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FAILURE OF TETRACYCLINE AS A BIOMARKER IN BATCH-MARKING JUVENILE FROGS

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ABSTRACT: Recent widespread amphibian declines call for better techniques to assess population dynamics. Tetracycline as a biomarker in capture-recapture studies is one technique used successfully in fish, reptiles, and mammals. A two-phase experimental study was conducted to evaluate tetracycline as a biomarker in green frogs (*Rana clamitans*) and pickerel frogs (*Rana palustris*). In the first experimental phase tadpoles were exposed to water containing either 250 mg/l or 500 mg/l tetracycline for a period of 24 hr. During the second phase, juvenile frogs were exposed to tetracycline in water at 500 mg/l or given injections of tetracycline at the dose rate of 100 mg/kg body weight. At selected times several weeks later, under tricaine methanesulfonate anesthesia, a toe was surgically excised from each animal, sectioned and viewed under an ultraviolet microscope. No significant differences were found between the various treatments and control animals (untreated). Therefore, the use of tetracycline as a biomarker in anurans using these techniques is not recommended.

Key words: Anuran, biomarker, green frog, pickerel frog, *Rana clamitans, Rana palustris,* tetracycline.

INTRODUCTION

Widespread population declines in many amphibian species, particularly anurans (Lips, 1998; Morell, 1999; Houlahan et al., 2000), demonstrate the need for better estimates of survival rates and population sizes. These parameters are required for assessing population dynamics using methods such as population viability analyses (Beissinger and Westphal, 1998). Various techniques have been developed to mark larvae and postlarval juveniles of anurans (Elmberg, 1989; Rice et al., 1998; Anholt et al., 1998). First-year survival rates for amphibians are difficult to determine because of the challenge of permanently marking juveniles. Toe-clipping in young frogs and toads can be stressful because of their small size, could cause an infection, and may not work because of potential regeneration of digits.

Several tetracycline compounds have been used as biomarkers in the bony structures of mammals (Milch et al., 1957) and to batch-mark larval fish (Hettler, 1984; Dabrowski and Tsukamoto, 1986; Secor et al., 1991a; Wastle et al., 1994). Although individual, unique marks or tags are more accurate for estimating survival based on capture-recapture methods, batch-marking a sample of individuals and releasing them back into the population also may be used to estimate this parameter (Burnham et al., 1987).

Tetracycline permanently marks the bone structure of vertebrates, particularly the otoliths of fish. Marks can be read by sectioning and polishing the otolith and viewed as characteristic yellow fluorescence under an ultraviolet microscope (Secor et al., 1991a). Although some incidences of increased mortality have been reported in fish dosed with tetracycline (Kobayashi et al., 1964), low doses generally cause few harmful effects, and may even reduce mortality (Weber and Ridgway, 1967).

Amphibians deposit bone layers (Smirina, 1972) in a similar manner to that which allows marking with tetracycline in fish. We tested various dosages and exposure times for tetracycline as a biomarking method for amphibians by exposing metamorphosing tadpoles of green frogs (*Rana clamitans*) and pickerel frogs (*Rana palustris*).

MATERIALS AND METHODS

Green frog tadpoles were obtained in September, 1996, from free-ranging stock found in experimental ponds on the Patuxent Wildlife Research Center in Laurel (Maryland, USA; 39°3′N, 76°49′W). Pickerel frog tadpoles were hatched from eggs collected in May 1996, from free-ranging stock found in a backyard fish pond, also in Laurel. Tadpoles were initially raised in aquaria and fed a mixture of Romaine lettuce, spinach, and tadpole chow (National Academy of Sciences, 1974). All water used for the frogs came from wells used as a source of drinking water on the Center and was aged at least 1 day before use. Green frog tadpoles were moved to a larger, shallow plastic pool as they increased in size.

