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Evaluation of a new technique for marking anurans

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Abstract

Amphibians can be very difficult to mark because of their extraordinary powers of regeneration. Although many amphibian marking techniques have been developed, few meet the rigorous assumptions of capture-mark-recapture models. Also, excessive toe-clipping may affect frog survivorship adversely. We tested the efficacy of a new hybrid marking technique (VIE-C) that combines Visible Implant Elastomer (VIE) and toe-clipping on four species of treefrogs in West-Central Florida. Of the 840 treefrogs recaptured over a 15-month period, only one mark was unreadable. A significantly higher percentage of VIE marks (80%) than toe-clips (55%) remained viable for the duration of the study. On average, toe-clips remained readable for 100 days, and VIE marks remained readable for 112 days. There were no significant species differences in the length of time that either type of mark lasted. The hybrid VIE-C method represents an improvement over either method used alone, but the VIE mark will be more helpful in correctly reading and clarifying toe-clipping errors than will toe-clips be helpful in reading and clarifying VIE marks.

Key words

Amphibian, Florida, mark-recapture, marking, toe-clip, treefrog, visible implant elastomer, VIE-C technique.

Introduction

Wildlife studies often require individual animals to be marked for future identification (Seber, 1982). Differentiating individuals allows biologists to estimate population size and density; calculate growth rate, survival, and other demographic parameters; study behaviors and activity patterns; and document movements, home range and habitat requirements. Wildlife managers monitor changes in these pa-

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rameters to manage game populations, reveal population trends in endangered species, document the effects of pollution or habitat destruction, and assess environmental restoration projects (Engeman, 2005). To meet the many assumptions of capture-mark-recapture (CMR) population models, marks must be unique, easy to interpret, long-lasting or permanent, and must not affect the behavior or survival of the animal being studied (Otis et al., 1978; Pollock et al., 1990).

Amphibians present special problems in studies where individual marks are required. Toe-clipping has frequently been used to mark amphibians; but, their regenerative abilities are well known, and rapid re-growth of clipped toes can result in loss of the mark (Donnelly et al., 1994; Halliday, 1996). From an ethical standpoint, this technique is controversial (Funk et al., 2005) and has even been deemed barbaric (May 2004). While some laboratory studies (Liner et al., 2007) and field studies (Ott and Scott, 1999) indicate toe-clipping has no effect on amphibian survival, others suggest toe-clipping causes inflammation (Golay and Durrer, 1994) and affects growth rate (Davis and Ovaska, 2001). Moreover, a direct relationship between the number of toes clipped and decreased return rates in frogs (McCarthy and Parris, 2004) suggests that a maximum of only one or two toes should be clipped from any individual — a significant constraint in amphibian population studies requiring thousands of animals to be marked with unique numbers.

Many alternatives to toe-clipping are available for marking amphibians, including freeze branding, wire or bead tags, and passive integrated transponder tags (Ferner, 2007; Phillott et al., 2007), but many of these techniques require expensive and cumbersome equipment. Visible Implant Elastomer (VIE) is a fluorescent rubber material that can be injected under the skin in various locations to create a unique mark (Northwest Marine Technology, 2000). This technique was originally developed for fisheries research but has been used successfully in many other taxa, including amphibians (Nauwelaerts et al., 2000). This method is relatively inexpensive, easily visible, long lasting and relatively simple to apply (Hoffmann et al., 2008); however, VIE marking requires extensive animal handling and accurate mixing of materials in the field under sterile or nearly sterile conditions (Phillott et al., 2007). Moreover, VIE marks injected under the skin of ranid frogs exhibited high rates of migration, resulting in the loss of frog identity in a high proportion of individuals (Moosman and Moosman, 2006). Thus, VIE marks are only a partial solution to myriad amphibian marking problems.

