Effects of Land Use on Hellbenders (*Cryptobranchus alleganiensis*) at Multiple Levels and Efficacy of Artificial Shelters as a Monitoring Tool

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ABSTRACT

Understanding how species respond to anthropogenic changes and why species respond in the way that they do can help focus conservation planning. Hellbenders (*Cryptobranchus alleganiensis*) are a freshwater species of increasing conservation concern that are suspected to have declined due to loss of forest cover. However, quantitative evidence of land use effects on hellbenders is lacking. I used a multilevel approach to understanding whether hellbenders respond to land use by examining physiological indices, demographics and patterns of species occurrence as endpoints. My study area included two major river drainages in Virginia which mark a largely understudied portion of the species' range. In Chapter I, I described hellbender distribution and tested the hypothesis that hellbenders would be more likely to occur in heavily forested landscapes. Surprisingly, hellbenders occupied a relatively wide land use gradient (range = 50-90% forest in an upstream catchment) and current land use was an unreliable predictor of occurrence. In Chapter II I examined hellbender abundance and demographics at a subset of study sites stratified across a land use gradient. Abundance of sub-adult/adult hellbenders increased as forest cover increased in collective upstream riparian areas, primarily as the result of increased recruitment of new adults to local populations (rather than increased apparent survival of adults). Populations in lesser forested areas were declining and composed largely of relatively old adults, indicating that land use can lead to changes in hellbender distribution given sufficient time. In Chapter III, I examined three indices of physiological condition (body condition, hematocrit and leukocyte profiles) in hellbenders captured across a land use gradient. I found evidence suggesting low reproductive success may explain reduced recruitment in areas of low forest cover and evidence suggesting hellbender endocrinology during the breeding season may vary with land use. In Chapter IV I examine efficacy of artificial shelters as a monitoring tool and demonstrate their potential as tool to further our understanding of mechanisms underlying demographic responses of hellbenders to land use. I synthesize my findings in Chapter V and conclude that loss of forest cover in riparian areas poses a formidable threat to hellbender population viability in Virginia.

DEDICATION

To my father, Robert Bodinof, who taught me that before you solve a problem you must study it.

To my mother, Janice Bodinof, who ensured I had the opportunity to fall in love with nature.

And finally, to all of my mentors, who encouraged and inspired me.

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CHAPTER I. Current Land Use is a Poor Predictor of Hellbender Occurrence: Why Assumptions Matter When Predicting Distributions of Data Deficient Species

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ABSTRACT

Understanding species distributions is fundamental to effective conservation planning. Data deficiency is common among rare and imperiled species, and poses challenges for conservation planning because status assessments become reliant on scant data that can introduce bias. We used occupancy modeling to evaluate support for commonly accepted, but previously untested, hypotheses regarding factors that drive the occurrence of an imperiled and data-deficient amphibian, the eastern hellbender (*Cryptobranchus alleganiensis)*. We investigated the potential for mismatch between areas likely to be identified as having high conservation priority based on the common assumption that hellbender occurrence corresponds to areas of high forest cover and those identified by well-informed models. We conducted triplicate surveys to detect *C. alleganiensis* in 49 stream reaches in Southwest Virginia, USA, stratified across a land use gradient and two major drainages. We used a Bayesian multi-model framework to investigate factors associated with *C. alleganiensis* occupancy. We used the best performing models to predict probability of occupancy at the scale of a 50 m stream reach throughout our study area and identify areas most likely to be occupied. In contrast with common assumptions, *C. alleganiensis* occurrence was explained primarily by differences in underlying geology and topography (i.e., physiography) and negative effects of agriculture were only modestly supported. Best performing models suggested ~35% of our study area was occupied. Our findings suggest that predictions from models informed by presence-only data and current land use would likely underestimate *C. alleganiensis* occupancy by as much as one-third and incorrectly classify over half the currently occupied area to be of little importance to the species. Our study highlights the potential danger of assuming that the distribution of data deficient species can be approximated using untested, but commonly accepted, species-habitat associations.

INTRODUCTION

Reliable knowledge regarding species distributions is fundamental to effective conservation planning. Biodiversity is declining at an unprecedented rate and at a global scale (Hoffman *et al.*, 2010; IUCN, 2014). Freshwater ecosystems, in particular, are biodiversity hotspots and face losses and threats exceeding those in many terrestrial systems (Sala *et al.*, 2000; Revenga *et al.*, 2005; Strayer & Dudgeon, 2010). A major challenge to conserving freshwater biodiversity is the dearth of knowledge regarding distributions of individual species, which directly limits our ability to reliably identify areas of high conservation priority (e.g., that contain high levels of biodiversity or endemic and imperiled species). The lack of distributional information for species can often be attributed to financial and logistical constraints associated with monitoring rare species that are difficult to detect (Thompson, 2004). Anecdotal accounts, presence-only (as opposed to presence-absence) data, data collected from surrogate species and expert opinion often represent the best available information available to estimate species distributions (e.g., Abell, 2002; Linke *et al.*, 2011) or assess risk (Mace *et al.*, 2007). The class of models (hereafter presence-only models) capable of predicting species distributions from sparse data and readily available spatial environmental data have grown in popularity in recent years, but have been criticized for their potential to both underestimate and overestimate distributions (Hermoso *et al.*, 2015). Underestimates of distribution can result from geographically biased and insufficient survey efforts that often plague sparse datasets (Yackulic *et al.*, 2013). Overestimates of distributions might result due to biases in opinion about environmental variables that are important for species (Donlan *et al.*, 2010) or due to failure to recognize the importance of hierarchical filters (e.g., physiological tolerance, dispersal ability, biotic interactions; Angermeier *et al.*, 2002) that drive biologically relevant discontinuity in species distributions (Hermoso *et al.*, 2015). This is important because species distribution models may be used to inform conservation planning either by identifying critical habitat or assessing species' status, and unreliable predictions can lead to under- or overestimation of a species' extant range and extinction risk (Linke *et al.*, 2011). Therefore, while it may be challenging to test assumptions regarding the occurrence and habitat requirements of imperiled species, doing so has the potential to improve accuracy of status assessments and the effectiveness of conservation strategies (Hermoso *et al.*, 2015).

We used an imperiled and data deficient amphibian, the eastern hellbender (*Cryptobranchus* alleganiensis, Daudin), to investigate the potential for well-accepted, but untested, species habitat relationships to introduce bias into predicted distributions for data deficient species. Hellbenders are stream dwelling salamanders that make an excellent model for our study, as they are cryptic and difficult to monitor, and their current distribution is poorly understood. Among experts, heavily forested landscapes are considered to be an indicator of suitable hellbender habitat, while sedimentation and water quality alteration associated with intensive land use are commonly suggested as a leading driver of recent precipitous declines (Williams *et al.*, 1981; Wheeler *et al.*, 2003; Briggler *et al.*, 2007; Foster *et al.*, 2009). However, quantitative evidence for land use effects on hellbenders is generally lacking (but see Quinn *et al.,* 2013). Our first objective was to investigate factors associated with hellbender occurrence across two major river drainages marking the eastern extent and an understudied portion of the species range and explicitly evaluate support for the hypothesis that occurrence at the scale of a 50 m stream reach is negatively influenced by fine sediment within streams and surrounding human land use. Our second objective was to develop a predictive model of hellbender occurrence to investigate how model based predictions of occurrence might differ from predictions based on well accepted, but untested, species habitat relationships.

METHODS

Study species

Hellbenders are large (up to 74 cm total length), fully aquatic, long lived (25+ years; Taber *et al.*, 1975) benthic stream specialists native to much of the eastern U.S. (Fig. 1.1). All life stages are highly cryptic, and typically remain hidden beneath rocky cover (Nickerson *et al.*, 2003; Bodinof *et al.*, 2012a-b).   Given their fully aquatic life history, longevity, highly permeable skin and reliance on cool and well oxygenated water (Guimond & Hutchison, 1973), hellbenders are often considered an indicator of long-term in-stream habitat quality. Hellbender populations have declined precipitously across the species historic range (Wheeler *et al.*, 2003;

Foster *et al.*, 2009; Burgmeier *et al.*, 2011; Graham *et al.*, 2011; USFWS, 2011). Hellbenders are typically associated with cool, swift flowing streams surrounded by forested landscapes and substrate and water quality alterations associated with deforestation are considered to be primary drivers of declines (Smith, 1907; Nickerson & Mays, 1973; Willliams *et al.*,1981; Wheeler *et al.*, 2003; Briggler *et al.*, 2007). A major challenge for conservation planning is a poor understanding of hellbender status, namely the dearth of information regarding the species distribution (Briggler *et al.*, 2007). However, studies designed to minimize bias in sampling locations, account for imperfect detection, and explicitly test hypotheses of land use effects on the species are lacking.

Study area

Our study took place within the New River and South Fork Holston River drainages in southwestern Virginia (Fig. 1.1). Together, these drainages mark the eastern extent of the hellbender's range in Virginia and a largely unstudied portion of the species' total range (but see Hopkins & DuRant, 2011; Hopkins *et al.*, 2011; 2014; 2016; DuRant *et al.*, 2015). Both drainages overlap portions of the Blue Ridge and Ridge and Valley physiographic provinces of the greater Appalachian Highlands (Fenneman, 1928; Fig. 1.1). Physiographic provinces are delineated by specific differences in geology and topography and are important determinants of fish communities in Virginia (Angermeier & Winston, 1999). Anecdotal reports indicate hellbenders historically occupied both drainages and both provinces (Mayasich *et al.*, 2003). The Ridge and Valley is characterized by a series of steep ridges paralleled by narrow valleys, bordered on the west by the Appalachian Plateau and on the east by the Blue Ridge. Maximum elevations reach 1,400 m and geology consists primarily of sedimentary rock, with uplands composed largely of sandstones and shales and lowlands composed heavily of limestone. The

Blue Ridge consists of relatively steep topography bordered by the Valley and Ridge to the west and by the Piedmont to the east. In southwest Virginia the Blue Ridge widens and forms a broad plateau-like upland hosting the highest elevation (1,749 m) in the state and a divide for waters draining to the Atlantic and Gulf of Mexico. In contrast to the Ridge and Valley, geology in the Blue Ridge is relatively impervious, composed primarily of metamorphic and igneous rock, including granites, schist and gneiss, with limestones and shales more common on western slopes. Relative to the Valley and Ridge, Blue Ridge slopes are steep and soils are thin (Nelms *et al.*, 1997).

Sampling design

To investigate effects of land use on hellbender occurrence, we used a spatially balanced random stratified design (Stevens & Olsen, 2004) to select a sample of 49 stream reaches for our study. We considered a sampling pool of all fourth order or larger stream segments delineated within the National Hydrography Dataset (NHD) Plus version 2.1 (1:100000 scale; USGS & USEPA, 2012). To ensure our sample represented the full range of land use in our study area we quantified the percent of forest in upstream catchments delineated for each of 661 segments in our sampling pool, and stratified our sample across bins representing a human activity gradient (Yates & Bailey, 2010) ranging from 50-89% forest in both drainages. While the range of land use in our study area was somewhat narrow, it largely overlaps the range of forested land use among stream reaches in Missouri where hellbenders have declined by an average of 77% (Wheeler *et al.*, 2003; % forest = 46-64% [Big Piney R.]; 28-57% [Gasconade R.]; 70-71% [Niangua R.]; 63-64% [North Fork White R.]; 70-79% [Eleven Point R.]). We used a Generalized Random Tesselation Stratified (GRTS) approach to reduce spatial autocorrelation among our sample reaches while still employing a true probability design, using the package ‘spsurvey’ (Kincaid & Olsen, 2013) in Program R (Team, 2013). Our final sample of 49 segments represented 16 streams (n = 1-6 segments per stream). For the purposes of our study, we investigated occupancy at the scale of a 50 m stream reach (widths were variable; Table 1.1). We selected the first 50 m reach that contained at least two boulders (Wentworth, 1922) or at least one irregular bedrock ledge during our first visit to each of the 49 pre-selected stream segments. Hellbenders are highly dependent on rocky cover and are rarely encountered far from shelter (Bodinof *et al.*, 2012b). Thus, our pre-requisite for the occurrence of rocky substrate in each reach was intended to avoid sampling habitats that were unavailable to hellbenders, while allowing for high variability in habitat quality among reaches.

We surveyed each reach on three occasions using snorkeling while turning rocks (Nickerson & Krysko, 2003) in 2013 (n=37 sites) or 2014 (n = 12 sites). On each occasion we ceased surveys once a hellbender was detected, to limit habitat disturbance. If no hellbender was detected we ceased efforts once the entire reach had been searched. For detailed survey methodology see Appendix A.

Predictors

We quantified all in-stream predictors (Table 1.1) from data collected during our initial visit to each sampling reach and prior to conducting hellbender surveys. To calculate reach size (Table 1.1) we used a measure of wetted width collected at a point visually estimated to represent average width of the sample reach, using a Nikon Prostaff© laser rangefinder. We quantified meso-habitat (pool, riffle, run) and substrate characteristics from data collected in a modified Wolman (1954) pebble count with 100 observations in each reach.

We quantified landscape scale predictors (Table 1.1) from remotely sensed data in ArcMap v.10.1 (ESRI; Redlands, CA). First, we delineated catchments for each sampling reach and used catchments to quantify contributing area (km^2) as a proxy of stream size as per Wenger *et al.* (2008). We quantified the proportion of each catchment that fell within the Blue Ridge and Ridge and Valley physiographic province, and quantified land use at three spatial scales (Table 1.1). We quantified land use at the catchment, catchment-riparian and immediate-riparian spatial scales (defined in Table 1.1). We used non-forested land use categories (hereafter agriculture and developed), as predictors in our analysis. We pooled crop and grassland categories from the national land cover dataset (NLCD; USGS, 2014) to represent agricultural land use, and pooled medium (50-79% impervious surface, typically single family residence) and high intensity development (80-100% impervious surfaces, typically residential, commercial and industrial) NLCD categories to represent developed land use (Table 1.1).

Model development

We used single season occupancy models to investigate factors associated with hellbender occurrence and detection while snorkeling. Briefly, occupancy models are hierarchical state-space models that are used to estimate species occurrence while accounting for imperfect detection. Occupancy is defined as the probability that the target species occurs within some area of interest (MacKenzie *et al.*, 2006). Failure to account for imperfect detection (i.e., probability of detecting a species during a single survey is < 1) can lead to negatively biased estimates of occupancy (MacKenzie *et al.*, 2006). Sampling approaches for occupancy modeling are varied (MacKenzie & Royle, 2005; Bailey *et al.*, 2007), but generally entail repeat surveys of sampling units; where the target species is either detected or not during each survey. Models allow for inclusion of predictor covariates and thus the potential to examine support for hypotheses concerning the effect of extrinsic factors on species occurrence or detection (MacKenzie *et al.*, 2002).

We used a multi-model framework, where we investigated relative support for multiple hypotheses concerning factors associated with hellbender occurrence. We considered fixed effects of 15 continuous variables in our analyses (Table 1.1). We hypothesized positive effects of visibility and either a positive (i.e., as a function of area surveyed) or negative (i.e., due to deep (> 2 m) water) influence of contributing area on detection. We also considered an interaction between contributing area and visibility, where we predicted increased importance of visibility in large rivers, where deep water was more common. We hypothesized negative effects of agriculture and developed land use on detection as a result of decreased local abundance due to altered water quality and increased sedimentation.

We hypothesized effects of physiography on hellbender occurrence, given that physiography corresponds to distinct differences in underlying geology and topography that has been associated with distributions of freshwater fish in the same region (Angermeier & Winston, 1999). We hypothesized positive effects of elevation, canopy cover, boulder substrate, and of riffle over pool, and negative effect of fine substrates and non-forest land use at both the catchment and immediate riparian scales based on habitat associations reported for hellbenders (Nickerson & Mays, 1973; Humphries & Pauley, 2005; Quinn *et al.*, 2013). We hypothesized a positive effect of contributing area, given that sample reach extent (m^2) increased with contributing area as a function of increasing wetted width. Finally, we considered an interaction between physiography and land use, to represent our hypothesis that susceptibility of reaches to land use effects would vary depending on underlying geology and topography associated with physiographic province.

In addition to fixed effects we considered random effects of the river where reaches occurred in both detection and occupancy models. While we designed our study to maximize independence between sample reaches, we recognized that stream networks are innately hierarchical in structure. As a result, we considered it likely that reaches within a stream may be more similar to each other than to reaches in other streams. Failure to account for such dependencies can lead to biased estimates in the response of interest and an increase in type I errors (Latimer *et al.*, 2006; Dormann *et al.*, 2007). Therefore, we wanted to investigate the possibility that nesting of reaches within streams may have introduced a problematic level of spatial autocorrelation into our data. On the contrary, we wanted to avoid over-fitting models if inclusion of random effects was not warranted, since doing so would be likely to reduce our power to detect effects from covariates of interest. To address our concerns, we fit candidate models both with and without a random effect term for river, in both steps of our analysis. We used model rank along with examination of fitted slope coefficients to determine whether river was an important source of variation in our study.

To aid model convergence and interpretation of fixed effects, we standardized $\left(\frac{x-\bar{x}}{sd(x)}\right)$ all continuous covariates other than proportions and multiplied developed land use proportions (due to very small values) by 10 prior to analysis. We screened predictor variables based on Pearson's correlation coefficients to avoid problems associated with coefficient estimation when predictors were collinear. We selectively discarded one of each pair of correlated predictors ($r \geq 0.7$ or ≤ -0.7). Catchment and catchment-riparian land use were strongly correlated (agriculture: $r = 0.87$, $p < 0.001$; developed: $r=0.84$, $p < 0.001$, $df = 47$), as were contributing area and size of the sample reach ($r = 0.86$, $p < 0.001$, $df = 47$). We retained catchment land use over catchment riparian land use, and contributing area over size of the sample reach based on fit statistics from models that included only one of the pairs at a time.

Given our small sample size relative to the number of covariates considered, we wanted to avoid spurious results due to fitting an overly large set of candidate models (Anderson & Burnham, 2002). To maximize parsimony we used a two-step approach (MacKenzie *et al.*, 2006), where we examined support for covariates hypothesized to influence detection while holding occupancy constant in step one. In step two, we modelled detection as a function of all covariates that occurred in models that out-ranked our null model in step one, while examining support for covariates hypothesized to influence occupancy.

Model fitting and selection

We used a Bayesian approach to fit and rank candidate models. Unlike maximum likelihood, Markov Chain Monte Carlo (MCMC) approaches to model fitting can accommodate random effects within hierarchical models (Royle & Dorazio, 2008). We fit all models in OpenBUGS v3.2.3 (originally written as WinBUGS [Lunn *et al.*, 2000]) using the package 'R2OpenBUGS' (originally written as R2WinBUGS [Sturtz *et al.*, 2005]) in program R (Team, 2013). In our model specification we employed non-informative priors to the extent possible. We specified uniform distributions between -10 and 10 on the logit scale for intercepts. When we specified uniform distributions (e.g., between -10 and 10) for coefficients, posterior distributions for some coefficients appeared 'cut-off' at either the upper or lower end. As a result we specified diffuse independent normal prior distributions for coefficients that were centered at zero with precision of $1x10^{-2}$, after determining that mean estimates and precision were generally insensitive to our choice of prior. We specified priors for the standard deviation of random effects as a uniform distribution between zero and 15. We obtained posterior distributions from every 25th iteration from three independent Markov chains, where each chain contained 20,000 iterations and the initial 2,000 iterations were discarded as burn-in. We visually inspected MCMC chains to ensure proper mixing and used the Brooks-Gelman-Rubin diagnostic as a criterion to ensure convergence, where we assumed convergence was reached when R-hat of all parameters reached 1.0 (Gelman & Rubin, 1992). We ranked models using the Wantanabe-Akaike information criterion (WAIC; Wantanabe, 2010) where we calculated WAIC as defined by Vehtari & Gelman (2014). Similar to other model ranking criteria, WAIC represents a measure of model fit corrected by a penalty for model complexity. Unlike other criteria (e.g., Deviance Information Criteria (DIC); Spiegelhalter, 2002) WAIC assesses fit based on full posterior predictive distributions rather than point estimates, and is thus recognized as the only fully Bayesian model ranking criteria (Hooten & Hobbs, 2015). Furthermore, unlike DIC, WAIC is considered valid in hierarchical and mixture models because of the manner in which the penalty term (i.e., number of effective parameters) is estimated for each model (see Vehtari & Gelman, 2014; Hooten & Hobbs, 2015). Currently, methods for handling model selection uncertainty in Bayesian analyses remains unclear, but model averaging approaches are not considered appropriate (Hooten & Hobbs, 2015). We considered models that outranked a null model (hereafter, top-ranked models) as being well enough supported to warrant further consideration. Prior to drawing inference, we subjected each top-ranked model to validation procedures to ensure each was useful.

Model validation

We used k-fold cross validation (Boyce *et al.*, 2002) and area-under-the receiver operating curve (ROC; Metz, 1978) to evaluate model performance. First, we randomly partitioned our original data five times according to Huberty's (1994) rule of thumb; each time excluding 33% of the data for testing. We refit models with each draw of training data and used newly fitted models to predict the parameter of interest (detection or occupancy) for each case in their complementary test data. For validation, we assumed that apparent occupancy after three surveys represented the true state of a reach. We pooled test results for each model to estimate area under the ROC curve using the package ROCR (Sing *et al.*, 2013) in Program R (Team, 2013). Briefly, the ROC curve is a plot of sensitivity (probability of correctly classifying a true positive) against specificity (probability of correctly classifying a true negative) across a range of cutoffs between zero and one (Metz, 1978). The area under the curve (AUC) is cutoff independent and defined as the probability that a model will score a randomly drawn positive sample higher than a randomly drawn negative sample. Values of AUC equal to 0.5 indicate a complete lack of predictive power (i.e., random), with values of 1.0 indicating perfect predictive performance (Cumming, 2000). We report posterior mean coefficients and model derived estimates of detection and occupancy along with their 95% credible intervals for our top-performing models based on AUC scores.

RESULTS

We detected hellbenders in both major drainages (New = 7 of 36 reaches; South Fork Holston = 6 of 13 reaches), in eight of 16 rivers, and in 26% of all reaches surveyed. We detected hellbenders during all three surveys in six reaches, during only two surveys in two reaches and during only one survey in five reaches. Most (26 of 27) individuals were sexually mature adults and there was no association between total length (proxy of age) and land use. However, our sample sizes were small ($n = \leq 3$ hellbenders per reach).

Detection

We pooled terms from three detection models that outranked the null model in step-one (Table 1.2) to define the detection model in step-two. Posterior distributions for fixed effect detection coefficients and AUC estimates for all models in step-two were similar and indicated excellent performance of our detection model (AUC = 0.97; Table 1.2). Predictions from the model supported our hypothesis that the threshold of visibility necessary to detect hellbenders depended largely on size of the river being surveyed (Table 1.2). Our model predicted that under average visibility (1.75 m) detection declined exponentially as river size increased (Fig. 1.2A). Once visibility exceeded about 3 m detection was predicted to remain extremely high even in large rivers; though uncertainty regarding the effects of visibility was considerable (e.g., Table 1.3; Fig. 1.2B). Notably, the river size effect appeared to be driven by a single river (main stem New R.) that contained all of the surveyed reaches with contributing areas > 800 km^2. In contrast to our predictions, the model suggested detection was positively associated with the proportion of the immediate riparian area classified as agriculture, though uncertainty regarding effects of land use was also considerable (Table 1.3; Fig. 1.2c). Though the final model included developed land in the immediate riparian area as a covariate of detection, the predicted effect of developed land was negligible (Table 1.3).

Occupancy

Six models met our criteria for further consideration based on model ranking. These included a physiography only model, a physiography X catchment land use model, and the duplicates of both models that included the random effect term for river; all of which performed similarly well (AUC = 0.83-0.86; Table 1.2). Additionally, two models that included local variables with and without the random effect term for river outranked a null model, but performed relatively poorly (AUC = 0.0.76-0.79; Table 1.2). Posterior distributions for fixed effect coefficients in the top-ranked models were virtually identical regardless of whether the random effect term was included (Table 1.3), suggesting negligible autocorrelation in the occupancy state of each reach as a result of site being nested within the same river. For simplicity, we focus our interpretation of results on the two best-performing models while ignoring random effects.

Collectively, results supported the hypotheses that hellbender occurrence was driven primarily by physiography, and demonstrated modest support for negative effects of agricultural and developed land use when quantified at the catchment scale (Table 1.2). The most parsimonious model, which included only physiographic province, predicted that reaches with catchments that fell entirely within the Blue Ridge ($\widehat{\Psi}$ = 0.67, 0.41-0.88) were about 16 times more likely to be occupied that those with catchments that fell entirely within the Ridge and Valley province ($\widehat{\Psi}$ = 0.04, 0.00-0.18; Fig. 1.3). Extrapolation of the physiography model across our entire study area predicted that only 36% of our study area was occupied by our target species (Table 1.4).

The equally well performing but less parsimonious model included an interaction between physiography and catchment land use. However, credible intervals for coefficients associated with land use variables overlapped zero (Table 1.3), suggesting a great deal of uncertainty regarding effects of agriculture and development on hellbender occurrence. This model predicted that, holding developed land use constant at the mean observed (0.2%), reaches with catchments entirely in the Blue Ridge were twice as likely to be occupied when 10% of land use was agricultural ($\widehat{\Psi}$ = 0.93, 0.46-1.00) compared to when 40% of land use was agricultural ($\widehat{\Psi}$ = 0.47, 0.13-0.84). Reaches with catchments entirely in the Ridge and Valley were predicted to have extremely low occupancy regardless of land use but were twice as likely to be occupied when agriculture composed just 10% of land use throughout the catchment ($\widehat{\Psi}$ = 0.05, 0.00-0.39) compared to 40% ($\widehat{\Psi}$ = 0.02, 0.00-0.17; Fig. 1.4a). The physiography X land use model also suggested a negative effect of developed land use on hellbender occupancy, but only in the Blue Ridge; and uncertainty regarding effects of development was considerable (Fig. 1.4b). Extrapolation of the physiography X land use model across our entire study area predicted that only 34% of our study area was occupied by our target species (Table 1.4).

Extrapolation of model predictions across our study area suggested that failing to account for physiography and assuming that high forest cover within a stream catchment functions as a reliable indicator of occurrence would yield numerically and spatially biased estimates of hellbender distribution. For example, our results indicated that over half (53-60%) of the habitat predicted to be occupied by hellbenders consisted of stream segments with catchments currently characterized by only moderate (≤ 65%) levels of catchment forest cover (Fig. 1.5). Furthermore, our model indicated that the majority of stream habitat characterized by heavily (≥ 70%) forested catchments was of little importance to our target species, given that over half (148 of 223 stream km) of the most heavily forested stream reaches fell within the Ridge and Valley physiographic province where occupancy was extremely low (≤ 0.05). Assuming that stream reaches with heavily (≥ 70%) forested catchments function as a proxy of hellbender distribution in our study area would yield erroneous conclusions for our target species in two ways. First, the proportion of area occupied would be underestimated by about one-third (i.e., 25% vs 34-36%). Secondly, over half of the area occupied by our target species (i.e., moderately forested stream reaches) would be misclassified as being of little importance to the species, while a narrow region (i.e., heavily forested Ridge and Valley reaches = 16% of study area) with extremely low occupancy would be misclassified as being of great importance (Fig. 1.5).

DISCUSSION

We found that the distribution of an imperiled and data deficient species was better predicted by broad scale patterns in underlying geology and topography, defined by physiographic province, than variables based on well accepted species habitat associations. Hellbenders were exceedingly rare in the Ridge and Valley physiographic province relative to the Blue Ridge, even among stream reaches with heavily (≥ 70%) forested catchments. The physiography effect we report is not surprising, as physiography has been shown to be an important determinant of both stream macro-invertebrate and fish distributions in several regions including Virginia (Angermeier & Winston, 1999; Feminella, 2000; Van Sickle *et al.*, 2004; Utz *et al.*, 2009, 2010; Pool *et al.*, 2010). However, the mechanisms by which physiography influences hellbender distribution remain unclear. Physiography is an important determinant of hydrological patterns and physicochemical properties in streams, and can directly influence habitat suitability for aquatic species. Ridge and Valley streams are characterized by lower base flows (Nelms *et al.*, 1997), higher levels of dissolved solids, harder water and slightly basic pH relative to Blue Ridge streams, due largely to innate differences in reactivity between water and underlying bedrock (Puckett and Bricker, 1992; Swain *et al.*, 2004). Therefore, although hellbenders were historically reported from many Ridge and Valley streams in our study area (Mayasich *et al.*, 2003), abundance may have always been low in the Ridge and Valley relative to the Blue Ridge due to inherent differences in physiography.

Other than possible differences in inherent habitat suitability of Blue Ridge versus Ridge and Valley streams, the higher current occupancy of hellbenders the Blue Ridge could be a legacy effect (Foster *et al.*, 2003) of historical land use intensity within each province. Physiography can influence the suitability of the terrestrial landscape for particular human land uses via topography, soil chemistry and vegetation community composition and structure (Godfrey, 1977), and thereby influence exposure of streams to various land use practices (Brown *et al.*, 2005). For example, the Blue Ridge is known to have experienced less exposure to agriculture and intensive logging relative to the neighboring Piedmont physiographic province (Brown *et al.* 2005). Agriculture and urbanization have been shown to negatively impact occurrence and abundance of many aquatic species (Wenger *et al.*, 2008; Barrett & Guyer, 2008; Utz *et al.*, 2009, 2010; Pool *et al.*, 2010; Price *et al.*, 2011; Ficetola *et al.*, 2010). Given their high degree of specialization and dependence on benthic microhabitat, it was somewhat surprising that hellbender occurrence did not respond more strongly to current land use or substrate characteristics. However, given longevity of our target species (25+ years), considerable lag could be expected to separate the onset of mechanisms of population decline (e.g., reduced recruitment) and extirpation. Utz *et al.* (2010) reported differential effects of land use on the distribution of fishes from different physiography provinces in Maryland, and hypothesized legacy effects similar to those we discuss here. If the Ridge and Valley province was historically exposed to higher levels of agriculture or logging or if physicochemical conditions in streams responded to land use differently than in the Blue Ridge, historical land use might be an equally strong (or better) predictor of current hellbender distribution than physiography. Unfortunately, high resolution (≤ 30 m) digitized land use data for our study area that pre-dates the 1990s is lacking, which precluded our ability to investigate the potential role of land use legacy effects.

Given our findings we began to speculate that water quality, whether driven primarily by physiography or by more complex interactions between physiography and land use, may function as an underlying mechanism linking physiographic province to current patterns of hellbender distribution. As a post hoc attempt to evaluate this hypothesis, we measured water quality at a subset of 30 stream reaches in December 2015. We found that conductivity decreased as the proportion of a catchment that fell within the Blue Ridge increased (Fig. 1.6a) and, regardless of physiography, decreased as forest cover within the surrounding area increased (Fig. 1.6b-c). Evidence of water quality variation along a physiographic gradient (Fig. 1.6a) supports our speculation that water quality may function as a contributing mechanism, or strong correlate of other mechanisms, defining current hellbender distribution. However, more comprehensive work is needed to understand effects of land use and physiography on water quality, and to determine whether water quality is associated with more sensitive metrics of hellbender population viability (e.g., abundance or reproductive success).

While our results indicate that about one-third of our study area is currently occupied by hellbenders, occurrence is not indicative of abundance or population viability. Occurrence of freshwater species is often less sensitive to land use than abundance (Utz *et al.*, 2009; Utz *et al.*, 2010), particularly over short time scales. Therefore, we caution against assuming probability of occupancy is an indicator of population viability in our study. Rather, given that stream segments subject to moderate or higher (< 65% forest) levels of human land use intensity currently represent the majority of occupied habitat in our study area (Fig. 1.5), additional research to determine how land use influences population viability may be particularly vital to conservation planning for hellbenders.

Our study highlights the potential danger of assuming that the distribution of data deficient species can be approximated using untested, but commonly accepted, species-habitat associations. Global changes likely to influence species distributions are occurring at an increasing rate. As a result, it is increasingly important to understand the current and potential future distributions for species. Our study focused on an imperiled habitat specialist whose occurrence is often readily assumed to correspond with current indicators of high quality in-stream conditions (i.e., heavily forested landscapes; Williams *et al.*, 1981). Similar assumptions are likely to exist for many imperiled freshwater species, given that land use has the potential to alter sedimentation input, water chemistry, channel morphology and flow regime (Allan, 2004). For our target species, however, we found current land use to be an unreliable predictor of distribution when physiography was ignored. Our findings are consistent with previous studies suggesting that models informed by presence-absence (rather than presence-only) data can reduce omission (false absence) and commission (false presence) errors when predicting species distributions (Hermoso *et al.*, 2015). Omission and commission errors can lead to inaccuracies regarding the status of species, and can reduce the efficiency of conservation planning when conservation targets include protecting discrete habitat units necessary to conserve a species (Hermoso *et al.*, 2015). For many imperiled species there are likely be important trade-offs between the desire for reliable data and the need to make conservation decisions in a timely manner. We acknowledge that in some cases the risks associated with waiting to acquire presence-absence data may outweigh the benefits. However, when possible we encourage randomized spatial sampling, multiple site visits and inclusion of absence data (even when only partially available; Hermoso *et al.*, 2015) when modeling distributions of species. Encouragingly, recent advances in molecular techniques to detect freshwater species from water samples (eDNA; reviewed by Goldberg *et al.*, 2015) offer a potentially powerful, more sensitive and relatively low-labor approach to presence-absence sampling in freshwater systems. Pairing presence-absence sampling via eDNA with an occupancy framework has great potential to improve our knowledge of distributions of rare species. However, because extrapolations of occupancy estimates are sensitive to the definition of the sampling unit, such studies should carefully consider and explicitly define the spatial unit represented by a water sample (e.g., see Jane *et al.*, 2015).

