OVERWINTERING HABITAT MODELING AND eDNA DETECTION FOR THE EASTERN
MASSASAUGA (*SISTRURUS CATENATUS*) IN A SOUTHWESTERN MESIC PRAIRIE

BY

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THESIS

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ABSTRACT

As global biodiversity declines, long-term and complete datasets become increasingly important for wildlife conservation. Collecting sufficient data can be difficult, especially for rare or endangered species. Many snake species face extinction, but due to their cryptic nature, limited activity patterns, and often inaccessible habitat, snakes represent one of the most data-deficient groups. Traditional monitoring methods, such as capture-recapture surveys, can be costly and time-consuming and may miss hard-to-detect species. Consequently, new surveying methods should be considered to reduce data deficiencies for snake species. To assess new surveying methods, I studied Eastern Massasauga (*Sistrurus catenatus*) overwintering sites in Carlyle, Clinton County, Illinois. The Eastern Massasauga is a small to medium-sized rattlesnake distributed throughout the Great Lakes region and is facing range-wide declines due to habitat loss, fragmentation, and synergistic effects. Extirpation rates are exceptionally high in southwestern populations because of the loss of important prairie and wetland habitats. To provide methods for reducing data deficiencies, I assessed habitat niche modeling and eDNA detection for the Eastern Massasauga in the southern portion of their range. I developed a habitat suitability model to identify vital overwintering sites in the Carlyle Lake region. I found soil characteristics and distance to water and medium-height vegetation were the strongest environmental predictors of species presence. I identified locations to prioritize for protection, restoration, and future surveys. My results provide information on the effectiveness of non-traditional survey methods and guide future conservation for Eastern Massasaugas in other southwestern mesic prairies. I also optimized an eDNA assay to detect target DNA in sediment and water samples collected from crayfish burrows. Due to low detections in my results and previous studies, I found eDNA is not as effective as visual surveys for monitoring the species.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

The current global biodiversity crisis is a phenomenon linked to anthropogenic activities. Pervasive habitat fragmentation, pollution, and overexploitation of natural resources have deeply affected the natural landscape and ecosystem function (Pievani, 2014). Estimates reveal most taxa have experienced at least a 5-20% biodiversity loss, suggesting an extinction rate 100-1000% higher than pre-human rates (Chapin et al., 2000). Reptiles, in particular, have faced high losses due to the combined influences of small ranges and strict survival requirements (Anderson, 1984; Bohm et al., 2013). Both factors make them vulnerable to habitat degradation through climate change and increased human-wildlife interactions caused by urban development (Schneider & Root, 2002). Current conservation rankings suggest 15-36% of known reptile species are threatened, with 5-26% near threatened (Bohm et al., 2013). Some studies have found snakes face lower levels of threats than other taxonomic groups, but the interpretation is likely due to data deficiencies in long-term population and global scale studies (Reading et al., 2010). Current estimates suggest that only 12% of global snake species face extinction (Bohm et al., 2013), whereas others have documented rapid declines globally with little chance of recovery (Reading et al., 2010; Lukoschek et al., 2013; Zipkin et al., 2020).

Snakes native to temperate grasslands have seen particularly high extirpation rates, likely due to habitat loss. Globally, 41% of temperate grasslands have been converted to agriculture, resulting in the loss of important habitats, breeding grounds, and foraging opportunities (Carbutt et al., 2017; White et al., 2000). Within North America, grassland conversion rates are as high as 50-99%, and the remaining habitat is highly fragmented and degraded (Hoeskstra et al., 2004; Henwood, 2010). Extreme habitat loss has threatened temperate snake populations, with

specialist snakes often experiencing more intense pressures. In some areas, only 44% of historic snake species still occur (Cagle, 2008).

