2018

Using Environmental DNA and Occupancy Modeling to Examine Drivers of Eastern Hellbender Extirpation and Sampling Method Efficiency in West Virginia

Sean Michael Wineland

USING ENVIRONMENTAL DNA AND OCCUPANCY MODELING TO EXAMINE DRIVERS OF EASTERN HELLBENDER EXTIRPATION AND SAMPLING METHOD EFFICIENCY IN WEST VIRGINIA

A thesis submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Master of Science
In
Biology
by
Sean Michael Wineland
Approved by
Dr. Jayme Waldron, Committee Chairperson
Dr. Shane Welch
Dr. Thomas Pauley
Dr. Jen Mosher

Marshall University
July 2018

ACKNOWLEDGMENTS

First, I would like to acknowledge and thank my family for their support and encouragement in pursuing a career in scientific research. I would not be the person I am today if they had not allowed me to roam around the woods and play in streams as a child which peaked my curiosity in the natural world. Second, I would like to thank my undergraduate research mentors, Drs. Brian Paulson, David Argent, and Carol Bocetti for their knowledge, guidance, support, and inspiration to keep moving forward. I would also like to thank Dr. Erica Kistner-Thomas who was my research mentor at the University of Notre Dame Environmental Research Center (UNDERC). My research experience and adventures across the country as an undergraduate student at UNDERC were unforgettable and have shaped me to be the biologist I am today. Next, I would like to thank my graduate advisor, Dr. Jayme Waldron, for the innumerable amount of work she does for her students, her guidance and help with field work on this project, and for greatly improving my writing and quantitative skills. I would also like to thank Dr. Shane Welch for his wealth of knowledge in aquatic ecology, his creative ideas, and for greatly improving the design and analysis of this project. I would also like to thank Drs. Waldron and Welch for allowing me to come out to their farm multiple times to hunt, herp, and enjoy nature (and escape from Huntington). I would like to thank Dr. Thomas Pauley for his knowledge and expertise in West Virginia Herpetology and for always being willing to talk about hellbenders. I thank Dr. Jen Mosher for allowing me to use (and break) her expensive equipment and lab space, and for her advice and support in delving into a research topic that I had no experience with.

This research would not have been possible without the help of many volunteers who helped with field work. I would like to thank Rachel Arrick for spending a whole summer doing field work with me and for putting up with my humor and sarcasm. Many thanks to Alex Murray who helped with field work numerous times and who has become a good friend. I will never forget the many times we went “rogue” and all of our herping adventures. Many thanks to numerous undergraduate students including Charles Chambers, Katie Taylor, and Cameron Cottrill, as well as Americorps volunteers for helping with fieldwork. I would like to thank the Herpetology and Applied Conservation Lab members: Mike Jungen, Kate Amspacher, Zach Ross, Rachel Arrick, Maggie Smith, Jessica Cantrell, and Emily Mausteller for their friendship, support, and for always being down to get lunch beers.

Finally, I would like to thank the Amphibian and Reptile Conservancy and NASA West Virginia Space Grant Consortium for the funding that supported my project. Many thanks to Cathy Johnson, Mike Owen, and Chad Landress of the U.S. Forest Service for permitting this research in the Monongahela National Forest.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

ABSTRACT

Amphibian population declines and range constrictions are widespread but poorly understood. Effective conservation planning relies on accurate distribution data to develop a fundamental understanding of causal changes in species distributions. However, conventional detection methods for cryptic and elusive amphibians suffer from imperfect detection. Environmental DNA (eDNA) has emerged as an innovative and powerful conservation tool for detecting aquatic species presence; however comparative studies aimed at fully understanding eDNA detection probabilities are lacking. In this study, I used environmental DNA (eDNA) sampling methods and detailed historical records to identify drivers of extirpation and sampling method efficiency for an imperiled, long-lived giant salamander, the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) in West Virginia, USA. I used a site occupancy and detection modeling framework (SODM) to test the influence of current land use, historical mining, hydrogeomorphic, and water quality variables on model-based predictions of occupancy and detection. Hellbenders are extirpated from 51% of the 49 historical sampling sites, and the top-ranked model indicated watershed-scale road density was the strongest predictor of Eastern Hellbender extirpation, and water turbidity and electrical conductivity were the best predictors of detection. Detection probability estimates for eDNA (84%) and conventional sampling methods (28%) suggest that eDNA provides a substantial performance advantage over conventional detection methods. Integrating eDNA data within a SODM framework allowed me to accurately and thoroughly assess causal changes in Eastern Hellbender distribution throughout their historical range in West Virginia, which will aid conservation planning. This study emphasizes the impacts of anthropogenic land alterations on freshwater ecosystems and the sensitivity of long-lived amphibian species to rapid environmental change.

CHAPTER 1

USING ENVIRONMENTAL DNA (eDNA) AND OCCUPANCY MODELING TO EXAMINE EASTERN HELLBENDER (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*) EXTIRPATION IN WEST VIRGINIA

INTRODUCTION

Monitoring the unprecedented population declines and extirpations of freshwater species has become a major focus of freshwater ecology and conservation (Strayer & Dudgeon 2010, Jackson *et al.* 2016). Freshwater species, among them amphibians, rank as some of the most threatened taxa because of rapid anthropogenic landscape change (Poff *et al*. 1997, Houlahan *et al.* 2000, Sala *et al.* 2000, Dudgeon *et al.* 2006). 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 are attributable to synergistic interactions among hydrologic modifications (i.e., channelization, damming), physical land-use changes, and declines in water quality and availability (Dudgeon *et al.* 2006). For example, the loss of watershed and riparian-scale forest cover in lotic systems are linked to population declines of several fish and amphibian species (Jones *et al.* 1999, Price *et al.* 2006, Surasinghe & Baldwin 2015, Bodinof Jachowski & Hopkins 2018). Soil erosion associated with forest cover loss can increase sediment loads, causing physiochemical changes to the lotic environment that range from increased water conductivity, substrate embeddedness, and clogging of the hyporheic zone (Likens *et al.* 1970, McBride & Booth 2005, Eaglin & Hubert 1993, Blaschke *et al*. 2003). Similarly, increased impervious surface cover can degrade water quality, increase sediment input, radically change discharge rates, and alter river flow regimes (Brabec *et al.* 2002, Poff &

Zimmerman 2010, de Souza *et al.* 2016). Thus, interactions between terrestrial and aquatic systems 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).

The temporal scale at which freshwater populations respond to habitat degradation across a species range can span decades, especially for long-lived species (Braulik *et al.* 2014, Bodinof Jachowski & Hopkins 2018). Consequently, few species are actively monitored at decadal-scales and thus appropriate data are generally lacking (Magurran *et al*. 2010, but see Wheeler *et al.* 2003). To fill information gaps, historical data can be incorporated with current distributions to examine long-term population trends (e.g., extirpation). While historical distribution data are limited (Hendricks *et al.* 2016), museums and natural resource management agency records can enable comprehensive assessment of changes in species distributions (Tingley & Beissinger 2009, Pitt *et al.* 2017).

Conventional sampling methods for freshwater fauna suffer from imperfect detection, particularly for rare, cryptic, and elusive species (Taberlet *et al.* 2012a, Fukumoto *et al.* 2015). Recent advancements in molecular-based indicators such as environmental DNA (eDNA) have allowed for rapid presence/absence detection of aquatic organisms (Thomsen *et al.* 2012, Spear *et al.* 2015, Wilcox et al. 2016, Barnes & Turner 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 (Thomson & Willerslev 2015); further, eDNA methods provide higher detection probabilities than conventional sampling approaches (Jerde *et al*. 2011, Dejean *et al*. 2012, Pilliod *et al.* 2013, Schmelzle & Kinziger 2016, Smart *et al*. 2015 Spear *et al.* 2015).

Thus, its widespread use and performance advantage as a sampling method highlights its effectiveness as a conservation tool (Thomson & Willerslev 2015, Goldberg *et al.* 2016).

In lotic systems, the application of eDNA has only recently grown in use, and there are still considerable knowledge gaps in understanding how various environmental conditions 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 aquatic eDNA detection is poorly understood (Pilliod *et al*. 2014, Barnes *et al.* 2014, Deiner & Altermatt 2014, Wilcox *et al.* 2016). Further, the physical and chemical characteristics of lotic systems that vary temporally throughout sampling seasons such as flow regimes, turbidity, and water chemistry likely influence DNA detection, but few studies employ proper modeling approaches to account for the influence of environmental covariates on detection probabilities (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 (Hunter *et al.* 2015, Ficetola *et al.* 2016, Boothroyd *et al.* 2016).

Eastern Hellbenders (*Cryptobranchus a. alleganiensis*) are cryptic, fully aquatic giant salamanders that have experienced precipitous population declines across their historical range (Wheeler *et al.* 2003, Graham *et al.* 2011, Burgmeier *et al.* 2011, Foster *et al.* 2009, Pitt *et al.* 2017). Eastern Hellbender current distribution in West Virginia has been largely unassessed, despite its listing as an imperiled species (S2 rank, West Virginia DNR 2018, but see Keitzer *et al*. 2013). This study is of conservation interest because Eastern Hellbenders are currently being considered for listing under the U.S. Endangered Species Act (J. Applegate, Personal Communication). Eastern Hellbenders inhabit swift-flowing streams with cobble/boulder rock cover and high-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 *et al.* 2016, Freake & DePerno 2017, Pitt *et al.* 2017, Bodinof Jachowski & Hopkins 2018). Causal factors of population declines are suspected to be habitat loss via siltation and filling of interstitial spaces because of anthropogenic landscape disturbances, water quality declines that impede successful reproduction, and lack of recruitment (Pitt *et al.* 2017). However, further insight into landscape-scale drivers of Eastern Hellbender population declines is needed.

In this study, I used historical and current distribution data to examine Eastern Hellbender extirpation in West Virginia (WV), USA. Specifically, I used eDNA to examine current Eastern Hellbender occupancy at locations that historically supported populations. I used a single species, single season SODM framework with watershed and riparian-scale predictors of occupancy that included hydrogeomorphic, current land cover, and historical mining data to determine possible drivers of extirpation. (Wenger *et al.* 2008, Pitt *et al*. 2017). I assumed populations were extirpated when the species no longer occupied a historical site. The species is well suited for this approach, as Eastern Hellbenders are very sensitive to water and habitat quality declines, have low vagility that inhibits recolonization, and exhibit a slow life history with great longevity (i.e., > 30 years, Taber *et al*; 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 provides a framework to examine a species decline at spatial and temporal scales suitable for quantifying extirpation while providing insight into causal agents.

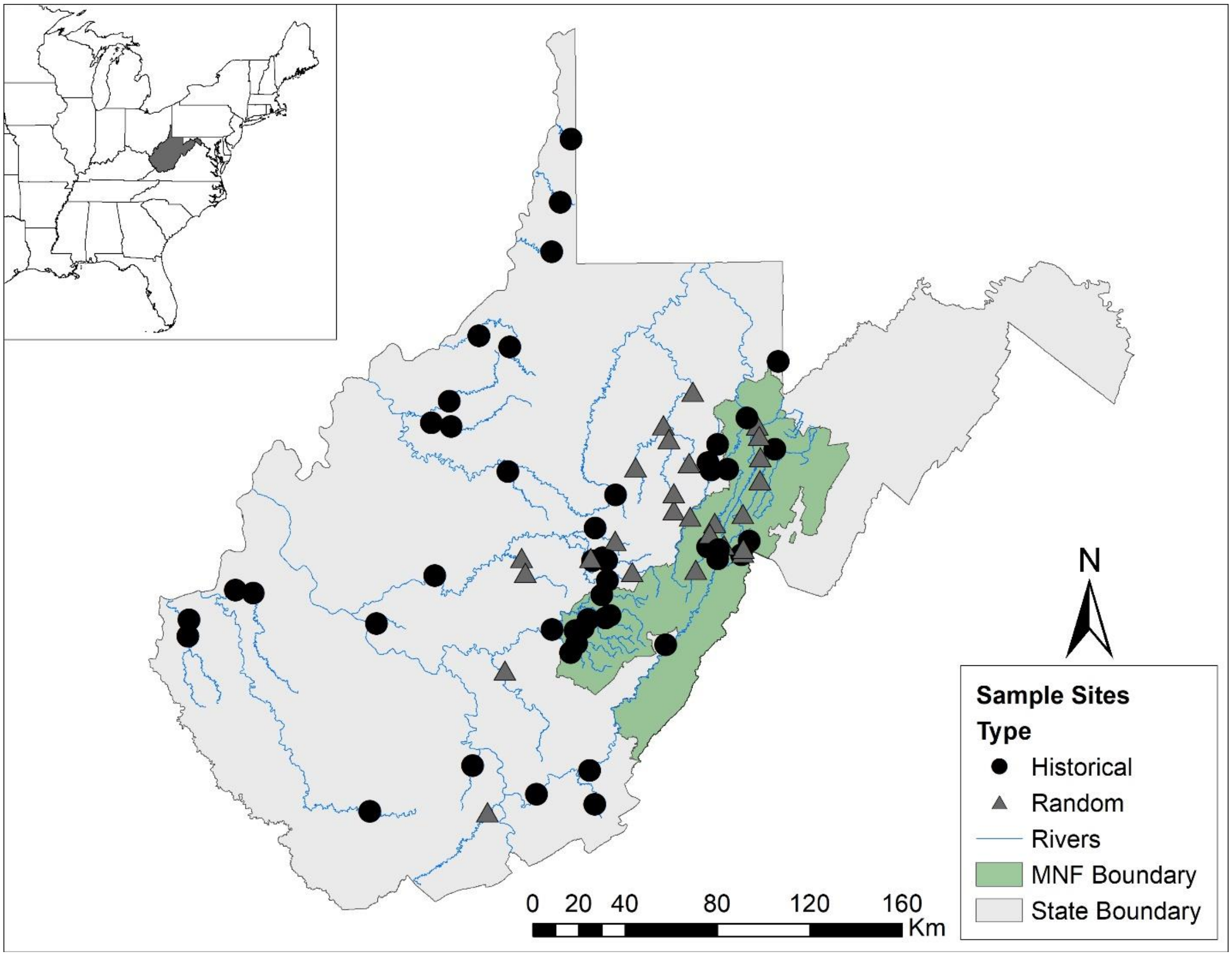

Figure 1. Study area. Eastern Hellbender (*Cryptobranchus a. alleganiensis*) sampling sites within the Ohio River drainage of West Virginia, USA (MNF = Monongahela National Forest).

METHODS

Study Area

My study area encompassed the historical range of Eastern Hellbenders within the Ohio River drainage in West Virginia, ranging 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 West Virginia. My study sites were within three major river drainages that contribute to the Ohio River in West Virginia: the Kanawha/New, Guyandotte, and Cheat. A historical account from Green

(1934) suggests that hellbenders were more abundant in West Virginia than any other region of the Ohio River drainage.

