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A New, Noninvasive Method of Batch-Marking Amphibians Across Developmental Stages

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***Abstract.*—Organisms that undergo complex morphological change like many amphibian species pose a particular challenge for marking individuals with labels that persist across ontogeny. Here, I detail a method for noninvasive, inter-stage, mass marking of Wood Frog, *Rana sylvatica* (= *Lithobates sylvatica*), larvae with calcein. Unlike other marking methods, such as tags or dyes, the calcein fluorochrome binds to bone and other calcified tissue. The mark is administered via short-duration (3.5 min.) submersion in liquid calcein solution. This study demonstrates that calcein marking is a fast and reliablemethod for mass marking amphibians that persists through metamorphosis with no adverse mortality or growth effects. This marking method is especially useful for species that are sensitive to handling and manipulation. The persistence of calcein labels correlated positively with the mass and developmental stage of the individual at the time of marking. Calcein fluorescence appeared throughout the larval integument for 3 d after administration. Afterward, I detected labels externally in skeletal tissue upon metamorphosis in all larvae marked at Gosner stage 30 or greater. I detected labels 146 d after administration in 100% of post-metamorphic individuals marked as larvae over Gosner stage 30 and marked within 19 d of metamorphosis. For larvae marked prior to Gosner stage 30, I detected the label in 75% of individuals after 8 d and 54% of individuals upon metamorphosis. Finally, I discuss the applicability of this technique in field studies and in other taxa.**

Key Words.—calcein; capture-mark-recapture; fluorescent label; mass marking; metamorphosis; monitoring; survey method; Wood Frog

Introduction

Organismal biologists and field researchers often need to recognize individuals or groups across space and time to, for instance, estimate population parameters (i.e., birth, death, immigration, survival, etc.) and spatial patterns (i.e., home range, dispersal distance, etc.; Nichols 1992; Heyer et al. 1994). Marking many individuals or working with small species with soft integument like amphibians is particularly challenging with current methods, all of which require invasive techniques and/or individual handling. Amphibians in early life stages have been particularly difficult to mark. However, these same stages are especially important to study demographically to understand population dynamics (Vonesh and De la Cruz 2002).

Practical factors (e.g., larvae may be easier to collect than adults) or experimental design may require animals marked as larvae to be recognized as adults. The complex life histories of amphibians challenges marking strategies across life stages, as none of the conventional methods persist reliably through metamorphosis (Grant 2008; Martin 2011; Courtois et al. 2013; Ringler et al. 2015). This is particularly true for anurans that undergo considerable change in morphology, including integument, internal organs, and skeletal structure (McDiarmid and Altig 1999). The regenerative ability of many amphibian species can yield tissue labels unreliable, adding further challenges (Brockes 1997). The few marking methods that may allow for inter-stage recognition, such as PIT (passive integrated transponder) tags or visible implants, require invasive administration and considerable handling of each individual, which can incur exorbitant time and monetary costs for large scale studies and which can bias results due to mortality or behavioral change (Grant 2008; Martin 2011; Courtois et al. 2013; Ringler et al. 2015).

A new method for marking larval amphibians that persists through metamorphosis is required. Ideally, such a technique would be permanent (or persistent enough for long-term studies), consistently and easily detectable with high accuracy, and incur no negative effects on the survival or development of the marked individuals. From a practical standpoint, the method should be easy and quick to administer in the field, cost-effective, and allow for marking many individuals at once. Fluorochrome dyes such as tetracyclines, calcein, xylenol orange, and alizarin red have been used as *in vivo* labels in animal anatomy studies for decades (reviewed in Frazier 1985; van Gaalen et al. 2010) and more recently employed in fisheries as a mass marking method (e.g., Wilson et al. 1987; Monaghan 1993;

Moran 2000; Crook et al. 2009; Becker et al. 2017). The fluorochrome tetracycline has been previously evaluated as a marking agent in amphibians, but generally found to be ineffective (Muths et al. 2000; Hatfield et al. 2001) and deleterious to health (Erben 2003; but see Francillon and Castanet 1985).