One such temperate snake species, the Eastern Massasauga (*Sistrurus catenatus catenatus*), has suffered range-wide population declines. The Eastern Massasauga is a small to medium-sized rattlesnake primarily associated with open-canopied habitat with access to wetlands. Its range extends from eastern Iowa to western New York and southern Illinois to southern Ontario (Jones et al., 2012). Listed as Threatened under the United States Endangered Species Act of 1973 and Canada's Federal Species at Risk Act, it is protected as threatened or endangered in all provinces or states occupied (COSEWIC, 2012; USFWS, 2016). Habitat loss, fragmentation, and synergistic effects, such as road mortality and human-wildlife conflict, are the primary causes of the Eastern Massasauga's decline (Szymanski, 1998; Durbian et al., 2008). Additional population-level threats include illegal collection, management-associated mortality, hydrological alterations, and disease (Dreslik, 2005; Shepard et al., 2008; Baker et al., 2016). Current estimates suggest Eastern Massasauga populations have decreased by 38% percent range-wide (USFWS, 2016). Known to occur in 588 populations historically, only 263 remain, with another 211 either extirpated or likely extirpated and 81 of unknown status (USFWS, 2016). Western populations of the Eastern Massasauga have suffered the heaviest extirpations at 70% (USFWS, 2016). Increased extinction events in these regions may be due to disparities in the effects of climate change, including heightened intensities of winter floods and summer droughts (Pomara et al., 2013). One study suggests that increased active periods due to temperature changes in southwestern areas may lead to a higher frequency of predation (Jones et al., 2012).

Within Illinois, Eastern Massasauga populations have declined by 96% (Szymanski et al., 2016). Where 25 known populations once occurred throughout the northern two-thirds of Illinois

(Szymanski, 1998), only one population cluster remains in Carlyle Lake, Clinton County. Road mortality, predation, and human persecution are major local sources of mortality (Shepard et al., 2008; Bailey et al., 2011; Baker et al., 2016); habitat loss and fragmentation within western states are the primary cause of broader regional declines. Illinois has lost 99% of its 2.2 million acres of tallgrass prairies (Corbett, 2004) and 90% of its 8.2 million acres of wetlands (Suloway & Hubbell, 1994). Habitat quality has suffered further degradation from invasive species, poor water quality, and fragmentation by human infrastructure (Suloway & Hubbell, 1994). Because of the severity of recent habitat loss, snake populations within Illinois have declined drastically, with temperate species at the edge of their range, such as the Eastern Massasauga, facing greater extirpation risks (Cagle, 2008).

Eastern Massasaugas prefer open, low-lying habitats with access to wetlands or marshes, such as wet prairies, peatlands, floodplain forests, and sedge meadows. Like most snakes, essential habitat changes clinally, ontogenetically, and seasonally (Syzmanski et al., 2016). Eastern Massasaugas establish home ranges within the broader landscape during the active period to accommodate dispersal, foraging, and reproduction (Harvey & Weatherhead, 2006b; Moore & Gillingham, 2006). Dry, open-canopied wetland habitats composed of emergent vegetation, short woody vegetation, and wet lowland hardwoods are used more frequently during the summer and fall when compared to spring emergence (Reinert & Kodrich, 1982; Moore & Gillingham, 2006). Across most of their range, Eastern Massasaugas rarely used upland or heavily forested areas (Danou, 1997; Dreslik, 2005; Moore & Gillingham, 2006; Marshall et al., 2006; Durbian et al., 2008); however, some studies reported coniferous forest use in the northern populations (Weatherhead & Prior, 1992; Harvey & Weatherhead, 2006b). Landscapes with a gradient of open to closed canopies, which provide thermoregulatory opportunities, protection

from predators, and prey abundance, appear to be preferred by these snakes (Syzmanski, 1998). Although not an aquatic species, Eastern Massasaugas rely on mesic habitats closer to wetlands (Johnson et al., 2000). While they occupy more xeric habitats in the summer and early fall, Eastern Massasaugas return to mesic habitats in the late fall for overwintering dormancy (Johnson et al., 2000; Bailey et al., 2012).

