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Factors Influencing Occupancy and Detection Probability of Larval *Cryptobranchus a. alleganiensis* in North Carolina, USA

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Abstract.—**Aquatic salamanders are faced with a variety of threats to their population persistence, including increased sedimentation, loss of habitat, and anthropogenic development. These threats, among others, have led to the decline in relative abundance of the Eastern Hellbender (*Cryptobranchus a. alleganiensis*) in many streams across its geographic range; however, many gaps exist in our knowledge related to species life history, particularly larval ecology, habitat selection, and occupancy. Conservation management of these Appalachian (USA) salamander populations requires policies informed by data-driven studies that improve our understanding of overall recruitment. To investigate this lesser studied, early life stage, we characterized the site factors associated with occupancy of larvae within stream reaches in North Carolina, USA. Our use of biologically relevant, environmental covariates and numerous *a priori* models revealed several factors as important predictors of larval occupancy including percent substrate embeddedness, percentage riffle, percentage cobble substrate, and percentage forest land cover within 1 km. Occupancy was greater at sites with increased forest land cover, higher elevations, and water quality parameters of decreased specific conductivity and total dissolved solids. Detectability decreased across most sites during late summer visits, likely due to some combination of increased mortality of juveniles or individual dispersal out of stream survey transects. Using occupancy modeling, we provide the first empirical evidence identifying key environmental variables correlated with larval Eastern Hellbender presence in North Carolina, which expands our existing knowledge of larval life history with important implications for conservation management of this unique, charismatic megafauna salamander.**

Key Words.—amphibians; conservation; Cryptobranchidae; Hellbender; modeling; larval salamanders

Introduction

Salamanders are important ecological components of aquatic ecosystems, as either larvae occupying shelters to avoid predators and consuming insect nymphs (Hecht et al. 2017), or as adults being consumers of crayfish and fish regulating food webs and contributing to ecosystem resilience (Peterson et al. 1989; Davic and Welsh 2004). Among these salamanders, the fully aquatic Eastern Hellbender (*Cryptobranchus a. alleganiensis*) is a species of conservation concern throughout its geographic range. Within North Carolina, USA, the Eastern Hellbender is found primarily in clear, cool flowing streams of the western portion of the state (Petranka 1998). The Eastern Hellbender has experienced precipitous declines throughout its geographic range (Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011), yet North Carolina appears to have some relatively stable populations with active recruitment of juveniles in some watersheds (Mayasich et al. 2003; Briggler et al. 2007). Regionally, recent conservation efforts and monitoring are focused on examining population trends (Wineland et al. 2019a), relative abundance of individuals (Pugh et al. 2018), captive propagation (Ettling et al. 2017), augmentation of shelter habitat in streams (Bodinof-Jachowski et al. 2020), use of shelters for nesting and habitat and resiliency of shelters over time (Button et al. 2020b,d), demographic response to land-use change (Jachowski and Hopkins 2018), and environmental DNA detection (eDNA) for monitoring populations (Spear et al. 2015; Pitt et al. 2017; Takahashi et al. 2018; Wineland et al. 2019b). There is a paucity of empirical information, however, on larval Eastern Hellbender life history, biology, and factors that influence occupancy and in-stream microhabitat selection in North Carolina and throughout much of the range of the species. In particular, little is known about larval ecology in watersheds in the southern Appalachian Mountains, which may represent some of the best remaining habitat for the species (Freake and DePerno 2017). For long-term population viability, it is likely larval presence is closely related to stability of riparian habitat. Therefore,

there is a dire need to determine biotic and abiotic variables that determine the presence of hellbender larvae and thus recruitment in streams within North Carolina, a potential stronghold within the geographic range of the species, to inform state-specific management and conservation actions.

Occupancy models are an ideal method to determine presence and single species monitoring in declining amphibians (Smith et al. 2014). These models provide an effective method for sampling rare and elusive species with imperfect detection probabilities (Nichols et al. 2008) and may be essential for large-scale monitoring programs of more elusive herpetofauna (Durso et al. 2011). Occupancy modeling involves repeated sampling of communities based on detection or non-detection (i.e., presence-absence), in species with inherently low detection rates (MacKenzie et al. 2006). This approach has been used across herpetological taxa (Smith et al. 2014; Walkup et al. 2018; Cassel et al. 2019; Kessler et al. 2019) but to a lesser extent for larval amphibians (Haggerty et al. 2019). Recent research has used occupancy modeling to identify predictors of adult Eastern Hellbenders based largely on adult presence and eDNA surveys (Pugh et al. 2015; Franklin 2016; Wineland et al. 2019b). Subsequently, Bodinof-Jachowski et al. (2016) found that predictions from Eastern Hellbender occupancy models incorporating land use, but not underlying geology and topology (physiography), may underestimate sites of conservation priority for the species. In addition, as estimates of occupancy and detection probability can increase with the number of surveys conducted (Crosby and Elmore 2012), application of occupancy modeling to hellbender larvae will help to ascertain the number of site visits necessary for detection. This pursuit is important given the critical nature of the larval life-history stage to the conservation of this threatened species.

Eastern Hellbender larvae may remain under adult shelter structures or under nest rocks with adult males until emergence in late spring to early summer (Groves et al. 2015). Larval surveys are rarely conducted across the geographic range of the Eastern Hellbender because most surveys are biased for adults, the primary target of traditional monitoring efforts (Nickerson and Krysko 2003; Nickerson et al. 2003; Hecht-Kardasz et al. 2012). These limited studies on larval biology, however, have identified macroinvertebrates as important food items for larval Eastern Hellbenders (Hecht-Kardasz and Nickerson 2013; Unger et al. 2020d) and larvae using gravel beds and cobble shelter (Nickerson et al. 2003; Freake and DePerno 2017; Hecht et al. 2019). Subsequently, further work is needed to quantify specific environmental factors associated with larval presence and persistence. To manage the species properly, an assessment of larval ecology and occurrence is an important component of long-term conservation of the species within watersheds, yet these essential natural history data are lacking. We sought to fill this knowledge gap by conducting surveys to identify important drivers of larval hellbender site occupancy and detection probability and thus, characterize local stream variables that may impact population recruitment within North Carolina.

