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Use of Fluorescent Visible Implant Alphanumeric Tags to Individually Mark Juvenile Ambystomatid Salamanders

Identification of individuals over time is essential for measuring growth, fecundity, survival, movement, and dispersal of amphibians. Such individual measures usually result in more robust estimates of processes affecting population growth and declines, and likely yield more effective management recommendations. There are a variety of marking and identification techniques that have been evaluated for use with amphibians including toe-clipping (McCarthy and Parris 2004; Otto and Scott 1999; Phillott et al. 2007; Waddle et al. 2008), passive integrated transponder (PIT) tags (Brown 1997; Gibbons and Andrews 2004; Otto and Scott 1999), coded wire tags (Sinsch 1997), radio-transmitters (Richards et al. 1994; Weick et al. 2005), pattern recognition (Gamble et al. 2008; Grant and Nanjappa 2006), various tattooing and branding techniques (Donnelly et al. 1994; Measey et al. 2001; Schlaepfer 1998), visible implant elastomer (VIE) marks (Grant 2008; Kinkead et al. 2006; Measey et al. 2001; Nauwelaerts et al. 2000; Ralston Marold 2001), and visible implant alphanumeric (VIAlpha) tags (Buchan et al. 2005; Gower et al. 2006; Heard et al. 2008; Measey et al. 2001). When selecting the most appropriate method, a researcher should consider impact on health and behavior of study animals, degree of invasiveness, ease and speed of use, handling time, mark longevity, expense, and number of unique marks necessary. Researchers should select tags or marking procedures with the least possible effect on behavior, growth, and survival.

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Ambystomatid salamanders at metamorphosis are typically less than 40 mm SVL and weigh < 2 g (Petranka 1998). Their small size limits the practical options for individual identification of juveniles. Most individuals are too small for currently available internal radio-transmitters. Although Otto and Scott (1999) demonstrated that PIT tags could be surgically implanted in juvenile Marbled Salamanders (*Ambystoma opacum*) weighing from 2 to 4 g, ambystomatid salamanders in Missouri are typically closer to 1 g at metamorphosis (Osbourn, unpubl. data). In addition to the body size constraints of PIT tagging, the necessary surgical procedure requires extra time and precautions, limiting the ability to mark a large number of individuals. Recent advancements in pattern recognition technology are very promising (e.g., Gamble et al. 2008), however Marbled Salamanders, Spotted Salamanders (*A. maculatum*), and Ringed Salamanders (*A. annulatum*) lack their characteristic patterns at metamorphosis, limiting its effectiveness. Toe-clipping is limited by the number of possible combinations, regeneration, and potential harmful effects (Brown 1997; May 2004; McCarthy and Parris 2004). Though there is considerable debate about the potential harmful effects of toe-clipping versus the effects of other marking techniques (e.g., Funk et al. 2005; Phillott et al. 2007), it is likely that stress associated with increased handling time impacts animal health regardless of marking technique (Kinkead et al. 2006). Visible implant elastomers are very effective with most species. Although often sufficient for marking individuals, VIEs are limited by the number of possible combinations of mark colors and lightly pigmented marking locations (Heemeyer et al. 2007). Visible implant elastomers can also be inconvenient for fieldwork because of their tendency to become solidified and inoperative when inadequately refrigerated. Perhaps the greatest limitation of VIEs is their potential for misidentification due to tag migration. Misidentifications of VIE markings were reported for 19% of Western Red-backed Salamanders (Davis and Ovaska 2001), 17% of Eastern Red-backed Salamanders (Heemeyer et al. 2007), and 31% of Wood Frogs (Moosman and Moosman 2006). Ralston Marold (2001) did not observe VIE mark migration in stream salamanders, however Bailey (2004) reported a misidentification rate of 13% in Blue Ridge Two-lined Salamanders which she attributed to color misidentifications, overlooking of small marks, and incorrect mark location.

