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Evaluation of Tagging Methods for Unique Identification of Individuals in Three Aquatic *Eurycea* Salamander Species

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Marking or tagging salamanders increases efficiencies in the operations and management of captive populations and facilitates techniques for monitoring demographics of wild populations. Tags facilitate monitoring of longitudinal data for individual animals such as age or estimated age, growth, reproduction, and health. We evaluated three different tagging methods on Texas Blind, San Marcos, and Comal Springs Salamanders to determine success of each as a long-term marker of individual animals. For this study, we selected visible implant elastomer (VIE), visible implant alphanumeric tags (VIA), and passive integrated transponders (PIT). Of these, only VIE tagging has been evaluated in San Marcos Salamanders; none have been evaluated for use in the other two salamander species studied; and comparison studies among these tags for fully aquatic salamander species have not been performed. These tag types were selected for their effectiveness with other salamander species, ability to be quickly and easily identified tank-side, minimal negative effects on organisms, and perceived ease of implanting the tags. The VIE tags were retained by all species and had high readability scores. However, the issues of tag breakage and deterioration should be considered when using VIE tags. VIA tags were rejected in all but one San Marcos Salamander and all Comal Springs Salamanders, but 90% of Texas Blind Salamanders retained them. PIT tags were not tested on San Marcos, Comal Springs, and smaller Texas Blind Salamanders due to size of tag and low retention rate in larger salamanders. Of the tags evaluated in this study, VIE was the most effective in terms of retention, readability, and ease of insertion.

THE ability to distinguish individuals and collective groups is essential for many ecological studies, most notably mark–recapture studies (Pollock, 1991). Individual, long-term tagging allows for effective species management by monitoring biological data over a period of time and possibly through an individual's lifespan (Donnelly et al., 1994). Tags facilitate monitoring of longitudinal data for individual animals such as age or estimated age, growth, reproduction, and health. These data can be used in population studies of wild individuals or to help manage captive populations. A variety of individual marking methods have been evaluated in amphibians, including coded-wire tags (Sinsch, 1997), radio tags (Richards et al., 1994), skin pattern or pigment recognition (Grant and Nanjappa, 2006; Gamble et al., 2008), and tattooing (Donnelly et al., 1994; Schlaepfer, 1998).

Salamanders pose unique challenges in marking methods due to their sensitive permeable skin, small body size, and some species' ability to regenerate clipped digits (Heemeyer et al., 2007). When choosing a method for tagging salamanders, Osbourn et al. (2011) recommended considering factors such as the impact on health, degree of invasiveness, mark longevity, and number of unique marks necessary. For this study, we tested visible implant elastomer (VIE), visible implant alphanumeric tags (VIA), and passive integrated transponders (PIT). These tag types were selected because they can be quickly and easily identified tank-side or in the field (as opposed to photo ID), have been effective in other salamander species, have low rates of negative health effects, and can be easily implanted and read (Davis and Ovaska, 2001; Bailey, 2004; Heemeyer et al., 2007; Osbourn et al., 2011; Welsh-Appleby, 2015; Whiteman et al., 2016; Lunghi and Veith, 2017; Mitchell et al., 2017). One tag type could be favored over others, depending on a species' size, skin coloration, retention of tag, habitat, and purpose of the tag.

The VIE tag is an attractive marking technique because multiple color combinations are available that can be used to create unique identifying marks, they are well retained in other species, and the small-gauge needles used to inject them are more suitable for small salamanders (Davis and Ovaska, 2001; Heemeyer et al., 2007). Common drawbacks using this method include tag migration or breakage, misidentification of tag color, elastomer product loss due to hardening, and the need for multiple marks to create unique tags (Davis and Ovaska, 2001; Ralston Marold, 2001; Heemeyer et al., 2007).

The VIA tags were originally developed for fishes and are the newest of the three tagging types tested in this study (Northwest Marine Technology, Inc., Anacortes, Washington, USA). The tags are made up of a small rectangular fluorescent piece of plastic containing an alphanumeric code on one side. Tags are placed by flat injector needle under the skin. The VIA tags are small in size (1.2 mm $\times$ 2.7 mm) and come pre-printed with 10,000 unique alphanumeric codes available. Tags require only a single injection site and tag colors do not confuse identification. Larger VIA tags (2 mm $\times$ 5 mm) have increased font size for easier reading and could be used in larger individuals. Studies have had conflicting results on the efficacy of VIA tags in salamanders but have been successfully used on salamanders weighing $\sim$1.2 g (Osbourn et al., 2011; Lunghi and Veith, 2017). Injector malfunction can disrupt tag insertion or can render a tag unreadable (Lunghi and Veith, 2017). If the skin pigmentation of the

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salamander is too dark or if the tag is inserted too deep, reliability of tag identification declines (Osbourn et al., 2011).

The PIT tags are microchips encased in glass that transmit a unique code when a specialized reader activates the tag at close range. This method has been extensively used in many organisms (fishes, mammals, birds, reptiles, amphibians) but is limited by the tag's size (Gibbons and Andrews, 2004). The smallest PIT tag currently on the market is 8.4 mm in length, 1.4 mm in diameter, and 30 mg (Biomark, Inc., Boise, Idaho, USA), and has been successfully implanted in salamanders weighing 1.5 to 3.9 g (Ott and Scott, 1999; Ousterhout and Semlitsch, 2014; Mitchell et al., 2017). When retained, detection of PIT tags is reliable, the tags can persist in an organism for years, even a life span, and each has a unique code from every other PIT tag (Gibbons and Andrews, 2004).