Historical Data & Study Site Selection

I obtained historical records through the West Virginia Biological Survey Museum housed at Marshall University, West Virginia Natural Heritage Database, Humphries & Pauley (2005), and Keitzer *et al.* (2013). To specifically examine Eastern Hellbender extirpation, I only used records that provided detailed location information (i.e., coordinates or landmarks). Of the 57 historical records I found, 52 sites were sampled based on my criteria and spanned a timeframe from 1932-2016 (Figure 1). Due to the close proximity (< 2 km) of some historical sites within a mainstem river channel, I conservatively removed three sites (n=49) from my 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 (Pilliod *et al.* 2013, Deiner & Altermatt 2014, Wilcox *et al.* 2016, Jane *et al.* 2016). I sampled 26 random sites within drainages that contained historical records of Eastern Hellbender presence to determine if populations persisted elsewhere within these watersheds. I generated random points every 2 km along major (fourth order or larger) stream features and used a select by location query in ArcMap 10.4 (ESRI Redlands, CA, USA) to select accessible sites (i.e. proximity to roads and bridges). Sites were georeferenced in the field using a Garmin GPSMAP ® 64st GPS unit.

Field Collection Protocol

During spring and summer 2017, I collected eDNA samples during four sampling periods for historical sites, and three sampling periods for random sites. Sampling periods ranged from (1) 17 April to 31 May (2) 02 June to 30 June (3) 06 July to 06 August (4) 08 September to 30

September (I sampled only historical sites during September). I used single-use disposable equipment in all my sample collections to avoid contamination between sites (Goldberg *et al.*, 2016). Forceps used for extracting filters were the only piece of equipment reused among sites and were treated with DNA Away Surface Decontaminant (Molecular Bio-products, Inc., San Diego, CA, U.S.A.) prior to filter extraction to avoid sample contamination. At each site, I used a sterile, disposable Whirlpak Stand-up Bag (36oz, 1065ml capacity, Nasco, Fort Atkinson, WI U.S.A.) to collect 1L water samples from the center of the stream. I used a Cole-Parmer Masterflex Peristaltic Pump (Model No. 7520-00, Cole-Parmer Instrument Co. Chicago, IL, U.S.A.) attached to a 1L Nalgene Vacuum Flask to filter water through sterile, disposable 250ml Nalgene Analytical Test Filter Funnels (pore size = 0.45 µm, cellulose nitrate membrane, Thermo Fisher Scientific Inc., Rochester, NY, U.S.A.). I placed filter membranes immediately in 1.5ml microcentrifuge tubes post-filtering and transported 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 4-23 ($\bar{x}$ = 13.14). I filled sterile Whirlpak bags with deionized 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, I filtered the negative field control after the last field sample using the same protocol and equipment as field samples. After each sampling period, I sterilized all equipment reused among sites (i.e., waders & water quality probes) using a 30% bleach solution.

Laboratory Methods

I 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). I divided filters in half and tore them into pieces, with the other half stored at -80˚C for potential later use. I followed the standard protocol for the extraction kit with the additional use of a Qiashredder (Qiagen, Inc.) spin column after the lysis step. I processed all samples in a separate and dedicated extraction and PCR setup section of the laboratory.

I amplified eDNA samples following the qPCR protocol from Spear *et al.* (2015). A 104bp 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').

I used a 20µL reaction volume consisting of 10µL of Luna universal probe qPCR master mix (New England Biolabs), 1µL of each primer at 10µM and probe at 5µM, 3.5µL nuclease free water, and 3.5µL 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 sec and 60˚ for 60 sec, with data collection during the annealing stage at 60˚C. I ran all extractions in triplicate and included a positive control from a captive Eastern Hellbender population water sample and negative control to ensure qPCR efficacy and any potential contamination. I used a 1:2 serial dilution of the 13ng/uL positive control to create a standard curve to determine concentration estimations for all of the eDNA samples.

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

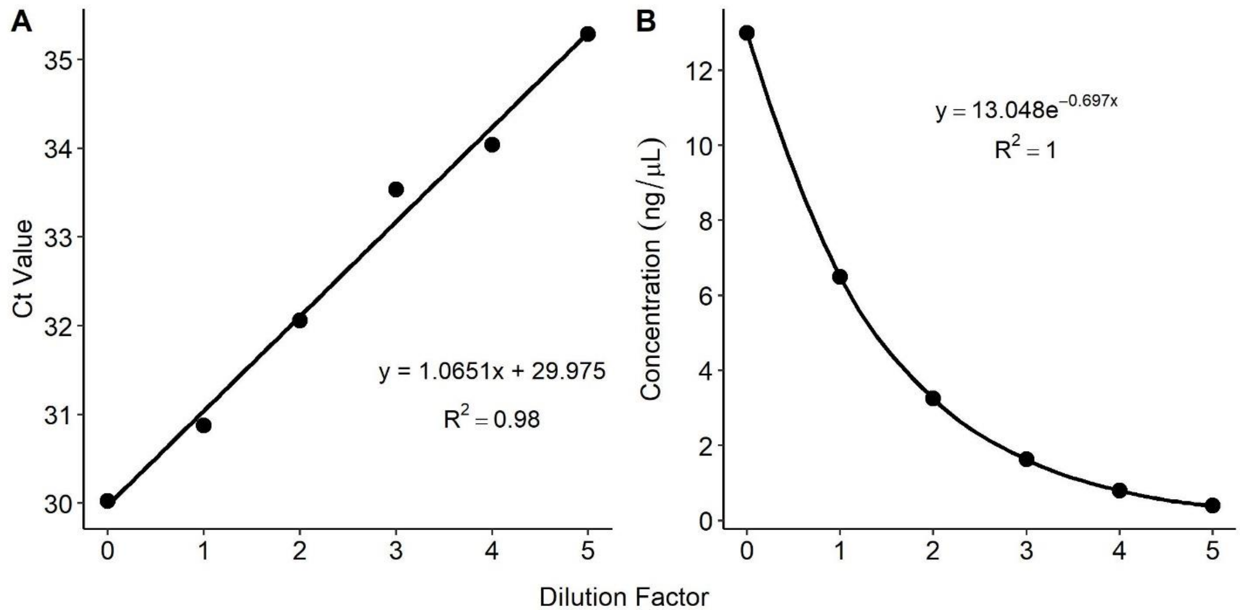

Figure 2. qPCR standard curve and CT values. Cycle threshold values from field samples vs. dilution factor of positive controls (A), and eDNA concentration vs. dilution factor of positive controls (B).

For the first three sampling periods, I found the deionized water used from the tap at Marshall University to be contaminated at the source, as about 1/3 of my 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. I determined that the deionized water was contaminated at the source by filtering three samples of it in a separate lab using all disposable single-use equipment, along with three samples of nuclease-free water for comparison. One out of the three samples of deionized water amplified with one qPCR replicate, and all nuclease-free water samples were negative. For the fourth round of surveys, I used nuclease-free water for all field negative controls to avoid further source contamination of negative controls. All contaminated filter blanks had only 1/3 qPCR replicates amplify, and all DNA concentration values were below 0.08 ng/µL. Therefore, I used field samples that had a minimum of 2/3 qPCR replicates amplify with concentrations above 0.08 ng/µL as an indicator of Eastern Hellbender presence.

Predictor Variables

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

For each site, I calculated tree canopy cover (2015 imagery) at the watershed and riparian scale using a freely available 30m resolution dataset (Sexton *et al.* 2013, www.landcover.org). Highly forested watersheds that protect instream habitat and water-quality have been associated with Eastern Hellbender site occupancy, but quantitative evidence is lacking, and the effect of tree cover loss may be time-lagged (Williams *et al.* 1981, Wheeler *et al.* 2003, Bodinof Jachowski *et al.* 2016). I chose not to include the National Land Cover Dataset (NLCD, Homer et al. 2015) classes regularly used in watershed-scale ecological studies because of the issues associated with highly correlated land cover classes (King *et al.* 2005). Pixel values ranged from 0-100, 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. I masked imagery to upstream watershed boundaries and 150m riparian buffers on both sides of the stream for each site, and computed summary statistics to obtain the mean pixel value for each watershed and buffer area used in my analyses (Table 1).

I quantified watershed and riparian-scale road density using the U.S Census Bureau Tiger/Line® Shapefiles. Roads permanently alter the physical landscape environment and contribute to sedimentation and chemical alteration of aquatic environments (Maltby *et al.* 1995, Trombulak & Frissel 2000, Kaushal *et al.* 2018). A study on the endangered Black Warrior Waterdog (*Necturus alabamensis*), a species with similar habitat and water quality requirements to Eastern Hellbenders, was negatively associated with impervious surfaces at the watershed scale (de Souza *et al.* 2016). I chose not to use the NLCD 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). I clipped road shapefiles to individual watershed boundaries and 150m riparian buffers, and calculated road density as a proportion of watershed and riparian area (km/km^2).

Due to the temporal scale of historical records (1932-2016) and unique land-use history of my study area, I included historical mining-related variables as predictors of Eastern Hellbender occupancy. Surface mining activities degrade in-stream habitat (via sedimentation) and water quality over time, even after mine reclamation (Lindberg *et al.* 2011). I digitized strip and deep mining features from a seamless digital raster graphic county mosaic of USGS topographic maps (1:24000 scale). Quadrangles varied in time from 1965-1987, as not all areas were surveyed at the same time. I calculated the proportion of the upstream watershed covered by surface mining, and density of deep mines per watershed (Table 1). I quantified the number of National Pollutant Discharge Elimination System (NPDES) mining-related outlets per watershed to assess the relative importance of point-source pollution on Eastern Hellbender occupancy. Data were freely obtained through the West Virginia Department of Environmental Protection (WV DEP) GIS server. I vetted outlets listed as storm water drainage and retained only mining-related outlets.

Sampling Covariates

I collected water quality data (Table 1) during each site visit. Variable flow conditions of lotic systems are known to influence environmental DNA detection probabilities (Jane *et al.* 2015). Further, Eastern Hellbender site occupancy is negatively associated with high conductivity, which could impede reproduction (Pitt *et al.* 2017). I collected water quality data using a Hanna Instruments HI98196 Multiparameter probe (Hanna Instruments, Woonsocket, RI). I measured water velocity (m/s) using a Marsh-McBirney Flo-Mate model 2000. I measured turbidity (FTU) using a YSI Ecosense 9500 Photometer (Yellow Springs Instruments, Yellow Springs, OH). I z-standardized all continuous site and sample covariates other than proportions.

Table 1. Covariate Summary. Summary of site and sample covariates considered in SODM to predict Eastern Hellbender occupancy and detection using environmental DNA (eDNA) in West Virginia, USA.

Variable	Data source	Definition	Unit	Abbr.
		Site Covariates		
		(1) Hydrogeomorphic		
Elevation	Field Measurement	Elevation of sample site (m above sea level)	meters	elev
Watershed Area	ArcMap Hydrology	Watershed 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	U.S. ACOE NID	Density of dams in tributary system	no./km	dam.den s
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 150m site covered by riffle habitat	%	rifl
Run	Field Measurement	% of 150m site covered by run habitat	%	run
Pool	Field Measurement	% of 150m 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				
Watershed Road Density	TIGER/Line	Total length of roads in watershed / watershed area	km/km^2	ws.road
Riparian Road Density	TIGER/Line	Total length of roads in 150m stream buffer / buffer area	km/km^2	rp.road
Watershed Tree Cover	Sexton et al. 2013	Mean % canopy cover (2015 imagery) in the watershed boundary	%	ws.ccov
Riparian Tree Cover	Sexton et al. 2013	Mean % canopy cover (2015 imagery) in a 150m stream buffer	%	rp.ccov
Public Land	WV DNR	% of upstream watershed covered by public land	%	pc.publ
(3) Historical Mining				
% Area Mined	USGS Topos	% of upstream watershed boundary covered by strip mines, quarries, or clay pits	%	pc.mine
Deep Mines	USGS Topos	Density of deep mines per watershed	$no./km^2$	deep
NPDES Outlets	WVDEP	Density of mining related NPDES outlets per watershed	$no./km^2$	npdes
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

Statistical Analysis

I used a single-species, single season SODM framework to examine covariates of Eastern Hellbender occupancy (Ψ) and detection (*p*). Occupancy models are used to estimate species occurrence while accounting for imperfect detection among multiple site visits (MacKenzie *et al.* 2002, Guillera-Arroita *et al.* 2010, MacKenzie *et al.* 2017). This modeling 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*) (Spear *et al.* 2015, de Souza *et al.* 2016, Schmelzle & Kinziger 2016).

I conducted two separate SODM analyses to account for differences in the number of sampling periods between historical and random sites: (1) Models with four temporal replicates and only historical (n=49) sites and (2) Models with three temporal replicates and only random (n=26) sites. My first analysis including only historical sites allowed me to specifically test hypotheses about Eastern Hellbender extirpation across their range in West Virginia.

I calculated Spearman's rank correlation coefficients for both datasets prior to model development to identify highly correlated variables ($r_s \geq 0.70$ or ≤ -0.70). For my historical site analysis, watershed and riparian-scale road density were highly correlated with both spatial scales of canopy cover, public land, and elevation. I omitted these predictors and retained road density because of the high anthropogenic impact roads can have on water chemistry and aquatic biota (Trombulak & Frissell 2000, de Souza *et al.* 2016). In models that included random sites, watershed-scale canopy cover was not correlated with road density, and I chose to add this predictor variable in my model set. Predictors of historical mining were also highly correlated (r_s

> 0.80) in my historical site analysis, and I retained only the proportion of watershed area covered by historical surface mining based on the severe impacts of this land-use practice on aquatic ecosystems (Lindberg *et al.* 2011, Wu *et al.* 2015). From the remaining set of variables, I developed biologically relevant *a priori* models in three model subsets: hydrogeomorphic, current, and historical land-use based on current knowledge of Eastern Hellbender life history and occupancy patterns to determine which variables best predict Eastern Hellbender occupancy and detection in West Virginia (Tables 3 & 6).

I 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. I used models with ΔAIC ≤ 2.0 for inference (Burnham & Anderson 2002). I evaluated model fit by examining the estimated variance inflation factors $(\hat{c})$ from 3 subglobal models among my 3 model subsets and used the smallest computed $(\hat{c})$ (Burnham & Anderson 2002). I performed statistical analyses using R (R Core Team, 2018), and used the package "unmarked" to conduct SODM analysis (Fiske & Chandler 2011).

As a post hoc analysis, I conducted a logistic regression to assess whether the years since the last Eastern Hellbender sighting at each historical site could predict the binary output of extirpation or occupancy. I used a Hosmer-Lemeshow goodness-of-fit test to evaluate model fit (Hosmer & Lemeshow 2000).

RESULTS

I detected Eastern Hellbender eDNA at 24/49 historical sites (naïve $\Psi = 0.49$), indicating that 51% (25) of the sites are locally extirpated. The majority of sites where populations persist are in or near the Monongahela National Forest in the eastern Allegheny Mountains and high Appalachian Plateau (Fig 3). I detected low eDNA concentrations at two sites near the northern panhandle of West Virginia. I detected Eastern Hellbender eDNA at 7/26 random sites (naïve $\Psi = 0.27$), with 73% of random sites unoccupied. Of the seven occupied random sites, five are within the mainstem of rivers, and two are within tributaries with historical presence of Eastern Hellbenders (Fig 4).