Soft VIAlpha tags, manufactured by Northwest Marine Technology, Inc., are individually coded, biocompatible tags measuring 1.0 × 2.5 mm. They are imprinted with one letter (A–Z) and two numerals (00–99) on a fluorescent red, orange, or yellow background (also available as fluorescent lettering on black background or larger 1.5 × 3.5 mm tags, although these were not tested here). The combinations of colors and alphanumeric codes yield 7800 possible individual fluorescent tags. VIAlpha tags have been used extensively in fish (e.g., Frenette and Bryant 1996) and are increasingly being used for individual identification in a range of amphibian species (e.g., Measey et al. 2001; Pittman et al. 2008; Spickler et al. 2006). In contrast to PIT tags and similar to VIE marks, VIAlpha tags are inserted just below the surface of the skin and do not require deep insertion into the abdominal cavity. This comparably less invasive tagging procedure could translate into decreased handling and recovery times for small salamanders. For VIAlpha tags the problem of tag migration often reported for VIEs seems only to be a problem in amphibians with loose skin. Some studies examining VIAlpha tags effectiveness with frogs reported tag migration (Kaiser et al. 2009) or having to manipulate inverted tags through the skin with forceps so that they could be read (Heard et al. 2008; Kaiser et al. 2009). These problems have not been reported in species with tight connective tissue between skin and muscle such as salamanders and caecilians (Gower et al. 2006; Measey et al. 2001). We conducted an experiment to assess the effectiveness of VIAlpha tags for individual identification of juvenile ambystomatid salamanders by evaluating ease of application, readability, tag retention, and effect on body mass gained by individuals.

Materials and Methods.—We collected Marbled Salamander larvae (*Ambystoma opacum*) from the Daniel Boone Conservation Area, Warren County, Missouri, and raised them in 1000 L cattle watering tanks until metamorphosis. Juvenile salamanders were placed in individual 17 × 12 × 9 cm plastic containers with moist sphagnum moss and fed 4–5 crickets weekly. Prior to and during the experiment, salamanders were housed in a temperature controlled (24°C) animal care facility at the University of Missouri. A total of 59 individuals were randomly assigned to one of four treatments. Fourteen untagged and non-anesthetized salamanders served as the control, 15 were anesthetized without a tag, 15 were tagged without anesthetization, and 15 were tagged with anesthetization. Of the salamanders receiving tags, three colors (red, orange, and yellow) were used.

Tricaine methanesulfonate (MS-222) is an effective and commonly used anesthetic for sedating and immobilizing fish and amphibians (Lowe 2004). We chose a concentration of 500 mg/L of MS-222 based on the mass of our juvenile salamanders (Peterman and Semlitsch 2006). To achieve a neutral pH, we buffered the solution with 800 mg of $NaHCO_3$ (sodium bicarbonate) (Cooper 2003). Salamanders were placed in the MS-222 bath until anesthetized (unresponsive to prodding and unable to right themselves) and for no more than 5 minutes. Once anesthetized, individuals were rinsed of MS-222 by dipping them in a shallow dish of dechlorinated tap water.

We individually marked juvenile salamanders with Northwest Marine Technology (NMT), Inc., (Shaw Island, Washington, USA) VIAlpha tags. During the marking procedure, one investigator (MSO) inserted all VIAlpha tags to insure consistency, while three others monitored anesthetization, weighed and measured, and monitored recovery of individuals. Tags were placed subcutaneously under translucent epidermis near the lateral base of the tail (Fig. 1). We administered VIAlpha tags by first making a 1 to 2 mm incision with a sterile pointed scalpel blade and then used the injector provided by NMT to insert the tag approximately 3 mm inward from the entry wound. Non-anesthetized individuals were restrained by hand during tag injection. To minimize infection, we wore nitrile gloves and sterilized all equipment with alcohol before each injection. During the four weeks of monitoring, each salamander was removed from its container at weekly intervals, weighed with an electronic balance, and fed. The same observer (MSO) examined tags weekly for retention and assigned a ranking according to the following readability index: 0, tag not visible or not present; 1, tag visible but only color discernable; 2, tag color and partial code visible or incorrect code read; 3, correct code only read with use of blue LED light and amber filter glasses; and 4, correct code visible without aid of amber filter glasses. This index was a useful tool for determining whether readability and accuracy decreased over time. Tags were initially read without the use of aids and then read while using a blue LED light and amber filter glasses to examine the usefulness of the light and glasses.