Our study assessed tags in three neotenic salamander species: Texas Blind Salamanders (*Eurycea rathbuni*), San Marcos Salamanders (*Eurycea nana*), and Comal Springs Salamanders (*Eurycea pterophila* = *Eurycea neotenes,* originally described as *neotenes* by Bishop and Wright 1937, reclassified as *pterophila* by Devitt et al., 2019). These salamanders were held in captive reassurance colonies at US Fish and Wildlife Service San Marcos Aquatic Resources Center (SMARC), San Marcos, Texas. Few studies testing tagging methods on aquatic, paedomorphic salamanders exist (however, see Bendik et al., 2013; Lunghi and Veith, 2017). Only VIE tagging has been evaluated in San Marcos Salamanders (Phillips and Fries, 2009) and no tagging methods have been evaluated for use in the other two salamander species. The average adult San Marcos Salamander held in our captive reassurance population weighs 0.37 g with an average snout–vent length (SVL) of 29.4 mm, Comal Springs Salamander weighs 0.59 g with 32.7 mm SVL, and Texas Blind Salamander weighs 1.6 g with 40.3 mm SVL. The 8.4 mm tag may make it possible to implant PIT tags in salamanders in the size range of San Marcos and Comal Springs Salamanders, but no published studies have tested it on salamanders of this size class.

We evaluated the utility of the three different, individual tagging methods based on tag insertion, tag retention, and tag readability by expert and naive readers over a 12-month period. Comparison studies among these three tag types in aquatic salamander species have not been performed. Studies have compared two tagging methods, PIT and VIE (Welsh-Appleby, 2015), VIE and photographs (Bailey, 2004), VIE and toe clipping (Kinkead et al., 2006), but with only one selected species. Beyond the use of tagging for the captive reassurance populations, the information gained on suitable tags can be applied to studies with wild populations of these species or other similar species.

MATERIALS AND METHODS

We randomly selected 20 individuals of each species held in our captive assurance populations to test VIE and VIA tags. For PIT tags, we first selected a small subset of our largest Texas Blind Salamanders and evaluated them for one month. After the initial assessment, we tested PIT tags on a smaller size class of Texas Blind Salamanders. Following our evaluation of PIT tags in Texas Blind Salamanders, we did not test PIT tags in San Marcos and Comal Springs Salamanders. Other salamanders not in the tagging study, but within our care are referred to as the refugia population and were used as a control to compare survival rates.

We anesthetized salamanders using a low dosage of tricaine mesylate (MS-222, 0.5 g/L) buffered with sodium bicarbonate to facilitate immobilization of salamanders during tagging (Wright, 2001). During handling, salamanders were kept wet with moist paper towels. We measured and recorded total length (mm), SVL (mm), and weight (g), and identified sex by the candling method (Gillette and Peterson, 2001). After measuring physical attributes, we inserted the selected tag type into the salamander. We completed all injections with the syringes facing away from the body cavity to reduce chances of injury to internal organs. After tag insertion, we photographed the tag (VIE and VIA only), placed the salamander into a container with flowing water to fully recover from anesthesia, and then returned tagged salamanders to captive tanks. We disinfected equipment, including needles, injectors, and forceps, after each salamander was tagged. Tagging was split equally between two people to represent the real-world situation that the same person would not be continuously doing all the tags in a captive reassurance colony or in a large mark–recapture study.

Tag insertion.—We tagged salamanders in this experiment during March–April 2019. We inserted VIE tags using 29-gauge needles subcutaneously posterior to the back hip of the salamander. We mixed the elastomer (Northwest Marine Technology, Inc.) according to the manufacturer's instructions (https://www.nmt.us/wp-content/uploads/2021/01/VIE-Manual-Kit-Instructions-.pdf). Individual VIE codes were created using three lines of colored elastomer. Colors used were fluorescent red, orange, pink, yellow, green, and blue, along with two non-fluorescent colors, black and purple. Due to the dark skin pigmentation of San Marcos and Comal Springs Salamanders, we only used fluorescent colors, but were able to use the two non-fluorescent colors in Texas Blind Salamanders.

In Texas Blind Salamanders, we used horizontal VIE tags (Fig. 1). However, in the smaller bodied San Marcos and Comal Springs Salamanders, we found horizontal tags were not feasible due to tail width restrictions. We used vertical VIE tags on the smaller bodied species and added a second group of Texas Blind Salamanders with vertical tags. Our Texas Blind Salamander population had less size heterogeneity, so for the VIA and first group of VIE, we selected from our 2- to 5-year-old members of the population to have similar size and growth rate within species, and to be in the same age/maturity category as the other two species. When we added the second group of Texas Blind Salamanders, we moved down to the nine months to 1.5-year size class, which were similar in body size to the San Marcos and Comal Springs Salamanders, but at different life stage and growth rate (Fig. 2).

We used fluorescent orange standard sized VIA tags (1.2 mm width by 2.7 mm length, Northwest Marine Technology, Inc.) in all species tested. We implanted VIA tags using the visible implant (VI) Alpha Injector subcutaneously posterior to the hip in the tail of the salamander. We targeted the left side of the salamander so that the tags would be upright based on the direction of tagging away from the body cavity, as the alphanumeric code is only on one side of the tag and the tag can only be loaded in one direction into the needle. For consistency of tag orientation, we adjusted salamander

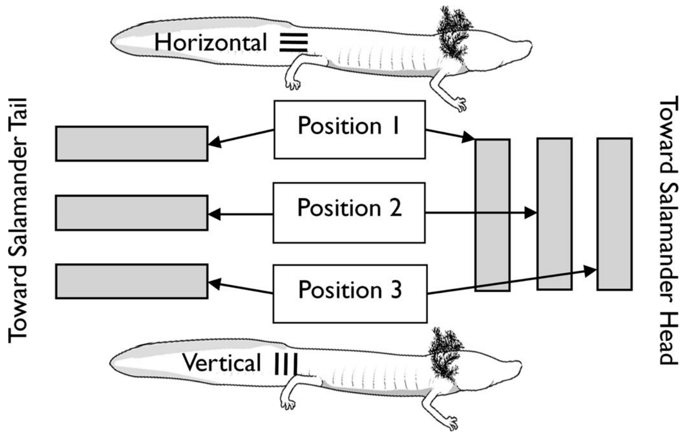

Fig. 1. VIE tag orientation used on three aquatic salamanders (*Eurycea* spp.) was either vertical or horizontal. Tags were read left to right (tail to head) for vertical orientation and top to bottom (dorsal to ventral) for horizontal orientation.