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TABLES

Table 1.1 Summary of covariates considered in modeling detection and occupancy of Cryptobranchus alleganiensis in Virginia, USA 2013-2014. NED = National Elevation Dataset, NHD = National Hydrography Dataset, NLCD = National Land Cover Dataset

Covariate	Description	Data Source	Median (Range)	Predicted Effect
Fixed effect covariates				
visibility	Lateral secchi distance (m) while snorkeling; measured prior to each survey	field measurement	1.6 (0.4-6.0)	+
size	Extent (m^2) of the sampling reach	wetted width X 50	1450 (400-9400)	+/-
fine	Proportion of gravel, sand and silt particles (b-axis ≤ 1.6 cm)	pebble count	0.34 (0.15-0.83)	-
boulder	Proportion of boulders (b-axis > 25 cm)	pebble count	0.08 (0.01-0.28)	+
riffle	Proportion of 100 stratified sampling locations that occurred in riffles	field measurement	0.30 (0.00-0.88)	+
pool	Proportion of 100 stratified sampling locations that occurred in pools	field measurement	0.16 (0.01-1.00)	-
elev	Elevation (m) of the sampling reach	NED	593 (464-797)	+
contr. area	Extent (km^2) of contributing area for the sampling reach	NHDPlus V2	409 (49-7631)	+

physiography	Proportion of the upstream catchment within the Blue Ridge physiographic province	Fenneman and Johnson, 1946	0.60 (0.00-1.00)	+/-
Catchment land use				
ag.c	Proportion of land use characterized as pasture, cultivated crop, and grassland within the upstream catchment	NLCD 2011	0.29 (0.09-0.41)	-
dev.c	Proportion of land use characterized as medium and high intensity development within the upstream catchment	NLCD 2011	0.00 (0.00-0.02)	-
Catchment-riparian land use				
ag.cr	Proportion of land characterized as pasture, cultivated crop, and grassland within a 50 m buffer on both sides of all delineated streams within the upstream catchment	NHDPlus V2, NLCD 2011	0.30 (0.11-0.47)	-
dev.cr	Proportion of land characterized as medium and high intensity development within a 50 m buffer on both sides of all delineated streams within the upstream catchment	NHDPlus V2, NLCD 2011	0.00 (0.00-0.02)	-
Immediate-riparian land use				
ag.ir	Proportion of land use characterized as pasture, cultivated crop, and grassland within a 50 m buffer on both sides of the sample reach and extending upstream for 300 m	NLCD 2011	0.25 (0.00-0.91)	-
dev.ir	Proportion of land use characterized medium and high intensity development within a 50 m buffer	NLCD 2011	0.00 (0.00-0.42)	-

	on both sides of the sample reach and extending upstream for 300 m
Random effect covariates	
RIVER	Random effect variable with 16 levels representing the river in which the sample reach was located

Table 1.2. Candidate models and area-under-the-curve (AUC) measures of performance used to evaluate support for factors influencing detection (step 1) and occupancy (step 2) of *Cryptobranchus alleganiensis* in Virginia, USA 2013-2014, ranked from highest to lowest according to a Wantanabe-Akaike Information Criterion (WAIC).

Model	WAIC	AUC (Ψ)	AUC (p)
Step one			
Ψ(.) p(visibility + ag.ir + dev.ir + RIVER)	144.65		
Ψ(.) p(RIVER)	144.72		
Ψ(.) p(visibility + contr. area + visibility*contr.area)	144.73		
Ψ(.) p(.)	144.79		
Ψ(.) p(visibility + contr. area + visibility*contr.area + RIVER)	145.94		
Ψ(.) p(visibility)	146.11		
Ψ(.) p(visibility + RIVER)	146.24		
Ψ(.) p(visibility + ag.ir + dev.ir)	146.84		
Ψ(.) p(visibility + ca)	147.42		
Ψ(.) p(visibility + ca + RIVER)	147.49		
Ψ(.) p(visibility + ag.c + dev.c + RIVER)	149.09		

Ψ(.) p(visibility + ag.c + dev.c)	149.23		
Step two			
Ψ(physiography) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	133.86	0.83	0.97
Ψ(physiography + ag.c + dev.c + physiography*ag.c+ physiography*dev.c) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	137.83	0.84	0.97
Ψ(pool + riffle + canopy + contrib. area) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	138.05	0.76	0.97
Ψ(physiography + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	139.12	0.85	0.97
Ψ(physiography + ag.c + dev.c + physiography*ag.c+ physiography*dev.c + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	142.68	0.86	0.97
Ψ(pool + riffle + canopy + contrib. area + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	142.79	0.79	0.97
Ψ(.) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	147.40		
Ψ(ag.c + dev.c) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	149.33		
Ψ(RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	152.03		
Ψ(ag.c + dev.c + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	153.37		

Ψ(boulder + fine) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	153.76
Ψ(ag.ir + dev.ir) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	154.43
Ψ(physiography + ag.c + dev.c + boulder + fine + contrib. area) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	154.97
Ψ(ag.c + dev.c + ag.ir + dev.ir) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	155.35
Ψ(elev + ag.c + dev.c + contrib. area) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	158.73
Ψ(boulder + fine + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	158.87
Ψ(ag.ir + dev.ir + contrib. area + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	159.17
Ψ(physiography + ag.c + dev.c + boulder + fine + contrib. area + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	159.36
Ψ(ag.c + dev.c + ag.ir + dev.ir + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	160.56
Ψ(elev + ag.c + dev.c + contrib. area + RIVER) p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	162.92

Ψ(boulder + fine + contrib. area)	p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	163.25
Ψ(boulder + fine + contrib. area + RIVER)	p(visibility + contr. area + visibility*contr. area + ag.ir + dev.ir + RIVER)	169.92

Table 1.3. Coefficients and their associated 95% credible intervals for top ranked models describing detection and occupancy of *Cryptobranchus alleganiensis* in the New River and South Fork Holston River drainages, Virginia, USA.

	Parameter	Estimate	95% CI		nEff[a]	R-hat[b]
Detection						
	Intercept	-2.3809	-7.0390	1.5350	54000	1.0010
	visibility	0.7464	-1.1690	2.8400	54000	1.0010
	contr. area	-9.1743	-16.4900	-3.3330	16000	1.0011
	visibility X contr. area	5.9220	0.8489	14.0300	54000	1.0010
	ag.ir	11.3555	0.4048	24.4603	7000	1.0013
	dev.ir	-0.3474	-19.9700	19.4700	13000	1.0011
	RIVER	.	.	.	.	
Occupancy						
	Intercept	-3.2932	-5.7480	-1.5650	14000	1.0011
	physiography	4.0368	1.7800	6.9910	18000	1.0011
	Intercept	-2.5040	-6.5250	0.8027	54000	1.0010
	physiography	5.9221	1.5060	10.9600	51000	1.0010
	ag.c	-3.8376	-15.9600	8.0991	54000	1.0010
	dev.c	0.4034	-15.8000	16.0503	54000	1.0010
	physiography X ag.c	-5.0249	-19.0000	8.6940	54000	1.0010
	physiography X dev.c	-2.4834	-21.5703	16.5003	54000	1.0010
	Intercept	-3.8036	-7.4640	-1.3590	5200	1.0013
	physiography	4.8044	2.0517	9.0324	3800	1.0014
	RIVER	.	.	.	.	.
	Intercept	-2.7563	-7.6100	1.3175	15000	1.0011
	physiography	6.0134	1.3115	11.3900	6700	1.0013
	ag.c	-4.0739	-16.9753	8.6845	33000	1.0010
	dev.c	-0.0757	-17.2000	16.5000	29000	1.0010

physiography X ag.c	-3.3325	-18.3453	12.0400	33000	1.0010
physiography X dev.c	-2.6717	-21.9853	16.3453	33000	1.0010
RIVER	.	.	.		

[a] Number of effective samples (independent iterations) produced from the MCMC chain

[b] Model convergence criteria, where at convergence R-hat = 1

Table 1.4. Extrapolated estimates of the total proportion of area occupied (PAO) by *Cryptobranchus alleganiensis* throughout fourth order and larger streams in the New and South Fork Holston river drainages in southwest Virginia, USA (assuming coarse substrate is present in every 50 m reach).

	Ψ(physiography)		Ψ(physiography X catchment land use)	
Estimated Occupancy	Stream km	PAO	Stream km	PAO
0-0.1	371.1	0.04	371.1	0.03
0.1-0.2	0	0	0	0
0.2-0.3	0.4	0.27	25.1	0.26
0.3-0.4	54.4	0.37	74.2	0.36
0.4-0.5	66.2	0.43	128.3	0.46
0.5-0.6	104.3	0.53	107.7	0.54
0.6-0.7	309.5	0.67	80.9	0.64
0.7-0.8			67.8	0.75
0.8-0.9			39.9	0.82
0.9-1.0			11.06	0.92
Total	**906.1**	**0.36**	**906.1**	**0.34**

FIGURE LEGENDS

Figure 1.1. Location of *Cryptobranchus alleganiensis* sampling reaches in southwest Virginia, USA.

Figure 1.2. Predicted detection probability for *Cryptobranchus alleganiensis* as a function of (a) contributing area upstream of the reach when holding visibility constant at 1.75 m and as a function of (b) visibility when holding contributing area constant at 1000 km^2, and (c) riparian land use while holding visibility at 1.75 m and contributing area the median observed value (400 km^2). Note the suggested need for visibility ≥ 2 m for even a minimal chance of detection when surveying large rivers (e.g., widths ≥ 50 m). Solid lines represent mean estimates and dashed lines represent 95% credible intervals based on 1000 random samples drawn from MCMC chains.

Figure 1.3. Predicted probability of occupancy for *Cryptobranchus alleganiensis* within a randomly selected 50 m stream reach, based on the proportion of the upstream catchment falling within the Blue Ridge physiographic province of southwest VA, USA. Solid lines represent mean estimates and dashed lines represent 95% credible intervals based on 1000 random samples drawn from MCMC chains.

Figure 1.4. Predicted probability of occupancy for *Cryptobranchus alleganiensis* within a randomly selected 50 m stream reach, based on the proportion of the upstream catchment that

occurs within the Blue Ridge physiographic province of southwest VA, USA, and is classified as agriculture (a) or developed (b). Predictions are the result of 1000 random samples drawn from MCMC chains, where effects of each land use type were generated while holding levels of the other category constant at the mean observed (developed = 0.02%; agriculture = 27%).

Figure 1.5. Distribution of the total area predicted to be occupied by *Cryptobranchus alleganiensis* across 4th order and larger stream reaches subject to varying levels of land use in South Fork Holston and New River drainages, VA, 2013-2014. Predictions are based on a best performing occupancy models (light grey bars = physiography only model; dark grey bars = physiography X land use model). Note that over half of the area predicted to be occupied falls within stream reaches with only moderately forested (≤ 65%) catchments, and only 17-20% of occupied habitat occurs in reaches with heavily (> 70%) forested catchments.

Figure 1.6. Relationship between water quality, physiography (a) and land use quantified at the catchment (b) and immediate riparian (c) spatial scales. Points are observations from a subset of 30 sample reaches with catchments that fell predominantly in the Ridge and Valley (circles) or Blue Ridge (triangles) physiographic provinces. Lines represent predicted effects of covariates, separately for each province (solid black lines = Ridge and Valley; dashed black lines = Blue Ridge), based on results from a least squares regression ($R^2_{adj} = 0.557$, $F_{1,28} = 13.15$, $p < 0.0001$). Covariate effects were predicted while holding other variables in the model at the mean observed value. Note that conductivity was generally higher in Ridge and Valley streams relative to those

from the Blue Ridge (a). Additionally, conductivity decreased as forest cover increased at both the catchment (b) and immediate riparian area (c) regardless of physiography.

FIGURES

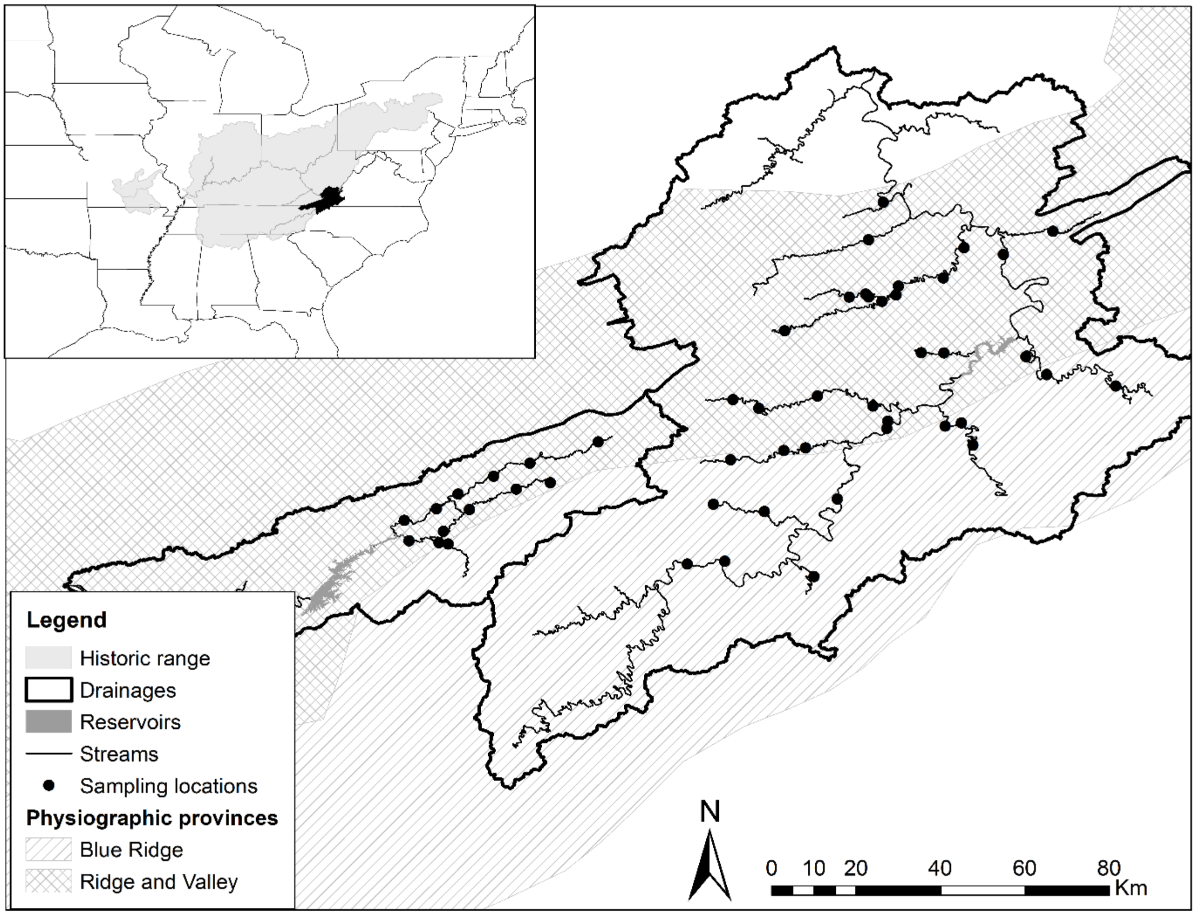

Figure 1.1. Location of hellbender sampling reaches in southwest Virginia, USA.

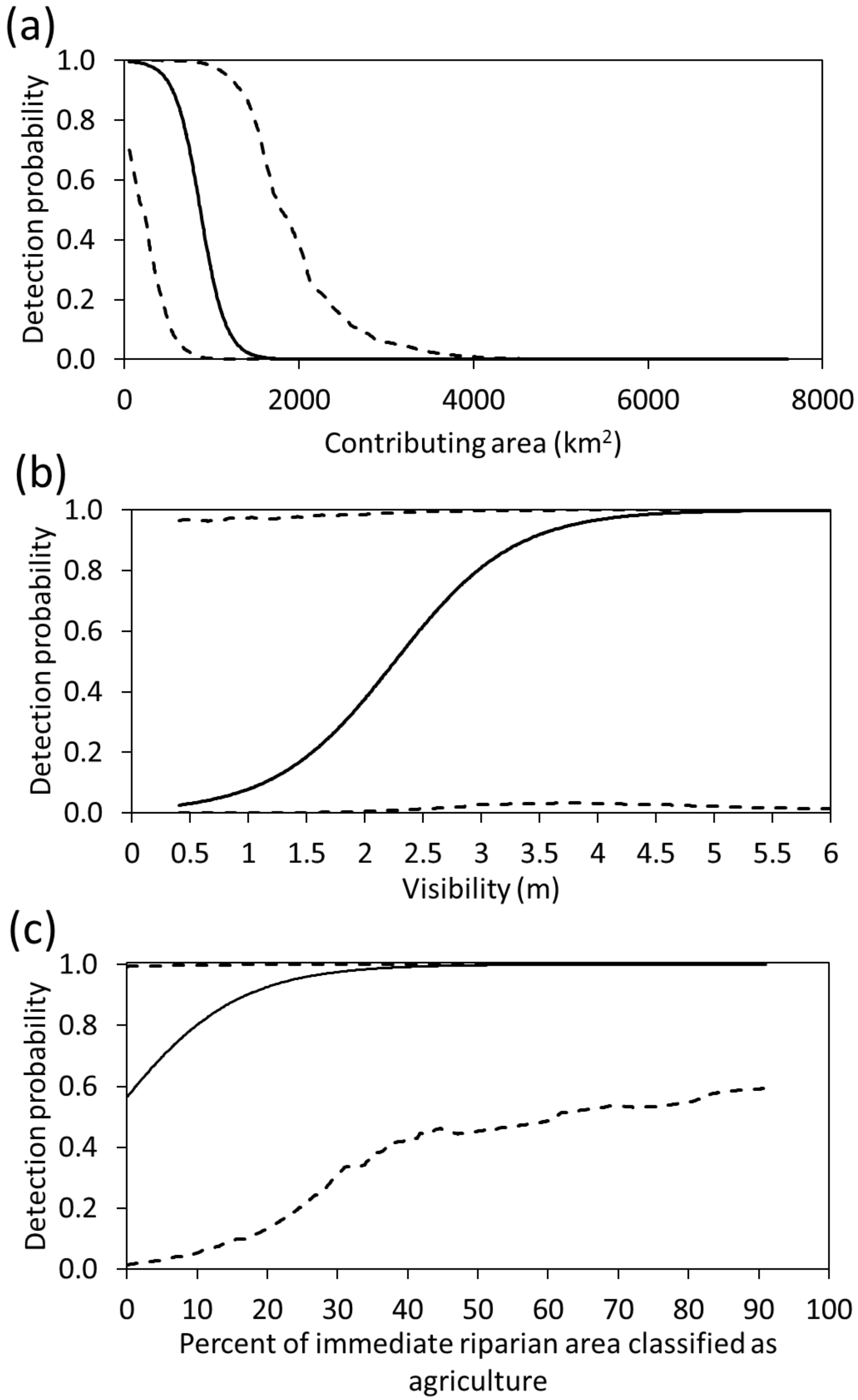

Figure 1.2. Effects of contributing area (a), visibility (b) and land use in the immediate riparian area (c) on detection probability of hellbenders while snorkeling.

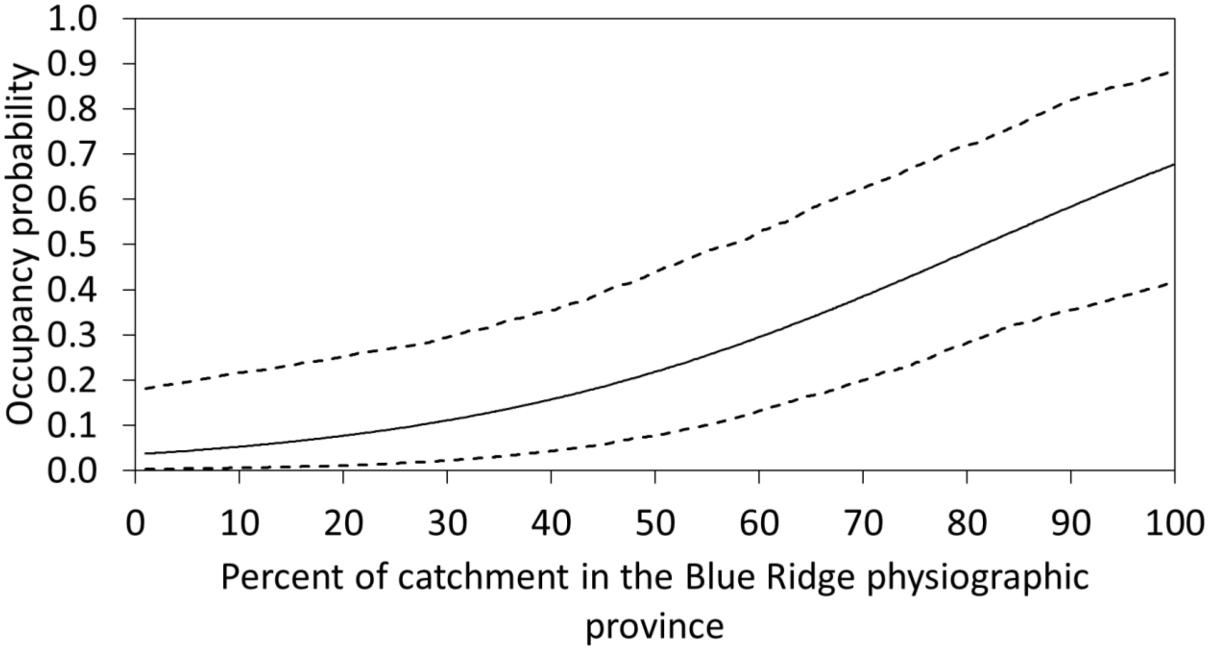

Figure 1.3. Effects of physiography on hellbender occupancy within a randomly selected 50 m stream reach.

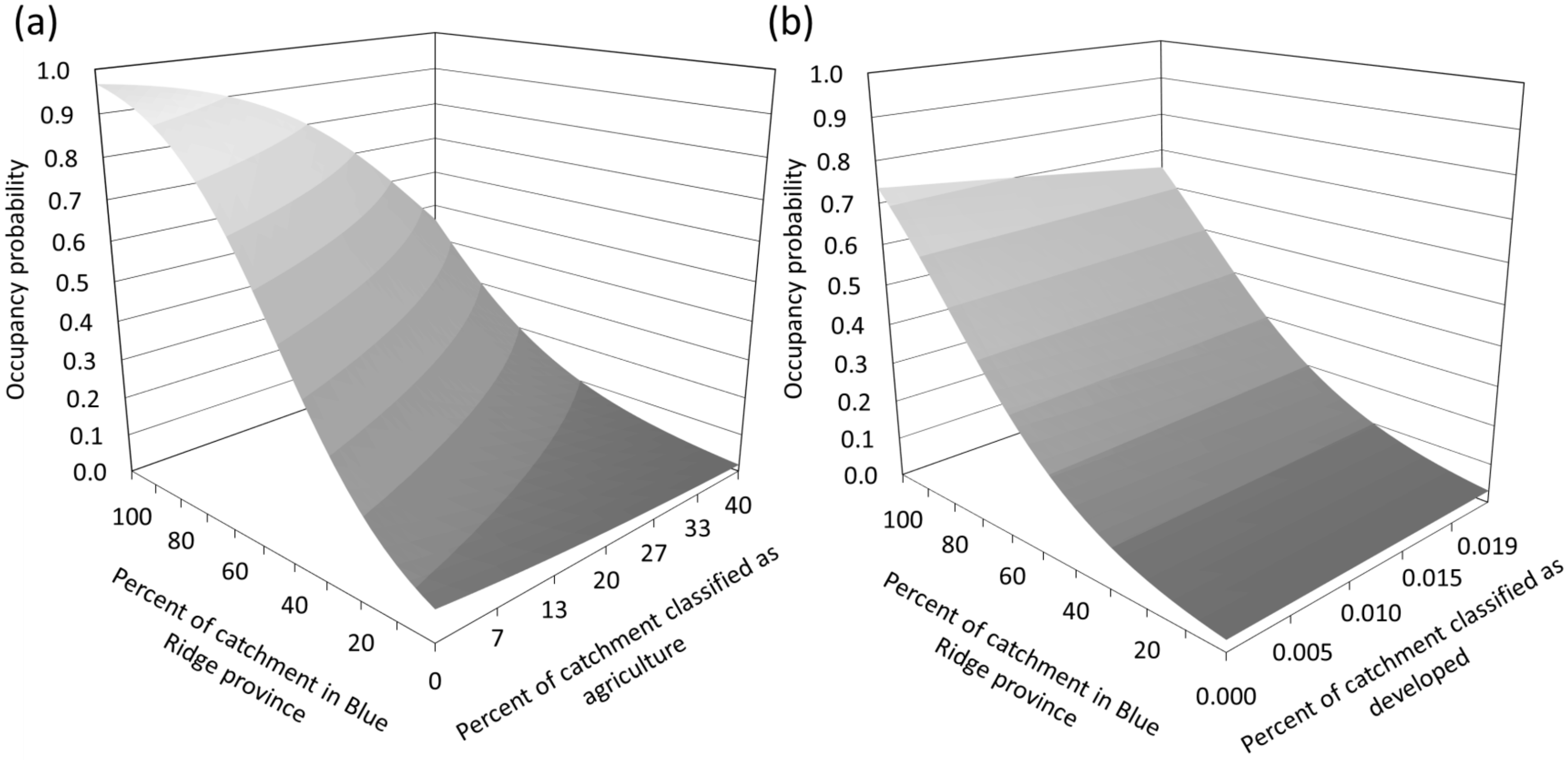

Figure 1.4. Predicted effects of agriculture (a) and medium-high intensity development (b) on hellbender occupancy.

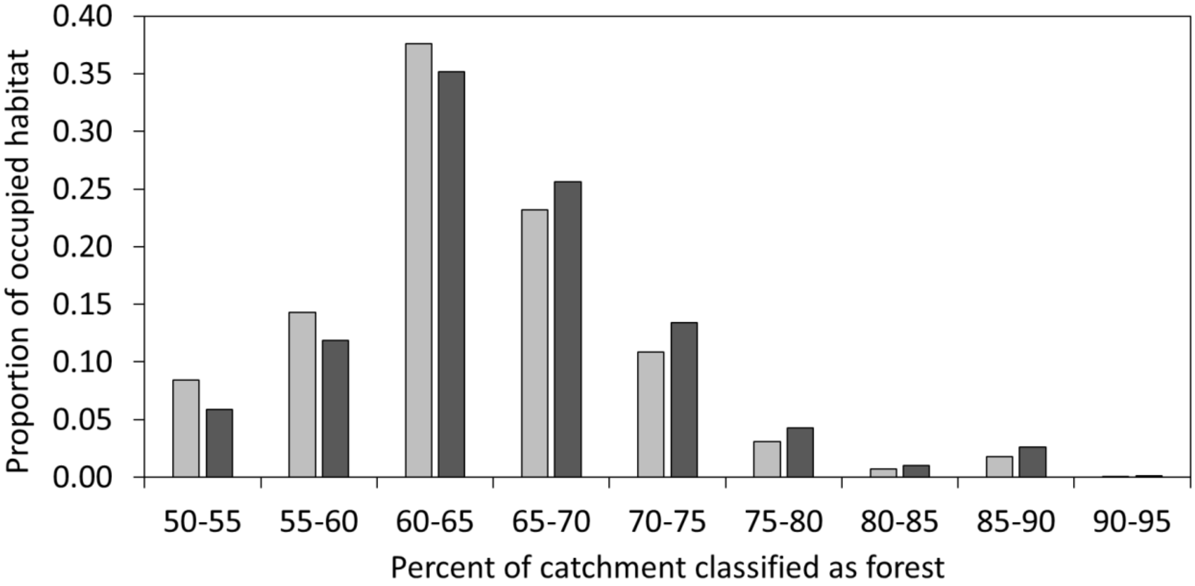

Figure 1.5. Relationship between land use and the relative proportion of habitat predicted to be occupied by hellbenders in South Fork Holston and New River drainages, VA, 2013-2014.

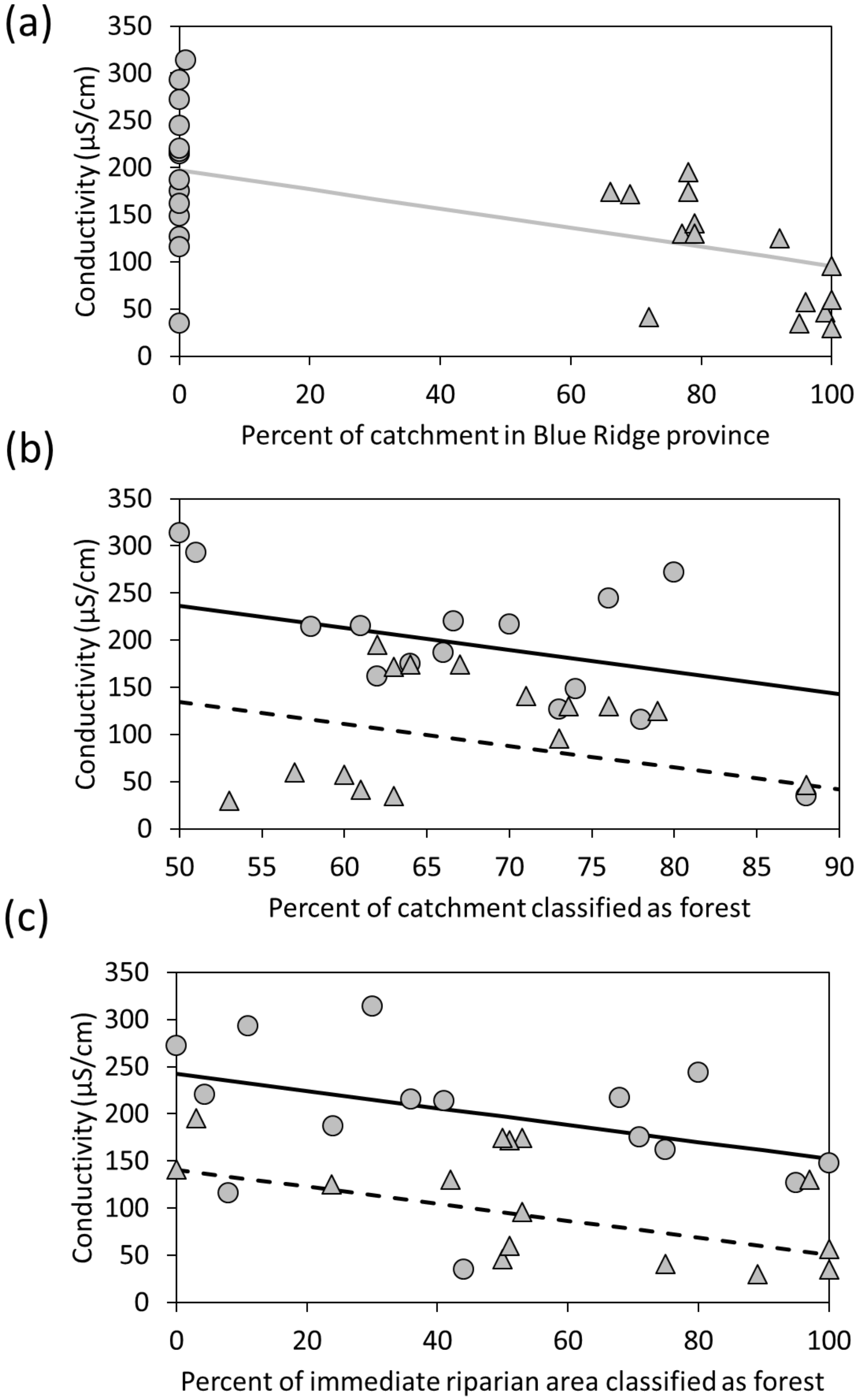

Figure 1.6. Relationship between water quality, physiography (a) and land use quantified at the catchment (b) and immediate riparian (c) spatial scales.

