Transactions of the American Fisheries Society 130:501–507, 2001

The Suitability of Calcein to Mark Poeciliid Fish and a New Method of Detection

J. LEIPS*[1]

Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695-7614, USA

C. T. BARIL

#202, 1107 West 14th Avenue, Vancouver, British Columbia, Canada V6H 1P5

F. H. RODD

Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 3G5, and Department of Biological Science, Florida State University, Tallahassee, Florida 32306-4340, USA

D. N. REZNICK AND F. BASHEY

Department of Biology, University of California, Riverside, California 92521, USA

G. J. VISSER

236 Green Oaks Drive, Riverside, California 92507, USA

J. TRAVIS

Department of Biological Science, Florida State University, Tallahassee, Florida 32306-4340, USA

Abstract.—The suitability of calcein as a marker for population studies depends on (1) the assumption that marked individuals have unaltered viability, (2) the fidelity of the calcein label, and (3) the facility with which calcein can be used. We examined the effects of calcein on survival, growth, and the timing and size at sexual maturity of least killifish *Heterandria formosa* and present a new method for detecting calcein. To test the assumption that marked individuals have unaltered viability, juvenile least killifish were immersed for 24 h in a 250-mg/L solution of calcein. A control group of same-aged juveniles was immersed in the same volume of water for 24 h without calcein. All individuals were then removed and reared individually in separate containers. Upon examination under an epifluorescent microscope, all calcein-treated fish showed fluorescent green marks on their scales and fin rays, whereas controls did not. The calcein treatment had no significant effect on growth and survival through 9 weeks nor on the age and size at maturity. We also designed a portable detector (uses InGaN blue LEDs as a light source) for distinguishing calcein-marked individuals; using either this new detector or a standard epifluorescent microscope, the fluorescent mark was visible on the test fish for up to 5 weeks in the laboratory, although some individuals required remarking (due to fading) at 2–3 weeks postimmersion. The calcein tag was also visible in the vertebrae of ethanol-preserved specimens for up to 6 years, provided specimens were stored in the dark.

* Corresponding author: jwleips@unity.ncsu.edu

[1] Current address: Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, Maryland 21250.

Received April 10, 2000; accepted November 10, 2000

In population studies, chemical markers such as calcein and tetracycline can be preferable to mechanical or genetic tagging techniques because they may take less time to apply and cost less (i.e., a large number of individuals can be marked in a short time with minimum handling). Chemical markers have three additional assets: (1) they can last for several weeks, (2) they can be applied to fish of all ages (larval to adult), and (3) they are not visible under normal sunlight, so should not affect visually dependent behaviors nor alter susceptibility to visually foraging predators.

One increasingly used chemical marker in population studies is calcein (2,4-bis-[N,N′-di(carbo methyl)-aminomethyl]fluorescein). Calcein binds to alkaline earth metals and can cause calcified parts of organisms (e.g., vertebrae, otoliths) to fluoresce when viewed under an appropriate light source (Wilson et al. 1987). Calcein has proved to be an effective nonlethal chemical marker in several fish species, including guppies *Poecilia reticulata* (Rodd and Reznick 1991; Reznick et al. 1996), red drum *Scianops ocellatus,* Atlantic croaker *Micropogonias undulatus,* spot *Leiostomus xanthurus* (Wilson et al. 1987), spotted seatrout *Cynoscion nebulosus* (Beckman et al. 1990), silver

perch *Bairdiella chrysoura* (Hales and Hurley 1991), summer flounder *Paralichthys dentatus* (Monaghan 1993), and Atlantic salmon *Salmo salar* (Mohler 1997).