Dormancy over winter is behaviorally driven in reptiles and characterized by low activity and reduced metabolic function meant to increase survival during periods of low temperature (Cobb & Peterson, 2008; Nordberg, 2013). Overwintering dormancy is particularly common in reptiles due to their inability to create heat, making them inherently vulnerable to environmental conditions. In addition, reptiles use a combination of both physiological and behavioral thermoregulation to reach optimal temperature and metabolic function (Dawson, 1975). Although past research delves deeper into poorly understood physiological methods (Seebacher & Franklin, 2005), behavioral methods, such as basking, posturing, and shifting activity patterns, have been well-studied in reptiles (Dawson, 1975). When temperatures fluctuate during the spring and fall, reptiles spend most of their active hours basking with little movement except between refugia and basking sites. When temperatures are highest, reptiles maintain high activity temperatures to maximize foraging efficiency and increase reproductive success (Dawson, 1975; Avery et al., 1982). Changes in photoperiod and decreased temperatures associated with winter months can prevent a reptile's ability to thermoregulate properly. Physiological and behavioral performances are ultimately compromised when body temperatures fall below the preferred range (Dawson, 1975). Decreased efficiency in thyroid and reproductive organ function, metabolic activities, immune response, digestive abilities, and muscle function can lead to increased mortality through high predation rates or shutdown of important organs, such as the

brain and heart (Bartholomew & Tucker, 1963; Dawson, 1975; Harlow et al., 1976; Huey & Kingsolver, 1989; Beaupre et al., 1993; Wood & Gonzales, 1996). When thermoregulation is no longer feasible and activity costs outweigh the benefits, reptiles may enter dormancy by downregulating metabolic processes (Seebacher, 2005).

Appropriate overwintering refugia influences survival; however, selection processes differ between species, populations, and habitats. Suitable overwintering conditions are especially important for temperate and high-latitude species, where extreme climates increase the risk of freezing. Cooler temperatures and longer winters limit overwintering refugia to microhabitats with highly specific characteristics, such as sufficient insolation and high solar exposure (Hamilton & Nowak, 2009). Thus, overwintering refugia selection is extremely important for species in temperate and high-latitude regions. To survive low temperatures during the winter, snakes must reach deep enough to maintain a resilience zone that includes sufficient amounts of unflooded space and temperatures above 0°C (Markle et al., 2020). Because of their subterranean refugia, they may be more susceptible to changes in environmental conditions, such as flooding and snowfall, which increase winter mortality (Yagi et al., 2020). Some studies have shown Eastern Massasaugas experience 11-43% overwinter mortality rates, suggesting suitable overwintering refugia may be a limiting factor for populations (Harvey & Weatherhead, 2006a). Because of the significance of overwintering habitat in long-term survival, conservation plans must include knowledge of overwintering refugia. However, more research is needed to address Eastern Massasauga's needs and preferences regarding overwintering sites.

Eastern Massasauga overwintering refugia show variation based on the availability of habitat. In New York, Eastern Massasaugas use sphagnum hammocks within peatlands to overwinter (Johnson, 1995; Johnson et al., 2000; Smolarz, 2018). In contrast, an Ontario

population favors root systems or rock crevices within wetlands or coniferous forests (Weatherhead & Prior, 1992; Harvey & Weatherhead, 2006b). Across most of their range, though, these snakes use crayfish or small mammal burrows within or near low wet habitats (Reinert & Kodrich, 1982; Johnson et al., 2000; Dreslik, 2005; Smith, 2009). Despite the obvious external differences, Eastern Massasauga refugia share similar characteristics. Specifically, overwintering sites must provide vital elements, including protection from freezing, desiccation, and predation during dormancy (Shoemaker, 2007). Across their range, Eastern Massasaugas employ similar strategies for winter survival; specifically, they retreat underground and partially submerge themselves in groundwater to prevent freezing (Markle et al., 2020). As climate change increases the intensity of winter conditions, overwintering site availability and suitability becomes more vital to Eastern Massasauga survival. In particular, southwestern populations have faced high levels of loss due to changing environmental pressures on their habitat (Pomara et al., 2013). Between Iowa, Wisconsin, and Illinois, researchers estimated only 13 isolated populations remain, with habitat loss, modification, and degradation noted as some of the primary threats (Szymanski, 1998; Dreslik et al., 2017). None of these populations are considered secure due to a lack of environmental stability, stressing the importance of protecting important habitats, such as overwintering refugia (Szymanski, 1998).

