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Searching for a Salamander: Distribution and Habitat of the Mudpuppy (Necturus maculosus) in Southeast Ohio Using eDNA as a Rapid Assessment Technique

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ABSTRACT.—Habitat destruction and anthropogenic drivers have led to amphibian population declines worldwide, but the conservation status of many species remains in question. This study reports on the distribution of Mudpuppies, *Necturus maculosus,* in southeast Ohio, where widespread acid mine drainage and other forms of habitat destruction have led to severe declines and extinction in many waterways. Within the last century, however, the region has reforested, and damage to some streams has been mitigated, providing opportunities for Mudpuppy recolonization and population recovery. However, being a relatively secretive species, Mudpuppies require difficult and time intensive field surveys to detect. Therefore, the current distribution and conservation status of Mudpuppies in southeast Ohio is unclear. As a first step in documenting the current distribution and abundance of Mudpuppies in southeast Ohio, we conducted a rapid species assessment using environmental DNA (eDNA) surveys (September–November 2016) at 10 stream sites. We detected Mudpuppies at six of 10 streams using eDNA, including four streams in which they were known to occur from historical records and two streams from which Mudpuppies had not been previously reported. We also collected habitat data at each site, including concentrations of heavy metals and nutrients, physical stream habitat, conductivity, pH, temperature, total dissolved solids, and oxygen levels. Using logistic regression, we found composite Qualitative Habitat Evaluation Index (QHEI) scores were the best predictor of Mudpuppy presence. Our results suggest Mudpuppy eDNA is not easily detected when they are at low density, and animals may need to be within approximately 182 m of sampling points to be detected.

INTRODUCTION

Globally, even in protected areas, amphibian populations have declined over the past 25 y, with over one-third of amphibian species having undergone rapid decline or extinction, and 41% of species currently threatened (International Union for Conservation of Nature, 2017).

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Species associated with stream and freshwater habitats are especially imperiled due to habitat loss and degradation (Blaustein *et al.*, 2011). Habitat destruction in aquatic ecosystems results in altered thermal regimes, flow patterns, biochemistry, and community composition, and can jeopardize native species (Collen *et al.*, 2014). Declines of native species are often difficult to track without long-term population monitoring and studies of habitat use (Wheeler *et al.*, 2003).

In southeastern Ohio some amphibian communities in freshwater systems have suffered from widespread habitat destruction, and contamination of underground aquifers has occurred through land use change, natural resource extraction, and organic enrichment (Ohio Environmental Protection Agency (EPA), 1994). Specifically, a long history of coal mining in the region has contaminated ground and surface waters by lowering pH, increasing heavy metal and dissolved solid loads, and elevating rates of conductivity (Ohio EPA, 2012b). Other land use changes, such as deforestation, construction of roadways/urban areas, and an increase in timber harvesting, have resulted in a reduction of biological health scores in many streams (Ohio EPA, 2012a). On the other hand, the region has largely reforested within the last century, and acid mine drainage problems have been mitigated in some streams. Therefore, it is possible the amphibian communities in southeast Ohio have rebounded in recent decades.

Here, we report on field survey results for an aquatic salamander, the Mudpuppy, *Necturus maculosus*, in southeastern Ohio. Mudpuppies are fully aquatic and present in a variety of habitats, including lakes, rivers, streams, and creeks. As nocturnal feeders, they seek refuge under rocks or logs and plant debris during the day (Matson, 2013). They commonly occupy shallow waters with low temperatures from autumn (when most breeding occurs in Ohio) to early spring (Craig *et al.*, 2015). Females lay fertilized eggs under rock slabs and other large cover objects during the spring and early summer (April–June) and brood the nests until the young hatch (Matson, 2013).

Historically, Mudpuppies were common across the midwestern United States, with a range spanning from Wisconsin to Vermont (Matson, 2013). Occurrence data suggest anthropogenic drivers, such as habitat destruction and altered water chemistry, have led to range contractions and the reduction of population sizes (Matson, 1998; Lannoo, 2005). The Ohio Department of Natural Resources (2007) ranked Mudpuppies 14th on a list of 39 Ohio amphibians of conservation concern and considered the species to be in decline due to habitat loss. However, a lack of recent data renders their distributional limits and population status in Ohio unclear. Data are limited in part because Mudpuppies are secretive and can be difficult to detect. Even when a combination of field survey methods is employed, low capture rates can be a problem (Murphy *et al.*, 2016). Therefore, we sought to develop a complimentary rapid assessment technique for Mudpuppies using environmental DNA (eDNA).