The study was conducted in two phases (experiment 1 and experiment 2). In experiment 1, tadpoles were assigned to one of three treatments: 0 mg/l, 250 mg/l or 500 mg/l of oxytetracycline (OTC, Medamycin 100, Fermenta Animal Health, Kansas City, Missouri, USA). To avoid potential effects due to delayed metamorphosis, every third tadpole that metamorphosed was randomly assigned to one of the three treatments. Tadpoles were placed in individual 946 ml mason jars (Kerr Group, Inc., Los Angeles, California, USA; Alltrista Corp., Muncie, Indiana, USA), with just enough treatment solution to cover the tadpole (30 ml for green frogs, 15 ml for pickerel frogs), and were left in solution for 24 hr. Each jar was then thoroughly cleaned, and replaced with aged tap water. After 2 days, damp sphagnum moss was added to allow froglets to emerge from the water. The moss was replaced every two weeks. Jars were placed on their side to provide more surface area for each developing froglet. Feeding was begun following metamorphosis using flightless fruit flies or crickets (Fluker Farms, Port Allen, Lousiana, USA) twice weekly. To prevent calcium and vitamin deficiencies, crickets were dusted with Vionate vitamin mineral powder (Henry Schein, Inc., Melville, New York, USA) or Herpcare calcium and vitamin D3 supplement (Mardel Laboratories, Inc., Harbor City, California, USA). Natural bone meal tablets (Henry Schein, Inc., Melville, New York, USA) were added to all water (1 tablet per 4 l) used to moisten the moss.

At 2 and 4 wk post treatment, one randomly selected toe of each frog was excised and stored in 70% ethyl alcohol in the dark. The foreleg thumbs and hindleg long toes were never removed as these toes have been shown to be critical for survival (Heyer et al., 1994). To select the toe to remove, we used a random numbers generator to randomize the order of the toes on each frog, not including the foreleg thumbs and hindleg long toes. Each time a toe was to be removed from a given frog, we removed the toe at the top of the randomized list for that frog, thus insuring random selection of the toes to be removed from each frog.

Initial microscopic observations of the 2 wk toe samples indicated no difference in detection of fluorescence between control and treated groups (see results below). This was believed to be due either to the use of the more diluted formulation of veterinary grade OTC or to the froglets inability to absorb the chemical from solution. To test these hypotheses, a second phase of the study was conducted (experiment 2). Previously treated (control or veterinary grade OTC) green and pickerel frogs were randomly assigned to one of the following: control (no OTC exposure); dermal OTC exposure for 6 hr in 500 mg/l of a more efficacious (Frazier, 1985a) research grade tetracycline hydrochloride crystalline (Sigma Chemical Co., St. Louis, Missouri, USA); intramuscular injection with 100 mg/kg body weight of OTC; or intramuscular injection with 100 mg/kg body weight of the research grade tetracycline. Only green frogs were given the intramuscular injections of the veterinary grade of OTC due to the limited numbers of pickeral frogs available. Injection would probably not be a viable method for batch-marking small frogs or toads due to the time involved in individually handling each animal. However, for the purpose of this research, the intramuscular injections were given to optimize the opportunity for discernable markings. The green and pickerel frogs were approximately 4 to 6 mo post metamorphosis for experiment 2; ages varied because tadpoles had metamorphosed over a wide time frame. Two wk post treatment, a randomly chosen toe was excised from each frog and preserved.

For toe removal, each frog was individually anesthetized in a bath of tricaine methanesulphonate (MS-222, Finquel, Argent Chemical Laboratories, Redmond, Washington, USA), a water-soluble white powder that, when diluted in water buffered with sodium bicarbonate, is absorbed through the skin of frogs, producing a surgical anesthetic plane (Crawshaw, 1993; Wright, 1996). A 1:2,000 dilution of MS-222 in water buffered with sodium bicarbonate resulted in ataxia in 2 to 3 min, and a surgical plane of anesthesia within 5 to 8 min. The appropriate randomly selected toe was excised using sterile ophthalmic surgical instruments. Any hemorrhage was controlled with electrocautery. Frogs were revived in aged tap water covering the body up to the head (with water changes every 5 min) until they recovered to a sitting

TABLE 1. Number of green and pickerel frogs scored with 0–4 positive detections of fluorescence out of a total of 4 scorers in experiment 1.

Number of positive detections	Treatment (dermal exposure)					
	0 mg/l OTC		250 mg/l OTC		500 mg/l OTC	
	Green	Pickerel	Green	Pickerel	Green	Pickerel
0	2	0	3	0	1	0
1	0	1	1	0	0	0
2	1	0	1	0	3	0
3	2	0	1	1	2	1
4	3	0	3	1	4	1

posture which usually occurred within 10 to 20 min.

