Using environmental DNA and occupancy modelling to identify drivers of eastern hellbender (Cryptobranchus alleganiensis alleganiensis) extirpation

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ORIGINAL ARTICLE

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Using environmental DNA and occupancy modelling to identify drivers of eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) extirpation

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Abstract

1. Population declines and local extirpation trends are widespread among freshwater species, but the responsible drivers of these trends are poorly understood. Identifying the potential drivers of population declines is essential to effective conservation planning. However, conventional detection methods used to monitor cryptic and elusive freshwater species are inefficient. Integrating new surveying and modelling techniques may allow for a more comprehensive assessment of population declines.
2. We used environmental DNA (eDNA) sampling methods and detailed historical records to identify drivers of local extirpation in a declining, long-lived giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in West Virginia, U.S.A. We used a site occupancy and detection modelling framework (SODM) to test the influence of current land use, historical mining, hydrogeomorphic and water quality variables on model-based predictions of hellbender extirpation and detection.
3. We failed to detect hellbender eDNA at 51% (naïve $1 - \Psi$) of historical sites, suggesting local extirpation at a broad spatial scale in West Virginia. Our best-supported SODM model suggested catchment-scale road density was the best predictor of hellbender extirpation, and that 38% (predicted $1 - \Psi$) of historical sites may be locally extirpated. Estimates of hellbender occupancy probability were extremely low in highly developed catchments. Water turbidity and conductivity were the best predictors of eDNA detection, both negatively influencing detection probability.
4. Roads can increase sedimentation rates and alter water chemistry of freshwater ecosystems, identifying landscape alteration/human development and water quality declines as possible drivers of hellbender extirpation trends in West Virginia. Our findings also suggest that water conductivity and turbidity may act as polymerase chain reaction inhibitors and decrease eDNA detection in lotic systems.

5. This study emphasises the negative impacts of urban development on freshwater ecosystems and the sensitivity of long-lived amphibian species to rapid environmental change. Our findings may aid in conservation planning by providing a sampling framework that integrates eDNA data within a SODM framework to rapidly and accurately assesses relational changes in aquatic species' occupancy at historical sites.

KEYWORDS
amphibian, conductivity, extirpation, population declines, turbidity

1 | INTRODUCTION

Monitoring the unprecedented population declines and extirpations of freshwater species has become a major focus of freshwater ecology and conservation (Jackson et al., 2016; Strayer & Dudgeon, 2010). Freshwater species, among them amphibians, rank as some of the most threatened taxa (Collen et al., 2014; Dudgeon et al., 2006; Houlahan, Findlay, Schmidt, Meyer, & Kuzmin, 2000; Poff et al., 1997; Sala et al., 2000). While documenting current species distributions is important for effective conservation planning and management (Groves et al., 2002), investigating the suspected causal agents responsible for species extirpations can further understanding of declines and benefit future conservation and habitat restoration efforts.

The declines of many freshwater species can be linked to synergistic interactions among hydrologic modifications (i.e. channelisation, damming), physical land-use changes, and declines in water quality and availability (Dudgeon et al., 2006). Additionally, the temporal scale at which freshwater populations respond to habitat degradation across a species range can span decades, especially for long-lived species (Bodinof Jachowski & Hopkins, 2018; Braulik, Arshad, Noureen, & Northridge, 2014). Consequently, few species have been actively monitored at decadal-scales and thus appropriate data are generally lacking (Magurran et al., 2010; but see Wheeler, Prosen, Mathis, & Wilkinson, 2003). To fill information gaps, historical data can be incorporated with current distributions to examine long-term population trends (e.g. extirpation, Tingley & Beissinger, 2009). Thus, interactions among terrestrial and aquatic systems and ecological responses are complex, can propagate, and operate at multiple scales, making it difficult to link changes in freshwater species distributions to landscape-scale processes (Stanfield & Kilgour, 2013).

Freshwater species monitoring programmes often employ physical capture sampling methods; however, these methods suffer from imperfect detection, particularly for rare, cryptic and elusive species (Fukumoto, Ushimaru, & Minamoto, 2015; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). Recent advancements in molecular-based methods such as environmental DNA (eDNA) have allowed for rapid presence/absence detection of aquatic organisms (Barnes & Turner, 2016; Spear, Groves, Williams, & Waits, 2015; Thomsen et al., 2012; Wilcox et al., 2016). The application of eDNA sampling methods in a variety of ecosystems has shown eDNA to be a time-and-cost-effective, non-invasive surveying approach (Thomsen & Willerslev, 2015). Further, eDNA methods may provide higher detection probabilities than conventional sampling approaches (Dejean et al., 2012; Jerde, Mahon, Chadderton, & Lodge, 2011; Pilliod, Goldberg, Arkle, & Waits, 2013; Schmelzle & Kinziger, 2016; Smart, Tingley, Weeks, van Rooyen, & McCarthy, 2015; Spear et al., 2015). Thus, its widespread use and performance advantage as a sampling method highlight the effectiveness of eDNA as a conservation tool (Goldberg et al., 2016; Thomsen & Willerslev, 2015).

In lotic systems, the application of eDNA has only recently grown in use. Consequently, there are considerable knowledge gaps in understanding how environmental variables influence DNA detection (Pilliod et al., 2013; Wilcox et al., 2016). Experimental studies have identified DNA persistence times and transport distances; however, the influence of hydrology and water quality characteristics on eDNA detection is poorly understood (Barnes et al., 2014; Deiner & Altermatt, 2014; Pilliod, Goldberg, Arkle, & Waits, 2014; Wilcox et al., 2016). Further, the physical and chemical characteristics of lotic systems that vary temporally such as flow, turbidity, and water chemistry likely effect eDNA detection, but few studies employ proper modelling approaches to account for variation in eDNA detection (Schmelzle & Kinziger, 2016). Integrating a site occupancy-detection modelling (SODM) framework with eDNA sampling methodologies can improve estimates of occupancy and detection derived from eDNA presence/absence data (Boothroyd, Mandrak, Fox, & Wilson, 2016; Ficetola, Taberlet, & Coissac, 2016; Hunter et al., 2015; Schmidt, Kery, Ursenbacher, Hyman, & Collins, 2013).

