Herpetological Review, 2015, 46(2), 192–196.

Evaluation of Two Individual Identification Techniques for Spotted Salamanders (*Ambystoma maculatum*)

Introduction.—Mark-recapture (MR) is a widely-implemented and effective method used to monitor amphibian populations over time (MacNeil et al. 2011; Waye 2013). Long-term MR studies allow researchers to collect valuable information about the growth, movement, health, and dispersal of populations (Osbourn et al. 2011) that can ultimately be used to inform decisions regarding management and conservation of amphibians and their habitat.

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Mark-recapture studies often require that each individual be identified as unique from others in the population. Common amphibian identification techniques include visible implant elastomer (VIE) tags (Bendik et al. 2013), visible implant alphanumeric (VIA) tags (Osbourn et al. 2011), toe-clipping (McCarthy and Parris 2004), passive integrated transponder (PIT) tags (Homan et al. 2008; Ousterhout and Semlitsch 2014), and pattern recognition (Loafman 1991; Foster et al. 2007). Each identification technique presents its own set of advantages and disadvantages, and therefore must be carefully considered before selecting a technique for long-term MR studies.

The scope and objectives of a study determine the most appropriate and feasible identification technique. The number of individuals that are likely to be captured, handling time per individual (i.e., amount of time necessary to identify each individual), potential effects of the marking technique on behavior and health of the organism, longevity of the mark or tag, duration of study, and budget must all be considered (Osbourn et al. 2011). For example, although VIE tags are well-suited for marking very small animals, they offer a limited combination of codes. Even with six possible locations for tags on the body, four colors, and three tags per individual, only 1280 possible combinations exist (MacNeil et al. 2011), which may be insufficient for a multi-year study. VIE tags can also migrate beneath the skin or fragment after injection, making the code difficult to determine (Osbourn et al. 2011). Many techniques, including VIE tags, often require anaesthetization, constant monitoring of anaesthetized individuals, and the injection of a tag, which is time-consuming, invasive, and may have negative effects on behavior and stress levels (Kinkead et al. 2006; Osbourn et al. 2011).

As an identification technique, pattern recognition minimizes the need for invasive tagging methods by using natural color patterns as the primary identifier for each individual. The use of natural skin or fur patterns as markers has been successfully implemented with many different taxa, including polar bear whisker spot patterns, whale shark spot patterns, zebra stripe patterns, and bobcat spot patterns (Anderson et al. 2010; Arzoumanian et al. 2005; Foster et al. 2007; McClintock et al. 2013, respectively). Pattern recognition has also been evaluated before for use in MR studies of amphibians (e.g., *Ambystoma tigrinum*; Grant and Nanjappa 2006; Waye 2013). Assuming an individual's pattern does not change over time once they have reached the adult life stage, pattern recognition can be used for the entire adult life of each individual (Grant and Nanjappa 2006).

Some species of wetland-breeding salamanders (e.g., *Ambystoma* spp.) can serve as model amphibians for long-term MR studies using pattern recognition because of their individually distinctive patterning, relatively low vagility, and predictable movement patterns associated with reproduction (Petranka 1998; Semlitsch 2008). We developed an affordable and less-invasive MR technique for identifying individual Spotted Salamanders (*Ambystoma maculatum*) using spot pattern recognition. Our primary goal for this study was to assess the feasibility of using this pattern recognition (henceforth referred to as "spot code") as an alternative MR technique to VIE. Our assessment included measurement of identification accuracy and handling time for both techniques, where identification accuracy was defined by the percentage of correctly identified individuals by newly trained observers, and handling time was defined as the amount of time necessary to identify each individual.

Methods.— We conducted our study at a 0.93-ha ephemeral wetland located on a peninsula in Mountain Island Lake within Cowan's Ford Wildlife Refuge in Mecklenburg County, North Carolina, USA (35.3775°N, 80.9658°W). The wetland is surrounded by deciduous and pine forest and is dominated by tall grassy vegetation (Pittman et al. 2008). Each day we sampled 40 aquatic traps and 38 pairs of pitfall traps (13-L plastic buckets) placed every 10 m on either side of a 400-m drift fence completely encircling the wetland (Strickland et al. 2014). We sampled Spotted Salamanders from 18 January to 27 February 2014. A total of 253 individuals were processed and given spot codes in 2014. Individuals with VIE markings from previous years were identified as recaptures using the VIE code, but no new VIE codes were administered. Of the 253 individuals processed, 139 were recaptures with VIE codes and 114 were new captures.

The VIE marking technique was used in collection years prior to the current study. Salamanders were anaesthetized by submersion in Orajel® diluted to 1.0 g/L in 1L of water (Brown et al. 2004). A 26.5-gauge needle was then used to inject elastomer in any combination of six body locations (VIEs; Northwest Marine Technology, Inc., Shaw Island, Washington, USA). Salamanders could receive zero, one, or two colors in each location. The colors used were red, orange, yellow, and pink (Cecala et al. 2007; Fig. 1). An ultraviolet flashlight was used when reading the codes to increase visibility of the elastomer marks.

