The Spatial and Temporal Variation of eDNA in Mudpuppy (*Necturus maculosus*) Detection in Alum Creek, Ohio.

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Abstract

Nearly 90% of Ohio's aquatic species are reliant on streams, which face many threats that contribute to habitat loss. The common mudpuppy (*Necturus maculosus*) is a native Ohio salamander that inhabits rivers and streams. The conservation status of mudpuppies is currently unknown. Due to widespread riparian habitat loss and alteration, as well as modification of river flows, there is potential for mudpuppy populations to be extirpated from waterways where they historically occurred. To assess the current distribution of mudpuppies, a reliable surveillance method is needed. One such method is environmental DNA (eDNA), which allows for the detection of organisms without seeing, hearing, or capturing the organism itself. eDNA is DNA that is extracted from any type of environmental sample, such as soil, water, or air, that is produced from shedding, excreting, or decaying. eDNA is becoming more commonly used for studying and monitoring aquatic species, such as amphibians, fish, and mammals. Many factors, such as organism mass, sun exposure, temperature, distance to the organism, and seasonal activity can impact detection probabilities. In order to accurately quantify mudpuppy distributions using eDNA, factors that influence detection seasonally and in relation to potential mudpuppy habitat must be understood. Through a greater understanding of these variables, eDNA methods can become more reliable and efficient, allowing for data to be gathered on mudpuppy populations faster than traditional methods. In this study, I assess temporal and spatial aspects of mudpuppy detection when using eDNA in relation to environmental factors, hydrological factors, and habitat suitability. Results from this work will help refine protocols for future statewide surveillance of mudpuppies.

Introduction

Biodiversity loss is occurring on a global scale in all types of ecosystems, including freshwater systems. Over the past 50 years, freshwater habitats have declined by 30% and freshwater species have declined by 83% (Climate Adaptation Science Centers, 2020). This decrease in freshwater species is occurring at a faster rate than that of terrestrial or marine species (Darwall, n.d.). Freshwater ecosystems face habitat loss from the creation of dams, drainage for development, water pollution, and increasing occurrences of floods and droughts due to climate change ("Freshwater Threats," n.d.). Approximately 33% of freshwater fish are at risk of extinction ("One-third of freshwater fish…," 2021). Of the mussels found in the eastern and midwestern United States, 70% have experienced population declines ("Lawsuit Launched…," 2018).

Aquatic species found in rivers and streams face various threats to their survival and longevity. Among the greatest threats to riverine habitats are alteration of stream flows, siltation, and increased temperatures (Titchenell and Lehnen, 2009). These changes arise from the damming of rivers and loss of natural vegetation surrounding rivers. For fully aquatic amphibian species, such as mudpuppies (*Necturus maculosus*) and hellbenders (*Cryptobranchus alleganiensis*), the increase in silt could impact ability to shelter in stream bed substrate and impact nesting success. Mudpuppies are found throughout southern central Canada and the midwestern United States, as far east as North Carolina and as far south as Georgia and Mississippi (Matson, n.d.). In addition to being within the 90% of Ohio aquatic species that rely on streams, they also inhabit lakes, ponds, and rivers ("Aquatic Habitat Stewardship," n.d.).

Mudpuppies are often found under rocks and logs when not actively looking for prey at night (Matson, n.d.). Hellbenders are endangered in the state of Ohio, so while the status of mudpuppies is unknown, the similarities between these species may suggest a vulnerable mudpuppy population overall (Phillips & Humphries, n.d.). Because of the hidden and underwater nature of aquatic species, they are inherently more difficult to study in comparison to terrestrial species. Physical means of studying these species often involve trapping, seining, and/or wading through waterways to lift rocks and disrupt habitat. Thus, there is a need for greater incorporation of study methods that minimize these time-intensive activities to study these aquatic species more efficiently and reliably.

To gain a better understanding of mudpuppy distribution within Ohio, a statewide survey of rivers and streams using eDNA has been proposed. eDNA uses the genetic signatures of different organisms to determine their presence or absence and eliminates the need to trap animals to know if they are at a given site. After an animal sheds, excretes, or decays, its DNA can remain in the environment. Many eDNA researchers are currently focusing their efforts on designing methods and creating guidelines for eDNA use through a better understanding of possible applications of DNA and the environmental characteristics that may hinder it (Seymour 2019). With a better understanding of when and where to sample eDNA, survey protocols can be optimized and allow for more accurate detections. The use of eDNA has the potential to reduce the costs of surveying mudpuppies by eliminating the need for trapping equipment (Yates et al. 2019). When comparing traditional sampling methods to eDNA methods, detection probability increased from 0.58 to 0.97, respectively, for amphibians.