Eastern Hellbenders (*Cryptobranchus a. alleganiensis*, hereafter hellbender) are cryptic, fully aquatic giant salamanders that have experienced precipitous population declines across their historical range (Burgmeier, Unger, Sutton, & Williams, 2011; Foster, McMillan, & Roblee, 2009; Graham et al., 2011; Pitt et al., 2017; Wheeler et al., 2003). Their current distribution in West Virginia (WV) has been largely unassessed, despite being listed as an imperilled species (S2 rank, WV Division of Natural Resources 2017, but see Keitzer, Pauley, & Burcher, 2013). This study is of conservation interest because hellbenders are currently being considered for listing under the U.S. Endangered Species Act (J. Applegate, personal communication,

March 14, 2018). Hellbenders inhabit streams with fast flow, cobble/boulder rock cover, and good water quality (Nickerson & Mays, 1973). Current research has focused on identifying quantitative relationships among suspected land use, habitat, and water quality variables associated with the species presence and changes in population demography (Bodinof Jachowski & Hopkins, 2018; Bodinof Jachowski, Millspaugh, & Hopkins, 2016; Freake & DePerno, 2017; Pitt et al., 2017). Causal agents of population declines are suspected to be habitat loss via siltation and filling of interstitial spaces because of anthropogenic landscape disturbances and water quality declines that impede successful reproduction via a lack of recruitment (Pitt et al., 2017). However, implementing a more effective sampling and modelling framework to better understand the possible causes of hellbender extirpation is needed.

In this study, we used historical and current distribution data to examine hellbender extirpation in WV, USA. Specifically, we used eDNA to examine current hellbender occupancy at locations that historically supported hellbender populations. We used a single species, single season SODM framework with catchment and riparian-scale predictors of occupancy that included hydrogeomorphic, current land cover, and historical mining data to determine possible drivers of extirpation (Pitt et al., 2017; Wenger, Peterson, Freeman, Freeman, & Homans, 2008). We assumed that populations were extirpated when the species no longer occupied a historical site. The species is well suited for this approach, as hellbenders are very sensitive to water and habitat quality declines, have low vagility that inhibits recolonisation, and exhibit a slow life history with great longevity (i.e. >30 years, Taber, Wilkinson, & Topping, 1975). Together these traits provide a strong and persistent signal of extirpation. This study is important because its use of historical data and ability to account for imperfect detection provide a framework to examine a species decline at spatial and temporal scales suitable for quantifying extirpation, while also providing insight into suspected environmental variables associated with declines.

2 | METHODS

2.1 | Study area

Our study area encompassed the historical range of hellbenders within the Ohio River drainage in WV, which ranges from high-gradient streams in the eastern Allegheny Mountains and Appalachian Plateau, to low gradient streams in the Ohio Valley (Figure 1). The Ohio River's drainage area is 490,600 km^2, of which 34% (168,827 km^2) lies within WV. Our study sites were within three major river drainages that contribute to the Ohio River in WV: the Kanawha/New, Guyandotte, and Cheat. A historical account from Green (1934) suggests that hellbenders were more abundant in WV than any other region of the Ohio River drainage.

2.2 | Historical data and study site selection

We obtained historical records through the WV Biological Survey Museum housed at Marshall University, WV Natural Heritage Database, Humphries and Pauley (2005), and Keitzer et al. (2013). To specifically examine hellbender extirpation, we only used records that provided detailed location information (i.e. coordinates or landmarks). Of the 57 historical records we found, 52 sites were sampled based on our criteria and spanned a timeframe from 1932 to 2016. Due to proximity (<2 km) of some historical sites within a mainstem river channel, we conservatively removed three sites from our analysis as a precaution for lack of site independence. Environmental DNA transport distances vary by species and environmental conditions, and the issue of site independence in eDNA studies within lotic systems is rarely discussed (Deiner & Altermatt, 2014; Jane et al., 2015; Pilliod et al., 2013). Sites were georeferenced in the field using a Garmin GPSMAP® 64st GPS unit.

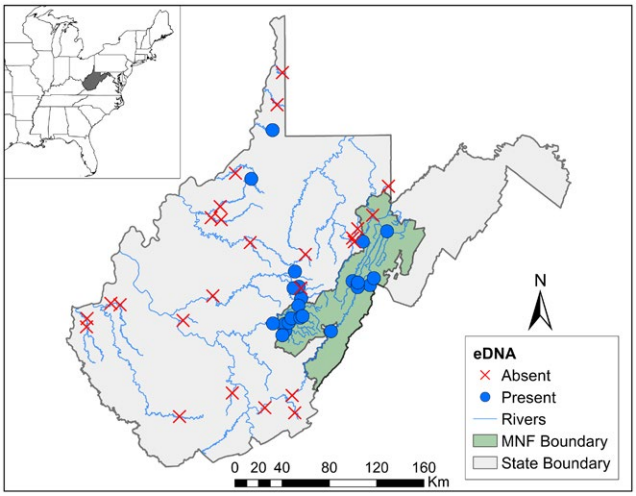

FIGURE 1 Naïve results of environmental (eDNA) sampling surveys at 49 sites with historical Eastern Hellbender records in the Ohio River drainage of West Virginia, U.S.A. Sites are shown as present if eDNA amplified in at least one out of four temporal sampling replicates with two out of three positive quantitative polymerase chain reaction replicates. MNF, Monongahela National Forest

2.3 | Field collection protocol

During spring and summer 2017, we collected 1-L water samples to use in our eDNA analysis at each of the historical 49 sites and temporally replicated sampling over four sampling periods to estimate the probability of eDNA detection. Sampling periods ranged from (1) 17 April to 31 May; (2) 02 June to 30 June; (3) 06 July to 06 August; and (4) 08 September to 30 September. We used single-use disposable equipment to collect samples to avoid contamination among sites (Goldberg et al., 2016). Forceps used for extracting filters were the only piece of equipment reused among sites and were first soaked in a 70% ethanol solution and flame sterilised, then treated with DNA Away Surface Decontaminant (Molecular Bio-products, Inc., San Diego, CA) prior to filter extraction to avoid sample contamination. At each site, we used a sterile, disposable Whirlpak Stand-up Bag (1065 ml capacity, Nasco, Fort

