A Novel Approach to Identifying Kirtland's Snake Habitat along Lake Erie

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PI: Matt Cross
The Toledo Zoo and Aquarium
P.O. Box 140130, Toledo, OH 43614

Abstract

The Kirtland's Snake (*Clonophis kirtlandii*) is a species on the decline and is considered threatened or endangered throughout its remaining range. This species is largely fossorial and is notoriously difficult to detect via traditional survey methods (i.e. visual encounter or cover object surveys). To address the paucity of knowledge regarding detection, distribution, and habitat associations of the Kirtland's Snake, we used a multi-technique approach to identify suitable habitat and conduct field surveys. Using the most up-to-date element occurrences for the state of Ohio, we created a preliminary Kirtland's Snake distribution model for 22 counties in the state. To test model predictions, we selected a gradient of likelihood of occurrence sites and conducted artificial cover object surveys and collected water samples to test for the presence of eDNA. Our distribution models suggest Kirtland's Snake probability of occurrence is highly influenced by proximity to wetland habitat and soil composition. Kirtland's Snake DNA was detected from water samples at 20% of our sites, but were not detected during cover board surveys. In addition to the Kirtland's Snake detections, we detected DNA from four other snake species. Our preliminary test of eDNA as a survey method for Kirtland's Snakes highlights the utility of this approach for detecting cryptic species.

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Technical Report

Activities and Timeline

Quarter 1

- Project planning and coordination: obtain permits and identify partner roles.
- Obtained all reported Kirtland's Snake element occurrences (EO) from the Ohio Division of Wildlife.
- Used EO data to create a preliminary distribution model for all counties where Kirtland's Snakes had been reported. Too few occurrences were from the initial study boundary of the Lake Erie watershed, so we constructed a distribution model for 22 counties.

Quarter 2

- Distribution models were refined and finalized to guide sample site-selection.
- 80 artificial cover objects (ACOs) were deployed at eight locations throughout the study area.
- Water sampling occurred at 20 sites throughout the study region in areas of high (n = 7), medium (n = 7), and low (n = 6) probability of occurrence, as predicted by our preliminary distribution model.

Quarter 3

- Artificial cover objects were monitored once every three days from July – August 2018.
- Water sampling continued throughout our study region.

Quarter 4

- Water samples were analyzed and DNA was extracted.
- Data were compiled and final analyses were concluded.

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Methods

Site Description

This study took place in northwestern Ohio, with sites occurring in the region located south of Toledo; north of Findlay; east of Napoleon, and west of Sandusky (Fig.1). This area was once the Great Black Swamp and the Oak Openings ecoregions. Furthermore, this area harbors numerous species of conservation concern and ecologically important areas. (Ricci 2006; Schetter and Root 2011). The Oak Openings region, in the north central part of this study area is considered a regional biodiversity hotspot with 177 federal and state listed rare species as stated by the Ohio department of Natural Resources Division of Natural Areas and Preserves (Ricci 2006). However, since European settlement, >95% of Ohio's wetlands have been lost to agriculture and urban development (Black Swamp Conservancy, 2018). Therefore, this sensitive and biodiverse ecoregion is also highly fragmented and degraded.