Numerous studies have demonstrated the use and applicability of eDNA for amphibian and other aquatic species surveillance. eDNA techniques have been used in detecting invasive rusty crayfish, *Orconectes rusticus*, in Michigan and Wisconsin in both known and previously unknown habitats (Dougherty et al. 2016). In a stream-dwelling amphibian, the Idaho giant salamander, researchers found that eDNA production was positively related to salamander mass, but unrelated to salamander length (Pilliod et al. 2014). Hellbenders, the largest North American amphibian, are currently listed as endangered under the U.S. Endangered Species Act (Phillips and Humphries, n.d.). eDNA has been used to detect this species at 6 sites that were previously unknown to be occupied by hellbenders (Spear et al. 2015). Spring salamanders, northern two-lined salamanders, and northern dusky salamanders were the subject of a study in Quebec, Canada, that resulted in the detection of target species in 9 streams where the target species was not visually seen during searches (Plante et al. 2021). Together, these studies demonstrate the value of eDNA to detect aquatic species and discover new populations. eDNA has also been used to detect mudpuppies in streams, rivers, and creeks throughout the Western Allegheny Plateau Ecoregion in Ohio (Collins et al. 2019).

While eDNA has great potential for detecting species, there are numerous challenges and uncertainties to consider. For example, detection tends to decrease with increased distance to the source. In Ohio, Collins et al. (2019) found detection of mudpuppy eDNA was unlikely more than 182-meters downstream from where individuals were located, highlighting the importance for appropriate spatial collection of samples (Collins et al. 2019). In another study, Pilliod et al. (2014) used five caged giant salamanders to determine that detection was limited beyond 50 meters. Detection of eDNA can also be affected by exposure to UV light, which degrades eDNA

(Pilliod et al. 2014). Additionally, higher temperatures promote the denaturing of DNA, while also increasing the rates of enzyme kinetics and the metabolism of microbes (Barnes et al. 2014).

In addition to spatial and environmental factors, species' life history can also affect detectability when using eDNA. For instance, organism activity and life cycle patterns may alter the detection of eDNA. One study showed that the Black Warrior Waterdog, an organism also of the genus *Necturus*, has a higher eDNA detection probability during the cooler months of October-April, when it is more active (de Souza et al. 2016). In a study performed on hellbenders, their breeding season during September produced a significant temporal increase in eDNA detection. The September detection value was two to three orders of magnitude higher than values from any other time (Spear et al. 2015).

In order to better understand how eDNA can be used to study mudpuppy populations, I designed a study to determine the variation in detection, both spatially and temporally. Specifically, I collected eDNA samples from near the Alum Creek Dam over the course of one year to determine seasonal variations in detection. I also collected samples throughout a 1-kilometer stretch downstream of Alum Creek Dam to compare detections to variations in habitat suitability. The goal of my study was to refine protocols for collecting mudpuppy eDNA to maximize detection of mudpuppy eDNA in future statewide surveys. Temporally, I predicted that detection of mudpuppy eDNA would be greatest in winter due to colder temperatures and less direct sunlight. These environmental conditions may act as a preservative to the DNA in the water prior to sampling. Mudpuppies are active in the winter and are therefore more likely to generate eDNA. Spatially, I predicted that eDNA detection would occur in association with suitable mudpuppy habitat. Specifically, eDNA detections are expected downstream of stream reaches containing suitable habitat

Methods

Study Sites

From January 2021 through January 2022, water samples were collected from Alum Creek spillway below the Alum Creek Dam in Lewis Center, Ohio, where mudpuppies are known to occur. Collections generally occurred every 1-2 weeks, except for May 1 to July 13, during which time samples were not collected. Two sample points were selected, each approximately 375 meters from the start of the spillway below the dam. These points were located on either side of the foot bridge crossing the spillway (Figure 1). The area below the spillway has been extensively surveyed for mudpuppies, with >160 individuals being observed.