A critical assumption of these studies is that the marker itself does not alter the viability of marked animals nor the phenotypic expression of key characters such as life history or behavioral traits. The primary objective of our study was to determine the suitability of calcein as a marker for use in behavioral and life history studies of the least killifish *Heterandria formosa.* One limitation of the use of chemical markers such as calcein is the method used for detecting tagged individuals. Most studies use an epifluorescent microscope designed for examining tissue sections. This setup usually consists of a microscope equipped with an excitation filter, an emission filter, a dichroic mirror, and a high-power (100–150 W) mercury or xenon light source, but it is difficult to use for detecting calcein on live fish (but see Leips et al. 2000) and impractical for use in the field. To address this issue, a second objective was to develop a new type of portable detector for use with live fish.

Methods

Least killifish experiment.—The least killifish is a small (average size at maturity = 8–10 mm for females and 10–14 mm for males) live-bearing poeciliid that occurs in fresh and slightly brackish water throughout the southeastern coastal plain of the United States. At maturity, females develop a small black dot on their anal fin (Fraser and Renton 1940) and males develop a gonopodium, a modified anal fin used for sperm transfer (Constantz 1984).

A 250 mg/L solution of calcein in aged (approximately one week old) well-water was buffered to a pH of 5.3 by adding a powder form of tris [hydroxymethyl]-amino-methane (Trizma rO base, reagent grade) to the solution. Four polyethylene bins (30 × 40 cm) were filled to a depth of 2 cm with the calcein solution, and juvenile least killifish were immersed in the solution (10 individuals/bin) for 24 h. Control juveniles were immersed for 24 h in four additional bins (10 individuals/bin) with the same volume of untreated aged well-water (pH 7.2). The pH of the calcein solution was adjusted upward to 5.3 (calcein is acidic in solution) to match the natural habitat of least killifish. The pH of the control solution was not adjusted downward to match that of the calcein solution because this would have required the addition of an acidic solution, creating yet another difference between the calcein and control treatments. Thus, the pH of the calcein and control solutions were not the same, and so any treatment differences detected may have been due, in part, to pH differences. After 24 h, all fish were rinsed in well-water and placed in individual 2-L polyethylene bowls containing 1 L of aged well-water. Forty fish from each treatment were evenly distributed across five vertical shelves in the laboratory, eight fish per treatment per shelf. Fish were maintained at 31°C on a 14 h light : 10 h dark photoperiod (conditions experienced by natural populations during the breeding season). Sylvanna Gro-Lux/Aquarium wide spectrum fluorescent lights (20 W) were used to provide lighting. One java fern *Microsorum pteropus,* was added to each bowl to provide cover for growing individuals.

Bowls were cleaned weekly throughout the study by replacing approximately one-third of the water with aged well-water. Water lost through evaporation was replaced with distilled water between cleanings to maintain constant water levels and minimize fluctuations in ion concentrations.

Fish were fed twice daily with initial rations of 2 mg of ground flake food at each feeding. After 28 d, rations were increased to 5 mg at each feeding. Food rations were increased further as necessary when the fish consumed all of the food available by the next scheduled feeding. All fish were checked daily for mortality and weekly for sexual maturity.

Standard lengths of all fish were measured every 2 weeks to estimate growth rates. Individuals were removed from their bowls with a turkey baster and placed in separate petri dishes (50 × 11 mm) containing a small volume of water. Petri dishes were fitted into holes in a piece of plexiglass (12 at a time) and photographed and from the photographs digitized measurements of body size were obtained using a scale on the plexiglass sheet for calibration. When an individual matured between measurement periods, its size at maturity was estimated by interpolating linearly from the measurements made before and after maturation. In the few cases where an individual matured after measurements were completed, an estimate of its size at maturity was extrapolated from its growth over the previous three measurement periods.

Growth rates for each juvenile were analyzed as the maximum achieved rate of growth (in millimeters per 14 d). This was done to avoid the problems inherent in estimating growth rate from nonlinear growth curves. Juveniles do not grow in a

linear fashion, which means that an estimate of growth rate can depend on the stage of development at which it is measured. Moreover, an estimate at any particular developmental stage can be derived only by fitting an arbitrary curve to the data and taking the fitted value at that point. An alternative is to use the parameters of these growth curves as dependent variables, but this method lacks statistical power. Use of the ''maximum observed growth'' as a metric avoids both problems. This is because it compares individuals across the treatment groups whenever they attain their maximum growth stage, even though these may not occur synchronously due to variation in the size and stage of development of fish when they were marked.