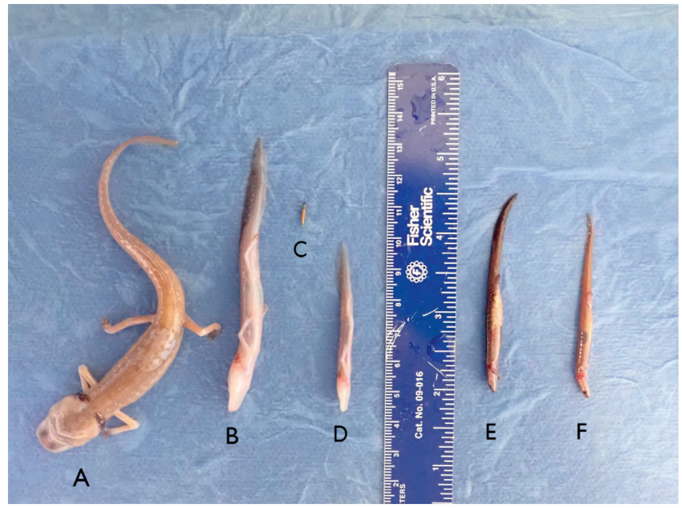

Fig. 2. Body size ranges for three aquatic salamanders (*Eurycea* spp.) varied by species and animal age. (A) Largest F1 Texas Blind Salamander, in the first group to receive PIT tags. (B) Average size representative of Texas Blind Salamanders to receive horizontal VIA and VIE tags, approximately 2–5 plus years of age. (C) 8.4 mm PIT tag. (D) Average size of vertical VIE-tagged Texas Blind Salamanders, approximately nine months to 1.5 years of age. (E) Average size of San Marcos Salamanders used in study, 2 plus years of age. (F) Average size of Comal Springs Salamanders used in study, 2 plus years of age.

body orientation to correspond with the handedness of the person tagging; one tagger was left-handed, the other, right-handed (Supplemental Fig. 1; see Data Accessibility). If there was an injector malfunction or issue with tag insertion, we then moved to the right side of the salamander though the lettering on tag would be upside down in relation to the dorsal side of the salamander (Supplemental Fig. 1; see Data Accessibility). Half of the injection sites of VIA tags in Texas Blind Salamanders were sealed with Vetbond tissue glue (Courtois et al., 2013). We stopped using the tissue glue after the long limbs of two Texas Blind Salamander individuals had to be extracted from the glue. We did not use glue in Comal Springs Salamanders after that, and observed excessive tag loss within a week of injection. To help increase retention rates, we used Vetbond tissue glue in a second treatment group of Comal Springs Salamanders and in San Marcos Salamanders to close the VIA injection site. These species have much shorter legs that could not easily reach the tissue glue, and we gently held them out of the way with a Kimwipe.

We implanted PIT tags (8.4±0.3 mm length by 1.4±0.1 mm diameter, 30 mg weight) posterior to the back hip subcutaneously into the tail musculature using a 16-gauge needle with syringe implanter (N165 & MK165 Biomark, Inc.). We tested implanting PIT tags subcutaneously into the upper tail musculature. We first placed six PIT tags in the tails of Texas Blind Salamanders that were 56–71 mm SVL. After one month of acclimation, we observed no infection at the insertion site and tolerance of the PIT tags (movement and swimming ability were not compromised). We then implanted PIT tags in five Texas Blind Salamanders that were 43–55 mm SVL. During the one-month monitoring period, in both groups the rejection rate of the PIT tags was high so we decided not to proceed with injecting smaller individuals since they would likely have poor retention. Based on the observed skin tearing and rejection of VIA tags in San Marcos and Comal Springs Salamanders and their smaller size, we decided not to inject PIT tags in those two species (Fig. 2).

Tag readability and retention.—We monitored tag retention and readability monthly and noted tag deterioration and overall animal health for one year. For consistency, one observer conducted this monitoring, and we designated them as the expert reader in this study. Naive readers also rated tags every three months (month 3, 6, 9, and 12). They were chosen for their inexperience with these tagging methods to represent the ease of reading tags for new staff or inexperienced personnel. At each time period, a new naive tag reader(s) was used. We provided instruction to the naive readers and gave them VIE and VIA color standard cards for reference. Tag readers scored the readability of VIE and VIA tags on a readability index (Table 1). A VI light projecting deep violet (405 nm) light (Northwest Marine Technology, Inc.) was used to cause fluorescence in the tags for better visibility when needed. Every three months, salamanders were weighed, measured, and a new photograph was taken of the VIE and VIA tags to evaluate potential tag migration and deterioration.

During the study, we observed that VIE tags can develop breaks and begin to deteriorate. We did not consider tag deterioration in the readability index, defined prior to the experiment based on an index in Osbourn et al. (2011). To measure deterioration, we examined tag photographs taken initially and every three months (month 3, 6, 9, and 12) and estimated the percent of tag remaining intact and numbers of breaks in a line (Table 1). Each line position was given a rating in both categories. Percent remaining categories were converted to midpoints and the average of all three lines used as an overall percent remaining rating for the tag. We used the midpoint of each breakage category for each line and totaled them for an overall number of breaks for the tag.

