Tools and Technology Note

Visual Implant Elastomer Mark Retention Through Metamorphosis in Amphibian Larvae

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ABSTRACT Questions in population ecology require the study of marked animals, and marks are assumed to be permanent and not overlooked by observers. I evaluated retention through metamorphosis of visual implant elastomer marks in larval salamanders and frogs and assessed error in observer identification of these marks. I found 1) individual marks were not retained in larval wood frogs (*Rana sylvatica*), whereas only small marks were likely to be retained in larval salamanders (*Eurycea bislineata*), and 2) observers did not always correctly identify marked animals. Evaluating the assumptions of marking protocols is important in the design phase of a study so that correct inference can be made about the population processes of interest. This guidance should be generally useful to the design of mark–recapture studies, with particular application to studies of larval amphibians. (JOURNAL OF WILDLIFE MANAGEMENT 72(5):1247–1252; 2008)

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Detailed understanding of animal populations requires precise recognition of captured individuals. Estimation of some ecological parameters, such as movement and survival, or population growth rate, often relies on tracking individuals. To estimate these parameters, marks must be permanent, consistently identified throughout the life of an individual, and have no effect on survival or development. Capturing and marking large numbers of amphibians may be most easily accomplished during the larval stage, especially for aggregate breeders where egg deposition sites are spatially concentrated (e.g., wood frog [*Rana sylvatica*]) or species whose terrestrial forms are less likely to be encountered than larvae (e.g., northern two-lined salamander [*Eurycea bislineata*] adults are frequently absent from stream surveys; Grant et al. 2005). Natural variation in color pattern may be useful in mark–recapture studies of adult amphibians (e.g., Bailey 2004, Grant and Nanjappa 2006) but may not be useful in larval animals because color patterns have not completely formed. Retention of marks added during the larval stage is a critical consideration in any study where individuals are to be tracked through metamorphosis.

Many species of amphibians are characterized by complex life cycles, with ontogenetic changes characterized by changes in body form, rapid growth, and transition from aquatic to terrestrial life forms (Wilbur 1980). In general, metamorphosis of salamanders differs from that of frogs and toads. Salamander morphology remains much the same through metamorphosis, whereas anurans undergo a drastic change in morphology between tadpole and adult stages, including development of limbs, resorption of the tail, and development of adult skin (Duellman and Trueb 1986). I hypothesized that these differences in mode of metamorphosis may affect retention of marks assigned to an individual during the larval stage.

Three errors can be made that would invalidate the assumption that marks are retained and recorded correctly. First, a mark can be lost physically (mark loss). Second, a mark may move from the initial marking location (mark migration), because the mark was administered into the body cavity or too deeply into the space between the skin and the underlying muscle. I consider both mark loss and mark migration as separate components of mark retention, and I discuss both components of this error herein. Finally, observers may fail to correctly recognize a mark, either by overlooking a mark completely or by misidentifying the mark code (i.e., observer bias).

My objectives were to 1) assess retention of marks through metamorphosis in 3 species of larval amphibians: northern dusky salamander (*Desmognathus fuscus*), northern two-lined salamander, and wood frog, and 2) to quantify bias in observer identification of marked salamander larvae (northern dusky salamander).

STUDY AREA

In April 2006, I collected 30 larval northern dusky salamanders from streams in the Chesapeake and Ohio Canal National Historic Park, Maryland, USA. In June 2006, I collected 30 larval two-lined salamanders from a different stream in the same park. In June 2006, I obtained 120 wood frog tadpoles from a backyard swimming pool in Silver Spring, Maryland, USA.