APPENDIX

Appendix A: Hellbender surveys

Within each year, sampling occurred between 1 May and 30 Sep. Occupancy models assume that sites are closed to changes in occupancy during the season when sampling occurs (MacKenzie *et al.*, 2006). Hellbenders are long-lived, typically have small home ranges (< 10 m activity radius [Hillis & Bellis, 1971]; 198 m^2 [Humphries & Pauley, 2005]; 10-30 m^2 [Bodinof *et al.*, 2012b]; but see Burgmeier *et al.*, 2011) and exhibit high site fidelity to specific stream reaches. Therefore, while our sample reaches were only 50 m long we feel confident that the extent of wetted channel within each reach (range = 400-9400 m^2) was appropriately large to contain at least one hellbender home range and thus remain robust to assumptions of closure in occupancy during the sampling season defined within each year. We made an effort to maximize independence between surveys of the same reach by stratifying surveys temporally across the sampling season. However, for 10 reaches that were logistically difficult to access and rarely offered conditions suitable for snorkeling, we conducted up to two surveys of a given reach on the same day. In cases where the same reach was surveyed twice in one day, surveyors were assigned to search different sections of the reach on each pass. We used snorkeling while turning rocks and dive lights to locate hellbenders (Nickerson & Krysko, 2003). Prior to each survey we measured visibility while snorkeling as lateral secchi distance, measured just beneath the water surface, to the nearest 0.1 m.

Two to three individuals, each equipped with snorkeling gear, participated in every survey. Surveyors worked parallel to each other, moving upstream in a zig-zag fashion, to identify and turn potential cover objects. Surveyors independently lifted, peered into and felt within crevices to locate hellbenders and assisted one another with lifting cover objects as necessary. Surveys continued until a hellbender was detected or the entire reach had been surveyed. We captured hellbenders by hand for processing, or required that at least two surveyors visually confirmed species identity when hellbenders were physically inaccessible. We released all hellbenders at the point of capture, after carefully restoring cover objects to the condition in which they were found.

CHAPTER II. Loss of Riparian Forest Cover Is Associated with Reduced Recruitment and Population Density of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*)

Catherine M. B. Jachowski and William A. Hopkins

ABSTRACT

Habitat loss due to deforestation is one of the greatest threats to freshwater biodiversity. Many studies have linked land use to changes in abundance and occurrence of freshwater biota. However, few studies have attempted to explicitly relate land use to demographic rates of fully aquatic species. We used mark-recapture data collected between 2007 and 2015 to describe demography of declining giant salamander (eastern hellbender [*Cryptobranchus alleganiensis alleganiensis*]) populations in six stream reaches stratified across a land use gradient in the upper Tennessee River basin in Virginia. We tested the hypotheses that loss of forest cover negatively affects hellbender density and population growth rates due to reduced recruitment (evidenced from demographic structure) and/or reduced adult survival. We also investigated relationships between surrounding forest cover and potential in-stream mechanisms (water quality and substrate) linking land use to hellbender demography. Mean sub-adult/adult densities in 2014-2015 were three- to nine-fold higher in reaches with ≥ 65% forest in catchment-wide riparian areas (range = 1.50 – 3.13 per 100 m^2) relative to reaches with 53-63% forest (0.34 – 0.45 per 100 m^2). Apparent survival of adults did not vary with forest cover. However, population structure reflected a decline in recruitment as surrounding forest cover within the catchment-wide riparian area decreased. In addition, populations composed of relatively few (≤ 10% of the population) young adults declined during our study. Multiple measures of in-stream habitat quality were correlated with forest cover, suggesting possible mechanisms of shifts in hellbender demography. Specifically, water temperature and ionic concentrations (e.g., conductivity) decreased and the size of dominant substrates increased as catchment-wide riparian forest cover increased. Maintaining forest in > 65% of collective riparian areas throughout our study area can benefit hellbenders and perhaps many of the freshwater fauna inhabiting the broader, and extremely biodiverse, Tennessee-Cumberland freshwater ecoregion.

INTRODUCTION

Effective conservation planning and policy for freshwater systems relies on understanding how global changes influence processes that drive population dynamics in aquatic systems. Freshwater habitats cover less than 1% of earth's surface but harbor nearly 10% of described species (Strayer and Dudgeon, 2010). Because of anthropogenic disturbances, species tied to freshwater ecosystems rank among the most threatened on earth (Sala *et al.*, 2000; Dudgeon *et al.*, 2006). Land use alteration is recognized as a major driver of aquatic habitat quality (Allen, 2004) and one of the greatest threats to freshwater biodiversity (Sala *et al.*, 2000). Deforestation can lead to altered water quality, sedimentation, and disruption of flow regimes (Allan, 2004). Numerous studies have shown that deforestation leads to reduced species richness, abundance and occurrence of native freshwater mussels (Poole & Downing, 2004; Arbuckle & Downing, 2002; Strayer *et al.* 2004), insects (Weijters *et al.*, 2009; Utz *et al.,* 2009), fishes (Morgan & Cushman, 2005; Hudy *et al.*, 2008; Wenger *et al.*, 2008; Utz *et al.*, 2010; DeWeber & Wagner, 2015) and amphibians (Barret & Guyer, 2008; Thompson *et al.*, 2015). However, relatively few studies explicitly relate land use to demographic rates that drive population dynamics of fully aquatic species (Paulsen & Fisher, 2001; Österlin & Högberg, 2013; Grantham *et al.*, 2012; Penaluna *et al.*, 2015). This is important because a stressor might act on one demographic rate (e.g., reproductive success) without affecting another (e.g., adult survival). Poinpointing which demographic rates are sensitive to land use can inform conservation decision making.

The hellbender (*Cryptobranchus alleganiensis*) is an imperiled and fully aquatic salamander whose declines are poorly understood. Hellbenders are endemic to streams in the eastern U.S. and both subspecies (eastern [*C. a. alleganiensis*] and Ozark [*C. a. bishopi*]) have undergone precipitous declines since the 1970s (Wheeler *et al.*, 2003). Hellbenders are protected under CITES and the Ozark hellbender is a federally endangered species (USFWS, 2011a-b). The eastern hellbender is currently being considered for federal protection. Habitat loss as the result of deforestation is commonly cited as a primary driver of hellbender population losses (Wheeler *et al.*, 2003; Briggler *et al.*, 2007; Foster *et al.*, 2009). However, attempts to explicitly test hypotheses regarding the effects of land use on hellbenders are lacking entirely.

The purpose of our study was to investigate the relationship between land use and demography of eastern hellbenders. We tested the hypotheses that reduced forest cover at one or more spatial scales (i.e., catchment, catchment-wide riparian or immediate riparian) would negatively affect population density and population growth rates of hellbenders as a result of reduced recruitment (evidenced by population structure) and/or reduced adult survival. To investigate potential mechanisms linking land use to hellbender demography, we evaluated support for the hypotheses that water quality and substrate variables considered to be important for our focal species would co-vary with land use and demographic responses of interest.

METHODS

Focal species

Hellbenders are one of three extant members of the Family Cryptobranchidae (giant salamanders). Hellbenders are endemic much of the eastern U.S. (Fig. 2.1) while other members of the family are restricted to Asia. The Chinese giant salamander (*Andrias davidianus*) is critically endangered and endemic to China (Wang *et al.* 2004) and the Japanese giant salamander (*A. japonicas*) is near threatened and endemic to Japan (Ota, 2000).

All Cryptobranchids are extremely long-lived (25+ y; Taber *et al.*, 1975), fully aquatic and are benthic habitat specialists that rely heavily on rocky crevices for shelter, nest sites and as microhabitat to support a prey base. Sub-adult and adult hellbenders consume crayfish as their primary prey (Peterson *et al.*, 1989) and exhibit high site fidelity to specific stream reaches and rock cavities (Nickerson & Mays, 1973a-b; Bodinof *et al.*, 2012). Age at sexual maturity is generally five to eight years (Peterson *et al.*, 1988) and individuals can exceed 25 years of age (Taber *et al.*, 1975). Spawning occurs annually (Smith, 1907) where females can deposit 500 or more eggs (Topping and Ingersol, 1981) in a nest cavity guarded by a single male. Larval hellbenders are rarely encountered in most populations, but are considered to largely inhabit interstitial spaces among cobble and gravel and consume a variety of small macroinvertebrates as prey (Nickerson *et al.*, 2003; Hecht-Kardasz, 2011). Following metamorphosis (~ 18 mo., characterized by external gill resorption), hellbenders rely primarily on cutaneous respiration (Guimond & Hutchison, 1973) and have rarely been observed out of water (Coe *et al.*, 2016). However, little is known regarding hellbender tolerance to water quality parameters. Given Cryptobranchid biology and habitat requirements, fine sediment loading and water quality alterations associated with land use alteration are often suspected to function as more proximate drivers of declines (Ota, 2000; Wang *et al.*, 2004; Wheeler *et al.*, 2003; Briggler *et al.*, 2007; Foster *et al.*, 2009; Okada *et al.*, 2009).

Study sites

Our study area included a portion of the upper Tennessee River basin in southwest Virginia. Streams throughout our study area function as headwaters of the greater Tennessee-Cumberland freshwater ecoregion which is recognized as a biodiversity hotspot (Abell *et al.*, 2008). We studied hellbender populations in six stream reaches (hereafter, R1-R6) staggered among three streams (n = 1-4 reaches per stream; Fig. 2.1). Due to the sensitive status of our focal species and threats of illegal collection we do not refer to waterbodies by name.

Study design

We selected our six study reaches from areas known to be occupied by hellbenders based on previous research (Hopkins *et al.*, 2009; Hopkins & DuRant, 2011; Jachowski & Hopkins, 2013; Jachowski *et al.*, in press) that would reflect the broadest range of land use intensity possible (Table 2.1). Three of our reaches (R1-R3) were first surveyed between 2007 and 2012 while the remainder (R4-R6) were part of a randomly selected pool of stream reaches first surveyed in 2013 in an effort to better understand hellbender distribution across a land use gradient. Reaches were similar in average wetted width (range = 13 – 18 m). We defined length (range = 93 – 129 m) of each reaches such that the extent of wetted stream channel was equal (~1,680 m^2) among reaches, thus facilitating comparison of hellbender sub-adult/adult density estimates.

We considered land use at three spatial scales including the catchment, catchment-wide riparian, and immediate riparian scales. We defined a catchment as all land within the contributing area upstream of a given reach. We defined the catchment-wide riparian area as all land within a 50 m corridor on each side of all upstream segments delineated in the National Hydrography Dataset (NHD) Plus version 2.1 (1:100000 scale; USGS & USEPA, 2012). We defined immediate riparian area as all land within a 50 m corridor on each side of the reach and the 1000 m of main water channel immediately upstream. At each spatial scale we quantified the percent of area classified as forest (deciduous, evergreen, mixed forest and shrub and brush land categories), agriculture (crop and grassland categories), and developed (areas with 50-100% impervious surface, including residential, commercial and industrial use categories) based on the 2011 national land cover dataset (NLCD; USGS, 2014). For simplicity we used percent forest cover at either the catchment, catchment-wide riparian or immediate riparian scale (hereafter Forest_{C} , $\text{Forest}_{\text{CR}}$ and $\text{Forest}_{\text{IR,}}$ respectively) to represent land use in all analyses.

Field sampling

We used a robust sampling design with two primary and two secondary occasions to estimate density and describe demographic structure of hellbender populations in each reach and in successive years. Primary occasions were defined by year (summer of 2014 and 2015) and each contained two secondary sampling occasions. We assumed populations were open between primary occasions but closed between secondary occasions in each year. To minimize potential for violating assumptions of closure we conducted secondary occasion surveys as close together in time as possible (median no. days between secondary occasions within a primary occasion = 2.5 [range = 1-34 days], n = 12 primary occasions). We conducted surveys between 6 July and 27 Aug in 2014 and between 23 June and 5 Aug in 2015. We took advantage of capture events recorded during prior and ongoing research conducted between 2007 and 2015 (Hopkins *et al.*, 2011; Hopkins and DuRant, 2011; Hopkins *et al.*, 2014; DuRant *et al.*, 2015; Hopkins *et al.*, 2016; Jachowski *et al.*, In press) to estimate apparent survival and population growth rates (Table 2.1).

We used snorkeling while turning rocks and tactile searches to locate and capture all size classes of hellbender (Nickerson *et al.*, 2003) and marked individuals ≥ 130 mm in total body length with uniquely coded passive integrated transponder (PIT) tags (models HPT8 or HPT12; Biomark Inc., Boise, ID, USA). Detailed survey and tagging procedures are outlined in Supporting Information (Appendix A).

In-stream conditions.---We deployed HOBO® (Onset Corp, Bourne, MA, USA) model Prov2-U22-001 temperature loggers set to record water temperature (accuracy = +/- 0.2 ˚C) at 1 h increments in each reach between Sep 2014 and Aug 2015. We visited each reach approximately monthly to measure dissolved oxygen (mg/L), conductivity (µS/cm), salinity, total dissolved solids (mg/L) and pH using a YSI 556 MPS portable water probe (Yellow Springs, OH). We quantified substrate characteristics during May 2015 using a modified Wolman Pebble Count (Wolman, 1954). In our modification we measured substrate variables at 100 points from 20 cross-sectional transects (five points per transect) stratified evenly across each reach. We recorded the size class (based on intermediate axis [mm]) of substrates at each sampling point. Because hellbenders are cavity dwellers, we measured cavity availability for 100 randomly selected boulders per reach, where we sampled the nearest boulder to each pebble count location (without replacement). We classified boulders as having a cavity when an observer was able to slide a 3 cm wide ruler beneath the boulder to a depth of at least 3 cm; otherwise we classified boulders as having no cavity.

Data analysis

Density.---We fit a Huggins (1989, 1991) version of the closed robust design model (Pollock, 1982) to our data to estimate abundance (N) and capture probability (p) in each reach in both 2014 and 2015. Due to the rarity of small age classes and our inability to permanently mark larvae, our density analysis and thus inference regarding density was limited to the pooled group of larger sub-adult and adult age classes (≥ 190 mm, hereafter sub-adult/adult). A detailed description of robust model development can be found in Supporting Information (Appendix B). The Huggins version of the robust design model estimates abundance as a derived parameter (Huggins, 1991) which precluded our ability model abundance as a function of forest cover directly. To investigate the relationship between land use and hellbender abundance, we used abundance estimates from the model to calculate sub-adult/adult density (no. per 100m^2) in each reach and used simple linear regression to test the hypothesis that loss of forest cover would negatively affect density. We predicted that density would increase linearly or up to some threshold (log-linearly) as forest cover increased at one or more spatial scale(s). Notably, R2 contained considerably more uninhabitable substrate, in the form of smooth bedrock, than other reaches (i.e., R2 = 45% versus 1-9% bedrock in other reaches). To account for bedrock, we adjusted the denominator used to estimate density as:

$$Area_{ADJ} = A \times (1 - proportion\ of\ reach\ characterized\ as\ bedrock)$$

where A is the approximate wetted extent (~ 1680 m^2) of a survey reach, and the proportion of the reach characterized as bedrock was estimated as the proportion of pebble count observations classified as bedrock.

Demographic structure and recruitment.--- We used the sample of sub-adult/adults captured during robust design sampling events to describe demographic structure in each reach.

We excluded gilled larvae and recent metamorphs from our demographic structure analysis due to low sample size (Table 2.3). We used the hellbender age-growth model of Taber *et al.* (1975) to classify remaining individuals into one of five age classes based on total body length: 2-5 y (190-289 mm), 5-10 y (290-420 mm), 10-15 y (> 420-480 mm), 15-20 y (480-515 mm) and > 20 y old (≥ 515 mm). We pooled captures from 2014 and 2015 but counted individuals only once. We assigned age class based on average body length for individuals captured in both years. We assumed individuals in the youngest age class were immature (2-5 y) and that age classes older than 5 y included only sexually mature adults (Nickerson and Mays, 1973b; Peterson *et al.*, 1988; Hecht-Kardasz *et al.*, 2012). We calculated the proportion of samples assigned to each age class where, for each reach, proportions summed to 1.0. We used simple linear regression to test our hypothesis that loss of forest cover altered population structures through reduced recruitment. We predicted that the proportion of individuals assigned to relatively young age classes would increase linearly or up to a threshold (log-linearly) as forest cover increased at one or more spatial scales.

Apparent survival and population growth rate.---We estimated monthly apparent survival (Φ_m) and the monthly per capita population growth rate (λ_m) for each reach using Pradel (1996) models. Pradel models are open population models, which allowed us to take advantage of capture events dating back as far as 2007 (Table 2.1) while accounting for variation in p among surveys. The lack of young age classes in our long-term dataset restricted our inference regarding Φ_m and λ_m to the sub-adult/adult population in each reach. A detailed description of Pradel model development can be found in Supporting Information (Appendix C).

In stream characteristics.---We used simple linear regression to quantify associations between eight water quality and four substrate variables. We averaged across months to obtain a single measure of average conductivity (µS/cm^3), total dissolved solids (mg/L), salinity, dissolved oxygen (mg/L) and pH for each reach. We calculated mean weekly mean, mean weekly minimum, and mean weekly maximum temperature by taking the mean value of mean daily values over a period of seven days. We used size class of particles encountered during our Wolman pebble counts to identify the diameter of particles marking the 50th (D_{50}), 75th (D_{75}) and 95th (D_{95}) percentiles. We estimated cavity availability as the proportion of boulders in each reach that had cavities.

We fit closed robust design and Pradel models in program MARK (White and Burnham, 1999) and ranked candidate models using Akaike's Information Criterion adjusted for small samples (AICc). When the upper 90% of AICc model weight was distributed across more than one model, we used model averaging (Burnham and Anderson, 2002) to account for model selection uncertainty. We report estimates of p, N , Φ and λ and their 95% confidence intervals unless otherwise noted. We fit all simple linear regression models in program R (Team, 2013) and observed statistical significance at the $\alpha = 0.05$ level.

RESULTS

Density

We captured 204 unique individuals during robust design surveys (Table 2.3). We captured only four larvae. The majority (198 of 199) of PIT tagged individuals were ≥ 190 mm long. A single model received the majority of support explaining differences in p and N among reaches ($w_i = 0.99$; Table 2.2). Capture probability increased with total body length in every reach, and ranged from 0.52-0.91 for average sized individuals (~ 400 mm; Fig. 2.2). We observed some evidence to suggest that temporal proximity of a primary occasion to the onset of breeding may have influenced abundance and sex ratios in each year. For example, $\widehat{N}$ and sex ratios were similar between years for the three reaches (R3, R5 & R6) that were sampled at approximately the same time each year (mid-late July); while $\widehat{N}$ varied by 30-40% between years in reaches (R1, R2 & R4) that were sampled at different times each year (Table 2.3). In all cases $\widehat{N}$ was higher and sex ratios more even when a given reach was sampled closer in time to the onset of breeding. The two reaches with the lowest levels of forest cover were characterized by the lowest density of sub-adult/adults in both years (Fig. 2.3). Density increased with $\text{Forest}_{\text{CR}}$ (but not with forest at any other spatial scale; Table 2.5) in 2014 but not in 2015 (Table 2.5; Fig. 2.3).

Demographic structure and recruitment

The two lowest density populations were composed primarily of large/old adults (Table 2.3; Fig. 2.4). Variation in population structure among reaches was driven primarily by differences in the relative proportion of individuals in the 5-10 y versus 10-15 y age classes. The proportion of a population composed of the youngest sexually mature age class (5-10 y olds) increased with Forest_{C} and $\text{Forest}_{\text{CR}}$ while the proportion of a population composed of middle aged adults (10-15 y olds) decreased with $\text{Forest}_{\text{CR}}$ (Table 2.5, Fig. 2.5). Proportions of other age classes were not significantly associated with land use at any spatial scale (Table 2.5).

Apparent survival and population growth rate

To facilitate comparison among reaches, we model-averaged estimates of apparent survival for the smallest size class present in all reaches (400 mm in total length). We found no evidence that apparent survival varied as a result of land use (Table 2.6). Sub-adult/adult populations declined during our study ($\hat{\lambda} < 1$) in the two reaches (R5 and R6) subject to the lowest levels of forest cover, but were stable or increased ($\hat{\lambda} \geq 1$) in three of the four others (Table 2.6). The population in R2 declined during our study despite having relatively high levels of forest cover (Table 2.6).

In stream conditions

Mean weekly mean and mean weekly maximum temperatures, measures of ionic concentration (conductivity, salinity and total dissolved solids) and pH were negatively correlated with Forest_{C} and/or $\text{Forest}_{\text{CR}}$ (Table 2.7; Fig. 2.6). D_{75} was positively associated with Forest_{C} and $\text{Forest}_{\text{CR}}$ (Table 2.7; Fig. 2.6). No water quality or substrate variables were significantly associated with $\text{Forest}_{\text{IR}}$ (Table 2.7). Dissolved oxygen, D_{50}, D_{95}, and cavity availability were not significantly associated with land use at any spatial scale (Table 2.7).

DISCUSSION

Our study provides novel and fundamental insights into the relationship between land use and hellbender demography. Sub-adult/adult hellbender densities were three- to nine-fold (range = 1.50 – 3.13 per 100 m^2) higher in areas subject to ≥ 65% $\text{Forest}_{\text{CR}}$ relative to areas subject to 53-63% $\text{Forest}_{\text{CR}}$ (range = 0.30-0.45 individuals per 100 m^2). Apparent survival of adults did not respond to land use. However, low density (< 0.50 individuals per 100 m^2) populations reflected a clear lack of adult recruitment and were declining over the course of our study. Our findings provide evidence that that loss of forest cover can result in decreased hellbender densities and suggests that loss of forest cover acts specifically on demographic rates that determine recruitment to the population of breeding adults (i.e., reproductive success, survival of larvae/sub-adults, reduced immigration, or some combination of the three).

Multiple measures of water quality were highly correlated with forest cover at the spatial scale most closely aligned with demographic changes (Forest$_{CR}$). Our findings are consistent with previous research showing that water temperatures increase as a result of deforestation in riparian areas (Swift & Messer, 1971). Water temperature can be an important determinant of metabolism, growth, reproduction and behavior of many fishes, especially cold-water species (Ficke *et al.*, 2007). Mean weekly temperatures in all of our reaches fell within the range of temperatures preferred by adult hellbenders (11-21 ˚C) according to Hutchison & Hill (1976). However, sublethal effects of thermal variation on hellbenders and thermal tolerance to immature life stages of hellbender have not been studied.

Conductivity, salinity and total dissolved solids are highly interrelated reflect the concentration of impurities, namely salts and other inorganic ions, in water (Allan & Castillo, 2007). Ionic concentration can vary as a result of underlying geology, weather patterns and addition of pollutants (Gibbs, 1970; Allan & Castillo, 2007). Freshwater organisms have evolved various traits to cope with fluctuations in ionic concentration. However, extreme fluctuations can result in mortality (Evans, 1980) and juvenile life stages are often more vulnerable than adults (James *et al.*, 2003). Ionic concentrations can elicit sub-lethal effects by altering cues to initiate spawning, and inhibiting sperm development, activation and motility and embryonic development (James *et al.*, 2003; Alavi and Cosson, 2006; Bonislawska *et al.* 2015). Hellbender *occurrence* has been shown to be negatively correlated with conductivity (Quinn *et al.*, 2013; Keitzer *et al.*, 2013; Pugh *et al.*, 2015; Jachowski *et al.*, in press) and recent captive breeding success for Ozark hellbenders was at least partially attributed to reduction of total dissolved solids in captive enclosures (Ettling *et al.*, 2013). Levels of total dissolved solids recommended for captive propagation (175-300 mg/L; Ettling *et al.*, 2013) are relatively high compared to levels we observed (Fig. 2.6c). However, Ettling *et al.* (2013) were developing husbandry protocols for Missouri hellbender populations that may be locally adapted to relatively high intrinsic levels of dissolved solids that are characteristic of their native karst-influenced habitat (Benke & Cushing, 2005). The fact that hellbender demography and water quality parameters were both closely associated with $\text{Forest}_{\text{CR}}$ emphasizes the need for more work to understand physiological responses of hellbenders, especially early life stages, to water quality alteration.

Our findings provide general support for the hypothesis that addition of fine substrates as a result of loss of riparian forest cover can be detrimental to hellbender population viability. The size of substrate particles dominating each reach increased as $\text{Forest}_{\text{CR}}$ increased (Fig. 2.7). Removal of riparian vegetation is known to increase fine sediment loading, decrease availability of interstitial spaces (Allan, 2004) and can lead to shifts in assemblages of freshwater species (Jones *et al.*, 1999). Substrate is known to be an important predictor of hellbender *occurrence* (Quinn *et al.*, 2013; Keitzer *et al.*, 2015; Pugh *et al.*, 2015). However, our study is the first we are aware of to document covariance between land use, substrate and hellbender demography (Fig. 2.7). We found no evidence that cavity availability (range = 56-69% of boulders per site had cavities) varied with D_{75}. However, it is possible that the lack of variation in cavity availability among reaches was an artifact of intensive mark-recapture surveys and associated boulder disturbance 10 months prior to substrate surveys.

CONSERVATION IMPLICATIONS

Our findings suggest that protection and/or enhancement of forest cover in riparian areas should be a focus of conservation actions for hellbenders and other Cryptobranchids. Specifically, we recommend a target of maintaining forests throughout > 65 % of collective riparian areas upstream of habitats occupied by hellbenders. The majority non-forested riparian areas in our study area is dedicated to agriculture (Table 2.1), often in form of livestock grazing where livestock are allowed direct stream access. Hellbender conservation should benefit from policies that encourage landowners to maintain riparian buffers and restrict livestock access to streams. Given headwaters for our study area originate in national forest, we recommend careful restriction of activities that might lead to loss of riparian vegetation on public lands. While our study focused on a single species, we suspect that protection and enhancement of riparian forest cover throughout our study area may contribute to biodiversity conservation more broadly. For example, our study area functions as the headwaters of the Tennesee-Cumberland freshwater ecoregion which is home to over 200 species of fish (67 of which are endemic), 125 species of mussel, 65 species of crayfish and is a global center of salamander diversity (Abell, 2000).

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TABLES

Table 2.1. Stream, elevation (m), land use statistics (percent forest [For], agriculture [Ag] and developed [Dev]) and number of sampling occasions used to estimate monthly apparent survival and per-capita increase in the sub-adult/adult hellbender (*Cryptobranchus alleganiensis*) population in six stream reaches (R1-R6) in southwest, Virginia, USA.

		Catchment scale			Catchment-wide riparian scale			Immediate riparian scale			No. monthly sampling occasions									
	Elevation	For	Ag	Dev	For	Ag	Dev	For	Ag	Dev	2007	2008	2009	2010	2011	2012	2013	2014	2015	Total
R1	652	92	5	0	83	7	0	83	3	0	1	1						3	5	10
R2	691	78	18	0	69	22	0	24	47	0						1		2	4	7
R3	642	74	23	0	69	23	0	45	46	0			1		2		6	9	7	25
R4	635	71	25	0	66	25	0	16	64	0							7	10	6	23
R5	645	67	25	2	53	30	3	4	2	30								4	4	8
R6	595	66	30	0	62	30	0	74	11	0							1	5	6	12

Table 2.2. Relative ranking among candidate Huggins closed-robust-design models used to estimate probability of capture (p) and abundance (as a derived parameter) of sub-adult/adult *Cryptobranchus alleganiensis* in six stream reaches in southwest Virginia, USA, 2014-2015.

Model	K[a]	AICc[b]	ΔAICc	w_i[c]	Likelihood	Deviance
Φ(Reach) $\gamma' = \gamma''(0)$ $p=c$(Reach + Total body length)	12	625.26	0.00	0.99	1.00	600.46
Φ (Reach) $\gamma' = \gamma''(0)$ $p=c$(Reach*Year + Total body length)	17	634.77	9.51	0.01	0.01	599.18
Φ (Reach) $\gamma' = \gamma''(0)$ $p=c$((Reach)	11	642.48	17.22	0.00	0.00	619.80
Φ (Reach) $\gamma' = \gamma''(0)$ $p=c$(Reach*Year)	16	651.86	26.60	0.00	0.00	618.45

[a] Number of estimated parameter in each model

[b] Akaike information criterion adjusted for small samples

[c] Akaike model weight

Table 2.3. Summary of captures and model derived estimates of abundance ($\hat{N}$ [95% CI]) of sub-adult/adult *Cryptobranchus alleganiensis* in six stream reaches (R1-R6) stratified across a gradient of percent forest cover at the catchment (Forest$_{C}$), catchment-wide riparian (Forest$_{CR}$) and immediate riparian (Forest$_{IR}$) spatial scale in southwest Virginia, USA.

				2014						2015					
	Forest$_{C}$	Forest$_{CR}$	Forest$_{IR}$	L^{a}	SA^{b}	F	M	$\hat{N}$	Sex ratio (F:M)	L^{a}	SA^{b}	F	M	$\hat{N}$	Sex ratio (F:M)
R1	92	83	83	0	2	16	12	41 [34-59]	1.3:1	1	2	15	6	31 [26-47]	2.5:1
R2	78	69	24	0	1	7	8	16 [16-19]	0.9:1	0	3	7	1	11 [11-16]	7.0:1
R3	74	69	45	1	7	18	19	48 [45-56]	0.9:1	1	7	20	21	52 [49-61]	1.0:1
R4	71	66	16	2	5	10	9	26 [24-32]	1.1:1	0	5	14	15	36 [35-44]	0.9:1
R5	67	53	4	0	0	4	2	4 [4-7]	2.0:1	0	0	3	4	6 [6-9]	0.8:1
R6	66	62	74	0	0	4	0	6 [6-11]	4.0:0	0	0	5	1	7 [7-12]	5.0:1

[a] Larvae; free-gills present

[b] Sub-adult; lacking gills, no cloacal swelling and total length < 290 mm

Table 2.4. Relative ranking of candidate Pradel models used to estimate apparent survival (Φ), probability of capture (p) and per-capita rate of increase (λ) for sub-adult/adult *Cryptobranchus alleganiensis* populations in six stream reaches (R1-R6) in southwest Virginia, USA.

	Model	K[a]	Likelihood	AICc[b]	ΔAICc	w_i[c]
R1	$\Phi(.)$ p (Survey) $\lambda(.)$	11	1.00	418.00	0.00	0.53
	$\Phi(.)$ p(Survey + Total body length) $\lambda(.)$	12	0.38	419.96	1.96	0.20
	Φ(Total body length) p (Survey) $\lambda(.)$	12	0.31	420.32	2.32	0.17
	Φ(Total body length) p(Survey + Total body length) $\lambda(.)$	13	0.18	421.39	3.39	0.10
R2	$\Phi(.)$ p(Survey) $\lambda(.)$	9	1.00	158.42	0.00	0.48
	Φ(Total body length) p(Survey) $\lambda(.)$	9	0.67	159.22	0.80	0.32
	$\Phi(.)$ p(Survey + Total body length) $\lambda(.)$	10	0.25	161.19	2.77	0.12
	Φ(Total body length) p(Survey + Total body length) $\lambda(.)$	11	0.15	162.26	3.83	0.07
R3	$\Phi(.)$ p(Survey + Total body length) $\lambda(.)$	27	1.00	1417.74	0.00	0.56
	Φ(Total body length) p(Survey + Total body length) $\lambda(.)$	28	0.79	1418.21	0.46	0.44
	$\Phi(.)$ p(Survey) $\lambda(.)$	27	0.00	1437.30	19.56	0.00

	Φ(Total body length) p(Survey) λ(.)	28	0.00	1439.75	22.01	0.00
R4	Φ(.) p(Survey + Total body length) λ(.)	26	1.00	581.56	0.00	0.73
	Φ(Total body length) p(Survey + Total body length) λ(.)	27	0.36	583.60	2.04	0.27
	Φ(.) p(Survey) λ (.)	25	0.00	609.17	27.61	0.00
	Φ(Total body length) p(Survey) λ(.)	26	0.00	609.32	27.76	0.00
R5	Φ(.) p(Survey) λ(.)	13	1.00	148.79	0.00	0.53
	Φ(.) p(Survey + Total body length) λ(.)	14	0.45	150.39	1.60	0.24
	Φ(Total body length) p(Survey + Total body length) λ(.)	15	0.24	151.65	2.87	0.13
	Φ(Total body length) p(Survey) λ(.)	14	0.22	151.84	3.05	0.11
R6	Φ(.) p(Survey) λ(.)	9	1.00	114.89	0.00	0.59
	Φ(.) p(Survey + Total body length) λ(.)	10	0.57	116.00	1.11	0.34
	Φ(Total body length) p(Survey) λ(.)	10	0.09	119.75	4.87	0.05
	Φ(Total body length) p(Survey + Total body length) λ(.)	11	0.04	121.47	6.59	0.02

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[a] Number of estimated parameter in each model

[b] Akaike information criterion adjusted for small samples

[c] Akaike model weight

Table 2.5. Summary statistics for simple linear models used to examine the effect of forest cover quantified within the catchment (Forest$_C$), catchment-wide riparian area (Forest$_{CR}$) or immediate riparian area (Forest$_{IR}$) and density and demographic structure of sub-adult/adult hellbender (*Cryptobranchus alleganiensis*) populations in southwest Virginia, USA.