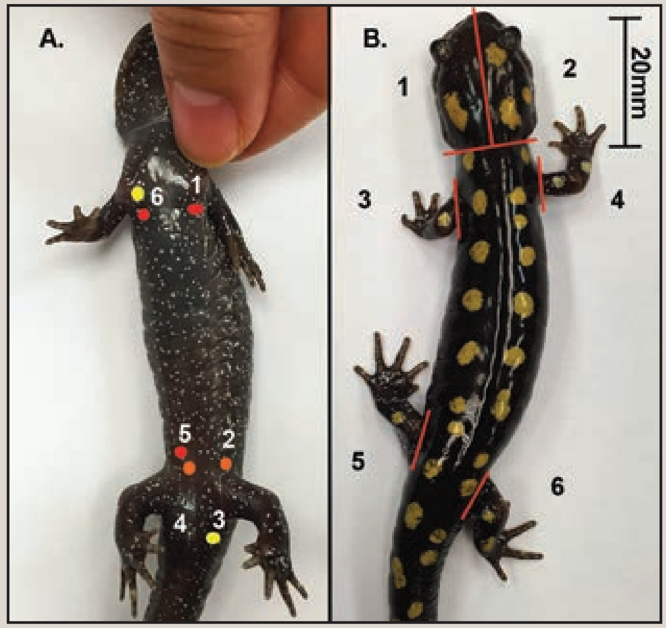

Fig. 1. Images of two Spotted Salamanders (*Ambystoma maculatum*) demonstrating two marking/identification techniques. A) Injection locations for the Visual Implant Elastomer (VIE) technique on the ventral side of a Spotted Salamander. This individual's code is 1R 2Or 3Y 5ROr 6RY—note that colored dots are not actual elastomer but only indicate locations and colors used. B) Demarcation of the six designated regions on the dorsal side of a Spotted Salamander developed for our spot pattern identification technique. This individual's spot code is: 131212. See Methods for a detailed explanation of both identification techniques.

We developed a spot pattern identification technique that relies on the number of spots in six designated regions on the dorsal side of each salamander. These regions are the left and right sides of the head anterior to the gular fold, and the four legs of the animal, from the feet (but not toes) to the top of the appendage where it joins the body (Fig. 1). Thus only head and limb spots, rather than spots on the trunk of the body, are considered in this technique. Orange and yellow spots ≥ 1 mm in diameter were counted in each of the six locations to create a spot pattern code. In order for a spot to be regarded as part of the official coding count, at least 50% of the spot's area must fall within one of the boundaries defined above. Irregularly shaped spots were counted as one spot unless a line of darker pigmentation passed through the spot. We recorded the spot code in a specific order: spot location 1 (left side of head), spot location 2 (right side of head), spot location 3 (left front leg), spot location 4 (right front leg), spot location 5 (left rear leg), spot location 6 (right rear leg). Thus, a salamander with two spots on each side of its head, one spot on each front leg, one spot on its left rear leg, and zero spots on its right rear leg was assigned the code 221110.

Of the 253 individuals processed in the 2014 season, 16 individuals had a shared spot code with at least one other individual,

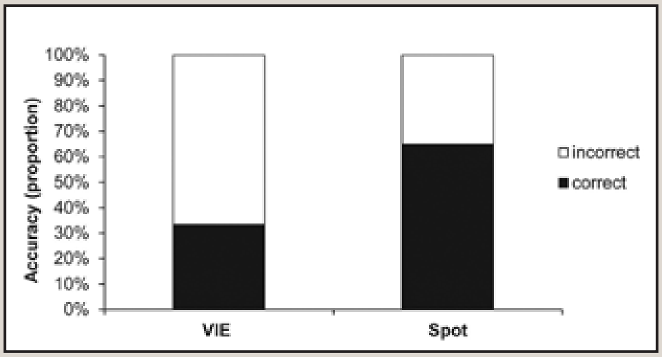

FIG. 2. Comparison of identification accuracy by identification technique. Proportion of trials correct and incorrect was derived from the total number of trials for each identification technique (N = 123 for VIE; N = 171 for Spot).