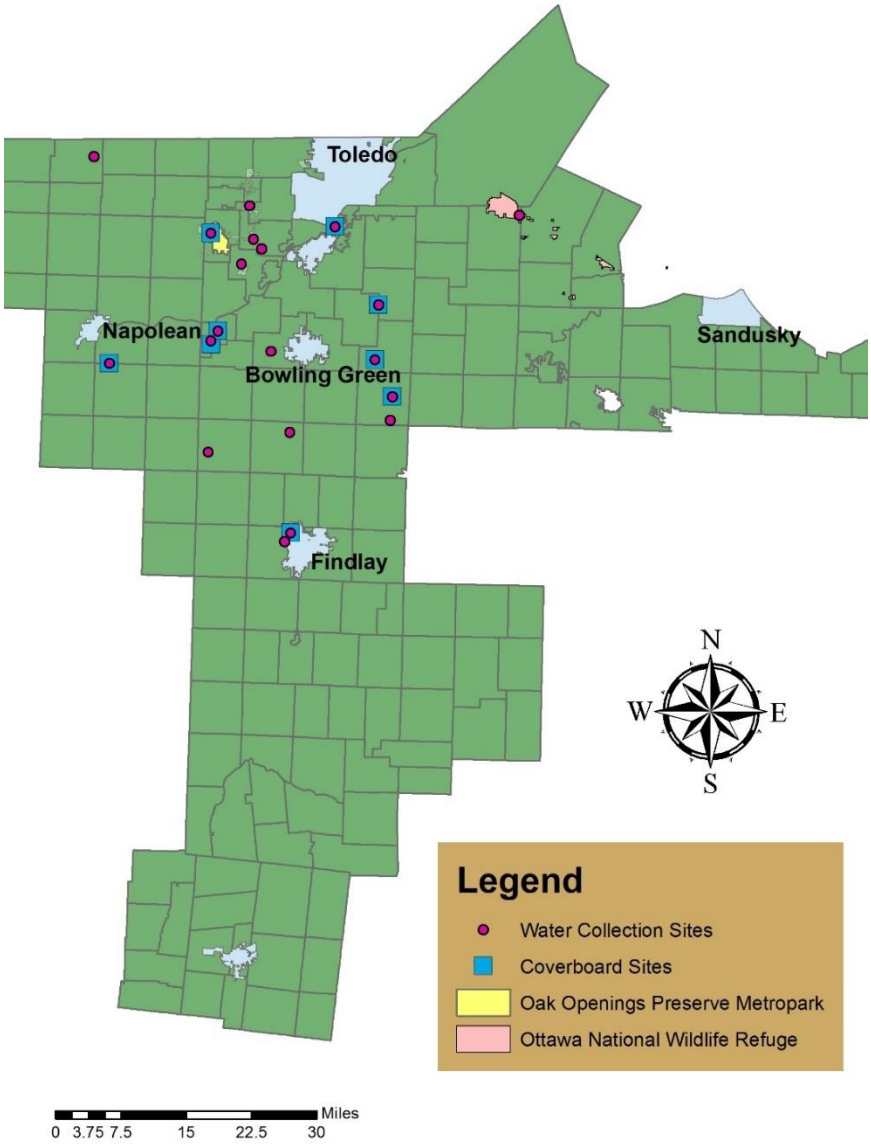

Fig. 1. Locations where ACO and eDNA surveys for Kirtland's Snakes took place in Northwest Ohio in 2018.

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Distribution Modeling

Element Occurrences and Data Layers

We worked with the Ohio Division of Wildlife and several other state partners to compile the most current list of all Kirtland's Snake sightings within the state. We were primarily interested in recent sightings, those within the last 25 years, so we excluded EOs prior to 1993. It was clear early on there were too few occurrences within the Lake Erie watershed to generate accurate distribution models, so we expanded our study area to cover all 22 counties where Kirtland's Snakes have been reported, and adjacent counties (Fig. 2). Many of the Kirtland's Snake EOs came from surveys where there were multiple snakes within a relatively small geographic area, so we spatially rarefied occurrence data using a buffer of 100 m. This value represents the higher end of known Kirtland's Snake movement (M. Seymour, USFWS, *pers. comm.*). The final dataset to be used for the distribution modeling consisted of 76 occurrences.

Landscape variables selected for use in the distribution models were based off the known ecology of the species and included: canopy cover, compound topographic index ("wetness index"; CTI), distance to wetland, elevation (digital elevation model; DEM), impervious surface %, National Land Cover, DEM-derived variables (slope, aspect, surface curvature), and soils (composition and water table depth). All data were obtained from the USDA NRCS (https://datagateway.nrcs.usda.gov/) and were at a resolution of 30 m. The number of candidate variables was reduced by removing highly-correlated layers in ENMTools (Warren et al. 2010). The resulting dataset included canopy cover, CTI, distance to wetland, impervious surface, soil composition (silt), soil pH, and water table depth (Fig. 2).