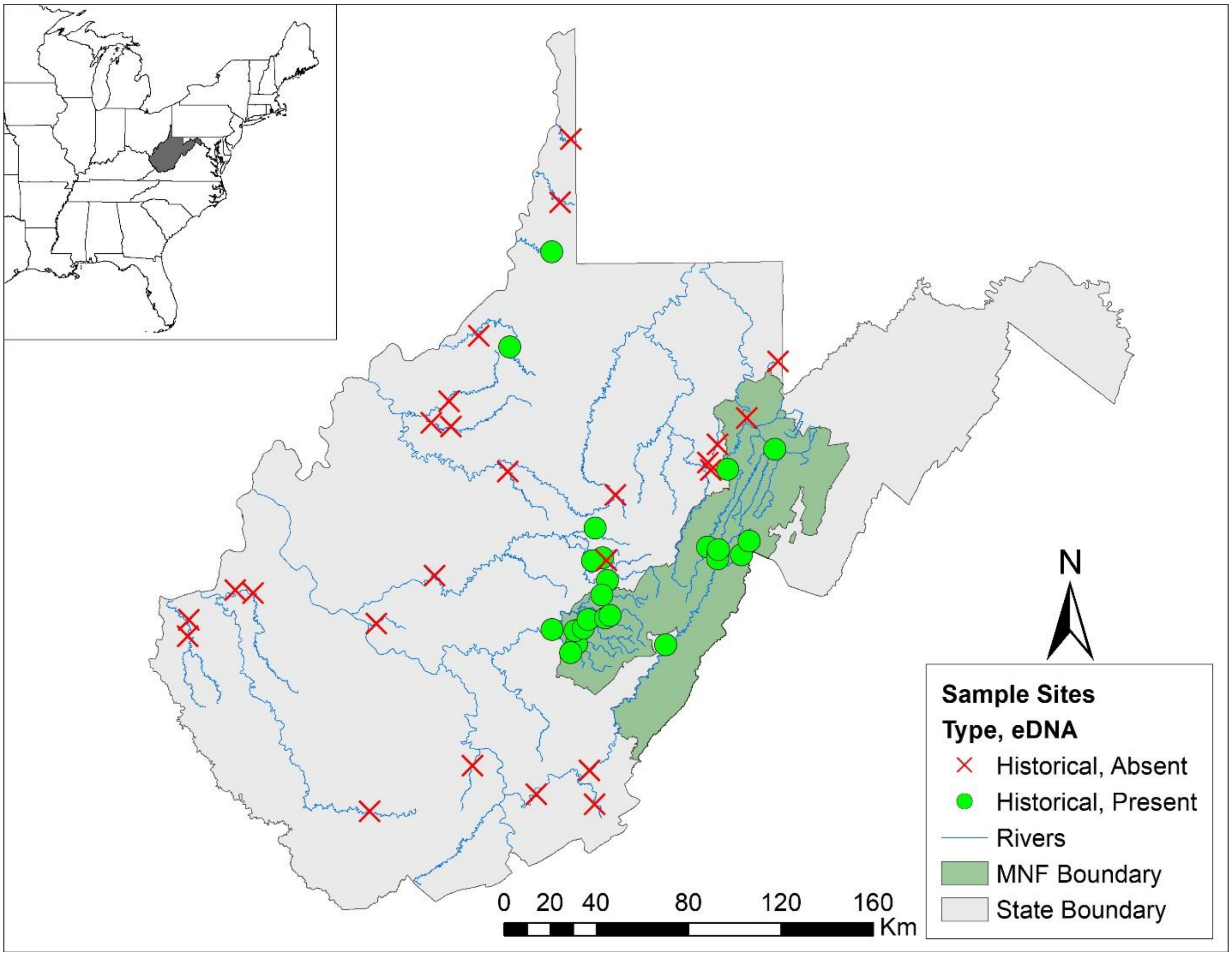

Figure 3. eDNA results: historical sites. Results of eDNA sampling surveys for sites with historical Eastern Hellbender records in West Virginia, USA (MNF = Monongahela National Forest).

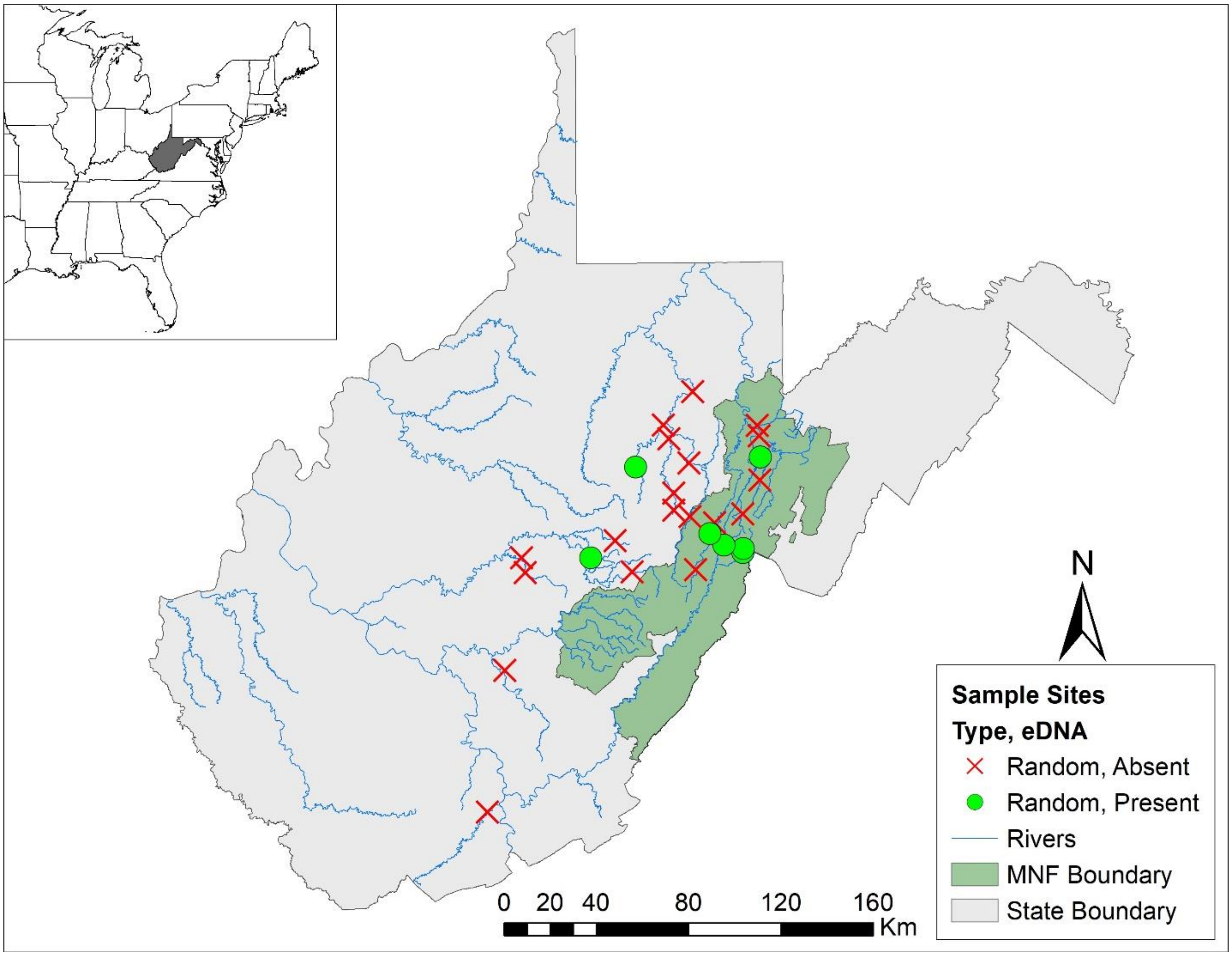

Figure 4. eDNA results: random sites. Results of eDNA sampling surveys at randomly selected sites within watersheds of Eastern Hellbender presence in West Virginia, USA (MNF = Monongahela National Forest).

Occupancy and Detection

I found no evidence for lack of fit in my historical site model set based on goodness-of-fit test ($\hat{c} = 0.93$) ; therefore I did not adjust my model ranking procedure for overdispersion (Table 3). While ($\hat{c}$) values of less than 1 indicate underdispersion, corrections are typically only made to overdispersed datasets, and it is recommended to set $\hat{c}$ = 1 in cases of underdispersion (Burnham & Anderson 2002, Mackenzie *et al.* 2017). I retained two models from the historical location analysis for inference (Tables 2 & 4). The top model, ψ(ws.road), *p*(cond+turb), indicated that occupancy was negatively associated with watershed road density (β = -2.525 ± 0.923; Fig. 5,Table 4). The second-ranking model had one additional occupancy covariate (% fine substrate) that was uninformative based on 95% confidence intervals (Table 4). Road density at the watershed scale was included in both models and negatively influenced Eastern Hellbender occupancy ($\widehat{\Psi}$ = 0.62, 0.05-0.98, Fig 5, Table 4). I failed to detect a relationship between the proportion of watershed area mined, dam density, and natural hydrogeomorphic variables on historical Eastern Hellbender occupancy.

I found evidence of overdispersion in my random site model set based on goodness of fit test ($\hat{c} = 1.42$); therefore, I used QAIC to rank models (Table 6). Watershed-scale canopy cover was the best supported model of Eastern Hellbender occupancy for random sites, positively influencing occupancy (β = 14.745 ± 8.961; Fig.5, Table 7). Six models were within ΔQAIC ≤ 2.0 of the best supported model (Tables 5 & 6), including proportion of area mined, watershed-scale road density, number of deep mines, and dam density as predictor variables. However, I considered only watershed- scale canopy cover an informative parameter in predicting Eastern Hellbender occupancy based on 95% confidence intervals (Table 7).

Supported models for Eastern Hellbender eDNA detection at historical and random sites included conductivity and turbidity of water as predictors (Tables 2,4,5,7). Both covariates negatively influenced detection probability (Fig. 5.). All equivalent models at random and historical sites included conductivity and turbidity as additive or single predictors for detection probability (Tables 2 & 5).

The post hoc logistic regression analysis indicated that the number of years since Eastern Hellbenders were last seen at historical locations was a significant predictor of extirpation (χ^2 = 18.49, df = 1, n = 49, P < 0.0001, Table 8). Hosmer-Lemeshow goodness-of-fit test indicated good model fit (χ^2 = 11.205, df = 8, n = 49, P = 0.08).

Table 2. Candidate models: historical sites. Candidate site occupancy and detection (SODM) predicting occupancy and detection of Eastern Hellbenders using environmental DNA (eDNA) at historical sites (n = 49 sites), ranked according to Aikake's Information Criterion (AIC), with Δ AIC, number of parameters (k), and AIC weight (ω)

Model	AIC	Δ AIC	k	*ω*
Ψ(ws.road), *p*(cond+turb)	127.28	0.00	5	0.56
Ψ(ws.road+fine), *p*(cond+turb)	129.28	2.00	6	0.20
Ψ(pc.mine+fine), *p*(cond+turb)	135.40	8.12	6	0.20
Ψ(pc.mine), *p*(cond+turb)	138.52	11.24	5	0.01
Ψ(pregion), *p*(cond)	155.29	28.01	4	0.01
Ψ(dam.dens), *p*(turb)	158.35	31.07	4	0.00
Ψ(dam.dens+fine), *p*(turb)	158.70	31.42	5	0.00
Ψ(dam.dens+pool), *p*(turb)	160.26	32.98	5	0.00
Ψ(fine), *p*(turb)	163.27	35.99	4	0.00
Ψ(cobl+boul), *p*(do+flow)	168.42	41.14	6	0.00
Ψ(dam.dens), *p*(wtemp)	175.84	48.56	4	0.00
Ψ(rifl+run), *p*(do+flow)	177.50	50.47	6	0.00
Ψ(width+sgrade), *p*(do+flow)	178.43	51.15	6	0.00
Ψ(.), *p*(.)	181.79	54.51	2	0.00
Ψ(width+depth), *p*(do+flow)	182.30	55.02	6	0.00
Ψ(pc.mine), *p*(ph)	185.47	58.19	4	0.00
Ψ(pregion), *p*(ph)	185.48	58.20	4	0.00
Ψ(pregion), *p*(flow+wtemp)	187.06	59.78	5	0.00

Table 3. Model subsets: historical sites. Model subsets,($\hat{c}$) from goodness-of-fit tests, number of parameters (k), and model weights (ω) from candidate site occupancy and detection (SODM) models predicting Eastern Hellbender occupancy and detection at historical sites (n=49 sites)

Model Subset	**Model**	$\hat{c}$	**k**	ω
Hydrogeo	Ψ(cobl+boul), *p*(do+flow)	1.35	6	0.00
	Ψ(dam.dens+fine), *p*(turb)		5	0.00
	Ψ(dam.dens), *p*(turb)		4	0.00
	Ψ(fine), *p*(turb)		4	0.00
	Ψ(pregion), *p*(ph)		4	0.00
	Ψ(pregion), *p*(cond)		2	0.01
	Ψ(rifl+run), *p*(do+flow)		6	0.00
	Ψ(pregion), *p*(flow+wtemp)		5	0.00
	Ψ(dam.dens), p(wtemp)		4	0.00
	Ψ(dam.dens+pool), *p*(turb)		5	0.00
	Ψ(width+sgrade), *p*(do+flow)		6	0.00
	Ψ(width+depth), *p*(do+flow)		6	0.00
Current	Ψ(ws.road+fine), *p*(cond+turb)	0.93	6	0.20
	Ψ(ws.road), p(cond+turb)		5	0.56
Historical	Ψ(pc.mine+fine), p(cond+turb)	1.02	6	0.20
	Ψ(pc.mine), p(cond+turb)		5	0.01
	Ψ(pc.mine), p(ph)		4	0.00

Table 4. Best supported models: historical sites. Best supported SODM predicting Eastern Hellbender occupancy (Ψ) and detection (p) for historical sites (n=49 sites) with associated coefficients (β), standard error (SE), and 95% confidence intervals (LCL, UCL). (ws.road = watershed-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

Table 5. Candidate models: random sites. Candidate SODM predicting occupancy and detection of Eastern Hellbenders using environmental DNA (eDNA), ranked according to Quasi Aikake's Information Criterion (QAIC), with Δ QAIC, number of parameters (k), and QAIC weight (ω)

Model	**QAIC**	**Δ QAIC**	**k**	***ω***
Ψ(ws.ccov), *p*(cond+turb)	42.31	0.00	5	0.12
Ψ(pc.mine), *p*(cond+turb)	42.80	0.49	5	0.09
Ψ(ws.ccov), *p*(cond)	42.94	0.63	4	0.08
Ψ(ws.road), *p*(cond+turb)	42.99	0.68	5	0.08
Ψ(deep.mine), *p*(cond)	43.07	0.76	4	0.08
Ψ(dam.dens), *p*(turb)	43.15	0.84	4	0.07
Ψ(.), *p*(.)	43.73	1.42	2	0.05
Ψ(ws.ccov+fine), *p*(turb)	44.00	1.69	5	0.05
Ψ(deep.mine), *p*(ph)	44.18	1.87	4	0.04
Ψ(ws.ccov+ws.road), *p*(cond+turb)	44.30	1.99	6	0.04
Ψ(fine), *p*(turb)	44.46	2.15	4	0.04
Ψ(pregion), *p*(cond)	44.49	2.18	4	0.04
Ψ(pc.mine+fine), *p*(cond+turb)	44.77	2.46	6	0.03
Ψ(ws.road+fine), *p*(cond+turb)	44.99	2.68	5	0.03
Ψ(dam.dens+pool), *p*(turb)	45.10	2.79	5	0.03
Ψ(dam.dens+fine), *p*(turb)	45.14	2.83	5	0.02
Ψ(dam.dens), *p*(wtemp)	46.29	3.98	4	0.01
Ψ(depth), *p*(do+flow)	46.87	4.56	4	0.01
Ψ(pc.mine), *p*(ph)	47.33	5.02	4	0.01
Ψ(pregion), p(ph)	47.35	5.04	4	0.01
Ψ(pregion), *p*(flow+wtemp)	48.15	5.84	5	0.00
Ψ(rifl+run), *p*(do+flow)	48.51	6.20	6	0.00
Ψ(cobl+boul), *p*(do+flow)	48.83	6.52	6	0.00