Atkinson, WI) to collect 1-L water samples from the centre of the stream. We used a Cole-Parmer Masterflex Peristaltic Pump (Model No. 7520-00, Cole-Parmer Instrument Co. Chicago, IL) attached to a 1-L Nalgene Vacuum Flask to filter water through sterile, disposable 250 ml Nalgene Analytical Test Filter Funnels (pore size = 0.45 μm, cellulose nitrate membrane, Thermo Fisher Scientific Inc., Rochester, NY). We immediately placed filter membranes into 1.5-ml microcentrifuge tubes post-filtering and subsequently stored them on dry ice prior to storage in a −20°C freezer. Due to the time constraint of keeping dry ice in the field and broad geographic spread of this study, sampling periods typically lasted 3–4 days, and the number of field samples taken during a sampling period ranged from 4 to 23 ($\bar{x}$ = 13.14). We filled sterile Whirlpak® bags with deionised water from a tap at Marshall University to use as a negative field control and kept them in the same container as all sample equipment. For each sampling period, we filtered the negative field control after the last field sample using the same protocol and equipment as field samples. After each sampling period, we sterilised all equipment reused among sites (i.e. waders and water quality probes) in a 30% bleach solution.

2.4 | Laboratory methods

We extracted DNA from filters using the protocol from Spear et al. (2015) with slight modifications of the DNeasy® Blood and Tissue Kit (Qiagen, Inc., Venlo, The Netherlands). We divided filters in half and tore them into pieces, with the other half stored at −80°C for potential later use. We followed the standard protocol for the extraction kit with the additional use of a Qiashredder (Qiagen, Inc.) spin column after the lysis step. We processed all samples in a separate and dedicated extraction and polymerase chain reaction (PCR) setup section of the laboratory.

We amplified environmental DNA samples following the quantitative (q)PCR protocol from Spear et al. (2015). A 104 bp region was amplified using primers;

CRALQ-F (5′ GTTTGCATGAGTATTRCGGATT 3′),

CRALQ-R (5′ TCGCTATRCATTATACAGCAGATACA 3′)

and probe: CRALQ-P (5′ VIC–CATCTCGGCAGATATG–MGB-NFQ 3′).

We used a 20 μl reaction volume consisting of 10 μl of Luna universal probe qPCR master mix (New England Biolabs), 1 μl of each primer at 10 μM and probe at 5 μM, 3.5 μl nuclease-free water, and 3.5 μl of sample extract on an Applied Biosystems 7900HT system. The qPCR protocol is as follows: 15 min at 95°C, 50 cycles of 94°C for 60 s and 60°C for 60 s, with data collection during the annealing stage at 60°C. We ran all extractions in triplicate with an internal positive control, a positive sample per plate from a captive hellbender population water sample, and negative control to assess qPCR efficacy and any potential contamination. Any samples that appeared to be inhibited were treated with OneStep-96™ PCR Inhibitor Removal Kit (Zymo Research) and re-run. We used a 1:2 serial dilution of the 13 ng/μl positive control to create a standard curve to determine concentration estimations for all eDNA samples.

We generated cycle threshold values (C_t), using SDS 2.4 software (Applied Biosystems). We used the C_t, known concentration, and dilution values for the positive control to generate two graphs; C_t versus dilution factor and dilution factor versus concentration. We plugged the averaged sample Ct values into the equation of the line for both graphs, $y = 1.0651x + 29.975$, and $y = 13.048e^{-0.697x}$ (Figure 2), to yield sample concentration.

For the first three sampling periods, we found the deionised water used from the tap at Marshall University to be contaminated at the source, as about one third of our negative field controls every sampling period amplified with one qPCR replicate. In some cases, all field samples were negative during the sampling period and the control was positive. We determined the deionised tap water to be contaminated at the source by filtering three samples of it in a separate laboratory using all single-use equipment, along with three samples of nuclease-free water for comparison. We found one out of the three samples of deionised tap water to be contaminated (one third of qPCR replicates for this sample amplified), and all nuclease-free water samples were negative. For the fourth sampling period, we used nuclease-free water sourced from outside the hellbenders

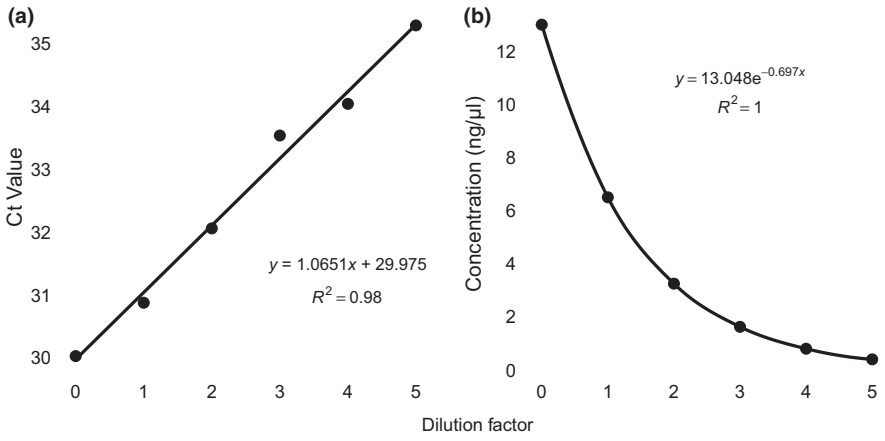

FIGURE 2 Cycle threshold (C_t) values from field samples versus dilution factor of positive controls (a), and environmental DNA concentration versus dilution factor of positive controls (b) used to estimate environmental DNA field sample concentrations

natural range for all field negative controls to avoid further source contamination of negative controls. All contaminated filter blanks from the first three sampling periods had only one third of qPCR replicates amplify, and all DNA concentration values were below 0.08 ng/μl. Therefore, field samples that had a minimum of two thirds of qPCR replicates amplify with concentrations above 0.08 ng/μl were used as an indicator of hellbender presence. There were only 12 occasions at a total of nine sites where field samples amplified with only one third of qPCR replicates that were excluded from being considered positive. Of those nine sites, five sites had significant amplification during other survey periods (see supplementary information). At the other four sites, this was the only occasion among all four survey periods that a sample amplified with one third of qPCR replicate. We deemed all 12 samples ambiguous and excluded them from being considered as a positive indicator of hellbender presence.

