Technology meets tradition: A combined VIE-C technique for individually marking anurans

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Abstract. We report on the use of a hybrid marking technique (VIE-C) combining Visible Implant Elastomer (VIE) marks with toe-clipping (C) to mark individuals of several species of treefrogs (Hylidae). Our marking strategy entailed injecting elastomer into the plantar surface of the digits and clipping only one toe. This method allows large numbers of frogs to be individually marked, reduces the potential for negative effects due to clipping multiple toes, and minimizes the frequency of elastomer migration from the injection site, a common problem with VIE marks on the body or limbs. We found retention rate of VIE marks in the digits to be similar to that of toe-clips, indicating that VIE provides a satisfactory alternative to multiple toe-clips. In addition, cost of materials, frog handling time, and ill effects were minimal. This VIE-C marking scheme is highly recommended when considering techniques for marking anurans, as it reduces potential negative effects of clipping multiple toes, and provides a large number of inexpensive and long-lasting individual marks that can be easily applied and quickly read in the field by trained observers.

Key words: Amphibian; anuran; hylid; marking; toe-clipping; visible implant elastomer; VIE.

Introduction

When conducting ecological studies it is often essential to identify individuals by applying a unique tag or mark. Although date-specific cohort marks can be used to obtain population estimates, individual-specific marks can provide a wealth of data on growth, movements, survival, and more (Krebs, 1999). Ideally, the mark should identify a study animal as an individual, be adaptable to individuals of all sizes, last indefinitely, and be easily and accurately read. In addition, the mark should cause only minimal pain or stress, and should not affect behavior or survivorship. Ease of use and cost are also important considerations, especially when conducting large-scale field studies (Ferner, 1979).

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Ecologists have employed a variety of techniques to individually mark amphibians, with varied success. These range from metal jaw tags (Raney, 1940; Woodbury, 1956) and reflective sheeting glued to the epidermis (Robertson, 1984), to photo recognition based on individual variation in pigment patterns (Bailey, 2004). Other creative marks include small beads and tags of surveying tape sewn to an amphibian's legs or tail (Nace, 1982; Rice and Taylor, 1993; Rice et al., 1998). Hot and freeze branding have also been tried (Clark, 1971; Daughtery, 1976), as have fluorescent pigments introduced into the dermis with compressed air (Taylor and Deegan, 1982; Nishikawa and Service, 1988; Schlaepfer, 1998). Problems with such methods include unacceptable tag loss, expensive and cumbersome equipment, and lack of efficiency applying tags and marks.

Advances in technology have allowed for longer-lasting, safer marks, although these techniques are often expensive and may require special equipment to read tags. Passive integrated transponders (PIT tags) can be quickly injected under the skin without anesthesia and are commonly used to mark amphibians. These tags do not appear to affect behavior or survivorship, often outlive the amphibian they are injected into, and provide virtually limitless codes that are easily read with a scanner (Donnelly et al., 1994; Brown, 1997; Ireland et al., 2003; Pyke, 2005). However, PIT tags are relatively expensive, especially when marking many individuals, and the size of the needle applicator precludes this method for small amphibians. Recently, alphanumeric fluorescent tags have been used to mark several species of amphibians of varying sizes (Buchan et al., 2005; Gower et al., 2006). These long-lasting tags are injected or inserted, with or without anesthesia, under the skin of the leg, tail, or body of the animal and the alphanumeric code is read through translucent skin (Buchan et al., 2005; Gower et al., 2006). However, although there are many benefits to using these advanced marking methods, the tags and other equipment required are rather expensive, especially when large numbers of individuals are involved.

Another relatively recent development in marking techniques for amphibians is Visible Implant Elastomer (VIE; Northwest Marine Technologies, Inc.). This novel method was initially developed for use in fish, but it has also been used to mark amphibians and reptiles (Anholt et al., 1998; Nauwelaerts et al., 2000; Penney et al., 2001; Bailey, 2004; Heemeyer et al., 2007). Colored elastomer and a clear curing agent are mixed to form a compound that can be loaded into a syringe and injected subcutaneously. In approximately one hour (curing time varies with temperature), the injected VIE compound solidifies into a flexible internal tag that can be viewed externally. This method has been used to produce cohort and individual marks in amphibians and reptiles by injecting small amounts of elastomer into a unique combination of locations on the body of the animal (Anholt et al., 1998; Marold, 2001; Penney et al., 2001; Bailey, 2004; Heemeyer et al., 2007).

Retention and readability of VIE tags has varied in laboratory and field studies (Nauwelaerts et al., 2000; Penney et al., 2001; Bailey, 2004; Heemeyer et al., 2007). Several of the available VIE colors fluoresce under black light, increasing readability of the marks. Although VIE marks may fade over time when viewed

under visible light, fluorescent color under black light remains true (Penney et al., 2001). Notably, Heemeyer et al. (2007) found marks with blue-colored elastomer more difficult to see under black light as compared to three other colors (red, orange, yellow). Perhaps most troubling is the possibility that marks placed in regions of the body with loose skin may migrate under the skin to new locations, rendering marks illegible and thus invalid (Nauwelaerts et al., 2000; Davis and Ovaska, 2001; Moosman and Moosman, 2006; Heemeyer et al., 2007). Estimates of migration rates of VIE marks located in the body and limbs of amphibians under laboratory conditions have ranged from 4% (*Plethodon cinereus*; Heemeyer et al., 2007) to 31% (*Rana sylvatica*; Moosman and Moosman, 2006). The extensive subcutaneous lymphatic system unique to anurans (Carter, 1979; Duellmann and Trueb, 1994) may exacerbate the problem of tag migration, requiring regional (rather than specific) placement of VIE tags, thus resulting in a relatively limited number of available individual marks.