Table 6. Model subsets: random sites. Model subsets,($\hat{c}$) estimates from goodness-of-fit tests, number of parameters (k), and model weights (ω) from candidate site occupancy and detection (SODM) predicting Eastern Hellbender occupancy and detection for random sites (n=26 sites)

Model Subset	**Model**	**ĉ**	**k**	***ω***
Hydrogeo	Ψ(cobl+boul), *p*(do+flow)	1.42	6	0.00
	Ψ(dam.dens+fine), *p*(turb)		5	0.02
	Ψ(dam.dens), *p*(turb)		4	0.07
	Ψ(fine), *p*(turb)		4	0.04
	Ψ(depth), *p*(do+flow)		5	0.01
	Ψ(pregion), *p*(ph)		4	0.01
	Ψ(rifl+run), *p*(do+flow)		6	0.00
	Ψ(pregion), *p*(flow+wtemp)		5	0.00
	Ψ(dam.dens+pool), *p*(turb)		5	0.03
	Ψ(pregion), p(cond)		4	0.04
	Ψ(dam.dens), p(wtemp)		4	0.01
Current	Ψ(ws.ccov+ws.road), *p*(cond+turb)	1.53	6	0.04
	Ψ(ws.road+fine), *p*(cond+turb)		6	0.03
	Ψ(ws.ccov), p(cond)		4	0.08
	Ψ(ws.road), *p*(cond+turb)		5	0.08
	Ψ(ws.ccov), *p*(cond+turb)		5	0.12
	Ψ(ws.ccov+fine), *p*(turb)		5	0.05
Historical	Ψ(pc.mine+fine), *p*(cond+turb)	1.65	6	0.03
	Ψ(pcmine), *p*(ph)		4	0.01
	Ψ(pcmine), *p*(cond+turb)		5	0.09
	Ψ(deep.mine), *p*(ph)		4	0.04
	Ψ(deep.mine), *p*(cond)		4	0.08

Table 7. Best supported models: random sites. Best supported SODM predicting Eastern Hellbender occupancy (Ψ) and detection (p) for random sites (n=26 sites) with associated coefficients (β), standard error (SE), and 95% confidence intervals (LCL, UCL). (ws.ccov = watershed-scale canopy cover, pc.mine = percent area of watershed mined, ws.road = watershed-scale road density, deep.mine = number of deep mines within watershed, dam.dens = density of dams within watershed, cond = conductivity of water, turb = turbidity of water)

Model	**Var**	**Parameters**	**β**	**SE**	**LCI**	**UCI**
Ψ(ws.ccov), *p*(cond+turb)	Ψ	intercept	-10.470	6.161	-22.546	1.606
	Ψ	ws.ccov	14.745	8.961	2.819	32.309
	p	intercept	-1.064	1.002	-3.028	0.900
	p	cond	-1.558	1.101	-3.716	0.600
	p	turb	-2.441	1.779	-5.928	1.046
Ψ(pc.mine), *p*(cond+turb)	Ψ	intercept	-0.508	0.643	-1.768	0.752
	Ψ	pc.mine	0.244	0.416	-0.571	1.059
	p	intercept	-1.541	0.920	-3.344	0.262
	p	cond	-2.080	1.076	-4.189	0.029
	p	turb	-2.729	1.755	-6.169	0.711
Ψ(ws.ccov), p(cond)	Ψ	intercept	-13.129	5.453	-23.817	-2.441
	Ψ	ws.ccov	18.492	7.957	2.896	34.088
	p	intercept	-0.361	0.827	-1.982	1.260
	p	cond	-1.629	1.165	-3.912	0.654
Ψ(ws.road), p(cond+turb)	Ψ	intercept	0.019	1.210	-2.353	2.391
	Ψ	ws.road	-0.248	-0.780	1.281	-1.777
	p	intercept	1.385	0.993	-0.561	3.331
	p	cond	-1.863	1.155	-4.127	0.401
	p	turb	-2.682	0.014	-2.708	-2.656
Ψ(deep.mine), p(cond)	Ψ	intercept	-4.088	5.270	-14.417	6.241
	Ψ	deep.mine	-10.397	14.849	-39.501	18.707
	p	intercept	-0.441	0.945	-2.293	1.411
	p	cond	-1.694	1.325	-4.291	0.903
Ψ(dam.dens), p(turb)	Ψ	intercept	-0.327	0.592	-1.487	0.833
	Ψ	dam.dens	-25.323	23.515	-71.412	20.766
	p	intercept	-0.375	0.829	-2.000	1.250
	p	turb	-2.602	1.670	-5.875	0.671

Table 8. Logistic Regression. Maximum likelihood estimate and odds ratio from the logistic regression analysis predicting the years since Eastern Hellbender presence was recorded on Eastern Hellbender extirpation (OR = Odds ratio)

Variable[1]	**Estimate ± SE**	**df**	**$\chi 2$**	**P**	**OR**
Years	0.0583 ± 0.0170	1	11.815	0.0006	1.06
Nagelkirke Pseudo R^2	0.42				

[1]Years = Years since Hellbender presence last recorded

Table 9. Probabilities and Odds. Predicted probabilities of Eastern Hellbender extirpation with varying years since the last sighting to show variable probabilities of extirpation, log-odds and odds from the logistic regression analysis.

Years	**Log-odds**	**Odds**	**Probability**
10	-1.233	0.29	0.23
20	-0.65	0.52	0.34
30	-0.067	0.94	0.48
40	0.516	1.68	0.63
50	1.099	3.00	0.75
60	1.682	5.38	0.84
70	2.265	9.63	0.91
80	2.848	17.25	0.94
90	3.431	30.91	0.97

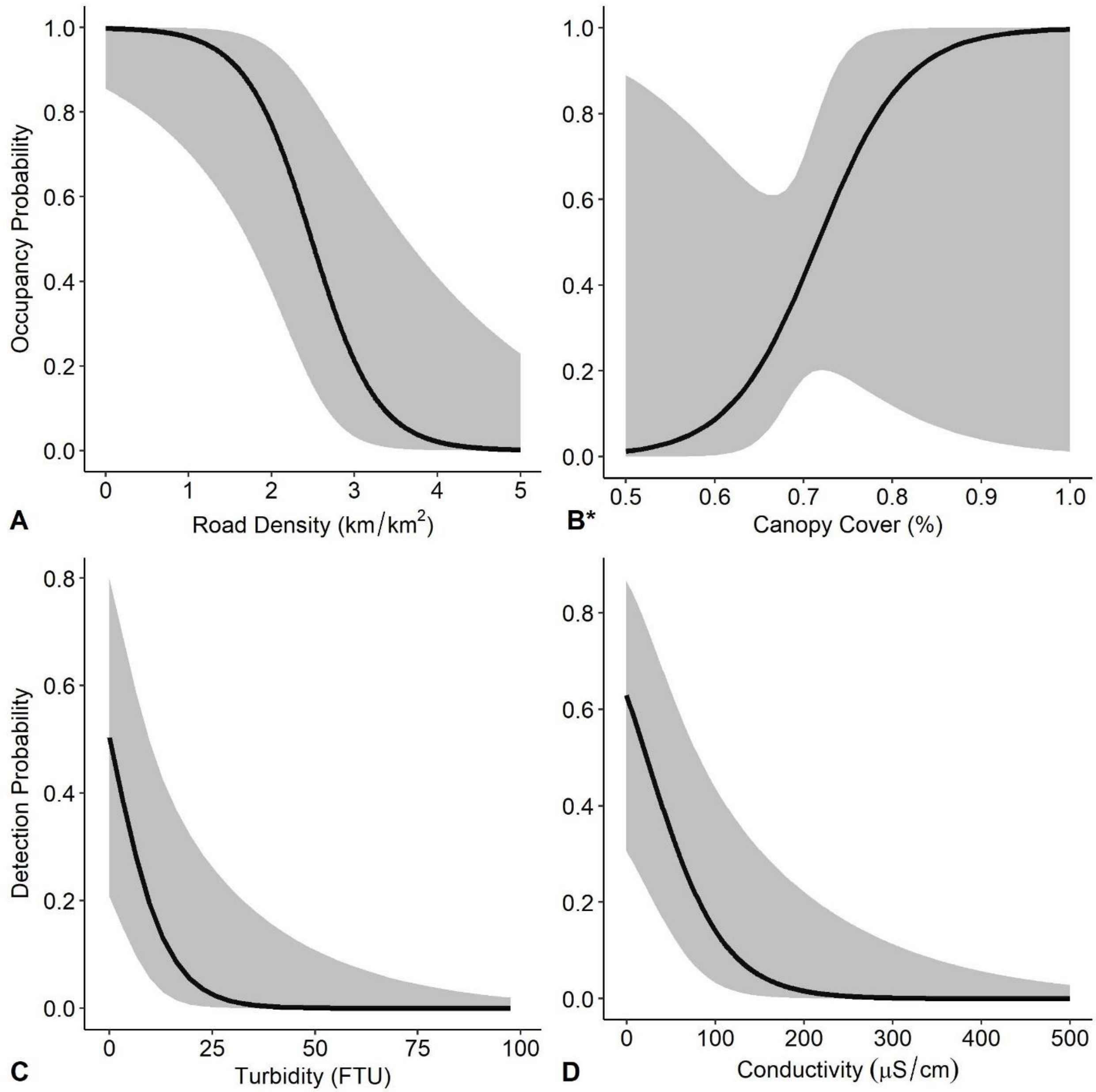

Figure 5. Predicted probabilities of occupancy and detection. Predicted occupancy (top) and detection (bottom) probability for Eastern Hellbenders as a function of (A) watershed-scale road density, (B) watershed-scale canopy cover (* denotes prediction is from top model with random only sites: Ψ(ws.ccov), p(cond+turb), all other predictions are from top model with historical sites only; Ψ(ws.road), p(cond+turb), (C) turbidity of water at sample site, and (D) conductivity of water at sample site. Gray shading indicates 95% confidence intervals.

DISCUSSION

My study fills a knowledge gap concerning causal factors that may influence population declines in fully aquatic salamander species that inhabit lotic freshwater ecosystems by using a novel sampling approach. My eDNA results indicate hellbenders are no longer present at 51% of the 49 historical sites, suggesting local extirpation and range constriction in the Ohio River drainage of West Virginia. Overall estimates of Eastern Hellbender occupancy and detection at historical sites based on the best supported model were 0.62 ± 0.12 SE, and 0.45 ± 0.06 SE, respectively. Despite source contamination issues with the deionized water used for my filter blanks, I believe my results accurately represent presence/absence of hellbenders at my sample sites. Given the lack of contamination in the fourth round of sampling using the same sampling protocol and no discrepancies among field samples, I believe the first three rounds of negative field controls did not reflect contamination in the field because contamination started at the source. Environmental DNA sampling is a relatively new technique that is prone to false positive/negative errors (Ficetola *et al*. 2016). While alternative modeling approaches exist to account for such errors in analysis of eDNA data, I believe my conservative approach of applying a DNA concentration threshold and a minimum of two qPCR replicates for eDNA presence consideration to be valid (Lahoz-Monfort *et al*. 2016).

I found that watershed-scale road density best supported model-based predictions of Eastern Hellbender extirpation, while historical mining, hydrogeomorphic, and current forest cover covariates received little support. My findings are consistent with a recent study by deSouza *et al.* (2016), who found that presence of the endangered Black Warrior Waterdog (*Necturus alabamensis*), a fully aquatic salamander species with similar habitat requirements to hellbenders, was negatively associated with watershed-scale impervious surface area. Roads have detrimental impacts on instream water quality and contribute to sedimentation (Maltby *et al.* 1995, Forman & Alexander 1998). The combined impacts of water quality declines and increased sedimentation may have degraded streams beyond the suitable conditions required for hellbenders to persist. Further, I could not include forest cover at both spatial scales in the historical site analysis because it was highly correlated with road density. However, watershed-scale forest cover was the best predictor of Eastern Hellbender occupancy in my analysis that included random sites, indicating that Eastern Hellbender extirpation in West Virginia may not be attributable to singular landscape-scale factors, but rather the possible synergistic effects of deforestation and anthropogenic landscape development. Though I could not include the percentage of watershed area covered by public land in my models because it was highly correlated with forest cover and road density, recent research highlights the importance of highly forested watersheds within public lands to preserve water quality and Eastern Hellbender populations (Freake & DePerno 2017, Bodinof Jachowski & Hopkins 2018). My findings suggest that Eastern Hellbender current distribution is 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 Eastern Hellbenders.

Hydrogeomorphic covariates were poor predictors of Eastern Hellbender occupancy; although the proportion of fine sediment was relevant in top performing models, it was an uninformative parameter. Fine sediment can fill interstitial spaces in cobble/boulder fields that are essential Eastern Hellbender habitat, especially for larvae (Hecht *et al.* 2017). It is possible that I failed to detect a relationship between proportion of fine sediment and Eastern Hellbender occupancy because pebble count techniques greatly underestimate fine sediment (Hedrick *et al.*

2013). Future habitat characterization studies should use alternative methods to quantify sedimentation and rock embeddedness.

Historical mining at the watershed scale was a poor predictor of Eastern Hellbender extirpation in my historical site analysis despite the large extent of strip mining throughout my study area. The low relative contribution of these landscape features proportional to watershed size could explain the poor predictive power of this site covariate. For example, the largest watersheds in my study (> 657 km^2, the mean size of HUC 10 watershed boundaries for which sample sites occurred) contained the largest areas of strip mining; therefore, the signal of historical mining features may become drowned out. However, in my random site analysis, the percent area mined was included in the best supported models but was uninformative. Further, due to Eastern Hellbender longevity (30 + years) and relatively high adult survivorship, time-lagged effects of declines and local extinctions could bias linking historical land use to presence/absence for long-lived species (Bodinof Jachowski & Hopkins 2018).

Long-lived species respond slower to environmental perturbations at the population level through the process of extinction debt (Kuussaari *et al.* 2009). Individuals will survive initial habitat change, and populations will slowly senesce at a future ecological cost (Tilman *et al.* 1994). Through a post hoc logistic regression analysis, we found that the number of years since the last Eastern Hellbender sighting at historical sites was a significant predictor of the binary outcome of extirpation or current occupancy (Table 8). The predicted probability of extirpation increased with increasing years since the last sighting (Table 9). Some historical records for Eastern Hellbenders in West Virginia go as far back as the 1910s (Green 1934, T.K. Pauley, Personal Communication), with the oldest date since last seen being 1932. Thus, using the best available historical site data that only extends back to 1965 could have inhibited my ability to detect a signal of extirpation using these data.