2.5 | Predictor variables

We used three categories of predictor variables to develop models of hellbender extirpation: hydrogeomorphic, current land cover, and historical mining (Table 1). We quantified all landscape-scale predictor variables using ArcMap 10.4 (ESRI, Redlands, CA). For each site, we delineated the upstream catchment area as the total area draining to the collection site (km^2). We calculated stream gradient using a Digital Elevation Model and stream network data from the National Hydrography Dataset (NHD, USGS 2017). We calculated dam density using the National Inventory of Dams (NID) dataset (U.S. Army Corps of Engineers 2017). We used physiographic region as a categorical predictor of whether the upstream catchment lay within the Appalachian Plateau or Appalachian Mountain physiographic region. We quantified in-stream habitat (pool, riffle, run) and substrate characteristics using a modified Wolman (1954) pebble count with 100 observations at each site. We measured stream wetted width and stream depth at three transects across each site, downstream (0 m), middle (75 m) and upstream (150 m) after our last eDNA surveys.

For each site, we calculated tree canopy cover (2015 imagery) at the catchment and riparian scale using a freely available 30 m resolution dataset (Sexton et al., 2013, www.landcover.org). Highly forested catchments that protect in-stream habitat and water quality have been associated with hellbender occurrence, but quantitative evidence is lacking, and the effect of tree cover loss may be time-lagged (Bodinof Jachowski et al., 2016; Wheeler et al., 2003; Williams, Gates, Hocutt, & Taylor, 1981). We chose not to include the National Land Cover Dataset (Homer et al., 2015) classes that are regularly used in catchment-scale ecological studies because of the issues associated with highly correlated land cover classes (King et al., 2005). Pixel values ranged from 0 to 100, indicating the percentage of the pixel area ground shaded by tree canopy. Pixel values above 100 denoted water, clouds, shadows or filled values, and were set as null values using a conditional input raster. We masked imagery to upstream catchment boundaries and 150 m riparian buffers on both sides of the stream for each site, and computed summary statistics to obtain the mean pixel value for each catchment and buffer area used in our analyses (Table 1).

We quantified catchment and riparian-scale road density using U.S. Census Bureau Tiger/Line® shapefiles. Roads permanently alter the physical landscape environment and contribute to sedimentation and chemical alteration of aquatic environments (Kaushal et al., 2018; Maltby, Forrow, Boxall, Calow, & Betton, 1995; Trombulak & Frissell, 2000). A study on the endangered black warrior waterdog (*Necturus alabamensis*), a species with similar habitat and water quality requirements to hellbenders, was negatively associated with impervious surfaces at the catchment scale (de Souza, Godwin, Renshaw, & Larson, 2016). We chose not to use the National Land Cover Dataset impervious surface dataset due to its underestimation of impervious cover at low development intensities and believed road density to be a finer-scale predictor for use in model development (Smucker et al., 2016). We clipped road shapefiles to individual catchment boundaries and 150 m riparian buffers, and calculated road density as a proportion of catchment and riparian area (km/km^2).

Due to the temporal scale of historical records (1932–2016) and unique land-use history of our study area, we included historical mining-related variables as predictors of hellbender occupancy. Surface mining activities degrade in-stream habitat (via sedimentation) and water quality over time, even after mine reclamation (Lindberg et al., 2011). We digitised strip and deep mining features from a seamless digital raster graphic county mosaic of USGS topographic maps (1:24,000 scale). Quadrangles varied in time from 1965 to 1987, as not all areas were surveyed during the same time. We calculated the proportion of the upstream catchment covered by surface mining, and density of deep mines per catchment (Table 1). We quantified the number of National Pollutant Discharge Elimination System (NPDES) mining-related outlets per catchment to assess the relative importance of point-source pollution on hellbender occupancy. Data were freely obtained through the WV Department of Environmental Protection GIS server. We vetted outlets listed as storm water drainage and retained only mining-related outlets.

2.6 | Sampling covariates

We collected water quality data (Table 1, also see supplementary information) during each site visit. Variable flow conditions of lotic systems have been shown to influence environmental DNA detection probabilities (Jane et al., 2015). Further, hellbenders have been negatively associated with high conductivity, which could impede reproduction (Pitt et al., 2017). We collected water quality data using a Hanna Instruments HI98196 Multiparameter probe (Hanna Instruments, Woonsocket, RI). Water velocity (m/s) was measured using a Marsh-McBirney Flo-Mate model 2000. Turbidity (formazin turbidity unit, FTU) was measured using a YSI Ecosense 9500 Photometer (Yellow Springs Instruments, Yellow Springs, OH). We standardised all continuous site and sample covariates by calculating *Z*-scores.

2.7 | Data analyses

We used a single species, single season site occupancy and detection modelling (SODM) framework to test the effects of environmental covariates of hellbender occupancy (psi,Ψ) and detection

TABLE 1 Summary of site and sample covariates considered in site occupancy and detection models to predict Eastern Hellbender occupancy and detection using environmental DNA in West Virginia (WV), U.S.A. DEP, Department of Environmental Protection; NID, National Inventory of Dams; DNR, Division of Natural Resources