METHODS

I marked animals with visual implant elastomer (VIE; Northwest Marine Technology Inc., Shaw Island, WA). This material has been used to mark frogs (e.g., Anholt et al. 1998, Nauwelaerts et al. 2000) and salamanders (e.g., Davis and Ovaska 2001, Marold 2001, Johnson and Wallace 2002, Bailey 2004) across a range of sizes and life history stages. The VIE is a 2-part silicone-based polymer that cures to a pliable consistency, which can be detected with ultraviolet or blue light with amber filtering glasses. The best method for

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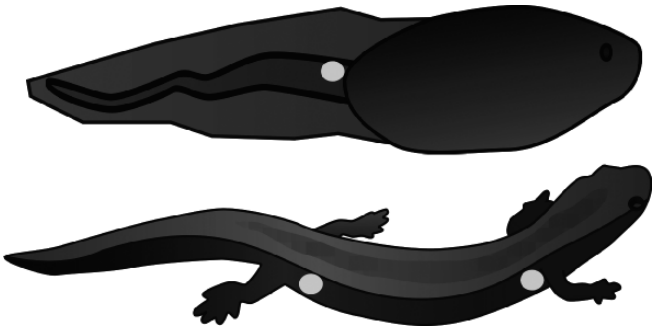

Figure 1. Marking locations on wood frog tadpoles (top) and northern dusky and northern two-lined salamander larvae (bottom) collected in April and June 2006 from a pond and streams in Maryland, USA, and used to assess mark retention through metamorphosis.

administering VIE marks to amphibians is to inject the VIE just under and parallel to the skin.

I gave 60 wood frogs both a red and a green mark at the base of the tail (Fig. 1) with a 29-gauge needle, just above the tail musculature on either side of the tail fin (following Anholt et al. 1998), and I handled but did not inject an equal number as unmarked controls. To administer marks, I anesthetized all individuals (including control animals) in a buffered (pH 7.0) 500 mg/L tricaine methylsulfonate (MS-222). I allowed all animals to recover in clean water and then I added them to 1 of 2 10-L tanks. I fed animals frozen, thawed organic romaine lettuce and fish food flakes. After 20 days, I captured 20 tadpoles at random from the marked treatment group, and assessed retention of marks. After each surviving animal metamorphosed I recorded marks for the marked group on 2 separate days following metamorphosis. I used a 2-tailed *t*-test to test the hypothesis that marking had no effect on either time to metamorphosis or size at metamorphosis in the wood frog tadpoles, and a linear regression to relate mark loss (as an explanatory variable with 3 levels: 0, 1, or 2 marks retained) to time to metamorphosis.

I chose 20 individuals of both the dusky salamander and the northern two-lined salamander at random to receive marks. I gave 8 northern dusky salamanders and 5 northern two-lined salamanders one mark and 12 northern dusky salamanders and 15 northern two-lined salamanders 2 marks. I gave animals a unique mark by combining 2 VIE colors (red, green) and 4 marking locations (anterior to each hind limb and posterior to each front limb; Fig. 1) with a 29-gauge needle. I handled, but did not inject, all animals that were not marked. I kept animals in individual containers in an environmental chamber maintained at 15° C and fed them frozen, thawed bloodworms until metamorphosis. I did not use a microscope while I marked either frogs or salamanders because I was interested in feasibility of the technique for marking large numbers of small animals in remote field locations. I conducted all work under approved protocols through the University of Maryland Animal Care and Use Committee (ACUC no. R-06-14) and the Patuxent Wildlife Research Center ACUC, and I obtained all applicable permits from the state of Maryland and the Chesapeake and Ohio Canal National Historic Park.