Many studies that have compared eDNA with other field techniques have found eDNA sampling tends to match or exceed detection rates of other methods (Jerde *et al.*, 2011; Dejean *et al.*, 2012; Thomsen *et al.*, 2012; Mächler *et al.,* 2014; Spear *et al.*, 2015; Hobbs *et al.*, 2019). Despite an overall high sensitivity, there are several factors that influence eDNA detection, including (but not limited to) organismal shedding rates (Klymus *et al.*, 2015), flow rate, distance from organisms (Wilcox *et al.*, 2016), pH, and temperature (Strickler *et al.*, 2015). Therefore, detection rates will be dependent both on species and aquatic system, and eDNA efficacy assessments need to be conducted for each system independently. However, due to the consistent ability of eDNA analysis to detect species of interest, even at low DNA concentrations, it has been increasingly used as a tool to locate habitats of rare or elusive freshwater species, or to determine if species are still present within a historic range (Rees *et al.,* 2014).

The objectives of this study were to develop an eDNA rapid assessment protocol for Mudpuppies and test for the presence or absence of Mudpuppies at 10 sites in southeast

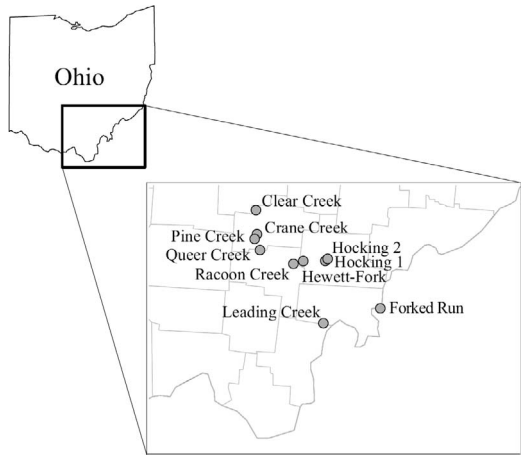

FIG. 1.—Environmental DNA sampling sites denoted by grey dots for Mudpuppies in 10 streams throughout southeastern Ohio

Ohio. Between 1952 and 1989, Mudpuppies were found in eight of the 10 streams we sampled, but sampling efforts in those locations have not been conducted since that time period (Matson, 2013). To quantify habitat, we conducted physical and chemical surveys at each locality, and we used our survey results to test for correlations between habitat characteristics and Mudpuppy presence/absence.

MATERIALS AND METHODS

SITE SELECTION

Our study was conducted in the Western Allegheny Plateau Ecoregion of Ohio, a mixed matrix of bottomland hardwood forests with patches of agricultural terrain, located in the southeast portion of the state (Armitage and Lipps, 2013). We selected 10 study sites in southeast Ohio based on historic museum records (Ohio EPA, 1994; Matson, 2013) and personal communications (A. Marietta, pers. comm. May 2016). Our sampling locations included two sites in the Hocking River in Athens County; Clear Creek, Pine Creek, and Crane Creek in Hocking County; Hewett -Fork, East Fork of Queer Creek, and Raccoon Creek in Vinton County; and Leading Creek and Forked Run in Meigs County (Fig. 1). Museum records show Mudpuppies were historically present at eight of these sites. There are no museum records of Mudpuppy presence in Crane Creek and Pine Creek, but communications with the Crane Hollow preserve manager suggested they may be present. Details on each study site, including summaries of each site's ecological history, and GPS coordinates for sample sites can be found in Collins (2017).

WATER AND HABITAT QUALITY SURVEYS

We used the Qualitative Habitat Evaluation Index (QHEI; Ohio EPA, 2006) to quantify the health of macrohabitats in lotic waters. The QHEI quantifies the quality of substrate, instream cover, channel morphology, riparian zone, riffles, and map gradients, with a maximum total score of 100. We used the QHEI to score the habitat of each study location, except Crane Creek

(due to its small size) and the East Fork of Queer Creek (due to its intermittent flow). We evaluated Crane Creek and the East Fork of Queer Creek using the Primary Headwaters Habitat Index (PHWHI), which is analogous to the QHEI, but adapted for headwater streams (Ohio EPA, 2009). We evaluated 700 m of stream stretch in larger streams and 300 m in smaller streams.