Results from my occupancy model analysis showed that turbidity and conductivity were the best predictors of Eastern Hellbender detection in both historical and random model sets. I considered both covariates equal in predicting Eastern Hellbender detection due to their additive effects and inclusion in all top performing models (Tables 2 & 5, Fig. 5). Conductivity is a water quality parameter that measures the concentration of salts and other organic ions. Lotic freshwater systems are becoming increasingly saline due to anthropogenic salt inputs (e.g. road salts, sewage, brines, irrigation runoff), and accelerated geologic weathering (Kaushal *et al.* 2018). Recent studies have identified negative associations between Eastern Hellbender occurrence and conductivity (Keitzer *et al.* 2013, Pugh *et al.* 2016, Pitt *et al.* 2017, Bodinof Jachowski & Hopkins 2018). Pitt *et al.* (2017) reported absence of hellbenders from the Susquehanna River drainage in Pennsylvania where conductivity exceeded 278 µS/cm. Similarly, hellbenders were completely absent from sites in my current study where conductivity exceeded 216 µS/cm. Average conductivity values for occupied historical sites were 42.33 (0-216) µS/cm, and 162.51 (70-546) µS/cm for extirpated sites. Increased water conductivity is suspected to impede Eastern 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, essentially rendering them functionally extinct (Wheeler *et al.* 2003, Briggler *et al.* 2007, Burgmeier *et al.* 2011, Pugh *et al.* 2016, Pitt *et al.* 2017). Conductivity levels are known to be greater in deforested and anthropogenically impacted (i.e., high impervious surface cover) watersheds (Likens *et al.* 1970, Trombulak & Frissell 2000). Thus, protection of highly forested watersheds is crucial for conservation planning and continued persistence of Eastern Hellbender populations. However, further research should aim to better understand how conductivity and other important water quality parameters influence Eastern Hellbender population demography (Bodinof Jachowski & Hopkins 2018).

Turbidity refers to the concentration of suspended sediments or organic particles that result in the cloudiness of water (Lloyd *et al.* 1987). Turbidity had a strong negative affect on eDNA detection, with estimates of detection probability reaching 0 around 25 Formazin Turbidity Units (FTU) (Fig. 5). Water turbidity could inhibit eDNA detection by increased filtering time which could further degrade DNA present in the sample, or by degrading suspended DNA particles that are present in the system (Lacoursière-Roussel *et al.* 2016, Williams *et al.* 2017). To my knowledge, no quantitative link between water turbidity in lotic systems and eDNA detection probabilities has been identified. Recent work by Schmelzle & Kinziger (2016) identified turbidity as a relevant covariate in detecting an endangered marine fish species in lagoon and estuarine environments. However, they used sample filtering time as a proxy for turbidity. Because the effects of environmental variables on the detection of aquatic organisms in lotic systems is poorly understood, this finding is significant to further understanding eDNA detection probabilities. Future eDNA studies in lotic systems should measure turbidity as a sampling covariate and consider its strong influence on DNA detection.

My study adds to a growing body of literature documenting substantial declines and extirpations of Eastern Hellbender populations in other portions of their range (Gates *et al.* 1985, Pfingsten 1990, Wheeler *et al.* 2003, Briggler *et al.* 2007, Foster *et al.* 2009, Graham *et al.* 2011, Keitzer *et al.* 2013, Quinn *et al.* 2013, Pitt *et al.* 2017). Range-wide Eastern Hellbender population declines, often described as enigmatic, are just now being fully investigated using eDNA to determine loss of area occupied, along with detailed demographic surveys to assess changes in population demography (Pitt *et al.* 2017, Freake & DePerno 2017, Bodinof Jachowski & Hopkins 2018). These range constriction trends warrant timely conservation action to ensure continued persistence of remaining Eastern Hellbender populations. Conservation action could induce an umbrella effect, protecting habitat and water quality for other sensitive freshwater species (Bodinof Jachowski & Hopkins 2018).

My study highlights the importance of preserving highly forested, low anthropogenically impacted watersheds for Eastern Hellbender conservation. By integrating high quality historical data, I was able to accurately and rapidly assess changes in Eastern Hellbender distribution and identify likely causes of extirpation over a broad portion of their historical range. My analyses integrated a novel sampling method with site occupancy and detection models, allowing me to determine the effects of landscape-scale and water quality factors on Eastern Hellbender extirpation and detection. This sampling approach has broad-scale applications and could be used to monitor changes in freshwater species distributions. My findings emphasize the sensitivity of freshwater species, particularly stream-dwelling amphibians, to land-use and water-quality changes. Conservation planning should consider limiting road/impervious surface development and protecting or restoring forested landscapes in headwater streams to preserve water quality for the multitude of species reliant on it. Given my results, I propose that more research is needed to assess the effects of roads on sedimentation and water quality changes in freshwater systems. Given increasing impacts on freshwater ecosystems and reports of population declines, I emphasize the need for further studies on species range constrictions that precede population demographic surveys for conservation monitoring.

CHAPTER 2

ENVIRONMENTAL DNA (eDNA) SAMPLING IMPROVES DETECTION OF EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*) OVER CONVENTIONAL SURVEYING METHODS

INTRODUCTION

The ability to effectively detect freshwater species has been facilitated by recent advances in molecular-based indicators such as environmental DNA (eDNA) approaches (Jackson *et al.* 2016). eDNA methods involve capturing and detecting DNA shed from organisms into aqueous environments using various field sampling, DNA extraction, and amplification methods to indirectly determine a species' presence/absence (Ficetola *et al.* 2008, Jerde *et al.* 2011, Deiner *et al.* 2015). eDNA applications have expanded across a range of species and environments, particularly for detecting invasive, threatened/endangered, cryptic, and elusive species, as well as for non-invasively detecting species that are difficult to sample using conventional methods (Taberlet *et al.* 2012b, Thomsen *et al.* 2012 Dejean *et al.* 2012, Goldberg *et al.* 2013, Moyer *et al.* 2014, Sigsgaard *et al.* 2015, Spear *et al.* 2015). Optimal sampling design must be considered in each study due to varying rates of DNA production, diffusion, and environmental conditions that influence eDNA detection (Bohmann *et al.* 2014, Goldberg *et al.* 2016).

eDNA approaches promise to improve freshwater species monitoring programs by increasing detection while decreasing sampling cost (Bohmann *et al.* 2014, Thomsen & Willerslev 2015, Valentini *et al.* 2016, Pitt *et al.* 2017). Freshwater species are declining rapidly as a result of multiple interacting anthropogenic stressors (Dudgeon *et al.* 2006, Strayer & Dudgeon 2010). Many cryptic and elusive freshwater species are understudied as a result of ineffective sampling methods that suffer from low detection (Abell 2002, Collen *et al.* 2014). Conventional surveying methods for freshwater species are often time and labor intensive, cost-ineffective, destructive to habitat, and potentially hazardous to researchers (Nickerson & Krysko 2003, Pregler *et al.* 2015). Mounting evidence suggests that eDNA sampling methods have a performance, logistical, and detection advantage compared to conventional surveying methods (Jerde *et al.* 2011, Dejean *et al.* 2012, Pilliod *et al.* 2013, Schmelzle & Kinziger 2016, Smart *et al.* 2015 Spear *et al.* 2015). However, few studies are designed to directly compare eDNA and conventional sampling method detection probabilities (Roussel *et al.* 2015, Smart *et al.* 2015). These comparisons also fail to account for spatial, temporal, and environmental variation that can influence detection probabilities (Pilliod *et al.* 2014, Wilcox *et al.* 2016). Comparative approaches allow decision-makers to weigh the costs and benefits of sampling methods to better understand population declines, fill data gaps, and improve monitoring programs (Nichols *et al.* 2008, Jackson *et al.* 2016).

In this study, I used a multi-method occupancy-modeling framework (Nichols *et al.* 2008) to compare method-specific detection probabilities between two sampling methods for Eastern Hellbenders, a cryptic and elusive species of fully aquatic salamander of conservation concern. Eastern Hellbenders inhabit swift-flowing streams with large cobble/boulder sized rocks used for shelter, feeding, and breeding in portions of the Ohio, Missouri, Mississippi, and Susquehanna river drainages in the United States, with an isolated population in central Missouri (Nickerson & Mays 1973). Range-wide population declines and range constrictions have been reported (Gates *et al.* 1985, Pfingsten 1990, Wheeler *et al.* 2003, Briggler *et al.* 2007, Foster *et al.* 2009, Graham *et al.* 2011, Keitzer *et al.* 2013, Quinn *et al.* 2013, Pitt *et al.* 2017), resulting in Eastern Hellbenders being considered as a candidate for listing under the U.S. Endangered Species Act (J. Applegate, personal communication, Spear *et al.* 2015). Conventional Eastern Hellbender sampling methods suffer from low detection due to the logistical constraints of physically searching under large rock cover in swift-flowing streams. Recent studies have been successful in using eDNA to detect Eastern Hellbender presence using this non-invasive method (Spear *et al.* 2015, Pitt *et al.* 2017). However, studies that aim to monitor population demography often use conventional sampling methods that involve physically turning rocks, which can be destructive to habitat and result in rock shelter abandonment (Nickerson & Krysko 2003, Olson *et al.* 2012 Pugh *et al.* 2016).

Additionally, I estimated relative concentration of Eastern Hellbender eDNA during each survey using qPCR standard curve analysis and compared these data to catch per unit effort (CPUE) estimates from conventional sampling methods. I hypothesized a greater probability of detecting Eastern Hellbender presence using eDNA than conventional sampling methods because of the species cryptic nature, the logistical constraints of physical sampling methods, and sensitivity of eDNA methodology.

METHODS

Study Area & Design

I sampled 22 sites within and around the Monongahela National Forest (West Virginia, USA, Figure 1). Of the 22 sites, 16 were locations with historical records of Eastern Hellbender presence within the last 15 years, and 6 were random sites either within the same mainstem river/stream, or within tributaries of watersheds in which Humphries & Pauley (2005) and Keitzer *et al.* 2013 located hellbenders. I selected random sites by generating random points every 2 km on stream line features and performed a select by location in ArcMap 10.4 (ESRI, Redlands, CA) based on proximity to access via trails or roads.

I defined sites as a 150m stream reach split into three 50m sections based on estimates of Eastern Hellbender home-range size (Humphries & Pauley 2005, Burgmeier *et al.* 2011). I sampled sites three times over a single season from April-August 2017. During each visit, I first collected an eDNA sample, followed by a conventional sampling method survey. After each site visit, I followed a decontamination protocol to prevent sample cross-contamination among eDNA surveys. I soaked sample equipment (i.e., waders, wetsuits, dip nets) in a 30% bleach solution and rinsed equipment with well water from a campground tap. This study was part of a larger study examining Eastern Hellbender occupancy across its range in West Virginia using eDNA only.

eDNA Sampling

I collected eDNA samples using single-use disposable equipment to avoid contamination between sites (Goldberg *et al.* 2016). Forceps used for extracting filters were the only piece of equipment reused among sites and were treated with DNA Away Surface Decontaminant (Molecular Bio-products, Inc., San Diego, CA, U.S.A.) prior to filter extraction to avoid sample contamination. At each site, I used a sterile, disposable Whirlpak Stand-up Bag (36oz, 1065ml capacity, Nasco, Fort Atkinson, WI U.S.A.) to collect 1L water samples from the center of the stream. I used a Cole-Parmer Masterflex Peristaltic Pump (Model No. 7520-00, Cole-Parmer Instrument Co. Chicago, IL, U.S.A.) attached to a 1L Nalgene Vacuum Flask to filter water through sterile, disposable 250ml Nalgene Analytical Test Filter Funnels (pore size = 0.45 µm, cellulose nitrate membrane, Thermo Fisher Scientific Inc., Rochester, NY, U.S.A.). I placed filter membranes immediately in 1.5ml microcentrifuge tubes post-filtering and transported them on dry ice prior to storage in a -20°C freezer. I filled sterile Whirlpak bags with deionized water from a tap at Marshall University to use as a negative field control and kept it in the same container as all sample equipment. For each sampling period, I filtered the negative field control after the last field sample was filtered using the same protocol and equipment as field samples.

Laboratory Methods

I 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). I divided filters in half and tore them into pieces, with the other half stored at -80˚C for potential later use. I followed the standard protocol for the extraction kit with the additional use of a Qiashredder (Qiagen, Inc.) spin column after the lysis step. I processed all samples in a separate and dedicated extraction and PCR setup section of the laboratory.

I amplified eDNA samples following the qPCR protocol from Spear *et al.* (2015). A 104bp 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').

I used a 20µL reaction volume consisting of 10µL of Luna universal probe qPCR master mix (New England Biolabs), 1µL of each primer at 10µM and probe at 5µM, 3.5µL nuclease free water, and 3.5µL 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 sec and 60˚ for 60 sec, with data collection during the annealing stage at 60˚C. I ran all extractions in triplicate and included a positive control from a captive Eastern Hellbender population water sample and negative control to ensure qPCR efficacy and any potential contamination. I used a 1:2 serial dilution of the 13ng/uL positive control to create a standard curve to determine concentration estimations for all of the eDNA samples.

I generated cycle threshold values (Ct) using SDS 2.4 software (Applied Biosystems). I used the Ct, known concentration, and dilution values for the positive control to generate two graphs; Ct vs. dilution factor and dilution factor vs. concentration. I plugged averaged sample Ct values into the equation of the line for both graphs, $y = 1.0651x+29.975$, and $y = 13.048e^{-0.697x}$ (Fig. 2 in Chapter 1), to yield sample concentration.

For the first three sampling periods, I found the deionized water used from the tap at Marshall University to be contaminated at the source, as about 1/3 of my 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. I determined that the deionized water was contaminated at the source by filtering three samples of it in a separate lab using all disposable single-use equipment, along with three samples of nuclease-free water for comparison. One out of the three samples of deionized water amplified with one qPCR replicate, and all nuclease-free water samples were negative. For the fourth round of surveys, I used nuclease-free water for all field negative controls to avoid further source contamination of negative controls. All contaminated filter blanks had only 1/3 qPCR replicates amplify, and all DNA concentration values were below 0.08 ng/µL. Therefore, I used field samples that had a minimum of 2/3 qPCR replicates amplify with concentrations above 0.08 ng/µL as an indicator of Eastern Hellbender presence.