After excision, each toe was stored in 70% ethyl alcohol and kept in a dark location. Toes were subsequently imbedded in Spurr epoxy resin (Electron Microscopy Sciences, Ft. Washington, Pennsylvania, USA). The epoxy block was cut into thin pieces using a jeweler's saw (Jeweler's Saw 7043, X-acto, Hunt Manufacturing Co., Statesville, North Carolina, USA), producing cross sections of the toe. Each epoxied piece was then glued (Crystalbond 509, Aremco Products, Inc. Ossining, New York, USA) to a 27 × 46 mm microscope slide (#2746, Ward's, Rochester, New York, USA), sanded with 300 and 600 grit paper and polished with 0.3 micron alumina polishing powder (Buehler Ltd., Lake Bluff, Illinois, USA), following the methods of Secor and Dean (1989).

Cross sections of the toes were viewed using a Zeiss Axiovert 135 Inverted Microscope with epifluorescence (Carl Zeiss, Inc., Thornwood, New York, USA). The light source was a mercury vapor, HBO 50, arc lamp. Ultraviolet emission range used was 390 nm (absorbance 390, excited 560) with 100× magnification. A blue filter helped screen out autofluorescence. Toes were evaluated and scored by four of the authors independently.

Results were analyzed using exact, nonparametric contingency table analyses, with separate analyses for experiments 1 and 2 (Lehmann, 1975). The frequency of 0–4 positive detections by scorers was calculated for each treatment level and each experiment. Fisher's exact test (FE) (Lehmann, 1975) was used to test for the independence in the number of positive detections (0–4) per toe versus treatment level. The Jonckheere-Terpstra test (JT) (Lehmann, 1975) was used to test for a trend in the treatments (i.e., more positive detections in higher treatment levels) in the order that the treatments are presented in their descriptions above. STATXACT software (Cytel Software Corp., Cambridge, Massachusetts, USA) was used to perform these tests. The JT test would be more powerful than the FE test for detecting differences among treatments in the number of 0–4 positive detections of fluorescence, if there were indeed a trend in the data. One-sided tests were used for the JT test, to give more power for detecting upward trends. We also used the JT test to test for differences in frog survival among the three treatments in experiment 1 for the first 2 wk post treatment.

RESULTS

The OTC exposure in the first experiment caused no significant mortality increase in treated frogs versus controls. In fact, we found significantly higher mortality (JT test, $P = 0.0172$, $n = 57$) up to age 2 wk in the control group of green frogs (32% mortality, $n = 22$) versus the two treatment levels (12% mortality for 250 mg/l OTC, $n = 17$; 6% mortality for 500 mg/l OTC, $n = 18$). No mortality was observed in the pickerel frogs ($n = 7$) during the first 2 wk of experiment 1 or in the green frogs during experiment 2 ($n = 27$).

There were no significant differences among treatments for green frogs in the number of positive detections (0–4) of fluorescence per toe in either experiment 1 (Table 1; FE test, $P = 0.8892$; JT test, $P = 0.3541$, $n = 27$) or experiment 2 (Table 2; FE test, $P = 0.5452$; JT test, $P = 0.4406$, $n = 27$). Similar negative results were obtained for pickerel frogs for experiment 1 (Table 1; FE test, $P = 1.000$; JT test, $P = 0.2333$, $n = 5$) and experiment 2 (Table 2; FE test, $P = 0.6000$; JT test, $P = 0.5000$, $n = 5$) although the lack of sta-

TABLE 2. Number of green and pickerel frogs scored with 0–4 positive detections of fluorescence out of a total of 4 scorers in experiment 2.

Number of positive detections	Treatment							
	0 mg/l OTC		Dermal exposure of 500 mg/l OTC		Injection of veterinary grade OTC[a]		Injection of research grade OTC	
	Green	Pickerel	Green	Pickerel	Green	Pickerel	Green	Pickerel
0	0	0	0	0	0	—	1	0
1	0	0	0	0	0	—	1	0
2	3	0	1	0	0	—	1	0
3	1	1	1	0	3	—	1	1
4	3	0	5	2	2	—	4	1

[a] Pickerel frogs were not given this treatment due to the small number of pickerel frogs available.

tistical power due to the small sample size of pickerel frogs is a problem.