Toe-clipping is a traditional method and is generally considered the standard means of marking amphibians and small reptiles. However, it has recently undergone increasing scrutiny as researchers have begun to evaluate the effects of toe-clipping on amphibian behavior and survival. Toe-clipping involves the removal of a unique combination of toes, each toe corresponding to part of a pre-defined numeric code system used to identify both cohorts and individuals (Martof, 1953; Donnelly et al., 1994; HACC, 2004). Inflammation, infection, and necrosis of clipped digits have been reported in several studies (Golay and Durrer, 1994; Davis and Ovaska, 2001; Liner et al., 2007). Susceptibility to infection and effects of such infections on survival in the field (and thus on recapture rates) are unknown, and may vary with species (Lemckert, 1996). Furthermore, clipped toes of amphibians often re-grow, and marks may be lost over time.

Whereas some studies suggest that toe-clipping decreases recapture probability (Clarke, 1972; Davis and Ovaska, 2001), perhaps as a result of decreased survival or trap avoidance, others argue that toe-clipping does not affect recapture rates (Williamson and Bull, 1996; Luddecke and Amezquita, 1999). Parris and McCarthy (2001) suggest that failure to find a significant effect of toe-clipping may, in many cases, be due to low statistical power. Re-analysis of data from four previous studies showed a significant linear decline in recapture rates with increasing number of toes removed (Parris and McCarthy, 2001; McCarthy and Parris, 2004). Phillott et al. (2007) questioned the mechanism underlying the findings of Parris and McCarthy (2001) and McCarthy and Parris (2004) and argued that toe-clipping or toe-tipping (a slightly modified method they advocate) is an acceptable method for individually marking amphibians. Regardless of the contradictory evidence as to the humaneness or ill effects of toe-clipping (or lack thereof), it seems prudent to eliminate the need for removing toes when possible. Because such is not always feasible, due to financial constraints or size of animals being marked, a marking method requiring minimal toe removal is preferred and development of such techniques undoubtedly merits investigation (May, 2004).

Here we evaluate a novel marking scheme that combines VIE with a single toe-clip (C). The aim of this "VIE-C" scheme is to provide a minimally invasive, efficient, and inexpensive method of marking large numbers of frogs while increasing the reliability of VIE marks, with the goal of improving the applicability of VIE as a common marking technique for amphibians. We present a detailed description of VIE use in the field as well as a brief analysis of the performance of VIE marks as compared to toe clipping.

Materials and Methods

We used a novel VIE-C scheme to mark individuals of several species of treefrogs, and briefly evaluated the efficacy of this technique (mark retention, ease of use/handling, cost effectiveness, and ill effects). The VIE marks were placed on the plantar surface of the digits of frogs in order to reduce VIE migration, facilitate mark interpretation, and reduce risks of mark detection by predators. To limit potential negative effects of toe-clipping, we only removed a single digit on each frog and we never removed the thumbs. By using a combination of VIE and removal of a single digit, we were able to generate thousands of individual identifications.

Treefrog capture sites and methods

To evaluate the efficacy of our hybrid VIE-C technique, we used this method during the course of an intensive mark-recapture field study in Central Florida. We applied the technique to four species of hylid frogs (*Hyla cinerea*, *H. femoralis*, *H. squirella*, *Osteopilus septentrionalis*) that we captured in 1-m PVC pipe refugia (Boughton et al., 2000; Zacharow et al., 2003) installed in the ground in flatwoods habitat at two sites — Wekiwa Springs State Park, Apopka, FL; Flatwoods/Hillsborough Wilderness Park, Tampa, FL. We checked PVC pipe refugia for three consecutive days every three weeks from July 2006 to June 2007. We used a sponge "plunger" (Boughton et al., 2000; Zacharow et al., 2003) — a sponge cut to fit the inner diameter of the pipe and glued to the end of a dowel rod — to gently transfer frogs into plastic zipper bags for measurement prior to marking.

VIE preparation

In order to maximize the number of marks obtained from the elastomer, we took several precautions to minimize the amount of waste. We refrigerated the unmixed compounds according to manufacturer recommendations (Northwest Marine Technology, Inc.) and avoided temperature fluctuations to maximize the shelf life of the unmixed components. We used a small, soft-sided cooler of ice to transport the unmixed compounds into the field, and mixed cooled VIE in a 0.3 ml insulin syringe as needed. We also mixed cooled components, as suggested by the manufacturer, in order to extend the working time of the prepared VIE. We were able to prepare small amounts (approximately 0.05-0.10 ml) of VIE by mixing equal-sized drops

of elastomer and curing agent (fig. 1a, upper left) directly in the widened portion of the syringe barrel above the finger flange (fig. 1b), rather than using a larger mixing container. We used a toothpick to mix the VIE and guide it into the barrel of the syringe for application, taking care to avoid creating air bubbles. Next, we loaded the syringe into the manual elastomer injector (fig. 1a, upper right) for ease of mark application. When not in use, we stored the syringe of mixed VIE on ice in a cooler. Additionally, we carried mixed VIE on ice in a small, soft-sided cooler among our capture sites, thus effectively extending the elastomer working time and reducing frog handling time to less than 2 min from capture to release.