Variable	Data source	Definition	Unit	Abbr.
Site covariates				
(1) Hydrogeomorphic				
Elevation	Field measurement	Elevation of sample site (m above sea level)	m	elev
Catchment area	ArcMap hydrology	Catchment area above sample site	km^2	ws.area
Stream gradient	ArcMap hydrology	Stream gradient above sample site (Δ Elev/stream length)	m/km	sgrade
Dam density	US ACOE NID	Density of dams in tributary system	no./km	dam.dens
Fine	Field measurement	% silt, sand, and fine gravel particles (*b*-axis 0.06–4 mm)	%	fine
Cobble	Field measurement	% Cobble substrate (*b*-axis 65–255 mm)	%	cobl
Boulder	Field measurement	% Boulder substrate (*b*-axis >256 mm)	%	boul
Riffle	Field measurement	% of 150 m site covered by riffle habitat	%	rifl
Run	Field measurement	% of 150 m site covered by run habitat	%	run
Pool	Field measurement	% of 150 m site covered by pool habitat	%	pool
Stream width	Field measurement	Mean width of stream at sample site	m	width
Stream depth	Field measurement	Mean depth of stream at sample site	m	depth
Physiographic region	USGS, Fenneman & Johnson 1946	Categorical predictor (Appalachian Plateau or Mountain region)	–	pregion
(2) Current land cover				
Catchment road density	TIGER/Line	Total length of roads in catchment/catchment area	km/km^2	ws.road
Riparian road density	TIGER/Line	Total length of roads in 150 m stream buffer/buffer area	km/km^2	rp.road
Catchment tree cover	Sexton et al. (2013)	Mean % canopy cover (2015 imagery) in the catchment boundary	%	ws.ccov
Riparian tree cover	Sexton et al. (2013)	Mean % canopy cover (2015 imagery) in a 150 m stream buffer	%	rp.ccov
Public land	WV DNR	% of upstream catchment covered by public land	%	pc.publ
(3) Historical mining				
% Area mined	USGS Topos	% of upstream catchment boundary covered by strip mines, quarries, or clay pits	%	pc.mine
Deep mines	USGS Topos	Density of deep mines per catchment	$no./km^2$	deep
NPDES outlets	WV DEP	Density of mining-related NPDES outlets in upstream catchment boundary	$no./km^2$	npdes
(4) Sample covariates				
Water velocity	Field measurement	Water velocity at sample site	m/s	flow
Ph	Field measurement	pH of water at sample site	pH	ph
Dissolved oxygen	Field measurement	Dissolved oxygen of water at sample site	%	do
Water temperature	Field measurement	Temperature of water at sample site	°C	temp
Salinity	Field measurement	Salinity of water at sample site	PSU	sal
Total dissolved solids	Field measurement	Total Dissolved Solids of water at sample site	ppm	tds
Conductivity	Field measurement	Conductivity of water at sample site	μS/cm	cond
Turbidity	Field measurement	Turbidity of water at sample site	FTU	turb

(p). In this study, we define psi (Ψ) as the probability that a site is occupied and p as the probability that eDNA will be detected in at least two of three qPCR replicates in at least one of four temporal replicates, given that the species occurs at the site. We assumed that there was no change in occupancy (i.e. immigration/emigration or colonisation/extirpation) between sampling periods. Occupancy models are used to estimate species occurrence while accounting for imperfect detection among multiple site visits (Guillera-Arroita, Ridout, & Morgan, 2010; MacKenzie et al., 2002, 2017). This modelling approach is robust to varying species detection probabilities, while allowing for the inclusion of covariates to test specific hypotheses about the factors that may influence species occurrence and detection (MacKenzie et al., 2017). The use of SODM in eDNA studies is imperative to account for imperfect detection and the seasonal activity of the study organism by estimating detection probability (p) (de Souza et al., 2016; Schmelzle & Kinziger, 2016; Spear et al., 2015). Since the goal of our study was to specifically examine the influence of environmental covariates on hellbender extirpation, negative estimates of the state variable occupancy (Ψ) were interpreted as positively influencing extirpation.

We assessed covariate independence using Spearman's rank correlation matrices for both sets of occupancy and detection covariates prior to model development to identify highly correlated variables ($r_s \geq 0.70$ or ≤ -0.70; Graham, 2003). Catchment and riparian-scale road density were highly correlated with both spatial scales of canopy cover, and numerous hydrogeomorphic variables such as catchment area and elevation. We removed these variables and retained road density because of the high anthropogenic impact roads can have on water chemistry and aquatic biota that could have contributed to hellbender extirpation (de Souza et al., 2016; Trombulak & Frissell, 2000). Predictors of historical mining were also highly correlated with each other ($r_s > 0.80$) and we retained only the proportion of catchment area covered by historical surface mining, based on the severe impacts of this land-use practice on aquatic ecosystems (Lindberg et al., 2011; Wu, Stewart, Thompson, Kolka, & Franz, 2015). From the remaining set of variables, we developed a candidate set of biologically relevant a priori models in three model subsets: hydrogeomorphic, current, and historical land use based on current knowledge of hellbender life history and site occupancy patterns to determine which variables best predict hellbender extirpation and detection in WV. We also included a model that varied detection by survey period to account for varying environmental flows and seasonal activity pattern differences throughout our sampling period (de Souza et al., 2016; Jane et al., 2015).

We ranked models using Akaike's information criterion (AIC) as recommended by MacKenzie et al. (2017) due to the ambiguity surrounding effective sample size of site occupancy models. We used models with ΔAIC $\leq$ 2.0 for inference (Burnham & Anderson, 2002). We evaluated model fit by examining the estimated variance inflation factors ($\hat{c}$) from three sub-global models among our three model subsets and used the smallest computed ($\hat{c}$) (Burnham & Anderson, 2002; MacKenzie & Bailey, 2004). We performed statistical analyses using R (R Core Team, 2018), and used the package *unmarked* to conduct SODM analysis (Fiske & Chandler, 2011).

3 | RESULTS

We detected hellbender eDNA at 24/49 historical sites (naïve $\Psi = 0.49$), indicating that 25/49 (naïve $1 - \Psi = 0.51$) of historical sites in WV may be locally extirpated. Many sites where populations persist are in or near the Monongahela National Forest in the Allegheny Mountains and high Appalachian Plateau (Figure 1). We detected low eDNA concentrations at two sites near the northern panhandle of WV. We detected no contamination in our negative field controls from the fourth sampling period.