Fish were anesthetized (including controls) in a sublethal dose of MS-222 (0.02 mg/100 mL) and examined for the chemical tag every 2 weeks through the end of the experiment and when fish became sexually mature. Fish were individually placed on a microscope slide and examined for the calcein mark under an epifluorescence microscope (Zeiss Axiovert 35) at 50× magnification. A 150-W xenon light source, an excitation filter at 495 nm, and an emission filter at 535 nm formed the correct spectrum for mark observation.

Analysis of variance (ANOVA; SAS Institute, Inc. 1989) was used to test for effects of calcein on the fish. Body size was used as a covariate in analyses of growth rate (using the measurement of size immediately preceding the period of most rapid growth) to account for any size-specificity of growth rate. This covariate was not significant for females and, so, was not used in the analysis of female growth rate. Body size was a significant covariate for males and therefore was used in analysis of male growth rate. The effect of shelf location (the shelf on which an individual was placed) was tested with the intent of using it as a blocking variable in the analyses. Shelf location affected only female growth rates, so the effects of shelf location are not reported here. No data were transformed prior to analyses except for the ages at maturity. These data were transformed to natural logarithms to satisfy assumptions of ANOVA. There were unequal sample sizes among treatments because some fish either jumped from their holding areas and died or because the sex of some fish could not be determined when the experiment was terminated. To account for these unequal sample sizes, type III sums of squares were used in all analyses.

Mark durability samples.—Reznick et al. (1996) applied calcein marks to guppies in field experiments conducted in Trinidad between 1993 and 1996. The guppies were marked in the same fashion as the least killifish, except that the pH of the 250-mg/L calcein solution was adjusted with NaOH to 7.2–7.8. This pH is similar to that observed in streams with natural populations of guppies in Trinidad. The marked guppies were released into the natural environment and recollected 20 d later. All recaptured guppies were preserved in 95% ethanol within 6 h of collection, then stored in the dark. These guppies were available to evaluate the long-term durability of calcein marks in the skeletons of alcohol-preserved fish.

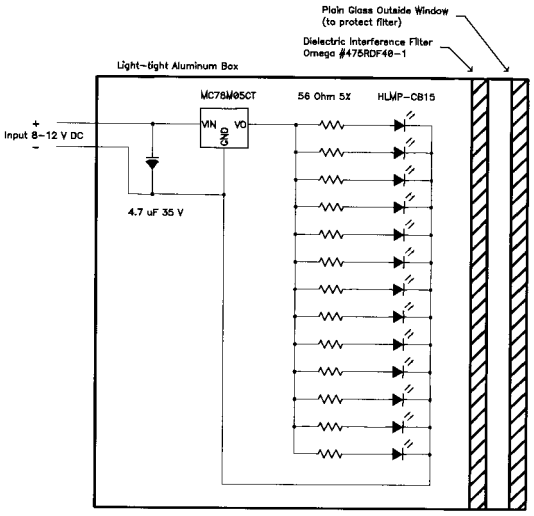

FIGURE 1.—Schematic diagram of the blue light source for the portable calcein detector.

Portable detector.—A dielectric interference bandpass filter at 535 nm, with a width of 35 nm (Omega Optical 535df35–1[d′]) was placed in one ocular of a dissecting microscope to act as a barrier filter. To excite the calcein, a light source was constructed from 13 indium gallium nitride blue light-emitting diodes (Agilent HLMP-CB15), which have a peak emission wavelength of 470 nm. Because the LED emission spectrum, although efficiently concentrated around 470 nm, overlaps the 535 nm filter, an excitation filter centered at 475 nm with a width of 40 nm (Omega Optical 475RDF40–1[d′]) was placed between the LEDs and the sample. The LEDs were arranged in a close-packed hexagonal lattice of holes in an aluminum plate that was mounted in a small light-tight aluminum box (2.5 × 3.8 × 7.6 cm; Figure 1). The filter was mounted to the LED plate, and the filtered blue light was allowed to exit the box

TABLE 1.—Results for comparisons (analysis of variance) of calcein-treated individuals and controls on three measures of growth and development. Least-square means (SEs) are presented for the two treatments.