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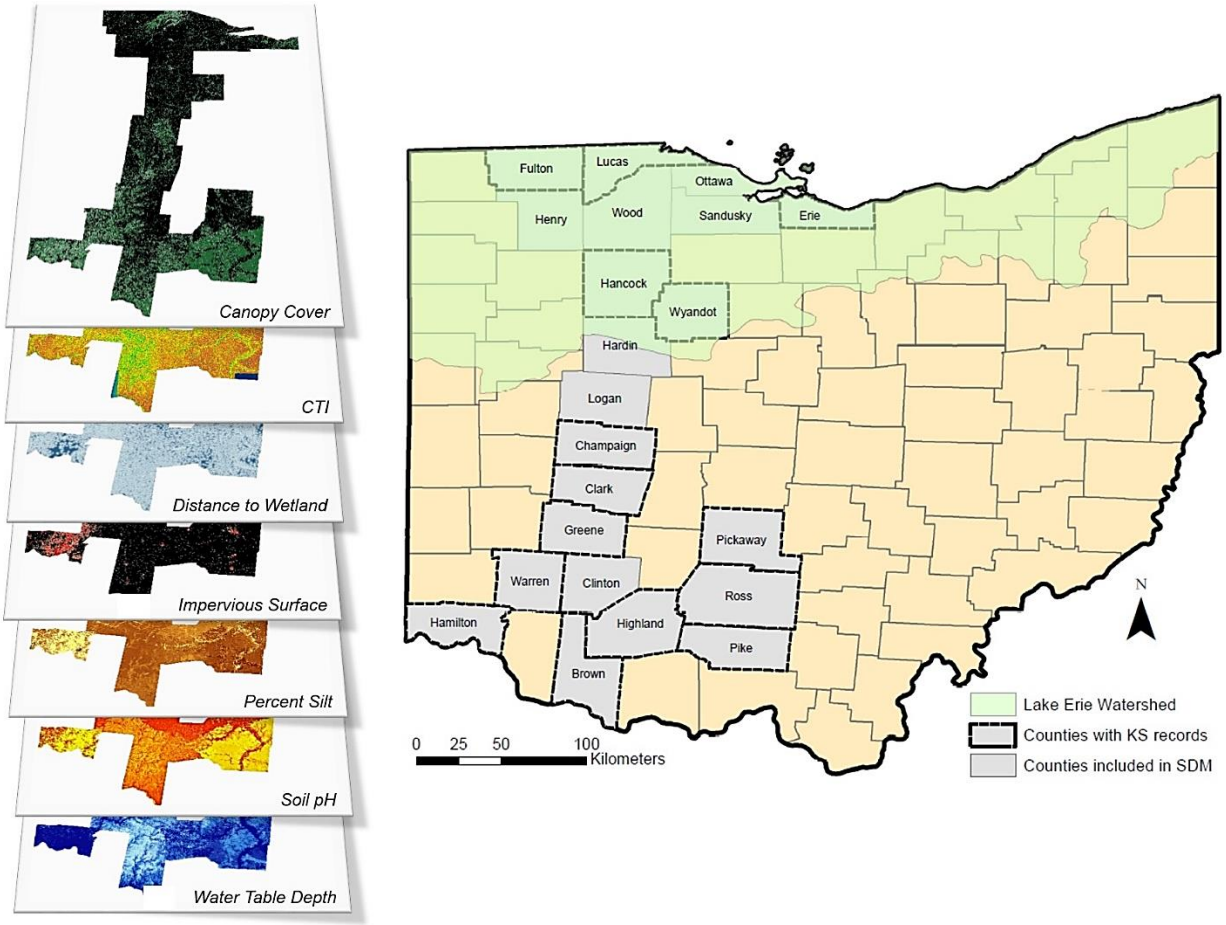

Fig. 2. Final set of data layers used for ensemble modeling (left), and a map of counties where Kirtland's Snakes had been sighted in the last 25 years, counties included in the study, and the boundary of the Lake Erie watershed (right).

Species Distribution Models (SDMs)

We employed an ensemble approach to predict the occurrence of Kirtland's Snakes in our study area. Ensemble modeling has the benefit of incorporating the predictions of multiple models to improve predictive accuracy (Araújo and New 2007; Grenouillet et al. 2011; Kindt 2018). We selected Maxent (Phillips et al. 2006), a generalized linear model (GLM), and classification tree analysis (CTA) as they represent three different algorithms used for species distribution modeling. The R package "SSDM" (Schmitt et al. 2017) was used to create individual models and the final ensemble model.

Cover Board and eDNA Surveys

Output from the preliminary distribution model was used to select field sampling sites for eDNA and artificial cover board (ACO) surveys (Fig. 1). Water samples were collected at 7 sites

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with high probability of Kirtland's Snake occurrence, 7 sites with medium probability of Kirtland's Snake occurrence, and 6 sites with low probability of Kirtland's Snake occurrence. Sample sites consisted of ditches, reserves, riparian zones, and private property.

Field Survey Methods

We placed 10, 60cm × 60cm ACOs at 8 (total = 80) locations throughout the study site (Fig. 1). Cover objects were checked once every 3 days from July 2018 to August 2018. All snakes located under the cover boards were identified to species and then immediately released on site.

Surveys for eDNA were conducted at 20 sites (Fig. 1), throughout the study region. At each site a ¼" rubber hose[1] attached to a 100 mL serological pipette was inserted into a cray fish burrow to siphon approximately 300-500 mL of water out of the burrow. At each sample location 3-5 crayfish burrows plus surface water (900-2,500 mL of water total) were collected (Fig. 3). Samples were placed in a cooler with ice packs until transported back to the lab for extraction. We surveyed 20 sites in this manner in the summer of 2018, from July-August 2018.