3.1 | Occupancy and detection

We found no evidence for lack of fit in our model set based on goodness-of-fit test ($\hat{c} = 0.93$); therefore, we did not adjust our model ranking procedure for overdispersion. While ($\hat{c}$) < 1 indicates underdispersion, adjusting model ranking procedures is suggested only for overdispersion, and it is recommended to set $\hat{c} = 1$ in cases of underdispersion (Burnham & Anderson, 2002; MacKenzie et al., 2017). We retained two models with ΔAIC $\leq$ 2.0 for inference (Table 2). The top model, ψ(ws.road), p(cond+turb), indicated that occupancy was negatively associated with catchment road density ($\beta = -2.525 \pm 0.923$; Figure 3, Table 3). The second-ranking model had one additional occupancy covariate (% fine substrate) that was uninformative based on 95% confidence intervals (Table 3). Road density at the catchment scale was included in both models and negatively influenced hellbender occupancy (Figure 3, Table 2). We failed to detect a relationship between the proportion of catchment area mined, dam density, and natural hydrogeomorphic variables on hellbender occupancy. Both equivalent models (ΔAIC $\leq$ 2.0) included conductivity ($\beta = -2.448 \pm 0.870$) and turbidity ($\beta = -4.772 \pm 1.837$) as additive predictors of detection probability (Table 3). Conductivity and turbidity both negatively influenced detection probability, with estimates of p reaching 0 around 200 μS/cm and 25 FTU, respectively (Figure 3). Overall predicted estimates of hellbender occupancy and detection based on the best-supported model were 0.62 ± 0.12 standard error, and 0.45 ± 0.06 standard error, respectively. We then estimate the predicted proportion of historical sites that may be locally extirpated to be 38% (20/52) sites.

4 | DISCUSSION

Our study adds to a growing body of literature that establishes associations among landscape-scale and in-stream variables influencing population declines in fully aquatic salamander species that inhabit lotic freshwater ecosystems. Our eDNA and SODM results predict that hellbenders are no longer present approximately 38% of the 49 historical sites, suggesting broad-scale extirpation and range

TABLE 2 Candidate site occupancy and detection predicting Eastern Hellbender occupancy and detection using environmental DNA at historical (n = 49) sites, split among three model subsets and ranked according to Akaike's information criterion (AIC), with Δ AIC, number of parameters (k), and AIC weight (ω), and ($\hat{c}$) from goodness-of-fit tests

Model subset	Model	AIC	Δ AIC	k	ω	$\hat{c}$
Current land cover	Ψ(ws.road), *p*(cond+turb)	127.28	0.00	5	0.72	
Current land cover	Ψ(ws.road+fine), *p*(cond+turb)	129.28	2.00	6	0.26	0.93
Historical mining	Ψ(pc.mine+fine), *p*(cond+turb)	135.40	8.12	6	0.01	1.02
Historical mining	Ψ(pc.mine), *p*(cond+turb)	138.52	11.24	5	0.00	
Hydrogeomorphic	Ψ(pregion), *p*(cond)	155.29	28.01	4	0.00	
Hydrogeomorphic	Ψ(dam.dens), *p*(turb)	158.35	31.07	4	0.00	
Hydrogeomorphic	Ψ(dam.dens+fine), *p*(turb)	158.70	31.42	5	0.00	
Hydrogeomorphic	Ψ(dam.dens+pool), *p*(turb)	160.26	32.98	5	0.00	
Hydrogeomorphic	Ψ(fine), *p*(turb)	163.27	35.99	4	0.00	
Hydrogeomorphic	Ψ(cobl+boul), *p*(do+flow)	168.42	41.14	6	0.00	1.35
Hydrogeomorphic	Ψ(dam.dens), *p*(wtemp)	175.84	48.56	4	0.00	
Hydrogeomorphic	Ψ(rifl+run), *p*(do+flow)	177.50	50.47	6	0.00	
Hydrogeomorphic	Ψ(width+sgrade), *p*(do+flow)	178.43	51.15	6	0.00	
	Ψ(.), *p*(.)	181.79	54.51	2	0.00	
Hydrogeomorphic	Ψ(width+depth), *p*(do+flow)	182.30	55.02	6	0.00	
Historical mining	Ψ(pc.mine), *p*(ph)	185.47	58.19	4	0.00	
Hydrogeomorphic	Ψ(pregion), *p*(ph)	185.48	58.20	4	0.00	
	Ψ(.), *p*(survey)	185.85	58.57	5	0.00	
Hydrogeomorphic	Ψ(pregion), *p*(flow+wtemp)	187.06	59.78	5	0.00	

constriction in the Ohio River drainage of WV. Despite source contamination issues with the deionised water used for negative field controls during the first three sampling periods, we believe our results accurately represent presence/absence of hellbenders at our sample sites. We used deionised water from a tap that was sourced within the hellbenders range (Ohio River), which should generally be avoided in eDNA sampling, and whether we were amplifying hellbender eDNA from this source of water or experiencing non-specific binding is unknown. Given the lack of contamination in our fourth sampling period using a different source of water under the same sampling protocol and had no discrepancies among field samples, we believe the negative field controls from the first three sampling periods do not reflect contamination in the field given that contamination started at the source. Environmental DNA sampling is a relatively new technique that is prone to false positive and false negative errors (Ficetola et al., 2016). While alternative modelling approaches exist to account for such errors in analysis of eDNA data, we believe our conservative approach of applying a DNA concentration threshold and a minimum of two qPCR replicates for eDNA presence consideration to be valid (Lahoz-Monfort, Guillera-Arroita, & Tingley, 2016).

We found that catchment-scale road density best-supported model-based predictions of hellbender extirpation, while historical mining and hydrogeomorphic covariates received little support. The impacts of roads on freshwater ecosystems and aquatic biota have been well documented (Forman & Alexander, 1998; Laurance et al., 2014; Maltby et al., 1995). Increased catchment urbanisation and impervious surface cover can contribute to sedimentation, flow alteration and water quality declines (Baruch et al., 2018; Brabec, Schulte, & Richards, 2002; Wang, Lyons, Kanehl, & Bannerman, 2001). In our study, the combined impacts of water quality declines and increased sedimentation may have degraded streams beyond the suitable conditions required for hellbenders to persist. Our findings are consistent with a recent study by de Souza et al. (2016), who found that presence of the endangered black warrior waterdog, a fully aquatic salamander species with similar habitat requirements to hellbenders, was negatively associated with catchment-scale impervious surface area. However, their study took place in a relatively flat area of Alabama compared to the complex terrain of WV, and therefore, the relative impacts of sedimentation and chemical runoff may differ. Further, we could not include forest cover at both spatial scales in our analysis because it was highly correlated with road density, indicating that hellbender extirpation in WV may not be attributable to singular landscape-scale factors, rather the possible synergistic effects of deforestation and urban development (Price, Dorcas, Gallant, Klaver, & Willson, 2006; Surasinghe & Baldwin, 2015). Although we could not include the percentage of catchment area covered by public land in our models because it was highly correlated with forest cover and road density, recent research highlights the importance of highly forested catchments within public lands to preserve water quality and hellbender populations (Bodinof Jachowski & Hopkins, 2018; Freake & DePerno, 2017). Based on our analysis of historical locations, current hellbender