Sex	*N*	Mean square	*F*-value	$P > F$	Calcein mean (SE)	Control mean (SE)
Maximum growth rate (mm/14 d)						
Male	31	0.0001	0.00	0.98	3.16 (0.12)	3.16 (0.13)
Female	37	0.044	0.06	0.80	3.69 (0.19)	3.62 (0.20)
Size at maturity (mm)						
Male	31	2.04	2.41	0.13	14.39 (0.23)	14.93 (0.26)
Female	37	0.24	0.12	0.73	12.86 (0.34)	13.04 (0.34)
Age at maturity ($\log_e$d)						
Male	33	0.0002	0.00	0.95	3.87 (0.05)	3.87 (0.06)
Female	36	0.034	0.41	0.53	3.43 (0.07)	3.36 (0.34)

through an aperture at the end. The LED current was set at about 26 mA by using a 5-V regulator integrated circuit and a 56-Ω series resistor for each LED. Input power was supplied from a 12-V DC power adapter. Alternatively, more sophisticated electronics could be used, allowing the light source to operate for about 30 h from four size-D alkaline batteries.

To test the portable detector, we used guppies marked as described above for the long-term retention samples. To read the marks with the portable detector, the live guppies were anesthetized with MS-222 (0.02 mg/100 mL) and observed under the dissecting microscope individually.

Results

Least Killifish Experiment

Four of the 80 least killifish died during the study period (two controls and two calcein-treated fish). There were no significant differences between the controls and the calcein-treated fish for rates of growth nor for female and male age and size at maturity (Table 1). A power analysis procedure (Zar 1984) was carried out after the data were obtained to determine the minimum difference between the treatment and control groups that would have been significant ($P < 0.05$, power = 0.90), given the sample sizes used and the amount of variation in the data. Based on these calculations, the difference in size at maturity would have had to have exceeded 1 mm for males and 2 mm for females for the data to have revealed a significant detrimental effect from calcein. This difference is small, only slightly more than the precision of measuring live fish, which suggests that this experiment had the power to detect even a very weak effect of calcein. For maximum growth rates, the difference would have had to have exceeded 0.6 mm/d for males and 0.7 mm/d for females. For the difference in time to maturity to be significant, ages at maturity would have had to differ by more than a day for both males and females. It should be noted that fish were only checked for sexual maturity on a weekly basis; if the effect of calcein altered the age at maturity by less than 1 week, differences between the control and calcein labeled fish would not have been detected. In general, the experimental design provided good power to detect treatment effects.

Under the epifluorescent microscope, the fin rays and scales of calcein-treated least killifish glowed fluorescent green (Figure 2a). The mark was especially prominent on the gonopodium of males. There was no evidence of any fluorescent mark in the control fish, which shown black under the light source. For most individuals, the mark was visible after 5 weeks, although in a few cases the mark began to fade noticeably after 2–3 weeks postimmersion. In such cases, individuals were remarked through calcein immersion (using the same concentration as their initial treatment) and later examined for the mark. Individuals that were remarked were not handled separately in the analyses from those marked only once.

Mark Durability

In 1999, the calcein label was still visible in the caudal fin rays of all 40 guppies collected in 1995 and in 23 of 25 fish from 1996. Guppies from the 1993 and 1994 field studies were also clearly labeled, but all individuals were not processed so the retention proportion is unavailable. These observations suggest that the calcein label can be retained in hard tissues of preserved specimens for up to 6 years, provided that the specimens have been stored in the dark.

