Long-term Retention, Readability, and Health Effects of Visible Implant Elastomer (VIE) and Visible Implant Alpha (VI Alpha) Tags in Larval Eastern Hellbenders (Cryptobranchus alleganiensis alleganiensis)

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ABSTRACT.—The ability to identify individuals is essential for determining population demographics of a species, but traditional marking techniques, such as passive integrated transponder tags, are often limited to individuals that meet minimum size thresholds. Visible implant elastomer (VIE) and visible implant alpha (VI Alpha) tags are promising methods for marking small-bodied individuals. However, the efficacy and health effects of VIE and VI Alpha tags are not established for many, increasingly imperiled, herpetofauna. Over a 12-mo period, we examined tag retention, tag readability, VIE tag color readability, and effects on growth and body condition of VIE and VI Alpha tags in larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*), a species of conservation concern. We observed 100% retention of VIE tags and 80% retention of VI Alpha tags over 1 yr. Readability degraded over time for both tag types but was consistently higher for VIE relative to VI Alpha tags. Degradation in readability over time increased our reliance on a 450-nm-wavelength VI light to read VIE tags but had more severe implications for VI Alpha codes, which were illegible after 4 mo. Pink- and green-colored VIE tags performed similarly well and we found that neither VIE nor VI Alpha tags negatively affected growth or body condition of larval hellbenders. Our findings collectively suggest that VI Alpha tags are an unviable tagging method, but VIE tags were safe and effective for identifying unique larval hellbenders up to 1 yr.

Techniques such as capture–mark–recapture give researchers valuable information about abundance, growth rate, survival, and movement patterns of a species but require unique identifiers to differentiate individuals (Pradel, 1996). Ideal tagging techniques should be long lasting and not influence the recipients' health, survival, movement, or behavior (Lettink and Armstrong, 2003). Traditional methods for uniquely identifying animals include injectable identifiers such as radioisotope markers or passive integrated transponder (PIT) tags, external identifiers such as ear tags, and body modifications such as branding or toe-clipping (Donnelly et al., 1994). However, each identification method is limited by certain parameters, such as body size (Carver et al., 1999), skin pigmentation (Robbins and Fox, 2012), behavioral tendencies (McFarlane et al., 2019), and regenerative capabilities (Ursprung et al., 2011). As a result, certain taxa are more prone to issues in implementing typical methods of unique identifiers than others (Silvy et al., 2012). Therefore, evaluating the efficacy of tagging techniques for individual species can advance our ability to monitor species and our knowledge about basic ecological processes such as dispersal, survival, and recruitment.

An estimated 30% of all amphibian species are considered threatened (Collins et al., 2009), making it critical to accurately monitor amphibian population dynamics to identify drivers of declines. However, amphibians possess several distinct traits that present difficulties in uniquely identifying them. For instance, regenerative abilities of salamanders thwart body modifications such as toe-clipping (Davis and Ovaska, 2001). Though traditional tagging methods may be effective for certain amphibian species, the development of taxa-unique identifying

methods is often required. For example, certain anuran species can be fitted with elastic waistbands containing numbered tags (Emlen, 1968) while the application of beryllium nitrate may inhibit digit regeneration post–toe-clipping in certain salamander species (Heatwole, 1961). Technological advancements have also improved identification methods such as digitally identifying unique patterns through photographs (Bendik et al., 2013). However, many of these techniques are expensive, time-consuming, or only applicable to larger bodied species or species with differentiated morphological patterns. As a result, there is strong demand for quick and inexpensive methods to uniquely identify smaller bodied amphibian individuals.

Visible implant elastomer (VIE) and visible implant alphanumeric (VI Alpha) tags are promising techniques for identifying small-bodied amphibians. Both VIE and VI Alpha tags are implanted subcutaneously, fluoresce when exposed to ultraviolet (UV) light, and are read through transparent skin or tissue, but they differ in application and code structure. Through VIE marking, a colored liquid polymer is injected subcutaneously under clear or transparent tissue to create distinctive patterns that solidify under the skin. Conversely, VI Alpha tags are solid, flat elastomer rectangles that are preprinted with a unique alphanumeric code and are injected underneath clear or transparent tissue. Visible implants can be effective identifiers for multiple amphibian species at different life stages, such as larval Common Midwife Toads (*Alytes obstetricans*; Courtois et al., 2013), adult Common Mistfrogs (*Ranoidea rheocola*; Sapsford et al., 2015), and adult Red-Backed Salamanders (*Plethodon cinereus*; Heemeyer et al., 2007). However, uncertainties remain regarding optimal VIE and VI Alpha application for small amphibians including implant location and color readability, while tag migration, metamorphosis, and read errors present further challenges.

Brannelly et al. (2013) found that 50% of VIE tags implanted in two species of adult anurans shifted subcutaneously, causing >70% of individuals to be potentially misidentified, while a field study performed by Bainbridge et al. (2015) found that VIE retention in larval anurans decreased through metamorphosis. Bendik et al. (2013) also reported high VIE read errors compared with computer-assisted photograph identification. Therefore, although implant-tagging is successful in certain amphibian species, efficacy and optimal implant application may need to be determined on a species-to-species basis.

One such species in which the efficacy and optimal application of visible implant-tagging remain uncertain are Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Eastern Hellbenders are large (up to 74 cm), aquatic salamanders and are a species of conservation concern throughout their range (Petranka, 1998). Adult hellbenders can be identified through PIT tags (Unger et al., 2012), but differentiating individual larvae is difficult because of their regenerative abilities and small size. Upon emergence from the nest, hellbender larvae measure only 5 cm in total length, and it may take ≥3 yr before hellbenders reach a size when PIT-tagging is feasible (~20 cm total length). Furthermore, hellbenders are a species of conservation concern, so care must be taken to minimize the health risks associated with tagging. For example, toe-clipping, a common method of uniquely identifying small herpetofauna, may pose risks to larval movement and health (Davis and Ovaska, 2001). Although Fouilloux et al. (2020) successfully used VIE tags to identify small larval anurans, the safety and efficacy of VIE-tagging has not been demonstrated with larval hellbenders. A lack of unique identifiers has also potentially hindered our understanding of basic hellbender ecology and conservation because information such as larval abundance, survival, and dispersal remains unclear (Bodinof Jachowski and Hopkins, 2018; Diaz et al., 2022).