In response to the abovementioned issues, Hoffmann et al. (2008) developed an easy and inexpensive hybrid VIE-C technique that combines injections of VIE on the plantar surface of the toes of the rear foot with a single toe-clip, resulting in 1920 unique codes for each VIE color used. Their technique reduces the negative aspects of clipping multiple toes while also providing thousands of unique identifiers. They utilized this technique in population studies of four species of treefrogs captured in PVC pipes deployed in upland habitats (pine flatwoods) at two Central Florida sites between July 2006 and June 2007. Although Hoffmann et al. (2008) were not able to assess species differences due to sample size constraints, the marks were found to be easy to apply, easy to read, relatively inexpensive, and accurate over a 33-week study period.

Simultaneously, authors TSC, KRC, and SAJ initiated a field experiment to assess the effects of surrounding development (Delis et al., 1996), groundwater pumping (Guzy et al., 2006), and introduced species on native anurans. In this ongoing study, all captured treefrogs were given unique identifiers using the VIE-C technique to track their growth, reproductive output, and survival, and to generate population estimates and demographic parameters. Before initiating the experimental manipulations in our study, we assessed whether or not frogs were captured often enough to ensure the marks would remain viable and persisted for similar lengths of time in each species. The following analysis is a statistical evaluation of the VIE-C marking method (Hoffmann et al., 2008) applied over a 15-month period to four species of treefrogs inhabiting PVC pipes deployed in 33 isolated wetlands.

Materials and methods

This study was conducted at Morris Bridge Wellfield (MBWF), a 2000-ha natural area in northeastern Hillsborough County, Florida. The site consists of hundreds of isolated and contiguous hardwood swamps and freshwater marshes embedded in an upland matrix of mostly pine flatwoods habitat. Green treefrogs (*Hyla cinerea*), pinewoods treefrogs (*H. femoralis*), squirrel treefrogs (*H. squirella*), barking treefrogs (*H. gratiosa*), and Cuban treefrogs (*Osteopilus septentrionalis*) are abundant throughout the site along with numerous other anuran species (Delis et al., 1996; Guzy et al., 2006). Treefrog CMR studies were conducted in 33 hydrologically isolated wetlands (18 freshwater marshes and 15 cypress domes) at MBWF.

In May and June 2006, we inserted 1-m long, 5-cm diameter, Schedule 40 polyvinyl chloride (PVC) pipes into the ground at 20-m intervals around each wetland (600 pipes total) and left them undisturbed for at least two weeks so that the treefrogs could acclimate to the pipes (Boughton et al., 2000; Bartareau, 2004). We removed treefrogs from the pipes using a ‘plunger’ consisting of a snugly-fitting paint roller mounted on a stick. Treefrogs were held individually in quart plastic zipper bags and transported in coolers to a marking station, where they were measured, weighed and marked. We applied all marks using the VIE-C method (Hoffman et al., 2008), where a single toe was clipped at the proximal joint using sterilized surgical scissors, and a narrow cord of VIE was injected into the plantar surface of the toes of the hind foot with an insulin syringe. All treefrogs were held for less than one hour during the abovementioned processing, and were released into the pipe in which they were captured.

We used data collected from July 2006 to September 2007 (15 months). CMR sampling sessions were conducted at two-week intervals for the first 3.5 months (mid-July to October 2006) to allow frequent qualitative assessments of the marking method, to minimize the risk of losing marks, and to improve our knowledge of local treefrog reproductive cycles. We switched to monthly intervals in November

2006 because the reproductive season for all four species had ended and preliminary analyses indicated that the majority of toe-clips and VIE marks remained readable for at least one month. Thus, we conducted 18 sampling sessions during the 15-month period: seven at two-week intervals and 11 at monthly intervals.