Toe clipping

Toe-clipping is a traditional method used to mark amphibians for ecological studies — for description and discussion of this method, see Donnelly et al. (1994) and May (2004). In brief, we used surgical scissors to remove toes at the proximal joint, and did not clip thumbs due to their role in mating. We did not anesthetize frogs prior to toe-clipping, as this practice has been shown to increase amphibian stress levels (likely due to increased handling time), requires a long recovery period, and may affect behavior (Kinkead et al., 2006). We disinfected surgical scissors with 70% isopropyl alcohol between frogs. In contrast to conventional toe-clipping code schemes requiring the removal of multiple toes, we clipped only one toe per frog.

Retention rate analysis

We calculated and compared the retention rates for VIE and toe-clipping. Our redundant marking scheme (VIE + toe-clip = VIE-C) frequently enabled us to identify recaptured frogs with failed marks by using the remaining mark in combination with the frog's species, sex, size, and specific location in the field. We defined "failed" marks as marks that had become illegible when toe-clips regenerated or VIE migrated, or marks that we believed would become illegible within three weeks (before the next sampling session). Our marking scheme allowed identification of individual frogs; therefore we were able to approximate the age (t) of each mark (retained or failed) to the nearest three-week sampling interval. We calculated mean mark retention rate (r) and standard error ($\hat{\sigma}_{\mathrm{r}}$) for both VIE and toe-clipping (Robson and Regier, 1966). Mark retention rate (r) can be defined as the maximum likelihood estimate of the probability that a frog will retain the mark until the next sampling session, and was calculated using the following formulae:

$$r = (X/n)^{(1/t)},$$
$$\hat{\sigma}_{\mathrm{r}} = \sqrt{[r(1-r)/nt]},$$

where:
t = age of mark (measured to the nearest 3-week sampling interval),
X = number of remaining marks at age t,
n = total number of previously marked frogs with tags of age t (marks retained or failed).

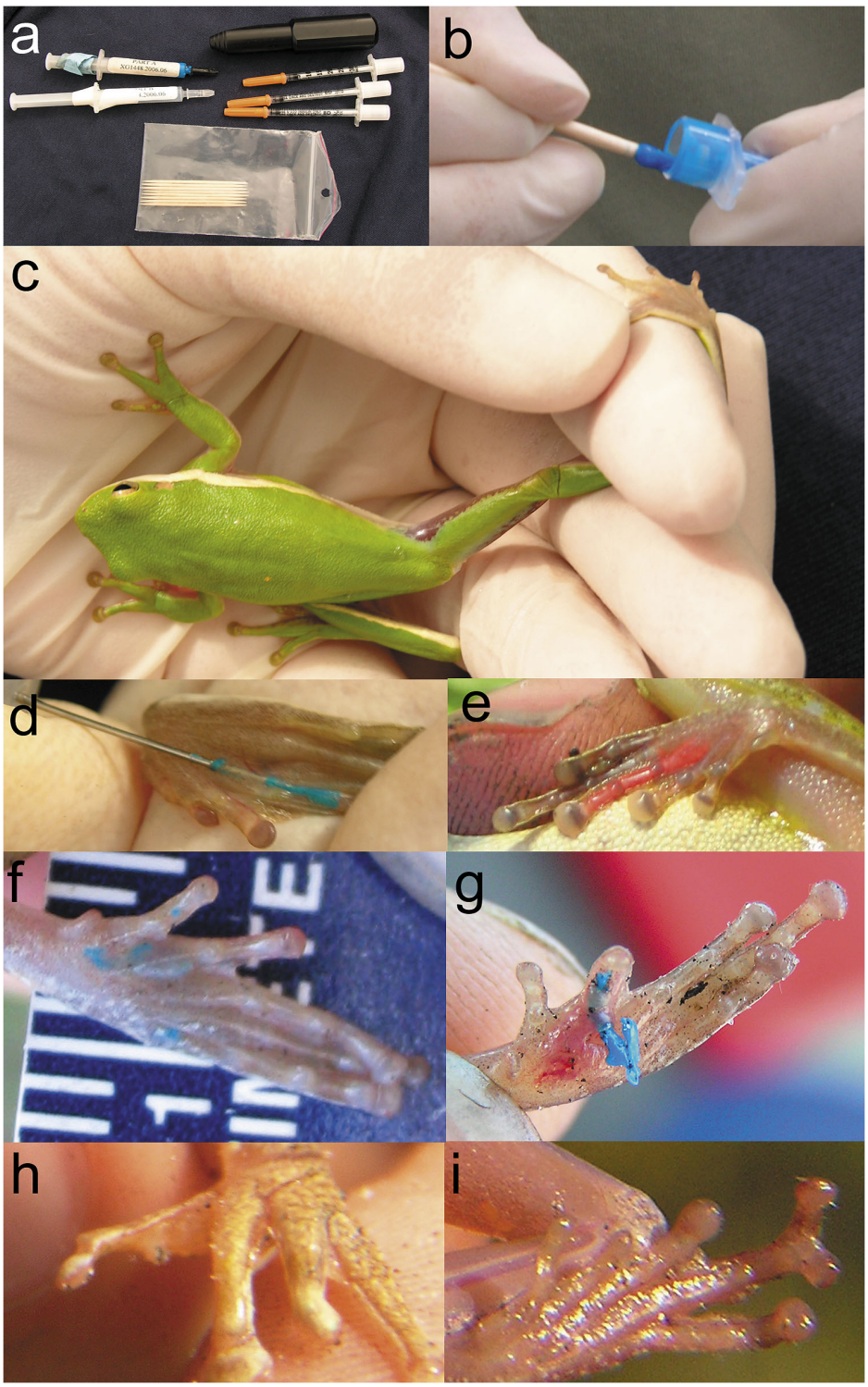
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Figure 1. VIE mixing supplies (a) can be purchased separately or as a kit; manual elastomer injector is optional (a, upper right). A small amount of elastomer is mixed directly in the syringe (b), which is then loaded into the manual elastomer injector. The investigator quickly immobilizes the frog in one hand (c), leaving the other hand free for VIE mark injection (d). The brightly colored mark (e) quickly identifies a frog as marked, and can be easily read by even novice markers. Minor problems associated with VIE and toe-clipping include: VIE marks may migrate (f), or may be rejected (g), and toe-clips may regenerate (h) or result in aberrant toe regeneration (i).