During the summer and fall of 2016, we collected water quality samples from stream sites in sterile 250 mL bottles and transported these on ice the same day to Ohio University's Biochemistry Research Laboratory. We tested water samples for concentrations of copper, lead, and iron, which are contaminants associated with acid mine drainage. We measured nitrogen and phosphorous levels on site using a handheld DR900 colorimeter (Hach, Loveland CO). The EPA (2014) found the median concentration of nitrate levels in warm water streams should be near 1.0 mg/L. The EPA has not established phosphorus limits for biological health, but research to determine the threshold has been initiated.

We measured conductivity, pH, total dissolved solids, oxygen reduction potential (ORP), and temperature from May–November 2016 using a Myron L Ultrameter II 6P (Myron L Company, Carlsbad CA). We averaged Myron data to establish a mean score for field parameters at each site.

ENVIRONMENTAL DNA

To collect eDNA, we followed the United States Geological Service (USGS) environmental DNA collection protocol 1 (Laramie *et al.*, 2015). We filtered water using a hand vacuum pump with a 1 L flask, 250 mL disposable cup, and 47 mm cellulose nitrate (0.45 microliter pore diameter) filter membrane (Thermo Fisher Scientific, Waltham, MA) attached to the top of the flask using a plastic hose. We submerged the filter cup directly into the stream at the streamside, or from an access point in the middle of the stream if available. After filtration we extracted filters from the cup using forceps sterilized with a 50:50 bleach:water solution and placed the filters in a 2 mL sterile tube filled with 95% ethanol for preservation, changing gloves after each sample. At each sample site, we collected three 1 L stream samples and one filter blank. Blank samples consisted of distilled water filtered in the field as a control to detect cross-contamination. We stored sample tubes at –80 C until processing. For most streams we collected eDNA samples from four sample sites. Exceptions included Crane Creek, where we collected 20 samples (every 182 m), with one blank every five samples, and Pine Creek, where we collected 20 samples every upstream and downstream from a known nest site (10 upstream, 10 downstream, 5 blanks; samples collected every 182 m). In total our sampling design resulted in 164 samples from 10 streams. We recorded coordinates for each sampling site using an eTrex GPS unit (Garmin Ltd. Lenexa, KS, U.S.A.).

EDNA DEVELOPMENT AND LAB PROTOCOL

We tore filters containing eDNA in half using sterile forceps, with one half of each filter stored in a 2 mL tube in ethanol as a backup. We extracted eDNA from filters using the protocol described in Goldberg *et al.* (2011), modified to repeat the DNA isolation step twice to increase potential DNA yields. We designed a qPCR primer and probe set using mitochondrial cytochrome *b* (cyt-*b*) sequence data from *N. maculosus* using RealTimeDesign software (LGC Biosearch Technologies, Petaluma, CA.). Our primer/probe combination amplified a 149-base pair region (Forward: 5′AGCAACAGCCTTTGTAGGGTA 3′; Reverse: 5′ TCGCCTT ATCGACGGAGAATC 3′; Probe: 5′ TXR-CGTACTACCATGAGGCCAAATATCCTTC-BHQ2). We tested our primer and probe combination *in silico* in GenBank and *in vitro* using tail tissue samples from *N. maculosus* from Pine Creek in Ohio and from samples in the New and Little

Tennessee River drainages in North Carolina. We also tested for *in vitro* amplification from tissues of several other salamander species sympatric with *N. maculosus*, including *Eurycea bislineata, E. wilderae, Gyrinophilus porphyriticus, Pseudotriton ruber, Desmognathus quadramaculatus, D. monticola, D. ocoee,* and *Cryptobranchus alleganiensis*. The taxa we tested include many of the salamander species endemic to North America that use the same microhabitats as *Necturus*, and most were more closely related to *Necturus* than other North America salamander species (Zhang and Wake, 2009; Pyron and Wiens, 2011).

Our qPCR reactions had a total volume of 15 μL and included 7.5 μL IDT qPCR master mix (Integrated DNA Technologies, Coralville Iowa), 2.85 μL of distilled H_2O, 0.75 μL of primer/probe mix at a concentration of 8 μM for primers and 4 μM for probe, 0.6 μL of TaqMan Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems), 0.3 μL of TaqMan Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems), and 3 μL of sample extract. We used a Nanodrop spectrometer to quantify a 40 ng/μL DNA tail clip sample and diluted the sample at five concentrations from 10^{-1} to 10^{-5}.