Figure 1. Map of Alum Creek Dam collection site. The green circles show where mudpuppies have been observed or trapped. The red stars show where temporal sampling took place.

On April 7, 2021, a series of 11 samples were taken every 100 meters along a one kilometer stretch of Alum Creek, about 1.5 km southeast of the Alum Creek Dam (Figure 2). Only 3 mudpuppies have been seen/trapped along this stretch in >500 trap nights, despite there being areas of good and excellent mudpuppy habitat. Habitat suitability was scored on a scale of 1–10. Heavily influential characteristics in assigning higher rankings include number of rocks, number of logs, and percent pool.

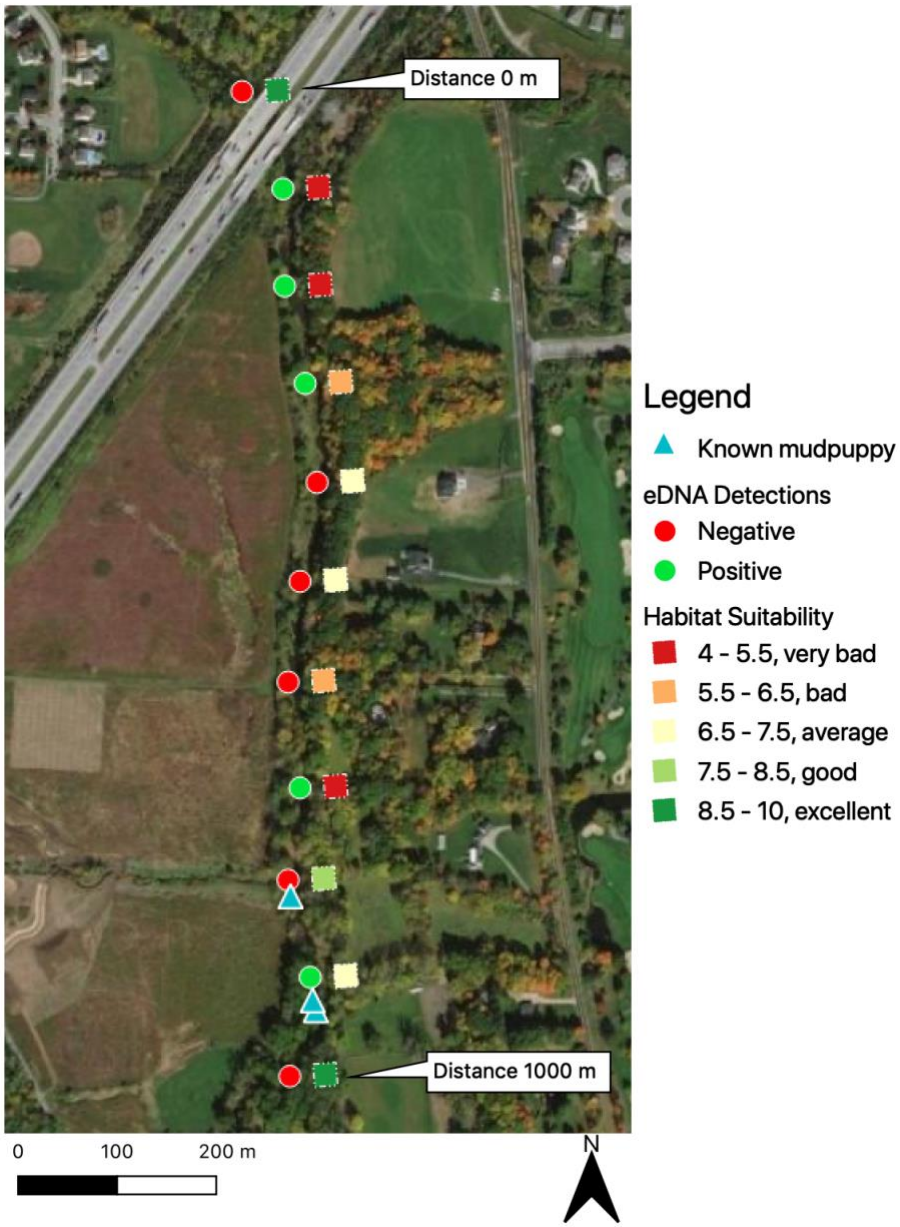

Figure 2. Map of 1- km reach of Alum Creek. "Distance 0" is approximately 1.5 km southeast of the Alum Creek Dam. Water flows from north to south. Each sample/habitat assessment occurred every 100 meters. Each 100-meter marking is ranked based on the average of the 100 meters of upstream habitat (except for distance 0, which is based on the 50 meters upstream).