Habitat suitability models have become popular tools in wildlife conservation and natural resource management. Researchers can predict species distributions by identifying spatial associations between occurrence data and environmental variables (Valencia- Rodriguez et al., 2021). Not only are ENMs useful for predicting potential occurrences of rare or cryptic species, but these tools provide a method to identify important habitats and associated environmental conditions. Conserving important habitats is vital for unstable or at-risk populations (Szymanski,

1998); however, protection can be difficult to obtain, especially for rare, cryptic, or difficult-to-study species with significant data gaps. One study suggests the time it takes to understand habitat needs for protection may threaten the long-term survival of the focal species (Martin et al., 2017). For Eastern Massasaugas, habitat protection is a priority. In particular, southwest populations have faced markedly high rates of loss due to habitat loss and degradation, which can be attributed to the effects of climate change and human presence (Szymanski, 1998; Dreslik et al., 2017). To receive protection, managers require data on spatial and biological requirements, habitat use, and suitable habitat availability. Previous work exists regarding Eastern Massasauga habitat preferences, but these studies primarily addressed microhabitat characteristics, and results were never repeated to identify range-wide trends (Reinert & Kodrich, 1982; Weatherhead & Prior, 1992; Johnson et al., 2000; Harvey & Weatherhead, 2006b; Moore & Gillingham, 2006; Smith, 2009; Smolarz et al., 2018; Yagi et al., 2020). Data regarding overwintering habitat is even more limited, with most focusing on northern populations (Smith, 2009; Smolarz et al., 2018; Yagi et al., 2020).

Future work should prioritize protecting these highly threatened southwestern areas and seek to understand overwintering site selection processes on a larger scale. However, studying trends in habitat may benefit from long-term data, which does not exist for many snake species. Scientists have attributed data deficiencies in snake populations to historical disinterest in studying reptile species (Gibbons et al., 2000). Even with increased attention, though, most reptiles lack formal status under the IUCN Red List. Listings often cite unknown population status (33%) and uncertain threats (23%) as the primary reasons for the lack of assessment (Bland and Böhm, 2016). Collecting the long-term data needed to address these deficiencies can be difficult. Snakes, in particular, present a challenge for researchers. In addition to their cryptic

natures and limited activity, snakes' slow life history makes studying long-term survival even more challenging (Seigel et al., 2001; Mazerolle et al., 2007). Reproductive physiology and behaviors are important considerations for population management (Dorcas & Willson, 2009), but for species reaching sexual maturity at 1-7 years, resource availability may prevent implementing such studies (Shine, 1978; Shine & Charnov, 1992; Rollings et al., 2017). Even with the challenges of capture, long-term datasets needed for population viability analyses, understanding demographic trends, and identifying threats will likely be the most useful in management efforts (Seigel et al., 1998). Despite data deficiencies, herpetologists have a consensus snake populations are declining worldwide (Gibbons et al., 2000). As extirpation rates continue to grow, alternate methods to collect long-term data may be necessary.