We ran all samples in triplicate on a Bio-Rad CFX qPCR machine (Bio-Rad, Hercules CA). Each plate included a series of standards and negative template controls consisting of DNase/RNase free distilled water. We used Exogenous Internal Positive Control to detect PCR inhibition or failure. Our qPCR cycling protocol followed Spear *et al.* (2015). We created a standard curve of DNA detection and analyzed samples with Bio-Rad CFX software. We considered a sample positive if the exponential amplification curve exceeded the C_t threshold. We set the C_t threshold at the fluorescence value that crossed the amplification curve of all standard curve dilutions and stayed above the baseline readings of negative samples. At each sampling locality, if two out of three wells amplified for a filtration, we scored the filtration as positive. If only one of three wells amplified, we scored this as ambiguous and omitted the sample from the analyses, as Goldberg *et al.* (2011) suggest single positives should be interpreted with caution. If no wells amplified, we considered this a negative result.

ENVIRONMENTAL CORRELATES

We ran mixed effects models in R v3.3.2 (R Core Team) using the package lme4 (Bates *et al.*, 2013) to test if water quality characteristics predicted DNA presence/absence while accounting for nonindependence due to multiple filtration locations along each stream. In our analyses we included the water quality variables conductivity, maximum water temperature, QHEI substrate score, QHEI instream cover score, and QHEI riparian zone score. These specific QHEI categories were selected based on the importance of instream cover and substrate in maintaining Mudpuppy habitat and the documented negative impact of poor riparian quality and sedimentation on reproduction (Lannoo, 2005; Mattson, 2013). We excluded several variables from our analyses: total dissolved solids, given its known correlation with conductivity (Thirumalini and Kurian, 2009); nitrogen, phosphorous, and heavy metals, because none of these crossed toxicity thresholds; and ORP, because it is highly correlated with pH (Hargrave, 1972). Using stream site as a random effect, we tested all variables in separate analyses given our low sample size ($n = 10$) and compared analyses using an Akaike information criterion corrected for small sample sizes (AICc; Burnham and Anderson, 2002).

RESULTS

WATER AND HABITAT QUALITY SURVEYS

Clear Creek received an "excellent" QHEI score (≥ 70), and sample sites within the unaltered channel of the Hocking River were ranked as "fair" (43–54). All other streams fell into the

TABLE 1.—Average and range for field readings of pH, conductivity (μs/cm), total dissolved solids (ppm), oxygen reduction potential (mV), and water temperature (C) taken at each field site during summer and fall 2016

Site	pH	Conductivity	TDS	ORP	Temp
Crane Creek	6.7	102.5	102.5	77.3	19.4
	(6.4–7.6)	(78.9–144.3)	(55.6–102.5)	(73.0–131.0)	(15.1–23.1)
Clear Creek	8.1	403.6	304.2	155.8	19.5
	(7.8–8.5)	(328.7–492.3)	(234.7–400.0)	(100.0–199.0)	(15.4–22.8)
Forked Run	7.8	138.3	98.0	111.1	27.8
	(7.2–8.4)	(104.0–164.3)	(67.5–122.0)	(90.0–169.0)	(25.5–30.2)
Hocking 1	8.1	961.7	658.5	111.4	28.4
	(7.7–8.8)	(698.0–1289.0)	(400.3–900.6)	(56.0–158.0)	(26.0–32.1)
Hocking 2	8.1	628.6	417.2	139.9	26.6
	(8.0–8.2)	(506.7–726.1)	(276.8–496.6)	(89.0–202.0)	(25.6–27.3)
Queer Creek	7.4	119.9	72.6	177.3	19.4
	(7.0–8.1)	(96.4–107.5)	(54.6–97.0)	(78.0–259.0)	(15.2–25.1)
Pine Creek	7.3	258.0	182.8	138.2	21.5
	(7.1–7.6)	(179.2–356.7)	(117.9–252.4)	(86.0–215.0)	(19.5–25.3)
Raccoon Creek	7.1	547.9	244.4	161.0	24.9
	(6.7–7.3)	(401.0–701.0)	(198.6–302.5)	(81.0–230.0)	(22.1–27.2)
Hewett-Fork	6.8	713.0	375.4	44.6	22.1
	(6.1–7.1)	(344.0–1122.0)	(201.0–588.7)	(4.0–101.0)	(20.6–24.6)
Leading Creek	7.6	1146.4	822.8	158.7	23.0
	(7.4–7.8)	(567.0–2557.0)	(230.0–1889.0)	(92.0–220)	(20.7–24.9)

"good" (55–69) category. A breakdown of QHEI scores by category can be found in Collins (2017). No stream had any heavy metal contaminant levels above established toxicity thresholds (Collins, 2017); however, four sites exceeded the healthy nitrate limit: both Hocking River sites, Forked-Run, and Pine Creek. Pine Creek nitrate levels were elevated at all sites downstream from farmland (Collins, 2017). Our highest phosphorous readings were from the Hocking River sites.