Our goal was to evaluate the long-term efficacy and optimal application of two different types of visible implant tags in larval hellbenders. Our specific objectives were to (1) compare retention and readability of VIE and VI Alpha tags implanted in larval hellbenders over 12 mo; (2) compare VIE tag color readability; and (3) investigate the relationship between larval hellbender growth, survival, and tagging method. We hypothesized that (1) VIE and VI Alpha tags in larval hellbenders would differ in readability, retention would be similar among tag types, and readability would change over time; (2) pink-colored VIE tags would differ in readability from green-colored VIE tags; and (3) both VIE and VI Alpha tags would affect larval hellbender growth and body condition. Under our hypotheses, we predicted that (1) readability of VI Alpha tags would be higher than VIE tags on account of their larger size and alphanumeric code, but that retention and the decline in readability over time would be similar for both tag types; (2) pink-colored VIE tags would be more readable than green-colored VIE tags because of their brighter fluorescence; and, (3) given that tagging is an invasive procedure, growth and body condition would be lower in tagged hellbenders than untagged hellbenders. We used a controlled ex-situ experiment to address each objective using larvae that hatched from eggs collected in the wild that were being reared as part of a conservation propagation effort.

<center>MATERIALS AND METHODS</center>

Husbandry.—We collected hellbender eggs from Brooke County, West Virginia, USA, on 28 September 2019, and all eggs hatched by early October 2019. We housed larval hellbenders at The Wilds Hellbender Conservation Center in Cumberland, Ohio, inside either 38- or 76-L glass aquaria filled with tap water originally transported to The Wilds from Zanesville, Ohio, as part of regular water service to the conservation center. We subsequently treated tap water with Kordon NovAqua water conditioner (Kordon) and allowed it to sit for ≥24 h before adding it to the tanks. Inside each tank, we placed three sponge filters and three small shelters made of polyvinyl chloride (PVC).

We initially fed hellbenders live blackworms (*Lumbriculus variegatus*) for the first 6 mo to account for hellbender gape limitations, but then transitioned to thawed mysis shrimp (order Mysida) as hellbenders grew larger. We fed hellbenders 3 times/wk, with each fed ad libitum. We manually netted tanks daily for waste and debris; tested water for pH, ammonia, nitrite, and nitrate; and performed partial water changes weekly. During each partial water change, we changed 50% of water volume, scrubbed hides to remove algae, and rinsed sponge filters. To avoid unnecessary stress, we only netted individuals for experimental procedures and not as part of daily care. We emulated light cycles for the tanks following the external day–night cycle, which changed seasonally. Finally, we maintained building air temperature at approximately 16°C, but water temperature was not maintained aside from ambient temperatures in the facility.

Experimental Design.—We used individual hellbender larvae as our experimental unit and assigned larvae to treatment groups using a randomized complete block design, with three treatments per block. The blocks consisted of 10 aquaria, labeled 1–10. We separated each aquarium into three compartments using perforated PVC board dividers labeled A, B, and C, to represent the three treatments. We placed one larval hellbender in each compartment ($n = 3$ larvae per block × 10 blocks = 30 larvae total). This design allowed us to identify individual hellbenders using an aquaria-treatment code (e.g., 6B) regardless of tag readability. Hellbenders assigned to treatment A received four VIE marks (one per foot), using a unique combination of green and pink tags (product number 1VIFE000002l; Northwest Marine Technology). We selected green and pink because we suspected these colors would provide maximum visibility and contrast against the darkly pigmented skin of larvae (Fig. 1A). We chose to apply VIE marks to the feet because larval hellbenders have a relatively uniform dark pigment throughout their body except for light pigment on the ventral surface of each foot. Hellbenders assigned to treatment B were implanted with a yellow, 1.2-mm × 2.7-mm VI Alpha tag (product number 1VIA000003; Northwest Marine Technology) with a three-digit alphanumeric code that was positioned on the lower abdomen just anterior to the right hind limb (Fig. 1B). Hellbenders assigned to treatment C were considered the control group and received no tag.