We used two-way analysis of variance (ANOVA, SAS version 9.1) to test for significant differences between our treatment groups in proportion increase of body mass after four weeks.

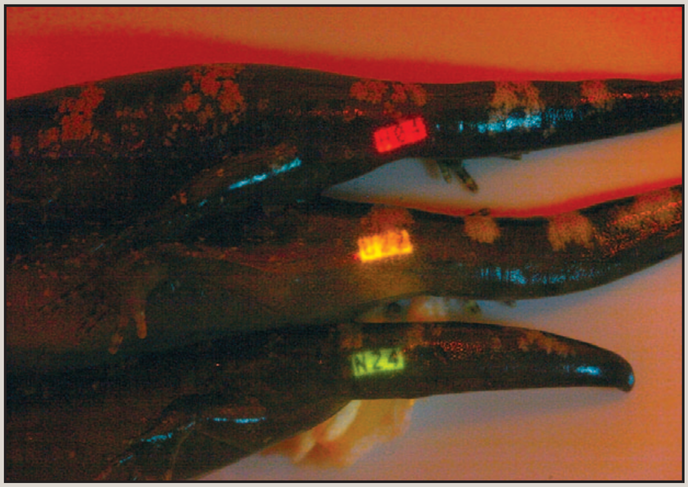

Fig. 1. *Ambystoma opacum* juveniles marked near the lateral base of the tail with VIAlpha tags as viewed with blue LED light and amber filter glasses.

Table 1. Mean initial body mass, mean final body mass, and mean proportion body mass increase (± SE) of Marbled Salamanders reared in the laboratory after being marked with VIAlpha tags and/or anesthetized with MS-222.

Treatment	N	Initial Mass (g)	Final Mass (g)	Proportion Mass Increase
Anesthetized	29	1.17	1.74	0.49 (± 0.15)
Non-anesthetized	29	1.25	1.80	0.46 (± 0.17)
Tagged	28	1.29	1.90	0.48 (± 0.14)
Non-tagged	30	1.13	1.63	0.47 (± 0.18)

To adjust for differences in mean initial body mass among our treatments, we used arcsine square-root transformed proportions. Anesthetization (anesthetized and non-anesthetized) and tag (tagged and non-tagged) treatments and their interaction (tag x anesthetization) were fixed effects in our model. We then used two-way ANOVA to test these same effects for significant differences in tag readability index scores after four weeks. Finally, we tested for random effects in tag retention and reading accuracy between anesthetized and non-anesthetized salamanders with a goodness of fit G-test (Zar 1999).

To supplement our laboratory experiment, we recorded additional observations on 122 Spotted Salamanders marked as recent metamorphs for ongoing mark-recapture experiments. Anesthetization, VIAlpha tagging procedures, housing, and care of these juveniles followed similar methods as in the laboratory experiment. After twelve weeks, our observer (MSO) assigned each salamander's tag a readability score and recorded tag retention. These additional observations were useful for examining tag retention over a longer time scale and comparing readability between species.