During each sampling session, we assessed the readability of each mark and repaired all damaged marks, following a rigid protocol. Once a mark was deemed unreadable, or once a mark reached the point that we feared that it would be unreadable during the next sampling session, the same toe was re-clipped and/or VIE re-injected. Toes were only re-clipped if (1) any portion of the toe-pad had grown back, (2) the toe had re-grown to more than about 25% of its original length, or (3) extra digits had grown from the clip site. The VIE marks were repaired using the following protocol: (1) if less than about 50% of the amount of elastomer that would constitute an acceptable mark (as per Hoffmann et al., 2008) remained in the marked toe, additional elastomer was injected into the marked toe; (2) if any portion of the elastomer mark was seen protruding from the skin, it was clipped off or removed entirely and new elastomer was injected into the previously marked toe, and; (3) if the elastomer had migrated to other toes or into the foot or leg, new elastomer was injected into the previously marked toe. We also made detailed comments about each defective mark, why it was unacceptable, and how it was fixed, in order to facilitate quality control and later conversion of this information to quantitative data that could be subjected to statistical analyses.

Two quantitative variables were generated from the qualitative data for every treefrog captured during each sampling event: whether or not a toe was re-clipped, and whether or not the VIE was re-injected. Thus, the number of toes that had to be re-clipped and the number of VIE marks that had to be repaired were the variables of interest in subsequent analyses.

Only recaptured treefrogs were included in data analyses. Recapture data were summarized by species to generate (1) the proportion of readable and unreadable marks and (2) the duration of time (in days) between the initial application of a mark and the first time that the mark had to be repaired. We calculated the proportion of individuals for which toes had to be re-clipped and VIE marks that had to be repaired during the study. Using Julian day, we calculated the mean, minimum, and maximum duration of time (in days) that each type of mark lasted.

Analysis of variance (ANOVA) was used to test for differences in the mean proportion of re-clipped toes and re-injected VIE by species, and to test for differences in the duration of time each type of mark remained readable by species for animals that needed to have their mark repaired. Differences in the mean proportion of toe-clips that remained readable throughout the study and VIE marks that remained readable throughout the study were assessed with Student's t-tests. Differences in the mean duration of time that a toe-clip remained readable and the duration of time that a VIE mark remained readable were also analyzed with Student's t-tests. All summaries and statistical tests were performed using JMP® Version 5.1 (SAS Institute Inc., Cary, NC, 1989-2004).

Results

During this study, 2,031 treefrogs from four species were captured, marked and released (table 1). Of those, 840 were recaptured at least once, but the mark of one recaptured Cuban treefrog was not readable upon recapture due to complete toe regeneration and VIE migration into the base of all the toes of the hind foot, so only 839 frogs were included in further analyses. The overall recapture rate was 41.4%, and 60.1% of the recaptured frogs were recaptured more than once (table 1).

Of the 839 recaptured treefrogs, 44.9% had to be re-clipped at least once, but only 20.1% had to be re-injected with VIE at least once during the study (table 2). Of the 377 frogs that had to be re-clipped during the study, the average time that a toe-clip remained readable was 99.8 days (table 3). There was no difference in the mean duration of time that toe-clips remained readable among species ($F_{3,373} = 0.805$; $P = 0.492$). Of the 169 frogs that had to be re-injected with VIE, the average time that the VIE mark remained readable was 111.7 days (table 3). There were no differences in the duration of time that VIE marks remained readable among species ($F_{3,165} = 0.501$; $P = 0.682$).

A significantly higher proportion of VIE marks than toe-clips (table 2) remained viable throughout the study ($t_{5.5} = 4.038$; $P = 0.008$). However, of the marks that needed to be repaired, the mean duration the marks remained viable (table 3) did not differ significantly between the two methods ($t_{5.5} = 1.552$; $P = 0.176$).

Table 1.
The number of times 839 marked treefrogs were recaptured between July 2006 and September 2007 at Morris Bridge Wellfield, Hillsborough County, Florida.

No. recaptures	Species			
	H. cinerea	*H. femoralis*	*H. squirella*	*O. septentrionalis*
1	19	91	80	145
2	15	64	47	66
3	9	33	37	34
4	5	23	27	17
5	2	13	25	11
6	2	7	10	5
7	1	7	8	4
8	1	9	0	2
9	0	2	1	4
10	0	4	0	0
11	0	3	1	1
12	0	3	1	0
Total no. recaptured	54	259	237	289
Total no. marked	117	460	451	1003
% recaptured	46.2	56.3	52.5	28.9

Table 2.
The percentage of marked treefrogs for which the mark was reapplied in four species of treefrogs marked and recaptured between July 2006 and September 2007 at Morris Bridge Wellfield, Hillsborough County, Florida.