Field Collection and Storage

Each sampling occurrence included the use of one one-liter field blank and the collection of two one-liter water samples from the water surface at each sampling site (Fig. 1). Water and weather conditions were recorded during each sampling period while in the field. In addition, gauge height and discharge records for the Alum Creek Dam were downloaded from the U.S. Geological Survey ("Alum Creek at Africa OH," 2022). Samples were transported back to Ohio State in a cooler in order to prevent eDNA degradation while keeping the samples dark and cool.

The water samples were vacuum filtered through a 45 µm paper filter via a vacuum flask. Each water sample required one or two paper filters depending on the turbidity of the water sample. The filters were placed in 1.5 ml centrifuge tubes and stored at -20°C until DNA was extracted. This same procedure was followed for the spatial samples.

DNA Extraction and PCR

From August 9–10, 2021, and January 3–5, 2022, DNA was extracted at the Wildlife Conservation Lab at The Wilds. In order to prepare the filters for extraction, each filter was cut in half using a scalpel; one half was be placed back in the tube and saved as a backup, while the other half was cut into fourths prior to extraction. I used Qiagen DNeasy Blood and Tissue Kits to extract DNA following protocols outlined by Goldberg et al. (2011).

Amplification Analysis

Following the DNA extraction, samples were placed in a ThermoFisher 7500 Real-Time PCR System to undergo a quantitative polymerase chain reaction (PCR) assay, consisting of denaturing, annealing, and extending. The cutoff number for replications was 40 replication cycles. Thirty-eight replications generally contain one copy of DNA, but due to the large amount of negative detections that we would go on to find, 40 was used to shape the threshold to match the scope of this project. There is no one-size-fits all threshold, so 40 was selected to give the best interpretation of the mudpuppy samples.

Samples were run in triplicate; those producing 0/3 positives were considered negative, those producing 1/3 were rerun, and those producing 2/3 or 3/3 were considered positive. Reruns were also performed in triplicate. After performing the reruns, all samples, including those only run once, were placed into final positive/negative categorizations based on normal distribution confidence intervals. The copy number, or the number of copies per reaction, was used to determine the intervals, and any sample producing a lower limit less than zero was considered a negative detection.

Results and Discussion

Temporal sampling

One temporal sample was collected at the near and far sample locations on each collection day (Fig.1). For data analysis, these samples were combined and treated as one, meaning that if only one of the two was positive, that entire day was considered to have an overall positive detection. Of 46 near and far samples, six were positive. Two of these occurred on 2/21, resulting in five overall positive detection days.

Field blanks from 3/15, 3/29, and 4/7 resulted in positive eDNA detections, indicating contamination. It is unknown as to when this contamination occurred. Field samples collected on 3/29 and 4/7 included positive detections, while field samples collected on 3/15 did not show any positive detections. The 4/7 blank was used for both spatial and temporal sampling, meaning that it had more opportunities to be exposed to DNA than any other field blank. The contamination of

the 3/15 sample likely occurred in the lab during DNA extraction, while the contamination of the 3/29 and 4/7 blanks could have occurred in either the field or the lab. Despite the evidence of contamination, all field samples collected on dates associated with contaminated blanks were included in the analyses of the results.

All positive detections occurred from January to April, with the final positive occurring in October (Table 1). This is consistent with the findings of de Souza et al. (2016), that showed the Black Warrior Waterdog, also of the genus *Necturus*, having a higher detection probability during their most active period from October-April. Factors that showed the greatest difference between positive and negative samples were gauge height and discharge (Table 2). Positive detections occurred when gauge height and discharge were lower. This suggests that positives are more likely when there is less water present to dilute the target DNA. This is further supported by all positives being detected during times of no precipitation (Table 1). Gauge height and discharge are the only two factors showing significant differences between the two detection categories when assessed with a two-sample t-test for unequal variances (Table 2).

Table 1. All temporal sample dates, weather conditions, and environmental and hydrological factors. Positive detections are highlighted.

