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EVALUATING A NOVEL TECHNIQUE FOR INDIVIDUAL IDENTIFICATION OF ANURAN TADPOLES USING CODED WIRE TAGS

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***Abstract.*—The ability to mark individuals and measure their performance and survival is an extremely valuable tool for studying the behavior, conservation, ecology, and evolution of reptiles and amphibians. However, there are currently few methods available to individually mark and identify anuran tadpoles. Here, I evaluate a novel technique for individually marking anuran tadpoles. I individually marked Mexican Spadefoot Toad (*Spea multiplicata*) tadpoles in the ventral tail membrane using coded wire tags. I then followed the growth, development, and survival of marked and unmarked tadpoles as well as tag retention for eight days in experimental mesocosms or until tadpoles reached metamorphosis (i.e., Gosner stage 42). Survival, size, and development (i.e., proportion of tadpoles reaching metamorphosis within 8 days) did not differ between marked and unmarked tadpoles. In addition, the coded wire tag was clearly visible in marked pre-metamorphic tadpoles, and in many individuals was retained well into metamorphosis. However, 20% of the marked tadpoles lost their tag by the end of the study. In general, the results of this study suggest that given the millions of unique identifications possible with coded wire tags, they are best suited for studies where large numbers of individuals need to be individually identified. Future studies should evaluate whether tag retention can be improved by injecting coded wire tags in different locations.**

Key Words.—anurans; individual identification; marking; microtags; *Spea multiplicata*; tadpoles

INTRODUCTION

The ability to mark individuals is an extremely valuable tool for studying the behavior, conservation, ecology, and evolution of reptiles and amphibians. As a result, there are many different techniques available for individually marking reptiles and amphibians (reviewed in Ferner 2007). However, because of their generally small size, fragility, and rapid development, there are relatively few techniques available for individual identification of anuran tadpoles (Donnelly et al. 1994; McDiarmid and Altig 1999; Ferner 2007).

An ideal marking technique should be permanent and have no impact on survivorship, performance, and behavior (Ferner 2007). In addition, useful marks and tags should be easily identifiable, usable across a large range of organism sizes, and relatively inexpensive (Ferner 2007). Few, if any marking techniques meet all of these criteria in practice. Nevertheless, non-ideal marking techniques can be useful if their shortcomings can be accounted for in a particular study. Current methods described in the literature for marking anuran tadpoles include whole body staining, tail clips, tail tags, and injected pigmented and/or fluorescent tags (for a review of marking techniques available in anuran larvae see Donnelly et al. 1994; Ferner 2007; Skelly and Richardson 2010). Of these methods, staining is likely the simplest method, and appears to cause relatively little mortality (Guttman and Creasy 1973; Travis 1981; Semlitsch and Kahli 2001). However, staining is temporary (Guttman and Creasy 1973; Travis 1981; Semlitsch and Kahli 2001), and has been shown to detrimentally affect growth rates under some conditions (Travis 1981). In addition, with only two available dye colors, its usefulness for individual identification is limited (Semlitsch and Kahli 2001). Similarly, tail clips are not useful for individual identification, and cause higher mortality than staining (Guttman and Creasy 1973). In contrast, tail tags as described by Rice and colleagues (1998) and injections of pigmented and or fluorescent marks (Seale and Boraas 1974; Cecil and Just 1978; Anholt et al. 1998; Grant 2008) allow for individual identification of numerous subjects. While tail tags cause little mortality and have high retention rates (Rice et al. 1998), Ferner (2007) suggests they may be best suited for use in laboratory research because of the risk of the tag snagging on objects in more complex natural environments. Several methods have been developed to inject pigmented and/or fluorescent marks under the skin of larval anurans, including tags composed of visual implant elastomer (VIE; Northwest Marine Technologies Inc., Shaw Island, Washington, USA; Anholt et al. 1998; Grant 2008), acrylic (Cecil and Just 1978), and organic dye-solvents (Seale and Boraas 1974). These methods appear to have little to no effect on survival, growth, or development. For each of these methods, however, the number of possible color and/or fluorescent combinations that can be used limits the

number of unique marks. For example, Cecil and Just (1978) used three color combinations of acrylic pigments to make six unique marks, and Anholt and colleagues (1998) used five color combinations and three marks per individual to uniquely mark 80 individuals. Both acrylic (Cecil and Just 1978) and organic dye-solvent marks had retention rates of 100% (Seale and Boraas 1974). In contrast, Anholt et al. (1998) found that 85% of individuals marked with VIE were identifiable after eight days. Furthermore, Grant (2008) found that 50% of tadpoles tagged with VIE lost one of two marks after 20 days.