Species	No. times re-marked											
	0	*n*	1	*n*	2	*n*	3	*n*	4	*n*	Total $\geqslant 1$	*n*
Hyla cinerea												
Toe-clip	66.6	36	31.5	17	1.9	1					33.4	18
VIE	92.6	50	3.7	2	3.7	2					7.4	4
Hyla femoralis												
Toe-clip	54.1	140	33.2	86	10	26	2.3	6	0.4	1	45.9	119
VIE	74.5	193	21.6	56	3.1	8	0.8	2			25.5	66
Hyla squirella												
Toe-clip	43.9	104	42.6	101	12.2	29			1.3	3	56.1	133
VIE	81.4	193	17.3	41	1.3	3					18.6	44
Osteopilus septentrionalis												
Toe-clip	63	182	32.5	94	4.2	12	0.3	1			37	107
VIE	81	234	16.6	48	2.4	7					19	55

Table 3.
The duration of time (in days) between initial mark application and the first time a mark had to be repaired in four species of treefrogs marked and recaptured between July 2006 and September 2007 at Morris Bridge Wellfield, Hillsborough County, Florida.

Species	*n*	mean	min.	max.	SE
Hyla cinerea					
Toe-clip	18	118.1	31	215	13.26
VIE	4	107.0	28	169	36.46
Hyla femoralis					
Toe-clip	119	95.2	28	308	6.09
VIE	66	111.3	14	392	11.25
Hyla squirella					
Toe-clip	133	93.7	14	398	5.70
VIE	44	124.5	28	301	11.95
Osteopilus septentrionalis					
Toe-clip	107	92.3	28	336	6.55
VIE	55	103.8	28	336	10.67

Discussion

This study presented us with a unique opportunity to assess the efficacy of a novel marking method using relatively large samples of four species of free-ranging treefrogs. Because treefrogs were not recaptured during every CMR sampling session, many toe-clips and VIE marks went unchecked for two or more months. Thus, a wide range of time intervals were available for assessing the length of time that toe-clips and VIE marks remained viable. Initial sampling frequency was conservative at semi-monthly intervals for the first three months, ensuring that the temporal resolution of the sampling effort was adequate to identify any problems arising from the marking technique. We then increased the length of the interval between monitoring events to sample only once per month, after having calculated that we were no more likely to lose the identity of an individual in this time period.

The mark of only one of the 840 recaptured frogs could not be deciphered, a good indication that the VIE-C technique of marking anurans is effective. However, the apparent effectiveness of the technique was increased because we were able to verify that we had the correct frog, even when the clipped toe had regenerated or the VIE mark had migrated, partially as a result of the morphology and behavior of the treefrogs themselves. First, mark redundancy was pivotal in that both marks were never completely lost, and a clipped toe often allowed us to decipher a problematic VIE mark and vice versa. Second, morphological information (e.g., sex, size, injuries, etc.) could be used to verify the identity of the treefrog in question. Third, hylid frogs also generally occupy a prescribed territory or home range and are repeatedly found in the same PVC refugia (Boughton et al., 2000; Bartareau, 2004). Fortuitously, all four species in this study exhibited high degrees of wetland site and pipe fidelity over the 15-month study; all but one of the 840 treefrogs remained in the same wetland, and over 90% of them occupied the same pipe (Irvin et al., 2007). These quality control features allowed us to verify the identity of all treefrogs in the study, and repair any partially failed VIE-C identifiers, in the field. Since this study was completed, we have been able to identify three dead frogs found in the pipes, largely due to the presence of the VIE mark.