The PIT tags had an operating frequency of 134.2 kHz and were identified using an HPR-Plus Biomark Radio Frequency Identification (RFID) reader (Biomark, Inc.). We evaluated PIT tags based on tag retention, detection of the tag using the RFID reader, and the time required by the RFID to read the tag number. Salamanders were individually removed from the water and the RFID wand waved over their body. Naive

Table 1. Categorical ratings for tag integrity. Two indices characterized tag breakage and two indices characterized tag readability. The readability indices were patterned after Osbourn et al. (2011).

	Tag breakage indices		Tag readability indices	
Index	Percent of VIE tag remaining	VIE breakage (no. of breaks)	VIE readability	VIA readability
0	0	NA	Tag not visible or not present	Tag not visible or not present
1	1–24	≥10	Tag visible but colors not distinguishable	Tag visible but only color discernable
2	25–49	7–9	Tag colors visible but incorrectly read	Tag colors visible and partial code visible or incorrect code read
3	50–74	4–6	Correct colors or code only read with use of VI Light (deep violet 405 nm)	Correct colors or code only read with use of VI Light
4	75–99	1–3	Correct colors or code visible without aid of VI Light	Correct colors or code visible without aid of VI Light
5	100	None	NA	NA

readers also used the equipment to read the PIT-tagged salamanders.

For further evaluation of the potential utility of PIT tags, we tested the distance tags can be identified through water, both above the water surface and in the tank. We placed a salamander with a PIT tag in a 57 L glass tank with water depth starting at 25 cm. We held the RFID reader parallel to the water surface, just above the water. If the tag could not be identified, we lowered the water level in 5 cm increments, until the tag was identified by the reader. Once the maximum water depth was found, we slowly moved the Biomark reader further from the surface of the water until the tag could not be read. In a full tank of water, we tested how close the submerged wand needed to be to a salamander to read the tag. We also tested if the tag reader could distinguish multiple PIT-tagged individuals in a tank. We placed two salamanders in a 57 L tank with a water depth of 10 cm. We then added tagged salamanders until the reader could not distinguish individual tags.

***Statistical analysis.*—**We used Program R (version 4.0.2) to analyze the data (R Core Team, 2020). The probability of tag retention was estimated using the Kaplan-Meier estimator (package Surv; Conover, 1999). We compared tag retention between species and tag types using χ^2 tests and used log-rank tests for pairwise comparisons (packages Surv and survminer). We also used the Kaplan-Meier estimator to estimate survival probability of tagged salamanders. We compared survival of tagged salamanders and their refugia populations. We also compared survival among tag types (except for VIA since few were retained beyond one month).

We modeled tag readability by the expert and naive observers using linear mixed-effects models (packages lme4; Bates et al., 2015). Each species by observer type was modeled separately. We used Akaike's Information Criterion adjusted for small sample size (AIC_c, Burnham and Anderson, 2002) to select between random effects where individual salamanders vary randomly in terms of their intercept (1|Salamander) or where individual salamanders vary randomly in terms of their intercept and their slope over time (Month|Salamander). After selecting the random effect, we conducted model selection using AIC_c among readability models that were combinations of tag type, tagger, and month. We considered models with $\Delta AIC_c \leq 2$ as competitive and excluded models with uninformative parameters ($P > 0.157$; Arnold, 2010). We conducted pairwise comparisons of the readability of tag types using Tukey-adjusted contrasts (package emmeans). We also used a paired *t*-test to determine whether readability differed between the expert and naive observers during the twelfth month.

We modeled percent of a VIE remaining during the twelfth month as a function of species, tag orientation (vertical or horizontal), tagger, and growth rate using beta regression (package betareg). We estimated growth rate as the average log difference in SVL over time. We modeled the number of breaks in VIE tags during the twelfth month using general linear regression. Since data were counts, we assumed a Poisson distribution. We used AIC_c for model selection.

RESULTS

We tagged 181 salamanders during this experiment. We inserted PIT tags into 11 Texas Blind Salamanders. We tested no other species with PIT tags. We inserted VIA tags into 40 Comal Springs Salamanders, 20 San Marcos Salamanders, and 20 Texas Blind Salamanders. We inserted vertical VIE tags into 20 Comal Springs Salamanders, 20 San Marcos Salamanders, and 30 Texas Blind Salamanders. We also inserted horizontal VIE tags in 20 Texas Blind Salamanders.

***Tag retention.*—**We found tag retention over the course of one year ranged from 0% (Comal Springs Salamander VIA) to 100% (all species VIE; Table 2). All VIE tags were retained for the year for all species. However, all but two VIA tags were lost after one month for San Marco and Comal Springs Salamanders; after two months, only one VIA tag remained in a San Marcos Salamander (none in Comal Springs Salamanders) and that one was retained the whole year (Table 2). In Texas Blind Salamanders, 90% (SE = 6.71%) of VIA tags were retained and only 54.5% (SE = 1.50%) of PIT tags were retained (Table 2). There was no difference in retention between the Comal Springs Salamanders with non-glued VIA injection sites versus salamanders with glued VIA injection sites, as all tags were shed by the second month, so all VIA tags for Comal Springs Salamanders were grouped in further retention analysis. Retention of VIA tags was significantly different among species ($\chi^2 = 65.2$, df = 2, $P \leq 0.001$). Texas Blind Salamander VIA retention was 90% (SE = 6.71%), and was greater ($P \leq 0.001$) than retention by San Marcos Salamanders (5%; SE = 4.87%) or Comal Springs Salamanders (0%). Only six of the 11 PIT tags placed in Texas Blind Salamanders were retained a month after their

Table 2. Estimates of tag retention probability for three aquatic salamanders (*Eurycea* spp.) over the course of one year.