In this study, I test the suitability of coded wire tags (CWT; Northwest Marine Technology Inc., Shaw Island, Washington, USA) for marking anuran larvae. As described by Jefferts et al. (1963), CWT are made of magnetized stainless steel wire, 0.25 mm in diameter, and can be cut as small as 0.5 mm in length. Each tag is etched with a number sequence that can be used for either batch identification or millions of unique individual identifications, when viewed under a low-powered microscope (20–40x magnification). Coded wire tags are injected, and tagged individuals are later identified by magnetic detection. Coded wire tags have been widely used in a variety of organisms including fish (Bergman et al. 1968), turtles (Schwartz 1981), lizards (Downes 2000), and metamorphic toads (Sinsch 1997), but to my knowledge their suitability for use in studies of larval amphibians has not been evaluated. The possible advantage of CWT over the other marking methods described above is the ability to uniquely tag a much greater number of individuals with a single mark.

MATERIAL AND METHODS

To test the suitability of CWT for marking anuran larvae, I evaluated CWT retention and compared the body size, development, and survival of marked and unmarked Mexican Spadefoot Toad (*Spea multiplicata*, family Scaphiopodidae) tadpoles in experimental mesocosms over the course of eight days. I collected 200 (Gosner stages ~33–35; Gosner 1960) *S. multiplicata* tadpoles from an ephemeral pond near Portal, Arizona, two weeks after a breeding chorus. I kept the tadpoles in a wading pool (1.5 m diameter × 0.25 m tall) filled with dechlorinated well water, which I set in an open field at the Southwestern Research Station (SWRS, American Museum of Natural History) near Portal, Arizona. After placing the tadpoles in the wading pool, I fed them shredded lettuce *ad libitum*. The day following their collection, I randomly chose 120 tadpoles from the wading pool and randomly assigned half to be marked with CWT and half to be unmarked controls. I used a single-shot injector (Northwest Marine Technologies Inc., Shaw Island, Washington, USA) to inject a pre-cut and magnetized CWT (1.1 mm long, 0.25 mm in diameter) into the ventral tail membrane of each tadpole assigned to the marked treatment (Fig. 1). In addition, I measured each tadpole's body size (snout-vent-length, SVL), from both the marked and unmarked treatments, using digital calipers. An analysis of variance revealed that the tadpoles used in the experiment did not differ in initial size across treatments ($F_{1,104}$ = 0.52, P = 0.47; mean SVL ± SD: marked = 17.99 ± 1.22 mm, unmarked = 17.81 ± 1.13 mm). I then released the tadpoles into two experimental mesocosms (1.5 m diameter × 0.25 m high), with 25 marked and 25 unmarked tadpoles assigned to each mesocosm. Five of the remaining 20 tadpoles were used to replace experimental tadpoles that died on the first day of the experiment (see below). I placed the remaining 15 tadpoles back into the wading pool with the 80 other unused tadpoles. I fed these tadpoles shredded lettuce *ad libitum* until the end of the experiment, at which point they were released back to their natal pond.

FIGURE 1. Preserved *Spea multiplicata* tadpole marked in the ventral tail membrane with a coded wire tag (indicated by arrow). (Photographed by Ryan Martin).

Two days prior to initiating the experiment, I had filled the bottom of each mesocosm to a depth of approximately 10 cm with 13 L of soil collected from a dry pond in which *S. multiplicata* typically breed. I then filled the mesocosms to a depth of 25 cm with dechlorinated well water. The soil served to provide the tadpoles with resources found in natural ponds such as detritus, algae, and fairy shrimp (order Anostraca; Pfennig et al. 2006). I allowed potential natural predators of *S. multiplicata* tadpoles, such as dragonfly larvae (order Odonata), beetle larvae (order Coleoptera) and giant water bugs (family Belostomatidae) to colonize the mesocosms. I checked the mesocosms for any mortality three times a day for eight days. I removed any tadpoles I found dead from the mesocosms and preserved them in 95% ethanol. After the first inspection for mortality in the mesocosms on the first day of the experiment, I replaced the dead tadpoles (four marked and one unmarked tadpole) with new tadpoles from the same treatment. I did not replace dead tadpoles found on later inspections with new, live tadpoles.

FIGURE 2. A live metamorphosing *Spea multiplicata* that was marked at the beginning of the study in the ventral tail membrane with a coded wire tag (indicated by arrow and oval). (Photographed by David Pfennig).