Here, we identify important environmental landscape predictors of stable, larval populations of Eastern Hellbenders within North Carolina streams. The objectives of our study were to estimate occupancy and detection probability of larval Hellbenders using a single-season occupancy modeling approach and characterize in-stream microhabitat factors related to individual presence of larvae (habitat selection and preference). Moreover, we relate these findings to varying levels of forest land cover in representative hellbender occupied streams across western North Carolina. These data represent the first quantitative occupancy modeling specifically for hellbender larvae, and our results have implications for the larval life history, ecology, and conservation of this unique, cryptic, giant salamander across its geographic range.

Materials and Methods

Study site.—We conducted our study in western North Carolina, USA, within selected watersheds in the Upper French Broad River and Hiwassee River sub-basins (Fig. 1). Sites were selected within watersheds that presumably represented ideal, potential larval Eastern Hellbender habitat and included both private land and public land (Pisgah and Nantahala National Forests). Streams in western North Carolina may

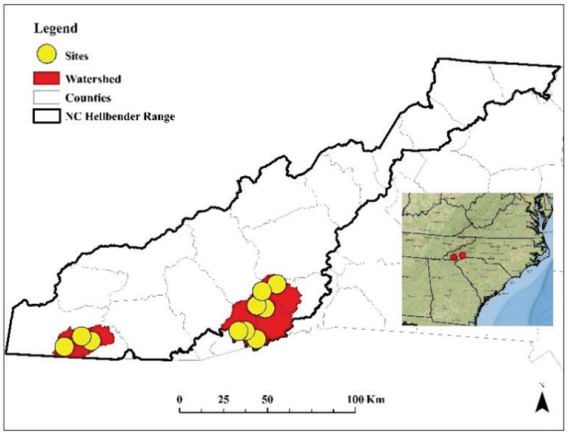

Figure 1. Sampling locations (n = 20 sites) for Eastern Hellbender (*Cryptobranchus a. alleganiensis*) larvae used in this study in western North Carolina, USA, within the Upper French Broad River and Hiwassee River sub-basins (shown in part as watersheds, in red).

Figure 2. Typical 50-m stream reach for cobble surveys (left) and a representative gilled Eastern Hellbender (*Cryptobranchus a. alleganiensis*) larva (right) from Upper French Broad River sub-basin, North Carolina, USA. (Photographed by Shem Unger).

contain relatively stable hellbender populations with some evidence of recruitment (Mayasich et al. 2003). Many of these sites selected for this study have evidence of presence of adult hellbenders based on previous snorkel and rock-flipping (peavey) surveys (Rossell et al. 2013; Spear et al. 2015), underwater video-camera surveys (Unger et al. 2020c) that documented breeding activity (Unger et al. 2020a), encounters by anglers (Williams et al. 2019), and diurnal activity of adults (Humphries 2007). Despite some knowledge of adult presence, demographic information on recruitment is lacking; no formal surveys that target larval habitat have been conducted previously in these stream reaches. The Upper French Broad River sub-basin (North Carolina and Tennessee) and Hiwassee River sub-basin (Georgia, North Carolina, and Tennessee) encompass an area of 4,868 km^2 and 5,326 km^2, respectively (Powell 2006). All sites were within the Blue Ridge physiographic province with similar underlying geography and to account for consistency for several covariates, including conductivity (Griffith et al. 2014).

Field methods.—We chose twenty 50-m stream reaches (sites), within 10 individual streams (two sites per stream) for this study. Sites we chose were logistically feasible for accessing and sampling multiple times in a single season and included areas with a history of adult and juvenile hellbender presence. In 2016, we selected seven tributaries within the Upper French Broad River sub-basin, representing 14 unique sites, while in 2017 we selected three tributaries of the Hiwassee River sub-basin representing six unique sites (site and stream names are not included due to conservation concerns but are on file with the North Carolina Wildlife Resources Commission). We chose the number of sites and repeated visits to provide sufficient power to ensure covariate effect on parameters following Wisniewski et al. (2013).

We were more restricted in the selection and placement of six sites in three of our Upper French Broad River streams than in the rest of our sites due to accessibility issues, trying to avoid disturbance from intense recreational pressure from the public, and/or sites having a confirmed history of juvenile presence (unpub. data). Therefore, we used a meter tape in the field to ensure a minimum of 200 m separated these sites to meet the assumption of independence for occupancy and detection probability analysis (MacKenzie et al. 2002). Most sites (14, within seven of 10 streams) were farther than 200 m apart and averaged 2.79 km in separation distance between the upper and lower sites per stream, as measured in ArcGIS® (Esri, Redlands, California, USA).

Teams surveyed sites on three occasions in the summer season; each year the first survey occurred in June, the second in July, and the third in August or early September. This design accounted for varying larval hellbender presence (MacKenzie and Royle 2005), as surveying stream sites multiple times during active seasons is an effective method for determining herpetofauna occurrence and presence of rare amphibian species (Bailey et al. 2019). Each site survey involved 4–8 surveyors snorkeling within a 50-m stream reach divided into five, 10-m transects established across the width of the stream (Fig. 2). All authors were present for surveys to ensure consistent survey effort methodology across all site visits. We performed area-constrained surveys covering all stream habitat from bank to bank, moving from downstream to upstream within each 10-m section, while another team member stood on the shoreline recording active search time for each transect. To keep survey effort consistent, this person also monitored survey progress and stopped individuals at transect boundaries until all surveyors were done before instructing the team to begin sampling the next upstream transect in unison. Surveyors were strategically placed

across the width of the transect to ensure full coverage for searches. Surveys consisted of cobble searches, a modification of visual encounter surveys where cobble-sized rocks (Wentworth size 64–255 mm; Wentworth 1922) were carefully lifted by hand to detect larval hellbenders underneath (Hecht-Kardasz et al. 2012), because larvae are associated with smaller rock shelters than adults (Hecht et al. 2019). The entire transect was searched for presence of hellbender larvae until an individual was encountered (Fig. 2), and at that point, time of capture and location (transect number) were noted, surveys were deemed complete, and no further habitat was searched. In cases where no individual was encountered, the entire 50-m stream reach (with all five transects) was surveyed. Lastly, all observers quantified how many other salamanders (e.g., brook salamanders, *Eurycea* sp.) and crayfish (Cambaridae) were encountered during each survey as a qualitative indicator of stream habitat and to collect additional ecological characteristics on other species present in hellbender occupied stream reaches. To inform how occupied sites differed for other species encountered during surveys, we used stream reach area to estimate the number of brook salamanders and crayfish per 100 m^2.