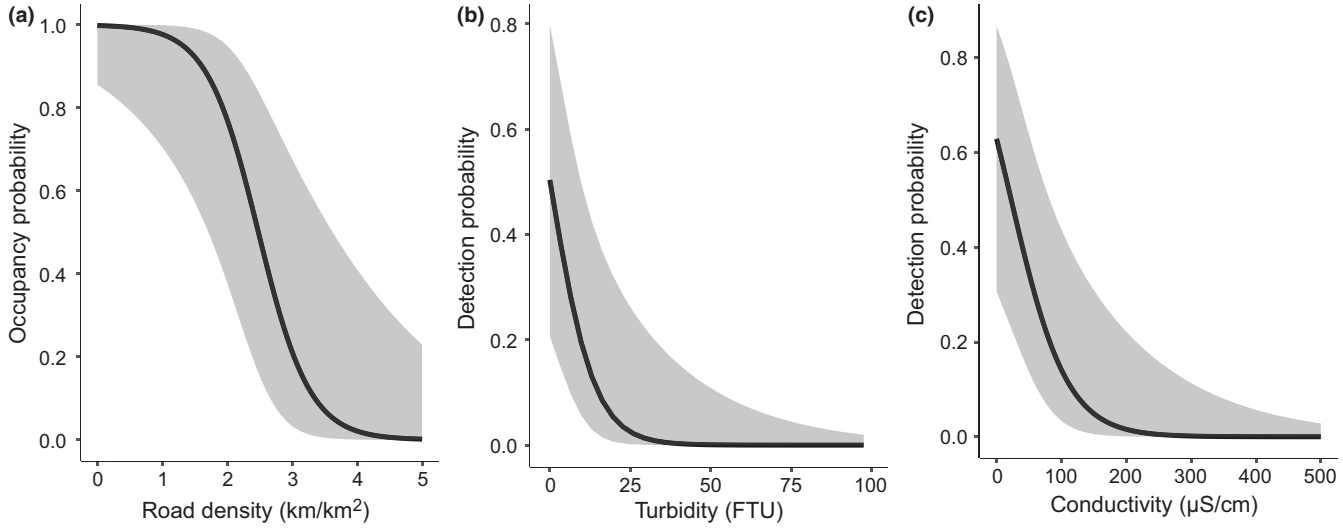

FIGURE 3 Predicted occupancy and detection probabilities for Eastern Hellbender environmental DNA based on the best-supported model: Ψ(ws.road), *p*(cond+turb) as a function of (a) catchment-scale road density, (b) turbidity of water at sample site, and (c) conductivity of water at sample site. Grey shading indicates 95% confidence intervals

TABLE 3 Best-supported site occupancy and detection models predicting Eastern Hellbender occupancy (Ψ) and detection (*p*) at historical sites (*n* = 49 sites) with coefficients (*β*), standard error (*SE*), and 95% confidence intervals (LCL, UCL). (ws. road = catchment-scale road density, fine = % fine substrate at sample site, cond = conductivity of water, turb = turbidity of water)

Model	Var	Parameters	*β*	*SE*	LCL	UCL
Ψ(ws.road), *p*(cond+turb)	Ψ	intercept	6.263	2.280	1.793	10.732
	Ψ	ws.road	−2.525	0.923	−4.334	−0.716
	p	intercept	−1.895	0.814	−3.490	−0.300
	p	cond	−2.448	0.870	−4.154	−0.743
	p	turb	−4.772	1.837	−8.373	−1.171
Ψ(ws.road+fine), *p*(cond+turb)	Ψ	intercept	6.274	2.477	1.419	11.129
	Ψ	ws.road	−2.525	0.924	−4.336	−0.715
	Ψ	fine	−0.068	5.587	−11.019	10.883
	p	intercept	−1.894	0.818	−3.498	−0.290
	p	cond	−2.449	0.871	−4.156	−0.741
	p	turb	−4.768	1.871	−8.436	−1.100

occupancy in WV may be constricted to high-quality headwater streams within and around the Monongahela National Forest, identifying this tract of managed public land as an important conservation area for hellbenders.

We failed to detect an influence of catchment-scale historical mining on hellbender extirpation in our analysis despite the large extent of contour strip mining throughout our study area, which could be due to the low relative contribution of these landscape features proportional to catchment size. For example, the largest catchments in our study (>657 km^2, the mean size of HUC 10 catchment boundaries for which sample sites occurred in) contained the largest area of strip mining. Alternatively, due to hellbender longevity (>30 years) and relatively high adult survivorship, relaxation time could inhibit linking historical land use to presence/absence for long-lived species (Bodinof Jachowski & Hopkins, 2018). Relaxation time is a delayed response of population declines in long-lived species as a result of some past habitat loss (Kuussari et al. 2009). While hellbender populations could be experiencing time-lagged effects of past land-use change, our failure to detect an influence of historical mining on hellbender extirpation could also reflect population resilience, given the return of suitable environmental conditions.

Hydrogeomorphic covariates were poor predictors of hellbender extirpation, and although the proportion of fine sediments was relevant in top performing models, it was an uninformative parameter. Fine sediment can fill interstitial spaces in cobble/boulder fields that are essential hellbender habitat, especially for larval age classes (Hecht, Freake, Nickerson, & Colclough, 2017). It is possible that we failed to detect a relationship between proportion of fine sediment and hellbender extirpation because pebble count techniques underestimate fine sediment (Hedrick, Anderson, Welsh, & Lin, 2013). Future habitat characterisation studies should use alternative methods to quantify sedimentation and substrate embeddedness.