Fig. 3. Active crayfish burrow at one of the eDNA sampling sites.

To reduce the risk of cross contamination of DNA samples between sites, all equipment was stored in a clean laboratory storage facility that did not have any living animals present. While handling animals or eDNA survey equipment, researchers wore a clean pair of nitrile gloves, and washed their hands with a mild detergent and rinsed with distilled water prior to and after handling any equipment/specimens. For each field sample collection, a new and sterile serological pipette and Sterivex filter was used for each burrow or surface water sample collected. A separate clean and sterilized hose was used for each burrow and surface water sample. Every day after field activities, hoses were thoroughly scrubbed and soaked in a mild detergent. Hoses were then soaked in a 3% bleach solution for ~30-45 minutes, then thoroughly rinsed with a 70% molecular grade ETOH. Lastly, the hoses

[1] In some instances, the crayfish burrow may be mudded-in or have collapsed. In these instances, a 1" soil bore was used to gently open up the burrow so that the filter hose could be inserted.

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were rinsed with distilled water and allowed to air dry for at least 24 hours, before being redeployed in the field. The soil bore was cleaned in the same manner.

During field sampling we followed the recommended PARC (Partners in Amphibian and Reptile Conservation) biosafety and sterilization guidelines. While in the field collecting data, researchers wore rubber boots. Prior to the start and end of each sampling site, researchers' boots were brushed off to remove any mud, dirt, or seeds using a wire brush. Boots were then submerged in a mild detergent to remove any chemical contaminants, then rinsed with distilled water, and allowed to air dry prior to storage or transport. All equipment was stored in a clean laboratory facility free from any live animals.

eDNA Isolation and Extraction

Water samples were immediately filtered after collection and concentrated onto a 45micron Sterivex filter (EMD Millipore Corp, Billerica, MA) with a sterile ¼" rubber hose attached to a 10 mL serological syringe. DNA was extracted using FastDNA TM SPIN kit protocol for soil (MPbio, Santa Ana, CA). The method is currently optimized to detect not only Kirtland's Snake, but 5 other Thamnophine or related snake species. In addition to Kirtland's Snake tissue (*Clonophis Kirtlandii*), the Toledo Zoo provided blood and/or tissue samples for 5 other snake species for use in this optimization protocol: Eastern Garter Snake (*Thamnophis sirtalis*); Butlers Garter Snake (*Thamnophis butleri*); DeKay's Brown Snake (*Storeria dekayi*); Northern Water Snake (*Nerodia sipedon*); and Eastern Fox Snake (*Pantherophis vulpinus*). DNA was extracted from these blood and tissues using DNeasy Blood & Tissue kit protocol (Qiagen, Venlo, Netherlands).

Genetic Analysis

DNA barcoding using a MinIon rapid sequencer (Oxford Nanopore Technologies, Oxford, UK) is ongoing. For barcoding we used a PCR-based, 16s Ligation protocol to barcode ~400ng of purified DNA PCR product. We then used DNA samples from the 6 snakes of known identity as reference samples to compare to DNA samples from our sample sites. DNA sample alignment and accuracy assessment were carried out using Oxford Nanopore Epi2me software (Oxford Nanopore Technologies, Oxford, UK). Alignment of samples to reference samples and genetic analysis of all samples are being carried out using Mega4 (Tamura et al. 2007) and Beast

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(Suchard et al., 2018). *Final Results of these analyses are still in progress and beyond the scope of this report.*

Results

Species Distribution Model

All models performed well with similar area-under-the-curve (AUC) values above the suggested discrimination threshold (i.e., AUC ≥ 0.75; Table 1). The weighted average ensemble model revealed potential Kirtland's Snake habitat to be widespread, but limited (10.7% of the study area) throughout the study area, often separated by large areas of unsuitable habitat (Fig. 4). **Larger map and location specifics are omitted due to collection concern.**

Variable contributions varied depending on model algorithm, but generally followed similar trends (Table 1). Overall distance to wetland and percent silt were the most important variables, followed by water table depth, and to a lesser extent, impervious surface, soil pH, and canopy cover.

Based on the small number of Kirtland's Snake detections from ACO and eDNA surveys (see below), we omitted the refined ensemble model as it was unlikely the addition of four potential sites, identified by eDNA, would dramatically change the predicted distribution.

Table 1. Results of the individual models used to create the ensemble species distribution model for Kirtland's Snakes, including AUC and relative importance of each environmental variable to specific models.