This study revealed a significant difference in the efficacy of the two techniques used in the VIE-C method. Differential error proportions and mark resilience over time indicated that the VIE mark will be more helpful in correctly reading and clarifying toe-clipping errors than will toe-clips in reading and clarifying questionable VIE marks. While about half of the recaptured treefrogs had to be re-clipped, only 20% had to be re-injected with VIE. Because most of the re-injected frogs in this study were only re-injected once (table 2), it appears that VIE mark errors were not exacerbated over time as additional VIE was injected to repair the mark. This is encouraging, given that VIE marks can migrate due to handling and even exit the body via needle holes (Northwest Marine Technology, 2000; Moosman and Moosman, 2006). Conversely, clipped toes regenerate; thus, the longer a frog goes without being re-captured, the more likely its toe will have to be re-clipped.

Although the VIE-C method is an improvement over marking methods that employ only toe-clips or only VIE marks, there are still certain limitations. In this study, the identity of only a single frog was lost, but the fact that toe-clips or VIE had to be repaired within a short time period from the initial VIE-C application could be problematic in long-term studies. Even the hybrid VIE-C identifiers may be lost in frogs that range widely, move frequently, or are otherwise unlikely to be recaptured every few months.

The treefrog sampling method we employed provided artificial microhabitats for the frogs, and so may have influenced our results and diminished the applicability to amphibians in natural areas without PVC pipe refugia. PVC pipe refugia are efficient and effective sampling devices for long-term studies of hylid treefrogs (Moulton et al., 1996; Boughton et al., 2000). However, PVC pipe refugia provide a stable microclimate and protection from predators, and may represent a safer environment for treefrogs relative to a natural area without PVC pipe refugia. This may in turn affect the viability of marks applied to vulnerable extremities. It seems unlikely that PVC pipe refugia would have affected the re-growth rate of clipped toes; however, they might have decreased the incidence of damage to the toes containing the elastomer, thereby inflating the efficacy of the VIE mark relative to studies not utilizing PVC pipe refugia.

In any CMR study, the tradeoff between the cost of frequent sampling and the cost of losing data on marked individuals must be considered. According to our data, the average clipped toe is at risk of regenerating and becoming unreadable in about three months, and the average VIE mark is at risk of migrating, disintegrating, or otherwise becoming unreadable in three to four months. Although most VIE marks remained readable for over three months, some had to be repaired after one month, and at least one toe-clip and one VIE mark was unreadable after only 14 days (table 3), but this occurred at beginning of the study when we intentionally sampled at 2-week intervals to minimize learning-curve errors. We switched to a one-month sampling interval only after completing four consecutive marking events without marking errors. A one-month sampling interval appears to be conservative for this method when used on treefrogs. This interval would ensure that very few identifiers will become unreadable and is reasonable in terms of funding and manpower.

Redundant marking methods may be the best way to ensure mark retention in small species with regenerative abilities. The VIE-C method combines a long-accepted but controversial (May, 2004; Funk et al., 2005) marking method and a more recently developed marking method (Nauwelaerts et al., 2000). This novel combination was effective for retaining identifiers, and the efficacy of this technique is probably greater than either of the techniques by themselves, especially given that clipping more than one or two toes results in possible decreases in return rate (McCarthy and Parris, 2004). Thus, the redundant VIE-C method allows toe-clipping to be used, but minimizes the number of toes clipped and therefore the likelihood of clip-related changes in behavior, and ultimately should minimize study-related decreases in return rates. Redundant marking techniques also provide a quality control check for one method by the other.

Carefully designed, long-term studies of amphibian populations utilizing CMR methods are needed to document as well as identify the causes and mechanisms of recent amphibian population declines (Stuart et al., 2004). Hopefully with increased understanding of amphibian declines, researchers will eventually develop the tools necessary to reverse this trend. Unfortunately, many amphibian marking techniques remain controversial, many do not minimize errors of interpretation, and many do not, by themselves, allow large numbers of individuals to be marked without altering behavior and decreasing the return rate. More studies of novel amphibian marking techniques are needed (Phillott et al., 2007), and rigorous field studies testing the accuracy of existing marking techniques are also required (Moosman and Moosman, 2006). We hope our analysis of the novel VIE-C marking technique will help in the design of future studies of hylids and other amphibian groups.

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