Environmental data, model covariates, and occupancy.—Following completion of three survey visits for all sites in the summer season, we collected stream morphology data using cross-sections, Wolman pebble counts (Wolman 1954), and habitat surveys to compare hydrologic characteristics of sites. At representative riffles, we established temporary cross-sections using 1.27 cm (½-inch) rebar and metric tape. To map cross-section elevations manually, we used a Bosch® BT160 Professional (26× magnification) automatic level (Bosch LLC, Farmington Hills, Michigan, USA) and a 3.96 m (13 foot) fiberglass stadia rod (Supplemental Information, Fig. S1). Water surface slope was measured in the field concurrent with cross-section surveys. We entered data into a reference reach spreadsheet to calculate flow rate (m^3/s) and cross-section area (m^2) and estimate flood and wetted width (m) across streams (Mecklenburg and Ward 2004). These calculations allowed us to create 2D models of detailed stream habitat for comparison across sites (Supplemental Information, Fig. S1).

Standing approximately in the thalweg (middle channel) of the stream, we recorded water velocity (m/s) for all sites using the standard Stream Gaging Surface Float Method (Hundt and Blasch 2019). Flow rate was derived from instream measurements coupled with modeled values from channel cross-sections and slopes. We corrected estimated discharge rates using nearby gages when applicable. We measured stream wetted width (m) and depth (cm) with a meter tape and meter stick along cross-sections.

Within each 50-m site, we characterized the stream substrate composition by randomly selecting and subsequently sampling three, 1-m^2 quadrats every 10 m, for a total of 15 quadrats per site. We stratified sampling across both stream width and survey transects by randomly throwing a pre-measured Polyvinyl Chloride (PVC) square within each transect to ensure characterization of right, middle, and left side of stream width. We estimated percentage sand, gravel, cobble, boulder, and bedrock within each quadrat and used a Wildco® gravelometer field sieve and sand card (Wildco Supply Company, Yulee, Florida, USA) as needed. Moreover, we visually estimated substrate embeddedness (mean percentage rock coverage of sediment) for five random rocks within each site following Platts et al. (1983) and Fitzpatrick et al. (1998), in which embeddedness is defined as the degree that the larger particles are surrounded or covered by fine sediment typically buried in silt, sand, or mud. The embedded height or depth on each sampled rock was discernably detectable by visually inspecting the gradation between fine sediment-stained portions (typically darker colored) and un-tinted clear substrate (buried).

We quantified the number of potential adult-sized shelters (minimum size of 0.41 × 0.41 m, 0.17 m^2) checked with a meter stick and potential nest shelters (0.61 × 0.61 m, 0.37 m^2) within all sites; minimum size guidelines were based on cover rock area and size reported in Rossell et al. (2013), Button et al. (2020d) of > 0.40 m, and according to North Carolina Wildlife Resources Commission survey data. In addition to size criteria, to count a particular rock as a potential adult shelter or a potential nest shelter available for hellbender use, we manually inspected the rock to ensure that it was not completely embedded by running a hand underneath to locate an opening. If no opening was identified, the rock was not counted regardless of its size. We conducted pebble counts using a zig-zag pebble count method (Bevenger and King 1995). We classified particle size using the gravelometer field sieve and sand card, and we calculated cumulative distribution, including D50 and D85, with the spreadsheet tool we used for cross-sections (Mecklenburg and Ward 2004).

To sample water chemistry, we used a YSI® PRO DSS water meter with four-port cable assembly and probes (YSI Incorporated, Yellow Springs, Ohio, USA) to record water temperature (°C), pH, conductivity (µS/cm), specific conductivity (µS/cm), dissolved oxygen (% or mg/L), and total dissolved solids (TDS; mg/L). We performed all water quality measurements three times immediately before surveys and averaged values for each site within the same season. To ensure

adequate visibility for visual surveys, we characterized turbidity or water clarity using a standard 120 cm transparency/turbidity tube equipped with a Secchi disc (Forestry Suppliers, Jackson, Mississippi, USA), which allowed for quantification of visibility either at or under 120 cm for each survey. We also followed the sampling methodology of Kincaid et al. (2018) and included estimates of Ephemeroptera, Plecoptera, and Trichoptera (EPT) richness as a covariate (Unger, unpub. data). In brief, we collected benthic macroinvertebrates (aquatic insects) across sites using a Surber sampler and identified EPT taxa.

We estimated percentage tree canopy cover at our study sites by using a spherical crown densiometer, a standard method for quantifying tree canopy cover of riparian stream systems (Warren et al. 2013). One person stood within 1 m of the edge of the water at a point near the downstream start of our 50-m stream reach, estimated percentage canopy cover in each of the four cardinal directions, and then took an average for that point. This process was repeated near the middle of the reach, and again near the end, for a total of six points, three along the right stream bank and three along the left stream bank for each of our sites. We then obtained a final percentage canopy cover estimate for the whole site by averaging the amounts from the six points.