The successful application of calcein fluorochrome to mark freshwater fish fry promises that this technique may be transferable to amphibians as an alternative to tetracycline (Leips et al. 2001; Mohler 2003; Negus and Tureson 2004). Calcein is a chelating agent that binds to the mineralizing surface of ossified tissue such as bones or scales (Wallach et al. 1959; Hefley and Jaselskis 1974). Calcein labels are not observable in sunlight; however, when marked tissues are exposed to light of a specific (excitation) wavelength it is reflected at a longer (emission) wavelength (van Gaalen et al. 2010). When viewed through a filter that cancels the excitation wavelength, only the fluorescent emission is visible as a green label (van Gaalen et al. 2010).

Labels can be applied to many individuals at once through batch immersion in calcein solution. Osmotic induction, wherein animals are immersed a hyperosmotic salt solution prior to immersion in calcein, greatly reduces the amount of time fish species must remain in the calcein solution and increases the longevity and intensity of the resultant labels in fish (Alcobendas et al. 1991; Mohler 2003; Hill and Quesada 2010). It is unknown if this technique will improve fluorochrome uptake in amphibians or impact the health of marked individuals.

In this study, I test a new technique for mass marking larval amphibians using a fluorochomatic calcein solution administered with and without osmotic induction. I consider the utility of this technique for inter-stage and intra-larval stage labeling by testing the persistence of the resultant mark over time in Wood Frogs, *Rana sylvatica* (= *Lithobates sylvatica*), larvae marked at various life stages. Unlike other studies of *in vivo* tissue marking techniques that require viewing cross sections of calcified tissue in the laboratory (e.g., Muths et al. 2000), I nonlethally detected calcein labels from external observation. At the end of the experiment, I compared the label detection probability between nonlethal, external observation and postmortem observations of skeletal cross sections. I also tested the effect of the marking procedure on mortality and growth.

Materials and Methods

Study population.—I collected the larvae used in this experiment as eggs within 48 h of oviposition from Yale Myers Forest ponds (Eastford, Connecticut, USA) on 9 and 10 April 2017 and subsequently hatched and reared the larvae in outdoor mesocosms (152 cm diameter, 1,500 L volume plastic stock tanks). Prior to stocking, I filled mesocosms to 1100 L with aged well water and seeded each with 60 g Kaytee Rabbit Chow (Kaytee Products, Inc.; Chilton, Wisconsin, USA), 600 g of leaf litter collected adjacent to the pond, and aliquots of phytoplankton and zooplankton collected from a nearby pond. I covered each mesocosm with a mesh screen to prevent colonization by predators.

Experimental design.—To test the utility and longevity of calcein labeling for amphibian larvae, I conducted two experiments. In the first experiment, I tested inter-stage persistence of calcein labels in postmetamorphic juvenile Wood Frogs that were marked as larvae (Gosner 1960; stages 30–42). In the second experiment, I tested intra-stage persistence of the label in larval Wood Frogs marked as early-stage larvae (< Gosner stage 30) up to metamorphosis (Gosner stage 42). In both experiments, I tested the effect of calcein and the osmotic induction technique in a cross-factorial design consisting of a control group that received neither salt solution nor calcein solution (Treatment A) and treatment groups that received salt solution only (Treatment B), calcein solution only (Treatment C), or salt solution and calcein solution (osmotic induction; Treatment D; Supplemental Information Table S1).

Marking method.—The osmotic induction marking technique requires submersion in a hypotonic solution prior to exposure to calcein (conditions in the saline bath are hyperosmotic to larval cells). Immediate, subsequent immersion in a calcein solution promotes osmotic uptake as the crenated cells equilibrate to become isotonic with the calcein bath. A 5% saline solution has been used for this technique with freshwater (Honeyfield et al. 2008; Crook et al. 2009) and anadromous (Mohler 2003) fish; however, salt is a known toxic agent for Wood Frog larvae even at weak concentrations (Sanzo and Hecnar 2006; Brady 2013). Prior to this study, I tested responses to 1.5%, 1.0%, and 0.5% saline solutions and found that Wood Frog larvae exhibited no pain-response (i.e., no reaction of strong muscular movement nor attempts to jump from the holding container) when exposed to salt concentrations at or below 1.0% and no subsequent growth malformities (A.Z. Andis, pers. obs.). I therefore chose to test osmotic induction using 1.0% salt solution.