		Variable Importance (% contribution)						
Model	**AUC***	**Canopy Cover**	**CTI**	**Dist. to wetland**	**Impervious Surface**	**Soil pH**	**Silt**	**Water table depth**
Maxent	0.89	3.3	0.2	67.1	0.6	1.6	15.8	11.4
GLM	0.84	3.3	0.7	21.4	1.1	4.7	53.7	15.1
CTA	0.78	14.3	12.6	38.9	8.9	0.0	17.4	7.8

*Values above 0.75 are considered informative (Swets 1988; Eskildsen et al. 2013).

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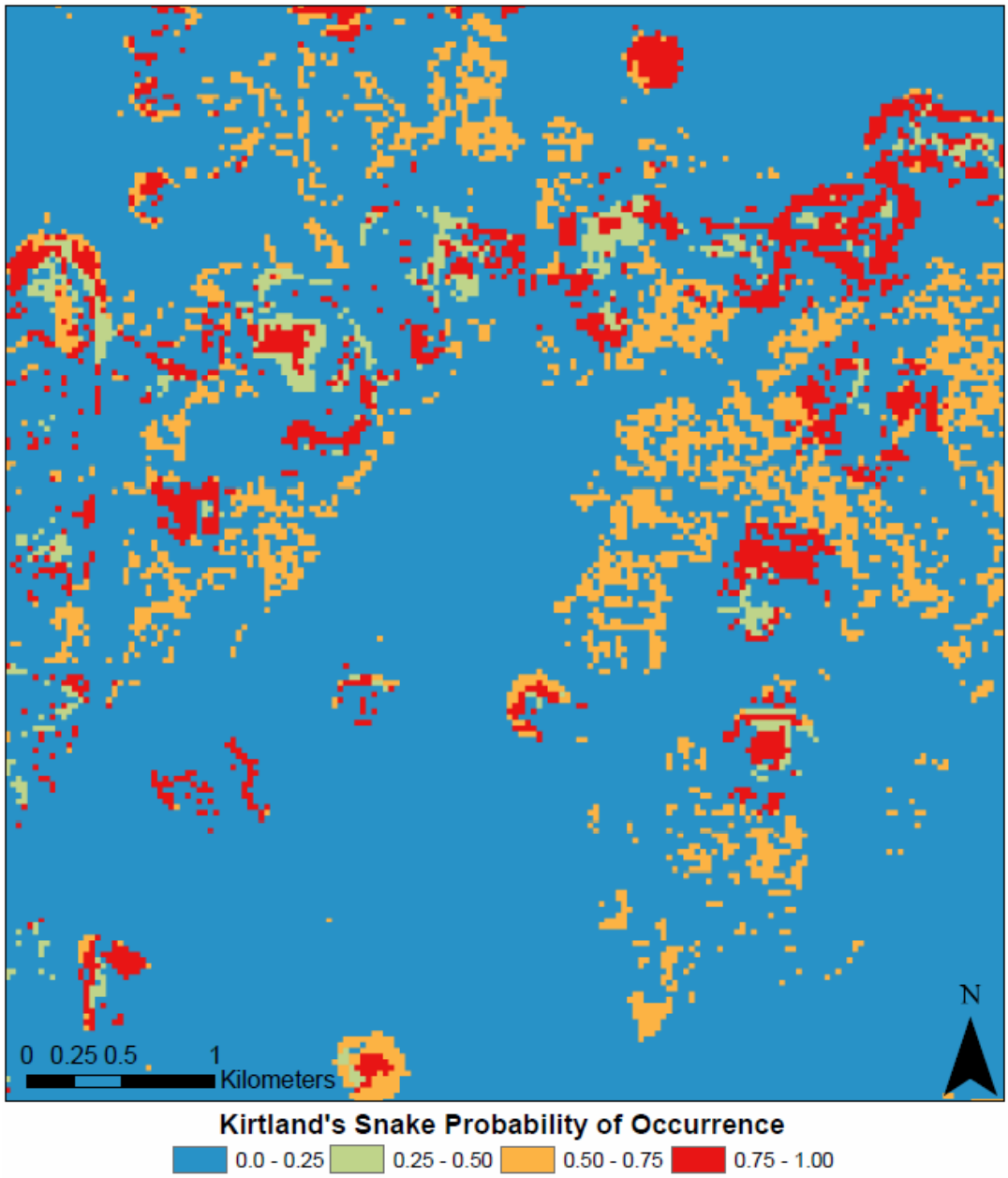

Fig. 4. An example of the ensemble distribution model output for Kirtland's Snakes in Ohio, highlighting low-high probability of occurrence areas.