	Forest$_C$			Forest$_{CR}$			Forest$_{IR}$			log(Forest$_C$)			log(Forest$_{CR}$)			log(Forest$_{IR}$)		
Response	F$_{1,4}$	R^2	p	F$_{1,4}$	R^2	p	F$_{1,4}$	R^2	p	F$_{1,4}$	R^2	p	F$_{1,4}$	R^2	p	F$_{1,4}$	R^2	p
2014 density	4.09	0.51	0.11	6.34	0.61	0.06	0.32	0.07	0.60	4.67	0.54	0.09	6.78	0.63	0.05*	0.96	0.19	0.38
2015 density	0.77	0.16	0.43	1.77	0.31	0.25	0.04	0.01	0.85	0.89	0.18	0.39	2.03	0.34	0.22	0.42	0.10	0.55
Proportion age 2-5y	0.32	0.07	0.60	0.86	0.18	0.40	0.22	0.05	0.66	0.45	0.10	0.54	1.15	0.22	0.34	0.06	0.01	0.82
Proportion age 5-10y	6.76	0.63	0.06	8.44	0.68	0.04*	0.09	0.02	0.78	7.99	0.67	0.04*	8.83	0.69	0.04*	0.48	0.11	0.52
Proportion age 10-15y	2.44	0.38	0.19	8.66	0.68	0.04*	0.61	0.13	0.47	2.78	0.41	0.17	12.08	0.75	0.02*	2.71	0.40	0.17
Proportion age 15-20y	1.29	0.24	0.32	0.28	0.06	0.62	0.86	0.18	0.40	1.42	0.26	0.29	0.20	0.05	0.67	0.83	0.17	0.41
Proportion age > 20y	2.31	0.37	0.20	2.18	0.35	0.21	0.10	0.02	0.76	2.71	0.40	0.17	2.26	0.36	0.20	0.01	0.00	0.94

*Indicates a statistically significance correlation at the $\alpha = 0.05$ level

Table 2.6. Model-averaged estimates of monthly apparent survival (Φ_m [95% CI]) of sub-adult/adult *Cryptobranchus alleganiensis* and monthly per-capita growth rate (λ_m [95% CI]) of populations in six stream reaches (R1-R6) stratified across a gradient of percent forest cover at the catchment (Forest$_{C}$), catchment-wide riparian (Forest$_{CR}$) and immediate riparian (Forest$_{IR}$) spatial scale in southwest Virginia, USA. To allow for comparison among reaches, apparent survival values are model averaged estimates for individuals measuring 40 cm total length.

	Forest$_{C}$	Forest$_{CR}$	Forest$_{IR}$	Time period	$\widehat{\Phi_m}$	$\widehat{\lambda_m}$
R1	92	83	83	2007-2015	0.97 [0.95-0.98]	1.00 [1.00-1.01]
R2	78	69	24	2012-2015	0.93 [0.87-0.96]	0.97 [0.87-0.99]
R3	74	69	45	2009-2015	0.97 [0.96-0.98]	1.01 [1.00-1.02]
R4	71	66	16	2013-2015	0.97 [0.94-0.98]	1.03 [1.01-1.06]
R5	67	53	4	2014-2015	0.95 [0.85-0.99]	0.98 [0.66-1.00]
R6	66	62	74	2013-2015	0.97 [0.90-0.99]	0.97 [0.85-0.99]

Table 2.7. Summary statistics for simple linear models used to examine the effect of forest cover quantified within the catchment ($Forest_C$), catchment-wide riparian area ($Forest_{CR}$) or immediate riparian area ($Forest_{IR}$) and in-stream habitat variables from six stream reaches occupied by hellbenders (*Cryptobranchus alleganiensis*) in southwest Virginia, USA.

	$Forest_C$			$Forest_{CR}$			$Forest_{IR}$		
Response	$F_{1,4}$	R^2	p	$F_{1,4}$	R^2	p	$F_{1,4}$	R^2	p
Mean weekly mean temperature [a]	44.47	0.94	0.01*	5.71	0.66	0.10	0.52	0.15	0.52
Mean weekly minimum temperature [a]	7.72	0.72	0.07	2.07	0.41	0.25	0.01	<0.01	0.91
Mean weekly maximum temperature [a]	91.65	0.97	<0.01*	13.23	0.82	0.04*	2.36	0.44	0.22
Conductivity	29.95	0.88	0.01*	494.70	0.99	<0.01*	3.45	0.46	0.14
Salinity	21.15	0.84	0.01*	206.90	0.98	<0.01*	2.75	0.41	0.17
Total dissolved solids	22.03	0.85	0.01*	0.60	0.99	<0.01*	2.72	0.41	0.17
Dissolved oxygen	0.02	0.01	0.89	0.04	0.01	0.85	0.50	0.11	0.52
pH	30.00	0.88	0.01*	11.86	0.75	0.03	3.09	0.44	0.15
D_{50}	1.18	0.23	0.34	0.89	0.18	0.40	<0.01	<0.01	0.97
D_{75}	31.35	0.89	<0.01*	10.48	0.72	0.03*	3.29	0.45	0.14
D_{95}	2.35	0.37	0.20	0.65	0.14	0.46	<0.01	<0.01	0.99
Cavity availability	0.12	0.03	0.74	0.13	0.03	0.74	1.63	0.29	0.27

[a] Data was only available for five of six stream reaches, thus F-statistic was calculated for 1 and 3 degrees of freedom ($F_{1,3}$)

*Indicates a statistically significance correlation at the $\alpha = 0.05$ level

FIGURE LEGENDS

Figure 2.1. Figure 2.1. Historic range (inset) of eastern (*Cryptobranchus alleganiensis* alleganiensis) and Ozark (*C. a. bishopi*) hellbenders, and land use surrounding study reaches (R1-R6) where *C. a. alleganiensis* were sampled between 2007 and 2015.

Figure 2.2. Estimated probability of capturing hellbenders (*Cryptobranchus alleganiensis*) during a single sampling event (assuming an individual is present and available for capture) in six stream reaches (R1-R6) as a function of total body length. Solid lines represent mean model-derived estimates and dashed lines represent 95% confidence intervals. Predictions are only generated across the range of total lengths observed in a given reach.

Figure 2.3. Model-derived estimates of sub-adult/adult hellbender (*Cryptobranchus alleganiensis*) density in six stream reaches subject to various intensities of human land use. Points represent mean estimates and error bars represent 95% confidence intervals. Dashed line represents a marginally significant log-linear trend evident in 2014 ($R^2 = 0.62$, $F_{1,4} = 6.78$, $p = 0.05$).

Figure 2.4. Relative distribution of size classes of *Cryptobranchus alleganiensis* in samples collected from six stream reaches (R1-R6) subject to varying levels of human land use intensity. Values of n indicate the number of individuals captured at least once in a given year. Figures are arranged in order of decreasing forest cover at the catchment scale (R1 = 92%, R2 =78%, R3 =

73%, R4 = 71%, R5 = 67% and R6 = 66%). Note the lack of young age classes, fewer overall captures and shift towards older adults with decreasing forest cover.

Figure 2.5. Proportion of the sub-adult/adult hellbender (*Cryptobranchus alleganiensis*) population made up of 5-10 year olds and 10-15 year olds in six stream reaches subject to varying levels of human land use intensity.

Figure 2.6. Associations between land use and mean weekly water temperature (a), mean ionic concentration of water (b-d), mean pH (e) and diameter of substrate in the 75th percentile (f) of six stream reaches subject to varying levels of human land use intensity. Temperature data was only available for five of six reaches.

Figure 2.7. Summary of estimated hellbender (*Cryptobranchus alleganiensis*) demographic parameters, water quality and substrate characteristics in six stream reaches stratified across a land use gradient in southwest Virginia, USA. Estimated demographic parameters are specific the population of sub-adult/adult hellbenders in each reach (i.e., individuals $\geq$ 190 mm total body length). The dashed horizontal line is provided as a reference for population growth rates, where growth rates < 1 indicate decline. Density was calculated as the average of 2014 and 2015 estimates, annual apparent survival and population growth rates were extrapolated from monthly estimates. Conductivity values are averages generated from monthly measurements over one year. Note co-variance between density, recruitment (proportion of population composed of 5-10 y olds), conductivity and diameter of substrate marking the 75th sample percentile (D_{75}).

FIGURES

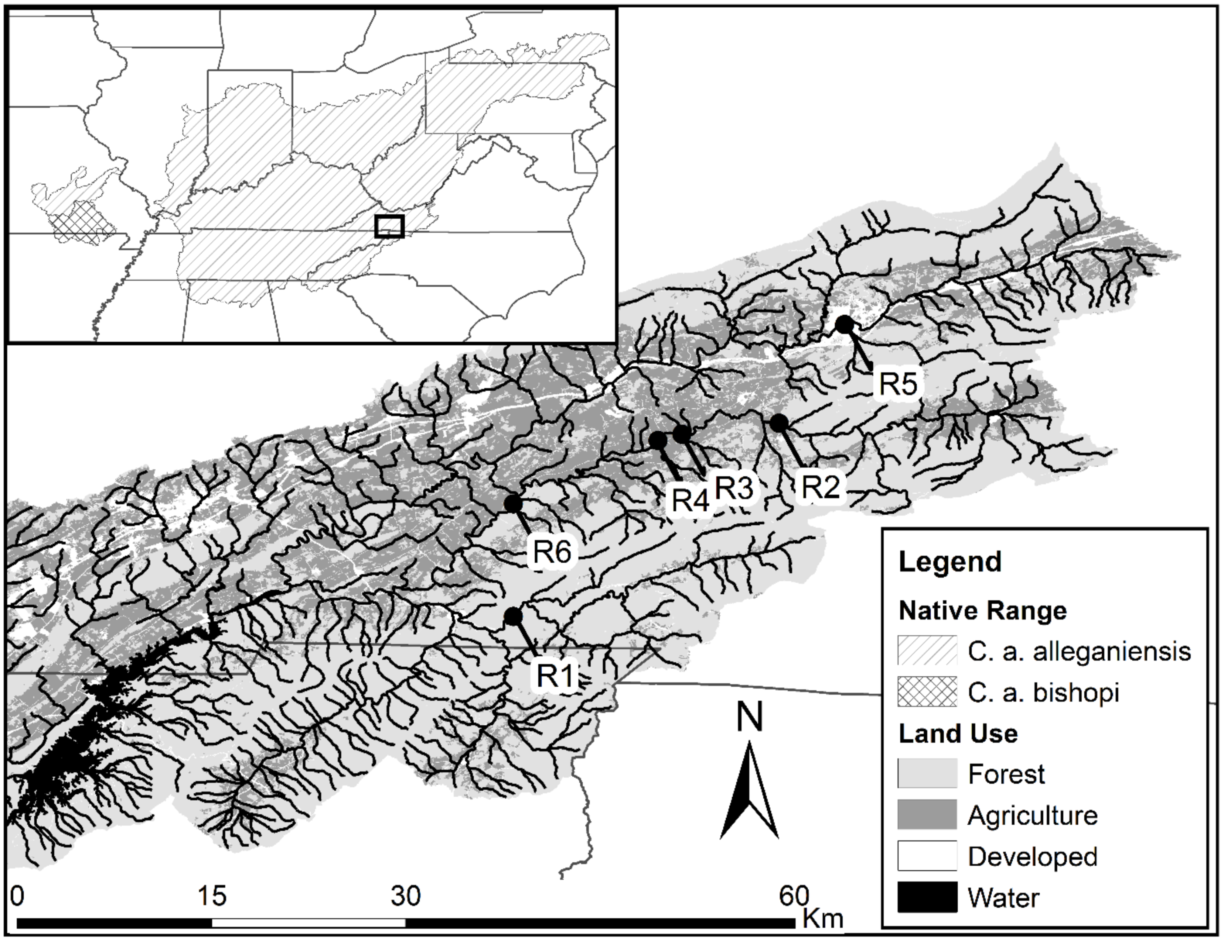

Figure 2.1. Study area and reaches (R1-R6) where hellbender density and demographics were estimated.

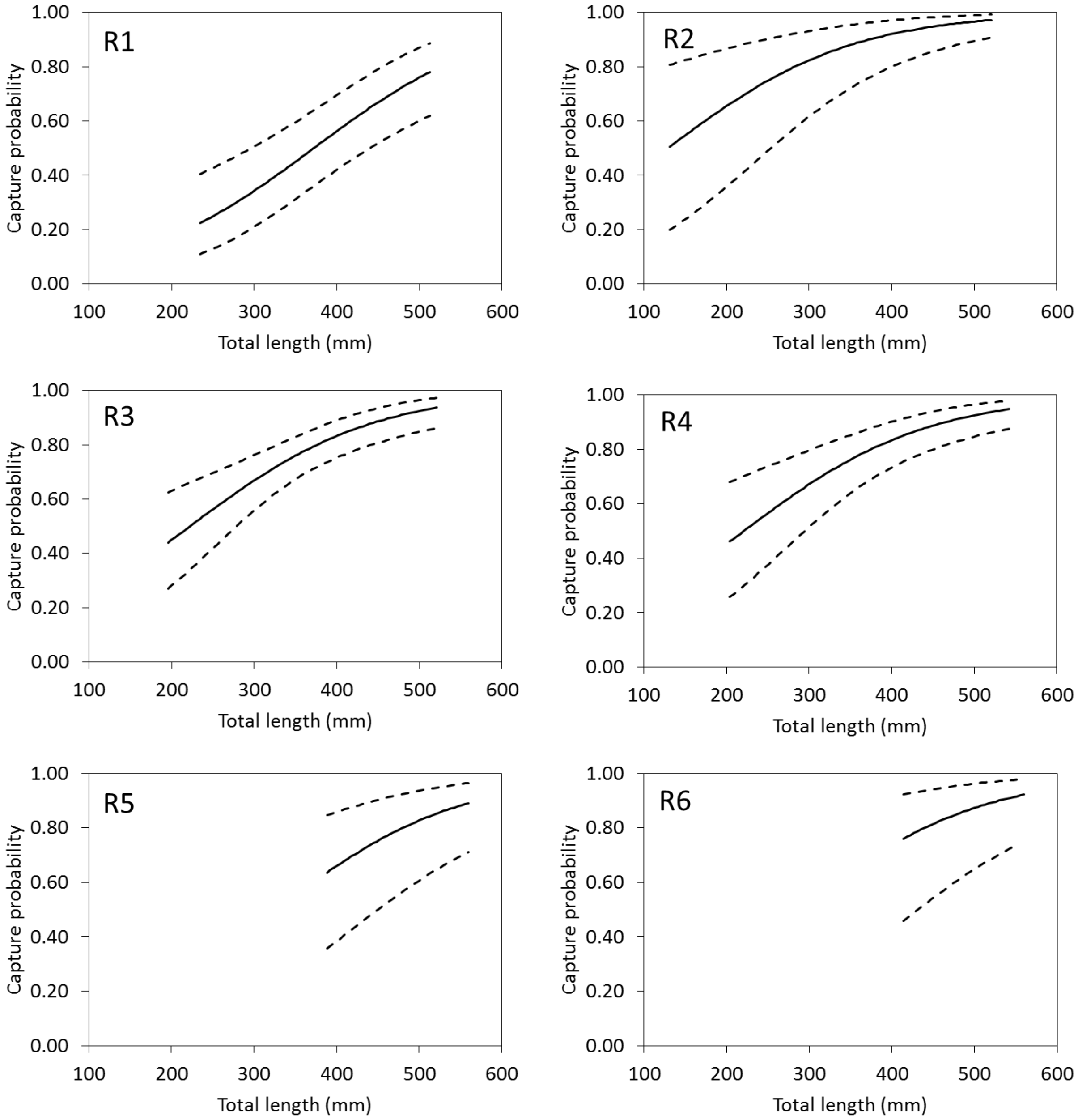

Figure 2.2. Effect of total body length on probability of capture for hellbenders during a single sampling event.

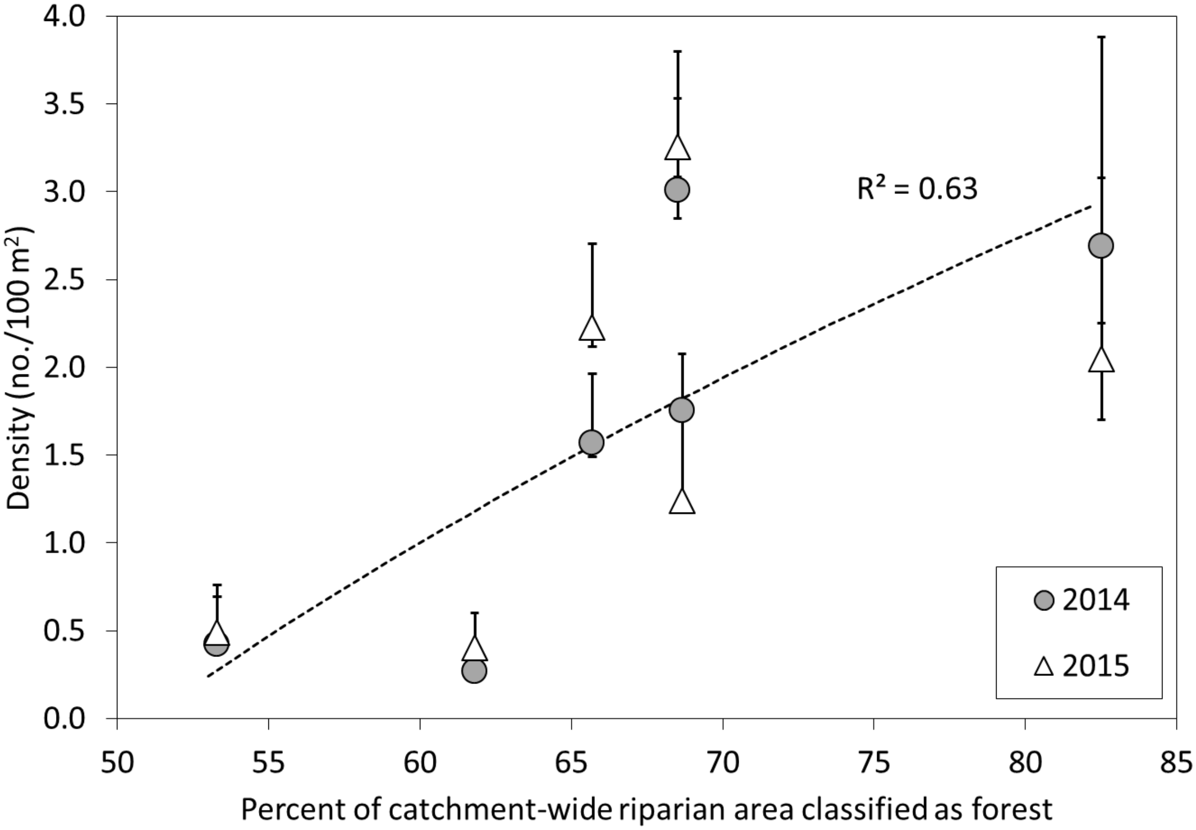

Figure 2.3. Relationship between sub-adult/adult hellbender density and percent of land classified as forest within the upstream catchment-wide riparian area.

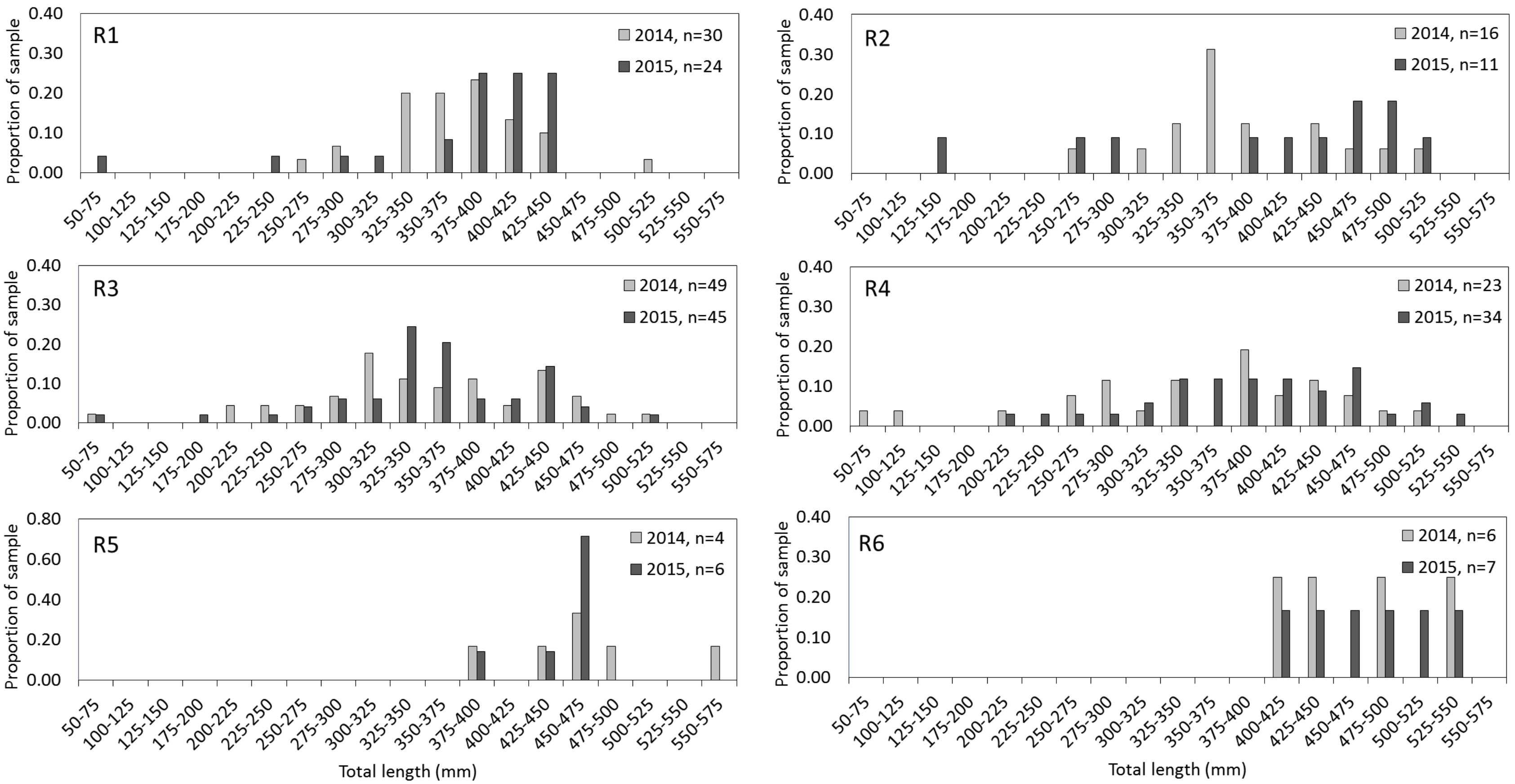

Figure 2.4. Size class distributions of hellbenders collected from six stream reaches (R1-R6) stratified across a land use gradient.

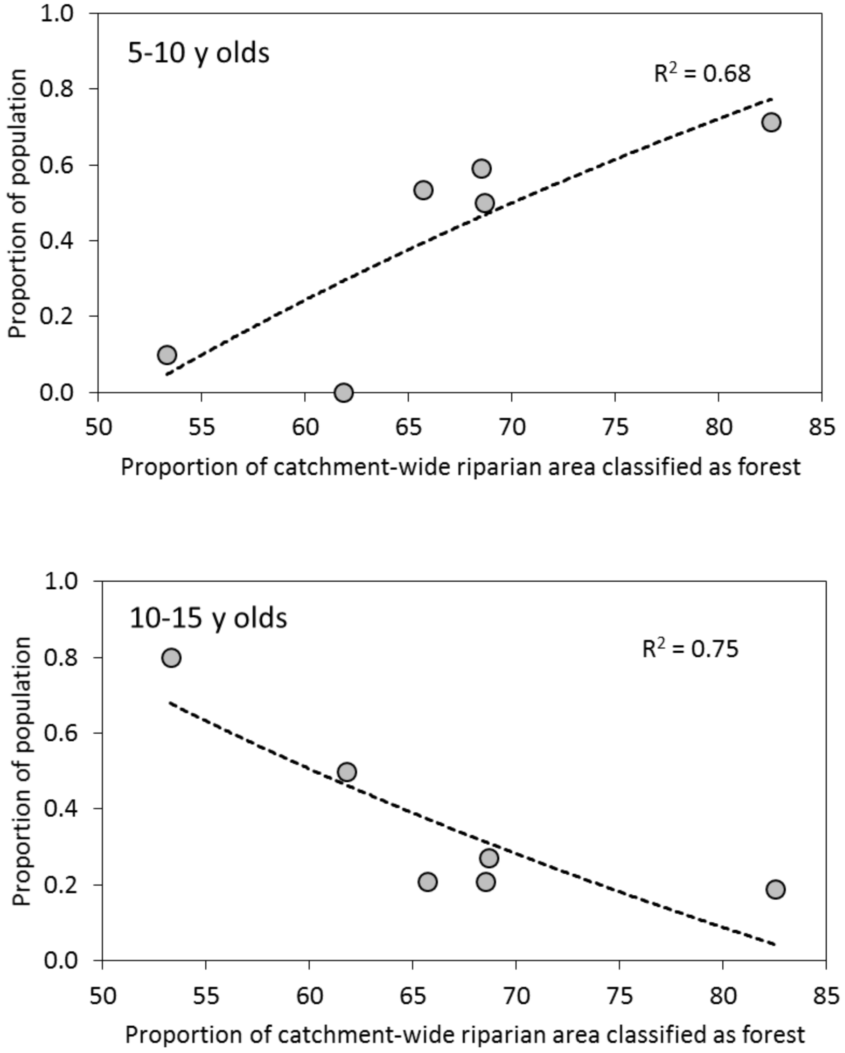

Figure 2.5. Relationship between forest cover in the catchment-wide riparian area and the proportion of hellbender populations made up of young (5-10 year olds) and middle aged (10-15 year olds) adults in six stream reaches.

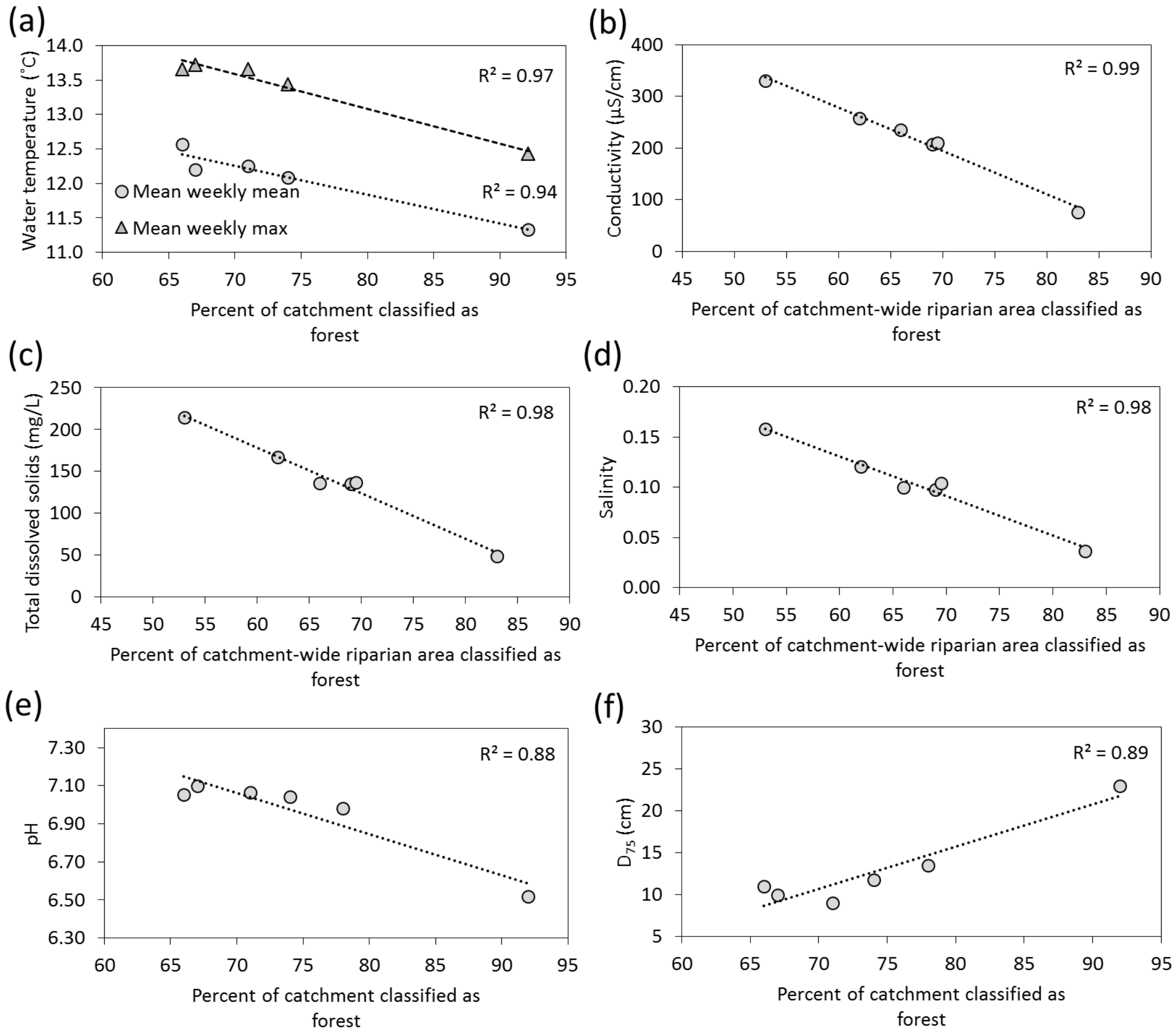

Figure 2.6. Relationship between land use and in-stream habitat characteristics of six stream reaches where hellbender density and demographics were studied.

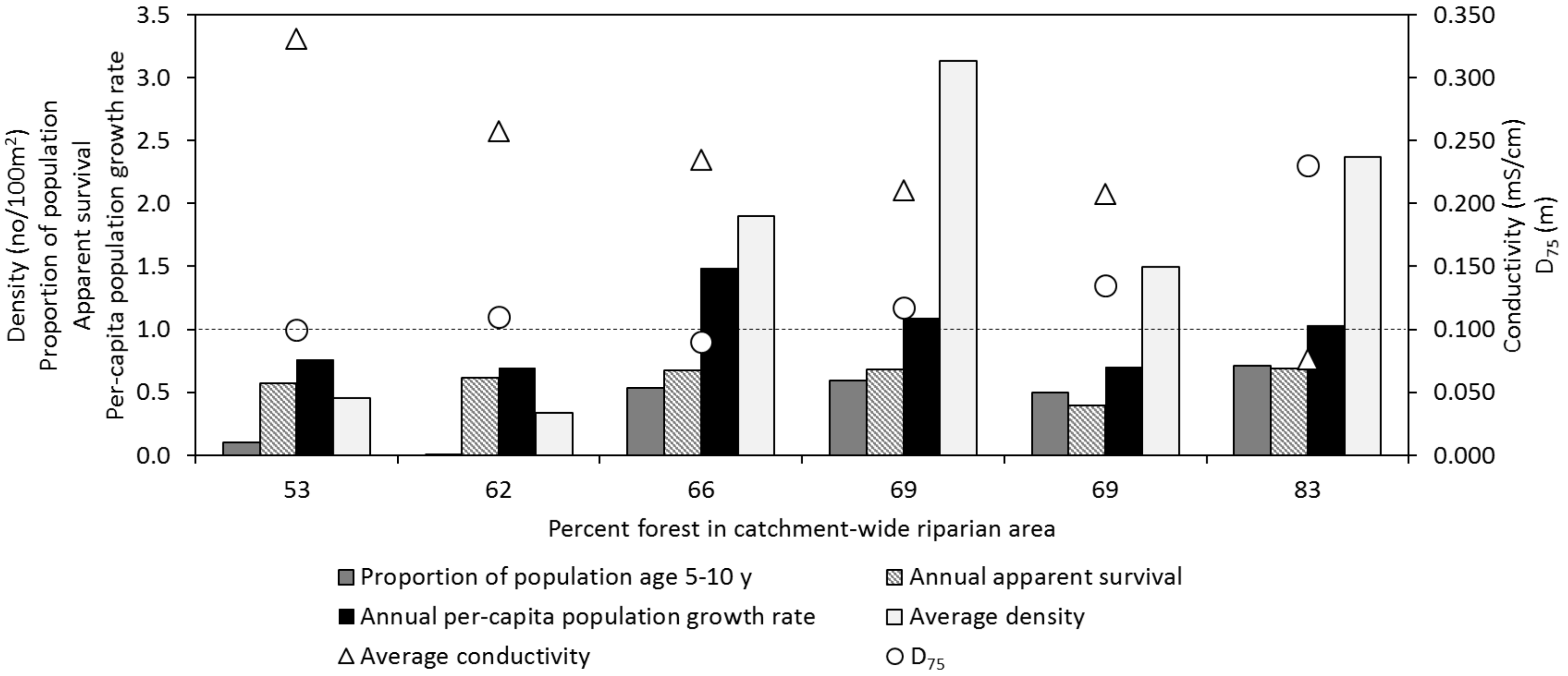

Figure 2.7. Summary of hellbender demographic parameters, water quality and substrate characteristics in six stream reaches stratified across a land use gradient in southwest Virginia, USA.