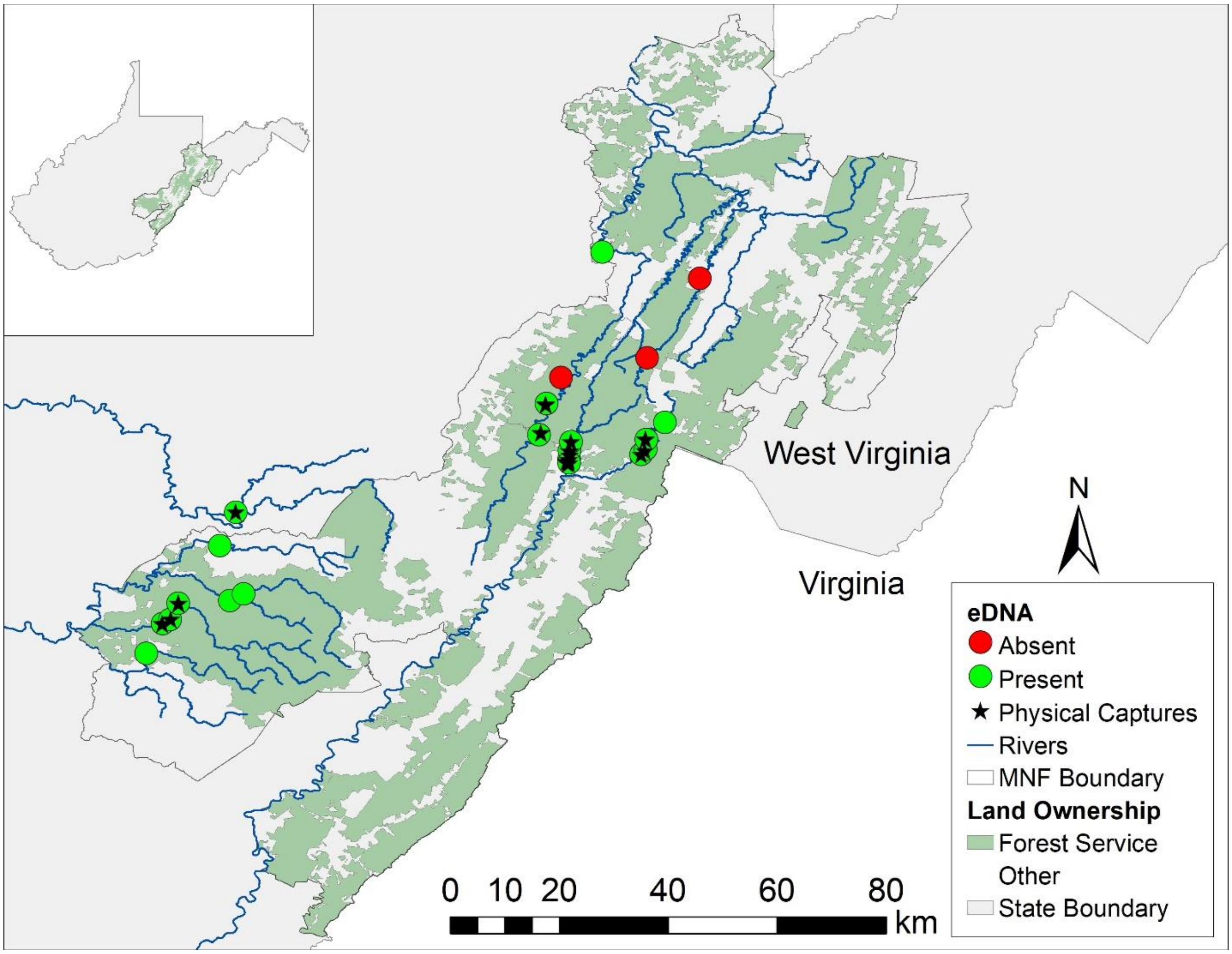

Figure 1. Study area, eDNA, and conventional sampling method results. Study area in the Monongahela National Forest (MNF), eastern West Virginia, USA showing the 22 sampling sites where paired eDNA and conventional surveys occurred. Green and red circles show the results of eDNA surveys, and black stars show where Eastern Hellbenders were physically captured.

Physical Field Surveys

Starting at the downstream end of the site, I turned cobble and boulder (*b*-axis ≥ 65 mm) sized rocks by hand or by using a log peavey to capture Eastern Hellbenders either by hand or with dip nets (Nickerson & Krysko 2003, Keitzer *et al.* 2013). One person turned rocks while one person was in the water snorkeling behind two others with dip nets. All observers carefully watched to see if Eastern Hellbenders swam out of the rock. The exposed area was carefully searched for Eastern Hellbenders by hand. Turned rocks were carefully replaced back to their original resting position to limit substrate and hydrologic disturbance. In areas with bedrock crevices and rocks too large to lift, snorkelers searched visually and tactilely for Eastern Hellbenders using dive lights (Pugh *et al.* 2016). To comply with the special-use permit granted by the USFS, I turned rocks at only 50% (75m) of the stream reach delineated for each study site to limit potential adverse effects to Eastern Hellbender habitat and populations. To increase the probability of detecting all size classes, I turned small cobble in riffle/run areas to search for larval individuals that often go undetected (Freake & DePerno 2017). I calculated catch per unit effort (CPUE) as the number of Eastern Hellbenders encountered per person hour.

I recorded capture location (using GPS), mass (grams), total and snout vent-length (TL, SVL, cm), and head width (cm) for each Eastern Hellbender I captured. I subcutaneously marked captured Eastern Hellbenders > 20 cm TL using a passive integrated transponder (PIT) tag injected at the base of the tail. I classified individuals as larvae if they had free gills and were TL < 9 cm, sub-adults if they lacked free gills and were TL < 29 cm, young adults TL 30-40 cm, and old adults (≥ 40 cm) based on Nickerson and Mays (1973). I replaced all captured Eastern Hellbenders carefully underneath their original shelter rocks.

Statistical Analysis

I used the multimethod occupancy modelling approach described by Nichols *et al.* (2008) to estimate method-specific detection probabilities (*p*). The multimethod framework deals with the lack of independence of detections within a sampling occasion by combining detection histories from all methods to estimate method-specific detection probabilities (Nichols *et al.* 2008, Haynes *et al.* 2013). While the goal of this study was to compare detection probabilities, these models are often used to estimate scale-specific occupancy using two parameters (Pregler *et al.* 2015). The first parameter Ψ estimates large-scale occupancy (i.e., probability of sampling unit being occupied), and the second parameter θ estimates small-scale occupancy (i.e., probability of species presence at immediate sample site). For the purposes of this study, I kept both occupancy parameters constant to compare the probability of detection for each method.

I examined the influence of environmental covariates on detection across methods to more accurately estimate detection probabilities (Table 1). I hypothesized that turbidity and water velocity would negatively influence detection probability due to increased filtering time for eDNA samples and reduced visibility during surveys (Schmelzle & Kinziger 2016). Physical characteristics of streams such as stream wetted width and depth were also included as detection covariates. I hypothesized that stream wetted width would negatively influence detection probability across methods because the relative concentration of eDNA could become diluted in larger dendritic systems, and search effort in larger rivers is limited by these variables. I hypothesized depth to have a neutral effect on detection because hellbenders can be found in relatively shallow or deep water. Continuous covariates were z-standardized.

I used the single season, multimethod model variant in program PRESENCE 12.7 (Hines 2006) to conduct occupancy analyses. I first ran a null model where all parameters were held constant (i.e., equal detection probabilities for each sampling method). I then allowed detection to vary by sampling method and created additive models with environmental covariates. (Table 1). I ranked models according to Aikaike's information criterion (AICc) corrected for small sample size (Burnham and Anderson 2002). Effective sample size for occupancy models is currently unclear (Mackenzie *et al.* 2017); however, I used the total number of sites. I used AICc model weights and 95% confidence intervals to evaluate best supported models (Δ AICc < 2) and considered models with Δ AICc < 2.0 equal. I evaluated goodness-of-fit using the most parameterized (global) model with 10000 simulations to check for overdispersion (Mackenzie & Bailey 2004).

I examined the relationship between eDNA concentration and Eastern Hellbender relative abundance at the site level by fitting a general linear model. I used CPUE estimates from conventional sampling method surveys and average eDNA concentration (ng/µL) when hellbenders were detected using both methods (n=20 occassions, Schmelzle & Kinziger 2016). Both variables were $\log_{10} + 1$ transformed.

As a post hoc analysis, I examined if the probability of detection varied by sampling period by building a single multimethod model: Ψ,θ(.) p(M+ sampling period). This model was not ranked with the candidate set.

Table 1. Covariate summary. Summary of covariates considered in multimethod occupancy models tested for their effects on Eastern Hellbender detection probability.

Covariate	Description	Unit	Abbr.
Stream Depth	Average depth of stream at sample site measured at 3 transects	m	depth
Stream Width	Average width of stream at sample site measured at 3 transects	m	width
Water Velocity	Water velocity measured at eDNA sample site	m/s	flow
Water Temperature	Water temperature measured at eDNA sample site	°C	wtemp
Turbidity	Turbidity of water measured at eDNA sample site	FTU	turb

RESULTS

I captured 32 hellbenders using conventional sampling method surveys at 13/22 sites in 4 separate watersheds (Fig. 1). I captured hellbenders at 3 random sites where their presence was previously undocumented. Hellbender sizes (TL) ranged from 4 cm gilled larvae to 57 cm adults (Fig 2). I detected most gilled larvae and subadults while snorkeling and turning small cobble, except for one gilled individual found under the same rock as an adult. Average CPUE was 0.029 (0.011 - 0.116) hellbenders per person hour for conventional sampling method surveys. I detected Eastern Hellbender presence using eDNA at 19/22 sites: at all 16 historical, and at 3/6 random sites. I also detected Eastern Hellbenders using eDNA at 6 sites where physical sampling methods failed to detect them (Fig 1).

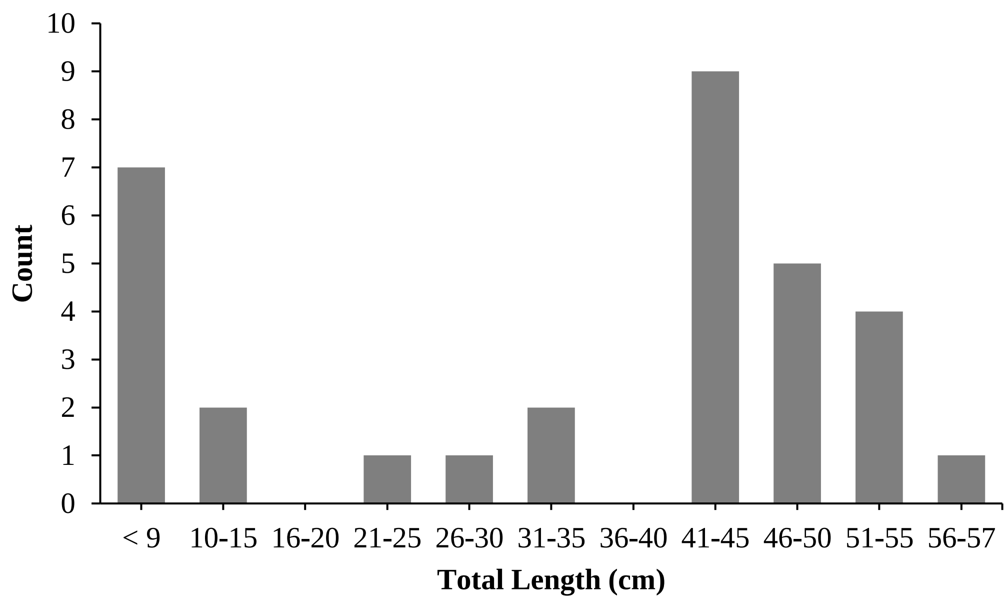

Figure 2. Eastern Hellbender length-count distribution. Length count distribution of Eastern Hellbenders (n=32) from conventional sampling method surveys conducted April – September 2017, West Virginia, USA.

Multimethod Occupancy Analysis

I found evidence of underdispersion in the global model based on goodness-of-fit test ($\hat{c} = 0.57$). While $\hat{c} < 1$ indicate underdispersion, corrections are typically only made to overdispersion, and it is recommended to set $\hat{c}$ = 1 in cases of underdispersion (Burnham and Anderson 2002, Mackenzie *et al.* 2017). The best supported models given the candidate set (Δ AICc < 2.0) incorporated method, turbidity, and turbidity and water velocity as additive effects (Table 2). However, water velocity was found to be an uninformative parameter based on the 95% confidence interval of the parameter estimate containing zero. Parameter estimates and 95% intervals indicated turbidity negatively influenced detection (Table 3). Turbidity, water velocity, stream depth, and stream width accounted for 68%, 26%, 12%, and 12% of the model weights given the candidate model set, respectively (Table 4). Overall detection estimates from the best supported model suggest eDNA detection was 56% greater than conventional sampling methods (Table 5).

Table 2. Candidate multimethod models. Candidate models predicting detection probability ranked using second order AIC (AICc).

Model	*K*	AICc	Δ AICc	*ω*	-2*l*
Ψ,θ(.) p(M+turb)	5	136.58	0.00	0.45	122.83
Ψ,θ(.) p(M+turb+flow)	6	137.88	1.30	0.23	120.28
Ψ,θ(.) p(M+width+depth)	6	139.29	2.71	0.11	121.69
Ψ,θ(.) *p*(M)	4	139.77	3.19	0.09	129.42
Ψ,θ(.) *p*(M+wtemp)	5	141.37	4.79	0.04	127.62
Ψ,θ(.) p(M+flow)	5	141.81	5.23	0.03	128.06
Ψ,θ(.) p(M+width)	5	143.17	6.59	0.01	129.42
Ψ,θ(.) *p*(M+depth)	5	143.22	6.64	0.01	129.47
Ψ,θ(.) *p*(M+depth+turb+flow+width+wtemp)	9	150.73	14.15	0.00	117.73

eDNA Concentration

General linear model indicated no association between Eastern Hellbender CPUE and eDNA concentration (P = 0.11, R^2 =0.33). A visual assessment of the fitted residuals plotted against the predicted residuals indicated heterogeneity of the variance. A visual assessment of residual distribution using a Q-Q plot indicated a non-normal distribution of residuals, indicating poor model fit based on the assumptions concerning data structure of linear models (Nimon 2012).

Table 3. Model parameter estimates. Model parameters, coefficients (β), standard errors (SE), and 95% confidence intervals (LCI, UCI) from best supported models (Δ AICc ≤ 2) estimating Eastern Hellbender detection probability (p) between sampling methods.

Model	**Parameters**	**β**	**±**	**SE**	**LCI**	**UCI**
Ψ,θ(.) p(M+turb)	Intercept 1	1.733	±	0.424	0.903	2.563
	Intercept 2	-1.238	±	0.346	-1.916	-0.560
	turb	-1.8431	±	0.744	-3.302	-0.384
Ψ,θ(.) p(M+turb+flow)	Intercept 1	1.807	±	0.536	0.756	2.858
	Intercept 2	-1.493	±	0.399	-2.274	-0.711
	turb	-2.256	±	0.828	-3.879	-0.633
	flow	0.441	±	0.279	-0.105	0.987

Table 4. Model weights. AIC weights (ω) and parameter weights from the candidate model set.

Model	**AICc *ω***	***ω* Turb**	***ω* Flow**	***ω* Depth**	***ω* Width**
Ψ,θ(.) p(M+turb)	0.45	0.68	0.26	0.12	0.12
Ψ,θ(.) p(M+turb+flow)	0.23				
Ψ,θ(.) p(M+width+depth)	0.11				
Ψ,θ(.) *p*(M)	0.09				
Ψ,θ(.) *p*(M+wtemp)	0.04				
Ψ,θ(.) p(M+flow)	0.03				
Ψ,θ(.) p(M+width)	0.01				
Ψ,θ(.) *p*(M+depth)	0.01				
Ψ,θ(.) *p*(M+depth+turb+flow+width+wtemp)	0.00				

Table 5. Detection probability estimates. Eastern Hellbender detection probability (p) estimates (with standard errors) from best supported models (Δ AICc ≤ 2) for each sampling method.