Results

Alphanumeric marking scheme

We developed an alpha-numeric marking scheme (VIE-C) similar to that used in traditional toe-clipping studies to create a series of unique codes. The toes of the hind feet are represented by letters (A-J), corresponding to VIE marks. We did not include the toes of the forefeet in our VIE marking scheme, due to their small size in some hylids; however, inclusion of these toes when practical would increase the number of potential codes. With the exception of the thumbs, which were not clipped due to their role in mating, each toe is also represented by a number (1-16) that corresponds to a toe-clip (fig. 2). In some instances, toes receiving a

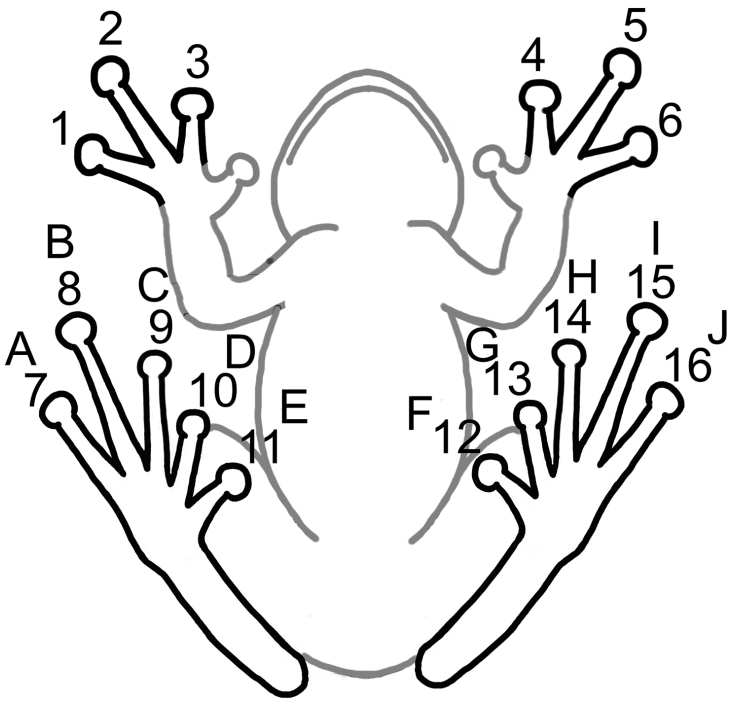

Figure 2. VIE-C marking scheme: Upper case letters correspond to VIE mark locations and numbers correspond to toe-clip locations. We did not clip thumbs, due to their role in reproduction, or inject VIE into the toes of the front feet.

Table 1. Number of unique codes available when applying up to four marks in up to three VIE colors, and with addition of one toe-clip. Numbers are not cumulative; additional marks and/or colors would yield additional unique codes according to the formula presented in the text.

	# Colors	One		Two		Three	
	# Toe-clips	None	One	None	One	None	One
# VIE Marks	One	10	160	20	320	30	480
	Two	45	720	180	2880	405	6480
	Three	120	1920	960	15360	3240	51840
	Four	210	3360	3360	53760	17010	272160

VIE mark might also be clipped. Codes were represented by an initial lowercase letter corresponding to the VIE color (e.g., b = blue), followed by uppercase letters corresponding to VIE mark locations and a number corresponding to a single toe clip (e.g., bAB13, rACF6, etc.). Using this alphanumeric system, three VIE marks of a single color will result in 120 unique codes without clipping any toes. The addition of one toe-clip multiplies this number 16-fold, producing 1920 codes. Additional colors or marks significantly increase the number of available codes (table 1); alternatively, a unique color can be used for each study site or species. The number of unique codes possible using our VIE-C marking scheme can be found by the formula:

$$[L!/(L - N)!]C^N 16^T,$$

where:
L = number of toes included in elastomer scheme (e.g., 10, here labeled A-J),
C = number of elastomer colors used,
N = number of elastomer tags,
T = number of toe-clips (16 = maximum toes available for clipping; here $T = 1$).

Alternatively, Northwest Marine Technology's free VIE Color Code Generator (http://www.nmt.us/support/software/viecodes/viecodes.htm) can be used to calculate the number of unique VIE codes possible (not including toe-clips) and generate a list of code numbers.

We used the VIE-C marking scheme described above, combining VIE with toe-clipping, to mark individuals of four species of treefrogs as follows: 372 *Osteopilus septentrionalis*, 259 *Hyla femoralis*, 80 *H. cinerea*, and 5 *H. squirella*. We marked each frog by injecting VIE in up to two toes (we used only red or blue VIE) and clipping one toe. We marked frogs sequentially by species across all plots at each site. This allowed us to use study plot in combination with known traits of marked frogs (e.g., species, sex, size) to corroborate identification of partially failed marks. This was especially useful for identifying individual frogs when VIE marks had begun to migrate or clipped toes were re-growing.