The procedure for batch marking was similar to Mohler (2003). I prepared a pre-bath of 1.0% saline solution by dissolving a ratio of 1 g of NaCl to 100 ml of laboratory-temperature distilled water and acclimatized a bath of 1.0% calcein solution (SE-MARK, Western Chemical, Inc., Ferndale, Washington, USA) to match enclosure water temperature. I prepared two baths of laboratory-temperature reconstituted distilled (RD)

water for null and control treatments. Each treatment finished with a rinse in laboratory-temperature RD water that I replaced after each batch to removed excess marking solution.

For each treatment group, I removed a larva from its enclosure, placed it in a strainer, and transferred it to the first bath. After 3.5 min, I quickly blotted the strainer on a paper towel and immediately transferred the larva and strainer to the second bath. After another 3.5 min, I blotted the strainer again and placed it in a RD water bath to rinse off the treatment solution before returning the individual to its enclosure. For the inter-stage experiment, I marked five larvae from each batch in a single strainer. I marked larvae individually in the intra-stage experiment. In total, fewer than 10 min elapsed between removing a larva from its enclosure until returning it.

Inter-stage experiment.—This experiment tested the detectability of calcein labels in terrestrial, post-metamorphosis Wood Frogs marked as larvae. For this experiment, I collected 80 larvae from the mesocosms on 24 May 2017 (six weeks after oviposition and approximately four weeks after hatching) and reared them in glass aquariums indoors for three weeks to allow for acclimation and further development. I predicted that the efficacy of the marking procedure will be influenced by the total amount of calcifying tissue present in the larvae. To standardize larvae by developmental stage and weight across treatments, I binned the larvae by stages (Gosner stages 30–32, 33–35, 36–38, 39–41, 42) and ranked them by mass within each stage bin. I blocked larvae into groups of four by mass within each stage-class and randomly assigned each individual in the block to one of the three treatment groups or control group. In total, the 80 larvae comprised 20 blocks with 20 larvae in each treatment group.

Marking occurred on 21 June 2017. After the treatment, I placed larvae individually in 1 L glass containers filled with laboratory-temperature RD water and arranged them by block in a climate-controlled animal room set at 15° C with a 12 h photoperiod centered at 1200 EST. Every two days, I fed larvae approximately 10% of body mass/day (powdered Kaytee Rabbit Chow from Kaytee Products, Inc.; Chilton, Wisconsin, USA, and TetraPro Goldfish Food from Tetra Gmbh; Melle, Germany in a 3:1 ratio). I performed complete water changes every four to five days.

I checked larvae daily. Once a larva reached metamorphosis (Gosner stage 42), I removed it from the aquatic housing, weighed it, and placed it in a 500 ml plastic container with 200 ml of RD water and set at an angle to provide access to aquatic and terrestrial habitat. After 3 d, I placed the metamorph in a leveled enclosure with a hide and damp paper towel as substrate. Metamorphs received 10 *Drosophila melanogaster* (fruit flies) every 2–3 d, with the number increasing by five flies once any animal in the experiment consumed all flies before the next feeding. I cleaned enclosures every 3–4 d.

I examined larvae to assess mark persistence weekly until all animals reached metamorphosis. I recorded mark detectability upon metamorphosis, followed by weekly examinations for six weeks and every two weeks thereafter. For detection trials, I randomly arranged animals from all treatment and control groups in a blind trial and a trained observer examined each animal in a dark, windowless room with a NIGHTSEA BlueStar handheld 440–460 nm flashlight and corresponding wavelength filtering glasses (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) as would be employed in the field. I did not remove animals from their enclosures for the examination. When experienced observers were not available, I recruited three volunteers. In this case, I considered a positive detection if at least one of three volunteers detected the mark.

The experiment terminated on 20 November 2017 (146 d post-treatment) as this is about the time Wood Frogs naturally begin hibernating in the northeastern United States. My assumption was that in the wild, mark quality would be consistent through hibernation and detectability in the fall should reflect detection rates expected in the spring. At the end of the experiment, I euthanized remaining animals and stored them in 70% EtOH. I dissected phalanges (4th digit of right hindfoot) and tibiofibula (right) from each specimen. I examined a transverse cross-section of each bone in a dark, windowless room under a dissecting scope to assess for evidence of a calcein label.