Model	**eDNA**			**Conventional**		
Ψ,θ(.) p(M+turb)	0.840	±	0.058	0.282	±	0.064
Ψ,θ(.) p(M+turb+flow)	0.852	±	0.065	0.293	±	0.082

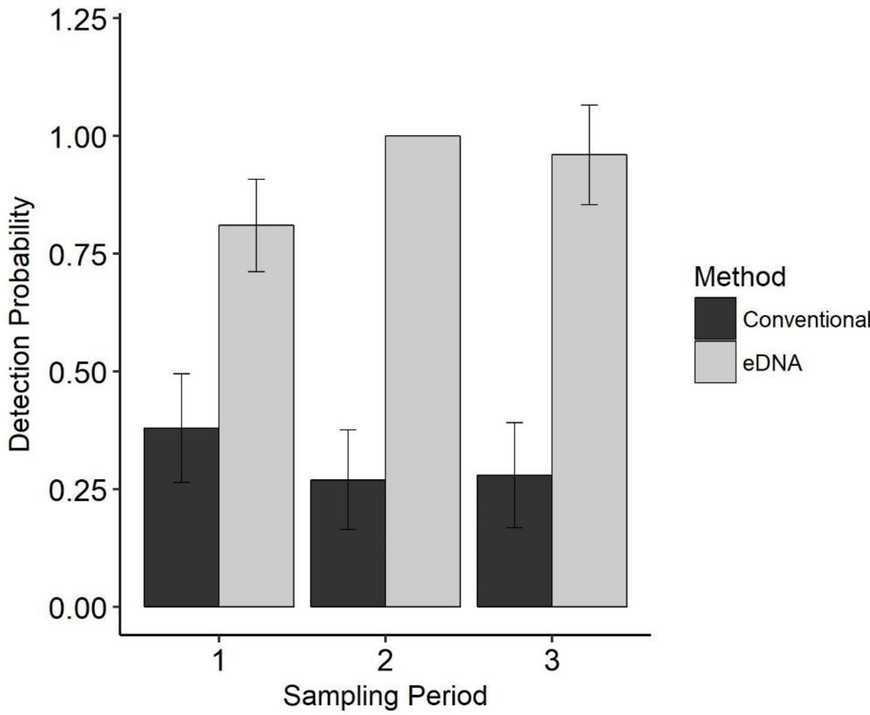

Figure 3. Detection probability by sampling period. Post hoc analysis of Eastern Hellbender detection probabilities varying by sampling period for each method. Note: the detection probability estimate for eDNA during the second sampling period was 1, thus the lack of a standard error bar.

DISCUSSION

My study provides novel insight into the performance advantage of eDNA over conventional sampling methods and the influence of environmental variables on Eastern Hellbender detection probabilities. eDNA sampling yielded higher detection probabilities than conventional sampling methods, supporting my hypothesis that eDNA provides a performance advantage over conventional methods to detect Eastern Hellbenders. My results demonstrate that: (1) eDNA detection probability was 3x higher than conventional sampling methods; (2) best supported models indicate water turbidity negatively influenced detection probability; and (3) eDNA concentration was not associated with Eastern Hellbender CPUE. This study adds to previous work that suggests eDNA is an effective tool for assessing Eastern Hellbender presence over conventional sampling methods and that studies aiming to assess broad-scale patterns of

Eastern Hellbender occupancy should implement this method due to its performance advantage (Spear *et al.* 2015, Pitt *et al.* 2017). This study also highlights the utility of comparative analyses to better understand detection methods for species monitoring programs.

The results of this study support those of Spear *et al.* (2015), who found eDNA sampling to outperform conventional surveys. However, Spear *et al.* (2015) did not repeat site visits and did not account for detection probability and environmental covariates that influence detection. Estimating detection probabilities from multiple site visits accounts for false negatives (i.e., when the animal is present at a site but goes undetected (Mackenzie *et al.* 2002). My estimates of 84% detection probability using eDNA compared to 28% for conventional sampling methods suggests that eDNA is a highly effective method to detect Eastern Hellbender presence. These results are consistent with other eDNA studies that report high estimates of detection (Schmidt *et al.* 2013, Schmelzle & Kinziger 2016, Jane *et al.* 2015, Wilcox *et al.* 2016). My results suggest that conventional Eastern Hellbender surveys likely underestimate overall Eastern Hellbender abundance at sites and that a large number of animals go undetected. Studies that employ these methods to assess population demography should account for low detection by using an occupancy modeling approach.

The best supported models indicated water turbidity negatively affected detection probability, supporting my hypothesis that turbidity would negatively affect detection (Table 3). Water turbidity, which refers to the cloudiness of water from varying amounts of suspended sediments and organic matter, likely influenced eDNA detection due to increased filtration time or faster degradation of DNA particles (Lacoursière-Roussel *et al.* 2016, Williams *et al.* 2017). Schmelzle & Kinziger (2016) identified turbidity as a relevant covariate in detecting an endangered marine fish species in lagoon and estuarine environments; however, they used sample filtering time as a proxy for turbidity. The influence of environmental variables on eDNA detection in lotic systems is poorly understood (Wilcox *et al.* 2016). My results suggest that water turbidity and flow should be considered when sampling for eDNA, and if an occupancy modeling approach is employed, these data should be collected to incorporate into estimates of detection probability. My findings are significant to furthering understanding of eDNA detection probabilities in lotic systems.

The general linear model failed to indicate an association between eDNA concentrations and Eastern Hellbender CPUE. These results are consistent with Spear *et al.* (2015) who failed to detect a correlation between eDNA concentration and Eastern Hellbender abundance but contrasts other previous studies that correlated eDNA concentrations with animal abundance or biomass (Thomsen *et al.* 2012, Takahara *et al.* 2012, Goldberg *et al.* 2013, Pilliod *et al.* 2013, Klymus *et al.* 2015, Schmelzle & Kinziger 2016). Low detection probablities using conventional sampling methods could be the reason why I failed to detect an association between eDNA concentrations and Eastern Hellbender CPUE. Further, as discussed by Spear *et al.* (2015), there is a limitation to running this analysis only using data from sites where detections occurred using both methods during the same sampling period. Finally, individuals occurring upstream of the sample site could increase eDNA concentration estimates relative to the animals present in the physically sampled area because of downstream diffusion of eDNA in lotic systems (Pilliod *et al.* 2013). Thus, I propose caution in using eDNA concentration estimates to infer Eastern Hellbender abundance and biomass relative to physical sampling methods.

My study highlights the effectiveness of eDNA as a conservation tool to detect Eastern Hellbender presence. My study builds upon previous work that suggest conventional sampling methods for this species suffer from low detection probabilities relative to sampling effort (Spear *et al.* 2015). For studies and monitoring programs aiming to examine Eastern Hellbender occupancy at many sites over a broad geographic area, eDNA is likely a more viable method to use than conventional sampling methods. Studies should account for the influence of environmental covariates on detection by employing an occupancy modeling approach where sites are visited multiple times. Further, seasonal effects on detection probabilities should not be overlooked. As a post hoc analysis, I examined if detection probability varied among my three sampling periods (Fig. 3). Estimates of eDNA detection probability for the first sampling period were slightly lower and increased through time. Temporal variation in detection probabilities could be due to higher water levels and increased turbidity during April/May when Eastern Hellbenders are actively foraging, which could explain the inverse relationship observed with conventional sampling method detection probabilities. Estimates of detection probability for conventional sampling methods were slightly higher during the first sampling period and decreased over time (Fig 3). Eastern Hellbender seasonal activity peaks bimodally in April/May when foraging occurs likely from changes in water temperature, and in the August/September breeding season (Humphries 2007). Spear *et al.* (2015) reported higher eDNA concentrations during the breeding season. Low water levels during the summer months seemed to increase eDNA detection, possibly due to increased concentrations of eDNA. Conversely, conventional sampling methods suffer from low detection during June/July, as hellbenders likely seek refuge in deeper areas of streams that are difficult to sample (Humphries 2007). Thus, Eastern Hellbender seasonal activity patterns and variable environmental conditions are an important factor to consider in sampling and monitoring programs.

While many Eastern Hellbender monitoring programs that aim to better understand population demography employ physical sampling methods to capture animals, alternative sampling methods such as nest boxes should be explored to avoid potential deleterious impacts on Eastern Hellbender populations (Olson *et al.* 2012). Nest boxes may allow for a non-invasive way to sample Eastern Hellbender population demography without having to physically alter stream substrate (Ettling et al. 2013, Jachowski 2016). Future studies should attempt to compare eDNA concentrations with abundance/density estimates from nest box sampling to determine the efficacy of this non-invasive method to detect hellbenders.

LITERATURE CITED

Abell, R. (2002). Conservation biology for the biodiversity crisis: a freshwater follow-up. *Conservation Biology*, 16(5), 1435-1437.

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L., & Lodge, D.M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*. *48* (3):1819-1827.

Barnes, M. A., & Turner, C.R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1-17.

Blaschke, A. P., Steiner, K. H., Schmalfuss, R., Gutknecht, D., & Sengschmitt, D. (2003). Clogging processes in hyporheic interstices of an impounded river, the Danube at Vienna, Austria. *International Review of Hydrobiology*, *88*(3-4), 397-413.

Bodinof Jachowski, C.M., Millspaugh, J. J., & Hopkins, W. A. (2016). Current land use is a poor predictor of hellbender occurrence: why assumptions matter when predicting distributions of data-deficient species. *Diversity and Distributions*, *22*(8), 865-880.

Bodinof Jachowski, C.M. & Hopkins, W.A. (2018). Loss of catchment-wide riparian forest cover is associated with reduced recruitment in a long-lived amphibian. *Biological Conservation*, *220*(2018), 215-227.

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., ... & De Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in ecology & evolution*, 29(6), 358-367.

Boothroyd, M., Mandrak, N. E., Fox, M., & Wilson, C. C. (2016). Environmental DNA (eDNA) detection and habitat occupancy of threatened spotted gar (*Lepisosteus oculatus*). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *26*(6), 1107-1119.

Brabec, E., Schulte, S., & Richards, P. L. (2002). Impervious surfaces and water quality: a review of current literature and its implications for watershed planning. *Journal of Planning Literature, 16*(4), 499-514.

Braulik, G. T., Arshad, M., Noureen, U., & Northridge, S. P. (2014). Habitat fragmentation and species extirpation in freshwater ecosystems; causes of range decline of the Indus River Dolphin (*Platanista gangetica minor*). *PloS one*, *9*(7), e101657.

Briggler, J.T., Utrup, J., Davidson, C., Humphries, J., Groves, J., Johnson, T., Ettling, J., Wanner, M., Traylor-Holzer, K., Reed, D., Lindgren, V. & Byers, O. (eds.) (2007) Hellbender population and habitat viability assessment: final report. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN.

Burgmeier, N. G., Unger, S. D., Sutton, T. M., & Williams, R. N. (2011). Population status of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *Journal of Herpetology*, *45*(2), 195-201.

Burnham, K. P., & D. R. Anderson. (2002). *Model selection and multimodel inference: a practical information-theoretic approach*. Second edition. Springer, New York, New York, USA.

Collen, B., Whitton, F., Dyer, E. E., Baillie, J. E., Cumberlidge, N., Darwall, W. R., ... & Böhm, M. (2014). Global patterns of freshwater species diversity, threat and endemism. *Global ecology and Biogeography*, *23*(1), 40-51.

Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PloS one*, *9*(2), e88786.

Deiner, K., Walser, J. C., Mächler, E., & Altermatt, F. (2015). Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*, 183, 53-63.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., & Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology*, *49*(4), 953-959.

de Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PloS one*, 11(10), e0165273.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., ... & Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews*, *81*(2), 163-182.

Eaglin, G. S., & Hubert, W. A. (1993). Management briefs: effects of logging and roads on substrate and trout in streams of the Medicine Bow National Forest, Wyoming. *North American Journal of Fisheries Management*, *13*(4), 844-846.

Ettling, J. A., Wanner, M. D., Schuette, C. D., Armstrong, S. L., Pedigo, A. S., & Briggler, J. T. (2013). Captive reproduction and husbandry of adult Ozark hellbenders, Cryptobranchus alleganiensis bishopi. *Herpetological Review*, *44*(4), 605-610.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423-425.

Ficetola, G. F., Taberlet, P., & Coissac, E. (2016). How to limit false positives in environmental DNA and metabarcoding? *Molecular Ecology Resources*, *16*(3), 604-607.

Fiske, I., & Chandler, R. (2011). Unmarked: an R package for fitting hierarchical models of wildlife occurrence and abundance. *Journal of Statistical Software*, 43(10), 1-23.

Forman, R. T., & Alexander, L. E. (1998). Roads and their major ecological effects. *Annual Review of Ecology and Systematics*, *29*(1), 207-231.

Foster, R.L., McMillan, A.M., & Roblee, K.J. (2009). Population status of hellbender salamanders (*Cryptobranchus alleganiensis*) in the Allegheny River drainage of New York state. *Journal of Herpetology*. 43, 579‑588.

Freake, M. J., & DePerno, C. S. (2017). Importance of demographic surveys and public lands for the conservation of eastern hellbenders *Cryptobranchus alleganiensis alleganiensis* in southeast USA. *PloS one*, *12*(6), e0179153.

Fukumoto, S., Ushimaru, A., & Minamoto, T. (2015). A basin‐scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. *Journal of Applied Ecology*, *52*(2), 358-365.

Gates, J. E., Hocutt, C. H., Stauffer, Jr, J. R., & Taylor, G. J. (1985). The distribution and status of *Cryptobranchus alleganiensis* in Maryland. *Herpetological Review*, 16, 17–18.

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., & Waits, L. P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (Potamopyrgus antipodarum). *Freshwater Science*, *32*(3), 792-800.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., ... & Laramie, M. B. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299-1307.

Graham, S. P., Soehren, E. C., Cline, G. R., Schmidt, C. M., Sutton, W. B., Rayburn, J. R., ... & Stiles, J. A. (2011). Conservation status of hellbenders (*Cryptobranchus alleganiensis*) in Alabama, USA. *Herpetological Conservation and Biology*, *6*(2), 242-249.

Green, N.B. (1934). *Cryptobranchus alleganiensis* in West Virginia. *Proceedings of the West Virginia Academy of Science*. 17:28-30.

Groves, C. R., Jensen, D. B., Valutis, L. L., Redford, K. H., Shaffer, M. L., Scott, J. M., ... & Anderson, M. G. (2002). Planning for Biodiversity Conservation: Putting Conservation Science into Practice: A seven-step framework for developing regional plans to conserve biological diversity, based upon principles of conservation biology and ecology, is being used extensively by the nature conservancy to identify priority areas for conservation. *AIBS Bulletin*, *52*(6), 499-512.

Guillera‐Arroita, G., Ridout, M. S., & Morgan, B. J. (2010). Design of occupancy studies with imperfect detection. *Methods in Ecology and Evolution*, *1*(2), 131-139.

Haynes, T. B., Rosenberger, A. E., Lindberg, M. S., Whitman, M., & Schmutz, J. A. (2013). Method-and species-specific detection probabilities of fish occupancy in Arctic lakes: implications for design and management. *Canadian Journal of Fisheries and Aquatic Sciences*, *70*(7), 1055-1062.

Hecht, K. A., Freake, M. J., Nickerson, M. A., & Colclough, P. (2017). Hellbender Salamanders (*Cryptobranchus alleganiensis*) Exhibit An Ontogenetic Shift In Microhabitat Use In A Blue Ridge Physiographic Region Stream. *bioRxiv*, 139766.

Hedrick, L. B., Anderson, J. T., Welsh, S. A., & Lin, L. S. (2013). Sedimentation in mountain streams: a review of methods of measurement. *Natural Resources*, *4*(01), 92.

Hendricks, S. A., Clee, P. R. S., Harrigan, R. J., Pollinger, J. P., Freedman, A. H., Callas, R., ... & Wayne, R. K. (2016). Re-defining historical geographic range in species with sparse records: implications for the Mexican wolf reintroduction program. *Biological Conservation*, *194*, 48-57.

Hines, J. E. (2006). PRESENCE2: Software to estimate patch occupancy and related parameters. USGS-PWRC. *http://www.mbr-pwrc.usgs.gov/software/presence.html*.