ACO Survey Results

Using standard ACO survey methods (Fig. 5) 8 snakes, including Eastern Fox Snake (*Pantherophis vulpinus*), North American Racer (*Coluber c. constrictor*), Dekay's Brown Snake (*Storeria dekayi*), and Eastern Garter Snake (*Thamnophis sirtalis*) were visually encountered at 3 out of 8 sites (Fig. 6; Table 1). No Kirtland's Snakes were encountered during ACO surveys.

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Fig. 5. The four snake species encountered during ACO surveys in 2018. Top left: Dekay's Brown Snake; Top right: Eastern Fox Snake; Bottom left: North American Racer; Bottom right: Eastern Garter Snake (melanistic and regular).

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Fig. 6. Artificial cover object locations (blue squares) in our study region and sites where snakes were visually encountered (green dots).

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Table 2. Results from ACO and eDNA surveys for Kirtland's Snakes in our study area during the 2018 field season.

Location	Type	Occurrence Probability*	eDNA Snake Detections	ACO Snake Detections
Site 1	Reserve	Medium	Kirtland's Snake	---
Site 2	Reserve	High	Kirtland's Snake and E. Fox Snake	---
Site 3	Margin	Medium	Kirtland's Snake and E. Garter Snake	---
Site 4	Ditch	Low	Kirtland's Snake and E. Garter Snake	---
Site 5	Ditch	Low	E. Garter Snake	---
Site 6	Reserve	Medium	E. Garter Snake and Dekay's Brown Snake	E. Garter Snake and N. American Black Racer
Site 7	Ditch	Low	---	Dekay's Brown Snake and E. Garter Snake
Site 8	Ditch	Medium	---	E. Fox Snake

*Based on the results of the Kirtland's Snake species distribution model.

eDNA Results

Two different researchers identified snake species putative genetic identity based on DNA genotypes and banding patterns. Each researcher scored the snake bands and identities of snakes at a site independently using a double-blind DNA band matching procedure. This resulted in >60% agreement on snake species present at each site. For those instances (N=2) where researchers disagreed on snake species presence, a consensus score was used based on $\geq$2 primers indicating the presence of a snake band at a site. All 6 identified reference samples were amplified.

A total of 10 snakes from 4 different snake species were detected at 6 out of 20 (30%) of the sites (Fig. 7). Kirtland's Snakes were detected at 4 of 20 (20%) sites (Fig.7; Table 2). Eastern Garter Snakes were detected at 3 of 20 (15%) sites (Figure 7; Table 1). Fox Snakes were detected at 2 of 10 (10%) sites (Fig. 7; Table 2). A single DeKay's Brown Snake was found at one site (1%). Out of the 4 sites that had Kirtland's Snake detections, one sites was high probability of occurrence (site 2) two sites were medium probability of occurrence sites (site 1 and site 3) and one site was a low probability of occurrence site (site 4) (Table 2). Sites 1, 2 and 6 are all reserves sites (Table 1), whereas site 3 is located on private property in what would otherwise be a marginal area (Fig. 7; Table 2). Sites 4 and 5 are ditch sites.