We marked sedated larval hellbenders using either a VI Alpha tag or a premixed aqueous solution of elastomer. For VIE-tagging, we thoroughly mixed a 10:1 ratio of colored elastomer (either pink or green) to curing elastomer for exactly 1 min, then used a 1-mL syringe to transfer 0.05 mL mixed VIE solution into a 0.3-mL syringe for tag injection. The larval hellbenders largely shared the same environment and are from the same brood, so we re-used syringes between hellbenders but used separate syringes for each color. We then sedated individuals using a 1-g/L mixture of regular strength Orajel® medicated (10% benzocaine) gel into dechlorinated distilled water (Cecala et al., 2007). We submerged larvae in the solution and monitored them until they lost the ability to right
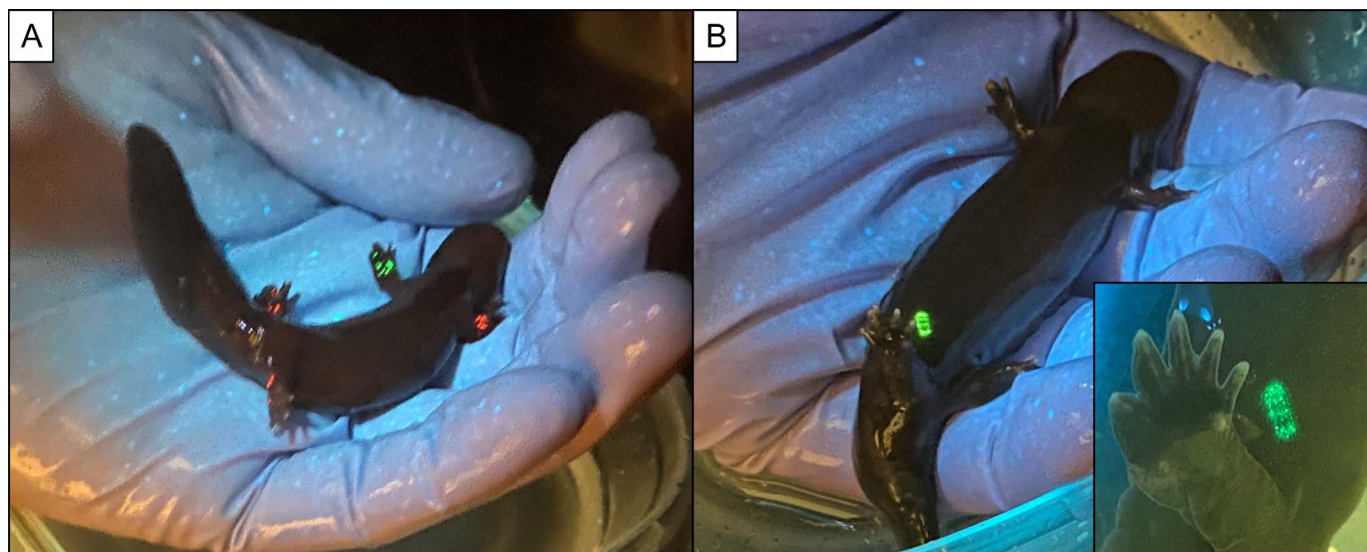
Fɪɢ. 1. Examples of larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) implanted with (A) pink and green visible implant elastomer (VIE) tags and (B) a yellow visible implant alpha (VI Alpha) tag after 12 mo. Inset in panel B is a close-up of the VI Alpha tag demonstrating the low readability of the alpha-numeric code. Images were taken under a VI light and yellow lens filter.

themselves, typically after 7–10 min (Unger et al., 2020). We chose to sedate the hellbenders to reduce physiological stress, reduce risk of injury associated with invasive tag implementation, and to maximize consistency of tag application across individuals. We also sedated hellbenders assigned to the control treatment to standardize handling effects that may lead to differences in growth and body condition among treatments. Once sedated, we measured the total length (mm; TL), snout–vent length (mm; SVL), and mass (g) of each hellbender. To tag larval hellbenders with VIE marks, one observer held the foot of the hellbender while the other injected the colored VIE-loaded 0.3-mL syringe into the ventral foot musculature according to the predefined color code. Then, the observer slowly depressed and withdrew the syringe leaving an approximately 1-mm-long line of elastomer into the ventral foot muscle. We used the VIE solution within 1 h of mixing. To tag larval hellbenders with VI Alpha tags, we inserted the VI Alpha tag into a water-moistened, beveled needle (included in the VI Alpha-tagging kit; product number 1VIA000003; Northwest Marine Technology) with both the VI Alpha tag code and bevel facing up. We cut a small path for the needle on the lower abdomen just anterior to the right hind limb using the needle tip. Then, we injected the needle bevel facing up into the clear tissue on the lower abdomen and depressed the plunger until the VI Alpha tag was fully underneath the shallow layer of clear tissue. The same observer tagged all larval hellbenders with both VIE and VI Alpha tags.

Once tagged, we recorded the initial readability of the tag on an objective 0–4 scale while viewing each hellbender with naked eye as well as under a fluorescent VI light (Northwest Marine Technology) in a dark environment. The VI light transmits a 450-nm-wavelength deep violet light ideal for fluorescing VI tags. We assigned a score of 0/NONE if the tag was not visible under any circumstance. We assigned a score of 1/POOR if the tag was not visible with the naked eye, was very small or had only a dull glow under UV, and if the alphanumeric code (VI Alpha) was not at all visible. We assigned a score of 2/MODERATE if the tag was not visible with naked eye but obvious with UV, the dot of color was smaller than ideal (for

VIE), and/or the alpha numeric code was not readable, but the tag presence could be confirmed. We assigned a score of 3/GOOD if the tag was barely visible with naked eye but clearly visible under UV with obvious color/glow, and/or the alphanumeric code was partially legible (≥1 of 3 characters clear). Finally, we assigned a score of 4/EXCELLENT if the tag was clearly visible with both naked eye and UV and/or the alphanumeric code easily readable with UV. Two independent surveyors quantified readability, and we used the average as the readability score for that occasion.

After the animal was measured and marked but still sedated, we took standardized photographs of each hellbender's marks under a VI light to compare tag shape, brightness, and location over time. We placed the hellbender in a dark environment (typically a black box with a black curtain opening, although a dark room was occasionally used), and we had one observer hold the animal and shine the VI light onto it while the other observer took photographs. The VI light oversaturates photographs, so we took all photos through a translucent yellow lens.

Initial tagging took place in early March 2020, and we monitored tag readability and hellbender growth at six time points over the course of our study: 0 (initial tagging), 3, 4, 5, 7, and 12 mo posttagging. On each occasion, we measured SVL, TL, and mass for each hellbender. Additionally, we scanned each hellbender twice (using independent observers) to determine whether any tags were detectable. When we detected a tag, we recorded tag type, color, location, and each observer scored readability separately. Readability scores were performed partially blind in which the primary observer recording tag information had no knowledge regarding what kind of tag the hellbender had, if any. Specifically, the order in which individuals would be observed was randomly generated; the secondary observer would net the individual for observation while the primary observer looked away. This process was done to mimic the experience of surveying wild marked and unmarked animals. At the 7-mo and 12-mo monitoring occasions we also collected standardized photos of the tags under a fluorescent VI light following the same protocol outlined above.