I chose 16 of the 30 northern dusky salamanders (11 marked, 5 unmarked) at random for the observer bias study. Thirteen observers participated in the study: 4 with prior experience identifying marks in the field and 9 naïve observers. Naïve observers were primarily graduate students at the University of Maryland and did not have experience with VIE marking techniques. I gave observers a brief introduction to VIE marking techniques and mark identification and allowed them to practice with color standards until they were comfortable using the VIE lights. Recently Northwest Marine Technology, Inc. switched from a blue-light emitting diode (LED) light and amber filtering glasses to a violet-LED light, which was intended to facilitate mark identification. I tested both lights for efficacy in mark identification. I informed observers of the marking procedure and that animals may have been given between zero and 4 marks, with 2 colors of VIE (red and green). I provided observers a schematic of the marking locations and samples of both VIE colors. After all observers had viewed the animals using one light, salamanders were rerandomized and presented to the same observers, who viewed the salamanders with the other light. Observers were unaware that they were presented with the same set of individuals for each light. An observation was scored as correct when an observer recorded all marks on an individual salamander that matched the true mark combination. Because I expected the probability of recording an incorrect mark to increase with the complexity of the marking code (1 vs. 2 marks), I used separate logistic regression models for each light (blue and violet) to determine if the proportion of correct identifications was related to the number of marks. I used a 2-tailed paired *t*-test to test whether observers took more time to identify marks by light or whether the light (blue vs. violet) was related to the proportion of correctly identified salamanders. I transformed the proportion of correctly identified salamanders with an arcsine square root. I separated observers into naïve and experienced observer groups to further evaluate the impact of training on correct mark identification.

I evaluated mark migration in northern two-lined salamanders on 4 occasions prior to their metamorphosis. On each occasion, I recorded the marks on each animal twice on the same day, which allowed me to separate mark migration from observer error in mark identification. After each animal metamorphosed, I recorded the position of marks remaining in each salamander. I also did this twice on one day to assess bias in mark identification. I used multistate modeling to evaluate my observations on mark retention in northern two-lined individuals. Mark migration is equivalent to an animal's code making a transition, with probability, Ψ_{ij}, among states correct and incorrect between 2 observation periods (Ψ_{CI}; Williams et al. 2002). Marks that are loose in the body cavity or administered too deeply under the skin may change position over different observation periods, resulting in some animals first experiencing mark migration and then recovering their marks (i.e., the mark returned to the original location on subsequent

Table 1. Results of model selection for 6 multistate models I analyzed using Program MARK, describing mark migration in northern two-lined salamanders collected June 2006 from streams in Maryland, USA.

Model	Model name[a]	ΔAIC_c[b]	AIC_c wt[b]	Model likelihood	K[c]	Deviance
5	S (t) Ψ (state, t_1,$t_{2–5}$)	0.000	0.460	1.000	7	16.694
2	S (t) Ψ (state)	1.018	0.276	0.601	6	20.037
6	S (t) Ψ (.)	2.325	0.144	0.313	5	23.618
4	S (t) Ψ (t_1,$t_{2–5}$)	3.517	0.079	0.172	6	22.536
1	S (t) Ψ (state×t)	5.773	0.026	0.056	11	12.620
3	S (t) Ψ (t)	7.994	0.008	0.018	8	22.310

[a] I estimated transition (Ψ) probabilities, which may occur between states correct (C) and incorrect (I), under several competing hypotheses. In all models, survival is assumed equal among states ($S_C = S_I$) but allowed to vary across observation periods (time, t), the capture probability (p) is set to one, and the transition probability, Ψ_{IC}, for the first time period is set to zero (because all individuals were known to have a correct mark code in the first observation period).

[b] AIC_c = Akaike's Information Criterion, adjusted for small sample size (Burnham and Anderson 2002).

[c] K = no, of parameters estimated in the model.

observations). Because I observed some animals that recovered their marks, I also estimated the transition probabilities from incorrect to correct mark codes (Ψ_{IC}). To estimate the transition probabilities Ψ_{CI} and Ψ_{IC}, I used the recaptures-only multistate model in Program MARK (White and Burnham 1999). I created a 5-period capture history for each animal, recording whether the read code matched the given code (on day 0) at 16 days, 43 days, 96 days, and 116 days after marking. I fit all models where survival was allowed to vary by time (because several animals died at each time period) but was equal for all animals (i.e., there was no effect of mark migration on survival), and I set the capture probability (p) equal to one. I set the initial transition probability from incorrect to correct (Ψ_{IC}) to zero for all models because all individuals started the study with a correct mark code.