Intra-stage experiment.—To test the short-term efficacy of marking Wood Frog larvae at early developmental stages, I collected 32 larvae from the outdoor mesocosms on 23 May 2017. After 24 h of acclimatization to the laboratory temperatures, I ranked individual larvae by mass and housed them individually in glass containers with 1 L of RD water. I ranked the larvae by mass and blocked them into groups of four. Within each block, I randomly assigned each larva to one of three treatment groups or the control group for a total of eight replicates. All larvae were between Gosner stages 26 and 30 with a mean mass of 0.35 g.

After treatment, I arranged larvae by block in an incubator (Precision Model 818, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) set at 20° C with a 12 h photoperiod centered at 1200 EST. Larval husbandry followed procedures outlined above with daily checks. When a larva reached metamorphosis, I euthanized it and recorded mass, total length, and snout-to-vent length (SVL) measurements.

Table 1. Mortality (65 d post-treatment), mean mass at metamorphosis (MM), and mean mass 65 d post-treatment (M65) of Wood Frog (*Rana sylvatica*) metamorphs treated as larvae in the inter-stage experiment. Sample size was 20 for all treatments. Treatments included a control (A), exposure to 1.0% salt solution (B), exposure to 1.0% calcein (C), and exposure to 1.0% salt and 1.0% calcein solutions (D, osmotic induction).

Treatment group	Mortality	MM (g)	SE	M65 (g)	SE
A (control)	3	0.70	0.10	0.74	0.08
B (salt only)	2	0.71	0.12	0.73	0.09
C (calcein only)	1	0.64	0.13	0.76	0.07
D (osmotic induction)	2	0.71	0.16	0.76	0.10

Analysis.—To assess the effect of the treatments on mortality and growth, I fit logistic and least squares regression models (respectively). For each analysis, I fit a minimal model with only treatment group as the independent variable, then subsequently included initial mass. Growth is nonlinear across the larval period, so for the growth analysis, I also considered the initial age (measured as time to metamorphosis) and the interaction of initial mass and initial age in the models. I used likelihood ratio tests using the ANOVA function to compare linear models and compared AIC statistics for logistic models to determine the most appropriate model in the hierarchy.

I tested the detectability of calcein over time for two marking techniques: osmotic induction and calcein immersion without the hypotonic pre-bath for both inter-stage and intra-stage detection. I evaluated the marking techniques simultaneously by fitting repeated measures mixed effect logistic regression models implemented in nlme package in R, with a subject identification factor as a random intercept (Pinheiro et al. 2017). As with the mortality and growth analysis, I fit a minimal model with only treatment type and days since marking (days since metamorphosis for inter-stage analysis) included as independent fixed effects. I subsequently included initial mass, initial age, and their interaction into the models. I compared AIC statistics for hierarchical model selection.

I estimated predicted detection probabilities from the best fit models by first predicting initial larval mass for a given age class via least squares regression on these values from the sample population. I then incorporated the predicted initial mass from this sub-model into the primary model to estimate detection probabilities for each age class. I conducted all statistical analysis in R version 3.4.1 (R Core Team 2017) with α for all tests 0.05.

Results

Marking.—Larvae reacted with minimal distress during transfer and initial immersion in salt treatment baths (i.e., short-duration vigorous swimming), but quickly calmed within the 3.5 min immersion window. Larvae showed no distress in the calcein bath and reacted similarly to those in the null treatment RD water bath. The calcein label was clearly observable externally in the epithelial cell of all larvae in calcein treatments (treatments C and D) after marking (Fig. 1A). Within 3–4 d after initial treatment, the mark attenuated externally but I easily observed it through the skin along the notochord and within skeletal structures in later stage larvae and metamorphs (Fig. 1B).

Mortality and growth.—There was no evidence that either the salt solution or calcein solution affected mortality up to 65 d post-treatment (Table 1). Furthermore, I observed no mortality among calcein-marked juveniles retained between 65 and 146 d post-treatment. For the mortality analysis, I modeled only treatment and initial mass because including more parameters led to complete separation among predictors and inflated coefficient estimates (Albert and Anderson 1984). None of the treatments nor initial mass were significant predictors of mortality (all $P > 0.300$; Supplemental Information Table S2).