To examine surrounding land use of the 10 individual streams in our study, we used ArcGIS® (Esri, Redlands, California, USA) to project the National Land Cover Dataset (NLCD; Homer et al. 2015) raster layer (30-m pixel resolution). We used stream flowline data (North Carolina Surface Water Classifications shapefile) from the North Carolina Department of Environmental Quality-Division of Water Resources and applied a stream buffer of 1 km to represent the immediate, surrounding landscape (including upstream contributing tributaries that fell within buffers). Then, we clipped the land cover layer to the 1-km buffer size and reclassified raster values to the following two, broader cover classes of interest: forest (deciduous, evergreen, mixed, and shrub forests) and agricultural/developed (grassland, herbaceous, pasture, hay, cultivated crops, open space, low, medium, and high intensity developed). Finally, we determined percentage cover for each class from the attribute table of the layer (Appendix 1). Stream access points of our sites primarily included Pisgah and Nantahala National Forests and private land. Definitions for all habitat parameters measured are presented in Appendix 2.

We ran Spearman's Rank Correlation among covariates to identify any highly correlated variables before running models. We removed conductivity (µS/cm) from our analysis, which was correlated with specific conductivity (µS/cm), as well as cross-section area (m^2), which was correlated strongly with average stream width (m; Appendix 2). We also removed the land cover classification of agriculture/developed as it was inversely correlated with the forest land cover class and represented minimal coverage. Forest was the predominant land cover type at our sites; thus, we chose to focus solely on the amount of forest within the 1-km buffers for our analyses.

We used single-season occupancy models (MacKenzie et al. 2003) and estimated occupancy (Ψ) and detection probability (P) in the program PRESENCE (Version 11.5; https://www.mbr-pwrc.usgs.gov/software/presence.html). We standardized all continuous covariates to z-scores using mean and standard deviations for use in occupancy (Hansen 2009; Long et al. 2011; https://www.uvm.edu/rsenr/vtcfwru/spreadsheets/occupancy/occupancy4.htm.). Encounter histories for each site visit were based on detection or non-detection (MacKenzie et al. 2002). We developed a series of *a priori* models that incorporated important factors (covariates) that may affect occupancy based on literature (Table 1) and used an iterative approach to investigate custom models using covariates. We used Akaike's Information Criteria (AIC) to rank and select models with lowest AICc scores considered the best explanatory models and with $\Delta AIC < 2$ (Burnham and Anderson 2002; Walkup et al. 2018; https://www.uvm.edu/rsenr/vtcfwru/spreadsheets/occupancy/occupancy4.htm.). We held P constant across all surveys and used an additive model approach to define model covariates (Smith et al. 2014). We converted AIC scores to AICc (corrected for sample size) following Burnham and Anderson (2002), with ΔAICc 0–2 regarded as the best supported models, while those with ΔAICc of 4–7 had reduced support. To test the overall fit of our top model to our data, we ran a parametric bootstrap test (n = 1,000) on the most global model (MacKenzie and Bailey 2004) and further assessed goodness-of-fit for our data (Chandler et al. 2015). Final custom models selected included only the lowest ranking ΔAICc scores and Akaike weights (ω) as the best supported, most parsimonious models and combination of biologically relevant covariates and parameters (K) similar to Gorman et al. (2009) and Cherukuri et al. (2018). Therefore, our informative models include a subset of best-fit covariates, but they do not include all possible additive or interactive environmental, habitat, or demographic factors likely to influence larval occupancy or detection probabilities. Further, to investigate the effect of survey window on occupancy, we took the top occupancy model and examined survey-specific P to allow detection probability to reflect differences for the first, second, and third survey windows over the course of the summer. We report these estimates for survey-specific detection probabilities.

To further investigate our parameters for modeling

larval presence, we compared factors of occupied sites (n = 14) with unoccupied sites (n = 6) using descriptive statistics and non-parametric Mann-Whitney U tests to explore habitat variables identified as important drivers of presence in our occupancy model using a significance level of 0.05. We also ran Spearman Rank Correlations for our top covariates to compare with total occupancy. Statistical analyses were performed in R, version 3.3.1 (R Development Core Team 2016). We compared medians of influential factors for sites where we detected larvae in all three surveys (n = 5 sites) to those where we failed to detect larvae in all three surveys (n = 6 sites).

Larval habitat selection.—In cases where individual larval hellbenders were present, we also recorded larval microhabitat selection variables including the following: occupied (shelter) rock length (cm), width (cm), and height (cm), water depth (cm), the distance of occupied rocks from nearest shore (m), and rock counts. For rock counts, as previously described, we counted the number of potential adult-sized, unembedded shelter rocks (boulders) and potential larval shelter rocks (cobble) within a 3-m radius around the spot of capture. We classified substrate under the occupied rock to size by measuring about 150 cm^2 of substrate from under the occupied shelter using a gravelometer (Floyd and Unger 2016; Hecht et al. 2019). We also measured tree canopy cover at larval capture sites in a manner similar to previously described methods; however, we only took one estimate with the spherical densitometer directly above capture shelter rock.

Results

We conducted surveys in summer 2016 and 2017 at 20 total sites within 10 streams (14 sites in 2016 and six in 2017). We noted some variation in duration of surveys (for the entire 50 m) across sites. The amount of survey time required to the first detection of an individual hellbender larva varied from 2–74 min. Total search time across sites was 2,693 min, with a mean (± one standard error) search time of 44.9 min (± 15.4). The maximum amount of time spent searching a stream reach was 124 min. In total, across three separate sampling events, we surveyed 212/300 (70.7%) transects across 20 sites.

Environmental data, model covariates, and occupancy.—We captured 30 Eastern Hellbender gilled larvae as part of occupancy surveys in 2016–2017. Larvae were detected at 14 of our 20 sites (naïve Ψ = 0.65), with larvae captured across all three surveys at only 5/20 sites (25%), captured in two of three surveys at 6/20 sites (30%), and captured in one of three surveys at 3/20 sites (15%). We observed reduced occupancy (Ψ) across the course of the summer season with 65% of sites occupied in the first survey, followed by 55% and 30% at second and third surveys, respectively. Further, we observed decreasing estimates over the summer for survey-specific detection probabilities (± standard error) modeled as follows: survey 1 (0.919 ± 0.078), survey 2 (0.778 ± 0.113), and survey 3 (0.434 ± 0.132; Fig. 3). The AICc value for the top survey-specific detection probability model, however, was larger than our top five standard occupancy models (with constant *P*), thus survey window appeared to have a weak effect (Table 1).