The pH values of all streams, including streams with a history of acid mine drainage impairment, were within neutral limits (Table 1). Crane Creek and Queer Creek averaged the lowest conductivity scores and had the lowest total dissolved solid scores. Conductivity and total dissolved solids are known to be correlated, with high conductivity readings often caused by pollutants (Thirumalini and Kurian, 2009). Leading Creek and Hocking Site 1 had elevated total dissolved solids and the highest conductivity readings, with Leading Creek's average conductivity 1116.4 μS/cm. Hocking Site 1 had an average conductivity of 961.7 μS/cm. No site exceeded heavy metal toxicity limits for iron (64 μg/L) (Besser and Leib, 2007), lead (65 μg/L) (EPA, 2016), or copper (1,000 μg/L) (EPA, 2016). All sites with eDNA present had mean water temperatures below 27C.

ENVIRONMENTAL DNA

Our *in-silico* tests with our selected primer/probe matched all *Necturus* sequences, indicating our protocol is genus specific for *Necturus*. Although DNA amplification specificity is on the genus level, *Necturus maculosus* is the only species of *Necturus* in our study area. No species outside of the genus *Necturus* matched our primers and probe or amplified during testing. In running qPCR reactions for our unknown samples, none of our negative controls

TABLE 2.—Average DNA quantity in ng/μL from eDNA positive surveys sites. Averages exclude ambiguous amplifications. We did not detect Mudpuppy eDNA at Hocking site 1, Queer Creek, Forked-Run, and Raccoon Creek, even though museum records indicate all selected sites sustained Mudpuppy populations in the past

Stream name	Historic record of presence	Number of sample sites	Positive amplifications	DNA ng/μL
Crane Creek	No	16	8	0.0977
Pine Creek	No	20	11	0.089
Hocking 2	Yes	4	2	0.00416
Leading Creek	Yes	4	2	0.00238
Clear Creek	Yes	4	3	0.000491
Hewett Fork	Yes	4	3	0.000106

amplified. All qPCR runs were within 100–120 in reaction efficiency and had a standard curve with an $r^2 \geq 0.90$.

ENVIRONMENTAL DNA AND HABITAT CORRELATES

We detected the presence of Mudpuppy eDNA in six of the 10 streams surveyed. The amount of DNA recovered varied widely among streams (0.000106 ng/μL — 0.0977 ng/μL) (Table 2). Four of the six sites with Mudpuppy eDNA present historically supported Mudpuppy populations (Matson, 2013), including the Hocking River, Leading Creek, Clear Creek, and Hewett-Fork. The remaining two sites in which we detected Mudpuppy eDNA, Crane Creek and Pine Creek, lacked museum records. We found eDNA was spatially variable, with eDNA positive sites only 182 m from areas with no eDNA detected in Crane Creek (Fig. 2). In our mixed effects models with filtration site number as a random effect and DNA presence per site as the response variable, the model using riparian zone score had the lowest AICc score (Table 3). Categories for scoring riparian zone included the extent of bank erosion (little, moderate, severe), riparian width (>5 m to <50 m), and flood plain quality (predominant land use of the flood plain: farming, conservation, urban, field, mining, or pasture) (OHIO EPA, 2006).