APPENDIX

Appendix A: Field surveys and hellbender processing

We worked from downstream to upstream, in zig-zag fashion to locate and search all cover objects > 25 cm in diameter. We searched smaller objects when we noticed obvious crevices or openings beneath them. Generally, one or more individuals lifted cover objects from the downstream end while a single snorkeler visually scanned for hellbenders. We relied on tactile searches when cover objects were impossible or dangerous to lift and when crevices could be easily accessed by hand without disturbing rocks. To reduce variability in hellbender detection and catchability due to surveyor experience, the same snorkeler was responsible for visually identifying and capturing hellbenders during every survey.

We inserted PIT tags subcutaneously along the dorsolateral region of the tail, approximately 5 cm posterior to the tail base. We sealed the PIT tag entry site with superglue prior to release to minimize tag loss. Unger et al (2012) demonstrated PIT tag retention rates of 100% over one year when tags were inserted into tail musculature in the same region. We recorded sex based on external morphology (cloacal swelling in males) and measured total length (mm) and mass (g) of each individual. We recorded capture locations using a hand held Garmin global positioning system (GPS; accuracy +/- 3 m). We carefully repositioned substrate to its original condition before returning individuals to the point of capture.

Appendix B: Robust design model development

We used the Huggins (1989; 1991) version of the closed robust design model so that we were able to model probability of capture (p) during a single survey as a function of individual covariates. In our case, preliminary data suggested that size of individuals would influence p. The closed robust design model allowed us to estimate apparent annual survival between primary occasions (the probability of surviving conditional on an individual remaining in the site from one year to the next). However, because we intended to use a more robust data set to investigate apparent survival across reaches we only report estimates of $\widehat{\mathrm{N}}$ and p.

We compiled capture histories for all PIT tagged individuals captured during primary occasions defined above. We coded capture histories as live encounters where on each occasion an individual was either alive or not-encountered, coded by a ‘1’ or ‘0’, respectively. We pooled data from all reaches and defined each reach as a separate group using dummy variable coding. We fit four candidate models to our data that differed only in how p was modeled; where we considered various combinations of reach, year and total body length of individuals as covariates of p (Table 2.2). Our ability to estimate all available parameters in the closed robust design model were limited by the number of sampling occasions and design of our study. For example, with only two primary and two secondary occasions, temporary immigration and emigration ($\gamma'$and γ'', respectively) and probability of a marked animal being recaptured in each primary occasion (c) were inestimable. As a result, we constrained $\gamma' = \gamma'' = 0$, and constrained $p = c$ in all models.

Appendix C: Pradel model development

Due to the variation in sampling effort across reaches (Table 2.1) we conducted a separate analysis for each reach. We pooled capture events such that each month represented a capture occasion, with the exception of robust design surveys that took place in 2014 and 2015 where we also included secondary occasions as a unique survey within a month. We coded histories as live encounters (coded by a ‘1’ or ‘0’) and defined time intervals between occasions in terms of months. We fit an identical set of four candidate models to data from each reach (Table 2.4). Because we were interested in average apparent survival and population growth rates across time, we constrained Φ_m and λ_m to be constant in each reach, but considered size of individuals at initial capture as a covariate of Φ_m to allow for variation in survival or emigration probability (i.e., Pradel models do not distinguish between permanent emigration and death) of different size/age classes. We allowed p to vary among occasions in all of our candidate models, and compared models with and without total body length a covariate of p.

Chapter III. Physiological Indices of Hellbender Salamanders in Response to Land Use and Parasitism

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ABSTRACT

Habitat loss due to land use alteration and exposure to parasites and pathogens are two of the most important and rapidly increasing threats facing amphibians but few studies have examined how land use and parasites exposure might interact. Hellbenders (*Cryptobranchus alleganiensis*) are a species of great conservation concern but are largely data deficient in terms of their response to environmental stressors. We investigated effects of season and two potential stressors (reduced forest cover and parasites) on three indices of adult hellbender physiological condition (body condition, hematocrit and differential white blood cell profiles). We used year-round sampling to examine variation in condition separately during the pre-breeding (1 June – 15 Aug; n = 381 captures), breeding (16 Aug – 31 Nov; n = 281 captures) and post-breeding seasons (1 Dec – 31 May; n = 124 captures). We recorded 786 hellbender capture events from 17 stream reaches stratified across a land use gradient (53-83% forest within a 50 m riparian buffer of streams throughout the catchment) between 2013 and 2015. We only detected parasites (trypanosomes and/or leeches) in areas with > 65% forest cover, where previous studies have found hellbender density to be relatively high (> 1 sub-adult/adult per 100m^2). All three condition indices responded to land use, parasites or both during at least one season. Body condition was not affected by parasites, but declined with forest cover (in contrast with our predictions) during every season and declined during the breeding season only in areas of relatively high forest cover (≥ 65%). Hematocrit was not affected by land use but declined during the post-breeding season as we predicted in response to cooler temperatures and higher dissolved oxygen concentrations in streams, and was lower in hellbenders infected with one or more parasites during every season. N:L ratios peaked during the coolest season (post-breeding) in areas of relatively low forest cover (≤ 65%), but peaked during the breeding season in more forested areas, and increased in response to parasite exposure during all seasons. Our study emphasizes the need for additional research to determine whether exposure to parasites that affect red blood cell parameters might influence vulnerability of amphibians to altered thermal regimes common in areas of reduced forest cover and whether variations in N:L ratios associated with land use might reflect disrupted modulation of hormones that are important mediators of reproductive behavior.

INTRODUCTION

Habitat loss due to land use alteration and exposure to parasites and pathogens are two of the most important and rapidly increasing threats facing amphibians (Gallant *et al.*, 2007; Collins, 2010; Blaustein *et al.*, 2012). Amphibians are currently one of the most endangered group of vertebrates on Earth (Stuart *et al.*, 2004). Human land use intensity can influence the integrity of aquatic habitats via alterations to water quality, substrate and hydrology (Allan, 2004) and can therefore function as an overarching driver of habitat loss for many amphibians. Parasites and pathogens such as *Batrachocytrium dendrobatidis, Ranavirus,* and *Icthyophonus* have been associated with morbidity, mortality and extinction of several amphibian species (Daszak *et al.*, 1999). Spread of newly identified pathogens, such as *B. salamandrivorans*, to novel regions poses a major threat to conservation of amphibian biodiversity (Yap *et al.*, 2015). While land use alteration and parasites/pathogens are broadly recognized as drivers of decline for many amphibians few studies have attempted to understand how land use and pathogen exposure interact in declining populations.

The hellbender (*Cryptobranchus alleganiensis*) is a species of giant salamander (Family Cryptobranchidae) that has recently undergone precipitous declines and is of great conservation concern. Crytobranchids are fully aquatic, long-lived (25 + years; Taber *et al.*, 1975) stream dwelling amphibians. Two of the three extant species of Cryptobranchids are restricted to Asia and are critically endangered in China (*Andrias davidianus*) and near threatened with extinction in Japan (*A. japonicas*; http://www.iucn.org; accessed February 19 2016). Hellbenders are unique to North America and both subspecies (Ozark hellbender, *C. a. bishopi* and eastern hellbender *C. a. alleganiensis*) are of great conservation concern. Several populations declined by an average of 70-80% between the mid 1970s and late 1990s (Wheeler *et al.*, 2003), and populations throughout the remainder of the species' range are largely suspected to have undergone declines of similar magnitude. Reasons for historic declines are unclear but are suspected to be driven by a multitude of factors including deforestation and increased fine sediment loads in streams, altered water quality, and exposure to parasites and pathogens (Williams *et al.*, 1981; Briggler *et al.*, 2007; Wheeler *et al.*, 2003; Foster *et al.*, 2009). Both hellbender subspecies are protected under CITES (Federal Register, 2011). The Ozark hellbender is listed as a federally endangered species under the Endangered Species Act (Federal Register, 2011) and the eastern hellbender is being considered for federal protection. Similar to many amphibian species, a major challenge to hellbender conservation is our limited understanding of factors responsible for declines.

The potential for land use alteration and parasite exposure to elicit deleterious effects on hellbenders has become increasingly clear. Recent studies have found that hellbender occurrence (Quinn *et al.*, 2013; Pugh *et al.*, 2016; Jachowski *et al.*, in review), population density, and recruitment (Jachowski *et al.*, in prep) are all negatively associated with loss of forest cover in the surrounding landscape. However, few studies have attempted to understand how land use might influence physiology of individual hellbenders (Hopkins & DuRant, 2011). Recent work has also highlighted noteworthy impacts of parasites on hellbenders (DuRant *et al.*, 2015; Hopkins *et al.*, 2016). For example DuRant *et al.* (2015) showed that a newly described species of parasitic leech (*Placobdella appalachiensis*, Hopkins *et al.*, 2014) detected in Virginia hellbender populations is capable of disrupting the normal adrenocortical response (i.e., increase in plasma glucorticoids in response to an acute stressor) of their hellbender host. This is important because the adrenocortical response is an adaptive response that mediates the mobilization of energy stores in response to acute disturbance and is modulated seasonally to support energetic demands such as reproduction (Wingfield *et al.*, 2013). Additionally, leeches are suspected to act as vectors for a sympatric blood parasite whose effect on hellbenders remains largely unclear (unknown sp. of trypanosome, Davis &Hopkins, 2013; DuRant *et al.*, 2015; Hopkins *et al.*, 2016). Given that hellbenders are known to respond to land use and parasite exposure when examined separately, more work is needed to understand how these factors might interact to elicit deleterious effects on hellbenders exposed to both factors simultaneously.

The purpose of our study was to determine whether land use and/or parasitism influenced measures of hellbender physiological condition (body condition, hematocrit, hemoglobin, and differential white blood cell profiles). Our first objective was to determine whether condition varied in a predictable fashion between seasons of biological interest (pre-breeding, breeding, post-breeding). We hypothesized that all measures of physiological condition would reflect the species' discrete annual breeding cycle and/or temperature variation. We predicted that body condition would be highest during the pre-breeding season and decrease during the breeding season as a result of egg deposition by females, and increased energy expenditure accompanied by reduced foraging by males focused on defense of breeding sites. We predicted that hematocrit and hemoglobin would be lowest during the coolest season (post-breeding; winter-spring) as a result of reduced metabolism of hellbenders and seasonal increases in dissolved oxygen. We predicted that white blood cell differentials would follow predictions from experimental work describing temperature effects on amphibian immunity, where neutrophils and neutrophil-to-lymphocyte (N:L) ratios would be highest and lymphocyte and eosinophil percentages lowest during the coolest season (post-breeding) and the reverse would be true during the warmest season (pre-breeding). Our second objective was to determine whether, within each season, measures of physiological condition could be explained by land use, parasite exposure or a combination of factors. We hypothesized that reduced forest cover and parasitic infection would negatively affect condition of hellbenders in all seasons, where we predicted that reduced forest cover and/or parasite exposure would lead to reduced body condition, reduced hematocrit and hemoglobin and elevated NL ratios (considered a proxy of baseline physiological stress in amphibians, Davis *et al.*, 2008). We predicted that eosinophil percentages would be similar across a land use gradient but higher in parasitized relative to uninfected individuals in all seasons.

METHODS

Species background

Hellbenders are large (up to 74 cm), long-lived, fully aquatic amphibians native to much of the eastern U.S. (Taber *et al.*, 1975; Fig. 3.1). Hellbenders rely intensively on rocky crevices for shelter and nesting (Nickerson *et al.*, 2003) and rely primarily on cutaneous respiration and thus require well-oxygenated environments (Guimond & Hutchison, 1973; Coe *et al.*, 2016). Typical habitat includes swift flowing, cool, rocky streams, but the species can also occur in large rivers with water temperatures that exceed 25 ˚C in summer. Larvae generally consume small macroinvertebrates such as *Ephemoptera* and *Trichoptera* inhabit interstitial spaces amongst larger cobbles and small boulders (Hecht-Kardasz, 2011). Sub-adults and adults consume crayfish as their primary prey (Peterson *et al.*, 1989) and exhibit high site fidelity to specific stream reaches and cavities beneath boulders and bedrock (Nickerson & Mays, 1973a-b; Bodinof *et al.*, 2012a). Individuals metamorphose at approximately 12.5 cm total length (~ 18 mo.; Nickerson & Mays, 1973b) and reach sexual maturity at approximately six to eight years (Peterson *et al.*, 1988). In our system, secondary male sex characteristics (cloacal swelling during the breeding season) become evident at approximately 29 cm total length. Hellbenders can exceed 25 years of age (Taber *et al.*, 1975). Though hellbender reproductive ecology has been poorly studied, it is well established that spawning occurs annually (Smith, 1907). In our system spawning spans a brief period (~ 14 d; unpublished data) in late August or early September. It is unclear how frequently females breed, but gravid individuals deposit up to 500 or more eggs (Topping & Ingersol, 1981) in a nest cavity guarded by a single male. Adults compete for nest sites, exhibit conspecific aggression in the form of biting, and members of both sexes are known to cannibalize eggs of conspecifics on occasion (Smith, 1907). In our system, hatching occurs in late November to early December (unpub data).

Physiological condition indices

Body condition.---Body condition refers to the state of an individual's energetic stores, where individuals with more energy reserves are interpreted as being in better condition. Indices used to quantify body condition are varied but are typically calculated as a measure of relative mass given an individual's structural size (Jakob *et al.*, 1996; Green, 2001; Shulte-Hostedde *et al.*, 2005; Peig & Green, 2009). Condition can vary as a result of resource availability/acquisition or an individual's ability to cope with environmental alterations (Jakob *et al.*, 1996) but can also fluctuate rapidly in response to spawning in some ectotherms (Brown & Murphy, 2004). Among amphibians, body condition has been linked to dispersal (Lowe *et al.*, 2003, 2006), survival, and reproductive success (Semlitsch, 1987; Reading & Clarke, 2005; Reading, 2007; Scott *et al.*, 2007; Garner *et al.*, 2011; Bodinof *et al.*, 2012b).

Red blood cell parameters.---The proportion of blood made up of red blood cells (hematocrit) and hemoglobin reflect the oxygen carrying capacity of blood (Hillman, 1976). Blood oxygen carrying capacity generally increases with hematocrit and can vary seasonally in some vertebrates (Withers *et al.*, 1991). Among ectotherms, increases in hematocrit can reflect thermal or handling stress (Roche & Boge, 1996; Biron & Benfey, 1994) and decreases in hematocrit may be indicative of anemia, dehydration, parasitic infection (Toque, 1993) or exposure to pollutants (Al-Attar *et al.*, 2005). Studies have shown that hematocrit levels of hellbenders vary across sampling localities (Solis *et al.*, 2007; Huang *et al.*, 2010). However, few studies have attempted to investigate the role of extrinsic factors (e.g., parasites, season) on hellbender red blood cell parameters (Hopkins *et al.*, 2016).

White blood cell parameters.---Differential white blood cell counts refer to the relative proportion of circulating white blood cells made up of each major white blood cell type and are a commonly used indicator of health for a wide range of vertebrates including amphibians (Davis *et al.*, 2008). In particular, the ratio of neutrophils-to-lymphocytes (hereafter N:L ratios) is recognized as a useful measure of relative physiological condition among individuals. N:L ratios of amphibians fluctuate predictably in response to temperature as a result proliferation of neutrophils and suppressed proliferation of lymphocytes at cool temperatures (Maniero & Carey, 1997; Raffel *et al.*, 2006). However, within a season, relatively heightened N:L ratios can indicate activation of an immune response in response to infection. However, N:L ratios are also considered to be a proxy of circulating glucocorticoid hormones (i.e., stress hormones) released via the hypothalamic-pituitary-adrenal axis in response to a range of factors (Davis *et al.*, 2008). Eosinophil percentages respond positively to infection but negatively to increases in circulating glucocorticoid hormones. Thus, examining the relative proportion of eosinophils alongside N:L ratios can help to tease apart the roles of infection versus other factors on individual state.

Study area

Our study area included the New River and South Fork Holston River drainages in southwestern Virginia (Fig. 3.1). Together, these drainages mark the eastern extent of the hellbender's range in the state. Our study sites included 17 stream reaches from eight streams (n = 1-8 reaches per stream). Percent forest cover within the catchment upstream of a reach ranged from 57-92% and forest cover within all riparian areas (50 m buffer on either side of main stems and tributaries) throughout the catchment ranged from 53-83% (2011 national land cover dataset;

USGS, 2014). We selected 50 m as the width of riparian areas based on findings from studies that indicate biotic impacts of forest removal are negated at buffer widths ≥ 30 m (Davies & Nelson, 1994; Frimpong *et al.*, 2005; Peterman & Semlitsch, 2009). We had estimates of sub-adult/adult (≥ 19 cm) density for six of the reaches that indicated hellbender population density generally increased with forest cover in our study area (Jachowski *et al.*, in prep; Table 3.1).

Sampling

We sampled hellbenders approximately monthly between Aug 2013 and Nov 2015. We used snorkeling while turning rocks (Nickerson *et al.*, 2003), tactile searches and surveys of artificial shelters (Briggler & Ackerson, 2012) deployed at a subset of study reaches to locate and capture hellbenders. All captures occurred between 0825 and 1730 h. Upon capture we quickly transported hellbenders to the bank for processing. We marked hellbenders with uniquely coded passive integrated transponder (PIT) tags (models HPT8 or HPT12; Biomark Inc., Boise, ID, USA). We inserted PIT tags subcutaneously along the dorsolateral region of the tail, approximately 5 cm posterior to the tail base. We sealed the PIT tag entry site with superglue prior to release to minimize tag loss. We recorded sex based on external morphology (cloacal swelling in males) when evident. We weighed each individual to the nearest gram using Pesola® spring scales (Pesola AG, Schindellegi, Switzerland) and recorded total length to the nearest mm. We conducted one to three independent (conducted by unique observers) visual scans to determine the presence of leeches and recorded the total number of leeches when they were present. We obtained whole blood samples (≥ 50 μL; but not more than 100 μL/100 g body mass) for trypanosome screening and measurement of red and white blood cell parameters within 20 min of capture following methods outlined by Hopkins & DuRant (2011).

Immediately after blood collection, we partially filled two 75 µL heparinized hematocrit tubes with whole blood from the syringe for measuring hematocrit and for trypanosome screening via a buffy coat technique (Hopkins *et al.*, 2016). Hematocrit tubes were stored on ice, transported to the laboratory and centrifuged at 5*g* for 5 min within 10 h of collection. We quantified hematocrit for each hellbender using a standard hematocrit capillary tube reader (McCormick Scientific, St. Louis, MO, USA). We averaged hematocrit values across tubes collected from each individual and used the averages in our analyses. We removed most of the plasma from each capillary tube using a Hamilton syringe, and made duplicate smears using the buffy coat (visible layer of WBC) and approximately 2-3 µL of plasma (to aid in evenness of smears) using a standard two-slide technique for screening trypanosomes (Hopkins *et al.*, 2016). We air dried buffy coat smears and stored them dry until fixing and staining with a Wright-Giemsa stain (Camco Quik Stain II) within one to six weeks of collection.

We measured hemoglobin for a subset of individuals captured between July-Aug 2014 and measured WBC parameters for a subset of individuals captured between Oct 2014-Aug 2015. We measured hemoglobin (g/dL) immediately after blood collection by placing one or two drops of whole blood on a cuvette and inserting it into a Hemocue Hb Analyzer Hb201, which relies on azide–methemoglobin reaction (Velguth *et al.*, 2010). We ran duplicate cuvettes for each individual and used the average reading in our analyses. At the same time hematocrit tubes were being filled and hemoglobin measured, we made duplicate blood smears using a standard two-slide technique for WBC counts. We air dried slides and stored them dry until fixing and staining with a Wright-Giemsa stain (Camco Quik Stain II) within three weeks of collection.

To determine whether hellbenders were infected with trypanosomes we examined buffy coat smears at x400 magnification. A pilot study indicated the probability of detecting at least one trypanosome after viewing 50 random fields of view from a buffy coat smear was 0.90 ± 0.02 SE (i.e., cumulative probability of detection after scanning two slides = 0.99; unpubl data). Slides were examined by one of three trained observers (C. Jachowski, V. Alaasam, A. Blumenthal), after confirming that detection probabilities for trypanosomes was robust to observer differences (detection probability ~ 0.90 for all observers). Observers were blind to the identity of each slide. We focused on quantifying presence/absence of trypanosomes since methods to quantify intensity of infections from buffy coat smears have not been validated. Observers scanned 50 random fields of view per slide and only screened a second slide if trypanosomes were not detected in the first slide. We considered individuals infected if at least one trypanosome was detected and uninfected if no trypanosomes were detected after scanning both buffy coat slides.

Body condition.--- We calculated body condition as a scaled mass index (SMI; Peig & Green, 2009). Briefly, scaled mass is interpreted as the estimated mass of an individual at a reference value of structural size. The SMI has been shown to be a more accurate indicator of 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 & Green, 2009; MacCracken & Stebbings, 2012). We used total length to quantify structural size of hellbenders given that urodeles store considerable energy reserves in the tail (Fitzpatrick 1976; Takahashi & Pauley, 2010). We calculated scaled mass for each individual as

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

where M_i was mass (g) and L_i was total length (cm) of individual *i*, L_0 was our selected reference category of total length (40 cm) 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 & Green, 2009). We used data from all known sex individuals collected from our study system during and prior to (since 2007) the current study in our regression (n = 1182 records (588 F; 594 M). Log-transformed mass and total length were positively correlated for females (r = 0.96) and males (r = 0.95). Coefficients from general linear mixed regression models (we included individual as random effect) were $\beta_0 = -2.39 \pm 0.07$ SE and $\beta_{Log10(TLcm)} = 3.10 \pm 0.04$ SE for females and $\beta_0 = -1.89 \pm 0.07$ SE and $\beta_{Log10(TLcm)} = 2.79 \pm 0.04$ SE for males. Scaled mass was strongly correlated with body condition estimated as residuals (Jakob *et al.*, 1995; Schulte-Hostedde *et al.* 2005) from each regression (r = 0.98 for both sexes). However we selected SMI as our measure of body condition in the current study due to the relative ease of interpreting SMI values and the ease of comparing values across studies (see Peig & Green, 2009).

White blood cell parameters.--- We examined whole blood smears at x400 magnification to determine differential white blood cell (WBC) profiles of hellbenders. All slides were examined by a single observer (V. Alaasam) who was blinded to the identity of each slide. At least 100 leukocytes were counted and only fields of view with even distributions of cells were used. Cells were identified as lymphocytes, neutrophils, eosinophils, basophils or monocytes following Turner (1988), Thrall *et al.* (2004) and Campbell & Ellis (2007). We determined the proportion of each cell type by dividing the number of cells of that type by the total number of WBC counted. We calculated N:L ratios by dividing the proportion of WBCs categorized as neutrophils by the proportion of WBCs categorized as lymphocytes. Monocytes (< 2%) and basophils (< 9%) were in very low abundance and are not discussed further.

Data analysis

Because we defined seasons in reference to hellbender reproductive cycles we restricted our analysis to sexually mature adults (≥ 29 cm) of known sex. Prior to running analysis, we checked for correlation among physiological measures. Hematocrit (%) and hemoglobin (g/dL) were positively correlated (r = 0.85) with each other in the subsample of individuals for which we had both measures (n = 198). Coefficients from a general linear mixed regression model (we included individual as a random effect) relating hemoglobin to hematocrit were $\beta_0 = 0.59 \pm 0.38$ SE and $\beta_{hematocrit(\%)} = 0.25 \pm 0.01$ SE. For simplicity, and because our hematocrit sample (n = 765) was considerably larger than our hemoglobin sample, we only considered hematocrit further. SMI was not correlated with hematocrit (r = - 0.03) or N:L ratios (r = 0.03) and hematocrit was only modestly associated with N:L ratios (r = - 0.43). Multiple WBC parameters were correlated with one another. To account for interdependence among WBC variables and reduce dimensionality of our dataset we performed a principal components analysis (PCA) based on a correlation matrix of WBC parameters. Prior to performing the PCA we applied square-root (eosinophils and neutrophils), power (lymphocytes) or cube-root (N:L ratios) transformations to WBC parameters in order to meet assumptions of normality. Following transformation all WBC parameters scaled between 0 and 1.25.

Model development

We used an information theoretic approach to investigate relative support for our hypotheses regarding factors that influence each measure of hellbender physiological condition. As a result, we used a nearly identical set of six candidate models (Table 3.2) in each analysis.

We defined biologically relevant seasons based on observed reproductive cycles of hellbenders in our system. We have observed nest establishment as early as 18 Aug and as late as 17 Sep and hatching as early as 16 Oct and as late as 1 Dec (unpubl data). Thus, we defined the breeding season as 16 Aug–31 Nov, to encompass spawning through hatching. We defined the pre-breeding season as 1 June–15 Aug, which correlates with the warmest time of year, maturation of oocytes in females and territory establishment for males. We defined the post-breeding season as 1 Dec-31 May, which includes the coolest time of year when individuals are expected to be recovering from any costs (e.g., injuries, reduced body condition) incurred during the breeding season. In our system forest cover throughout the catchment was strongly correlated with forest cover within the catchment-wide riparian area (r = 0.93). To facilitate comparison of our results with studies that describe effects of forest cover on hellbender demography (Jachowski *et al.*, in prep) we used catchment-wide (hereafter Forest_R) as the land use variable in all of our models and divided percentages by 100 to reduce the range of covariate values prior to analysis. We used a categorical term with three levels (uninfected, single infection with leeches or trypanosomes, or coinfected with leeches and trypanosomes) to represent parasite state in all analyses. Our decision to use parasite state was based on preliminary analyses conducted for each physiological variable, where models including parasite as a state were as well or better supported (based on model ranking criteria) than models including separate terms for trypanosomes (always a binomial indicating presence-absence) and leeches (either a continuous intensity term or a binomial term). We also conducted preliminary analyses to examine effects of sex, size (total length) and SMI (for hematocrit and WBC parameters) on each measure of condition. We standardized total length $\left(\frac{x - \bar{x}}{sd(x)}\right)$ prior to analyses. We included variables that were supported in every model within the given candidate set. Finally, we included a random effect term for individual in all models.

Our simplest model (season only) represented a null hypothesis that hellbender condition was driven entirely by predictable seasonal fluctuation in temperature, breeding activity and availability of resources but would be similar across a land use gradient and among parasitized and un-parasitized individuals. Our additive land use model (Season + Forest) represented our hypothesis that condition would increase with forest cover. Our season-land use interaction model (Season*Forest) represented our hypothesis that land use effects are season-specific. Our additive parasite model (Season + Parasites) represented our hypothesis that condition of parasitized individuals would be lower than that of uninfected individuals but otherwise similar across sites. Finally, our two most complex models included all effects (Season + Forest + Parasites and Season*Forest + Parasites) and varied only by whether season and land use effects were additive or interactive. We were unable to consider land use-parasite interactions due to the fact that parasites were not detected across the land use gradient in our study.

We performed all analyses in program R (Team, 2013). We plotted data and used Shapiro-Wilk tests to ensure our response variables were normally distributed. SMI data for females appeared to be leptokurtic but were not skewed, thus we did not attempt to transform them. We fit generalized linear mixed models using an identity link via maximum likelihood using the package ‘lmer’ in program R (Team, 2013). We used Akaike Information Criterion corrected for small samples (AIC_c) to rank competing models in each analysis. When the upper 90% of AICc weight was distributed over more than one model, we used model averaging

(Burnham & Anderson, 2002) to obtain model averaged coefficients, their unconditional standard errors and 95% confidence intervals. For each analysis we report predictions from the top-ranked model or the confidence set of models (after model averaging) along with their 95% confidence intervals unless otherwise noted.

RESULTS

We obtained a full parasite dataset for 786 adult hellbenders (382 F, 404 M, 379 unique individuals) between 2013 and 2015 (Table 3.1). Samples sizes varied by analysis (range of n = 320-741; Table 3.2). Parasites were patchily distributed among sampling reaches. We detected at least one parasite in only four of 17 reaches and in two of eight streams (Table 3.1). We detected trypanosomes and leeches in three reaches and detected only trypanosomes (but not leeches) in the fourth reach. We never observed leeches or trypanosomes in reaches subject to less than 65% Forest_R (Table 3.1). Reaches with parasites contained relatively high densities of sub-adult/adult hellbenders (Table 3.1). As a result, nearly 70% (550 of 786) of captures were characterized as infected by at least one parasite (Table 3.3). We detected leeches and trypanosomes in every season. Among captures from reaches where each parasite occurred, infection state was not associated with total length of adults but appeared to vary among sexes in some seasons (Table 3.3). Prevalence of leeches was lower for females only during the post-breeding season and prevalence of trypanosomes was lower for females only during the pre-breeding season (Table 3.3). Otherwise, trypanosome prevalence was relatively consistent among seasons relative to leech prevalence that peaked during the breeding season (Table 3.3). Single infections by trypanosomes (295 of 550 or 53% of parasitized individuals) were over 10 times more common than single infections by leeches (31 of 550 or 5% of parasitized individuals). Most cases of leech infection (224 of 255 or 88%) were coinfections.

Body condition

Body condition varied with land use, sex and season but did not vary among parasitized and un-parasitized individuals (Table 3.4-3.5). In contrast to our predictions, condition declined with forest cover for both males and females regardless of season (Fig. 3.1). Among reaches with less than about 65% Forest_R females and males were in similar condition during each season, whereas in areas with ≥ 65% Forest_R males tended to be in better condition than females during every season (Fig. 3.1). Partially following our predictions, condition was lowest during the breeding season, but only in areas with more than about 65% Forest_R (Fig. 3.1). Furthermore, the relative difference between pre-breeding and breeding season condition increased in magnitude as forest cover increased. The average mass lost between the pre-breeding and breeding season for a 40 cm long individual was predicted to be 11 g for females and 13 g, for males when Forest_R was 53% versus 35 g and 39 g for females and males, respectively, when Forest_R was 83%.

Hematocrit

Hematocrit varied with season, parasite state and size of individuals, but was not affected by land use (Table 3.4-3.5). As predicted, hematocrit was lowest during the coolest time of year (post-breeding season) and lower in individuals infected with at least one parasite relative to individuals that were uninfected (Fig. 3.2a). Regardless of parasite state, hematocrit increased with size of an individual in every season (Fig. 2b). We found no evidence that hematocrit differed between individuals infected with a single parasite and individuals that were coinfected (Table 3.5; Fig. 3.2a).

White blood cell parameters

The first principal component in our WBC PCA explained 83% of the variance in hellbender WBC parameters and loaded positively with neutrophils, N:L ratios and eosinophils and negatively with lymphocytes (Table 3.6). Given the large proportion of variance explained by a single principal component we used scores (hereafter WBC PC1) from the first principal component as a response variable in our WBC analysis and do not discuss other principal components further. White blood cell PC1 increased exponentially as N:L ratios increased and linearly as eosinophil percentages increased, indicating individuals with high N:L ratios also had relatively high eosinophil percentages (Fig. 3.3).

Hellbender WBC parameters varied with land use, season, parasites and total length of individuals, as indicated by unanimous model ranking support for our most saturated model (Table 3.4-3.5). N:L ratios and eosinophils increased with hellbender total length in all seasons regardless of land use and parasite state (Fig. 3.4). After accounting for total length, N:L ratios and eosinophil percentages were higher in hellbenders that were infected by at least one parasite than in hellbenders that were uninfected (Fig. 3.5). We found no evidence that N:L ratios or eosinophil percentages differed between individuals infected with a single parasite and individuals that were coinfected (Table 3.5). As predicted, N:L ratios were lowest during the warmest time of year (pre-breeding season) regardless of land use. The season when N:L ratios peaked varied with land use (Fig. 3.5). In areas below about 65% Forest_R, N:L ratios peaked during the post-breeding season as we predicted based on temperature. In contrast to our predictions, however, N:L ratios peaked during the breeding season in areas with > 65% Forest_R, even after parasites and total length were accounted for (Fig. 3.5).

DISCUSSION

Our study provides fundamental insight into the potential for land use and parasite exposure to alter physiological condition of individual amphibians. All measures of hellbender physiological condition that we examined responded to land use, parasite exposure or both. We found no evidence of parasites in areas subject to relatively human high land use intensity (Forest_R < 65%), which is consistent with findings that parasite prevalence is correlated with host density (Arneberg *et al.*, 1998) and findings that parasite communities are often depauperate in heavily altered systems (Hudson *et al.*, 2006; Blanar *et al.*, 2009; Marcogliese *et al.* 2009; Koprivnikar *et al.*, 2012). Among more heavily forested areas, hellbenders were exposed to parasites year-round but prevalence varied by season and host sex (Table 3.2). In all seasons, hellbenders exposed to parasites exhibited altered WBC profiles (increased N:L ratios and eosinophils) and decreased hematocrit, the latter of which is consistent with anaemia caused by trypanosomiasis in fishes (Woo, 1979; Khan *et al.*, 1980). Hellbenders exposed to low forest cover were in better body condition than hellbenders in more heavily forested areas but exhibited static body condition across seasons. Additionally, N:L ratios of hellbenders in areas of low forest cover were also relatively static among seasons (especially between the pre-breeding and breeding season) when compared to hellbenders in areas of relatively high forest cover. Collectively, our findings suggests that effects of reduced forest cover and parasite exposure on amphibians can be complex and context dependent and that these stressors have the potential to act in an additive, synergistic or perhaps agonistic (if reduced forest cover reduces parasite exposure) manner in free-living amphibian systems.