A popular tool in wildlife conservation is environmental DNA (eDNA), or identifiable genetic material left in water, air, or sediment (Barnes & Turner, 2016; Beng & Corlett, 2020). Previous work with the method has primarily been successful in aquatic environments where broad DNA dispersal and slow degradation result in higher detectability (Beng & Corlett, 2020). In particular, researchers have found excellent detection rates in fish and amphibians, whose wet skin and mucous coating shed abundant eDNA into water (Ratsch, 2019). Terrestrial eDNA studies using soil or sediment samples are limited, but recent work suggests high levels of success, particularly in mammals and arthropods (Leempoel et al., 2020; Valentin et al., 2020; Valentin et al., 2021; Allen et al., 2021). eDNA monitoring for semi-aquatic or terrestrial snakes has had mixed results. Researchers found higher levels of success when working with larger or more common species and when using greater volume samples from flowing water sources (Piaggio et al., 2014; Hunter et al., 2019; Adams et al., 2019). Soil also proved to be a relatively successful sampling medium for terrestrial eDNA detection in several snake species

(Kucherenko et al., 2018; Katz et al., 2021; Matthias et al., 2021). Detection rates are often higher in sediment than in water samples (Bairoliya et al., 2022), likely due to the ability of DNA to bind to mineral or humic particles in the soil, protecting the DNA from degradation (Sakata et al., 2020). Conversely, studies on smaller, rarer species or those using small-volume samples from standing water bodies, such as crayfish burrows, consistently had low detection levels (Halstead et al., 2017; Kucherenko et al., 2018; Rose et al., 2019; Ratsch et al., 2020). Sediment and water samples collected from crayfish burrows often showed low detection rates and high levels of inhibition from these groundwater systems (Halstead et al., 2017; Merkling, 2018; Niemeiller et al., 2018; Ratsch et al., 2020). High levels of degradation in loamy soils (Buxton et al., 2018), reduced stability in moist soils (Bairoliya et al., 2022), and the introduction of inhibitors by humic substances (Buxton et al., 2017) may reduce detection in certain sediment samples.

Burrows used for overwintering or summer refuge should provide samples with high DNA concentrations because of long periods of occupation. However, studies using burrow samples for species such as the Kirtland's Snake (*C. kirtlandii*), Burmese Python (*Python bivittatus*), and Giant Gartersnake (*Thamnophis gigas*) had poor results (Halstead et al., 2017; Kucherenko et al., 2018; Ratsch et al., 2020). eDNA studies involving samples from Eastern Massasauga overwintering refugia had low detection levels compared to traditional monitoring methods (Baker et al., 2018; Merkling, 2019). In Illinois, Baker et al. (2018) detected Eastern Massasauga DNA in only two of 100 crayfish burrows. Similarly, Merkling (2019) found only one positive detection out of 60 burrows in a Michigan nature preserve. Lack of detection could result from environmental factors, such as high degradation rates, dilution due to spring rains, presence of inhibitory organic material, or extraction/assay errors (Buxton et al., 2017). The

Eastern Massasaugas' small size, reduced shedding during inactive periods, and dispersal

behaviors may contribute to poor results (Ratsch, 2019). Despite difficulties with the method,

eDNA studies for the Eastern Massasauga remain limited and present the opportunity to improve

sampling and extraction techniques and increase detection rates. For example, Baker et al. (2018)

centrifuged their burrow samples to separate water from sediment, which may have reduced

eDNA concentrations in the water (Katz et al., 2021) but did not include sediment samples in

further steps. Merkling (2019) did test sediment samples; however, only one sample was

collected from each burrow and separated into phases, meaning there were no true sample

replicates. Increasing sample replicates and including sediment samples may improve eDNA

detection rates. eDNA testing should continue with the hope of providing a relatively quick and

inexpensive method of collecting presence data.

My research addresses gaps in knowledge for the Eastern Massasauga by applying two

alternate detection techniques to a population cluster located in Carlyle, Clinton County, Illinois.

In Chapter 2, I developed a presence-only ENM for the Eastern Massasauga in a mesic prairie

ecosystem to identify important environmental characteristics and predict suitable overwintering

sites in the Carlyle Lake region. In Chapter 3, I improved a previously described eDNA assay

protocol by incorporating additional sampling techniques and comparing detection capabilities to

traditional capture-recapture surveys.