TABLE 1. Candidate models used to represent alternative hypotheses regarding the effects of time, tag type (VIE = visible implant elastomer; VI Alpha = visible implant alpha), and treatment (VIE, VI Alpha, Control = no tag) on tag readability, growth (TL = total length), and body condition (SMI = scaled mass index) of larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*).

Analysis/Response	Model	Alternate hypothesis
Effects of tag type on tag readability	tag type + time[a]	Readability declines over time, readability of VI Alpha is higher, the rate of decline in readability is similar among tag types.
	time[a]	Readability is similar among tag types but declines over time.
	tag type	Readability is higher for VI Alpha tags and constant over time.
Effects of tagging on growth (TL) and body condition (SMI)	treatment × time[b]	Growth and SMI decline immediately following tagging but only for tagged hellbenders, but effects of tagging on each endpoint are gradually minimized over time
	treatment + time[b]	Growth and SMI decline immediately following tagging in tagged hellbenders but not in controls, but effects of tagging on each endpoint are constant over time
	time[b]	Growth and SMI vary over time but not among treatments
	treatment	Growth and SMI vary with treatment but do not vary over time

[a] Time was defined as a continuous covariate (range: 0–12 mo) during modeling to represent the hypothesis that readability would decline quickly and immediately following tagging before eventually stabilizing.
[b] Time was defined as a categorical covariate (0, 3, 4, 5, 7, or 12 mo) during modeling.

Notably, between the final two surveys (7 and 12 mo), dividers were removed and hellbenders were consolidated into six aquaria because of space limitations in the husbandry facility. During consolidation, we pooled larvae by treatment and each treatment group was divided in half ($n = 5$ larvae from the same treatment per aquaria). This approach facilitated our ability to track outcomes by treatment, but it precluded our ability to distinguish among some individuals within a treatment during our final survey (e.g., we could not distinguish among individuals in the control group or among individuals whose tags were illegible).

DATA ANALYSIS

Tag retention: To compare retention of VIE and VI Alpha tags, we summarized the frequency of larval hellbenders with a detectable tag on each sampling occasion, separately for each treatment group. On each occasion, we considered a tag to be retained if at least one surveyor detected its presence, even if the readability was poor. We quantified retention at each time point as the proportion of individuals with a detectable tag and plotted raw retention rates for each treatment across monitoring occasions to evaluate how retention changed over time.

Tag readability by type: We used linear mixed logistic regression models within a multimodel framework to evaluate support for our hypotheses concerning factors that influenced tag readability. We only included observations of hellbenders assigned to the VIE and VI Alpha treatments in our analysis because hellbenders in the control group were not tagged. Prior to analysis we converted each mean readability score (0–4) on a given occasion to a binomial (0 = nonlegible; 1 = legible) response, where we considered tags legible if the individual mark could be read correctly with the naked eye or under VI light and illegible if any of the four VIE marks or any of the alpha-numeric characters in a VI Alpha tag could not be determined with certainty. We considered effects of tag type (VIE or VI Alpha; as a categorical variable) and time since tagging (0, 3, 4, 5, 7, or 12 mo; as a continuous variable) on our response.

We considered a nested set of three candidate models (i.e., tag type + time; time-only; tag type-only) in our analysis. We did not consider an interaction between tag type and time because the model failed to converge when we attempted to fit it. Each model represented an alternative to the null hypothesis that

neither tag type nor time influenced VIE or VI Alpha tag legibility (Table 1). We included hellbender identity as a random effect in all models to account for repeated measurements collected from individuals over time.

Tag readability by color: We did not perform further analyses on tag color because there was no difference in legibility between colors.

Growth and body condition: We investigated effects of tagging and tag type on larval hellbender growth and body condition in two separate analyses. Our inability to distinguish all individuals during the final survey because of the consolidation of treatment groups (see Experimental Design) precluded our ability to explicitly quantify growth rates of individuals during the final time step in our experiment and thus our ability to use growth rates of individuals in each time step as the response variable in our analysis. Therefore, we used raw values of size and body condition on each survey occasion as endpoints and used linear mixed regression models within a multimodel framework to assess how the mean value of each response varied among treatments and over time. We included hellbenders from all three treatment groups (control, VI Alpha, VIE) in both analyses. In both analyses we considered a nested set of four candidate models that were similar in linear structure to those used in our analyses of tag readability (i.e., treatment × time; treatment + time; time-only; treatment-only), with the exception that we treated time as a categorical variable (0, 3, 4, 5, 7, or 12 mo) rather than as a continuous variable. We chose to treat time as a category because we were less certain that growth followed a linear or log-linear pattern over time.

Growth.—In our analysis to investigate effects of tagging on hellbender growth, we used TL of individual hellbenders on each sampling occasion as our response variable. Each model represented an alternative to the null hypothesis that neither treatment group nor time influenced hellbender growth (Table 1). We included hellbender identity as a random effect in all models to account for repeated measurements collected from individuals over time.