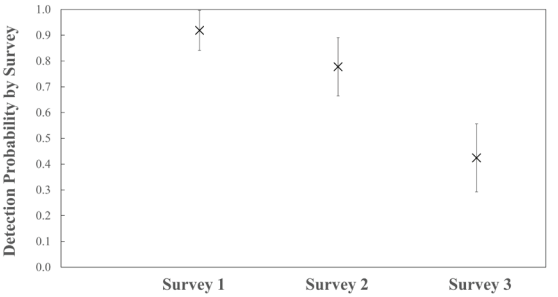

Figure 3. Larval Eastern Hellbender (*Cryptobranchus a. alleganiensis*) detection probabilities averaged across all sites (n = 20) showing reduced detection over the course of consecutive surveys in western North Carolina, USA, 2016–2017.

We were able to develop several biologically relevant and parsimonious models to explain larval occupancy in North Carolina. Our top four models accounted for about 99% of Akaike weight (ω), with the top two models receiving 92.7% of total AICc weight (ω). Results of the parametric bootstrap test indicated a variance inflation factor (VIF) of 1.63 indicating some overdispersion within our data and model. Estimated

Table 1. Top candidate occupancy models with covariates identified in PRESENCE for larval Eastern Hellbender (*Cryptobranchus a. alleganiensis*) in North Carolina, USA, with ΔAICc (difference from top ranked AIC model corrected for samples size), model Ψ (occupancy), *P* (detection probability), ω (model weight), and *K* (number of parameters).

Model	AICc	ΔAICc	ω	*K*
Ψ(% Riffle, % Cobble, % Forest), *P*(.)	54.58	0	0.678	4
Ψ(% Embeddedness, % Riffle, % Cobble, % Forest), *P*(.)	56.25	1.67	0.249	5
Ψ(% Embeddedness, % Riffle, % Cobble), *P*(.)	59.62	5.04	0.055	4
Ψ(% Embeddedness, % Riffle), *P*(.)	62.95	8.37	0.013	3
Ψ(% Cobble, % Forest), *P*(.)	65.12	10.54	0.004	3
Ψ(% Riffle, % Cobble, % Forest), *P*(Survey)	73.04	14.79	< 0.001	4
Ψ(.), *P*(.)	75.36	20.78	< 0.001	2

Table 2. Habitat metrics (mean ± one standard error) for all sites pooled (n = 20) from occupied (n = 14) and unoccupied (n = 6) sites for larval Eastern Hellbender (*Cryptobranchus a. alleganiensis*) in the Upper French Broad River and Hiwassee River sub-basins, western North Carolina, USA. An asterisk (*) denotes significant difference in occupied versus unoccupied sites from Mann-Whitney U test comparisons.

Covariate	(all sites)	(Occupied)	(Unoccupied)
Elevation (m)*	676.7 ± 27.9	706.1 ± 33.9	608 ± 39.2
Riffle (%)*	21.9 ± 3.7	15.8 ± 4.2	36.3 ± 2.3
Run (%)	52.7 ± 7.0	57.3 ± 8.5	42.0 ± 12.7
Pool (%)	12.9 ± 4.1	10.6 ± 3.9	18.3 ± 10.5
Glide (%)	12.5 ± 5.1	16.4 ± 7.1	3.30 ± 2.1
Stream width (m)	12.9 ± 0.8	12.9 ± 1	13.2 ± 1.5
Cross-section area (m^2)	3.58 ± 0.3	3.70 ± 0.3	3.40 ± 0.6
Stream slope (m)	0.003 ± 0.0006	0.002 ± 0.0005	0.005 ± 0.002
Steam velocity (m/s)	1.10 ± 0.1	1.10 ± 0.2	1.00 ± 0.1
Embeddedness (%)*	14.0 ± 1.6	11.1 ± 1.4	20.8 ± 2.7
Sand/Fines (%)	23.1 ± 2.7	20.5 ± 2.9	29.2 ± 5.5
Gravel (%)	14.8 ± 1.5	13.2 ± 1.5	18.4 ± 3.3
Cobble (%)*	47.3 ± 3.2	52.4 ± 3.4	35.2 ± 4.7
Boulder (%)	10.7 ± 2.2	9.70 ± 2.8	13.2 ± 3.1
Bedrock (%)	4.20 ± 1.4	4.20 ± 1.7	4.10 ± 3.0
D50	57.2 ± 5.6	59.0 ± 6.4	52.8 ± 12.2
D84	240.2 ± 39.3	248.6 ± 45.4	220.7 ± 83.5
Canopy cover (%)	23.2 ± 2.8	19.3 ± 1.9	53.4 ± 9.2
# Adult shelters (min 0.17 m^2)	37.8 ± 6.7	40.3 ± 8	32.0 ± 12.8
# Potential nest shelters (min 0.37 m^2)	18.5 ± 4.1	22.0 ± 5.4	10.3 ± 3.7
Water temperature (°C)	13.9 ± 1.1	14.4 ± 0.9	12.8 ± 3.3
Dissolved oxygen (%)	94.6 ± 0.6	94.3 ± 0.7	94.5 ± 0.7
Dissolved oxygen (mg/L)	9.90 ± 0.2	9.70 ± 0.2	10.4 ± 0.6
Conductivity (µS/cm)	14.9 ± 1.9	12.7 ± 0.7	20.1 ± 6.3
Specific conductivity (µS/cm)	18.3 ± 1.9	15.8 ± 0.9	24.0 ± 5.8
TDS (mg/L)	11.9 ± 1.3	10.3 ± 0.6	15.6 ± 3.7
pH	7.10 ± 0.1	7.10 ± 0.1	7.2.0 ± 0.1
Forest land cover within 1 km (%)*	87.8 ± 1.9	90.7 ± 1.5	81.3 ± 4.4
% EPT taxa	78.1 ± 1.4	79.1 ± 1.9	75.9 ± 0.5

occupancy probability (Ψ) was 0.7143 ± 0.0697. Among site covariates in our top models, percentage cobble and percentage forest land cover had a positive effect on occupancy, while percentage embeddedness and percentage riffle were each negatively associated with larval hellbender occupancy.