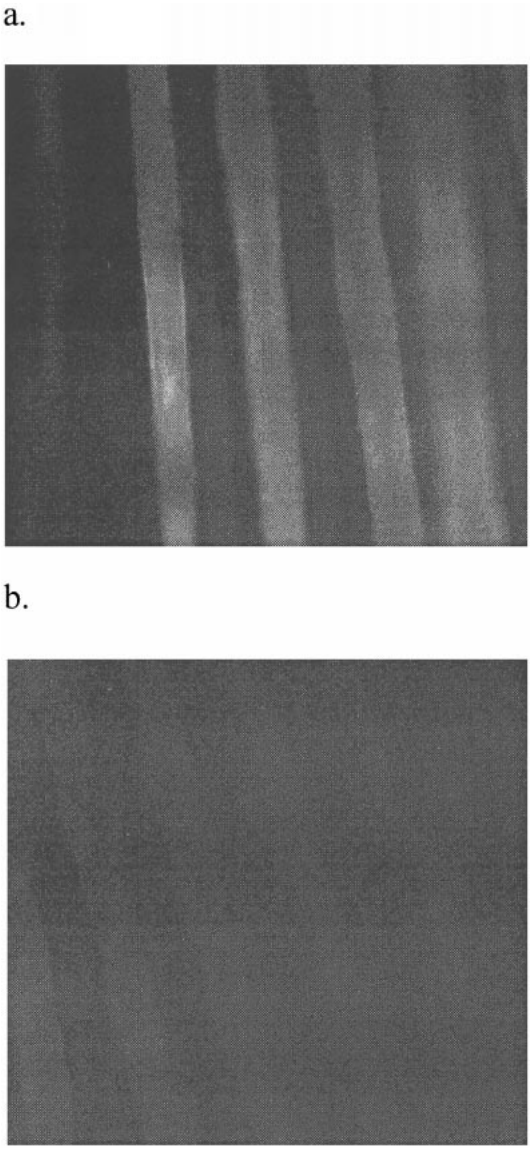

FIGURE 2.—Black-and-white images showing the intensity of the calcein label in the tail fin of **(a)** a field-marked guppy, and **(b)** a field control or unmarked guppy. These images were taken at 200× magnification with a standard epifluorescence setup (excitation at 495 nm, emission at 535).

Portable Calcein Detector

The detector worked best when used in a dark room. Newly marked guppies fluoresced throughout their bodies. After 2 weeks, calcein in the scales was not as apparent, but the entire skeleton fluoresced. Most marks could still be detected after 5 weeks. A small percentage (<1%) of individuals did not appear to be marked after 2 weeks; however, upon examination with an epifluorescent microscope, a very faint mark could still be detected. Therefore, the new detector was not as powerful as the standard setup.

It was easier, however, to use the new detector on live fish than to use an epifluorescent microscope on ethanol-preserved fish. A fish preserved in ethanol emits a bright yellow autofluorescence when viewed under an epifluorescent microscope. When a calcein-marked fish preserved in ethanol is viewed, the coloration is bright yellow-green. As a result, detecting a calcein mark on a preserved specimen poses the difficulty of distinguishing between yellow and yellow-green. With live fish, one needs only to determine the presence or absence of the green fluorescence.

Discussion

This study further establishes the suitability of using calcein as a chemical marker in population studies, and the new detector provides a relatively low-cost (approximately $500), portable alternative to standard epifluorescent microscopy. Calcein produces a distinct mark on specimens, which can be readily observed without harming the fish. There is no indication that immersion of juvenile *H. formosa* in calcein at the concentrations used in this study affects their survival, growth rates, or age and size at maturity. The sizes at maturity were within the range of the smallest mature individuals (an estimate of the size at maturity) observed in the field over the course of 2 years of sampling (Leips 1997 and unpublished data).