None of the treatments demonstrated effects on Wood frog growth (change in mass) up to metamorphosis or 65 d post-treatment; however, there was a slight trend for lower final mass at metamorphosis in Treatment C (calcein only) differing from the control groups ($P = 0.058$; Supplemental Information Tables S3 and S4). The best fit model ($r^2 = 0.80$) predicting growth up to metamorphosis included the main effects of initial mass and time to metamorphosis ($P < 0.001$) but did not include an interaction between these variables ($P = 0.594$; Supplemental Information Table S4). In considering growth up to 65 d post-treatment, regardless of the model applied, only a single variable (initial mass) influenced final mass, as expected ($P < 0.001$; Supplemental Information Table S3).

Inter-stage detection.—All larvae retained a detectable label through metamorphosis (Supplemental Information Table S5). After metamorphosis, I most easily detected the label in the tibiofibula, followed by the front phalanges and metacarpals, hind phalanges and metatarsals, and fronto-parietal bones (Fig. 1). I found no type I errors in detection. At the end of the experiment, 146 d after marking, I noninvasively detected the label in 79% of animals marked by calcein immersion without osmotic induction (n = 19) and 83% of animals marked by osmotic induction (n = 18; Supplemental Information Table S5). Detectability did not differ statistically between calcein administered with or without osmotic induction after accounting for initial mass and initial age (Table 2). Ignoring the method of