Date	eDNA	Gauge Height (ft)	Discharge (cubic ft/sec)	Air Temp (°F)	Cloud Coverage (%)	Weather Conditions	pH	Conductivity (µs/sec)
1/15/21	Negative	1.66	35.28	37	100%	rain and snow	N/A	N/A
1/22/21	Negative	1.66	35.57	34	87%	sunny/cloudy	8.86	513
1/29/21	Positive	1.68	36.94	27	21%	mostly sunny	9.23	555
2/7/21	Negative	1.66	35.62	16	65%	sunny	8.95	471
2/21/21	Positive	1.52	21.98	28	70%	sunny/cloudy	8.54	536
3/1/21	Negative	2.49	416.41	37	30%	partly cloudy	8.62	598
3/5/21	Negative	3.29	598.10	28	0%	sunny	8.12	457
3/15/21	Negative*	1.44	16.61	37	100%	rainy/drizzle	8.68	559
3/22/21	Negative	2.97	450.24	70	65%	partly cloudy	8.71	540
3/29/21	Positive*	1.48	19.12	50	0%	sunny	9.13	547
4/7/21	Positive*	1.47	18.89	81	80%	cloudy	8.65	
4/14/21	Negative	1.47	18.50	52	90%	very cloudy, sun peeking through	8.25	
4/30/21	Negative	1.38	13.49	57	10%	few, scattered clouds	8.96	591
7/14/21	Negative	3.38	637.59	84	5%	sunny	7.81	537
8/8/21	Negative	1.40	15.91	81	10%	clear skies	8.15	516
8/31/21	Negative	1.34	13.90	73	100%	cloudy	7.82	495
9/12/21	Negative	1.14	5.70	82	0%	sunny	8.36	520
9/27/21	Negative	2.04	104.19	81	35%	partly cloudy	7.73	524
10/10/21	Positive	1.98	86.78	79	5%	sunny	7.72	496
11/7/21	Negative	2.33	184.58	57	0%	sunny	8.23	528

11/21/21	Negative	2.58	275.58	43	100%	raining	8.1	473
12/5/21	Negative	1.51	28.17	39	90%	cloudy	8.52	514
1/2/22	Negative	3.53	714.49	29	100%	cloudy	8.3	536

*Field blanks from these dates had positive eDNA detections.

Table 2. Temporal eDNA results and environmental and hydrological factors, presented as mean (standard deviation). P-values are reported from two-sample t-tests for unequal variance.

eDNA	Gauge Height (ft)	Discharge (cubic ft/sec)	Air Temp (F)	Cloud Coverage (%)	Conductivity (µs/sec)	pH
Negative	2.07 (0.79)	199.97 (249.42)	52.06 (21.79)	54.83 (42.23)	525.97 (35.66)	8.37 (0.37)
Positive	1.63 (0.21)	36.74 (28.95)	53.00 (26.32)	35.20 (37.32)	536.88 (28.36)	8.64 (0.50)
t-test P-value	0.045	0.014	0.944	0.346	0.539	0.308

The average cloud coverage for positive samples was lower than negative samples (Table 2), contradictory to the findings of Pilliod et al. (2014), which showed that sun exposure resulted in faster degradation than shade exposure. However, the sun/shade study showed that sun samples allowed for positive detections for 8 days, while shade samples allowed for positive detections for 11 days. This, in combination with my findings, may suggest that sun exposure is of less concern as long as samples are filtered quickly after collection.

Both pH and conductivity showed non-significant differences between the two groups (Table 2). These factors cannot be controlled for within the system of interest. Even if there had been a difference or a clear pattern in the pH or conductivity between positive and negatives samples, it would be nearly impossible to select sampling dates around desirable pH and conductivity levels.

There were no positive detections from August through September, despite low gauge height and discharge (Table 1). During this time, temperatures ranged from 73°F to 82°F. Although the average temperature did not differ between positive and negative samples, these late summer negatives may indicate that temperature could hinder detection even during desirable hydrological conditions.

Spatial sampling

Of 11 samples, five positive detections occurred along the 1-km reach at distances 100 m, 200 m, 300 m, 700 m, and 900 m (Fig. 2). Positive detections were located at habitat scores of very bad, bad, and average. Distance 0 m has an excellent habitat suitability, which may suggest that this location resulted in all or some of the positive detections at distances 100 m, 200 m, and 300 m as eDNA traveled downstream. However, this suggests that eDNA can be detected far outside of the 50 m and 182 m ranges proposed by Pilliod et al. (2014) and Collins et al. (2019), respectively. In addition, an alternative explanation for detecting mudpuppies in stream reaches with lower suitability scores is that my assessment of habitat does not accurately reflect what is preferred by mudpuppies.