<div align="center">General Methods</div>

Study Species

Only one cluster of Eastern Massasauga populations remains in Illinois, centered around Carlyle

Lake, Clinton County, Illinois. Researchers considered Carlyle's Eastern Massasaugas one highly

fragmented population, with recent work suggesting at least three genetically distinct populations exist around the lake (Anthonysamy et al., 2022). Since 1999, capture-mark-recapture monitoring has occurred at sites around Carlyle Lake, providing decades of population and demographic data. The long-term data have addressed demographic trends, population viability, and sources of mortality within the Carlyle Lake region (Shepard et al., 2008; Baker et al., 2016). Additionally, researchers conducted work on physiological processes, including reproduction (Dreslik, 2005; Aldridge et al., 2008; Baker et al., 2016), thermal behaviors (Dreslik et al., 2003), disease (Allender et al., 2011; Allender et al., 2016) and genetic diversity (Baker et al., 2016; Baker et al., 2018; Anthonysamy et al., 2022). Radio-telemetry research has examined spatial ecological factors such as daily movements and home ranges (Dreslik et al., 2017) and mate acquisition (Jellen et al., 2007). Although previous work identified active season habitat preferences (Dreslik, 2005), there is limited focus on understanding overwintering refugia selection. Data regarding overwintering refugia typically exists for use in other research, such as focusing search efforts (Dreslik et al., 2011) or calculating range and daily movement (Dreslik, 2005).

Study Area

Carlyle Lake was built in 1967 and is the largest impounded reservoir in Illinois, with 10,500 ha of open water surrounded by a narrow band of 4,500 ha of degraded grasslands, wetlands, and some upland and bottomland forests (Crawford et al., 2020). However, human development and agricultural conversion have fragmented the larger landscape, creating smaller isolated sites, with some not even a mile wide (Dreslik, 2005). Public land in Carlyle is managed by either the Illinois Department of Natural Resources (IDNR) or the U.S. Army Corp of Engineers

(USCOE). Management practices include prescribed burns to maintain early successional habitats, native grass planting for prairie restoration, and chemical control of invasive plant species. The area is also popular for outdoor recreationists, receiving up to three million visitors annually for hunting, fishing, camping, and boating.

Around the southern portion of the lake, Eastern Massasauga populations occur in three distinct areas: South Shore State Park (SSSP), Eldon Hazlet State Park (EHSP), and Dam East/West Recreation Area (DERA/DWRA). SSSP is approximately 123.4 ha, covering 3 miles of Carlyle Lake's southeastern shoreline and managed by the IDNR and USCOE. It comprises a fire-managed grassland habitat characterized by seasonal flooding, sparse upland woodlands (Dreslik, 2005) and picnic areas. Portions of the park are managed for hunting, consisting of multiple grassland fields with scattered woodlands, scrub areas, and restored savanna habitats. Currently, it contains the largest Eastern Massasauga population within the Carlyle Lake cluster.

EHSP is located on the western shoreline of Carlyle Lake. The 944.9 ha park consists primarily of mature oak-hickory forests, upland riparian zones, fallowed grassland, and restored tallgrass prairie (Dreslik, 2005). The IDNR also manages fields for dove and pheasant hunting. Extensive areas of pine-oak flatwoods have been invaded by olive trees (*Eleagnus* sp.), wild rye species (*Elymus*), and Stoutwood Reed (*Cinna arundinacea*). Habitats are largely fire-managed grasslands located along or near roads and have a high frequency of human-wildlife interactions.

DERA is a 96.7 ha recreation area comprised of fallowed grasslands, restored prairies, and bottomland forest habitats (Dreslik, 2005) managed by the USCOE. It is located on the southern shore of Carlyle Lake, directly east of the Kaskaskia River channel. The Kaskaskia River's old channel forms the site's eastern and southern boundary. Second and third-growth woodlands and

a road on the northeastern edge define the boundaries of this site. A small RV campsite is in the center of the site, surrounded by fire-managed grasslands.