Hellbenders infected with parasites exhibited a shift in leukocyte profiles consistent with previous findings (Hopkins *et al.*, 2016). In contrast to previous findings however, we found that hellbenders exposed to parasites also exhibited reduced hematocrit relative to uninfected individuals. Our ability to detect effects of parasites on hematocrit was probably the result of our relatively large sample size and inclusion of capture events from sites where leeches and trypanosomes were absent or occurred at very low prevalence. For example, because prevalence can be high where parasites occur (Table 3.2), individuals that do not harbor current infections are likely to have been exposed in the recent past and may exhibit pathology from recent exposure; potentially making it difficult to detect effects within a single locality (e.g., as in Hopkins *et al.*, 2016). Hopkins *et al.* (2016) reported that parasite exposure was associated with total length of individual hellbenders, while we found no association between total length and parasite state; most likely because our study was restricted to mature adults while Hopkins *et al.* (2016) considered juveniles and subadults. Because hematocrit can function as a critical determinant of blood-oxygen capacity in amphibians (Hillman, 1976), more work is needed to determine whether trypanosomes and/or leeches may compromise the ability of hellbenders to tolerate stressors that reduce the availability of oxygen in the environment (e.g., reduced tree canopy cover in combination with a warming climate) and to understand factors that might increase hellbender exposure to leeches and trypanosomes.

Our study is the first to report any evidence of sublethal physiological effects of land use on hellbenders. A growing number of studies have documented effects of land use on hellbender occurrence (Quinn *et al.*, 2013; Pugh *et al.*, 2015; Jachowski *et al.*, in press) and density and demographics (Jachowski *et al.*, in prep), emphasizing the potential for land use to act as an overarching driver of population level changes. However, we aware of only one other study that has investigated effects of land use on individual hellbender physiology (Hopkins & DuRant, 2011). Hopkins & DuRant (2011) found no effect of land use on measures of innate immunity and stress physiology in hellbenders from two stream reaches that varied in terms of surrounding land use. Importantly, both reaches studied by Hopkins & DuRant (2011) were characterized by relatively high forest cover (i.e., 70% and 78% catchment forest cover) in comparison to the greater number of sites distributed across a much broader land use gradient examined in the current study. Surprisingly, we found that hellbenders exposed to the highest intensity of human land use were in better condition, during all seasons, than hellbenders in more forested areas. Among amphibians, body condition is generally interpreted as a proxy of energy reserves and has been recognized as an indicator of habitat quality and food availability (Sztatecsny & Schabetsberger, 2005), reproductive investment (Ryser, 1989; Castellano *et al.*, 2004), conspecific density and climatic changes (Reading & Clarke, 1995). The fact that density of sub-adult/adult hellbenders is correlated with forest cover in our system (Jachowski *et al.*, in prep) suggests that the trend we observed may be the result of increased conspecific density (see Table 3.1) and perhaps top-down effects of hellbenders and other predators on the prey base in areas of high density/forest cover. Alternatively the prey base within sites subject to higher land use intensity may have been elevated for reasons other than low density. For example, recent work has demonstrated that an increased prey base resulting from nutrient enrichment and altered thermal regimes can function as a mechanism linking land use to increased growth of aquatic salamanders (Barrett *et al.*, 2010; Bumpers *et al.*, 2015).

Hellbender body condition did not decline during the breeding season in areas of relatively low forest cover (< 65% Forest$_R$) as it did elsewhere. Among amphibians and fish, decreases in body condition during the spawning season are expected as a result of oviposition, increased metabolism and decreased feeding (Ryser, 1989; Brown & Murphy, 2004). Because individuals in better condition generally invest more resources in reproduction than those in poorer condition (Castellano *et al.*, 2004), it is noteworthy that body condition did not decline more during the breeding season in areas of low forest cover where individuals were generally in the best body condition. The mechanism for variation in seasonal body condition dynamics across our land use gradient remains unclear. The increasing magnitude of difference between pre-breeding and breeding body condition along a land use gradient that we observed may be the result of a change in the proportion of adults that are participating in breeding. Alternatively, breeders in less forested areas may be able to recover expenses incurred during reproduction more rapidly relative to individuals in areas of high forest cover where resources may be more limited as a result of relatively high conspecific density. Reproductive biology of hellbenders is virtually unstudied. The body condition trends that we report here, especially when considered alongside evidence that recruitment is lacking in populations subject to low forest cover (Jachowski *et al.*, in prep) emphasize the need for additional work to determine extrinsic and intrinsic factors that influence hellbender breeding and reproductive success.

Seasonal fluctuations in N:L ratios followed our predictions only in areas of low forest cover. Amphibian N:L ratios generally increased at cooler temperatures as a result of increased production of neutrophils and decreased proliferation of lymphocytes (Maniero & Carey, 1997; Raffel *et al.*, 2006). Within a single season, a relative increase in N:L ratios can indicate an activated immune response (i.e., infection) or elevated levels of circulating glucocorticoid hormones (i.e., stress hormones) released via the hypothalamic-pituitary-adrenal axis in response to a range of stressors (Davis *et al.*, 2008). While N:L ratios peaked during the coolest (post-breeding) season in areas of relatively low forest cover as predicted based on temperature, N:L ratios peaked during a relatively warm season (i.e, breeding) in more forested areas even after parasite state was accounted for (Fig. 3.5). The explanation for why N:L ratios peaked during the breeding season in heavily forested areas remains unclear. One possibility is that N:L ratios reflected a condition-dependent hormonal response associated with reproductive investment. For example, glucocorticoid hormones are an important mediator of amphibian breeding behaviors that can be especially costly (Moore, 1983) and typically become elevated during breeding in a range of amphibians (Moore & Jessop, 2003). However, glucocorticoid hormones are also negatively correlated with body condition in reptiles and amphibians (Moore & Jessop, 2003). Thus the positive association between N:L ratios and forest cover during the breeding season may reflect the combined stresses of breeding and relatively poor body condition in heavily forested areas. Alternatively, relatively low N:L ratios in areas of low forest cover during the breeding season might indicate that glucocorticoid hormones and reproductive behaviors modulated by hormones were suppressed in areas of low forest cover.

Our study demonstrates the potential for loss of forest cover and parasite exposure to negatively affect individual amphibians and emphasizes the need for additional research to understand the potential for both stressors to act in combination to elicit negative effects on amphibian populations. Specifically, our work highlights the need to understand whether loss of forest cover might function to reduce host exposure to parasites indirectly as a result of land use driven shifts in host demographic structure (e.g., reduced host density). Additionally, more research is needed to determine whether exposure to parasites that affect red blood cell parameters might influence vulnerability of amphibians to altered thermal regimes common in areas of reduced forest cover and whether variations in N:L ratios associated with land use might reflect disrupted modulation of hormones that are important mediators of reproductive behavior in amphibians and other aquatic vertebrates.

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TABLES

Table 3.1. Summary of known sex adult hellbender (*Cryptobranchus alleganiensis*) captures between 2013 and 2015 with complete parasite histories from 17 stream reaches stratified across a land use gradient.

		Parasites[c]				
Forest[a]	Density[b]	Tryps	Leech	n	Total length (cm) ± SE [range]	Mass (g) ± SE [range]
53	-	N	N	4	45.2 ± 1.6 [41.5 - 48.4]	567 ± 71 [420 - 730]
54	0.46	N	N	46	48.2 ± 0.6 [39.1 – 64.0]	709 ± 32 [425 - 1750]
55	-	N	N	1	45.0	580
57	-	N	N	6	51.2 ± 2.6 [38.4 - 55.3]	919 ± 12 [420 - 1275]
57	-	N	N	8	42.8 ± 2.2 [33.7 - 54.5]	506 ± 71 [254 - 850]
58	-	N	N	1	40.6	425
60	-	N	N	1	32.0	240
60	-	N	N	1	52.5	675
62	0.34	N	N	25	50.0 ± 1.1 [39.5 - 59.4]	851 ± 82 [365 - 1625]
62	-	N	N	11	44.6 ± 1.4 [32.7 - 50.2]	545 ± 39 [225 - 720]
66	1.90	Y	Y	309	42.0 ± 0.3 [29.4 – 58.0]	488 ± 11 [135 - 1420]
68	-	-	-	8	44.1 ± 1.4 [39.5 - 48.7]	583 ± 52 [410 - 745]
69	3.13	Y	Y	260	40.3 ± 0.3 [29.7 - 57.5]	421 ± 11 [140 - 1200]
69	1.50	Y	Y	39	42.7 ± 0.9 [31.1 - 52.4]	495 ± 29 [200 - 830]
69	-	-	-	5	40.6 ± 2.6 [34.5 – 48.0]	432 ± 83 [247 - 620]
71	-	-	-	1	33.5	225
83	2.37	Y	N	60	39.4 ± 0.5 [29.3 - 51.9]	312 ± 14 [130 - 770]
Total				786		

[a] Percent of area in the catchment-wide riparian (50 m buffer on both sides of streams) area classified as forest in the 2011 national land cover dataset

[b] Mean estimated number of sub-adult/adult (> 19 cm total length) per 100 m^2 within the stream reach as per Jachowski *et al.* (in prep)

[c] Indicates whether trypanosomes (Tryps) or leeches were ever detected in captures from a reach

Table 3.2. Sample sizes, broken down by land use (Forest$_R$ = percent forest in catchment-wide riparian area) and season, used to investigate the effects of land use and parasites on physiological condition indices of hellbenders (*Cryptobranchus alleganiensis*). Note that samples used to investigate hematocrit and white blood cell (WBC) profiles are both subsets of the 786 samples used to investigate body condition.

Condition index	Forest$_R$^{a}	Years	Pre-breeding 1 June – 15 Aug	Breeding 16 Aug- 31 Nov	Post-breeding 1 Dec – 31 May	Total
Female body condition	53-59	2013-2015	23	3	2	28
	60-65		86	54	39	179
	66-69		77	44	17	138
	70-83		17	19	1	37
	Total		203	120	59	382
Male body condition	53-59	2013-2015	19	17	4	40
	60-65		65	67	34	166
	66-69		84	64	26	174
	70-83		10	13	1	24
	Total		178	161	65	404
Hematocrit	53-59	2013-2015	41	19	6	66
	60-65		145	91	71	307
	66-69		161	105	43	309
	70-83		25	32	2	59
	Total		372	247	122	741
WBC	53-59	2014-2015	23	10	6	39
	60-65		62	22	44	128
	66-69		86	10	28	124
	70-83		26	1	2	29
	Total		197	43	80	320

Table 3.3. Comparison between size (TL = mean total length (cm)) and sex (F = female; M = male) of adult hellbenders (*Cryptobranchus alleganiensis*) with and without parasite infections each season based on pooled captures from reaches where each parasite was detected (n = 4 reaches for trypanosomes; n = 3 reaches for leeches). Note that infected status does not distinguish between single and coinfections.

	Trypanosomes						Leeches					
	Uninfected		Infected		Prevalence		Uninfected		Infected		Prevalence	
Season	n	TL ± SE	n	TL ± SE	F	M	n	TL ± SE	n	TL ± SE	F	M
Pre-breeding	74	41.4 ± 0.6	232	40.3 ± 0.3	0.68	0.85	196	40.9 ± 0.4	83	40.0 ± 0.5	0.32	0.28
Breeding	58	41.7 ± 0.8	192	41.1 ± 0.4	0.74	0.79	85	41.2 ± 0.6	134	41.9 ± 0.5	0.61	0.61
Post-breeding	17	43.1 ± 1.2	95	42.3 ± 0.5	0.86	0.84	72	42.2 ± 0.6	38	42.8 ± 0.8	0.26	0.42

Table 3.4. Candidate models, ordered by rank, used to investigate effects of land use and parasites on hellbender (*Cryptobranchus alleganiensis*) condition indices.

Response	Model	K^a	LogLikelihood	$AIC_c{}^b$	ΔAIC_c	$w_i{}^c$
Female body condition	Season + $Forest_R$ + Parasites	8	-213.48	443.34	0.00	0.39
	Season*$Forest_R$ + Parasites	10	-211.55	443.70	0.35	0.33
	Season*$Forest_R$	8	-214.21	444.82	1.47	0.19
	Season + $Forest_R$	6	-217.02	446.26	2.91	0.09
	Season + Parasites	7	-235.68	485.65	42.30	0.00
	Season	5	-237.98	486.12	42.78	0.00
Male body condition	Season*$Forest_R$	8	-198.94	414.25	0.00	0.48
	Season + $Forest_R$	6	-201.32	414.86	0.60	0.36
	Season*$Forest_R$ + Parasites	10	-198.46	417.49	3.23	0.10
	Season + $Forest_R$ + Parasites	8	-200.89	418.15	3.90	0.07
	Season	5	-209.35	428.85	14.60	0.00
	Season + Parasites	7	-208.25	430.78	16.53	0.00
Hematocrit	Season + Parasites+ Total body length	8	968.43	-1920.67	0.00	0.51
	Season + $Forest_R$ + Parasites + Total body length	9	968.74	-1919.23	1.44	0.25
	Season*$Forest_R$ + Parasites+ Total body length	11	970.62	-1918.87	1.80	0.21
	Season + Total body length	6	963.13	-1914.15	6.52	0.02
	Season + $Forest_R$ + Total body length	7	963.64	-1913.12	7.55	0.01
	Season*$Forest_R$ + Total body length	9	964.92	-1911.59	9.08	0.01
WBC PC1	Season*$Forest_R$ + Parasites+ Total body length	11	-540.67	1104.20	0.00	1.00
	Season + $Forest_R$ + Parasites + Total body length	9	-550.37	1119.33	15.13	0.00
	Season + Parasites+ Total body length	8	-553.11	1122.68	18.47	0.00
	Season*$Forest_R$ + Total body length	9	-561.46	1141.51	37.31	0.00

Season + Forest_R + Total body length	7	-575.34	1165.05	60.85	0.00
Season + Total body length	6	-580.63	1173.53	69.33	0.00

[a] Number of estimated parameters

[b] Akaike information criterion corrected for small samples

[c] Akaike model weight

Table 3.5. Model averaged or top-ranked model coefficients and their 95% confidence intervals for analyses of five hellbender (*Cryptobranchus alleganiensis*) physiological indices.

Response	Variable[a]	β	SE	Lower CI	Upper CI
Female body condition	Pre-breeding (intercept)	5.84	0.38	5.08	6.59
	Breeding	0.31	0.65	-0.96	1.58
	Post-breeding	0.36	0.77	-1.14	1.87
	Forest$_R$	-3.01	0.58	-4.15	-1.86
	Parasite$_{Single}$	0.12	0.06	0.00	0.23
	Parasite$_{Coinfected}$	0.16	0.07	0.03	0.29
	Forest$_R$*Breeding	-1.53	0.76	-3.02	-0.04
	Forest$_R$*Post-breeding	-1.17	1.38	-3.88	1.54
Male body condition	Pre-breeding (intercept)	4.95	0.44	4.09	5.80
	Breeding	0.33	0.60	-0.86	1.51
	Post-breeding	0.04	0.61	-1.15	1.24
	Forest$_R$	-1.48	0.65	-2.76	-0.20
	Parasite$_{Single}$	-0.02	0.06	-0.14	0.10
	Parasite$_{Coinfected}$	0.02	0.06	-0.10	0.14
	Forest$_R$*Breeding	-1.49	0.69	-2.84	-0.15
	Forest$_R$*Post-breeding	-0.31	1.18	-2.63	2.00
Hematocrit	Pre-breeding (intercept)	0.36	0.03	0.31	0.42
	Breeding	0.02	0.05	-0.08	0.12
	Post-breeding	-0.04	0.07	-0.17	0.09
	Forest$_R$	0.00	0.06	-0.13	0.12
	Parasite$_{Single}$	-0.02	0.01	-0.03	0.00
	Parasite$_{Coinfected}$	-0.02	0.01	-0.04	-0.01
	Forest$_R$*Breeding	-0.15	0.08	-0.32	0.01
	Forest$_R$*Post-breeding	-0.16	0.16	-0.47	0.15
	Total length	0.01	0.00	0.00	0.01
WBC PC1	Pre-breeding (intercept)	-2.64	0.90	-4.40	-0.87
	Breeding	-9.72	2.63	-14.88	-4.57
	Post-breeding	3.76	2.25	-0.66	8.18
	Forest$_R$	1.88	1.33	-0.73	4.49
	Parasite$_{Single}$	1.08	0.19	0.71	1.45
	Parasite$_{Coinfected}$	1.31	0.21	0.91	1.72
	Forest$_R$*Breeding	17.17	4.05	9.24	25.10
	Forest$_R$*Post-breeding	-3.07	3.40	-9.73	3.58
	Total length	0.70	0.08	0.53	0.86

[a] Forest_R = Proportion of catchment-wide riparian area classified as forest; $\text{Parasite}_{\text{Single}}$= infected with trypanosomes or leeches; $\text{Parasite}_{\text{Coinfected}}$= infected with trypanosomes and leeches

Table 3.6. Loadings and proportion of variance explained by principal components generated from a correlation matrix of hellbender (*Cryptobranchus alleganiensis*) white blood cell parameters.

White blood cell parameter	PC1	PC1	PC3	PC4
Neutrophils	0.53	-0.34	0.05	0.78
Lymphocytes	-0.55	0.04	0.76	0.34
N:L ratios	0.53	-0.26	0.62	-0.51
Eosinophils	0.38	0.90	0.16	0.13
Cumulative proportion of variance explained	0.83	0.99	1.00	1.00

FIGURE LEGENDS

Figure 3.1. Model averaged predicted effects of land use on seasonal variation in females (a) and male (b) hellbender (*Cryptobranchus alleganiensis*) body condition calculated as a scaled mass index (i.e., estimated mass at a reference size of 40 cm total length). Error bars represent 95% confidence intervals.

Figure 3.2. Model averaged predicted effects of season, parasite state (a) and size of individual (b) on percent of hellbender (*Cryptobranchus alleganiensis*) blood (by volume) made up of red blood cells. Parasite effects (a) are predicted for individuals measuring 40 cm total length and total length effects (b) are predicted for uninfected individuals. Error bars represent 95% confidence intervals.

Figure 3.3. Associations between white blood cell principal component one scores (WBC PC1) and the original correlated variables used in the principal components analysis (neutrophil-to-lymphocyte (N:L) ratios and eosinophil percentages). Figures were generated specifically to aid interpretation of WBC PC1 scores. Points are observed values for individuals that are uninfected (circles) or infected (triangles) with at least one parasite.

Figure 3.4. Effect of hellbender total length on hellbender (*Cryptobranchus alleganiensis*) white blood cell parameters based on a top-ranked model. White blood cell parameters are summarized by a single principal component (PC1) that explained 83% of the variance in the white blood cell parameters and was positively correlated with neutrophils, eosinophils and neutrophil-to-lymphocyte (N:L) ratios and negatively correlated with lymphocytes. Predictions were generated for non-parasitized individuals while holding forest cover within the catchment-wide riparian area constant at 63%. Error bars represent 95% confidence intervals.

Figure 3.5. Predicted effects of land use and parasite state on white blood cell (WBC) parameters of hellbenders (*Cryptobranchus alleganiensis*) based on a top-ranked model. White blood cell parameters are summarized by a single principal component (PC1) that explained 83% of the variance in the white blood cell parameters and was positively correlated with neutrophils, eosinophils and neutrophil-to-lymphocyte (N:L) ratios and negatively correlated with lymphocytes. Predictions were generated for individuals measuring 40 cm total length. Dashed lines represent 95% confidence intervals. Predicted effects of parasites are not extended across the entire land use gradient because parasites were never observed in reaches with < 65% forest cover.

FIGURES

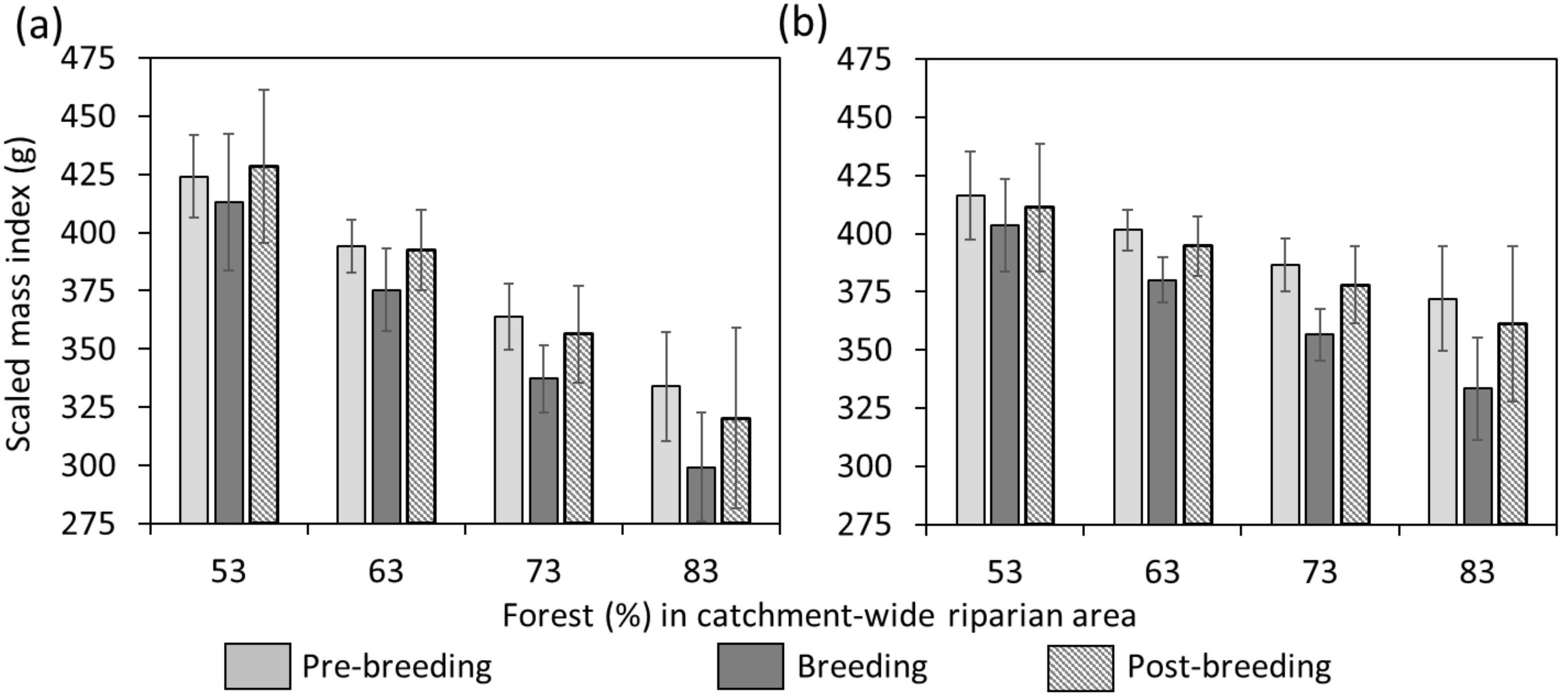

Figure 3.1. Effect of land use on seasonal variation in females (a) and male (b) hellbender body condition.

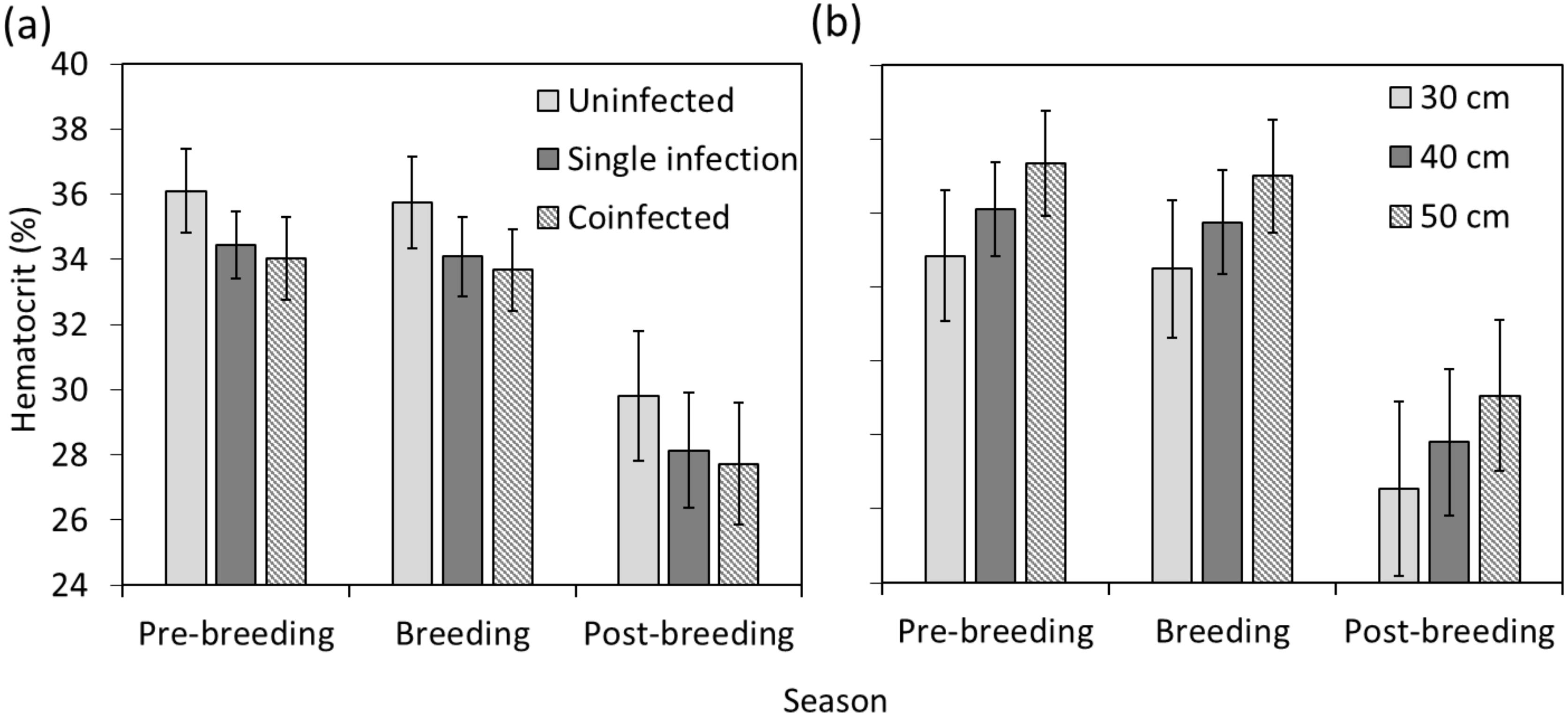

Figure 3.2. Effects of season, parasite state (a) and size of individual (b) on percent of hellbender blood (by volume) made up of red blood cells.

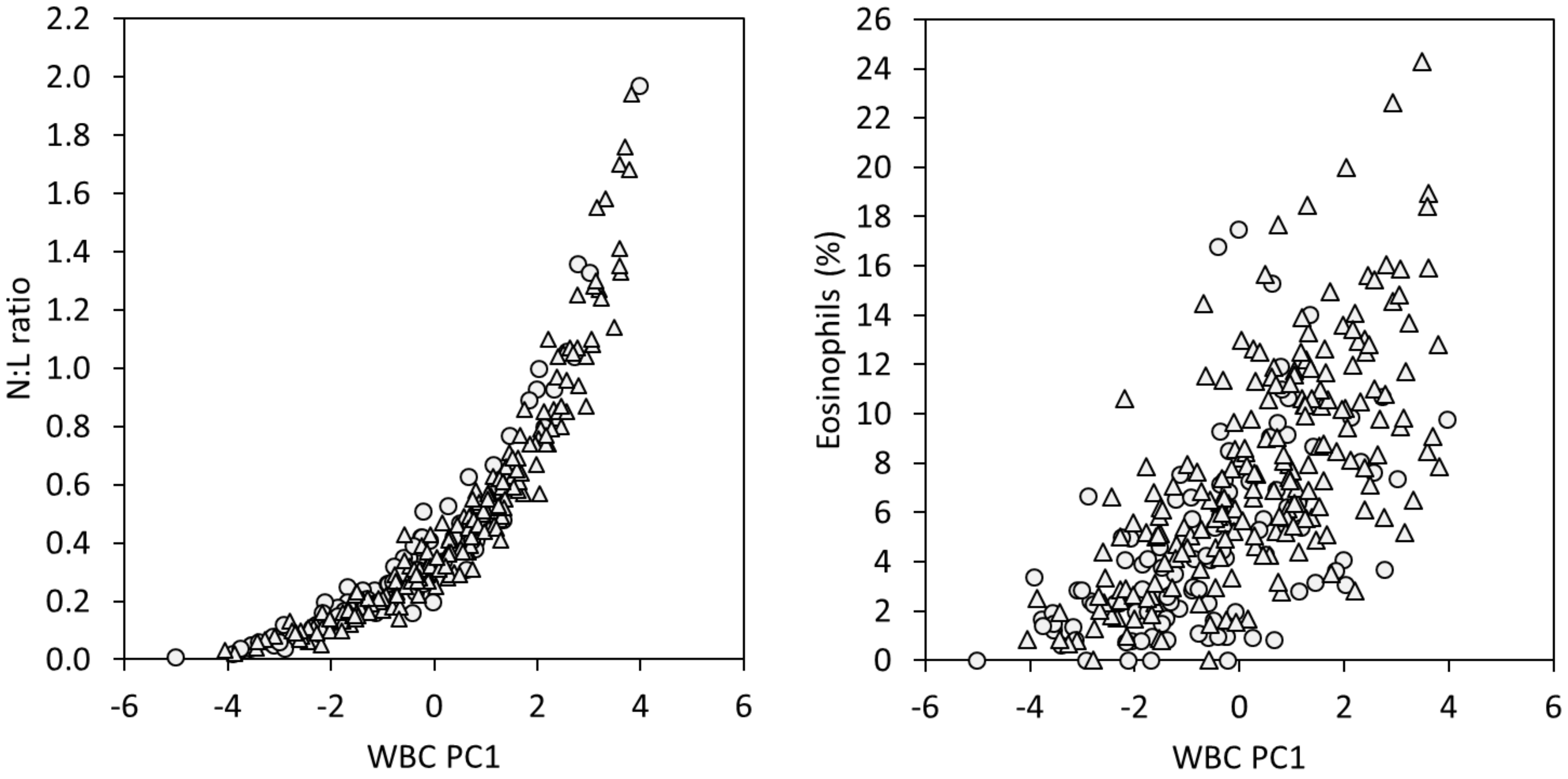

Figure 3.3. Relationship between WBC PC1 and original correlated variables used in the PCA.

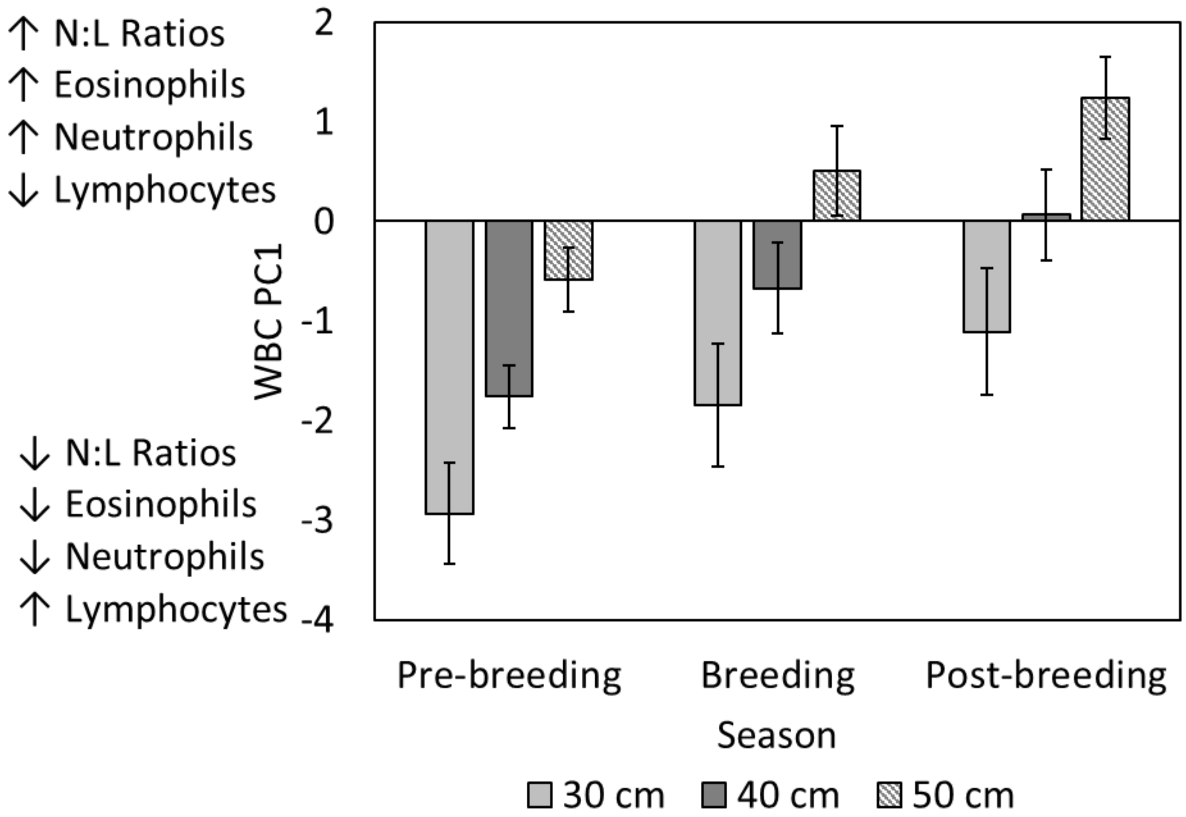

Figure 3.4. Effect of total length and season on hellbender white blood cell parameters.