I used 6 multistate models (Table 1), representing different hypotheses of the nature of mark migration in larval salamanders. Model 1 is the most general model, with transition probabilities state independent and allowed to vary among observation periods. Model 2 allows transition probabilities to vary by state only (constant across all observation periods). Model 3 specifies a constant transition probability among states but allows variation across observation periods. Model 4 allows a different Ψ_{CI} in the first time period (vs. time periods 2–5) and $\Psi_{CI} = \Psi_{IC}$ for each subsequent time period. Model 5 is similar to model 4 except that it does not require $\Psi_{CI} = \Psi_{IC}$. Model 6 specifies a constant transition probability among states (i.e., $\Psi_{CI} = \Psi_{IC}$) with no time variation. I use Akaike's information criterion corrected for small sample size (AIC_c; Burnham and Anderson 2002) to rank the candidate models. I considered models within 2.0 ΔAIC_c units to have support. I expected model 4, which specified a different transition probability (Ψ_{CI}) in the first time period and equivalence among states ($\Psi_{CI} = \Psi_{IC}$) to be the most likely (e.g., have the lowest AIC_c).

RESULTS

Of the 120 wood frog tadpoles, 42 of the 60 marked and 45 of the 60 unmarked tadpoles successfully metamorphosed. After 20 days, 50% of marked tadpoles had lost 1 of the 2 marks, though no individuals lost both marks. Among marked individuals that metamorphosed, 67% lost ≥1 mark and 21% lost both marks. There was no relationship between time to metamorphosis and number of marks retained ($R^2 = 0.03$). Location of marks did not change in any individual, though as the tail was resorbed, marks moved further up the animal's dorsum. Marks were visible for ≥2 weeks following metamorphosis. Marks in 2 individuals were still visible 6 months after metamorphosis under darkened dorsal pigment. There was no difference between marking treatments in time to metamorphosis ($\bar{x} \pm 1$ SE: unmarked = 28 ± 10 days, marked = 31 ± 13 days; $t_{88} = 0.132$, $P = 0.188$) or size (snout–vent length) at metamorphosis ($\bar{x} \pm 1$ SE: [for both marked and unmarked groups] = 14 ± 2 mm; $t_{68} = 0.67$, $P = 0.542$).

Naïve and expert observers correctly identified 69% and 83% of marked northern dusky salamanders, respectively. In all but 2 of 130 total observations of unmarked individuals (13 observers × 5 unmarked northern dusky × 2 lights), observers correctly identified unmarked individuals. Overall, observers using the blue light and amber glasses took less time ($t_{10} = 3.24$, $P = 0.008$) and had a higher proportion of correctly identified marks ($t_{10} = 2.54$, $P = 0.029$; 0.80 ± 0.07% for the blue light vs. 0.66 ± 0.05% for the violet light). The difference between lights was larger in naïve observers (0.79 ± 0.08% for the blue light, 0.60 ± 0.04% for the violet light) than in experienced observers (0.84 ± 0.08% for the blue light, 0.82 ± 0.10% for the violet light). Whereas naïve observers had higher correct identification under the blue light ($t_{10} = 2.38$, $P = 0.039$), experienced observers did not prefer either light ($t_{10} = 0.15$, $P = 0.882$). Probability of correct identification was not related to the number of marks on an individual; the variable describing number of marks (1 vs. 2 VIE marks) was not significant in a logistic regression model conducted separately for each light (blue light: $\chi^2 = 0.290$, $P = 0.591$; violet light: $\chi^2 = 0.601$, $P = 0.438$). Odds ratios were small for 2 marks with both lights (blue light 1.255, 95% Wald CI = 0.549–2.870; violet light 1.317, 95% Wald CI = 0.656–2.643). In 5 of 11 marked northern dusky salamanders, marked animals were incorrectly identified as not having a mark by ≥1 observer

Table 2. Marks moved in the following marked northern two-lined salamanders collected June 2006 from streams in Maryland, USA, that survived through ≥2 sample periods (43 days postmarking).