Results and Discussion.—The use of VIAlpha tags on small juvenile salamanders was successful. All tagged salamanders survived and grew during the four weeks of the experiment, although one small anesthetized untagged individual died after the first week. Our 100% survival of tagged individuals was comparable to the 96% survival of VIAlpha-tagged Pacific Treefrogs (*Pseudacris regilla*) reported by Buchan et al. (2005). Entry wounds created during tag insertion closed rapidly, lacked discoloration or swelling, and were typically considered healed within 24 hours. Our selected MS-222 concentration of 500 mg/L buffered with 800 mg of sodium bicarbonate was very effective, causing anesthetization in ~5 minutes with no detectable decline in health or behavior over four weeks. All surviving salamanders (N = 58) consistently fed and grew during the four-week period, increasing their mass by an average of 48%. There were no significant differences in the proportion of mass increase between tagged and untagged individuals ($F_{1,54}$ = 0.03, P = 0.869), anesthetized and non-anesthetized individuals ($F_{1,54}$ = 0.009, P = 0.621), nor was there an anesthetization by tag interaction ($F_{1,54}$ = 0.030, P = 0.3681; Table 1).

Through experience tagging over 2000 juvenile Spotted Salamanders (*Ambystoma maculatum*) and Ringed Salamanders (*A. annulatum*) in the field, we have observed the advantages of VIAlpha tags and potential problems which can arise during tag injection. The effectiveness of VIAlpha tags appears to be primarily limited by the skill and experience of the operator. Although tedious and requiring some initial practice to use efficiently, VIAlpha tags can be individually loaded in approximately 10 sec and injected into properly immobilized salamanders in less than 15 sec by a lone operator in the field. After a 5 min anesthetization period, handling time is often no more than 20 sec for immobilized salamanders. A recovery period of 10 to 20 min is also needed for anesthetized individuals. Our VIAlpha marking procedure time for juvenile salamanders is very similar to caecilians (< 1 min plus anesthetization and recovery; Measey et al. 2001) and Pacific Treefrogs (< 15 sec with no anesthetization; Buchan et al. 2005). Marking procedure times for VIAlpha tags are very similar to VIEs. Ralston Marold (2001) reported that it took 30 sec to inject four VIE marks and the entire procedure, including anesthetization and recovery, took about 20 min per individual. The VIAlpha tagging procedure can be streamlined with one technician loading tags and anesthetizing batches of five or more individuals while a second technician simultaneously injects and measures others.

Attempting to inject tags into improperly restrained individuals can result in agitation and tearing in the entry wound, potentially affecting tag readability by tags being placed too deep, too shallow, or folded. Despite difficulties encountered while trying to administer VIAlpha tags to non-anesthetized juvenile salamanders, we observed no significant differences in tag readability between anesthetized and non-anesthetized individuals ($F_{1,22}$ = 0.30, P = 0.591). If anesthesia is not preferred, restraining the salamander in a plastic bag and injecting the tag through the side of bag directly into the tail of the salamander is an effective technique with practice. The tradeoff of attempting to insert VIAlpha tags into non-anesthetized individuals is the potential for increased stress from prolonged handling time (however, no one has yet measured the level stress induced by exposure to MS-222 solution).

The mean readability score for Marbled Salamander juveniles after four weeks was 3.33 ± 0.16 (± SE; N = 24), indicating that a typical alphanumeric code could be read clearly by employing both the blue LED light and amber filter glasses. Marbled Salamanders are particularly darkly pigmented compared to other ambystomatids and we would expect their readability scores to be lower than more lightly pigmented species. For example, the comparably paler Spotted Salamander juveniles we monitored for twelve weeks had a mean readability score of 3.91 ± 0.03 (N = 103). A typical Spotted Salamander alphanumeric code could be clearly read without amber filter glasses. To ensure optimal readability, tags should be positioned just below translucent epidermis and inserted far enough away from the entry wound to allow it to heal and to prevent scar tissue from obscuring the alphanumeric code. Although we found that shining a blue LED or UV light and wearing amber filter glasses provided by NMT greatly improved tag detection in more heavily pigmented salamanders, for individuals with little translucent skin or abundant patterning, VIAlpha tags may not be a viable marking technique.