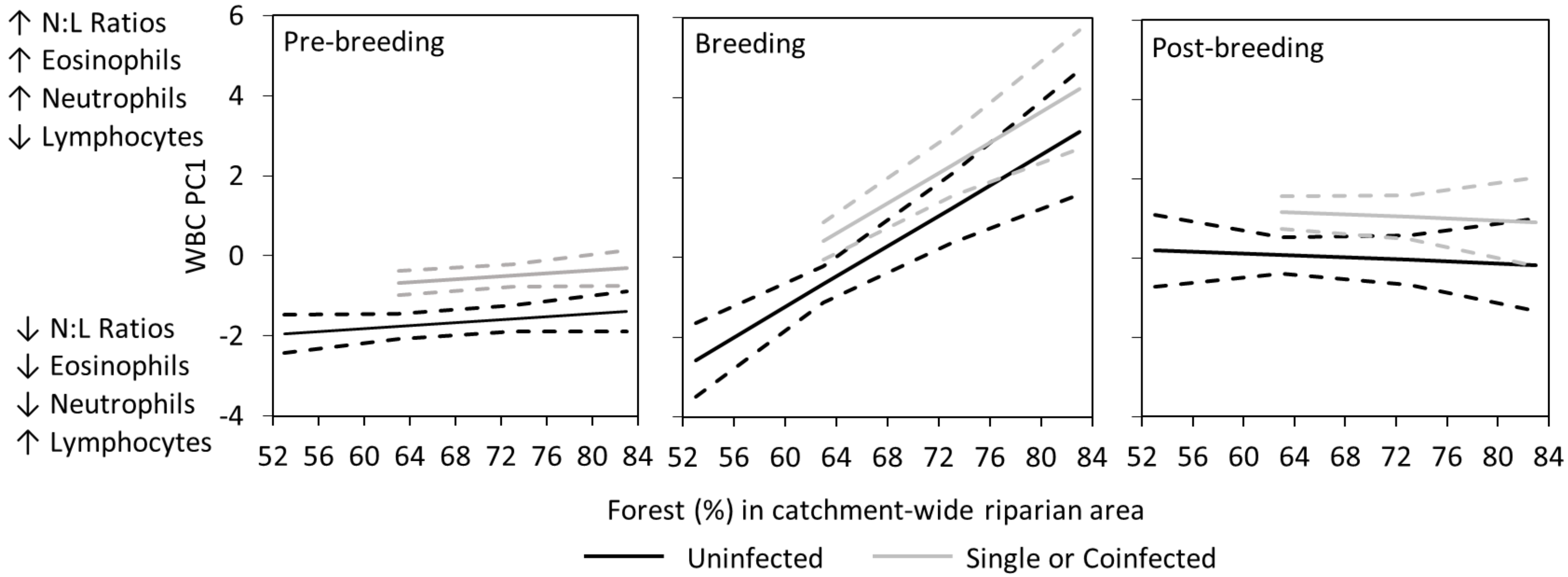

Figure 3.5. Effect of land use and parasite state on hellbender WBC parameters.

Chapter IV. Efficacy of Artificial Shelter Arrays as a Monitoring Tool for Hellbenders

Catherine M. B. Jachowski and William A. Hopkins

ABSTRACT

Eastern and Ozark hellbenders (*Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi*, respectively) are among the most imperiled amphibians in North America. A major challenge to hellbender conservation is the dearth of information regarding the stability of extant populations and their response to stressors. Currently, rock-lifting surveys are the standard approach used to monitor hellbender populations but can cause considerable habitat disturbance and yield unreliable estimates of relative abundance when imperfect detection is unaccounted for. We investigated the efficacy of artificial shelter arrays (ASAs) as a monitoring tool for hellbender populations and specifically investigated the potential for ASA occupancy rates to function as a reliable index of relative abundance. We constructed 180 artificial shelters and installed arrays (30 shelters per array) in six stream reaches stratified across a hellbender population density gradient (range = 0.3 – 3.0 sub-adult/adult hellbenders per 100 m^2). We surveyed shelters approximately monthly between 2013 and 2015. We used an information theoretic approach and generalized linear mixed models to evaluate support for our hypothesis that shelter occupancy increased with hellbender density while evaluating effects of season, time since shelter deployment and availability of natural shelter. Over the course of our study we detected 156 unique individuals (n = 6-66 individuals per array) and 33 nests during ASA surveys. The probability of a shelter being occupied increased with time since deployment and peaked each year during July – Aug. Shelter occupancy rates functioned as a reliable index of relative abundance only when natural shelter was relatively limited (≤ 30 % of reach characterized by boulder and bedrock). When natural shelter covered > 30% of a reach occupancy rates were unable to distinguish even a five-fold increase in hellbender density (0.3 vs 1.5 hellbenders per 100 m^2). Our findings highlight the potential for carefully designed ASAs to become a valuable component of long-term hellbender monitoring programs. However, or findings also emphasize the necessity to validate common assumptions that count data obtained from artificial shelters function as a reliable proxy of abundance for birds, mammals, reptiles and other amphibians.

INTRODUCTION

The hellbender (*Cryptobranchus alleganiensis*) is cryptic and imperiled amphibian that is of great conservation concern and largely data deficient in terms of population status and viability. Hellbenders are fully aquatic, large (up to 74 cm total length) stream dwelling salamanders that are endemic to eastern North America (Nickerson and Mays 1973a). Both subspecies (Ozark hellbender, *C. a. bishopi* and eastern hellbender *C. a. alleganiensis*) have undergone precipitous declines since the 1970s (Williams *et al.* 1981, Wheeler *et al.* 2003, Foster *et al.* 2009, Burgmeier *et al.* 2011b, Graham 2011). Reasons for historic declines are unclear but are suspected to be driven by a multitude of factors including deforestation and increased fine sediment loads in streams, altered water quality, and exposure to parasites and pathogens (Williams *et al.* 1981, Wheeler *et al.* 2003, Briggler *et al.* 2007, Foster *et al.* 2009, Jachowski and Hopkins In prep). Both subspecies are protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the Ozark hellbender is listed as a federally endangered species under the Endangered Species Act (USFWS 2011a;b). The eastern hellbender is being considered for federal protection. A major challenge to hellbender conservation is a lack of understanding on the current trajectory of hellbender populations in most states. Due to their considerable longevity of 25+ years (Taber *et al.* 1975) small groups of old hellbenders can persist in areas for several years while recruitment suffers (Jachowski and Hopkins In prep). Therefore, while hellbenders still occur in most states throughout their native range, it remains unclear whether most populations are stable, declining, or increasing.

Development and adoption of standardized long-term monitoring protocols for hellbenders can improve our knowledge regarding the species status and inform conservation decision making. Monitoring is a fundamental component of fish and wildlife conservation but can be extremely challenging for cryptic species like hellbenders. In most states snorkeling while rock-lifting is the most popular survey method (Nickerson and Krysko 2003, Browne *et al.* 2011) but trapping(Foster *et al.* 2008, Briggler *et al.* 2013) and analysis of environmental DNA (Olson *et al.* 2012, Spear *et al.* 2015) are also used. The primary advantage of rock lifting surveys are the relative efficiency at detecting all age classes of hellbenders (Nickerson and Krysko 2003, Foster *et al.* 2008) and the ability to estimate abundance from individual encounters. Drawbacks to rock-lifting include ethical concerns over habitat disturbance as a result of disturbing large cover rocks (Goode *et al.* 2005, Pike *et al.* 2010, Browne *et al.* 2011). Additionally, detection and capture probabilities during rock-lifting surveys vary among study sites, across time, among hellbender size classes, and in response to surveyor experience (Bodinof *et al.* 2009; 2010; 2011, Jachowski and Hopkins In prep). As a result, commonly used measures of relative abundance (i.e., catch-per-unit effort) can be unreliable and multiple surveys per site and relatively advanced statistical approaches are typically required to estimate hellbender abundance without bias (Mazerolle *et al.* 2007). Given the imperiled status of hellbenders, widespread adoption of standardized survey methods that minimize observer bias can provide managers and policy makers with better information on the status of populations.

Artificial shelters have been recently developed as a conservation tool for hellbenders, but their potential as a monitoring tool have not been examined. Artificial shelter arrays (ASAs) in the form of nest/roost boxes and cover boards have been widely applied as tools to monitor a variety of birds, mammals, and herpetofauna (Kibler 1969, Bolton *et al.* 2004, Fokidis and Risch 2005, Harper *et al.* 2005, Beyer and Goldingay 2006, Hampton 2007, Willson and Gibbons 2010, Hesed 2012, Heyer *et al.* 2014, Goldingay *et al.* 2015). Artificial shelters for hellbenders were originally designed by Briggler and Ackerson (2012) to mimic suitable nesting cavities, considered to be a limiting factor for many populations. Since their debut, artificial shelters have been augmented into streams in several states (pers. obs.) and in captive breeding facilities (Ettling *et al.* 2013), primarily to attract spawning hellbenders for the purpose of collecting eggs for captive propagation. However, observations that hellbenders use artificial shelters outside the nest brooding period (Briggler and Ackerson 2012) suggest that artificial shelter arrays have potential as a broader monitoring tool for hellbenders. More specifically, monitoring artificial shelter arrays may be a viable alternative to rock-lifting surveys if shelter occupancy rates function as a reliable index of local abundance.

The purpose of our study was to examine the efficacy of using ASAs as a tool for monitoring hellbender populations. We tested the hypothesis that shelter occupancy rates increase as a function of hellbender density while investigating effects of season, time-since-deployment, and availability of natural shelter sites on artificial shelter occupancy. We discuss the advantages and drawbacks of ASAs as a monitoring approach for hellbenders and provide recommendations for researchers and conservation practitioners interested in adopting ASAs to replace or supplement ongoing monitoring efforts.

METHODS

Focal species

The hellbender (*Cryptobranchus alleganiensis*) is a benthic habitat specialists that is heavily dependent on rocky crevices for shelter and nesting (Nickerson *et al.* 2003). The Ozark subspecies (*C. a. bishopi*) is endemic to southern Missouri and northern Arkansas while the Eastern subspecies (*C. a. alleganiensis*) is native to 15 states ranging as far north as New York and Pennsylvania, south to Georgia and west to Missouri. Sub-adults and adults consume crayfish as their primary prey (Peterson *et al.* 1989) and exhibit high site fidelity to specific stream reaches and rock cavities (Nickerson and Mays 1973b, Bodinof *et al.* 2012). Age at sexual maturity is generally six to eight years (Peterson *et al.* 1988) and individuals can exceed 25 years of age (Taber *et al.* 1975). Spawning occurs annually (Smith 1907) where females can deposit 500 or more eggs (Topping and Ingersol 1981) in a nest cavity that is guarded by a single male. Behavior and habitat requirements for larvae are poorly known.

Study sites

Our study sites included six stream reaches from three streams (n=1-4 reaches per stream) in the upper Tennessee River basin in southwestern VA, USA. Average wetted width of stream reaches ranged from 10-18 m and lengths ranged from 300–645 m. All reaches were known to be occupied by hellbenders based on previous surveys. Density of sub-adult/adult hellbenders (i.e., $\geq$ 190 mm total length) in each reach was unknown at the onset of our study.

Construction and design of artificial shelter arrays

We constructed artificial shelters by hand following methods outlined Briggler and Ackerson (modified boot-design; 2012). Briefly, we constructed internal frames out of galvanized hex-mesh and hardware cloth and covered each frame with a mixture of sand, portland cement and quick-crete. Each shelter consisted of a rectangular cavity (mean length = 40 cm [range = 29-49 cm]; mean width = 38 cm [range = 23-48 cm], mean height = 14 cm [range = 7-23 cm]) with a single entrance via a tunnel (mean length = 24 cm [range = 20-34 cm], mean entrance width = 11 cm [range = 8-18 cm], mean entrance height = 10 cm [range = 6-14 cm]). Each shelter had a single removable lid to allow researchers to access the main chamber. Most shelters were also outfitted with a PVC cleanout plug (5 cm diameter) in one corner of the main chamber ceiling that could be unscrewed to allow access of the chamber via a camera. Unlike Briggler and Ackerson (2012) we selected not to cut out a hole in the floor of each shelter. Once dry, we drilled three holes (4-5 mm diameter) through the rear wall of each shelter to allow for some water flow through the main chamber. We soaked shelters outdoors in cattle tanks for ≥ 30 d prior to installing them in streams (Fig. 4.2). Due to initial high rates of lid loss during high flow events, we modified all existing shelter lids with a lid-locking mechanism in May 2015. Briefly, we modified lids by first securing a stationary zinc-plated mending brace with a rubber washer to the underside of the upstream edge of each shelter lid that extending ~ 5 cm under the shelter ceiling. Additionally, we installed a second mending brace with a rubber washer to the underside of the downstream edge of each shelter lid that could be rotated to lock/unlock each lid from above via a stainless steel eye-bolt.

Prior to constructing a large number of shelters we conducted a pilot study involving 30 artificial shelters augmented into two stream reaches where we knew hellbenders were relatively abundant based on previous surveys (Hopkins and Durant 2011, Hopkins *et al.* 2011, Davis and Hopkins 2013, Hopkins *et al.* 2014, DuRant *et al.* 2015, Hopkins *et al.* 2016, Jachowski and Hopkins In prep). In our pilot study, we installed 30 shelters across the two reaches (n= 13 and 17 shelters per reach) between June and Sep 2013. Rapid colonization (i.e., within 1 week of installation in some cases) of artificial shelters in our pilot study provided sufficient justification for us to build additional shelters and expand our study across multiple stream reaches. As a result, we constructed an additional 150 shelters and installed them across our six stream reaches (two of which were involved in the pilot study) between June and Aug 2014.

Ultimately we installed six full arrays consisting of 30 artificial shelters each (30 shelters x 6 reaches = 180 shelters total; Fig. 4.2). Arrays were contiguous in two reaches (R1 and R6), but were partitioned into either two (R2, R4, R5) or three (R3) sub-arrays separated by up to 200 m in four reaches (Fig. 4.2). In most cases our reason for partitioning arrays was to avoid sections of stream that were inhospitable to shelter installation or monitoring (e.g., continuous bedrock or deep (>1.5 m) water). Partitioning of the array in R4 was simply to avoid moving shelters from our pilot study that were regularly occupied by hellbenders. When partitioned, sub-arrays consisted of either 10 or 20 shelters each. In each array/sub-array we installed shelters at a density of 0.6 shelters per 100 m2 of wetted in-stream habitat, such that each full array effectively sampled ~ 5000 m^2 of wetted in-stream habitat. We viewed habitat in-between sub-arrays as outside of our sampling area. The lateral location of each shelter within the stream channel was based on the best judgment of installers regarding factors that would maximize resilience of shelters to low water levels and high flow events.

We followed shelter installation methods outlined by Briggler and Ackerson (2012) where we 1) excavated a hole in existing substrate, 2) positioned the box in the hole such that the tunnel entrance was oriented 90˚-180˚ from oncoming current, 3) surrounded the periphery of the shelter with gravel, sand and pebbles, 4) lined the tunnel and chamber with sand and gravel to a depth of approximately 2 cm, and 5) placed one or more large rocks on top of each shelter for concealment and to anchor lids (Fig. 4.1). We attached an aluminum tag with a unique shelter identification number to the handle of each lid. When a shelter was relocated for any reason (outlined in sampling methods below) we assigned it a new identification number. Thus, shelter identity reflected a unique shelter-by-location combination.

Field sampling

Density.---We randomly selected one sub-reach from each array for hellbender density sampling via mark-recapture surveys (Fig. 4.2). We assumed hellbender density within a randomly-selected sub-reach was representative of density throughout the associated ASA. Our decision not to sample all three sub-reaches in each array was based on logistical constraints, a desire to minimize habitat disturbance from rock lifting, and concern that excessive habitat disturbance might inflate occupancy of artificial shelters. We used a robust sampling design with two primary and two secondary occasions to estimate the density of sub-adult/adult (≥ 190 mm total length) in each sub-reach while accounting for imperfect detection. Detailed methodology regarding mark-recaptures surveys can be found in Jachowski *et al.*, (in prep). Briefly, primary occasions were defined by year (summer of 2014 and 2015) and each contained two secondary sampling occasions. We assumed populations in each sub-reach were open between primary occasions but closed between secondary occasions in each year. To minimize potential for violating assumptions of closure we conducted secondary occasion surveys as close together in time as possible (median no. days between secondary occasions within a primary occasion = 2.5 [range = 1-34 days], n = 12 primary occasions). We conducted mark-recapture surveys to estimate density between 6 July and 27 Aug in 2014 and between 23 June and 5 Aug in 2015. We used snorkeling while turning rocks, tactile searches and artificial shelters to locate and capture all size classes of hellbender (Nickerson *et al.*, 2003). We worked from downstream to upstream, in zig-zag fashion to locate and search all cover objects > 25 cm in diameter. We searched smaller objects when we noticed obvious crevices or openings beneath them. Generally, one or more samplers lifted cover objects from the downstream end while a single snorkeler visually scanned for hellbenders. We relied on tactile searches when cover objects were impossible or dangerous to lift and when crevices could be easily accessed by hand without disturbing rocks. To reduce variability in hellbender detection and catchability due to surveyor experience, the same snorkeler was responsible for visually identifying and capturing hellbenders during every survey designed to inform density estimates.

When encountered, we captured hellbenders by hand and marked individuals with uniquely coded passive integrated transponder (PIT) tags (models HPT8 or HPT12; Biomark Inc., Boise, ID, USA). We inserted PIT tags subcutaneously along the dorsolateral region of the tail, approximately 5 cm posterior to the tail base. We sealed the PIT tag entry site with superglue prior to release to minimize tag loss. We recorded sex based on external morphology (cloacal swelling in males) when evident. We weighed each individual to the nearest gram using Pesola® spring scales (Pesola AG, Schindellegi, Switzerland) and recorded total length to the nearest mm. We released all hellbenders at the point of capture.

Artificial shelter occupancy.----We used snorkeling to visually inspected shelters approximately monthly from the time of installation through early Sep 2015. Shelters involved in the pilot study were monitored for over two years (July 2013-Sep 2015) while other shelters were monitored for just over one year (Jul or Aug 2014-Sep 2015).

On each occasion we classified each shelter as either 1) present and available, 2) present but unavailable, 3) permanently missing, or 4) censored. We classified shelters as present and available if we could physically locate the shelter, if it had not been dislodged since or previous survey and if the tunnel was at least partially open to hellbenders. We recorded whether the lids of shelters that were present and available were on or had been washed off. We replaced lids that had been washed off and re-anchored them with cobble/boulders prior to completing each survey. We classified shelters as present and unavailable if we were able to physically locate the shelter but it had either been dislodged since our previous survey or the tunnel had become completely blocked or buried by gravel since our previous survey. We restored present but unavailable shelters to an available condition before finishing each survey by reinstalling them in more sheltered microhabitat within ~ 15 m of their original location and/or clearing the shelter entrance by hand. We classified shelters as permanently missing if we were sure the shelter had been dislodged from its original location and we never relocated it during the course of the study. Finally, we classified shelters as censored if we were simply unable to survey the shelter due to poor visibility or high flows. When shelters were present and available, we searched each shelter by tactile methods where we simultaneously used one hand to block the tunnel entrance while feeling within the chamber and tunnel with a second hand. We recorded whether each shelter was occupied or unoccupied. When encountered we captured hellbenders by hand and processed individuals as during mark-recapture surveys for density estimation, with the exception of our final survey in Aug-Sep 2015 when we recorded occupancy status of each shelter but did not capture hellbenders from shelters in order to avoid disrupting breeding. Following processing we returned hellbenders to the shelter where they were captured.

Availability of natural shelter.---To account for variation in natural shelter availability among reaches we quantified substrate characteristics of each reach in May 2015 using a modified Wolman Pebble Count (Wolman 1954). We only sampled substrate in sub-reaches that were used to estimate density (Fig. 4.2), and we assumed that substrate characteristics within a sub-reach were representative of the reach as a whole. In our modified pebble count we quantified the size of substrate particles at 100 points distributed among 20 cross-sectional transects (five points per transect) stratified evenly across a sub-reach. We recorded the intermediate axis (mm) of the first particle encountered by a meter stick placed vertically at arms-length distance upstream of an observer. We classified substrate at each location as either bedrock (smooth or irregular), boulder (≥ 256 mm), cobble (64-256 mm), pebble (2-64 mm) or fine (≤ 2 mm). We defined availability of natural shelter as the percent of observations classified as either bedrock or boulder, given that the majority of hellbender captures (1,006 of 1,067 or 94%) recorded from our study system between 2007 and 2015 were associated with bedrock or boulders.

Data analysis

Density.---We fit a Huggins (Huggins 1989;1991) version of the closed robust design model (Pollock 1982) to our data to estimate hellbender abundance in each sampled sub-reach in both 2014 and 2015. A detailed description of methods used to estimate density is outlined in Jachowski *et al.* (Chapter II above), as density estimates used in the current study were originally generated as part of a separate study used to investigate effects of land use on hellbender demography. Briefly, due to the rarity of small age classes and our inability to permanently mark larvae, our density analysis was specific to pooled sub-adult/adult age classes (≥ 180 mm). Prior to fitting models, we compiled capture histories for all PIT tagged individuals captured during primary occasions defined above. We coded capture histories as live encounters where on each occasion an individual was either alive or not-encountered, coded by a ‘1’ or ‘0’, respectively. We pooled data from all reaches and defined each sub-reach as a separate group using dummy variable coding. We fit four candidate models to our data that differed only in how capture probability (the probability of capturing a randomly selected individual during a single survey) was modeled. We allowed capture probability to vary among reaches in every model, but fit models with and without fixed effects of year and body size (total length) to evaluate support for year and size effects on capture probability. We fit closed robust design models in program MARK (White and Burnham 1999) and used model averaged estimates of abundance ($\hat{N}$) in both 2014 and 2015 in our calculation of hellbender density, where we divided average $\hat{N}$ for each reach by the extent of a sub-reach:

$$\text{No. hellbenders per} 100\ \text{m}^2 = \left[\frac{(\hat{N}_{2014} + \hat{N}_{2015})}{2}\right] \times \frac{1}{1{,}680\ \text{m}^2} \times 100$$

Artificial shelter occupancy.--- We used individual shelter surveys as the sampling unit in our study and used the outcome (occupied or not occupied) of each survey of each shelter as a binomial response variable in our shelter occupancy analysis. We coded outcomes of each survey as ‘0’ if a shelter was not occupied and as a ‘1’ if it was occupied. Prior to analysis we removed survey records where a shelter was classified as present but unavailable, permanently missing or censored.

We used an information theoretic approach to evaluate support for our hypothesis that shelter occupancy rates would increase with hellbender density. We constructed a candidate set of eight models that included various combinations of four continuous and one categorical fixed effect variable (Table 4.1). We included shelter ID as a random effect in all models. We screened predictor variables for correlation and plotted our data to examine support for non-linear effects of covariates prior to fitting models. Pearsons correlation coefficients indicated no evidence of problematic correlation between continuous predictors ($-0.31 \geq r \leq 0.35$). We observed clear support for a quadratic (x + x2) form of 'Day of Year' which we used in all models. We predicted that the probability of a shelter being occupied would increase with hellbender density, and with the number of days since a shelter was deployed (Table 4.2). We predicted that shelter occupancy would peak at the onset of the breeding season (late August) when hellbenders are actively seeking out mates and interacting with conspecifics (Smith, 1907). We predicted that hellbenders would be less likely to use artificial shelter if natural shelter was readily available, thus we predicted negative effects of coarse substrate (Table 4.2). We predicted that hellbenders would be less likely to occupy shelters if the lid had been washed off since our previous survey (Table 4.2).

We fit generalized linear mixed models with a logit link using Laplace approximation via package 'lme4' in program R (Team 2013). We ranked candidate models according to AICc and report mean estimates for fitted parameter coefficients and model predictions based on our top-ranked model. We used five-fold cross validation (Boyce *et al.* 2002) and area-under-the receiver operating curve (ROC; (Metz 1978) to evaluate performance of our shelter occupancy model. First, we randomly partitioned our original data five times using an 80:20 ratio of training to testing data. We refit models with each draw of training data and used newly fitted models to predict the probability that a shelter was occupied by a hellbender for each case in the complementary test data. We pooled test results to estimate area under the ROC curve using the package ROCR (Sing *et al.* 2013) in Program R (Team 2013). Briefly, the ROC curve is a plot of sensitivity (probability of correctly classifying a true positive) against specificity (probability of correctly classifying a true negative) across a range of cutoffs between zero and one (Metz 1978). The area under the curve (AUC) is cutoff independent and defined as the probability that a model will score a randomly drawn positive sample higher than a randomly drawn negative sample. Values of AUC equal to 0.5 indicate a complete lack of predictive power (i.e., random), with values of 1.0 indicating perfect predictive performance (Cumming 2000).

RESULTS

Density

We captured 204 unique individuals during mark-recapture surveys designed to estimated density. Five individuals were larvae which could not be PIT tagged and were therefore excluded from our density analysis. The majority (198 of 199) of PIT tagged individuals were ≥ 190 mm long. Our top-ranked abundance model (w = 0.99) assumed capture probability was similar among years but unique to each reach, and increased with total body length. Mean estimates of capture probability for average sized individuals (~ 400 mm) ranged from 0.52-0.91. Detailed results of our density analysis can be found in Chapter II (this document). Briefly, abundance estimates for each mark-recapture sub-reach were similar between years and ranged from 4-47 individuals in 2014 and from 6-51 individuals in 2015. Final average density estimates ranged from 0.31-2.97 individuals per 100 m2 (Table 4.2).

Artificial shelter occupancy

During our study, we recorded observations from 2,061 surveys of our 180 shelters. Shelters had been shifted or dislodged on 44 occasions (range = 0-16 occasions per array) and in 16 instances shelters were permanently lost or destroyed (range = 0-7 shelters/array; Table 4.1).

Among surveys where a shelter was classified as present and available (n = 1,804) lids were ajar/off on 156 occasions (range = 20-44 occasions per array; Table 4.1). However, installation of locking mechanisms secured lids and reduced lid loss. Lid loss among available shelters was about five times more common prior to installation of lid-locking mechanisms (146 of 1,375 or 11% of occasions) than after (10 of 429 or 2% of occasions).

Hellbenders were observed using artificial shelters in all six arrays (Fig. 4.1). On average, hellbenders were actively occupying about one out of every four available shelters (460 of 1,804 or 25.4% of observations). Collectively, we detected 156 unique individuals (61 F, 81 M, 14 Unknown sex) from our arrays (range = 6-66 unique individuals per array; Table 4.1). Seventy-seven individuals were captured only once, 35 were captured twice and 44 were captured three or more times from shelters. One individual was captured on 18 occasions from only two different (but neighboring) shelters. Hellbenders occupying shelters were all mature adults (≥ 285 mm; Table 4.1) with the exception of larvae originating from nests established in shelters. We detected 33 established nests inside artificial shelters (range = 0-15 per array and n= 5-19 per year; Table 4.1).

Model weights indicated that our global model (w = 0.99) fit our data far better than any of the alternative models we considered (Table 4.3) and model validation suggested the model performed relatively well (AUC = 0.78). Estimated covariate effects from the fitted model largely matched our predictions (Table 4.4). The probability of a shelter being occupied was highest during the months of July and August (just prior to breeding) and increased with density as well as time since a shelter was deployed (Fig. 4.3). Odds ratios (e^β) indicated that the odds of a shelter being occupied increased by about 30% for every 100 days since deployment and more than doubled for each additional sub-adult/adult hellbender per 100 m2 (Table 4.4). Our model predicted that at one-year post-deployment, the probability of a shelter being occupied during August in a reach with 3 hellbenders/100 m2 (0.52 [0.40-0.64 95% CI]) was about five-times higher than a shelter in a reach with only 0.3 hellbenders/100 m2 (0.09 [0.05-0.16 95% CI]; Fig. 4.3). Furthermore, occupancy rates during August at two years post-deployment were (depending on density) one-and-a-half to two-fold higher than at one year post-deployment (Fig. 4.3). However, availability of natural shelter also appeared to be an important determinant of shelter use, where the odds of occupancy decreased by about 30% for every additional 10% of the reach that was characterized by bedrock and boulder, across a wide range of hellbender densities (Table 4.4; Fig. 4.4). As a result, shelter occupancy rates were reliable index of relative abundance of sub-adult/adults when natural shelter was somewhat limited (e.g., R1 vs R6; Table 4.1) whereas shelter occupancy was an unreliable indicator of relative abundance when natural shelter was abundant (e.g., R5 vs. R6; Table 4.1). Absence of a lid decreased the odds of occupancy by around 85%, but did not entirely eliminate the probability that as shelter would be occupied (Table 4.4).

DISCUSSION

Our findings provide novel insight into the efficacy of ASAs as a monitoring tool for hellbenders. We found that artificial shelter arrays were useful for detecting our extremely cryptic target species across a wide range of population densities. The proportion of shelters occupied by hellbenders increased over time and peaked each year just prior to the onset of breeding (Jul-Aug) in every reach. Our findings provide preliminary evidence that carefully designed ASAs may represent a viable approach for monitoring multiple aspects of hellbender biology, changes in abundance of adult hellbenders at a single location and detecting differences in abundance among locations under some circumstances.

Population density and natural habitat were both critical determinants of ASA occupancy by hellbenders. Our finding that hellbender occupancy of artificial shelters was inversely related to the availability of natural cover was consistent with findings for some arboreal cavity-dependent mammals (Beyer and Goldingay 2006, Durant *et al.* 2009). Surprisingly few studies have investigated the effect of population density on occupancy of artificial shelters by birds (Wesolowski 2011), mammals (Priol *et al.* 2014) or herpetofauna (Siddig *et al.* 2015). We found that occupancy rates of artificial shelters generally increased with population density. However, the inability of ASA occupancy to discriminate a five-fold increase in density (0.3 vs. 1.5 hellbenders per 100 m^2) when natural habitat was relatively abundant (≥ 30% bedrock and boulder; Fig. 4.4) emphasizes the necessity to account for factors influencing variation in species detection/capture probabilities among sites when using count data from artificial shelters to infer patterns of occurrence and abundance. Our findings are of particular interest given that uncorrected count data for other species obtained from artificial shelters are often interpreted as an index of relative abundance to infer changes in population density across space and time (Morneault *et al.* 2004, Mathewson 2009, Smallwood *et al.* 2009, Shutler *et al.* 2012). Our findings emphasize the need to validate assumptions that count data obtained from artificial shelter surveys function as a reliable index of relative abundance in other systems (birds, mammals, reptiles and amphibians) given that in our system a decrease in shelter occupancy could just as easily reflect an increase in natural shelter availability (with no change in abundance) as it could a decline in abundance (when natural shelter availability is constant).

Shelter occupancy by hellbenders increased over time and peaked each year just prior to breeding, regardless of population density or availability of natural habitat. Multiple studies have found that target species use well-established shelters more often than newly-installed shelters for unclear reasons (Grant *et al.* 1992, Monti *et al.* 2000, Madikiza *et al.* 2010). Given that hellbenders are highly sedentary during most of the year (Bodinof *et al*. 2012) we suspect the increased use of shelters over time was the result of additional time required for hellbenders to encounter new shelters (rather than avoidance of new shelters). Grant *et al.* (1992) found that cover board use by herpetofauna increased during the first two months following installation but thereafter remained stable. Additional monitoring is necessary to determine if and when hellbender occupancy of ASAs might plateau, which could be an important consideration when comparing occupancy rates among ASAs of varying age since establishment. Hellbender movement peaks (Burgmeier *et al.* 2011a) and aggressive conspecific interactions become more common during the brief breeding season (Smith 1907). We speculate that exploration of potential nesting sites and displacements by conspecifics as the result of aggressive interactions explain the peak in shelter occupancy just prior to breeding each year. Seasonal variation in artificial shelter use by other species is common but patterns are species-specific and attributed to seasonal variation in resource demand, resource availability, population fluctuations or some combination of factors (Mainwaring 2011). The fact that hellbender occupancy of artificial shelters varied seasonally but peaked around the same time each year in every reach indicates that late summer surveys should provide the most robust estimate of hellbender relative abundance (assuming natural shelter has been accounted for). Additionally, our findings warrant the need to control for season when making comparisons between occupancy rates of artificial shelters across sites and years.