Body condition.—To investigate effects of tagging on hellbender body condition, we used a scaled mass index (SMI) as our response variable representing body condition (SMI; Peig and Green, 2009). Briefly, scaled mass is interpreted as the estimated mass of an individual at a reference value of structural size. We selected SMI as our measure of body condition because of the relative ease of interpreting SMI values and the ease of
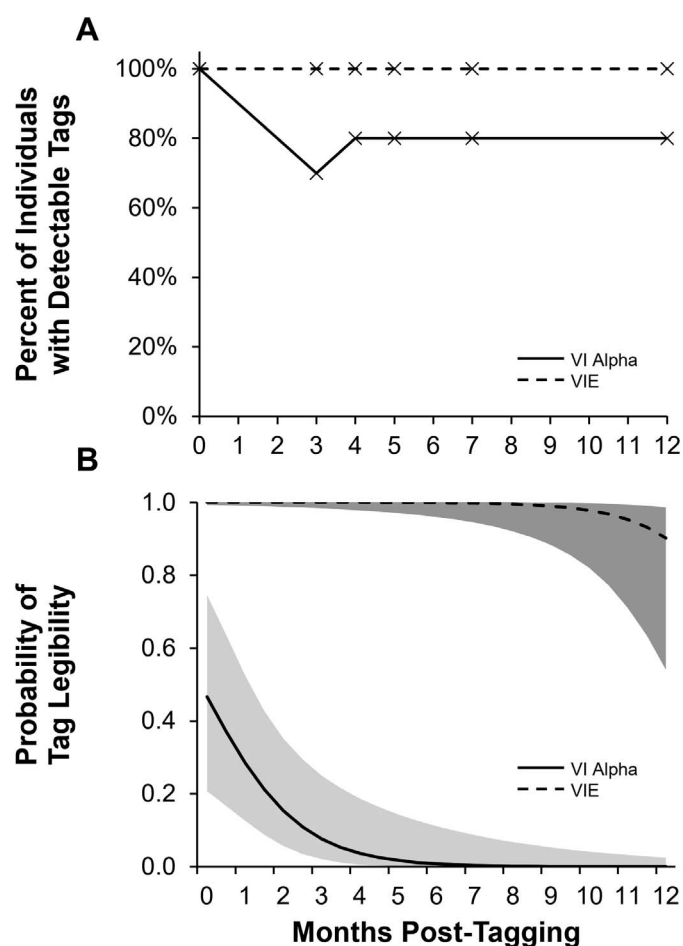
A

B

FIG. 2. Retention (A) and probability of tag legibility (B) of visible implant elastomer (VIE) and visible implant alpha (VI Alpha) tags implanted in larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) over a 12-mo period. "×"s mark time points at which we assessed detection and readability of tags. Tag legibility was scored as a binomial response (0 = nonlegible; 1 = legible).

comparing values across future studies (see Peig and Green, 2009). It has been shown that SMI more accurately indicates relative energy reserves than more commonly used indices of condition (e.g., residuals from an ordinary least squares regression) for a wide range of taxa including amphibians (Peig and Green, 2009; MacCracken and Stebbings, 2012). We used total length to quantify structural size of hellbenders given that salamanders store considerable energy reserves in the tail (Fitzpatrick, 1976; Takahashi and Pauley, 2010). We calculated scaled mass (SM) for each individual separately on each occasion as

$$\widehat{SM}_i = M_i \left[\frac{L_0}{L_i}\right]^b,$$

where M_i was mass (grams) during that occasion, L_i was total length (millimeters) of individual i during that occasion, L_0 was our selected reference category of total length (90 mm), and b was a scaling exponent. We calculated b by dividing the slope from a log–log regression of mass against total length by the Pearson's correlation coefficient (r; Peig and Green, 2009). We used all measurements collected from larvae during the current study in our regression ($n = 180$ records). Log-transformed mass and total length were positively correlated ($r = 0.81$), and coefficients from general linear mixed regression models (we included individual

TABLE 2. Models and pairwise model comparison ranking results as determined by likelihood ratio testing to evaluate effects of time and tagging type on readability, larval Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) growth, and larval hellbender body condition and time. Model 1: Treatment × Time, Model 2: Treatment + Time, Model 3: Time, Model 4: Treatment.

Analysis	Model comparison	Chi-square	P-value	df
Readability by tag type	1[a] vs. 2	17.813	<0.001	4
	1[a] vs. 3	20.531	<0.001	4
Growth	1 vs. 2[a]	3.865	0.953	10
	2[a] vs. 3	8.017	0.018	8
	2[a] vs. 4	263.240	<0.001	5
Body condition	1 vs. 2	7.657	0.662	10
	2 vs. 3[a]	3.556	0.169	8
	2 vs. 4	93.242	<0.001	3

[a] Indicates the top-ranking model in each analysis upon which inference was based.

as random effect) were $\beta_0 = -2.20 \pm 0.32$ standard error (SE) and $\beta_{Log10(TLcm)} = 1.46 \pm 0.16$ SE.

The hypotheses represented by each of our candidate models were identical to those described in our description of our analysis of growth, with the only difference being that the response was change in body condition over time rather than change in total length (Table 1).

Model Ranking and Selection.—We used likelihood ratio testing (LRT) to rank nested models for each analysis. To rank models, we first arranged them in order of decreasing complexity, and used LRTs to make pairwise comparisons between each sequential pair of models. Briefly, the LRT statistic is calculated based on the overall fit for two nested models to an identical data set, and the number of additional parameters that appear in the more complex model of the pair. It allows one to evaluate whether inclusion of the variable(s) present in the more complex model yields a statistically significant improvement in model fit to the data. We interpreted statistical significance at the $\alpha = 0.05$ level, such that when $P \leq 0.05$ we concluded that the more complex model was better supported by the data than was the simpler model. Our model ranking procedure ultimately facilitated our ability to identify the most parsimonious model in our candidate set. For each endpoint (readability, growth, body condition) we based inference on the best supported model, as indicated by LRTs. We performed all analyses using Program R Version 4.1.1 (R Core Team, 2021). We used the DHARMa package (Hartig, 2022) in Program R to evaluate the fit of each top-ranking model to our data, where we checked specifically for the correct distribution of residuals, under- and overdispersion of residuals, outliers, and zero-inflation (for binomial models). We report least square mean estimates for each treatment level $\pm$ SE unless otherwise noted.