Species	Tag type	*n*	Retention	SE	95% CI Lower	95% CI Upper
Texas Blind	VIA	20	0.900	0.067	0.778	1.000
	VIE	50	1.000	0.000		
	PIT	11	0.545	0.150	0.318	0.936
San Marcos	VIA	20	0.050	0.049	0.007	0.338
	VIE	20	1.000	0.000		
Comal Springs	VIA	40	0.000	0.000		
	VIE	20	1.000	0.000		

injections. However, those six PIT tags were retained for the remainder of the year. In Texas Blind Salamanders, PIT tags were not retained as well as VIA ($P = 0.077$) and VIE tags ($P \leq 0.001$).

Survival.—Survival of tagged salamanders and salamanders in their refugia populations was similar for all tag types ($P > 0.145$; Table 3). Survival of Texas Blind Salamanders with vertical VIE tags (survival = 93.3%, 95% CI = 84.8–100%) was similar to ones with horizontal VIE tags (survival = 100%). Survival of Texas Blind Salamanders with VIE tags were similar to ones with VIA tags ($\chi^2 = 0.808$, df = 1, $P = 0.369$; Table 3).

Readability.—All tags but two VIA were consistently readable by the expert, and only two VIE tags were misidentified at one tag check by the expert throughout the study, though some required the use of a VI light. We excluded VIA tags from the readability analysis for San Marcos and Comal Springs Salamanders since only one was retained. For statistical modeling of San Marcos Salamander VIE tag readability by the expert reader, we selected the random effect that assumed individual salamanders varied randomly in terms of their intercept and their slope over time. The best model indicated VIE tag readability was constant (null model; Table 4). For San Marcos Salamander VIE tag readability by naive readers, we selected the random effect that assumed individual salamanders varied randomly in terms of their intercept. The best model suggested readability decreased through time (Table 4). We found readability of San Marcos Salamander VIE tags was better during the twelfth month for the expert reader than naive readers ($t = 3.667$, df = 13, $P = 0.003$).

For Comal Springs Salamander VIE tag readability by the expert reader, we selected the random effect that assumed individual salamanders varied randomly in terms of their intercept. The best model ($AIC_c = 199.0$, $w = 0.626$) suggested readability increased through time ($\beta = 0.019$, SE = 0.007). For Comal Springs Salamander VIE tag readability by naive readers, we selected the random effect that assumed individual salamanders varied randomly in terms of their intercept. The best model indicated VIE tag readability was constant for naive readers (null model; $AIC_c = 173.0$, $w = 0.360$). We found readability of Comal Springs Salamander VIE tags was not different in the twelfth month for the expert reader and naive readers ($t = 1.756$, df = 18, $P = 0.096$).

For Texas Blind Salamander VIE and VIA tag readability by expert reader, we selected the random effect that assumed individual salamanders varied randomly in terms of their intercept and their slope over time. The best model ($AIC_c = 430.3$, $w = 0.450$) suggested expert readability was a function of tag type and tagger ($\beta = 0.467$, SE = 0.161, $z = 2.907$, $P = 0.004$). We found VIA tags without glue were more readable by the expert than those with glue ($t = -3.072$, df = 79.0, $P = 0.015$). Also, VIE tags were more readable by the expert than VIA tags with glue (VIE vertical, $t = -3.326$, df = 78.4, $P = 0.007$; VIE horizontal, $t = -3.747$, df = 79.6, $P = 0.002$) but readability was similar between VIE tags and VIA tags without glue (VIE vertical, $t = 0.121$, df = 77.2, $P = 0.999$; VIE horizontal, $t = -0.209$, df = 77.1, $P = 0.997$). For Texas Blind Salamander VIE and VIA tag readability by naive readers, we selected the random effect that assumed individual salamanders varied randomly in terms of their intercept. The best model ($AIC_c = 525.7$, $w = 0.545$) suggested tagger ($\beta = 0.831$, SE = 0.212, $z = 3.919$, $P < 0.001$) and month ($\beta = 0.048$, SE = 0.020, $z = 2.388$, $P = 0.017$) interacted ($\beta = -0.058$, SE = 0.024, $z = -2.456$, $P = 0.014$) to affect readability by naive readers. The interaction suggested readability declined through time for one tagger but increased through time for the other tagger. We found readability of Texas Blind Salamander tags was better during the twelfth month for the expert reader than naive readers ($t = 2.193$, df = 65, $P = 0.032$).

Table 3. Estimated survival of tagged salamanders (*Eurycea* spp.) over the course of one year compared to control salamanders in captive assurance populations during the same period.

		Tagged				Control population			
Species	Tag type	Survival	SE	LCL	UCL	Survival	SE	LCL	UCL
Texas Blind	VIE	0.960	0.028	0.907	1.000	0.881	0.035	0.815	0.954
	VIA	1.000	0.000			0.896	0.047	0.809	0.993
	PIT	1.000	0.000			0.895	0.070	0.767	1.000
San Marcos	VIE	0.700	0.102	0.525	0.933	0.674	0.030	0.617	0.735
Comal Springs	VIE	0.950	0.049	0.859	1.000	0.842	0.064	0.727	0.977

Table 4. Competitive statistical models for readability by species and treatment. For each model, we provide number of parameters (K), Akaike's Information Criterion adjusted for small sample size (AIC_c), difference in AIC_c compared to lowest AIC_c of the model set (ΔAIC_c), and AIC_c weight (w).

Model set	Beta (SE)		K	AIC_c	ΔAIC_c	w
	Month	Tagger				
VIE expert readability San Marcos Salamanders						
Null	NA	NA	5	120.3	0.000	0.286
Tagger+(Month|ID)	NA	0.173 (0.117)	6	120.5	0.239	0.254
Month+(Month|ID)	0.008 (0.007)	NA	6	121.1	0.767	0.195
VIE naive readability San Marcos Salamanders						
Month+(Month|ID)	–0.047 (0.028)	NA	4	160.2	0.000	0.278
Tagger+Month+(Month|ID)	–0.047 (0.028)	0.287 (0.192)	5	160.5	0.255	0.245
Null	NA	NA	3	160.7	0.524	0.214
Tagger+(Month|ID)	NA	0.291 (0.197)	4	161.0	0.753	0.191

We were able to identify PIT tags within 15 seconds by waving the Biomark reader's wand 10 cm or closer over the animal. There was no difference in readability over time or by expert or naive reader. Most PIT tags were not notable to the naked eye except with close inspection. During our water depth tests, we found that PIT tags could be identified with the RFID reader when salamanders were at depth of 10 cm, but not at greater depth. In 10 cm deep water, we could not identify a tag with the RFID reader farther than 2 cm above the water surface. When the RFID reader was submerged in water, we could only identify a PIT tag when the RFID reader was within 10 cm of the salamander. The RFID reader could not read more than two tags at any given time.