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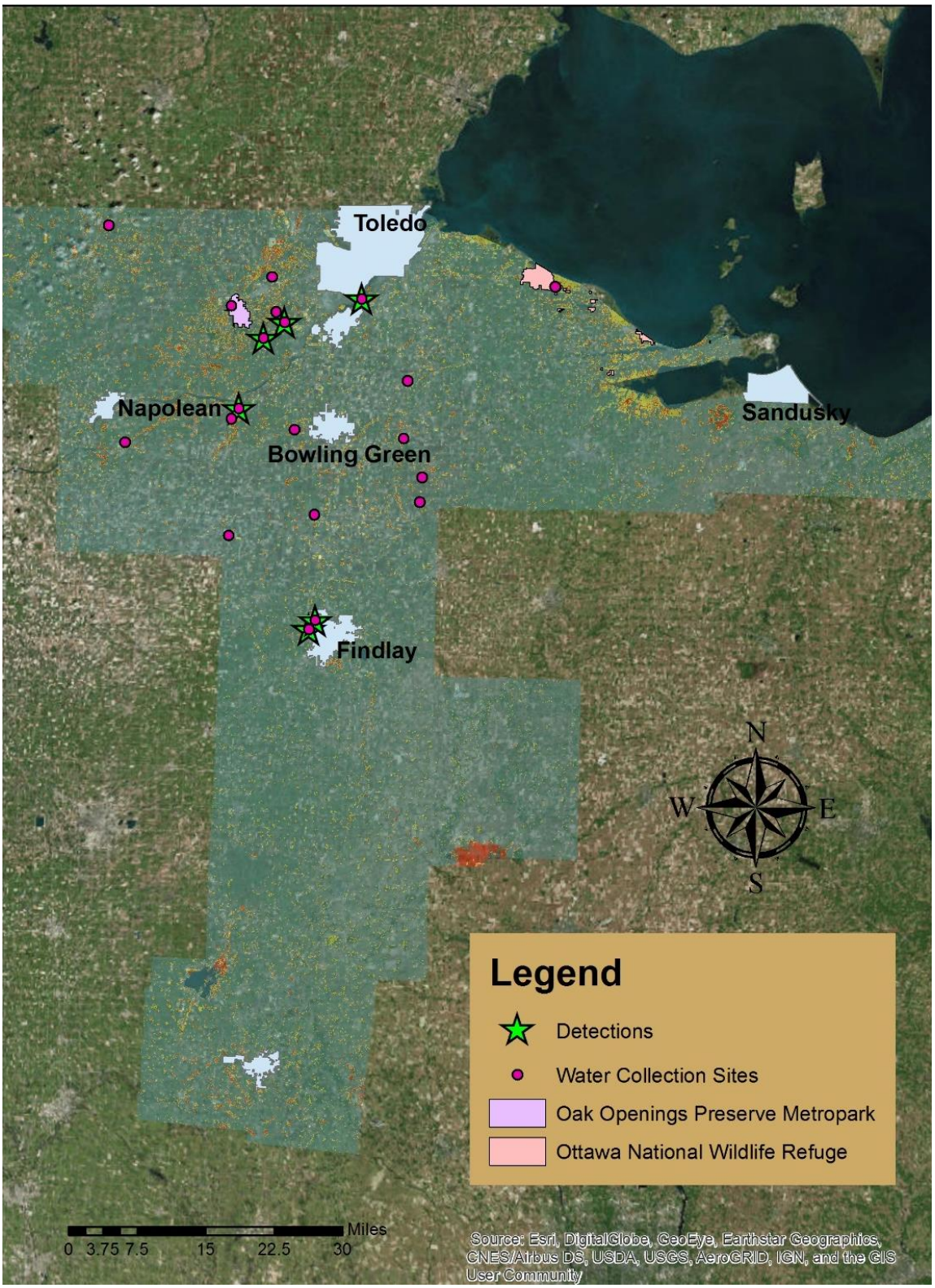

Fig. 7. Map of water sample locations (magenta dots) and sites where snakes were detected (green stars) via eDNA.

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Discussion

Conservation plans for Kirtland's Snakes are often hindered by the lack of even the most basic information regarding local distributions. Our multi-technique approach of using SDMs and eDNA surveys has produced a product managers can use to identify priority conservation areas, and tested a method of detection that could be incorporated into future survey efforts. The results demonstrate that the eDNA procedures used in this study successfully amplified all 6 identified reference samples. Furthermore, the results verify that the eDNA procedures used in this study are effective to detect *Thamnophine* snakes, including Kirtland's Snakes, under natural conditions.

We found that distance to wetland habitat, soil texture (silt), and water table depth were the most important predictors of Kirtland's Snake probability of occurrence throughout our study region. Throughout their range, Kirtland's Snakes can be found in variety of habitats including wet meadow/prairie, forested riparian areas, and even urban and suburban habitats, with a generic preference for open, moist areas being a minimum requisite (Conant 1951, Ernst and Ernst 2003, USFWS 2017). Despite the widespread loss of wetland habitat in Ohio, small, isolated wetlands and "wetland-like areas" are a relatively common feature across the landscape. Some reports from within the state have found Kirtland's Snakes in and around roadside ditches (G. Lipps, Ohio Biodiversity Conservation Partnership, *pers. comm*.; M. Cross, *pers. obs*.). We initially had combined ditches and wetlands into a generic "distance to water" variable, but preliminary models suggested there was a ditch bias and strongly predicted ditches as highly-ranked habitat. Ditches are likely used by Kirtland's Snakes in the absence of quality habitat, but in terms of identifying priority wetland habitat, we decided to exclude ditches from our model.

Soil texture and water table depth are likely surrogates of habitat type as these factors strongly influence vegetative characteristics of a site (Mack 2001; FGDC 2013). Silt particles play an important role in movement and retention of capillary water, which is responsible for moving groundwater within soils (Jensen 2007). While wet habitats are generally a prerequisite for Kirtland's Snakes, soil texture and water table depth are also likely to influence crayfish presence. Kirtland's Snakes have long been associated with crayfish and use burrows for shelter and a source of food (Bavetz 1994, Anton et al. 2003; Cross et al. 2018). Specifically, at three geographically distinct locations in Ohio, digger crayfish (*Fallicambarus fodiens*) have been regurgitated by Kirtland's Snakes (Cross et al. 2018). Digger crayfish are predominantly found

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in the western portion of Ohio and prefer wetlands not typically found in stream-associated wetlands (Thoma and Jezerinac 2000). Accounting for the relationship with crayfish, specifically digger crayfish, could help improve the accuracy of Kirtland's Snake models.