Tag reading accuracy is a potential problem when reading 1 mm alphanumeric codes. Our observer incorrectly read 4% of 227 total tag reading attempts and 10% of 30 VIAlpha tags were misread at least once. Our results are similar to those of Heard et al. (2008) who reported a VIAlpha tag misidentification rate by naïve observers in 3% of attempts. By comparison, Bailey (2004) reported a lower reading accuracy of 13% for VIEs due difficulty finding marks or color misidentifications. PIT tags probably have the lowest rate of inaccurate readings, although Pyke (2005) attributed a 1.8% error rate to human recording error.

Placement of tags too close to the entry wound was the primary cause for tags being dropped. For the first two weeks we recorded 100% tag retention, but by the end of week four 20% of 30 tags were dropped. Though not significant (P = 0.218), all six dropped tags were from non-anesthetized salamanders and could be the result of less than optimal tag placement while attempting to restrain them by hand. We found similar results in tag retention over twelve weeks for anesthetized Spotted Salamander juveniles (16% of 122 were dropped), suggesting that the problem of tag retention may be independent from the use of anesthesia. Tag loss may be the greatest potential limitation of VIAlpha tags. Heard et al. (2008) observed rates of tag loss of ~8% in frogs, which they attributed to expulsion of tags from slow-healing entry wounds. Tag loss has also been reported in PIT tags (Gibbons and Andrews 2004) however it has not been observed for VIEs (Bailey 2004; Moosman and Moosman 2006; Nauwelaerts et al. 2000) except when individuals are marked prior to metamorphosis (Grant 2008). Heard et al. (2008) suggest mark duplication as possible insurance against tag loss. Adding a cohort toe clip or a VIE mark in conjunction with a VIAlpha tag could be helpful, however at a potential cost in handing time and stress. During the twelve weeks we made observations, a small scar was almost always detectable upon close inspection, allowing us to identify individuals with lost tags in the field.

For individually identifying large numbers of small juvenile salamanders, VIAlpha tags are a reliable and effective marking system. They have both advantages and disadvantages when compared to VIE marks and PIT tags. While PIT tags are very effective for individual identification of larger individuals, they are not yet available in small enough sizes to be practical for most recently-metamorphosed salamanders and require a more invasive surgical procedure. VIAlpha tags and VIE marks are both effective marking systems for small amphibians and each has trade-offs. One key advantage of using VIAlpha tags in salamanders, however, is that they do not appear prone to migrate to other regions of the body as reported with VIE marks. While VIEs do not appear to be as prone to tag loss as VIAlpha tags (8–20%), the reported misidentification rates for VIEs (13–31%) appear to neutralize this advantage. According to the estimation of Nauwelaerts et al. (2000), VIEs cost about $0.06 per mark which is much less expensive than ~$1.00 per VIAlpha tag or ~$6.00 per PIT tag. The price advantages of VIEs may be somewhat diminished when multiple colors and locations are used to create individual marks, plus a proportion of a VIE batch is often wasted due to premature hardening in the field. Ralston Marold (2001) reported material costs for her project increased substantially because of the need to mix multiple batches per day despite keeping VIEs on ice. VIAlpha tags are very practical for fieldwork because they are extremely compact, lightweight, and are as visible as VIEs without requiring refrigeration.

Ultimately, which marking technique is the most appropriate depends on the question being asked. Here, we were concerned with individual identification of thousands of small, recently metamorphosed salamanders. Our field experiments required identification of recaptures over relatively short time periods (1–12 weeks). For our short-term projects, VIAlpha tags worked well, however long-term retention and readability has yet to be tested and is of concern. Marking juvenile salamanders with any method can be problematic because of their high rate of growth and development of pigmentation and patterning. We recaptured several Ringed Salamander juveniles the following field season with their tag color still discernable but their alphanumeric code mostly obscured. Although often tedious and slow when first used, an experienced operator can load and accurately inject tags swiftly with progressively decreasing handling time. When compared to other available marking techniques, the speed of use, cost, detection and readability, number of individual combinations, and lack of observed negative effects on survival or behavior, suggests that VIAlpha tags are highly effective for individual identification of small juvenile salamanders.

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