Animal identification	Days after marking[a]			
	16	43	96	146
15	−	−	xx	xx
12	−	+	+	xx
18	−	−	+	xx
09	−	+	+	−
23	−	−	−	−
17	+	+	+	−

[a] + indicates that the mark matched the original mark given, whereas − indicates the mark did not match the given mark (i.e., that a mark had moved elsewhere in the body). xx indicates that the animal died prior to the sample period.

Table 3. Estimates for the survival and transition probabilities for marks assigned to northern two-lined salamanders collected June 2006 from streams in Maryland, USA, under the best multistate model (model 4; S(t) Ψ (state, t_1,t_{2-5})) in Program MARK.

Days after marking (t)	Parameter	Estimated p[a]	SE (Estimate)[a]
16	Survival (S)	0.964	0.036
43		0.926	0.050
96		0.680	0.093
146		0.471	0.121
Initial	Transition (Ψ_{CI})	0.185	0.075
> day 16		0.048	0.033
Initial	Transition (Ψ_{IC})	−	−
> day 16		0.375	0.171

[a] For the analysis, I fixed the capture probability (p) equal to one and the transition probability between incorrect and correct marks (Ψ_{IC}) equal to zero for the first period (day 16 after marking), indicated by − in the table.

(range 1–5 observers), but ≥1 observer correctly identified marks in all animals.

Most of the northern two-lined salamanders died prior to metamorphosis, likely caused by a *Saprolegnia* fungus (D. E. Green, National Wildlife Health Center, personal communication). Marking did not contribute to mortality or susceptibility to the disease because control animals died at the same rate. Marks migrated from their original position at some point over the study period in 5 of 17 marked individuals that survived through the second observation period (43 days after marking; Table 2). There was no consistent pattern of mark migration, but marks tended to accumulate near the vent, which has been observed in adult animals in the field. A sixth individual (animal 17) had one mark migrate after 146 days. In 3 individuals, a mark was recorded in a different position in one time period, only to return to the original location in a later period (Table 2; animals 09, 12, 18). Viewing each salamander twice in the same day (i.e., during each observational period) ruled out the possibility that this effect was due to mark misidentification. Large marks (i.e., approx. ≥2 mm) were more likely to migrate; I gave 7 individuals large marks, of which 5 experienced mark migration over the course of the study. All individuals with mark migration had one mark that split into two, and one of these 2 marks then migrated to a different position.

The most general model (model 1), with transition probabilities different in each state and time period, was not well-supported (e.g., $\Delta AIC_c > 0$; Table 1), whereas models 5 and 2 had similar levels of support ($\Delta AIC_c < 2.0$). The estimated transition probability (Ψ_{CI}, from correctly marked to incorrectly marked) was highest during the first observation period (estimate ± SE; $\Psi_{CI} = 0.185 \pm 0.075$) and was smaller in the subsequent observation periods ($\Psi_{CI} = 0.048 \pm 0.033$) under model 5, with a different Ψ_{CI} in the first time period (vs. time periods 2–5), and $\Psi_{CI} = \Psi_{IC}$ for each subsequent time period (Table 1). Recovery probability (Ψ_{IC}, transition probability between an incorrect and a correct mark) was high (estimate ± SE; $\Psi_{IC} = 0.375 \pm 0.171$). The model I expected a priori to be most likely (model 4) was not well supported (Table 1; $\Delta AIC_c > 2.0$, AIC_c wt = 0.079), though the top model (model 5) still allows a different transition in the first observation period, (with Ψ_{CI} not equal to Ψ_{IC}; Table 3).

Probability of mark migration in the two-lined salamander larvae resulting in an incorrect mark decreased after the initial marking period (Tables 1, 3). Consider an example: if the 6 animals (in Table 2) are recaptured 16 days after marking, 5 of the individuals would be misidentified (due to mark migration). If the same 6 animals were recaptured 43 days after marking, only 3 of the individuals would be misidentified (due to mark migration). Some individuals recovered their marks over time, which may be unique in the mark–recapture literature.