Total occupancy across sites was significantly and negatively correlated with percentage embeddedness (r = –0.706, P = 0.005; Table 2). Mean percentage substrate embeddedness was significantly higher at unoccupied sites (20.8 ± 2.7%) compared to occupied sites (11.1 ± 1.4%; U = 7, df = 1, P = 0.002; Table 2). Mean percentage cobble across occupied (52.4 ± 3.4%) and unoccupied (35.2 ± 4.7%) sites also differed (U = 10, df = 1, P = 0.005; Table 2). Mean elevation was significantly higher (U = 16, df = 1, P = 0.018) between occupied (706.1 ± 33.9 m) and unoccupied (608 ± 39.2 m) sites (Table 2). Mean percentage riffle habitat was significantly less (U = 12, df = 1, P = 0.007) between occupied sites (15.8 ± 4.2%) and unoccupied sites (36.3 ± 2.3%; Table 2). Lastly, for land cover, mean percentage forest was significantly different between occupied and unoccupied stream reaches, (U = 16, df = 1, P < 0.020), with occupied sites having significantly more forest within 1-km buffer (90.7 ± 1.5%) than unoccupied sites (81.4 ± 4.4%; Table 2). For the five sites where we detected larvae for each survey (3/3 surveys), what we considered our best sites, median values for percentage

riffle, cobble, forest, and embeddedness, were 32.8%, 47.3%, 89.8%, and 6.5%, respectively. At the six sites where we failed to detect larvae in any survey (0/3 surveys), median values for those same parameters were 40%, 38.1%, 86.1%, and 20.1%, respectively.

Riparian zones consisted of combinations of roads, forests, agricultural fields, herbaceous cover, and rural residential land. Percentage dissolved oxygen was high overall, ranging 89.9–100%, or 8.5–12.3 mg/L, with pH ranging 6.6–7.6 across sites. TDS ranged from 7.1–13.3 mg/L with two sites in one stream that never had larval occupancy at a higher range of 24.2–29.7 mg/L. Specific conductivity was low across most sites ranging 11.0–20.7 µS/cm to a high of 37.3–45.8 µS/cm for two sites in one stream that had no occupancy for any survey. Turbidity was low with visibility high or 100% at 120 cm at all sites with the exception of one site, where visibility was 116.7 cm at site 11. The number of adult shelters and number of potential nest shelters across sites ranged from eight to 110, and three to 63, respectively. Percentage cobble ranged from two sites with no occupancy, 17.7–25.7, to a high of 73 for a site that had occupancy for each site visit.

We observed 1,418 other salamanders (brook salamanders) and 3,058 crayfish during this study. The mean number of salamanders and crayfish per site for all three survey events for occupied sites was 97.1 ± 27.0 and 145.4 ± 46.1, respectively, compared to 9.7 ± 3.3 and 170.3 ± 72.5, respectively, at unoccupied sites. We observed up to 227 brook salamanders and up to 217 crayfish while conducting cobble surveys at any individual site. The number of brook salamanders and crayfish observed per 100 m^2 across sites ranged from 0.06 to 40.9 and zero to 23.5, respectively.

Larval habitat selection.— Among larval captures, 85.7% were in runs, followed by 7.1% in riffles, and 3.6% each for pool and glide habitat. Habitat data for 25 individual captures (subtracting larvae for which habitat data were not available, i.e., we were unable to determine from which specific cobble shelter they came or they were escapees) resulted in a mean range of values for occupied rock length of 23.2 ± 2.4 cm, for width of 14.9 ± 1.4 cm, for height of 3.6 ± 0.4 cm), for water depth of 34.2 ± 2.3 cm, for number of larval shelters (cobble) within 3 m of 193.7 ± 22.2, and for the number of adult shelters (boulders) within 3 m of 9.1 ± 1.2. The mean distance to the nearest adult shelter was 0.852 m (± 0.1 m), with mean canopy cover 71.5 % (± 5.1%) and a mean distance to shore 3.74 m (± 0.3 m). Most occupied rocks were < 20% embedded. Mean dominant substrate within 3 m of individual larval capture location was cobble (56%) followed by sand/fines (16.8%), gravel (15%), boulders (10.6%) and bedrock (1.6%). Percentage composition of the substrate sizes were 45 mm (1.6%), 32 mm (2.9%), 22.6 mm (5.1%), 16 mm (8.3%), 11 mm (9%), 8 mm (12.9%), and 5.6 mm (60.2%), with smaller substrate (< 5.6 mm) dominant.

Discussion

Results of our top occupancy models highlight several important drivers of Eastern Hellbender larval detection and occupancy, including percentage substrate embeddedness, percentage cobble, percentage forested land cover within 1-km buffers, and percentage riffle. Our study provides the first comprehensive occupancy model and habitat assessment for hellbender larvae in North Carolina. Our results align with previous studies that found positive associations between cobble habitat and stream salamander density in Appalachian mountain streams (Davic and Orr 1987) as well as larval hellbender preference for cobble with decreased ability to burrow under embedded substrate (Unger et al. 2020b). Increasing substrate embeddedness has been identified as a factor linked to potential population declines in other stream salamanders, including dusky salamanders, *Desmognathus* spp. (Bank et al. 2006), and Spring Salamanders, *Gyrinophilus porphyriticus* (Lowe and Bolger 2002). Other stream species, including sculpin (Cottidae) and Plecoptera macroinvertebrates have a positive association with unembedded cobble substrate (Haro and Brusven 1994), which presumably provides greater interstitial microhabitat. As we observed for our five sites with detections across all three surveys, what we would consider our best larval sites, percentage embeddedness was low (< 10%); the relationship between available cover under non-embedded substrate (cobble) as a refuge from predators should be studied further as it is likely important microhabitat. Stream bottom substrate may provide important larval habitat as a factor affecting occupancy of salamanders in streams (Kroll et al. 2008) and may help maintain recruitment of adult populations (Nickerson et al. 2003). Moreover, habitat for adults, such as large rocks needed for cover (Hillis and Bellis 1971), may be concomitantly important as a factor affecting their presence within local stream populations.