The calcein mark appeared to fade over time in some individuals, so treated fish should be checked at regular intervals (every 2–3 weeks) and remarked, if needed, through subsequent immersion. Although the reason for this fading is not known, calcein is a derivative of fluorescein, which experiences photobleaching. Photobleaching can occur if the dye is exposed to intense light and oxygen-free radicals are present. Once this occurs, the dye's ability to be excited is lost. To minimize this possibility, preserved specimens that will be checked for the mark at a later time should be stored in the dark.

A calcein solution between 125 and 250 mg/L is an effective concentration range for many fish species (Wilson et al. 1987; Monaghan 1993, Mohler 1997). Effective concentrations for calcein are generally lower than those for oxytetracyline hydrochloride (tetracycline), which vary between 50 and 500 mg/L (Hettler 1984; Brooks et al. 1994, Bumguardner and King 1996). Tetracycline, which

is also used as a chemical marker in fish, is often compared to calcein when testing for the effectiveness of these chemicals as nonlethal tags. Tetracycline has been a successful nonlethal marker for Pacific salmon *Oncorhynchus* spp. (Weber and Ridgway 1967), American shad fry *Alosa sapidissima* (Lorson and Mudrak 1987), larval and juvenile striped bass *Morone saxatilis* (Bumguardner and Colura 1991; Secor et al. 1991, Bumguardner and King 1996) and proved to be a better marker than calcein for larval walleyes *Stizostedion vitreum*, which experienced mortalities when immersed in calcein (Brooks et al. 1994).

The use of calcein as a chemical marker may be preferred over tetracycline because, for many species of fish, it produces a detectable mark without apparently affecting their health. While tetracycline marks can persist for up to 10 years after marking (McFarlane and Beamish 1995), it has been reported to cause deleterious effects, such as increased mortality, decreased activity, and reduced appetite in two Australian fish species (Whitley's silverside *Hypoatherina tropicalis* and blue sprat *Spratelloides delicatulus*; Schmitt 1984), red drum and Atlantic croaker (Wilson et al. 1987), and summer flounder (Monaghan 1993). Unlike calcein, tetracycline is an antibiotic that might also potentially enhance growth (see Weber and Ridgway 1967). If these effects are significant over the time course of observation, they may bias the estimates of growth and survival rates obtained from marked individuals and thereby limit the inferences that can be made about individual or populations in nature. An additional safety benefit of the use of calcein is that the calcein mark can be viewed under visible blue light, whereas tetracycline requires the use of ultraviolet light.

The chemical concentrations and immersion times that will produce the best results differ among species (Wilson et al. 1987; Mohler 1997). Therefore, before the application of calcein in a specific study, it should be tested for any adverse (or even potentially beneficial) effects on the species in question, followed by a study to determine the ideal concentrations and exposure times required to produce a nonlethal mark. In addition, if calcein-marked fish are to be released in the waters of the United States, calcein must go through the approval process from the Federal Food and Drug Administration. This process includes gathering data under an investigational new animal drug (INAD) exemption to obtain an approved new animal drug application (NADA). At this time, calcein is not the subject of an approved NADA.

Acknowledgments

We thank N. Martin for help with the least killifish experiment and K. Riddle for help with the epifluorescence microscope at Florida State University. Thanks to M. Bryant for suggesting that we make a portable calcein detector and for encouraging us throughout the process. D. DeMason provided access to an epifluorescent scope at University of California, Riverside. R. Schnick at the Federal Food and Drug Administration provided us with legal information regarding the use of chemical tags in U.S. waters. S. Rollmann and three anonymous reviewers made suggestions that greatly improved the manuscript. Research was supported by the National Science Foundation (DEB 92-20849 awarded to J.T.), Sigma Xi Grants-in-Aid of Research (to J.L.), the Natural Sciences and Engineering Research Council and the Center for Population Biology at the University of California at Davis postdoctoral fellowships (to F.H.R.), and the University of Toronto (to F.H.R.). Financial support for the development of the detector was provided by National Science Foundation DEB-9801492 awarded (to D.N.R. and F.B.).