Environmental factors that may influence eDNA degradation and detection are understudied in lotic systems (Jane et al., 2015; Stoeckle et al., 2017). Results from our SODM analysis suggest that turbidity and conductivity negatively influenced hellbender eDNA

detection. We considered both covariates equal in predicting hellbender detection due to their additive effects and inclusion in all top performing models (Table 3, Figure 3). Conductivity is a water quality parameter that measures the concentration of salts and other dissolved organic ions and is related to total dissolved solids, which was removed from consideration in our candidate model set because it was highly correlated with conductivity. Conductivity has been suggested to negatively influence eDNA detection; however, most field studies report insignificant findings in lotic systems (Keskin, 2014; Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012; Takahashi et al., 2018). However, increased conductivity and dissolved solids have been linked to PCR inhibition and decreased eDNA detection in mesocosm experiments and ponds (Buxton, Groombridge, & Griffiths, 2017; Harper et al., 2018). Further, substrate type and suspended sediments may increase conductivity and dissolved solids, which could lead to increased PCR inhibition (Buxton et al., 2017). The relationship between conductivity and turbidity (suspended sediments) recently described by Buxton et al. (2017) could explain why both variables strongly negatively influenced eDNA detection in our study. However, increased turbidity could also influence eDNA detection due to increased filtering time which could further degrade DNA present in the water sample or increase binding to suspended DNA particles that are present in the system (Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2016; Stoeckle et al., 2017; Williams, Huyvaert, & Piaggio, 2017). Because the influence of environmental variables on the detection of aquatic organisms in lotic systems is poorly understood, our findings are significant to furthering understanding of eDNA detection and degradation in lotic systems. Future eDNA studies in lotic systems should consider conductivity and turbidity as sampling covariates and consider their strong influence on eDNA detection.

Even though we included conductivity as a detection covariate, we suggest that this environmental variable may act on both state variables (i.e. occupancy and detection). Our results support mounting evidence linking increased conductivity to hellbender population declines (Bodinof Jachowski & Hopkins, 2018; Keitzer et al., 2013; Pitt et al., 2017; Pugh, Hutchins, Madritch, Siefferman, & Gangloff, 2016). Pitt et al. (2017) reported absence of hellbenders from the Susquehanna River drainage in Pennsylvania where conductivity exceeded 278 μS/cm. Similarly, hellbenders were absent from sites in our current study where conductivity exceeded 216 μS/cm. Average conductivity values for occupied sites were 42.33 (0–216) μS/cm, and 162.51 (70–546) μS/cm for extirpated sites pooled across four temporal replicates. Conductivity is suspected to impede hellbender recruitment by inhibiting sperm motility, which can result in decreased reproductive success (Ettling et al., 2013). Decreased fertility could explain why, in some areas, populations are comprised of primarily large, old adult individuals, which could make populations reproductively extinct (Briggler et al., 2007; Burgmeier et al., 2011; Pitt et al., 2017; Pugh et al., 2016; Wheeler et al., 2003). Conductivity levels are known to be greater in deforested and anthropogenically impacted (i.e. high impervious surface cover) catchments (Likens, Bormann, Johnson, Fisher, & Pierce, 1970; Trombulak & Frissell, 2000). Additionally, lotic freshwater systems are becoming increasingly saline due to anthropogenic salt inputs (e.g. road salts, sewage, brines, irrigation runoff), and accelerated geologic weathering, which could further explain why road density was a strong predictor of hellbender extirpation (Kaushal et al., 2018). Thus, protection of highly forested catchments is crucial for conservation planning and continued persistence of hellbender populations. However, further research should aim to better understand how conductivity and other important water quality parameters influence eDNA detection and hellbender population demography (Bodinof Jachowski & Hopkins, 2018).

Our study is one of the first to specifically identify factors associated with local hellbender extirpation and implicates anthropogenic development combined with declining water quality. Additionally, our study adds to a growing body of literature documenting substantial declines and extirpations of hellbender populations in other portions of their range (Briggler et al., 2007; Foster et al., 2009; Gates, Hocutt, Stauffer, & Taylor, 1985; Graham et al., 2011; Keitzer et al., 2013; Pfingsten, 1990; Pitt et al., 2017; Quinn, Gibbs, Hall, & Petokas, 2013; Wheeler et al., 2003). Range-wide hellbender population declines, often described as enigmatic, are just now being fully investigated using eDNA to determine the loss of area occupied by the species. Combining eDNA sampling with detailed demographic surveys to assess changes in population demography could provide a more mechanistic understanding of population declines (Bodinof Jachowski & Hopkins, 2018; Freake & DePerno, 2017; Pitt et al., 2017). Hellbender range constriction trends warrant timely conservation action to ensure continued persistence of remaining hellbender populations. Conservation action could induce an umbrella effect, protecting habitat and water quality for other sensitive freshwater species (Bodinof Jachowski & Hopkins, 2018).

Our study highlights the importance of preserving highly forested, low anthropogenically impacted catchments for hellbender conservation. By integrating high-quality historical data and eDNA sampling methods in a robust SODM framework, we were able to accurately and rapidly assess changes in hellbender presence and identify likely associations between environmental variables and extirpation over a broad portion of their historical range. This sampling approach has broad-scale applications and could be used to monitor changes in historical occupancy for any freshwater species. Our findings emphasise the sensitivity of freshwater species, particularly fully aquatic stream-dwelling amphibians, to land-use and water quality changes. Conservation planning should consider limiting road/impervious surface development, decommissioning unnecessary roads, and protecting or restoring forested landscapes in headwater streams to preserve water quality for the multitude of species reliant on it. Given our results, we propose that more research is needed to assess the effects of roads on sedimentation and water quality changes in freshwater systems. Because of increasing impacts on freshwater ecosystems and reports of freshwater species population declines, we emphasise the need for further studies on species extirpations that precede population demographic surveys for conservation monitoring.

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CONFLICT OF INTEREST

The authors confirm that there are no known conflicts of interest associated with this publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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