Tag deterioration and breakage.—The best model describing the percent of a VIE tag remaining during the twelfth month (Table 5) suggested it was influenced by species, tag orientation (vertical or horizontal), and tagger. Horizontal tags had less deterioration than vertical tags. We found VIE tags in San Marcos Salamander deteriorated less than VIE tags in Comal Springs Salamanders (San Marcos Salamander 82.5%, SE = 2.95%; Comal Springs Salamander 73.3%, SE = 3.35%; $z = -2.312$, $P = 0.054$) or Texas Blind Salamanders (74.5%, SE = 2.55%; $z = 1.994$, $P = 0.114$). Deterioration in VIE tags was similar for Comal Springs and Texas Blind Salamanders ($z = -0.272$, $P = 0.960$). The best model describing the number of breaks in VIE tags at the twelfth month (Table 5) suggested it differed by tagger and was greater in vertical tags. On average at 12 months, horizontal VIE tags had 2.9 breaks (SE = 0.052) and vertical tags had 3.1 breaks (SE = 0.030).

DISCUSSION

Our study is the first study to evaluate three tagging techniques (VIE, VIA, and PIT) in three completely aquatic salamander species and to monitor tag retention, readability, and deterioration for a full year. Previous studies only follow tagged individuals for six months or less (though see Davis and Ovaska, 2001 and Heemeyer et al., 2007). The increased time we followed individuals provides valuable information for application to mark–recapture studies in the field and for captive reassurance colonies on retention rates, readability (with suggestions on how to increase readability), and the absence of behavioral interference or reduced survival due to tags.

Salamanders tagged with VIE color combinations resulted in the highest readability and retention rates in all three species of aquatic salamanders when compared to VIA and PIT tags. Skin texture and thickness of each species affected the retention and readability scores of the three different tagging methods used. Of the three tags tested, we found VIE tags best for our purposes of quick, tank-side identification, ease of insertion, retention, and readability.

Elastomer lines breaking from solid lines did reduce the clarity of some tags, but the majority of the tags could be accurately read and distinguished even with the breaks (Fig. 3). Taggers should practice injecting continuous lines with even flow of the elastomer and steady hands to potentially

Table 5. Competitive statistical models for percent of VIE tag remaining and number of breaks in VIE tag at the twelfth month. For each model, we provide number of parameters (K), Akaike's Information Criterion adjusted for small sample size (AIC_c), difference in AIC_c compared to lowest AIC_c of the model set (ΔAIC_c), and AIC_c weight (w).

Model set	Beta (SE)			K	AIC_c	ΔAIC_c	w
	Orientation	Tagger	Growth				
Percent VIE tag remaining							
Species+Orientation+Tagger	–1.369 (0.284)	1.214 (0.228)	NA	6	–108.4	0.000	0.471
Growth+Orientation+Tagger	–1.149 (0.231)	1.268 (0.215)	–5.201 (3.168)	5	–107.3	1.134	0.267
Orientation+Tagger	–1.219 (0.233)	1.099 (0.193)	NA	4	–107.3	1.174	0.262
Number of breaks in VIE tag							
Orientation+Tagger	0.152 (0.060)	–0.105 (0.052)	NA	2	471.7	0.000	0.398
Growth+Orientation+Tagger	0.147 (0.061)	–0.120 (0.057)	0.535 (0.859)*	4	473.6	1.826	0.160
Orientation	0.130 (0.059)	NA	NA	2	473.6	1.867	0.156

*This parameter might be considered spurious or uninformative as its $P > 0.15$ (Arnold, 2010).