RESULTS

Tag Retention.—All (100%) VIE tags were retained throughout the 12-mo survey period while only 80% of VI Alpha tags were detected at 12 mo posttagging (Fig. 2A). Retention of VI Alpha was lowest (70%) during the 3-mo survey, but we attribute this result to observer error at a single time point given the increase in frequency of detectable VI Alpha tags at later time points (80%; Fig. 2A).

Tag Readability.—Likelihood ratio testing indicated that the additive model (treatment + time) describing tag readability was best supported by the data (Tables 2, 3). Evaluation of model

TABLE 3. Coefficient estimates and associated standard error (SE) for top-ranked models in analyses to investigate factors associated with tag readability, and growth and body condition of larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). TL = total length; SMI = scaled mass index.

Analysis	Parameter	Mean estimate	SE
Readability by tag type	Intercept (Tag Type$_{VI\ Alpha}$)	−0.133	0.617
	Tag Type$_{VIE}$	11.793	3.148
	Time	−0.786	0.275
Growth (TL)	Intercept (0 mo & Treatment$_{None}$)	78.633	1.280
	3 mo	7.100	0.771
	4 mo	12.167	0.771
	5 mo	14.052	0.779
	7 mo	13.508	0.771
	12 mo	28.867	1.844
	Treatment$_{VI\ Alpha}$	3.787	1.628
	Treatment$_{VIE}$	−0.585	1.628
Body condition (SMI)	Intercept (0 mo)	1.515	0.027
	3 mo	0.027	0.037
	4 mo	−0.072	0.037
	5 mo	−0.083	0.038
	7 mo	−0.171	0.037
	12 mo	0.351	0.042

output suggested no evidence of zero inflation, under- or overdispersion, outliers, or incorrect distribution of residuals; this suggested that the model fit our data well. Our top ranked model indicated that the probability that a VIE tag was legible was consistently high (>0.9), whereas the probability of a VI Alpha tag being legible was only 0.5 immediately following tagging and degraded exponentially over time (Fig. 2B). At the end of our study we were able to accurately identify 100% of
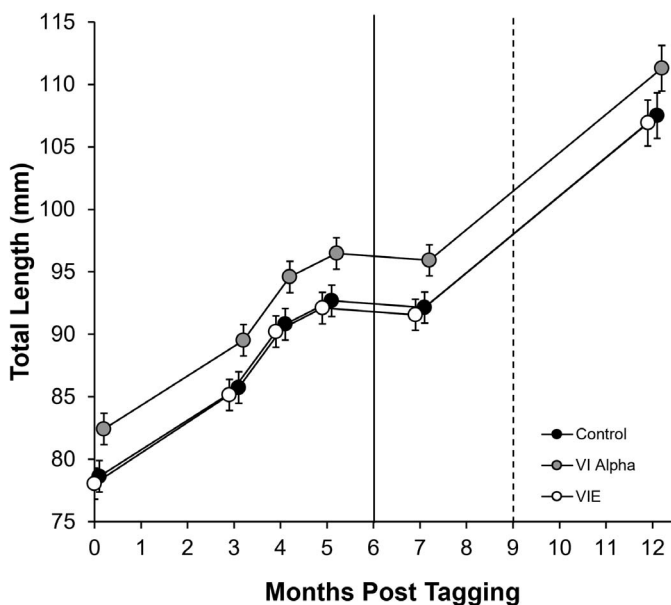

FIG. 3. Changes in estimated total length of larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) across three tagging treatments over a 12-mo period. Points represent mean estimates and error bars represent standard error. Points at each time point are staggered slightly (left-to-right) to better illustrate standard error. The vertical solid line represents the time point when hellbender diet was switched from live blackworms (*Lumbriculus variegatus*) to thawed mysis shrimp (order Mysida) and vertical dashed line represents the time point when hellbenders were consolidated from three hellbenders to five hellbenders per 38-L or 76-L aquaria.
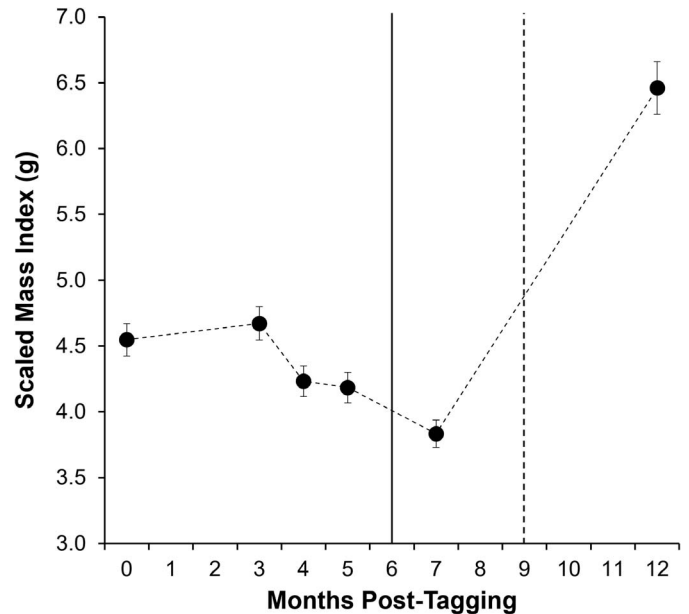

FIG. 4. Mean body condition, calculated as scaled mass index (SMI), of larval Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) over a 12-mo period. Scaled mass represents an estimate of an individual's body mass if it were of a reference structural size (defined here as 90 mm total length). Average larval hellbender SMI did not differ across treatments at any sampling occasion. Points represent mean estimates, averaged across treatments, and error bars represent standard error. The vertical solid line represents the time point when hellbender diet was switched from live blackworms (*Lumbriculus variegatus*) to thawed mysis shrimp (order Mysida) and vertical dashed line represents the time point when hellbenders were consolidated from three hellbenders to five hellbenders per 38-L or 76-L aquaria.

individual hellbenders in the VIE treatment, while we were unable to identify any unique individuals in the VI Alpha treatments.