References

Beckman, D. W., C. A. Wilson, F. Lorica, and J. M. Dean. 1990. Variability in incorporation of calcein as a fluorescent marker in fish otoliths. American Fisheries Society Symposium 7:547–549.

Brooks, R. C., Heidinger, R. C., and C. C. Kohler. 1994. Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. North American Journal of Fisheries Management 14:143–150.

Bumguardner, B. W., and R. L. Colura. 1991. Chemical marking of fingerling striped bass otoliths. Texas Parks and Wildlife Department, FWD, Management Data Series 64, Austin, Texas.

Bumguardner, B. W., and T. L. King. 1996. Toxicity of oxytetracycline and calcein to juvenile striped bass. Transactions of the American Fisheries Society 125: 143–145.

Constantz, G. D. 1984. Sperm competition in Poeciliid fish. Pages 465–485 *in* R. L. Smith, editor. Sperm Competition and the Evolution of Animal Mating Systems. Academic Press, Millbrae, California.

Fraser, E. A., and R. M. Renton. 1940. Observation on the breeding and development of the viviparous fish, *Heterandria formosa*. Quarterly Journal of Microscopical Science 81:403–413.

Hales, L. S., Jr. and D. H. Hurley. 1991. Validation of daily increment formation in the otoliths of juvenile

silver perch, *Bairdiella chrysoura*. Estuaries 14: 199–206.

Hettler, W. F. 1984. Marking otoliths by immersion of marine fish larvae in tetracycline. Transactions of the American Fisheries Society 113:370–373.

Leips, J. W. 1997. Genetic differentiation in life history traits: influence on population dynamics in the least killifish, *Heterandria formosa*. Doctoral dissertation. Florida State University, Tallahassee, Florida.

Leips, J., Travis, J., and F. H. Rodd. 2000. The effects of genetic differentiation in life-history traits on population characteristics. Ecological Monographs 70:289–309.

Lorson, R. D., and V. A. Mudrak. 1987. Use of tetracycline to mark otoliths of American shad fry. North American Journal of Fisheries Management 7:453–455.

McFarlane, G. A., and F. W. H. Beamish. 1995. Validation of the otolith cross-section method of age determination for sablefish (*Anoplopoma fimbria*) using oxytetracycline. Pages 319–329 *in* D. H. Secor, J. M. Dean, S. E. Camapana, editors, A. B. Miller, associate editor. Recent Developments in Fish Otolith Research. University of South Carolina Press, Columbia.

Mohler, J. W. 1997. Immersion of larval Atlantic salmon in calcein solutions to induce a non-lethally detectable mark. North American Journal of Fisheries Management 17:751–756.

Monaghan, J. P., Jr. 1993. Comparison of calcein and tetracycline as chemical markers in summer flounder. Transactions of the American Fisheries Society 122:298–301.

Reznick, D. N., Butler, M. J., Rodd, F. H., and P. Ross. 1996. Life history evolution in guppies: VI. Differential mortality as a mechanism for natural selection. Evolution 50:1651–1660.

Rodd, F. H., and D. N. Reznick. 1991. Life history evolution in guppies: III. The impact of prawn predation on guppy life histories. Oikos 62:13–19.

SAS Institute Inc. 1989. SAS/STAT User's Guide, Version 6, 4th edition, volume 2. Cary, NC.

Schmitt, P. D. 1984. Marking growth increments in otoliths of larval and juvenile fish by immersion in tetracycline to examine the rate of increment formation. Fishery Bulletin 82:237–242.

Secor, D. H., White, M. G., and J. M. Dean. 1991. Immersion marking of larval and juvenile hatchery-produced striped bass with oxytetracycline. Transactions of the American Fisheries Society 120:261–266.

Weber, D., and G. J. Ridgway. 1967. Marking Pacific salmon with tetracycline antibiotics. Journal of Fisheries Research Board of Canada 24:849–865.

Wilson, C. W., Beckman, D. W., and J. M. Dean. 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. Transactions of the American Fisheries Society 116:668–670.

Zar, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Inc., New Jersey.