The positive detection at distance 700 m is downstream of a series of bad to average habitats. The positive detection at distance 900 m is downstream of a very bad and good habitat. It is closely downstream of one known mudpuppy location and even more closely upstream of two known mudpuppy locations. There is no clear relationship between mudpuppy presence and what was considered suitable habitat for this study. These results further suggest that this study was limited by a disconnect between what is perceived as high-quality habitat and actual mudpuppy habitat selection. Basing higher ranked locations largely on the number of rocks, number of logs, and percent pool may have led to what are actually inaccurate habitat suitability rankings. There may be other factors that should carry a heavier weight when determining best habitats, or mudpuppies may not be as selective as previously assumed when choosing habitats. It is also possible that the habitat rankings were accurate, but that eDNA was being carried farther than anticipated by the current, resulting in positive detections far from suitable habitats.

Limitations

Overall, the findings of this study are limited by the low positivity rate. Given the large population known to be present below the dam, it was surprising to see only six positive detections on five different days throughout the temporal study. This small positive grouping makes it difficult to draw sound conclusions from the environmental and hydrological data (Table 2).

The unknown distance to the salamanders is also a limitation (Pilliod et al. 2016 and Collins et al. 2019). Despite the many physical sightings and/or captures of mudpuppies near the Dam site (Fig. 1), those locations may not reflect the locations of the individuals at the time of sampling. It is possible that since sampling was performed between dawn and dusk, the mudpuppies were either too far away or too inactive for more positive detections to occur.

Another limitation, specifically for the spatial study, was the relatively coarse scale that suitability was evaluated. Habitat assessments occurred every 50 meters, and samples were collected every 100 meters. This large scale overlooks the potential for microhabitats to support mudpuppies, which may have led to the inconsistencies between what was considered suitable habitat and positive detections. The other limitation is the impact of stream velocity and the rate of eDNA production. These two factors likely combine to influence the amount and distribution of DNA at any given time.

Conclusion and Future Directions

After analyzing the temporal results of the application of eDNA to study the mudpuppy population at Alum Creek, it is clear that dilution effects from high water flows influence the ability to detect DNA. Even with a large known population present at Alum Creek Dam, there is not enough eDNA produced to result in positive detections when there are large volumes of water moving at a high velocities. While not definitive, it appears from my study that environmental factors such as cloud cover, air temperature, pH, and conductivity have little to no influence on detection.

The spatial study showed that mudpuppy habitat preferences are likely too poorly understood to successfully develop a habitat suitability index that will align with positive detections. Another possibility is that assessments were too coarse, resulting in overlooking microhabitats and failing to assign habitat suitability rankings frequently enough along the stretch.

In order to further refine eDNA methods for temporal sampling, future studies should involve collection during times of low gauge height and low discharge. This could add in a control for low water flow so that environmental factors can be better studied and understood. Another suggestion would be to collect from more than 2 locations below the Dam, which will ensure some samples are collected in close proximity to individuals.

To better understand mudpuppy habitat selection, the spatial study can be expanded by collecting water samples in smaller increments (e.g., every 50 meters instead of 100) to allow for a finer scale assessment of detection in relation to suitability. Mudpuppies likely do not need 50-100 meters worth of great habitat, but rather smaller stretches of just 5-10 meters or even microhabitats such as single rocks or holes in an undercut bank may be sufficient to support individuals. This study could also be expanded by having more than one sample date. By combining additional spatial and temporal sampling, movement of individuals throughout the stretch can be better understood. Ultimately, this could lead to a better understanding of habitat characteristics that the mudpuppies prefer.

Overall, eDNA can be incredibly useful in detecting aquatic species. However, there are many refinements that need to be made to ensure it is being used to its fullest potential. Understanding the limitations caused by dilution effects, as shown in this study, is a step in the right direction, but further studies are needed to continue to both refine methods and gain a better understanding of the habitat preferences of mudpuppies. Adapting protocols following findings from my study will help ensure that future statewide surveys for mudpuppies are effective and reliable.

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