Growth.—The additive model (treatment + time) describing variation in hellbender size best fit our data (Tables 2, 3). Evaluation of model output suggested no evidence of zero inflation, under- or overdispersion, outliers, or incorrect distribution of residuals; this suggested that the model fit our data well. Our model indicated that mean TL of larvae varied by time and among treatments. Estimated TL increased throughout the 12-mo study at similar rates across treatments (Fig. 3) but VI Alpha-tagged hellbenders (0 mo: 82.42 mm TL ± 2.46 mm 95% CI) were on average 1.04 times longer when compared with control (0 mo: 78.63 mm TL ± 2.46 mm 95% CI) and VIE-tagged hellbenders (0 mo: 78.05 mm TL ± 2.46 mm 95% CI) at every time point in the study, including at the time of initial tagging.

Body Condition.—Model ranking indicated that the univariate model with time as the only predictor of larval body condition fit our data best (Tables 2, 3). Evaluation of model output suggested no evidence of zero inflation, under- or overdispersion, outliers, or incorrect distribution of residuals; this suggested that the model fit our data well. Mean SMI did not differ among treatment groups at any time point, suggesting that for every time point, hellbenders in all three treatments exhibited similar body masses when structural size was controlled. While average body condition decreased slightly throughout the first 7 mo of the study, SMI increased by approximately 70% for all hellbenders between the 7- and 12-mo time points (Fig. 4).

DISCUSSION

Our study is the first to quantitatively evaluate alternative tagging methods for larval hellbenders and highlights the safety and potential utility of visible implant tags in early life stages of this species. Contrary to our hypotheses, VIE tags had higher readability than did VI Alpha tags, VIE tag retention was higher than was VI Alpha tag retention, VIE tag color had no effect on readability, and tag type did not influence hellbender growth or body condition. Furthermore, our results suggest that VIE tags are a safe and effective method for facilitating identification of unique individuals for ≥12 mo regardless of VIE tag color. Conversely, we found that VI Alpha tags are not a viable method for tagging larval hellbenders because of their lower retention than that of VIE tags and illegibility of alphanumeric codes. Our findings collectively highlight the potential for VIE tags to facilitate novel capture–mark–recapture studies to address the dearth of knowledge concerning the ecology of early hellbender life stages.

Our results were consistent with previous findings regarding the efficacy and health risks of visible implants in salamanders (Marold, 2001; Bailey, 2004; Heemeyer et al., 2007; Phillips and Fries, 2009; Osbourn et al., 2011). Heemeyer et al. (2007) found that VIE tags injected into eastern red-backed salamanders were 100% retained throughout a 53-wk period and did not negatively affect mass between treatments. The rates of VIE retention that we observed over 1 yr (100%) were higher than those observed by Phillips and Fries (2009), who reported 86% VIE retention in San Marcos Salamanders (*Eurycea nana*) over a 244-d period when injected either dorsally or ventrally on the body. The cause for this discrepancy in VIE tag retention is unclear, though differences in tagging location (injected into foot for larval hellbenders and body for San Marcos salamanders) and salamander sizes when first tagged (∼8 cm total length for larval hellbenders and 5.4–7.5 cm total length for San Marcos salamanders; Phillips and Fries, 2009) may influence VIE retention. For example, foot-tagging reduces the tissue space for tags to migrate, relative to a location on the body trunk or limb. Further testing of the efficacy of VIE foot-tagging across different body sizes may provide viable methods for increasing tag retention in other salamander species. Like our findings, Osbourn et al. (2011) found that VI Alpha tags did not influence survival or growth of juvenile Marbled Salamanders (*Ambystoma opacum*) over a 4-wk period. Osbourn et al. (2011) also found that the alphanumeric code of VI Alpha tags implanted in marbled salamanders remained legible over a 4-wk period. We did not record readability at the 4-wk period for our study, but only 20% of the alphanumeric codes were legible in larval hellbenders after 3 mo. We tracked code readability for 1 yr compared with the 4-wk study of Osbourn et al. (2011); therefore, the reported performance of VI Alpha tags in marbled salamanders could potentially decline over prolonged observation as well. We found no effects of visible implants on growth and body condition, but there are several other physiological and behavioral responses that we did not consider that could potentially be influenced by tagging. For example, we did not inject hellbenders from the control group with saline or another harmless fluid to control for the effects of tagging. However, injection is a relatively invasive process, so we would have expected to see lower growth rates in tag-injected hellbenders than in hellbenders from the control group. We observed no difference in growth rates among treatments, so we conclude that tag presence did not affect growth rates; however, further tag retention and readability studies should incorporate a method to control for tag injection. We also did not directly observe changes in mobility throughout our study, but injecting VIE tags into larval hellbender feet may have unintended maneuverability consequences in a natural setting and should be further investigated. Furthermore, limited studies on the effects of visible implants on the physiology of other taxa have been encouraging (Antwis et al., 2014; Neufeld et al., 2015), but the effects on behavior have been mixed as evidence suggests visible implants may influence certain social interactions (Frommen et al., 2015; Fürtbauer et al., 2015; Ruberto et al., 2018; Padget and Thompson, 2021). Though hellbenders are a nonsocial species, it may still be important to determine whether visible implants influence other traits, especially given their designation as a species of conservation concern.