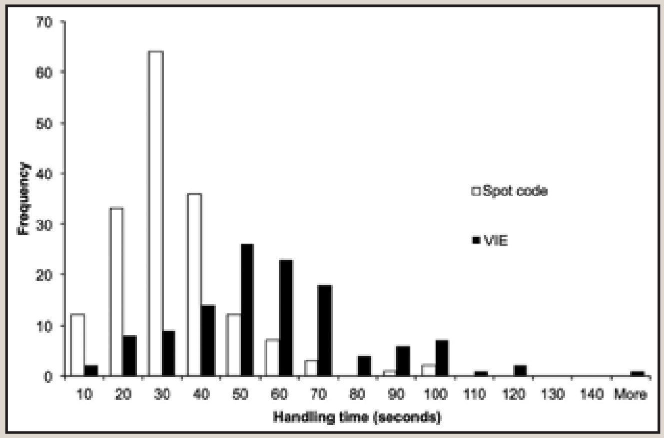

FIG. 3. Frequencies of time in seconds spent identifying individual salamanders (i.e., handling time) for each identification technique.

and one spot code was repeated four times. We accounted for these scenarios when two or more salamanders correctly receive the same spot code by adding a lower-case letter to the end of the spot code. Thus, the first individual captured with a given code would be 111111a, and a subsequent individual salamander with the same spot pattern would be 111111b. To help us differentiate between salamanders with the same spot code upon recapture, we took standardized photographs of the dorsum of each individual. Photographs were consistent with respect to background, angle, orientation, lighting, and distance from salamander. A ruler was also included in each photo to provide scale. These standardized photos were categorized in a computer database by spot code. Each individual spot code had its own folder in the database, and all photographs of a salamander with this spot code were stored in the folder for reference. Each photograph of a recaptured salamander was compared to database photographs to determine the identity of the individual captured. Photographs of each individual were also filed in the computer database under any alternate codes that might be mistakenly assigned to the salamander. Alternate codes were included when individuals had spots slightly smaller or larger than 1 mm or when spots lay on the boundaries of the designated regions, because these individuals are more likely to be misidentified. In the event of a misidentification, the identifier could find the salamander by looking up the mistaken code, but the image file would point them to the correct code.

To measure identification accuracy and overall handling time, we haphazardly selected salamanders that had been captured at our study site and brought back to the laboratory for processing. Processing individuals involved checking for a VIE code, reading the VIE code if it existed, assigning a spot identification code, taking a photograph of the dorsum, and taking the following measurements: mass (g), total length to the nearest mm (TL), and snout-to-vent length to the nearest mm (SVL). After processing, salamanders were haphazardly chosen to be used in our identification test (ID test) in which fifteen participants were asked to individually identify each salamander using both VIE and spot pattern techniques and record their results and handling time. Each identification of a salamander using one of the techniques was considered to be one trial. All participants had been trained in both techniques prior to ID testing, but participants were not informed of the correct VIE and spot codes for the salamanders they were identifying and were not permitted to crosscheck their identifications with the spot code or photo database. Finally, when participants identified an alternate spot code of a salamander, rather than the official code, this trial was still counted as a correct identification. Alternate codes are considered correct because when processing, identifiers would have access to the database where salamanders are cross-listed under both their official code and their alternate code(s).

We used program R (R Core Team 2013) to perform a mixed-effects logistic regression to determine whether identification technique was a significant predictor of identification accuracy. The binary response variable in this model was whether or not the salamander was identified correctly (yes or no) and the fixed predictor variable of interest was the identification type (VIE or spot code). We specified individual salamander as a random effect because individual salamanders were tested repeatedly. We also specified individual observer as a random effect because not all observers identified each salamander. The odds ratio for identification accuracy was used to determine the relative likelihood of correctly identifying individuals with each technique. We conducted a Student's t-test to determine if there was a difference in handling time (i.e., amount of time necessary to identify each individual) between VIE and spot code MR techniques. Alpha was 0.05 for all tests.

Results.—We conducted 171 trials for spot code and 123 trials for VIE on 41 *A. maculatum* individuals, totaling 294 trials. VIE trials matched the correct database codes 33% of the time, while 65% of spot code trials matched the correct database codes (Fig. 2).

We found that overall handling time using the spot code technique was significantly less than handling time using the VIE technique (Fig. 3; mean VIE = 54.2 ± 25.6 sec, mean spot code = 29.6 ± 14.9 sec; t-stat = 29.48; $p \leq 0.0001$). We found identification technique (e.g., VIE and spot code) to be a statistically significant predictor of identification accuracy (z-value = -5.092, $p \leq 0.0001$), with an odds ratio of 0.24 (95% CI 0.14–0.42). The odds ratio indicates that the participants were less likely to correctly identify a salamander when using the VIE technique than when using spot codes.

Discussion.— Our newly developed pattern recognition (spot code) technique for individually identifying Spotted Salamanders resulted in reduced handling time and improved accuracy compared to VIE techniques. Salamanders identified using the spot code technique took, on average, 25 fewer seconds than VIE and were twice as likely to be identified correctly than with VIE. Differences in standard error of handling time revealed our spot code technique to be more consistent than the VIE technique.