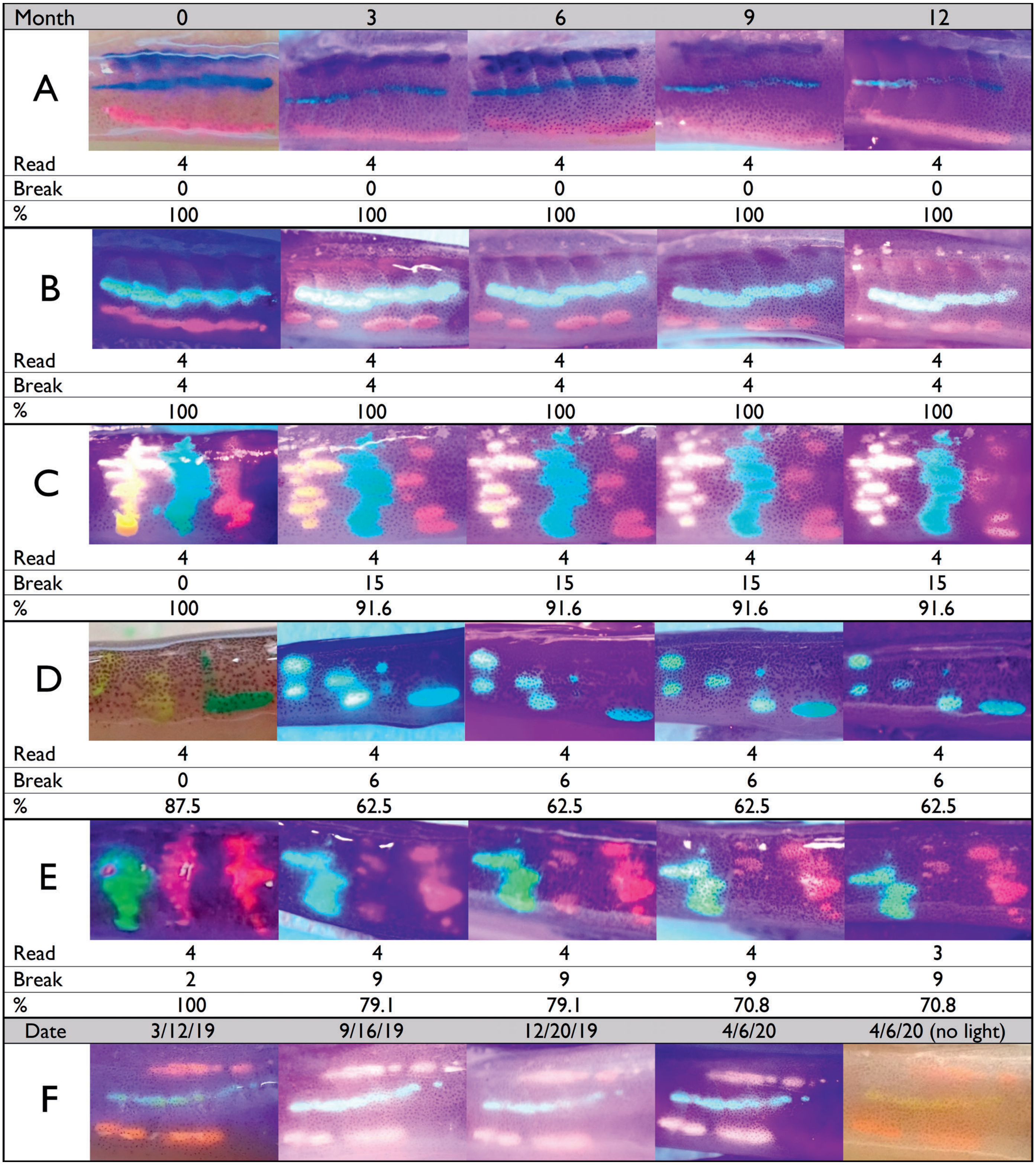

Fig. 3. Examples of the different type of scenarios with tags in the study with time series photos and corresponding scores for expert readability (Read), breakage (Break), and percent remaining (%). (A) Depicts *E. rathbuni* with near faultless horizontal VIE tag, black-blue-pink. (B) A typical horizontal VIE tag on Texas Blind Salamander, purple-yellow-pink. (C) A vertical VIE tag on Texas Blind Salamander showing how the elastomer spreads into costal grooves and breaks along those lines, orange-green-red. (D) San Marcos Salamander with vertical VIE tag showing partial tag migration between month 6 and 9. Bottom portion of second yellow line migrates toward third green line, yellow-yellow-green. This individual had a previous horizontal green mark for sex. (E) Comal Springs Salamander with vertical VIE tag portraying partial tag loss of pink line between month 6 and 9, yellow-pink-red. (F) A Texas Blind Salamander, part of pilot tagging group tagged in June 2018, that was followed during the study, depicting a long-term horizontal VIE tag, orange-yellow-orange.

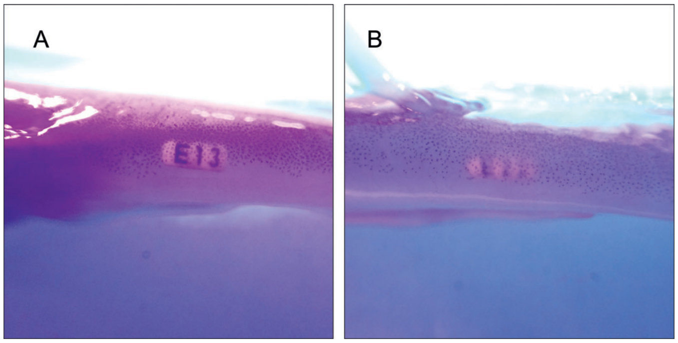

Fig. 4. (A) "E13" illustrates an ideal subcutaneous VIA tag. (B) "E22" illustrates a poor-quality VIA tag, with the code being blurred beyond legibility by both the angle and depth of tag insertion and melanophores that blur the numbers.

reduce the amount of breakage in the tags. Our tagger that used longer elastomer lines had higher readability scores. It should be noted that the expert reader was also the tagger with the higher score, so the ability to read one's own tagging technique might increase recognition of tags. In studies with multiple taggers there will be heterogeneity in readability due to taggers. Readability scores by the expert reader increased over time, suggesting they became better at identifying specific animals possibly due to learning the different quirks of tags, increased ability to differentiate colors with increased exposure, and/or improved technique/angle to view the tags. Even though we included both pink/red and green/yellow, as to not bias readability of this technique versus the others *a priori*, we support the manufacturer's recommendation and findings of other studies to not use those color combinations together if possible. The colors could be distinguished, with care, from each other and could be used if more individual combinations were needed for a study. We did find that these colors could be more easily distinguished without the VI light than with when in skin with pale pigment or near the skin surface. Naive readers were able to identify most tags, both VIE and VIA, but had greater difficulty on tags that were flawed. On these flawed tags, the expert reader generally was able to identify with a higher score. There is likely to be heterogeneity in readability scores with multiple readers, but the learning curve is low for a reader to become familiar with the tags.

When injecting the VIE polymer, researchers observed the polymer spreading into the lateral line and costal grooves, causing misshapen polymer lines or breaks (Fig. 3). VIE tags tended to break at costal grooves, which was not surprising since the elastomer lines were observed separating at initial injection (Fig. 3), and this factor should be taken into account. If a species has large enough space to avoid costal grooves, then horizontal lines may be preferred. Vertical line injection varied from horizontal line injections by adjusting the needle and salamander body position to account for the convex shape of their tails.