Unlike some studies, we did not encounter noticeable VIE tag migration or fragmentation. Once VIE tags solidify, there is a slight possibility for tags to fracture or shift underneath the skin, thus altering the unique identifying pattern and causing misidentification errors. Bailey (2004) reported 6% VIE tag misidentification in Blue Ridge Two-Lined Salamanders (*Eurycea wilderae*), while Davis and Ovaska (2001) reported 19% VIE tag misidentification in Western Red-Backed Salamanders (*Plethodon vehiculum*). Bendik et al. (2013) also found that VIE tags were 2.5 times more likely to generate false rejections (failing to identify a tagged individual) and more likely to generate false acceptances (falsely identifying a tagged individual) as a result of tag misidentification than photo identification methods. Although we report no VIE migration or fragmentation, our experimental tanks did not contain substrate, and tag fragmentation may occur when tags are injected into wild hellbenders that subsequently travel over coarse substrates such as gravel or cobble. Furthermore, larval hellbender density and dispersal are largely unknown, in part because of the lack of a reliable tagging method, which creates difficulties in replicating natural density and space use of larval hellbenders under ex-situ conditions. As such, hellbender movement and individual activity could have been constrained by the size of the aquaria and larval density. For example, increased movement or interactions with other larval hellbenders could have increased the risk of VIE tag loss, migration, or fragmentation in our foot-injected tags. The mechanism responsible for the complete loss of two (20%) VI Alpha tags in our study remains unclear but could have included tag expulsion or migration deeper into pigmented tissue.

Our primary focus was evaluating efficacy of using visible implant tags in larval hellbenders, but our repeated observations in an experimental setting provided potentially valuable information on larval hellbender growth rates in captivity. The only published study to report larval hellbender growth rates that we are aware of involved results from following larvae only through 25 wk posthatching (Unger and Mathis, 2013), which was the approximate age when larvae entered our study. At the time of tagging, larvae in our study were similar in total length (70–80 mm) to 25-wk-old larvae reared from eggs collected in Missouri, North Carolina, and Georgia (Unger and Mathis, 2013). Interestingly, all larvae in our study experienced a substantial increase in body mass (once structural size was accounted for) between 7- and 12-mo posttagging, which, to our knowledge, has not been reported elsewhere. The cause for this increase remains unclear but we speculate that it could be related to dietary shifts, changes in housing density, ontogenetic shifts in metabolic processes, or some combination. For example, larval diets shifted from blackworms to mysis shrimp

at 6 mo posttagging, just prior to the large increase in body condition we noted. Furthermore, we inadvertently altered the density of larval hellbenders by removing dividers and consolidating larval hellbenders from 10 aquaria to 6 aquaria during this time period. However, Petranka and Sih (1986) found that growth rates in salamanders are negatively affected by density; we observed an increase in growth rate across treatments so it is unlikely that this change in density influenced the effects of tagging on hellbender growth. Ontogenetic events similar to those observed in other salamander species, such as the ossification of structural (Fröbisch and Shubin, 2011) or feeding elements (Worthington and Wake, 1971), may also explain the patterns in SMI that we report, though the timing of such events is unknown in hellbenders. Further research into optimal diets and ontogeny may help understand the health and allometric trends of larval hellbenders and could be used to inform captive propagation programs (Ettling et al., 2017).

Our findings indicate that VI Alpha tags are a safe but ineffective method for marking individuals for this species. The probability of detecting a VI Alpha tag was relatively low compared with VIE tags within the first year posttagging and the alpha-numeric codes were illegible after 4 mo, which limited the possibility of identifying individual larval hellbenders. We also found that VI Alpha tags were relatively large and required more time and precision to apply when compared with VIE tags. Finally, VI Alpha tag implant requires making an incision, so hellbenders should be anesthetized prior to tagging and allowed extended recovery time, which can be logistically challenging in the field. Thus, we believe that VI Alpha tags do not provide a viable marking method for larval hellbenders. We did observe that larval hellbenders injected with VI Alpha tags had slightly longer bodies than did those with VIE tags or the control hellbenders from the onset of our study, which we speculate may have been attributable to an unconscious bias of surveyors toward relatively large larvae when selecting individuals for VI Alpha tagging.

Conversely, we found VIE tags relatively easy to apply, with an experienced individual able to tag one foot within 60 sec on average. Since completion of the current study, we have successfully applied VIE to larval hellbenders of >60 mm length in a natural setting without the need for anesthesia, as long as one surveyor is available to help restrain the salamander's foot while another applies the tag. Furthermore, a wide range of VIE tag colors (six fluorescent and four nonfluorescent) provide versatility for researchers performing long-term mark–recapture studies or when many unique identification combinations are required. We found no difference in fluorescent pink and green VIE tag color legibility, but we recommend similar retention and readability testing for both the nonfluorescent and other fluorescent colors in larval hellbenders.

Our findings provide evidence that VIE tags may be useful for estimating population-level demographic parameters that are needed to inform hellbender status assessments and population modeling. Currently, hellbender monitoring efforts typically focus on mature adults, though adult abundance and survivorship may not always accurately reflect the threats facing hellbender populations (Bodinof Jachowski and Hopkins, 2018). For example, because hellbenders exhibit considerable longevity, adult abundance may be slower to respond to environmental change than are abundance and survivorship of larvae and juveniles. Although VIE tags have occasionally been used to mark hellbender larvae in the wild (Hecht-

Kardasz et al., 2012; Pugh et al., 2013, 2016), our understanding of retention and readability over time was poorly understood. Diaz et al. (2022) reported the first confirmed recapture of a VIE-tagged immature hellbender in the wild using the techniques in our study, which occurred approximately 3 wk following initial capture and tagging. Our findings build upon these anecdotal reports by providing quantitative evidence that VIE tags are highly likely to remain detectable even 1 full yr posttagging. This result means that, in streams where immature hellbenders have sufficiently high detectability, VIE tags could facilitate novel investigations into abundance, apparent survival, and movement rates of a relatively vulnerable age class.

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