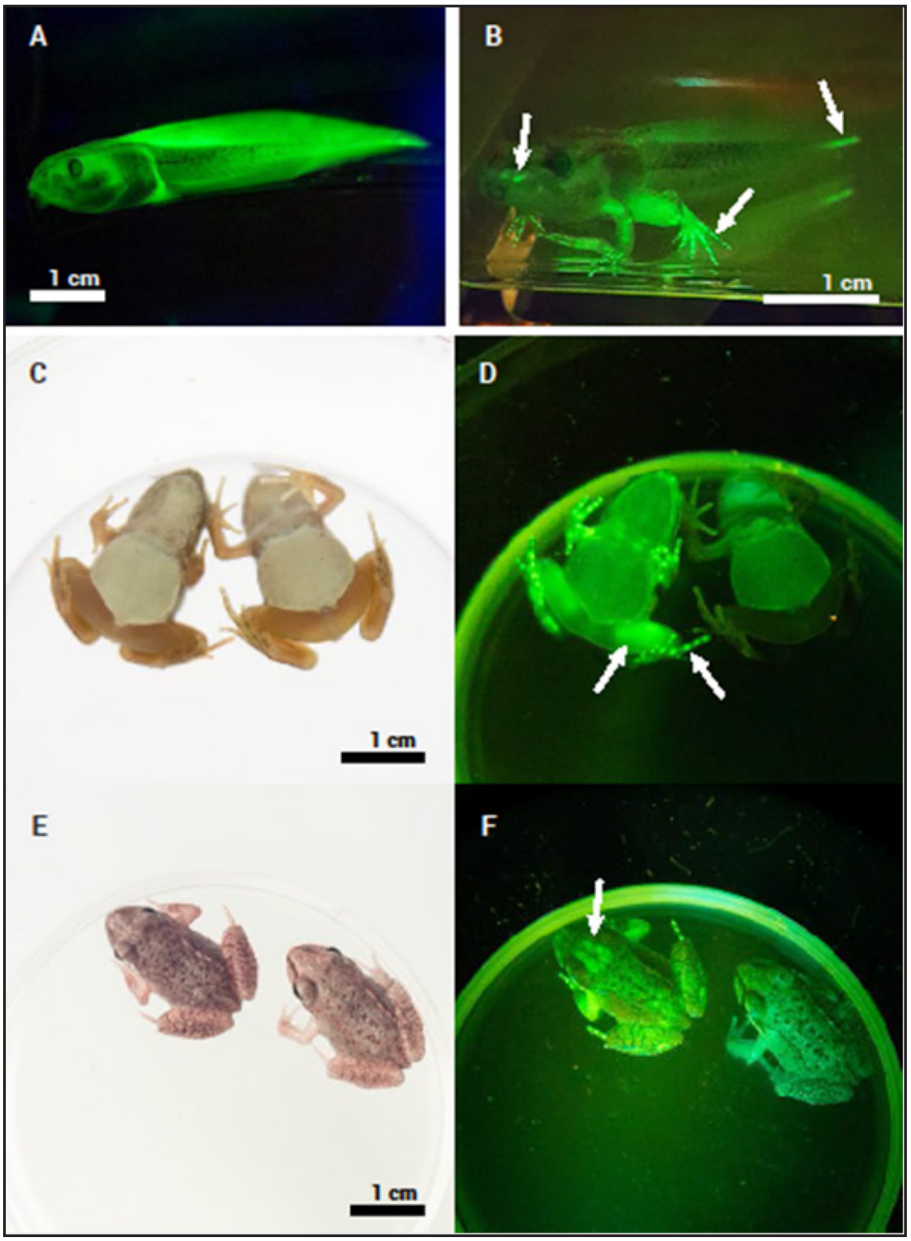

Figure 1. Photographs of a living calcein-labeled Wood Frog (*Rana sylvatica*) larva within 24 h of marking (A), a calcein-labeled Wood Frog metamorph approximately 10 d after marking (B), and ventral (C and D) and dorsal (E and F) views of a calcein-labeled Wood Frog (left) juvenile 63 d after marking and unmarked individual of the same age (right). Calcein fluoresces green in marked tissue when lit by a NIGHTSEA BlueStar handheld 440–460nm flashlight through a cancellation filter (A, B, D, F) but is not apparent in white light (C, E). In larval and metamorph stages the label is visible through the overlying tissue in the distal end of the tail along the notochord and in skeletal structures (arrows in B). In juveniles, the calcein label is most obvious from the ventral view in the bones of the limbs and feet (arrows in D) and from the dorsal view, in the parietal bones (arrow in F). Scale bar is approximate. Photographs taken with a Canon 6D DSLR (A, C, D, E, F) and Samsung G6 (B) in a dark, windowless room. Photographs have been corrected for exposure and white balance. (Photographed by A.Z. Andis).

marking, I detected the calcein label externally after 146 d in all animals marked 19 d or less before they metamorphosed.

The best fit model indicates that the initial age of the tadpole at the time of marking can impact the detectability and persistence of the calcein label (P = 0.003; Table 2). In general, the model predicts that a Wood Frog larva, of an average mass within its age class, marked within 10 d of metamorphosis, has a > 99% chance of detection post-metamorphosis even 146 d after marking (Fig. 2). Larvae marked 16 d prior to metamorphosis have > 90% chance of detection as juveniles after

Table 2. Summary information for the inter-stage experiment of coefficients from hierarchical repeated measures mixed logistic regression models predicting detection probabilities for Wood Frog (*Rana sylvatica*) juveniles marked as larvae via calcein with (Treatment C) and without (Treatment D) osmotic induction; subject identification is included as a random intercept (n = 37, obs = 481).

	Model 1			Model 2			Model 3		
Variable	*β*	SE	*P*	*β*	SE	*P*	*β*	SE	*P*
Intercept (Treatment C)	7.19	2.24		9.38	4.31		9.31	4.42	
Treatment D	0.18	1.84	0.921	0.61	0.73	0.408			
Days since meta	0.00	0.01	0.943	0.00	0.01	0.986	0.00	0.01	0.985
Initial mass				-3.82	5.58	0.493	-3.36	5.71	0.556
Initial age				-0.72	0.23	0.002	-0.71	0.24	0.003
Initial mass* Initial age				0.64	0.35	0.066	0.64	0.36	0.075
DF	477			474			475		
AIC	195.7			150.9			149.5		

146 d, but the detection probability of larvae marked earlier in development (> 17 d prior to metamorphosis) declines rapidly and was greatly contingent on the mass of the individual at time of marking (Supplemental Information Fig. S1). The model predicts no decline in detection probabilities over time (Table 2). Of the 30 marked animals detected via live, external observation at the end of the experiment (146 d post-treatment), I observed the calcein label via tibiofibula cross-section in 28 (93%) animals, and via toe-clip cross-section in 18 (60%) animals (Supplemental Information Table S6, Fig. S2).