Treefrog handling and marking technique

We developed a simple technique to immobilize and quickly mark treefrogs. The frog is held loosely in the palm of one hand, while the observer's thumb and forefinger are used to immobilize the appropriate foot. The frog's foot is then gently flattened against the forefinger of the observer with the plantar surface exposed, and the thumb or middle finger is used to spread the toes (fig. 1c). The observer's free hand is then used to manipulate the insulin syringe (0.3 ml, 28-ga needle) containing mixed elastomer, which is held in the manual elastomer injector (Northwest Marine Technology, Inc.). The needle is inserted at the base of the toe with the bevel pointed up and then advanced distally along the side of the toe to separate the skin from the toe. The needle is then slowly withdrawn as the VIE is simultaneously injected into the cavity created by the needle (fig. 1d). If necessary, the observer can manipulate VIE further into the distal portion of the toe by applying gentle pressure with a thumbnail. One toe is then clipped with surgical scissors at the proximal joint. The surgical scissors and needle are disinfected with alcohol between frogs.

Ease of use and handling time

We found this marking method to be quick and easy to use. The bright colors of the elastomer (fig. 1e) allowed field technicians to quickly recognize most frogs as marked or unmarked. An experienced technician could usually mark a frog in approximately 30 s, with a total handling time of >2 min from capture to release, including identification of marked individuals, weighing, and measuring snout to vent length (SVL). When provided with a diagram of the marking scheme (fig. 2), technicians quickly learned to read the marks and almost always identified their first recaptured frog correctly. However, we found that short training sessions using live animals were necessary in order to train technicians in the handling and marking techniques. Untrained technicians require a longer handling time to inject VIE marks, and lack of training can result in repetitive punctures or illegible marks. Technicians were often able to mark frogs proficiently after practicing the technique on 3-5 frogs.

Cost effectiveness

Our marking scheme provides a low cost method of reliably marking frogs for field or laboratory research. The cost of VIE is minimal: US$105 will purchase a single color refill kit (6 ml) complete with mixing supplies. The unmixed VIE can be refrigerated for up to one year, carried on ice into the field, and mixed in the end of the syringe barrel in small (<0.1 ml) amounts to prevent waste. Storing the elastomer components on ice prior to mixing and carrying the mixed elastomer on ice in a cooler can increase the working time of the mixed elastomer to three or more hours. A manual elastomer injector (which we highly recommend for use with small anurans) can be purchased from Northwest Marine Technology, Inc. for $32, as

can insulin syringes for $10 (20/pack). Fine-point surgical scissors for toe-clipping can be purchased from a scientific supply company for under $20, and additional supplies (70% isopropyl alcohol, small cooler, ice packs) can be purchased from any drug store for about $10. When mixed and stored properly, with minimal waste, one 6-ml tube of VIE can be used to create approximately 1500 marks, at a total cost of <$0.15 per mark (not including labor).

Problems and ill effects

We experienced minor problems with toe-clipping and VIE tags, most common of which were swollen digits, minor bleeding from clipped toes, deformed regenerating toes, VIE tag migration, and VIE tag rejection. The incidence of these problems is reported as percentage of frogs recaptured at least once (RF), rather than percentage of marks, as the coding system and number of frogs marked required a few frogs to receive double VIE tags. However, less than 2% of recaptured frogs had been given double VIE tags; hence, these results are comparable to percentage of marks. Migration of VIE tags was observed in 54 frogs (20.5% RF; fig. 1f). Of these 54 cases, 25 tags migrated into the sole or heel of the foot, eight tags migrated laterally into the webbing between the toes, and two tags migrated into the lower leg. Additionally, in nine cases only a dot of VIE was visible upon recapture, in eight cases a dot of migratory VIE was visible in an unmarked toe, and in two cases the tag was no longer visible. However, in 39 of these 54 cases of VIE tag migration, the tag was still legible; only in 15 cases (5.7% RF) were the VIE tags deemed illegible. Swelling of the digit was observed in six marked frogs, although swollen toes were also observed on one unmarked frog, and this may not be related to VIE injection. Rejection of the VIE tag (elastomer extruded through the skin/injection site) was observed in only 13 frogs (4.9% RF; fig. 1g). Minor bleeding from toe-clips was observed in four frogs (1.5% RF). Re-clipping due to some degree of toe regeneration (or anticipated toe regeneration) was necessary in 30 frogs (11.4% RF; fig. 1h). We did not make the distinction between complete and partial toe regeneration, hence complete toe regeneration was not quantified. Aberrant toe regeneration was observed in four frogs (1.5% RF; fig. 1i); regenerated toes grew multiple toe pads or grew from an abnormal location on the foot. Upon initial observation, the marks did not seem to noticeably hinder the mobility of the frogs, although one frog was observed rubbing its hind feet together as if trying to remove the elastomer.

Retention rate

The retention rate of VIE tags and toe-clips followed a similar pattern over time — retention rate was lowest for younger tags, but quickly stabilized and remained stable. Initial retention rate of toe-clips was slightly lower than that of VIE tags, although this difference is likely insignificant. Retention rates rapidly improved to 98% or better by the second (VIE tags) or third (toe-clips) sampling period after marking (6-9 wk) and stabilized at near 100% (table 2, fig. 3). The majority

Table 2. Age and numbers of retained and failed toe-clips and visible implant elastomer (VIE) marks. The age of the tag (t) is measured in 3-wk sampling intervals (for example, a tag at age $t = 11$ is 33 weeks old). It is important to note that tags evaluated at a given age belong to frogs captured at various times during the study; thus, the declining numbers of tags (n) with increasing tag age (t) is not necessarily indicative of a temporal trend in frog captures, survival, etc. Variation in total number (n) of tags of each type at a given age (t) is due to variability in tag failure — both tags did not fail simultaneously, thus one frog might possess a VIE mark and toe clip of different ages.