Only 7 (4 marked and 3 unmarked) northern two-lined salamanders survived and successfully metamorphosed; thus, there was no apparent effect of marking on survival to metamorphosis. All metamorphosed animals retained their marks in the correct positions. All northern dusky salamanders successfully metamorphosed, but marks migrated in 25% (5 of 20) of individuals.

DISCUSSION

Identity of an individual changed for every observation period after the mark migrated (unless the mark was recovered in a later period; e.g., Table 2, animals 09, 12, 18), which is unlike observer (e.g., resighting) errors that are independent among time periods. Size of the VIE mark may influence retention of a given mark code; large marks split and migrated to different positions within an animal. Therefore, I suggest taking care to assign marks that are small, if the assumption that marks are retained is to be met.

The effect of tag migration on estimates of demographic parameters may depend on goals of the study and, thus, the capture–recapture model being considered. For example, in 2-sample, closed population abundance estimators such as the Lincoln–Petersen model (Williams et al. 2002), only identification of the number of previously marked individuals is required. In this model, individual marks are not needed (and mark migration is not an issue if the mark is not physically lost). In a study lasting >2 capture occasions, estimation of survival from period t to $t + 1$ generally

requires individually marked animals, especially when investigating factors that influence survival (e.g., size, sex, age). Tag migration may result in an individual having a lower capture probability after initial marking (similar to a trap-shy response; Pollock et al. 1990), leading to a positive bias in abundance estimators under open population models (e.g., the Jolly–Seber model; Williams et al. 2002), due to a negative bias in the capture parameter estimate, p (Weiss et al. 1991, Schwarz and Stobo 1999). Survival estimates under the Jolly–Seber model are robust to heterogeneity in capture probabilities, and in long-lived species, the bias in survival estimates declines over time (Schwarz and Stobo 1999). Mark migration that results in a new individual can be identified by comparison with the list of known marks in the population, provided that the investigator added the marks. It is important to consider ways of reducing or detecting the error when mark migration results in an individual that has a mark matching a true code of a different animal in the population.

Errors in recording marks may be common (e.g., Stevick et al. 2001, Milligan et al. 2003). Mark loss or misidentification has the potential to bias estimates of demographic parameters; however, field studies may have insufficient power to detect these errors (Schwarz and Stobo 1999). When mark misidentification, mark loss, or mark migration cannot be controlled during the design phase of a capture–mark–recapture study, these errors must be incorporated into the modeling of the capture histories (e.g., Lukacs and Burnham 2005), which can deal explicitly with biases caused by incorrectly identifying marked animals (at a cost of reduced estimator precision). Reducing the potential for these errors in the data collection phase of a capture–mark–recapture study is advised, because an increase in estimated variance of population size, for example, may occur when error rates are as small as 5% in genetic mark–recapture studies (Lukacs and Burnham 2005).

Mark retention and observer bias in mark identification have not been routinely tested or reported in amphibian mark–recapture studies, despite potential for bias in demographic parameter estimates caused by mark loss or misidentification (Bailey 2004). Tracking amphibians through metamorphosis is an important component of investigations into factors limiting amphibian populations, and care must be taken when using VIE marks to individually identify larval amphibians. In addition, despite the cost associated with additional observers (or observations by one observer) of each captured animal, errors in mark identification can be identified only with repeat observations of each individual. Finally, care must be taken in the design phase of a mark–recapture study to ensure that all model assumptions are met, because violation of any of the assumptions of mark recapture models can result in either large variances of estimated parameters or incorrect inference.

Management Implications

For larval amphibians, I suggest 1) assigning small VIE marks if individual identifiers are needed because large marks may split and migrate, leading to incorrect mark codes, and 2) using 2 observers (or having one observer view an animal's mark twice) to check both the application of mark codes and recording of codes on recaptured animals. Further, assigning marks with a small number of locations or VIE colors (especially early in the study) and recording age and detailed size information may allow subsequent identification of suspect marks. This type of data may allow estimation of the joint probability of a mark migrating and being read as an invalid code.

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