DISCUSSION

Prior to our work, Mudpuppies had not been documented in Crane Creek, which has been surveyed for amphibians on multiple occasions (H. Stehle, Pers.Comm., May 2016). In

TABLE 3.—Results of the mixed effects model used to test if QHEI water quality characteristics correlate with Mudpuppy presence/absence, based on AICc scores and AICc weight for each model after comparison with a null model. Riparian zone score was the best predictor of presence

Model	AICc	AICw	r^2
Riparian Zone Score	30.5	0.35	0.84
Instream Cover Score	32.7	0.23	0.79
Maximum Temp	33.5	0.19	0.75
Substrate Score	34.3	0.14	0.64
Conductivity	35.1	0.05	0.40
Rip x Cover x Temp x Sub x Con	37.1	0.04	0.24

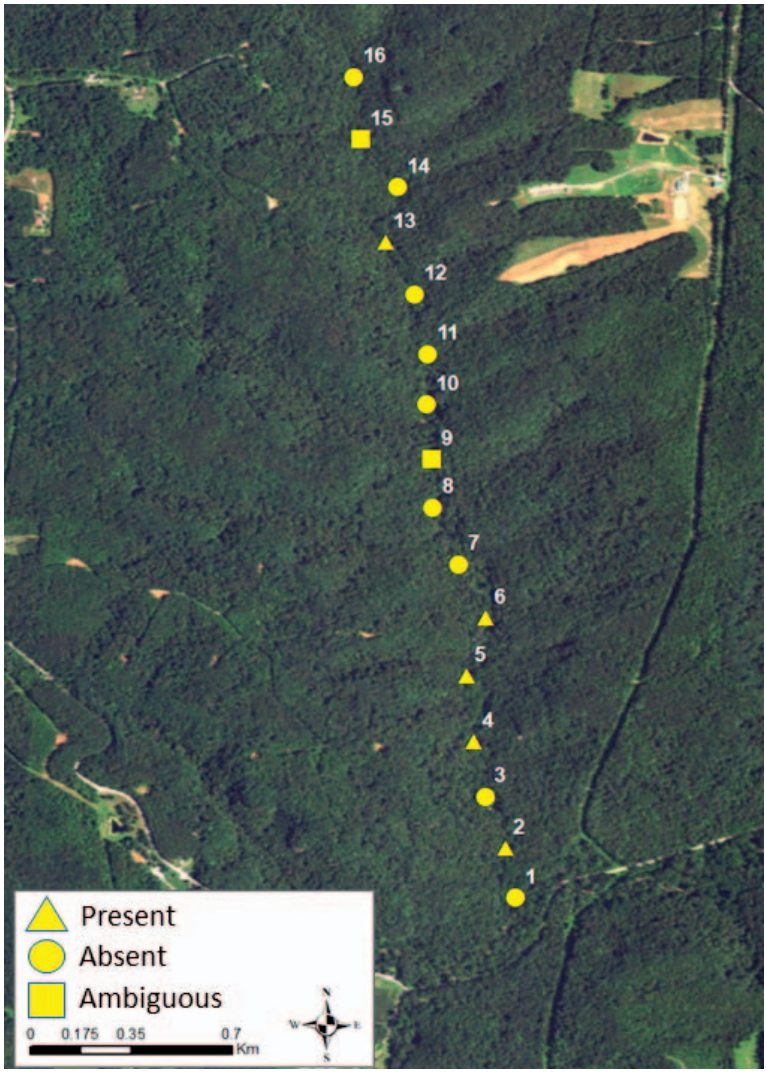

FIG. 2.—Environmental DNA survey sites in Crane Creek, Crane Hollow, Hocking County, Ohio. There are no previous records of Mudpuppies occupying Crane Creek. Triangles indicate presence of Mudpuppy eDNA, circles indicate absence of eDNA, and squares indicate ambiguous qPCR results

addition we reaffirmed the presence of Mudpuppies at five sites in which they were known from historical records: Pine Creek, Hocking River site 2 (unaltered channel), Clear Creek, Leading Creek, and Hewett-Fork. On the other hand, our eDNA surveys failed to detect Mudpuppies at four historical sites: Raccoon Creek, Forked-Run, Hocking River site 1 (altered channel area), and the East Fork of Queer Creek. As persistence of eDNA in lotic systems is dependent on the organism, flow regime, water temperature, and other factors (Pilliod *et al.*, 2013; Strickler *et al.*, 2015), it is possible we failed to detect eDNA from Mudpuppies at some sites. Our eDNA sampling was conducted during the autumn, which is

the primary mating season for Mudpuppies in Ohio (Matson, 1998). We chose to sample in the autumn given Spear *et al.* (2015) found an increase in eDNA during the fall breeding season in the Hellbender, *Crypotobranchus alleganiensis*. A study of *N. alabamensis* found increased eDNA detection in late winter as compared to summer, but autumn samples were not collected (de Souza *et al.,* 2016). A study on the optimal eDNA sampling time for Mudpuppies would be beneficial.