Houlahan, J. E., Findlay, C. S., Schmidt, B. R., Meyer, A. H., & Kuzmin, S. L. (2000). Quantitative evidence for global amphibian population declines. *Nature*, *404*(6779), 752.

Homer, C.G., Dewitz, J.A., Yang, L., Jin, S., Danielson, P., Xian, G., Coulston, J., Herold, N.D., Wickham, J.D., & Megown, K., (2015). Completion of the 2011 National Land Cover Database for the conterminous United States-Representing a decade of land cover change information. *Photogrammetric Engineering and Remote Sensing*, *81*(5), 345-354.

Hosmer, D. W. & S. Lemeshow. 2000. *Applied Logistic Regression Analysis*. Second Edition. John Wiley and Sons. New York, New York, U.S.A.

Humphries, W. J., & Pauley, T. K. (2005). Life history of the hellbender, *Cryptobranchus alleganiensis*, in a West Virginia stream. *The American Midland Naturalist*, 154(1), 135-142.

Humphries, W. J. (2007). Diurnal seasonal activity of *Cryptobranchus alleganiensis* (Hellbender) in North Carolina. *Southeastern Naturalist*, *6*(1), 135-140.

Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., ... & Hart, K. M. (2015). Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PloS one*, *10*(4), e0121655.

Jachowski, C. M. B. 2016. Effects of land use on hellbenders (*Cryptobranchus alleganiensis*) at multiple levels and efficacy of artificial shelters as a monitoring tool. Doctoral Dissertation. Department of Fish and Wildlife Conservation, Virginia Tech.

Jackson, M. C., Weyl, O. L. F., Altermatt, F., Durance, I., Friberg, N., Dumbrell, A. J., ... & Leadley, P. W. (2016). Recommendations for the next generation of global freshwater biological monitoring tools. In *Advances in Ecological Research* (Vol. 55, pp. 615-636). Academic Press.

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., ... & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, 15(1), 216-227.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. & Lodge, D.M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters.* 4, 150–157.

Jones, E. B., Helfman, G. S., Harper, J. O., & Bolstad, P. V. (1999). Effects of riparian forest removal on fish assemblages in southern Appalachian streams. *Conservation Biology*, *13*(6), 1454-1465.

Kaushal, S. S., Likens, G. E., Pace, M. L., Utz, R. M., Haq, S., Gorman, J., & Grese, M. (2018). Freshwater salinization syndrome on a continental scale. *Proceedings of the National Academy of Sciences*, 201711234.

Keitzer, S. C., Pauley, T. K., & Burcher, C. L. (2013). Stream characteristics associated with site occupancy by the eastern hellbender, *Cryptobranchus alleganiensis alleganiensis*, in southern West Virginia. *Northeastern Naturalist*, *20*(4), 666-677.

King, R. S., Baker, M. E., Whigham, D. F., Weller, D. E., Jordan, T. E., Kazyak, P. F., & Hurd, M. K. (2005). Spatial considerations for linking watershed land cover to ecological indicators in streams. *Ecological Applications*, *15*(1), 137-153.

Kuussaari, M., Bommarco, R., Heikkinen, R. K., Helm, A., Krauss, J., Lindborg, R., ... & Stefanescu, C. (2009). Extinction debt: a challenge for biodiversity conservation. *Trends in Ecology & Evolution*, *24*(10), 564-571.

Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation*, *183*, 77-84.

Lacoursière-Roussel, A., Côté, G., Leclerc, V., & Bernatchez, L. (2016). Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *Journal of Applied Ecology*, *53*(4), 1148-1157.

Lahoz-Monfort, J. J., Guillera-Arroita, G., & Tingley, R. (2016). Statistical approaches to account for false-positive errors in environmental DNA samples. *Molecular Ecology Resources*, *16*(3), 673-685.

Likens, G. E., Bormann, F. H., Johnson, N. M., Fisher, D. W., & Pierce, R.S. (1970). Effects of forest cutting and herbicide treatment on nutrient budgets in the Hubbard Brook watershed-ecosystem. *Ecological Monographs,* 40, 23–47.

Lindberg, T. T., Bernhardt, E. S., Bier, R., Helton, A. M., Merola, R. B., Vengosh, A., & Di Giulio, R. T. (2011). Cumulative impacts of mountaintop mining on an Appalachian watershed. *Proceedings of the National Academy of Sciences*, *108*(52), 20929-20934.

Lloyd, D. S., Koenings, J. P., & Laperriere, J. D. (1987). Effects of turbidity in fresh waters of Alaska. *North American Journal of Fisheries Management*, *7*(1), 18-33.

MacKenzie, D. I., & Bailey, L. L. (2004). Assessing the fit of site-occupancy models. *Journal of Agricultural, Biological, and Environmental Statistics*, *9*(3), 300-318.

MacKenzie, D. I., Nichols, J. D., Lachman, G. B., Droege, S., Andrew Royle, J., & Langtimm, C. A. (2002). Estimating site occupancy rates when detection probabilities are less than one. *Ecology*, *83*(8), 2248-2255.

MacKenzie, D. I., Nichols, J. D., Royle, J. A., Pollock, K. H., Bailey, L., & Hines, J. E. (2017). *Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence*. Elsevier.

Magurran, A. E., Baillie, S. R., Buckland, S. T., Dick, J. M., Elston, D. A., Scott, E. M., ... & Watt, A. D. (2010). Long-term datasets in biodiversity research and monitoring: assessing change in ecological communities through time. *Trends in Ecology & Evolution*, *25*(10), 574-582.

Maltby, L., Forrow, D. M., Boxall, A., Calow, P., & Betton, C. I. (1995). The effects of motorway runoff on freshwater ecosystems: 1. Field study. *Environmental Toxicology and Chemistry*, 14(6), 1079-1092.

McBride, M., & Booth, D. B. (2005). Urban impacts on physical stream condition: effects of spatial scale, connectivity, and longitudinal trends. *JAWRA Journal of the American Water Resources Association*, *41*(3), 565-580.

Moyer, G. R., Diaz-Ferguson, E., Hill, J. E., & Shea, C. (2014). Assessing environmental DNA detection in controlled lentic systems. *PloS one*, *9*(7), e103767.

Nichols, J. D., Bailey, L. L., Talancy, N. W., Campbell Grant, E. H., Gilbert, A. T., Annand, E. M., ... & Hines, J. E. (2008). Multi-scale occupancy estimation and modelling using multiple detection methods. *Journal of Applied Ecology*, *45*(5), 1321-1329.

Nickerson, M. A., & Mays, C. E. (1973). A study of the Ozark hellbender *Cryptobranchus alleganiensis bishopi*. *Ecology*, *54*(5), 1164-1165.

Nickerson, M. A., & Krysko, K. L. (2003). Surveying for hellbender salamanders, *Cryptobranchus alleganiensis* (Daudin): A review and critique. *Applied Herpetology*, *1*(1), 37-44.

Nimon, K. F. (2012). Statistical assumptions of substantive analyses across the general linear model: a mini-review. *Frontiers in Psychology*, *3*, 322.

Olson, Z. H., Briggler, J. T., & Williams, R. N. (2012). An eDNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water. *Wildlife Research*, 39(7), 629-636.

Pfingsten, R. A. (1990). The status and distribution of the hellbender, *Cryptobranchus alleganiensis* in Ohio. *Herpetological Review*, 21, 48–51.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*, *70*(8), 1123-1130.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream‐dwelling amphibian. *Molecular Ecology Resources*, *14*(1), 109-116.

Pitt, A. L., Shinskie, J. L., Tavano, J. J., Hartzell, S. M., Delahunty, T., & Spear, S. F. (2017). Decline of a giant salamander assessed with historical records, environmental DNA and multi‐scale habitat data. *Freshwater Biology*, *62*(6), 967-976.

Poff, N. L., Allan, J. D., Bain, M. B., Karr, J. R., Prestegaard, K. L., Richter, B. D., ... & Stromberg, J. C. (1997). The natural flow regime. *BioScience*, *47*(11), 769-784.

Poff, N. L., & Zimmerman, J. K. (2010). Ecological responses to altered flow regimes: a literature review to inform the science and management of environmental flows. *Freshwater Biology*, *55*(1), 194-205.

Pregler, K. C., Vokoun, J. C., Jensen, T., & Hagstrom, N. (2015). Using multimethod occupancy estimation models to quantify gear differences in detection probabilities: is backpack electrofishing missing occurrences for a species of concern? *Transactions of the American Fisheries Society*, *144*(1), 89-95.

Price, S. J., Dorcas, M. E., Gallant, A. L., Klaver, R. W., & Willson, J. D. (2006). Three decades of urbanization: estimating the impact of land-cover change on stream salamander populations. *Biological Conservation*, *133*(4), 436-441.

Pugh, M. W., Hutchins, M., Madritch, M., Siefferman, L., & Gangloff, M. M. (2016). Land-use and local physical and chemical habitat parameters predict site occupancy by hellbender salamanders. *Hydrobiologia*, *770*(1), 105-116.

Quinn, S. A., Gibbs, J. P., Hall, M. H., & Petokas, P. J. (2013). Multiscale factors influencing distribution of the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*) in the northern segment of its range. *Journal of Herpetology*, *47*(1), 78-84.

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Roussel, J. M., Paillisson, J. M., Treguier, A., & Petit, E. (2015). The downside of eDNA as a survey tool in water bodies. *Journal of Applied Ecology*, *52*(4), 823-826.

Sala, O. E., Chapin, F. S., Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., ... & Leemans, R. (2000). Global biodiversity scenarios for the year 2100. *Science*, *287*(5459), 1770-1774.

Schmelzle, M. C., & Kinziger, A. P. (2016). Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. *Molecular Ecology Resources*, *16*(4), 895-908.

Schmidt, B. R., Kery, M., Ursenbacher, S., Hyman, O. J., & Collins, J. P. (2013). Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, *4*(7), 646-653.

Sexton, J. O., Song, X. P., Feng, M., Noojipady, P., Anand, A., Huang, C., ... & Townshend, J. R. (2013). Global, 30-m resolution continuous fields of tree cover: Landsat-based rescaling of MODIS vegetation continuous fields with lidar-based estimates of error. *International Journal of Digital Earth*, *6*(5), 427-448.

Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, *183*, 46-52.

Smart, A. S., Tingley, R., Weeks, A. R., van Rooyen, A. R., & McCarthy, M. A. (2015). Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications*, *25*(7), 1944-1952.

Smucker, N. J., Kuhn, A., Charpentier, M. A., Cruz-Quinones, C. J., Elonen, C. M., Whorley, S. B., ... & Wehr, J. D. (2016). Quantifying urban watershed stressor gradients and evaluating how different land cover datasets affect stream management. *Environmental Management*, 57(3), 683-695.

Spear, S. F., Groves, J. D., Williams, L. A., & Waits, L. P. (2015). Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation*, 183, 38-45.

Stanfield, L. W., & Kilgour, B. W. (2013). How proximity of land use affects stream fish and habitat. *River Research and Applications*, *29*(7), 891-905.

Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society*, *29*(1), 344-358.

Surasinghe, T. D., & Baldwin, R. F. (2015). Importance of riparian forest buffers in conservation of stream biodiversity: responses to land uses by stream-associated salamanders across two southeastern temperate ecoregions. *Journal of Herpetology*, *49*(1), 83-94.

Taber, C. A., Wilkinson Jr, R. F., & Topping, M. S. (1975). Age and growth of hellbenders in the Niangua River, Missouri. *Copeia*, 633-639.

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012a). Environmental DNA. *Molecular Ecology*, *21*(8), 1789-1793.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012b). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, *21*(8), 2045-2050.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. I. (2012). Estimation of fish biomass using environmental DNA. *PloS one*, *7*(4), e35868.

Thomsen, P., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., ... & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565-2573.

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, 183, 4-18.

Tilman, D., May, R. M., Lehman, C. L., & Nowak, M. A. (1994). Habitat destruction and the extinction debt. *Nature*, *371*(6492), 65.

Tingley, M. W., & Beissinger, S. R. (2009). Detecting range shifts from historical species occurrences: new perspectives on old data. *Trends in Ecology & Evolution*, *24*(11), 625-633.

Trombulak, S. C., & Frissell, C. A. (2000). Review of ecological effects of roads on terrestrial and aquatic communities. *Conservation Biology*, *14*(1), 18-30.

U.S. Army Corps of Engineers. (2015). National Inventory of Dams: U.S. Army Corps of Engineers Dataset. URL http://nid.usace.army.mil/.

U.S. Census Bureau. (2015). TIGER/Line Shapefiles (machine readable data files) / prepared by the U.S. Census Bureau. URL https://www.census.gov/geo/maps-data/data/tiger-line.html.

U.S. Geological Survey. (2007-2014). National Hydrography Dataset. URL https://nhd.usgs.gov.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., ... & Gaboriaud, C. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, *25*(4), 929-942.

Wenger, S. J., Peterson, J. T., Freeman, M. C., Freeman, B. J., & Homans, D. D. (2008). Stream fish occurrence in response to impervious cover, historic land use, and hydrogeomorphic factors. *Canadian Journal of Fisheries and Aquatic Sciences*, *65*(7), 1250-1264.

West Virginia Department of Natural Resources. (2018). List of Rare, Threatened, and Endangered Species. URL http://www.wvdnr.gov/wildlife/endangered.shtm.

West Virginia Department of Environmental Protection. (2015). Technical Applications and GIS Unit, GIS Data Server. URL https://tagis.dep.wv.gov.

Wheeler, B. A., Prosen, E., Mathis, A., & Wilkinson, R. F. (2003). Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biological Conservation*, 109(1), 151-156.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Sepulveda, A. J., Shepard, B. B., Jane, S. F., ... & Schwartz, M. K. (2016). Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char *Salvelinus fontinalis*. *Biological Conservation*, *194*, 209-216.

Williams, K. E., Huyvaert, K. P., & Piaggio, A. J. (2017). Clearing muddied waters: Capture of environmental DNA from turbid waters. *PloS one*, *12*(7), e0179282.

Williams, R.D., Gates, J.E., Hocutt, C.H. & Taylor, G.J. (1981) The hellbender: a nongame species in need of management. *Wildlife Society Bulletin*, 9, 94–100.

Wolman, M. G. (1954). A method of sampling coarse river-bed material. *EOS, Transactions American Geophysical Union*, *35*(6), 951-956.

Wu, J., Stewart, T. W., Thompson, J. R., Kolka, R. K., & Franz, K. J. (2015). Watershed features and stream water quality: Gaining insight through path analysis in a Midwest urban landscape, USA. *Landscape and Urban Planning*, 143, 219-229.

APPENDIX A: IACUC APPROVAL LETTER

MARSHALL UNIVERSITY®
www.marshall.edu

Animal Resource Facility

DATE:	November 8, 2016
TO:	Jayme Waldron, PhD
FROM:	Marshall University IACUC
IACUC #:	
PROJECT TITLE:	[957095-2] Evaluating the use of environmental DNA (eDNA) as a supplemental method of detecting Eastern Hellbenders (Cryptobranchus a. alleghaniensis)
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE: 2016	November 8,
EXPIRATION DATE:	June 1, 2018
REVIEW TYPE:	Full and Designated Member Review

Thank you for your submission of Revised materials materials for this research project. The Marshall University IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

This submission has received Full and Designated Member Review.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

This project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Monica Valentovic at (304) 696-7332 or valentov@marshall.edu. Please include your project title and reference number in all correspondence with this committee.

Monica A. Valentovic, Ph.D.
Chairperson, IACUC