After six years of marking 2644 individuals from this *A. maculatum* population using VIE, we had exhausted many of the available code permutations. Our VIE marking technique yields a theoretical total of 6528 code permutations, but the more tags each salamander has, the longer its handling time, making the use of code combinations too time-consuming and expensive to be feasible for a daily trapping schedule. However, the theoretical maximum number of spot code permutations is limited only by the maximum number of spots in each body region. Of the 253 individual salamanders processed and assigned spot codes in the 2014 season, the maximum number of spots observed on any head region was seven, and the maximum number of spots observed on any appendage was five. If we assume these numbers to be the maximum number of spots possible in each location, then spot code yields a theoretical total of 82,944 possible permutations.

Furthermore, we found that the spot code technique is relatively easy to learn. Newly trained laboratory members had little difficulty with spot pattern but struggled through the duration of the study to correctly identify salamanders using VIE. One issue was that VIE colors (e.g., red and pink) were often difficult to discern. Migration and fragmentation of elastomer marks occurred as early as one year after VIE injection, causing some marks to be overlooked and others to move to ambiguous locations outside of the marking areas. With these difficulties, only experienced lab members with access to the full *A. maculatum* database could successfully identify VIE codes on a consistent basis.

Because the spot code identification technique was shown to have a shorter and more predictable overall handling time than the VIE identification technique, it is easier to predict how long processing will take. Low identification accuracy of our technique (65% accuracy) was an unexpected result. Other studies of VIE report identification accuracies of 87%, 83%, 81%, and 69% (Osbourn 2011). Other studies of manual pattern recognition report identification accuracies of 96.5%, 96%, or 97% (Loafman 1991; Grant and Nanjappa 2006). However, our observers were newly trained and thus had little experience in identification. We believe that as participants become more experienced with identification in future sampling seasons, the level of identification accuracy and standard error of handling time will improve rapidly. Further, we predict that ability of participants to crosscheck their observations through access to the database and standardized photographs would greatly improve identification accuracy.

Unlike other commonly used mark-recapture techniques such as toe-clipping, VIE, VIA, or PIT tagging, spot pattern is noninvasive and requires no use of anesthesia. Implementation of our technique is feasible, especially when standardized photos are taken of the dorsal side of each animal and additional alternate codes are given to animals whose spot code represents a borderline case (e.g., spot close to the 1-mm cutoff mark). These standardized photos are categorized in a computer database by spot code, so comparison of photographs to recaptured salamanders is straightforward when confirming salamander identity. The spot code technique is applicable to large populations and provides an easy and more effective way to identify individuals.

One concern with using spot pattern as a mark-recapture technique is that an individual's spot pattern could change over time, rendering spot pattern ineffective as an identification technique. Jonas et al. (2011) emphasize the importance of knowing whether or not body markings are variable before relying on these markings to identify individuals in a study. There are no published studies evaluating ontogenetic change in spot pattern in *A. maculatum*, although such studies do exist for other species: both *A. tigrinum* and *Eurycea longicauda longicauda* exhibit variation in spot pattern and coloration over time (Jonas et al. 2011; Waye 2013). As we accumulate images of recaptured individuals in future years, we can ensure whether or not spot patterns remain consistent over time by comparing images of salamanders identifiable by preexisting VIE tags and noting any changes.

We recommend further investigation into computer-assisted pattern recognition technology for Spotted Salamanders. Many studies have demonstrated the success and effective use of computer software in assigning identities based on unique natural patterns (Arzoumanian et al. 2005; Foster et al. 2007; Gamble et al. 2008; Anderson et al. 2010; Bendik et al. 2013; McClintock et al. 2013), but no software has been developed for this ambystomatid species. This technology could be applied to the standardized photographs taken of each salamander. Development of software to analyze spot pattern and assign individual identities to salamanders would reduce the issue of repeat codes and alternate codes and therefore has the potential to reduce human error in the identification process. A suggested approach would be to emulate the algorithm employed by Gamble et al. (2008) with *Ambystoma opacum*, which compared images based on visual similarity rather than on discrete geometric features. Software systems that extract shapes or distinct patterns and then apply a matching algorithm can struggle to identify large, irregular shapes like the spots of *A. maculatum*, so this visual comparison approach is more likely to yield successful results (Anderson et al. 2010).

Acknowledgments.—We thank members of the Davidson College Herpetology Laboratory, and in particular Kristen Cecala and Jacquelyn Guzy, for assistance in construction of the drift fence and data collection. We thank Michael Osbourn and Justin Strickland for comments that improved this manuscript. We also thank Mecklenburg County Department of Natural Resources, especially Don Seriff, Gary Marshall, Lenny Lampel, and Chris Matthews for site access and logistical support. Funding was provided by Davidson College Department of Biology. All work was carried out in accordance with Davidson College IACUC and a permit from the North Carolina Wildlife Resources Commission.

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