Age of tag (t) (3-wk intervals)	Type of tag					
	Toe-clip			VIE mark		
	Retained	Failed	Total (n)	Retained	Failed	Total (n)
1	135	14	149	189	17	206
2	61	6	67	102	3	105
3	54	2	56	84	3	87
4	29	2	31	52	1	53
5	36	3	39	49	3	52
6	44	1	45	72	1	73
7	38	2	40	56	2	58
8	23	0	23	57	0	57
9	13	0	13	37	0	37
10	14	0	14	33	0	33
11	15	0	15	31	0	31

(68-73%) of the marks that failed (both VIE tags and toe-clips) did so before the mark was six weeks old, and marks 24-33 weeks old were not observed to fail. This overall trend in mark retention was similar across the three species and the two VIE colors we used (blue and red), although further investigation would be needed to determine if this trend holds true for other anuran families and VIE colors. Chi-squared analysis indicated that VIE failure did not significantly vary among injected digits ($\chi^2 = 0.237$, df $= 4$, $P = 0.993$).

Discussion

Our VIE-C marking scheme allows for a large number of low-cost, individual codes that are easily created and read and may reduce negative effects on frog behavior, mobility, health, and survival associated with clipping multiple toes. Traditional toe-clipping can require as many as six toes to be clipped (Hero, 1989), and has the potential to negatively affect mobility, health, and even survival (Clarke, 1972; Golay and Durrer, 1994; Davis and Ovaska, 2001; Parris and McCarthy, 2001; McCarthy and Parris, 2004). The effects of toe-clipping likely vary among taxa — the potential effects of toe-clipping on mobility might be a significant source of bias when studying arboreal frogs as compared to aquatic species. In comparison, VIE has not been found to negatively affect body condition, movements, or survival (Davis and Ovaska, 2001; Bailey, 2004; Heemeyer et al., 2007), suggesting the potential to reduce negative effects (and thereby bias) associated with traditional

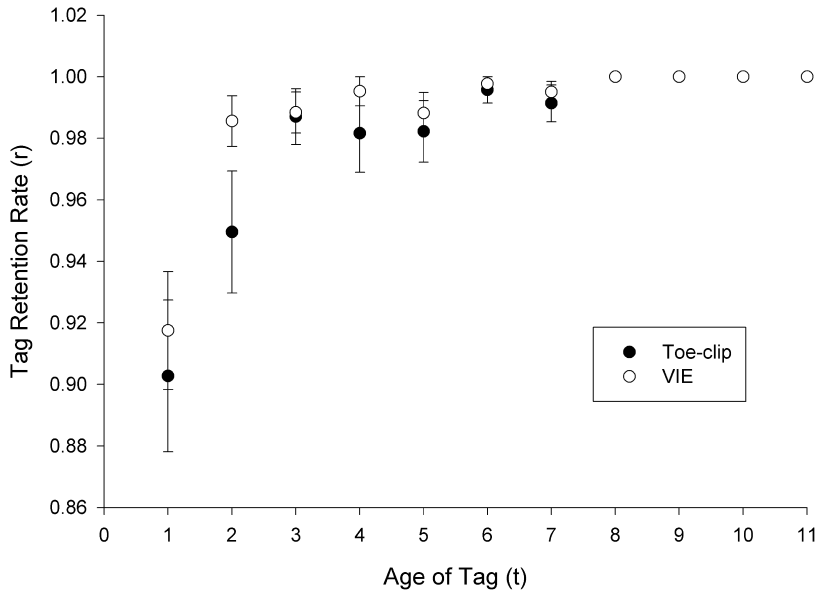

Figure 3. Comparison of mean retention rates of VIE tags and toe-clips over time. The age of the tag (t) is measured in 3-wk sampling intervals (for example, a tag at age $t = 11$ is 33 weeks old). The retention rates of both VIE tags and toe-clips follow a similar pattern, with tag failure being relatively high during the first six weeks (up to age $t = 3$) and older tags being comparatively stable. Retention rates of the oldest tags of both types were equivalent, thus the toe-clip symbol is obscured by that of VIE tags at the four oldest ages ($t \geqslant 8$). Numbers of VIE tags or toe-clips used to calculate retention rate at each age are not equal (see table 1). Error bars represent one standard error of the mean; standard error at $t \geqslant 8 = 0$.

toe-clipping. Our VIE-C marking scheme can provide 160 unique codes with only one VIE mark and one toe-clip, and this number can be increased by varying VIE color rather than adding marks (or toe-clips).

Some amphibian marking methods, such as PIT tagging, pressurized florescent marking, and branding require expensive supplies or cumbersome equipment. In contrast, we found VIE-C to be cost effective, and the small, light-weight marking kit was easily carried in the field — allowing us to mark frogs immediately upon capture. Our handling technique further increased marking efficiency, minimized handling time (and thus stress to the frog), and was easy to perform with minimal training. The bright VIE colors quickly alerted the observers to the presence of a mark, which was then easily read, even by novice technicians. It is important to note that the use of additional VIE colors, while allowing site or species-specific marks and increasing the number of available codes, can also increase costs, handling time, and potential for marking error. Hence, the maximum feasible number of VIE colors must be determined on a case by case basis.

