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

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

2.4 | Laboratory methods

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

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

CRALQ-F (5′ GTTTGCATGAGTATTRCGGATT 3′),

CRALQ-R (5′ TCGCTATRCATTATACAGCAGATACA 3′)

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

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

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

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

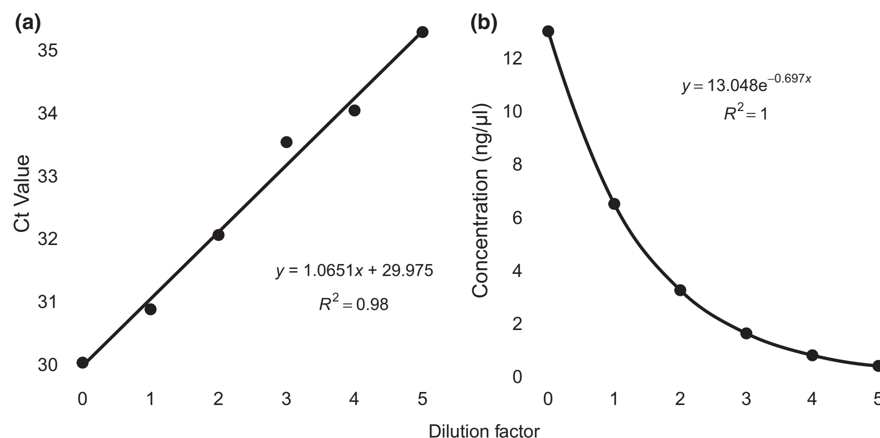

FIGURE 2 Cycle threshold (C_t) values from field samples versus dilution factor of positive controls (a), and environmental DNA concentration versus dilution factor of positive controls (b) used to estimate environmental DNA field sample concentrations

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

2.5 | Predictor variables

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

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

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

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

2.6 | Sampling covariates

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

2.7 | Data analyses

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