Our SDM predicted an abundance of Kirtland's Snake habitat throughout our study area, more so than expected given the species' threatened status. While sites with a middle-high probability of occurrence were widespread, most of these sites were separated by large areas of unsuitable habitat and unfavorable matrix. This patchy distribution of habitat is expected, given the degree of historical landscape modification, and is consistent with the decline of Kirtland's Snakes being primarily attributed to habitat loss, modification, degradation, and fragmentation (Gibson and Kingsbury 2006; USFWS 2017). As historic habitat decreased, the snakes have had to make use of suboptimal sites. For example, two of the most well-known historic Kirtland's Snake populations in Ohio occurred in and around urban areas in Toledo and Cincinnati (Conant 1951). This also occurred during the course of this study when two separate Kirtland's Snakes sightings came from residential areas in Perrysburg (M. Cross, *pers. obs.*; G. Lipps, *pers. comm.*). Despite these occurrences it is unlikely that Kirtland's Snakes are seeking out habitat in urban sites, but are instead making use of what little suitable historic habitat remains within these developed areas. The Kirtland's Snakes' ability to persist, even thrive, in small, isolated, generic moist habitats adds a level of uncertainty to distribution modeling since previously unconsidered habitats may prove useful for this species. Features like ditches, that provide intermediate-quality habitat, may serve as important stepping stones or corridors. Future Kirtland's Snake modeling and survey efforts may wish to examine how ditches and historic habitat influence model predictions.

Kirtland's Snakes were detected at 20% of the total sites surveyed, which is high given the cryptic nature of this snake species and its typically low detection probability (Ernst and Ernst 2003, USFWS 2017). However, it may not be surprising that Kirtland's Snakes were found as frequently as they were in this study because we structured our sampling to survey areas that were likely to contain Kirtland's Snakes based on historical EO data. It is interesting to note however, that sites with moderate probability of occurrence form the Kirtland's Snake SDM had the highest number of detections (both in total and per capita). There are multiple non-mutually exclusive explanations for this: First, sites were assigned to probability classes based on inflection points in the site probability of occurrence distribution histogram, which may not be

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the most accurate way to predict Kirtland's Snake likelihood of occurrence. A binary response variable might be more appropriate predicting yes or no to Kirtland's Snake presence or absence. Second, moderate probability sites might require the snakes to be more active, moving from burrow to burrow hunting and increasing the opportunity for us to detect eDNA in burrows. Therefore, even a lower density of snakes would yield a higher eDNA success rate. Without the ability to assign individual identity to positive Kirtland's Snake detection we have no way of testing this hypothesis. Finally, as discussed above, in the absence of preferred habitats, Kirtland's Snakes may be making use of non-traditional areas. In that context, creating the species distribution map using previous sighting data might well underestimate the importance of some areas, resulting in moderate areas being used more intensively. This same mechanism can also explain why we had such a high per capita detection rate in low probability of occurrence areas. Regardless of mechanism, this study demonstrated that eDNA surveying can be an effective means of surveying, especially for fossorial species.

eDNA surveying methods have many potential advantages compared to traditional cover board surveys. Cover board surveying require physically seeing Kirtland's Snakes and numerous other species, whereas eDNA surveying does not and can be done much less invasively. Furthermore, cover board surveying requires researchers with taxonomic expertise going into the field on multiple occasions, which is both time and labor intensive, i.e. costly. However, it is worth noting the USFWS initiated a program using ACOs and volunteers to survey for Kirtland's Snakes in the southern portion of the state and caught 89 at 13 sites in seven counties (M. Seymour, USFWS, *pers. comm.*). In this study, we were able to survey more sites with less effort using eDNA surveying than with cover board surveying. Using eDNA methods, researchers can detect multiple species that used an area sometime over the past several months during a single surveying bout. This offers numerous cost savings in terms of the field effort. Furthermore, our approach could also be modified to work for other reptile species, including species of conservation concern such as: Eastern Massasauga (*Sistrurus catenatus*), Queen Snake (*Regina septemvittata*), Spotted Turtles (*Clemmys guttata*), or Blanding's Turtles (*Emydoidea blandingii*). Regardless, the eDNA approach has great potential and should be used more regularly in conservation monitoring.

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Final Accounting

See Attached

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