Readability scores might have been different if we had included tag deterioration as a factor on our scale. Neither breakage nor percent tag remaining alone completely encompasses deterioration. The number of breaks can remain the same or decrease if portions of the tag disappear, and even with many breaks, tags can be easily identifiable (Fig. 3). The percent of tag remaining can reduce readability if a tag line can no longer be seen, but a tag could have a portion disappear yet still be clearly read. However, both can reduce identification or signal that a tag may not be clearly identifiable in the future. The few tags that showed migration of pieces could be tracked in photographs we took; therefore, individuality of the tag could still be retained through the recognition of this change. Individual identity could be retained for captive animals, but might be difficult to follow for wild animals in mark–recapture experiments.

There was some evidence of growth affecting both VIE tag breakage and deterioration in competitive models (Table 5). Higher growth rate translates into expanding skin tissues, which would be consistent with more breaks in tag lines. The youngest salamanders used in the experiment were the Texas Blind Salamander vertical VIE group. We observed this species had greater growth rates and increased deterioration of VIE tags than the other species. Fortunately, these tags could still be identified, but we suggest that tags may need to be touched up as an individual grows.

Readability of VIA tags depends on the depth and angle of tag injection and the presence of melanophores, which can obscure the printed number (Fig. 4). We had similar injector malfunctions and difficulties as other studies (Lunghi and Veith, 2017). The plunger of the flat VIA injection needle tended to stick to the tag or go over the top of the tag instead of pushing it out, forcing us to disassemble the needle to remove before continuing. We had three needles on hand when tagging, so that if the injector malfunctioned, we could quickly use another to tag the salamander. Without an extra disinfected and loaded needle, too much time would elapse in correcting the injecting needle for the safety of the salamander.

Difficulties in injections tearing the skin and almost zero retention rate discourage us from recommending VIA tags for marking San Marcos or Comal Springs Salamanders. San Marcos and Comal Springs Salamanders' skin was thinner and tore easily compared to Texas Blind Salamanders; their skin did not tear. Both San Marcos and Comal Springs Salamanders' skin was thin and fragile, similar to tissue paper, when using the larger VIA injection needle as opposed to the VIE needles. We also found that the VIA tags themselves easily pierced the skin during injections, creating an exit wound as the tag was pushed out of the injector in some individuals. It could be that the thinner skin of these two species played a role in their lack of retention. Alternatively, these species could have a higher propensity to reject objects from their skin. Biologists should consider skin thickness and fragility when considering VIA tags for other species.

In the species with more robust skin, the Texas Blind Salamander, VIA retention rate was high and not significantly different from VIE tags. Therefore, VIA tags may be appropriate for some species but not others. These tags would only require one larger injection, do not deteriorate, and the material does not have a usable life span like mixed elastomer VIE, thus may be more appropriate for field studies of larger species. Though VIA tag retention in Texas Blind Salamanders was high, some tags were lost. The less than 100% retention rate should be accounted for in mark–recapture studies (Cowen and Schwarz, 2006). We have found that the skin of Texas Blind Salamanders thickens as they age and becomes increasingly difficult to see through (they are not transparent, rather light pigmented). This might decrease the ability to read a tag as the organism ages, but would likely take serval years to see if this occurs. One drawback of VIA tags in Texas Blind Salamanders is that they could not be used on small juvenile Texas Blind Salamanders, whereas small VIE dots and lines could. If VIA tags are used, additional readability research examining multiple fluorescent colors along with codes that begin with varying letters may be needed (we knew that all our codes began with E, but if B was also in the mix, misidentification might have occurred). Further, larger sized VIA tags could be used on larger Texas Blind Salamanders to increase readability (e.g., larger font).

PIT tags were not injected on any of the smaller Texas Blind Salamanders and are not recommended for San Marcos or Comal Springs Salamanders due to the animals' small size. Should a smaller PIT tag be developed in the future, additional studies could be warranted with caution. The high rejection rate of the tags in Texas Blind Salamanders does not make them a tenable long-term marking technique for the salamanders we studied. Retention might be higher if we had injected the PIT tags into the body cavity as is traditionally done in other organisms. However, the small size of these salamanders makes it difficult to avoid piercing internal organs when injecting into the body cavity, and this was a risk we thought too high to take. When trying to read PIT tags in a large tank with several marked individuals, if more than two salamanders were close together, the PIT tag reader could only identify two tags at any given time (even with the reader in the water). Sometimes the display would stay with the same two tag numbers initially read; other times it would have one constant tag number and rotate through two other tag numbers. We were unable to leave a salamander in the water and read the tag with the wand more than 2 cm above the water (even on a lone salamander) if the salamander was in a water depth greater than 10 cm. The value of PIT tags would be in conjunction with submerged detection arrays in caves/wells for movement or population analysis studies. This species is less likely to form groups, as in tanks, that would swamp the reading ability of an array. Low retention rates and concerns about injection site infection in the wild should be considered. We did not see any infection in our salamanders, but there could be a higher chance in the wild.

In general, we found that VIE tags and un-shed VIA tags could easily and accurately be identified by both the expert and naive readers. This study was the first of its kind to compare three different tagging types on three species of salamanders simultaneously. It is one of the few studies to evaluate tags on salamanders that do not metamorphose and remain aquatic in all life stages. This study extends the size range of salamanders in tagging research for VIA and PIT tags. Our research lays the foundation for using these tag types for mark–recapture studies as it quantifies retention rates. These tags would be useful in a variety of situations: individual tagging, batch tagging, cohort identification, sex identification, and mark–recapture studies.

DATA ACCESSIBILITY

Supplemental material is available at https://www.ichthyologyandherpetology.org/h2021042.

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