Although other studies have examined performance of VIE marks in the body (Moosman and Moosman, 2006; Heemeyer et al., 2007) and toe webbing (Nauwelaerts et al., 2000), ours is apparently the first investigation of VIE marks applied

to amphibian digits. When injected into the digit of the frog, the VIE mark is effectively contained by the digit, thus reducing VIE mark migration. Marking the plantar surface is preferable, as the textured skin on the underside of the toes aids in stabilizing the syringe. The plantar surface of the foot is also less pigmented than the dorsal surface, resulting in enhanced visibility of the mark for ease of interpretation. Perhaps most importantly, marks on the plantar surface are presumably hidden from potential predators when the frog is at rest.

We experienced only minor problems with this method and found VIE retention rate over time to be similar to that of toe clipping, suggesting that VIE-C may provide a suitable alternative to traditional, yet controversial toe-clip marking. Initial VIE retention rate, although not 100%, was slightly higher than that of toe-clips, and the VIE tags stabilized within a relatively short time and remained stable. The VIE-C double tag effectively increased mark retention, as the two types of marks were unlikely to fail both completely and simultaneously, and one remaining mark could identify a frog as recaptured and potentially identifiable. For example, a clear VIE mark could be used in combination with known traits (i.e., species, sex, size) of a marked frog, to verify a partially re-grown toe clip. Furthermore, the philopatric tendency of hylids in PVC pipe refugia (Boughton et al., 2000) allowed us to use the capture location in addition to species and sex to assist in identification of frogs that had partially or completely lost one of their marks (VIE or toe-clip) — only four recaptured frogs were not individually identifiable (but were recognizable as marked frogs). We recommend that researchers restrict the time intervals between sampling periods to reduce mark loss due to greater initial migration and rejection of VIE marks, and regeneration of clipped toes. We found swollen or infected digits in a few marked frogs, as well as one unmarked frog. Because of this problem, researchers should take care to properly disinfect the syringe and scissors between uses on different frogs. We found VIE retention to be slightly lower in toes that were both clipped and injected, thus we suggest omitting such codes.

Our VIE-C technique should also be effective for use in studies of non-hylid frogs, many species of salamanders, and possibly some lizards. The application of VIE marks has not been found to negatively affect body condition, movements, or survival in laboratory and field studies of amphibians (Davis and Ovaska, 2001; Bailey, 2004; Heemeyer et al., 2007). Evaluating the potential effects of the combined VIE-C marks on behavior, mobility, health, and survival was beyond the scope of this study, although these topics warrant further investigation. In particular, we recommend evaluation of the effects of VIE marks in digits on locomotion and climbing ability of arboreal hylids. We also did not evaluate the effects of such variables as frog size and marker skill level on mark retention. Finally, although we found frogs with legible marks more than a year after marking, we do not yet know the ultimate duration of the marks. We suggest these as topics for future research as well.

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References

Anholt, B.R., Negovetic, S., Som, C. (1998): Methods for anaesthetizing and marking larval anurans. Herpetol. Rev. **29**: 153-154.

Bailey, L.L. (2004): Evaluating elastomer marking and photo identification methods for terrestrial salamanders: marking effects and observer bias. Herpetol. Rev. **35**: 38-41.

Boughton, R.G., Staiger, J., Franz, R. (2000): Use of PVC pipe refugia as a sampling technique for hylid treefrogs. Am. Midl. Nat. **144**: 168-177.

Brown, L.J. (1997): An evaluation of some marking and trapping techniques currently used in the study of anuran population dynamics. J. Herpetol. **31**: 410-419.

Buchan, A., Sun, L., Wagner, R.S. (2005): Using alpha numeric fluorescent tags for individual identification of amphibians. Herpetol. Rev. **36**: 43-44.

Carter, D.B. (1979): Structure and function of the subcutaneous lymph sacs in the Anura (Amphibia). J. Herpetol. **13**: 321-327.

Clark, D.R. Jr. (1971): Branding as a marking technique for amphibians and reptiles. Copeia **1971**: 148-151.

Clarke, R.D. (1972): The effect of toe clipping on survival in Fowler's toad (*Bufo woodhousei fowleri*). Copeia **1972**: 182-185.

Daughtery, C.H. (1976): Freeze-branding as a technique for marking amphibians. Copeia **1976**: 836-838.

Davis, T.M., Ovaska, K. (2001): Individual recognition of amphibians: effects of toe clipping and fluorescent tagging on the salamander *Plethodon vehiculum*. J. Herpetol. **35**: 217-225.

Donnelly, M.A., Guyer, C., Juterbock, J.E., Alford, R.A. (1994): Techniques for marking amphibians. In: Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians, p. 277-284. Heyer, W.R., Donnelly, M.A., McDiarmid, R.W., Hayek, L.C., Foster, M.S., Eds, Washington D.C., Smithsonian Institution Press.

Duellmann, W.E., Trueb, L. (1994): Biology of Amphibians. New York, McGraw-Hill.

Ferner, J.W. (1979): A Review of Marking Techniques for Amphibians and Reptiles. Herpetological Circulars 9, 42 pp. Salt Lake City, Society for the Study of Amphibians and Reptiles.

Golay, N., Durrer, H. (1994): Inflammation due to toe-clipping in natterjack toads (*Bufo calamita*). Amphibia-Reptilia **15**: 81-96.