I ended the experiment eight days after it began by removing every remaining tadpole from both mesocosms. I euthanized the tadpoles by immersion in a 0.1% aqueous solution of tricane methanesulfonate and preserved them in 95% ethanol. I then checked each preserved tadpole for the presence of a CWT and measured each tadpole's SVL using digital calipers. In addition, I removed live metamorphic tadpoles from the mesocosms as they appeared during the course of the experiment (identified by the initial emergence of a front limb: Gosner stage 42; Gosner 1960). I maintained them in a plastic terrarium filled with dechlorinated water and a sand beach until they were released to their natal pond at the end of the experiment. As I removed each metamorphic tadpole from a mesocosm, I checked for the presence of a CWT and immediately measured SVL using digital calipers and mass (after removal of excess water) with a digital balance.

I was able to identify tadpoles that had lost their tag from the incision made by the injector into their ventral tail membrane. I evaluated the retention of CWT by calculating the percentage of marked tadpoles that lost their tags. I then statistically evaluated if marking with CWT affected the survival, body size, and development of *S. multiplicata* using linear mixed models, and generalized linear mixed models. Development was represented as the proportion (*p)* of tadpoles reaching metamorphosis (i.e., Gosner stage 42) during the course of the experiment. I evaluated body size separately for pre-metamorphic tadpoles (i.e., Gosner stage ≤ 41) and metamorphic tadpoles (i.e., Gosner stage ≥ 42). First, I fitted separate linear mixed models to test if body size (SVL for pre-metamorphic tadpoles, SVL and mass for metamorphic tadpoles) differed between marked and unmarked treatments. Next, I fitted separate generalized linear mixed models to test if survival (including both those tadpoles that died on the first day of the experiment and their replacements) or development differed significantly between marked and unmarked treatments. I used generalized linear models to evaluate survival and development because they were measured as binomial responses (survival: alive/dead, development: pre-metamorphic tadpole/metamorphic tadpole), and therefore were best modeled using a binomial probability distribution and a logit link function. For all models, I fit marking treatment as a fixed factor, and the mesocosm identity as a random factor. I confirmed that the data met assumptions of normality for all tests (Shapiro-Wilk test: $P > 0.01$) and all statistical analyses were performed using R (version 2.9.2; R Development Core Team, 2009) with $\alpha = 0.05$.

RESULTS

I found that CWT were clearly visible in the ventral tail membrane of *S. multiplicata* in both preserved (Fig. 1) and live tadpoles. Magnification is needed to individually identify CWT, and I was unable to read many of the numerical sequence identification of the CWT while the tags were still implanted within the ventral tail membrane of either live or preserved tadpoles. In these cases, I had to remove the CWT from the ventral tail membrane before I could read its unique numerical sequence under a microscope. In addition, I found that CWT were retained and clearly visible in metamorphic tadpoles even after tail resorption had begun (Fig. 2).

The CWT had a retention rate of 80%. I recovered 43 pre-metamorphic or metamorphic tadpoles with coded wire tags out of the 54 marked tadpoles released into the two wading pools; 34 at the end of the experiment and nine that died during the course of the experiment. I identified 11 tadpoles that lost their tags.

Overall tadpole mortality was 13% (14 of 105). However, 36% of that mortality occurred within the first few hours of the experiment, likely due to handling. I found that survival (unmarked tadpoles = 90%, marked tadpoles = 83%) did not significantly differ between marked and unmarked tadpoles (Table 1). In addition, marked and unmarked pre-metamorphic tadpoles did not differ in body size (mean SVL ± SD: marked individuals = 19.95 ± 0.99 mm, unmarked individuals = 20.00 ± 1.22 mm) by the end of the experiment (Table 1). Similarly, marked and unmarked metamorphic tadpoles did not differ in either SVL (mean SVL ± SD: marked individuals = 17.51 ± 0.96 mm, marked individuals = 18.09 ± 1.38 mm) or mass (mean mass ± SD: marked individuals = 0.67 ± 0.13 g, unmarked individuals = 0.70 ± 0.11 g; Table 1). Finally, I found that the proportion of tadpoles reaching metamorphosis did not differ between the marked and unmarked treatments (Table 1, *p* marked treatment = 0.24, *p* unmarked treatment = 0.26).

TABLE 1. Summary of statistical models and results used to evaluate the effect of coded wire tags on the survival, growth, and development of *Spea multiplicata*. In all cases, I tested the null hypothesis that individuals marked with coded wire tags did not differ from unmarked individuals. Development is defined as proportion of tadpoles reaching metamorphosis (i.e., Gosner stage 42). Mass is in grams and abbreviations are n = sample size, df = degrees of freedom, and SVL = snout-vent length.