Our study provides preliminary evidence that ASAs offer several advantages over more traditional survey methods used to monitor hellbenders. Despite the notoriously problematic nature of CPUE as an index of abundance (Maunder *et al.* 2006), CPUE estimated from uncorrected count data obtained from rock lifting surveys remains a popular method for reporting relative abundance of hellbenders. While snorkeling surveys may increase the probability of detecting larvae and sub-adults (Nickerson and Krysko 2003), multiple snorkeling surveys per site are required to obtain reliable abundance estimates of any age class (Mazerolle *et al.* 2007). In contrast, although ASAs were only effective at detecting adult age classes, we found that occupancy rates during a single monthly survey functioned as a reliable index of relative sub-adult/adult abundance within our study reaches when natural cover was relatively limited. Other advantages include the fact that surveying ASAs generally requires fewer personnel, less training and is less dangerous that snorkeling while rock lifting (Browne *et al.* 2011). Snorkeling surveys require multiple individuals in the water, one of which is charged with reaching beneath heavy objects while they are elevated, and participation in multiple snorkeling surveys is generally required to train new surveyors to safely and effectively locate, capture, and process hellbenders (pers. exp.). In contrast, shelters can be checked by a single individual, and training can be completed within a day. Finally, ASAs offer the first non-destructive method to monitor breeding and reproductive ecology of hellbenders, which is virtually unstudied. The number of nests detected during our study surpasses the number of nests reported in any peer-reviewed hellbender study ([n = 12] Smith, 1907; [n = 2] Nickerson and Tohulka, 1986). While nest ecology was not the focus of the current study, our observations during monthly shelter surveys indicated great potential for ASAs to function as a novel tool to monitor hellbender reproductive success.

As with any alternative monitoring approach there are also potential drawbacks associated with relying on ASAs as monitoring approach for hellbenders. Construction of shelters as per Briggler and Ackerson (2012) is time consuming. Aside from materials (~ $30 per shelter), labor associated with construction and installation require considerable up-front costs. Due to the weight of shelters, installation in remote areas is challenging and once installed routine maintenance is required to ensure shelters and lids remain intact and to keep tunnels cleared. Furthermore, the lack of shelter use by juvenile hellbenders in our study suggests that ASAs are most useful for monitoring adults in a population or early developmental stages (i.e., egg and pre-emergent larvae).

MANAGEMENT IMPLICATIONS

Our findings suggest that ASAs may be best suited for long-term research programs focused on monitoring hellbender occurrence, adult health, relative abundance, reproductive success or egg/larval development. We recommend careful consideration of substrate characteristics, accessibility, and typical conditions (e.g., visibility and flows) during the season when monitoring is intended to occur at sites where ASAs might be installed. When obtaining a relative index of abundance is a monitoring goal we recommend installing ASAs in sites where ≤ 30% of substrate within the reach is classified as boulder or bedrock. In order to maximize occupancy rates and use of shelters for nesting in year one post-installment we recommend installing shelters in early spring (March-May). The ideal number of shelters to install per array depends on hellbender abundance, substrate within the reach and the desired number of hellbenders that researchers hope to collect from an array during each sample (Table 4.5). There is generally a decreasing return for investment in additional shelters when hellbender density is relatively low (< 1 sub-adult/adult per 100 m^2; Table 4.5). Ten shelters per array may be adequate if the goals of monitoring are to ensure that hellbenders continue to occupy the reach, while 20 or more shelters per array will improve reliability of counts as an index of relative abundance (Table 4.5). All of our recommendations assume shelters are installed at a density of approximately 0.6 shelters per 100 m^2 of wetted stream habitat. It remains unclear whether shelter density has an appreciable impact on ASA occupancy rates.

Our findings highlight the potential for ASAs to function as a valuable addition to the suite of sampling approaches developed for hellbender monitoring. Additional research aimed at identifying microhabitat characteristics within stream reaches that maximize resilience of shelters to high flow events, minimize tunnel blockage and maximize the likelihood of nest establishment could be especially valuable in developing ASAs as a monitoring and conservation tool for hellbenders.

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TABLES

Table 4.1. Summary of stream reach characteristics where artificial shelter arrays for hellbenders (*Cryptobranchus alleganiensis*) were installed and statistics from hellbenders captured during shelter surveys between July 2013 and Sep 2015.

				Shelter surveys[b]				No. nests established			
Reach	Density[a]	Boulder and bedrock cover (%)	No. shelters installed (missing)	Total	Available[c] (occupied)	No. unique individuals	Mean (range) total length (mm)	2013	2014	2015	Total
R1	0.31	7	30 (4)	318	260 (17)	6 (4 F; 2 M)	516 (421-594)	-	1	1	2
R2	0.41	17	30 (5)	270	227 (22)	11 (4 F; 7 M)	495 (425-640)	-	3	2	5
R3	0.82	51	30 (7)	270	207 (15)	7 (4 F; 1 M; 2 U)	430 (291-515)	-	0	2	2
R4*	1.84	10	30 (0)	483	442 (213)	53 (26 F; 24 M; 3 U)	426 (285-580)	4	3	8	15
R5	2.13	27	30 (0)	294	265 (22)	13 (3 F; 8 M; 2 U)	405 (340-471)	-	0	0	0
R6*	2.97	5	30 (0)	426	403 (171)	66 (20 F; 39 M; 7 U)	419 (310-575)	1	2	6	9
Total			180 (16)	2061	1804 (460)	156		5	9	19	33

* Indicates reaches where pilot work (July 2013-July 2014) took place. Shelters were not deployed in other reaches until July/Aug 2014.

[a] Estimated number of sub-adult/adult hellbenders per 100 m^2

[b] Shelter surveys were calculated as the sum of independent (i.e., approximately monthly) surveys for all shelters in a reach; where shelters that were defined as missing were ignored in all future surveys

[c] A shelter was only considered available during a survey if it had not been dislodged since the previous survey, if the tunnel was at least partially open, and if high water or poor visibility did not prevent the shelter from being surveyed. Dislodged shelters and shelters with blocked tunnels were restored to an available state at the end of each survey.

Table 4.2. Summary and description of covariates used to model occupancy of artificial shelters by hellbenders (*Cryptobranchus alleganiensis*).

Variable	Description	Mean (range)	Transformation	Predicted effect
Density	Mean density of sub-adult/adult hellbenders per 100 m^2	1.62 (0.31-2.98)	None	+
Day of Year	Days since 1 January of the given year (1-365)	200 (35-353)	$x - \bar{x} / _{\mathrm{sd}(x)}$	Peak at approx. day 230 (onset of breeding)
Day of Year^2	Day of Year squared (after transformation)			
Time	Number of days since shelter was installed in its current location; Note that number of days since deployment was reset to zero on the day a shelter was moved to a new locality	243 (6-834)	$x \times 0.01$	+
Lid	Categorical variable with two levels indicating whether the shelter lid was on (0) or ajar/off (1) at the time a shelter was surveyed	n_0 = 1647; n_1=154	None	−
Coarse	Variable representing availability of natural shelter sites; Percent of random points in a	17 (5-51)	$x \times 0.1$	−/+

	modified Wolman (1954) pebble count that were classified as boulder or bedrock
ID	Factor with 243 levels representing random effects of shelter ID

Table 4.3. Candidate set of models used to investigate factors associated with artificial shelter occupancy by hellbenders (*Cryptobranchus alleganiensis*), arranged in order of relative support according to model selection criteria.

Model Structure	K[a]	LogLikelihood	AICc[b]	ΔAICc	w_i[c]
Density + (Day of Year + Day of Year^2) + Time + Coarse + Lid + ID	8	-761.59	1539.26	0.00	1.00
Density + (Day of Year + Day of Year^2) + Time + Lid + ID	7	-768.00	1550.06	10.80	0.00
(Day of Year + Day of Year^2) + Days Out + Coarse + Lid + ID	7	-777.40	1568.88	29.62	0.00
Density + (Day of Year + Day of Year^2) + Coarse + Lid + ID	7	-786.87	1587.80	48.54	0.00
(Day of Year + Day of Year^2) + Time + Lid + ID	6	-793.86	1599.77	60.51	0.00
Density + (Day of Year + Day of Year^2) + Lid + ID	6	-796.36	1604.78	65.52	0.00
(Day of Year + Day of Year^2) + Coarse + Lid + ID	6	-805.62	1623.30	84.04	0.00
(Day of Year + Day of Year^2) + Lid + ID	5	-828.43	1666.91	127.65	0.00

[a] Effective number of estimated parameters

[b] Akaike information criterion corrected for small samples

[c] Akaike model weight

Table 4.4. Mean coefficients, standard errors, 95% profile confidence intervals and odds ratios for fixed effects in the best supported model describing variation in artificial shelter occupancy by hellbenders (*Cryptobranchus alleganiensis*).

	β	SE	95% CI		e^{β}[a]
(Intercept)	-2.908	0.436	-3.817	-2.092	
Density	0.870	0.164	0.562	1.211	2.39
Day of Year	0.046	0.079	-0.109	0.202	
Day of Year^2	-0.389	0.072	-0.532	-0.248	
Time	0.291	0.042	0.209	0.374	1.34
Coarse	-0.398	0.115	-0.633	-0.178	0.67
Lid	-1.882	0.414	-2.756	-1.110	0.15

[a] Odds ratio, interpreted as the increase in the odds of shelter occupancy (probability occupied/probability not-occupied) for every one-unit increase in the associated predictor (scaled for analysis).

Table 4.5. Model derived estimates of the number of hellbenders that would be available for capture from shelters (installed at a density of 0.6 shelters per 100 m^2 of wetted channel) during August, approximately one year post-installation as a function of population density, number of shelters and availability of natural shelter within a reach.

	10% boulder & bedrock				30% boulder & bedrock				50% boulder & bedrock			
	Sub-adult/adult density (no./100 m^2)				Sub-adult/adult density (no./100 m^2)				Sub-adult/adult density (no./100 m^2)			
No. shelters	0.5	1	2	3	0.5	1	2	3	0.5	1	2	3
5	1	1	2	3	0	0	1	1	0	0	0	1
10	1	2	4	6	1	1	2	3	0	0	1	1
15	2	3	6	9	1	1	3	4	0	1	1	2
20	3	4	8	12	1	2	3	5	1	1	2	2
25	4	5	9	15	2	2	4	7	1	1	2	3
30	4	6	11	18	2	3	5	8	1	1	2	4
35	5	7	13	21	2	3	6	9	1	1	3	4
40	6	8	15	24	3	4	7	11	1	2	3	5
45	6	9	17	27	3	4	8	12	1	2	3	5
50	7	10	19	30	3	5	9	13	1	2	4	6

FIGURE LEGENDS

Figure 4.1. Photographs of artificial shelters installed into natural stream habitat and occupied by hellbenders (*Cryptobranchus alleganiensis*) in southwest Virginia, USA.

Figure 4.2. Arrangement of hellbender (*Cryptobranchus alleganiensis*) artificial shelter arrays augmented into six stream reaches (R1-R6) in southwest VA, USA. Black dots represent original shelter locations (30 per array) and rectangles encompass the randomly selected sub-reach (10 shelters per sub-reach) where hellbender density and substrate characteristics were quantified. Note that locality of some shelters shifted slightly (within a ~15 m radius) during the course of the study.

Figure 4.3. Estimated probability of artificial shelter occupancy by hellbenders (*Cryptobranchus alleganiensis*) as a function of population density, time since installation and season. Note that occupancy peaks in late summer and increases with hellbender density and time since shelter installation. Estimates assume average levels of natural shelter availability within the stream reach (16% of reach characterized by boulder and bedrock). Height of bars represents mean model-derived estimates and error bars represent 95% confidence intervals.

Figure 4.4. Predicted effect of natural shelter (boulder and bedrock) availability on hellbender (*Cryptobranchus alleganiensis*) occupancy of artificial shelters during the season of peak shelter occupancy (August) at one year post-shelter installation. Note that shelter occupancy is only a

reliable index of hellbender density when natural shelter is relatively limited, and occupancy rates can be extremely disparate at high density sites depending on natural shelter ability.

FIGURES

Figure 4.1. Photographs of artificial shelters installed into natural stream habitat.

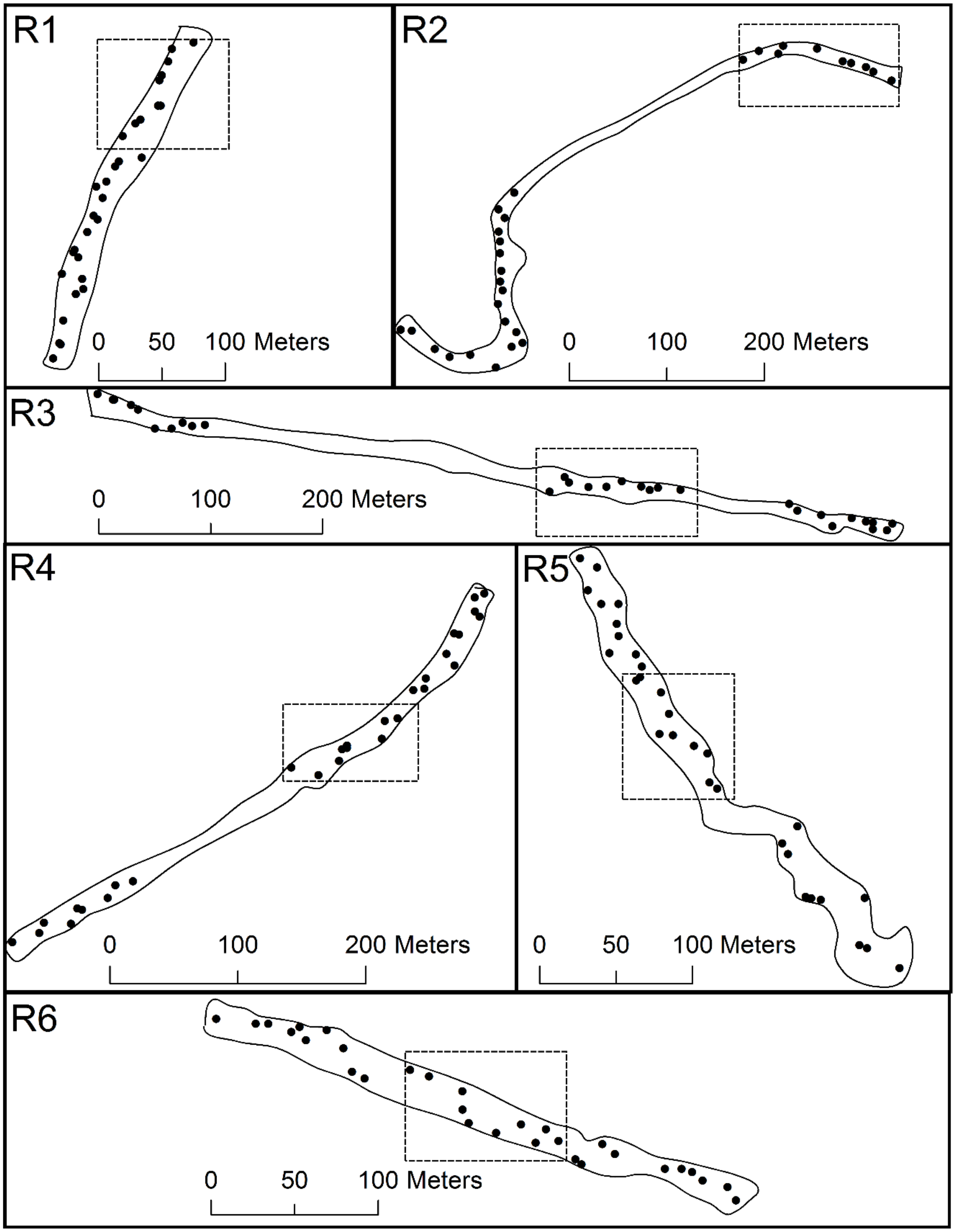

Figure 4.2. Arrangement of hellbender artificial shelter arrays augmented in six stream reaches (R1-R6) in southwest VA, USA.

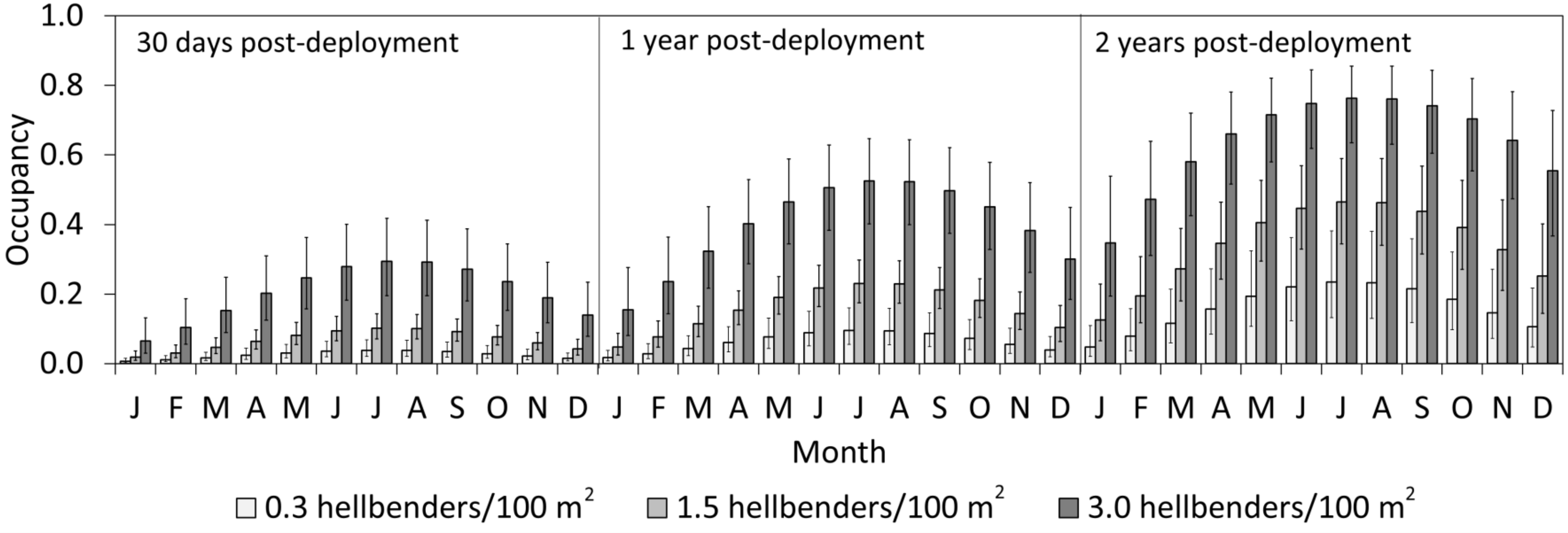

Figure 4.3. Effect of season and time since installation on occupancy of artificial shelter occupancy by hellbenders.

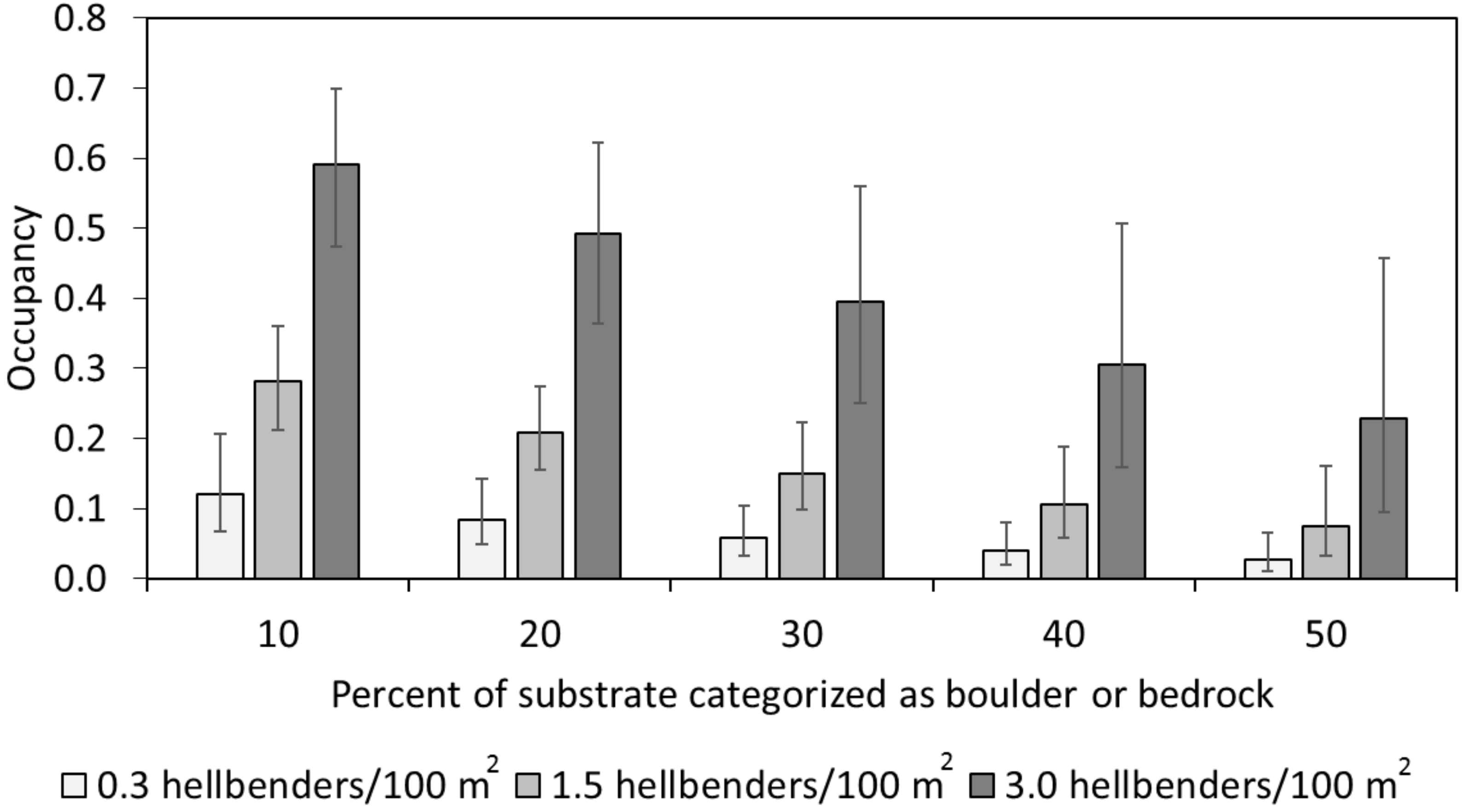

Figure 4.4. Effect of natural shelter (boulder and bedrock) availability and sub-adult/adult density on hellbender occupancy of artificial shelters during the season of peak shelter occupancy (August) at one year post-shelter installation.

CHAPTER V. Conclusions and Synthesis

Catherine M. B. Jachowski

Freshwater ecosystems and the species tied to them currently rank among the most threatened on Earth as a result of global changes associated with a growing human population (Stuart *et al.*, 2004; Bland *et al.*, 2012; Burhead, 2012). Overarching drivers of freshwater biodiversity losses include land use alteration, climate change, pollution, disease and introduced species (Cardinale *et al.*, 2012; Hooper *et al.*, 2012). Land use alteration is recognized as a particularly important determinant of aquatic habitat quality (Allan, 2004) and is predicted to be the most important determinant of freshwater biodiversity loss during the 21st century (Sala, 2000). Development of effective conservation strategies for freshwater ecosystems rely heavily on our understanding of the vulnerability of individual species to land use alteration, and how other stressors (e.g., parasite and pathogen exposure) might interact with land use to elicit negative effects on individuals and populations of freshwater organisms.

Understanding whether and how a species responds to a stressor across hierarchical levels (Fig. 5.1) can provide novel insight into ecology of a species while providing information that can be used to directly inform and focus conservation planning. At the finest scale, individual-level responses such as physiological condition provide a snapshot of how an animal is interacting with its environment and may highlight specific pathways (e.g. immunity, growth, reproduction) by which a stressor challenges individuals. Physiology is an important determinant of decisions made at the individual level that function as more proximate determinants of fitness, such as whether or not to forage, disperse or breed. Thus, effects of a stressor on individual physiology can result in scaled up effects at the local population scale. Thus, altered physiology can function as a mechanism of shifts in demographic rates that result in changes in local population abundance. Understanding whether local abundance responds to a stressor is fundamental for conservation planning because declines in abundance threaten persistence of a minimum viable population. Understanding mechanisms (e.g., altered demographic rates) responsible for change in abundance can highlight specific life history characteristics that make a species vulnerable to a stressor and can aid in development of targeted management strategies (e.g., reducing mortality versus increasing reproductive success). Finally, at the broadest scale, understanding whether patterns of species occurrence across a landscape are likely to shift in response to a stressor can identify areas of high conservation priority and may identify additional threats to a species. For example, as local population abundance declines in patches of occupied habitat the distributional range of a species might contract or become increasingly fragmented; resulting in increased risk of inbreeding depression via isolation, increased risk of species extinction, and/or shifts in biotic exchange as a result of altered community composition.

This body of work represents the first quantitative evidence of land use effects on hellbender demographics in any portion of their range and contribute valuable information regarding the spatial scale of land use that hellbenders are most sensitive to. Deforestation has long been suspected to act as a driver of hellbender declines, but previous attempts to detect effects of land use on hellbender demography have been unsuccessful. Findings herein suggest that protection and enhancement of forest cover in riparian areas of headwater streams as well as main stem water bodies is one approach to conserving and improving the status of hellbenders throughout their range. Furthermore, as little as 65% of upstream riparian areas need to be dedicated to forest in order for hellbenders to persist. The threshold of 65% catchment-wide riparian forest cover may be useful for identifying areas suitable for hellbender reintroduction. Repatriation/reintroduction has become an increasingly popular conservation strategy for hellbenders but has been criticized for attempting to improve low density populations before correcting drivers of decline. Findings presented here suggest areas where hellbenders were extirpated as a result of historic land use may be suitable reintroduction sites once riparian forest cover has been restored; and that reintroductions into areas with restored riparian areas may be more successful than attempts to repatriate remnant populations in areas with < 65% catchment-wide riparian forest cover $_{R}$.

Findings from Chapter I represent the first spatially explicit estimates of probability of occurrence for hellbenders anywhere throughout their range. Prior to this work, hellbender distribution was only understood at a relatively coarse scale (i.e., presence/absence among states, within streams or among pre-delineated habitat patches in a handful of streams). Thus, these findings contribute valuable information regarding the status of hellbenders in Virginia and perhaps elsewhere. A popular opinion among hellbender biologists is that the southern Appalachian region, and upper Tennessee River basin in particular, represent the core of remaining suitable habitat for hellbenders and contain the majority of viable extant populations. The findings herein, however, question the assumption that southern Appalachian populations are any less prone to extinction that populations in other regions. For example, the discontinuous distribution of hellbenders (i.e., isolation by physiography) reported in Chapter I suggests that hellbender distribution is likely to be patchier than previously estimated even in the species' assumed "stronghold". Furthermore, given the effects of riparian land use on local population structure reported here, hellbenders in the southern Appalachians are likely facing relatively high risk of local extirpation in most currently occupied areas. For example, while about 35% (323 of 906 stream km) of the study area described in Chapter I was estimated to be occupied by hellbenders in 2013-2014, a post-hoc investigation into riparian land use statistics throughout the study area indicated that only about 6% of the study area (54 of 906 stream km) was both occupied *and* characterized by ≥ 65% catchment-wide riparian forest cover.

Chapter III contributes novel information regarding seasonal variation in hellbender physiology and suggests variation in hellbender reproductive success may explain low recruitment in areas of low catchment-wide riparian forest cover. Interestingly, individuals in less forested areas were in better body condition and thus should have been relatively well suited to invest in reproduction in any given year. However, seasonal body condition profiles in areas of relatively low catchment-wide riparian forest cover did not reflect investment in reproduction as they did in more forested areas. This raises the question of whether hellbenders were unable or less likely to participate in reproduction in any given year, which is particularly intriguing when viewed in combination with the fact that low population densities in the same areas were indicated to be result of low recruitment. Effects of land use on N:L ratios during the breeding season raised related questions or whether individuals in less forested areas exhibited altered hormone profiles that were in some way related to decisions regarding reproductive investment. Given that hormones are important mediators of breeding behavior in amphibians, these findings warrant additional work to determine the intrinsic and extrinsic determinants of hellbender reproductive success.

Until now, a major impediment to understanding hellbender reproductive ecology and reproductive success has been the difficulty associated with detecting and monitoring active nests. Findings from Chapter IV emphasize the tremendous value of artificial shelters as a monitoring tool, specifically to facilitate research aimed at better understanding reproductive physiology of hellbender parents and success of reproductive attempts. The effects of global changes such as land use alteration on reproductive success of freshwater vertebrates is generally poorly understood. With advancements in artificial shelters, hellbenders now represent one of the only fully aquatic stream dwelling vertebrates that can be permanently marked, repeatedly captured and monitored at multiple time points during a single reproductive attempt.

As a collective, this work contributes novel information regarding how, and also why, some freshwater species respond to land use in the manner that they do. Occurrence and abundance of many freshwater species are known to respond to land use alterations. However, comprehensive studies that attempt to describe underlying mechanisms responsible for these changes are exceedingly rare. I found that hellbender population densities were low and densities were declining in areas with relatively low forest cover throughout collective upstream riparian areas primarily as the result of recruitment rather as opposed to variation in adult survival. This is important because it highlights the fact that remnant populations of long-lived aquatic species can persist for long periods of time even after recruitment has halted and that the simple measure of presence of long-lived species in an area can be an extremely unreliable indicators of biotic integrity, local population viability or suitability of habitat patches for population augmentation (repatriation).

Findings from Chapters I-III emphasize the value of a multilevel approach when attempting to understanding whether species respond to stressors and why they respond as they do. Examining patterns of hellbender occurrence as well as abundance improved my ability to detect land use effects and provided valuable insight into mechanisms responsible for patterns observed at higher levels. Integration of information obtained from each level of inquiry suggests that loss of forest cover in riparian areas threatens the persistence of local hellbender populations and indicates that although current patterns of hellbender occurrence do not correspond to current land use conditions, patterns of hellbender occurrence do respond to land use given sufficient time. Patterns in individual physiology are consistent with the hypothesis that variation in reproductive success is at least partially responsible for reduced recruitment in areas of low catchment-wide riparian forest cover. More work is needed to determine whether other long-lived species respond to land use in a manner similar to hellbenders and whether longevity is a life history trait indicative of particular vulnerability to land use alteration.

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FIGURES

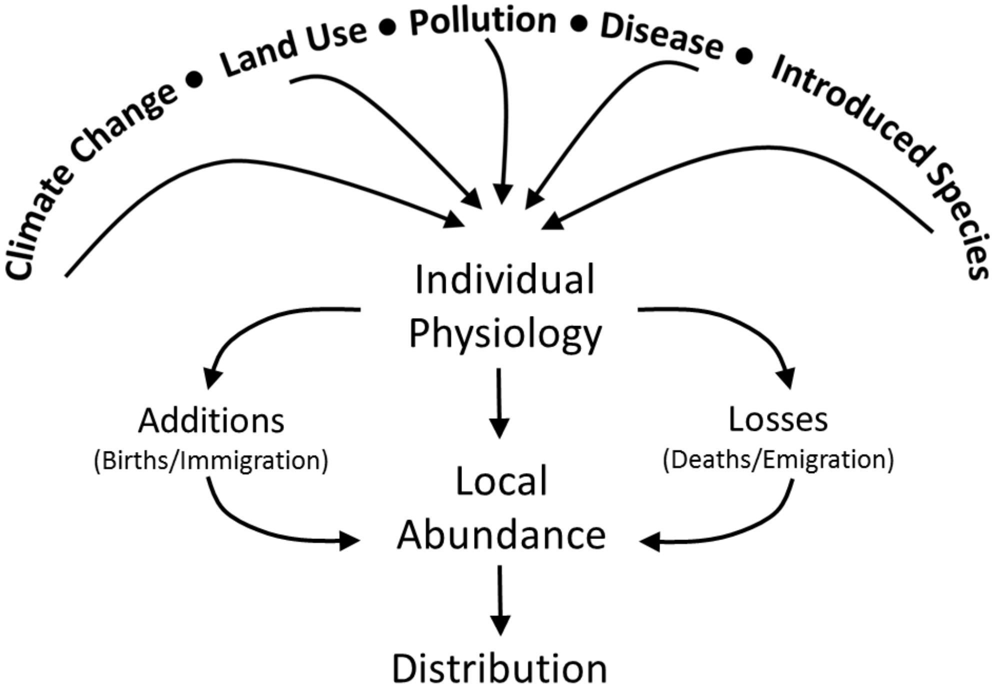

Figure 5.1. Conceptual diagram outlining mechanistic links between a species' response to a proposed stressor at various levels (individual physiology, local abundance, distributional patterns).