Because most sites consisted primarily of run habitat (52.7%), the negative association of percentage riffle and larval occupancy is likely an artifact of riffles encompassing sub-optimal conditions for larvae. This association could be due to multiple factors, including structural habitat conditions because adult hellbender nests and cobble habitat tend to be structured in runs or relationships between habitat and larval physiology because larvae may exhibit decreased swimming ability in high velocity habitats such as riffles. Alternatively, this correlation could be due to detection probability

differences in riffles versus runs. Riffle habitats are more difficult to snorkel due to higher velocities and often more shallow water depth, making detection unlikely if larvae were present. Runs are more ideal for snorkel surveys, as larvae can easily be seen and captured after lifting cobble substrate. Our findings of increased occupancy for lower percentage riffle habitat is consistent with other studies that found larval Blue Ridge Two-Lined Salamanders (*Eurycea wilderae*) were negatively associated with higher portions of fast-flowing stream habitats (Cecala et al. 2018); however, salamander macroinvertebrate prey biomass may be higher in riffle habitats compared to slower moving stream reaches (Trice et al. 2015). Therefore, these habitats may also provide a source of food for hellbender larvae (EPT taxa), especially if they use riffles when drifting or dispersing within streams.

Surprisingly, several covariates that we expected to be included in our top occupancy models (i.e., pH, specific conductivity, TDS, etc.) were not influential. Elevated levels of conductivity (Hutton et al. 2020) or elevated sediment or TDS (Hawkins et al. 1983; Lowe and Bolger 2002) can negatively affect occupancy and abundance of stream salamanders. We caution interpretation of non-significant covariates within our study (e.g., specific conductivity, TDS, etc.), however, as they have been identified as potential factors in declines across the geographic range (Keitzer et al. 2013; Wineland et al. 2019a). Moreover, conductivity has been associated with the presence of Eastern Hellbenders at the forest catchment scale (Jachowski and Hopkins 2018) and as a predictor of population persistence (Pitt et al. 2017). Also, it is possible that weather events (i.e., high flow between survey visits) may have impacted our point estimates and measurement of specific conductivity or other water quality variables, as has been noted for seasonal variation in streams (Timpano et al. 2018); however, we expect variation due to weather was minimal as flow was similar across surveys. Therefore, we recommend further quantification of water quality variables across sample seasons. The pH levels across stream reaches varied little and were well above acid sensitivities of other similar sized, larval, stream salamanders in the Appalachian Mountains (Green and Peloquin 2009).

Tree canopy cover can be an important parameter in occupancy modeling, reflecting large-scale land use for dusky and brook salamanders (Sweeten and Ford 2016). In some studies, open canopy cover can increase salamander occupancy in wetland complexes if herbaceous vegetation cover is available (Gorman et al. 2009). While percentage canopy cover was not identified as a primary parameter in our top models, canopy cover was higher at unoccupied sites versus occupied sites (53.4% versus 19.3%). This observation was likely a result of overall stream size or variation across stream reaches. Other studies have found less of a relationship between site canopy cover and salamander abundance, with densities more correlated with substrate composition (Hawkins et al. 1983). It is likely riparian canopy cover may impact stream productivity at a local scale with some studies showing an increase in macroinvertebrate biomass with more open canopy (Riley et al. 2009). Alternatively, allochthonous organic matter input varied across our study streams impacting macroinvertebrate presence and feeding groups (Aguiar et al. 2018).

At a larger scale, our study is consistent with other research that found extensive forest cover in the surrounding landscape to be a strong predictor of salamander presence (Southerland et al. 2004; Quinn et al. 2013; Jachowski and Hopkins 2018), particularly larvae (Crawford and Semlitsch 2008; Cecala et al. 2018). Studies on salamander occupancy for stream associated species, dusky and brook salamanders, found abundance and occupancy high with increasing riparian or streamside management zones and forest buffer along streams (Sweeten and Ford 2016; Guzy et al. 2019). As our five sites with detections across all three surveys (i.e., sites with 100% occupancy) indicated about 89.8% forest cover at a scale of 1- km, there is clearly a relationship between high forest cover, larval occupancy, and abundant, available in-stream habitat conditions (i.e., embeddedness). Moreover, multiple species occupancy modeling of declining larval amphibians has identified forest cover linked to greater salamander reductions in occupancy compared to anurans (Haggerty et al. 2019). In Virginia, USA, researchers identified lower catchment-wide riparian forest reduced population recruitment of younger age classes of hellbenders (Jachowski and Hopkins 2018). In the Watauga drainage of North Carolina, researchers found a relationship between forest cover at the catchment scale and hellbender captures (Pugh et al. 2015). Furthermore, the absence of forest land cover has been previously associated with lower occupancy in the stream salamanders dusky, brook, and mud (*Pseudotriton* spp.) salamanders (Grant et al. 2009). When combined, these studies further illustrate the importance of forest cover to benefit potentially both aquatic and terrestrial salamanders within national forests.

We noted detection probability estimates from our models decreased each consecutive survey window (1, 2, and 3) suggesting fewer larvae might be present as summer progressed. There is a chance that our repeated surveys, and thus repeated lifting of cobble substrate, disturbed larvae causing them to disperse from our sites, but we suspect any effect from our survey methods was negligible because we still found larvae at most sites.

Moreover, it is unknown to what extent individual larvae may have dispersed out of sites between site visits or potentially dispersed between sites. Potential dispersal may have violated assumption of independence based on minimal site distance of 200 m. Future studies could mark larvae with visible implant elastomer (VIE) to investigate this possibility further. Occasional, but short-lived, high flow events from summer storms could have added a potential source of error (Hecht-Kardasz et al. 2012); however, we noted minimal flooding between site visits, so this risk was minimal in our study. The reduction in detection probability over the summer may indicate that either larvae moved out of our survey reaches, avoided sampled habitat (cobble), or more likely, suffered increased mortality.