In several of the streams we examined, eDNA detection was spatially variable. For example, in Crane Creek, one site tested positive for DNA, whereas another site 182 m upstream did not. Additionally, in Pine Creek, where we surveyed upstream and downstream from a nesting site discovered during field work in May 2016 (Collins, 2017), our autumn sampling did not obtain positive results for eDNA at the nest site location, but we did detect Mudpuppy eDNA 182 m downstream from the nest site. More work on detection limits is needed, but our study suggests using eDNA to monitor for Mudpuppies may require dense sampling, within approximately 182 m or less from individuals. Other eDNA studies have also reported variable eDNA detection rates in lotic systems (Deiner and Altermatt, 2014). For instance one study found stream salamanders were best detected when an individual was within 5 m of a sampling point, and salamander DNA was not detectable 1 h after the animal was removed (Pilliod *et al.*, 2013). In another study, field surveys for the Hellbender (*C. alleganiensis*) located nine individuals at 23 sample sites, but Hellbender eDNA was found at 21 of 23 sites, including six new sites (Spear *et al.*, 2015). This result suggests environmental DNA, while not a replacement for field surveys, can in some situations function as a rapid assessment tool for evaluating the presence/absence of target species. This can help to define field survey areas and increase the probability of capture in other more detailed ecological studies.

Estimates of heavy metal concentrations in our study did not exceed the known limits for biological health (EPA, 2016). Even streams with acid mine drainage pollutants historically were not associated with elevated levels of any metals, which is likely a result of successful reclamation efforts by the Ohio EPA and local nonprofit partners. Caution is warranted, however, as single samples are not likely to detect pulses of contamination, which may be detrimental to Mudpuppies and stream health in general; for example, heavy rainfall can impact metal concentrations in lotic systems (Sarmiento *et al.*, 2009). It is best if water samples are collected year-round throughout the length of a stream system, including associated tributary streams, and analyzed for contaminant concentrations. In streams in which Mudpuppies were not detected, yet have good habitat and acceptable water quality, other factors, such as natural barriers to dispersal or limited dispersal ability, may prevent Mudpuppies from recolonizing available habitats. Alternatively, episodic pollution may also impact population persistence following successful colonization.

Nitrogen levels in both Hocking River locations as well as Forked-Run and areas of Pine Creek were higher than the EPA (1997) threshold (1.0 mg/L) for ecological integrity. Phosphorous levels were elevated at both Hocking River sites, although a toxic level of phosphorous has yet to be determined by the EPA (2016). Despite elevated nutrient levels, we found no correlation between nitrogen concentrations and Mudpuppy presence/absence. Although pH in all streams was neutral, conductivity was elevated in sites with a history of coal mining, especially Leading Creek, which had the highest average conductivity (Table 1). Heightened ion concentrations in waters impacted by acid mine drainage are common and can affect the presence and health of aquatic biota (Armstead *et al.*, 2016).

In this study we found high QHEI scores, specifically riparian zone scores, were associated with Mudpuppy eDNA presence. No site that tested positive for Mudpuppy eDNA scored below seven in our riparian zone assessment (Collins, 2017). Choosing streams with high

QHEI scores could assist in locating populations in future studies. While we only surveyed 10 streams, it is not surprising that Mudpuppy presence was associated with elevated habitat scores. Among other advantages, healthy riparian habitat limits erosion and stream sedimentation due to root systems stabilizing streambanks (Naiman and Decamps, 1997; Anbumozhi *et al.*, 2005), which is important for Mudpuppy survival given sedimentation can suffocate juveniles and eggs, as well as clog important nesting habitat (Casper, 1998; Minton, 1998). Riparian habitat also buffers water temperatures by limiting sunlight through canopy cover. The critical thermal maximum for Mudpuppies is 30° C (Hutchison and Hill, 1976), and the Forked-Run and Hocking River site 1 exhibited temperatures $>$ 30° C at several sample points at which QHEI riparian zone scores were also low.

It is important to continue to compile data on the distribution, habitat, and population status of Mudpuppies to evaluate their conservation status. In addition studies of the underlying causes of population decline and recovery are needed, including juvenile recruitment and population growth rates, the impact of pulses of pollution on population persistence, and the role of connectivity in population persistence and metapopulation dynamics. This study pioneers an effective framework for establishing the presence/absence of Mudpuppies through rapid assessment, which can be used in other regions to update population status and locate areas for further ecological work.

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