model	response	stage	probability distribution	link function	test statistic	n	df	*P*
linear mixed					F			
	SVL	pre-metamorphic tadpole	Gaussian	—	0.03	68	1,65	0.85
	SVL	metamorphic tadpole	Gaussian	—	1.37	23	1,20	0.26
	mass	metamorphic tadpole	Gaussian	—	0.54	23	1,20	0.47
generalized linear mixed					χ^2			
	survival	—	binomial	logit	1.08	105	1	0.30
	development	—	binomial	logit	0.03	91	1	0.85

DISCUSSION

Individual marking of organisms is a common tool in herpetological studies, yet few techniques are available for marking anuran tadpoles (Donnelly et al. 1994; McDiarmid and Altig 1999; Ferner 2007). The ability to assess growth and survival of anuran tadpoles would be advanced by the development of additional methods for easily and effectively identifying individual tadpoles in experimental and natural populations. My results suggest that CWT is a useful additional tool for marking and identifying anuran tadpoles. In particular, I found that CWT could be injected relatively quickly into *S. multiplicata* tadpoles using a single-shot injector (1–2 minutes per tadpole). Furthermore, I found that marking tadpoles did not significantly affect their survival, growth, or development in experimental mesocosms. In contrast, tag retention was not perfect in this study. Indeed, 20% of marked tadpoles lost their CWT. Any tag loss can result in biased estimates of survival, and population size in mark-recapture studies. However, these issues can be mitigated by large sample sizes, models that explicitly factor in some amount of tag loss, and double tagging individuals (McDonald et al. 2003). Therefore, it may be advisable to use CWT in conjunction with a second, visible tag to identify tadpoles whose CWT have been lost. Although I did not document exactly when CWT were lost in this study, it seems likely that they were lost soon after injection. Coded wire tag retention may be improved by holding tagged individuals for at least one day to remove tadpoles that have lost their CWT.

Marking techniques rarely meet all the criteria for an ideal tag (Ferner 2007). Other tadpole marking techniques have both strengths and weaknesses, and the choice of a marking technique will likely depend on the goals of a particular study. Because of its speed and simplicity (Guttman and Creasy 1973; Travis 1981; Semlitsch and Kahli 2001), whole body staining seems well suited for short-term studies where large numbers of tadpoles need to be marked quickly, and individual identification is not needed. Injected pigmented and/or fluorescent tags have smaller effects on growth and survival than staining and also allow for individual identification in the field (Seale and Boraas 1974; Cecil and Just 1978; Anholt et al. 1998; Grant 2008). Based on the results of my study, CWT has both advantages and disadvantages over other tadpole marking methods. First, with CWT it is possible to uniquely mark millions of individuals with a single tagging system. In contrast, the number of color combinations available and the number of locations a mark can be placed on an individual limit the number of unique identifications possible with injected pigmented and or fluorescent tags. Tag retention was 80% in my study and this retention rate was similar to (Anholt et al. 1998) and sometimes greater than (Grant 2008) the retention rates reported for VIE tags. In addition, unlike staining methods, CWT did not negatively affect growth or survival. However, CWT are likely not as visible in the field as either staining or injected pigmented tags. Furthermore, individual identifications are not possible from a distance and are often not possible without removing the tag. Finally, the cost of CWT is likely greater than that of other marking methods. For example, Hoffman and colleagues (2008) estimated the total cost of VIE tags at < $0.15 per mark. For CWT, with a manual injector and 200 tags, I estimated the average cost at $0.91 per individual for batch marking, and $1.35 per individual for unique identification (for individual identification every third tag must be archived for reference). However, the ability to uniquely mark individuals with a single tag helps mitigate this difference in price. Indeed, CWT seems best suited for studies where large numbers of individuals need to be uniquely identified.

It would be useful to further evaluate and ideally improve the use of CWT for marking anuran larvae in future studies. For example, because the loss of tags was not trivial in this study, the retention rates of CWT injected at different locations should be tested. One possibility is to inject CWT into the back legs of developing tadpoles, or into the base of the tail. However, injecting CWT into these locations may have

detrimental effects on performance. Therefore, directly testing the effect of CWT on tadpole swimming performance, predator escape, and competitive ability should be another focus of future research. Furthermore, because *S. multiplicata* develop rapidly, the duration of the present study was necessarily short. Experimental marking of species with slower-developing tadpoles is needed to assess CWT effectiveness for these species and to evaluate retention rates over longer periods of time. Finally, while my mesocosm results are informative, the suitability of CWT for marking anuran larvae should also be investigated in natural populations.

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RYAN MARTIN received his Ph.D. in biology from the University of North Carolina at Chapel Hill and is currently a Postdoctoral Research Associate in the Department of Biology at North Carolina State University. His research focuses on the ecological and evolutionary causes of phenotypic divergence. Ryan is shown collecting tadpoles from an ephemeral pond near Portal, Arizona. (Photographed by David Pfennig).