Elevation of streams occupied versus unoccupied differed in our study, and elevation has been previously shown to predict salamander occurrence in southern Appalachian streams (Bailey et al. 2004; Cecala et al. 2018). Therefore, we encourage researchers to consider elevation in surveys for hellbenders. In addition, monitoring of stream elevation and its potential impact of temporal stream temperature may impact hellbender distributions in the future in North Carolina, as projections for global climate change are predicted to threaten salamander diversity in the southern Appalachian Mountains (Milanovich et al. 2010). Stream flow variability may affect survival of larval stream salamanders (Lowe et al. 2019), and managers should consider incorporating site surveys across multiple years to account for potential changes in hydrologic regime. While researchers have found increased temperature variability may elicit increased immune system activity in hellbenders (Terrell et al. 2013), it is unknown to what extent temperature and stream flow variability will impact hellbender populations across the range of the species.

Further research should investigate the relationship between surrounding land use and the threat of increased sediment (i.e., the potential for less forest cover to increase sediment in streams) for occupancy of hellbender larvae. Suspended sediment and stream substrate embeddedness (Barr and Babbitt 2002; Sweeten and Ford 2016) can negatively influence occupancy of larval, aquatic salamanders such as brook salamanders. Similarly, larval Eastern Hellbenders should be monitored using our methods and in-stream microhabitat metrics (e.g., rock shelter embeddedness) assessed for management. Historical hellbender populations in West Virginia, USA, were found to be locally extirpated, potentially linked to increased sediment rates from roads, urban development, or altered water chemistry (Wineland et al. 2019a). Consequently, conservation managers should consider continued monitoring of larvae as a proxy for population recruitment and stability on both public and private lands. Managing long-lived species, such as the Eastern Hellbender, at both the landscape and range-wide scale should also include managing vital habitat factors within streams which may impact larvae. Surveys that focus on sampling across age classes from larval to adult stages may add to our body of knowledge of long-term species trends in abundance and recruitment. Future research could incorporate within-stream population size estimates in occupancy modeling approaches to inform conservation management of this enigmatic giant salamander of North America.

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Appendices

Appendix 1. Percentage composition of National Landcover Data (NLCD) cover classes within a 1-km stream buffer at sites surveyed (n = 20) for larval Eastern Hellbenders (*Cryptobranchus a. alleganiensis*) across 10 streams in the Hiwassee and Upper French Broad River sub-basins, western North Carolina, USA.

Site	Forest	Agricultural	Developed	Barren	Open Water	Wetland
Sites 1, 2: 1 km buffer	94.57%	0.15%	5.00%	0.29%	0.00%	0.00%
Sites 3, 4: 1 km buffer	86.52%	1.35%	11.72%	0.00%	0.00%	0.41%
Sites 5, 6: 1 km buffer	89.80%	3.91%	6.15%	0.03%	0.00%	0.11%
Sites 7, 8: 1 km buffer	93.29%	4.41%	1.76%	0.01%	0.02%	0.52%
Sites 9, 10: 1 km buffer	90.68%	2.94%	6.27%	0.00%	0.00%	0.12%
Sites 11, 12: 1 km buffer	91.58%	3.83%	4.39%	0.00%	0.09%	0.12%
Sites 13, 14: 1 km buffer	87.30%	8.61%	3.87%	0.00%	0.00%	0.21%
Sites 15, 16: 1 km buffer	67.44%	20.55%	11.68%	0.00%	0.00%	0.33%
Sites 17, 18: 1 km buffer	79.92%	13.30%	6.24%	0.00%	0.03%	0.51%
Sites 19, 20: 1 km buffer	97.37%	0.98%	1.65%	0.00%	0.00%	0.00%

Appendix 2. Description of stream habitat data collected across Eastern Hellbender (*Cryptobranchus a. alleganiensis*) larval surveys in western North Carolina, USA, 2016–2017; sites (n=20) were 50-m long stream reaches, and substrate quadrats were 1 m^2.

Covariate	Description
Elevation (m)	Elevation of site in meters
% Riffle	Percentage of site characterized as riffle habitat
% Run	Percentage of site characterized as run habitat
% Pool	Percentage of site characterized as pool habitat
% Glide	Percentage of site characterized as glide habitat
Stream width (m)	Wetted width of site in meters
Cross-section area (m^2)	Bankfull, cross-sectional area in meters squared
Slope (m)	Site slope in meters
Velocity (m/s)	Water velocity measured in meters per second
% Embeddedness	Percentage embeddedness of substrate within quadrats
% Sand/Fines	Percentage of sand or fine within quadrats
% Gravel	Percentage of gravel within quadrats
% Cobble	Percentage of cobble within quadrats
% Boulder	Percentage of boulder within quadrats
% Bedrock	Percentage of bedrock within quadrats
D50 (mm)	Median particle size of distribution (mm) from Wolman pebble counts
D84 (mm)	84^{th} percentile of particle size distribution (mm) from Wolman pebble counts
% Canopy cover	Percentage of left and right stream bank tree canopy cover measured by a spherical crown densiometer
# Adult shelters (minimum 0.17 m^2)	Number of adult Hellbender rock shelters (boulders; minimum of 0.41 x 0.41 m, 0.17 m^2)
# Potential nest shelters (minimum 0.37 m^2)	Number of potential Hellbender nest shelters (boulders; minimum 0.61 x 0.61 m, 0.37 m^2)
Temp (°C)	Water temperature measured in °C
DO (%)	Dissolved oxygen measured in percent
DO (mg/L)	Dissolved oxygen measured in mg/L
Conductivity (µS/cm)	Conductivity measured in microSiemens per centimeter
Specific Conductivity (µS/cm)	Specific conductivity measured in microSiemens per centimeter
TDS (mg/L)	Total Dissolved Solids in milligrams per liter
pH	Potential of Hydrogen measured with a water quality meter
% Forest land cover	Percentage of land cover classified as forest within a 1-km buffer around streams using the National Land Cover Dataset
% EPT taxa	The percentage of insect Order Ephemeroptera, Plecoptera, and Trichoptera relative to the total amount of aquatic insects identified