Gower, D.J, Oommen, O.V., Wilkinson, M. (2006): Marking amphibians with alpha numeric fluorescent tags: caecilians lead the way. Herpetol. Rev. **37**: 302.

HACC (2004): Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research. Herpetological Animal Care and Use Committee. http://www.asih.org/files/hacc-final.pdf.

Heemeyer, J.L., Hoymack, J.L., Haas, C.A. (2007): Retention and readability of visible implant elastomer marks in eastern red-backed salamanders (*Plethodon cinereus*). Herp. Rev. **38**: 425-428.

Hero, J.-M. (1989): A simple code for toe clipping anurans. Herpetol. Rev. **20**: 66-67.

Ireland, D., Osbourne, N., Berrill, M. (2003): Marking medium-to large-sized anurans with passive integrated transponder (PIT) tags. Herpetol. Rev. **34**: 2003.

Kinkead, K.E., Lanham, J.D., Montanucci, R.R. (2006): Comparison of anesthesia and marking techniques on stress and behavioral responses in two *Desmognathus* salamanders. J. Herpetol. **40**: 323-328.

Krebs, C.J. (1999): Ecological Methodology, 2nd ed. Menlo Park, Addison Wesley, Longman.

Lemckert, F. (1996): Effects of toe-clipping on the survival and behaviour of the Australian frog *Crinia signifera*. Amphibia-Reptilia **17**: 287-290.

Liner, A.E., Smith, L.L., Castleberry, S.B. (2007): Effects of toe-clipping on the survival and growth of *Hyla squirella*. Herpetol. Rev. **38**: 143-145.

Luddecke, H., Amezquita, A. (1999): Assessment of disc clipping on the survival and behavior of the Andean frog *Hyla labialis*. Copeia **1999**: 824-830.

Martof, B.S. (1953): Territoriality in the green frog *Rana clamitans*. Ecology **34**: 165-174.

Marold, M.-A.R. (2001): Evaluating visible implant elastomer polymer for marking small, stream-dwelling salamanders. Herpetol. Rev. **32**: 91-92.

May, R.M. (2004): Ethics and amphibians. Nature **431**: 403.

McCarthy, M.A., Parris, K.M. (2004): Clarifying the effect of toe clipping on frogs with Bayesian statistics. J. Appl. Ecol. **41**: 780-786.

Moosman, D.L., Moosman, P.R. Jr. (2006): Subcutaneous movements of visible implant elastomers in wood frogs (*Rana sylvatica*). Herpetol. Rev. **37**: 300-301.

Nace, G.W. (1982): Marking individual amphibians. J. Herpetol. **16**: 309-311.

Nauwelaerts, S., Coeck, J., Aerts, P. (2000): Visual implant elastomer as a method for marking adult anurans. Herpetol. Rev. **31**: 154-155.

Nishikawa, K.C., Service, P.M. (1988): A fluorescent marking technique for individual recognition of terrestrial salamanders. J. Herpetol. **22**: 351-353.

Parris, K.M., McCarthy, M.A. (2001): Identifying effects of toe clipping on anuran return rates: the importance of statistical power. Amphibia-Reptilia **22**: 275-289.

Penney, K.M., Gianopulos, K.D., McCoy, E.D., Mushinsky, H.R. (2001): The visible implant elastomer marking technique in use for small reptiles. Herpetol. Rev. **32**: 236-241.

Phillott, A.D., Skerratt, L.F., McDonald, K.R., Lemckert, F.L., Hines, H.B., Clarke, J.M., Alford, R.A., Speare, R. (2007): Toe-clipping as an acceptable method of identifying individual anurans in mark recapture studies. Herpetol. Rev. **38**: 305-308.

Pyke, G.H. (2005): The use of PIT tags in capture-recapture studies of frogs: a field evaluation. Herpetol. Rev. **36**: 281-285.

Raney, E.C. (1940): Summer movements of the bullfrog, *Rana catesbeiana* Shaw, as determined by the jaw-tag method. Am. Midl. Nat. **23**: 733-745.

Rice, T.M., Taylor, D.H. (1993): A new method for making waistbands to mark anurans. Herpetol. Rev. **24**: 141-142.

Rice, T.M., Walker, S.E., Blackstone, B.J., Taylor, D.H. (1998): A new method for marking individual anuran larvae. Herpetol. Rev. **29**: 92-93.

Robertson, J.G.M. (1984): A technique for individually marking frogs in behavioral studies. Herpetol. Rev. **15**: 56-57.

Robson, D.S., Regier, H.A. (1966): Estimates of tag loss from recoveries of fish tagged and permanently marked. Trans. Am. Fish. Soc. **95**: 56-59.

Schlaepfer, M.A. (1998): Use of a fluorescent marking technique on small terrestrial anurans. Herpetol. Rev. **29**: 25-26.

Taylor, J., Deegan, L. (1982): A rapid method for mass marking of amphibians. J. Herpetol. **16**: 172-173.

Williamson, I., Bull, C.M. (1996): Population ecology of the Australian frog *Crinia signifera*: adults and juveniles. Wildl. Res. **23**: 249-266.

Woodbury, A.M. (1956): Uses of marking animals in ecological studies: marking amphibians and reptiles. Ecology **37**: 670-674.

Zacharow, M., Barichivich, W.J., Dodd, C.K. Jr. (2003): Using ground-placed PVC pipes to monitor hylid treefrogs: capture biases. Southeast. Nat. **2**: 575-590.

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