Intra-stage detection.—In this experiment, I marked larvae before hind limbs were well developed, so I could only detect the calcein label reliably in the tail. Label retention diminished up to metamorphosis. Initially, the entire integument fluoresced up to 3–4 d after marking. After the integumentary calcein attenuated, I was able to easily distinguish the calcein labels in tails of larvae along the notochord. Over time, the fluorescence in the tail diminished from anterior to posterior until the mark was only visible in the posterior tip of the tail. In some individuals, I was able to detect the label in the tail up to 30 d after marking. Upon metamorphosis, I positively detected the label in 62.5% and 40% of larvae marked with and without osmotic induction (respectively; Supplemental Information Table S7; Fig. S3). I found no significant difference in detectability between the calcein treatments ($P > 0.200$ in all models). Ignoring treatment type, the best fit model predicted that calcein labels should persist up to 20 d after marking with > 90% detectability in larvae marked < 34 d prior to metamorphosis and over 99% detectability in those marked < 28 d before metamorphosis.

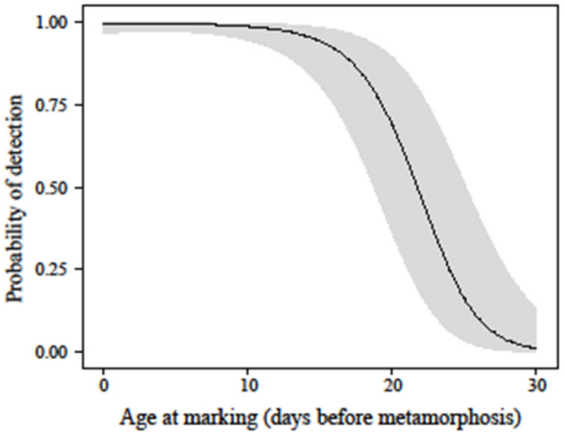

Figure 2. Inter-stage predicted probabilities of detecting a calcein label 146 d after administration in Wood Frog (*Rana sylvatica*) juveniles of average initial mass within a given age class marked at an initial age from 0 to 30 d prior to metamorphosis. Predicted values estimated from the data with a repeated measures mixed effect model. Shading indicates 95% confidence interval.

Discussion

Calcein labeling is an effective means of noninvasively marking amphibian larvae en masse. Detection of the label in postmetamorphic juveniles remained above 90% for individuals marked < 17 d prior to metamorphosis. Although model predictions cannot account for the exogenous factors discussed below, there was no discernable decline in detection probabilities over time projecting past the duration of the experiment. Short-term, external marking of a few days or fewer can be successfully applied to larvae at any stage with 100% detection. There was no significant advantage to the osmotic induction technique in comparison to immersion in calcein without a saline pre-bath. Although I found no adverse effects of the salt solution, given the demonstrated toxicity of salt to larval amphibians, it may be safer to use calcein alone for marking.

Timing is critical for effective marking via calcein immersion. Short-term external marking of larvae is effective in even early stages of development. In early stage larvae (prior to Gosner stage 30) of average mass for this age class, the calcein can remain visible with >

90% detectability for up to 30 d in the tail. Short-term marking could be useful for rapid dispersal or movement studies that do not require permanent labels and is an alternative to other short-term marking techniques that may be deleterious (Carlson and Langkilde 2013). While in this experiment I administered calcein labels in small batches, the marking technique could be scaled up to mark many animals at once.

It is important to note that amphibians as a class exhibit diverse life histories and developmental strategies. The use and optimal timing of marking with calcein is likely to vary across amphibian taxa. The optimal stage to mark larvae to ensure long-term persistence is when the skeletal structure is well developed. For species that undergo a developmental history similar to Wood Frogs, this optimal timing is near the end of the larval period within approximately 10 d of metamorphosis. Larvae marked with substantially ossified skeletal tissue retain easily detectable labels indefinitely. If a permanent label is required, but larvae must be marked prior to an optimum developmental stage, it may be feasible to mark individuals in the interim, then recapture and remark again at a later stage.