DISCUSSION

We exposed green and pickerel frogs to various levels of two different tetracycline products and used two different routes of administration (immersion or intramuscular injections). In experiment 1, with dermal exposure to veterinary grade OTC solution, route of exposure and timing were selected to maximize marking under field conditions. Amphibians absorb a large percentage of their required water dermally. The vascularized ventral pelvic regions of many anurans are sites for increased water uptake and are important rehydration areas during a frog's behavioral water absorption response (Duellman and Trueb, 1986). Exposing metamorphs based on similar appearance of the forelimbs minimized potential sources of variability between animals due to differences in developmental stage or growth. A 24 hr exposure time should take into account the cyclic periods of maximal water uptake by an animal, be it morning or afternoon. Dermal exposure was considered the least stressful method of treating a frog or toad in the field.

By the time of experiment 2, the frogs were 4 to 6 mo post metamorphosis. If experiment 1 failed because frogs had not taken up the OTC because of a hyperosmotic problem (calcium binding is greater in surrounding solution) or because of the weaker veterinary grade formulation, then the redefined hypothesis was that the more direct exposure by injection and use of a higher grade technical formulation of tetracycline hydrochloride would overcome problems in detecting fluorescence.

However, even after this second experiment, frogs that were definitely exposed to some form of tetracycline still had inconclusive results. Neither trial produced distinct, discernable labels in the bone cortex. Among the non-treated frogs, some autofluorescence was detected, producing false positives that render the technique useless for capture-recapture population studies. Among known treated frogs, scorers were still unsure whether what was observed was autofluorescence or a weak positive response. Based on this more complete picture, we concluded that the use of tetracycline is not effective for field use in first-year anurans.

This marking technique has been extensively applied in fish (Secor et al., 1991a; Wastle et al., 1994), reptiles (Frazier 1985b), and mammalian batch-marking (Frazier 1985a), and in studies using frogs (Smirina, 1972; Frazier, 1985a). However, the method is not without problems (Dabrowski and Tsukamoto, 1986; Koenings et al., 1986). Treated salmon do not always fluoresce under ultraviolet light, and a certain percentage of untreated fish have autofluoresence. Koenings et al. (1986) exposed fish to OTC, and concluded that marking was successful only at a certain

development stage. In other studies variability in results was attributed to insufficient exposure time (Bilton, 1986; Ruhlé and Grieder, 1989; Secor et al., 1991b) or differences in scoring the fluorescence (Rien and Beamesderfer, 1994).

In fish studies, skeletochronology and fluorescent markings depend on the marking and growth of a tissue such as the otolith. Unlike the otolith bone tissues which do not metabolize or metabolize very slowly, growing appendages and bones such as phalanges can be subjected to resorption (Kusano et al., 1995). In our study, toes to be clipped were randomly selected to avoid picking a toe that would not absorb the tetracycline.

However, there is still a need to be able to mark young frogs and toads in the field for population studies. We found a significant, albeit slight, beneficial effect of the OTC at these doses on survival, indicating that tetracyclines may not cause noticeable harm. Other available tetracyclines may be more tissue specific, or less prone to metabolism (Koenings et al., 1986). Dyes or flurochromes such as calcein, alizarin, and even tetracyclines such as OTC dehydrate may be better suited for anurans, and could be tested (see Frazier, 1985a; Koenings et al., 1986; Alcobendas et al., 1991; Monaghan, 1993), as could methods to increase uptake and binding. In the metamorph, the forelimb is already formed as a bud (Duellman and Trueb, 1986). Perhaps exposure either earlier in development or in the late tadpole phase would maximize calcium binding (Dabrowski and Tsukamoto, 1986; Ruhlé and Grieder, 1989; Ruhlé and Winecki-Kühn, 1992). Alcobendas et al. (1991) exposed fish eggs to hyperosmotic solution for a limited time, then to fluorochrome to maximize uptake. Given the sediment nature of some of the water bodies in which frogs and toads breed, this experimental exposure may well be tolerated in amphibians as well. Finally, methods to digitize fluorescence and record results with a computer may help better evaluate the incidence of fluorescence.

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