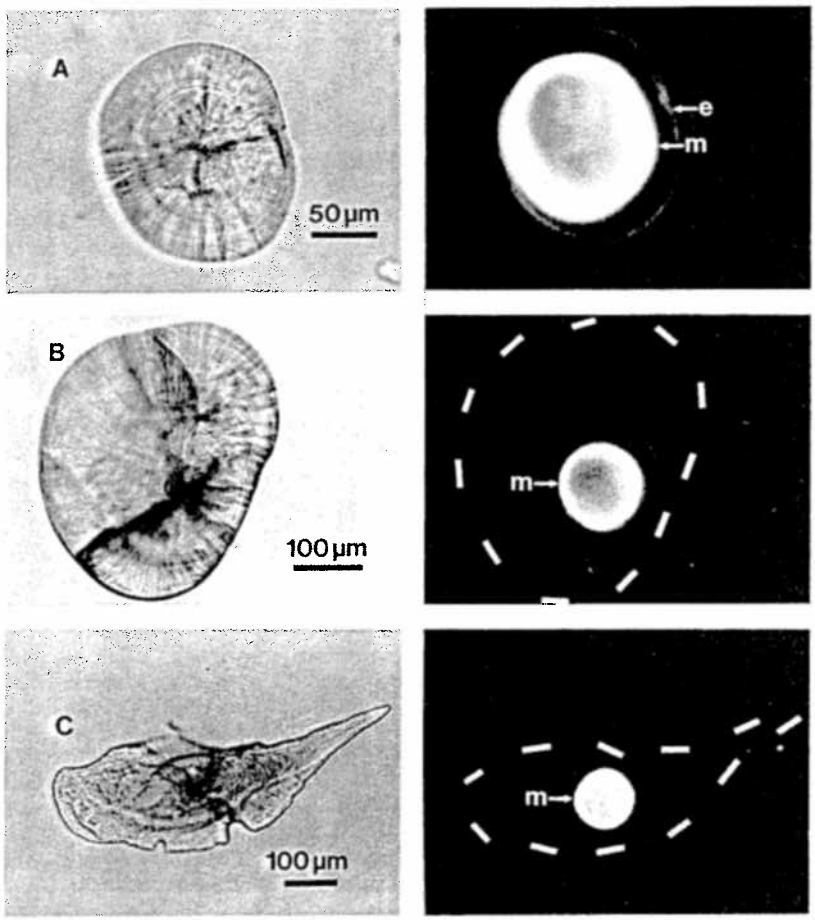

FIGURE 2.—Microphotographs of unground otoliths under white (left) and ultraviolet (UV) light (right). Otoliths from Colorado squawfish larvae immersed in a 350-mg tetracycline hydrochloride/L solution for 4 h. **A.** Sagitta from a 9.1-mm total length (TL) larva, 7 d after treatment. m = fluorescent mark; e = edge of otolith under UV light. **B.** Lapillus and **C.** Sagitta from a 18.2-mm TL larva, 56 d after treatment. m = fluorescent mark; edge of otoliths under UV light indicated by dashed line.

light, and its absorption capacity increases with increased amounts of dissolved solids and organic matter (Koller 1965). Under natural conditions, the small amount of solar UV radiation that penetrates the atmosphere and strikes the earth is attenuated in the surface layer of natural waters (Nicol 1974; Golterman 1975; Reid and Wood 1976; Ali and Klyne 1985; Kirk 1986). Also, the skull and skin of fish should directly protect internal structures from UV radiation (Koller 1965). Skin pigments, particularly melanin on the dorsal surface (including the head and nape), afford protection from light. Melanin helps protect underlying tissues from UV radiation by scattering rays, absorbing damaging radiation (less than 400 nm), and dissipating absorbed energy as heat (Pathak and Stratton 1969). Finally, the successful use of tetracycline-marked fish has been reported in field mark–recapture studies. Blackler (1974) indicated that tetracycline marks may persist for at least several years in internal structures such as bones and otoliths. Pacific salmon *Oncorhynchus* spp., fed pelleted food that contained tetracycline and then released into the wild, retained fluorescent marks in internal bone for at least 3.5 years after treatment (Weber and Ridgway 1967). Kokanee *Oncorhynchus nerka*, fed OTC-bearing food as larvae and early juveniles and then released into Lake Granby, Colorado, retained fluorescent marks in caudal vertebrae for 5–6 years (W. J. Wiltzius, Colorado Division of Wildlife, personal communication).

The utility of external fluorescence produced by exposure to tetracycline as a mark for young fish appears limited by its relatively short time of retention and detectability after treatment. Brothers (1985) reported observations similar to ours for larvae and early juveniles of lake trout *Salvelinus namaycush* exposed to TC and suggested that the presence of external fluorescence after treatment is a good indicator of otolith marking success. Hettler (1984) noted that the head and fins of larval spot and pinfish preserved 8 d after immersion in OTC solutions fluoresced yellow under longwave UV light. In mark–recapture studies of short duration in which larvae are released and sampled soon after marking, external fluorescence might permit identification of marked fish without sacrificing them.

Specimen Preservation

In this study, the otoliths removed from Colorado squawfish larvae that were fixed and preserved in 95% ethanol (as recommended by Brothers 1987) were in excellent condition, but the larvae themselves were shrunken and deformed due to dehydration. Conversely, larvae that were fixed and preserved in formalin solutions buffered to pH 6.8 were in good morphological condition, but their otoliths were either disintegrated or were significantly degraded and useless. The specific cause for loss or degradation of the otoliths is not known. Because the skeletal features of larvae preserved in formalin solutions buffered nearly to neutrality were successfully retained, we considered that 10% formalin fixative and 3% formalin preservative, both made with distilled water and buffered to pH 6.8 with phosphate (Markle 1984), would be adequate for the preservation of otoliths. Perhaps alkaline formalin solutions (about pH 8.0) are required as suggested by Steedman (1976) and McMahon and Tash (1979), or water content in the solutions (greater than 96%) was too high (Steedman 1976), or both. Perhaps, calcium ion concentration in the preservative should have been near saturation rather than almost absent (as in solutions made with distilled water). For the present, although we prefer and recommend formalin solutions buffered nearly to neutrality for most other purposes, we can only follow other researchers and recommend that specimens be preserved in concentrated alcohol (e.g., 95% ethanol) or frozen for otolith analysis.

Acknowledgments

This study was funded under Federal Aid in Fish Restoration matching grant 02-01-035 SE-3 administrated through the Wildlife Research Unit, Colorado Division of Wildlife. We thank J. Hamill (U.S. Fish and Wildlife Service, Denver, Colorado) for arranging the required endangered species permits, R. Hamman (Dexter National Fish Hatchery, Dexter, New Mexico) for providing Colorado squawfish larvae, and D. Keefe (Environmental Research and Technology, Incorporated, Fort Collins, Colorado) for providing fathead minnow larvae. R. Behnke, C. Carlson, and D. Snyder reviewed earlier drafts.

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