For some anurans, there may also be a critical juncture just prior to metamorphosis, before the animals enter their aphagic period, wherein developmental morphology aids mark retention. As larvae approach metamorphosis, excess calcium is stored in lime sacs where it may be transported to developing skeletal tissue during metamorphosis, essentially acting as a time-released delivery of the calcein label to newly ossifying tissue (Guardabassi 1963; Narbaitz and Jande 1974). If this is indeed the case, environmental conditions may also impact the effectiveness of calcein marking. In highly acidic environments or hypoxic environments, anuran larvae mobilize calcium from lime sacs to neutralize acidity in the blood, which may result in a wasting of the calcein label to the environment (Narbaitz and Jande 1974).

This marking technique is restricted to presence/absence detection which limits its use in studies that need to distinguish between multiple groups. Serially marking individuals to create banded labels may be an option for expanding the range of detectable groups. Juvenile Atlantic Salmon (*Salmo salar*) marked twice in a 90-d interval displayed two distinct bands on scales (Mohler 2003). Further testing would be needed to determine a marking regime that could accommodate the short larval period of many amphibians. Alternatively, other fluorescent dyes such as xylenol orange, alizarin red, and calcein blue could be used sequentially with calcein to produce a variety of color codes. However, the persistence and detectability of these dyes may be much lower than calcein (Brooks et al. 1994).

Future studies should test the applicability of calcein marking and detection in natural settings. Photodegradation due to sunlight may reduce the brightness and longevity of calcein labels (Leips et al. 2001; Bashey 2004). Honeyfield et al. (2008) tested the persistence and intensity of calcein label in Lake Trout (*Salvelinus namaycush*) under artificial sunlight (mercury halide lamp). They found that photodegradation decreased the intensity of the label on external features compared to animals housed in complete darkness, but was still discernible after 28 d of constant, direct light. The mark was retained internally on the ribs and fin rays despite light exposure (Honeyfield et al. 2008). However, the extent of photodegradation will be influenced by both the behavior and morphology of the species to be marked. For instance, Mohler (2003) found that Atlantic Salmon marked and released as fry, retained the calein label and the mark was readily observed in the field with handheld detector after 17 mo. Fossorial and nocturnal species will likely retain labels longer than diurnal, heliothermic species. Similarly, species or individuals with greater pigmentation will likely retain marking on internal tissue longer than those with translucent integument.

Skeletal growth and development post-marking can also lead to degradation of calcein labels (Negus and Tureson 2004). This dilution effect occurs when marked tissue is overlaid with opaque tissue as the animal grows (Frenkel et al. 2002). Invasive mark detection efforts by viewing skeletal cross-sections were less reliable than whole-body noninvasive assessment in this experiment. Thus, the allometry of a species will impact the use of calcein as a marking technique. Future studies to test the applicability of this technique on juvenile and adult amphibians with more developmentally stable skeletal structure would be useful. While this study found no negative effects of calcein marking on larval growth and development in the lab, the label or labelling procedure could have negative effects in more realistic conditions (Carlson and Langkilde 2013). Furthermore, although nonsignificant, larvae treated with the calcein technique in this study showed a tendency toward greater final post-metamorphic mass. Other studies in fish found similar results of elevated growth and survival in calcein-treated fry (Brooks et al. 1994; Mohler 2003; Crook et al. 2009). It may be that the saline bath and calcein provide a prophylaxis to disease, thereby increasing growth (Mohler 2003); however, no studies have shown this conclusively. This technique should be tested in the field or in predation trials before it is applied in practice.

In addition to the quality of the calcein label itself, the amount of ambient light, the intensity of the excitation light beam, and experience of the observer can confound accurate detection. Observations in the field should mitigate ambient light inasmuch as possible, perhaps by constructing a temporary darkroom, draping a light impenetrable barrier over the observer, or placing the subject and excitation light unit inside of a light-sealed

container with an observation portal (Jerre Mohler, unpubl. data). The intensity of the excitation beam can be calibrated by comparing marks against a colorimetric key and ensuring that observations are made with the light position at a fixed distance from the subject (Honeyfield et al. 2008). Finally, observers should be trained prior to data collection and/or misdetection rates should be calibrated for observers over time. While false negative rates were generally low and false positives were absent in this study, false negative observations may be a greater problem in field conditions, especially for subjects marked at sub-optimal mass and developmental stages.

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