DWRA is an 85.4 ha recreation area comprised of fallowed grasslands, restored prairie, and degraded bottomland forest habitats (Dreslik, 2005), also managed by the USCOE. Located on the southern shore of Carlyle Lake, the site is across the river channel from Dam East. The site is bordered by second and third-growth woodlands, with a road along the northeast edge. It contains large group picnic areas, a boat ramp, 118 campsites, and a visitor center.

Literature Cited

Adams, C.I.M., Hoekstra, L.A., Muell, M.R., and Janzen, F.J. 2019. A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA under field conditions. Diversity 11:50.

Aldridge, R.D., Jellen, B.C., Allender, M.C., Dreslik, M.J., Shepard, D.B., Cox, J.M., et al. 2008. Reproductive biology of the massasauga (*Sistrurus catenatus*) from South-Central Illinois. The Biology of Rattlesnakes (Hayes, W.K., Beaman, K.R., Cardwell, M.D., and Bush, S.P., eds.). Loma Linda University Press, Loma Linda, California 9 p.

Allen, M.C., Nielsen, A.L., Peterson, D.L., and Lockwood, J.L. 2021. Terrestrial eDNA survey outperforms conventional approach for detecting an invasive pest insect within an agricultural ecosystem. Environmental DNA 3:1102—1112.

Allender, M.C., Dreslik, M., Wylie, S., Phillips, C., Wylie, D.B., Maddox, C., et al. 2011. *Chrysosporium sp*. infection in Eastern Massasauga rattlesnakes. Emerging Infectious Diseases 17:2383—2384.

Allender, M.C., Phillips, C.A., Baker, S.J., Wylie, D.B., Narotsky, A., and Dreslik, M.J. 2016. Hematology in an Eastern Massasauga (*Sistrurus catenatus*) population and the emergence of *Ophidiomyces* in Illinois, USA. Journal of Wildlife Diseases 52:258—269.

Anderson, S. 1927. Areography of North American fishes, amphibians, and reptiles. American Museum Novitates 2802:15—16.

Anthonysamy, W.J.B., Dreslik, M.J., Baker, S.J., Davis, M.A., Douglas, M.R., Douglas, M.E., et al. 2022. Limited gene flow and pronounced population genetic structure of Eastern Massasauga (*Sistrurus catenatus*) in a Midwestern prairie remnant. PLOS ONE 17:e0265666.

Avery, R.A., Bedford, J.D. and Newcombe, C.P. 1982. The role of thermoregulation in lizard biology: Predatory efficiency in a temperate diurnal basker. Behavioral Ecology and Sociobiology 11:261—267.

Bailey, R.L., Campa, H., Harrison, T.M., and Bissell, K. 2011. Survival of Eastern Massasauga rattlesnakes (*Sistrurus catenatus catenatus*) in Michigan. Herpetologica 67:167—173.

Baker, S.J. 2016a. Life and death in a corn desert oasis: reproduction, mortality, genetic diversity, and viability of Illinois' last Eastern Massasauga population. Ph.D. Dissertation. University of Illinois Urbana-Champaign, Urbana-Champaign, Illinois 131 p.

Baker, S.J., Dreslik, M.J., Wylie, D.B., and Phillips, C.A. 2016b. Sources of mortality in the endangered Eastern Massasauga (*Sistrurus catenatus*) in Illinois. Herpetological Conservation and Biology 11:335—343.

Baker, S.J., Anthonysamy, W.J.B., Davis, M.A., Dreslik, M.J., Douglas, M.R., Douglas, M.E., et al. 2018. Temporal patterns of genetic diversity in an imperiled population of the Eastern Massasauga rattlesnake (*Sistrurus catenatus*). Copeia 106:414—420.

Barnes, M.A. and Turner, C.R. 2016. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics 17:1—17.

Bartholomew, G.A. and Tucker, V.A. 1963. Control of changes in body temperature, metabolism, and circulation by the Agamid lizard, *Amphibolurus barbatus*. Physiological Zoology 36:199—218.

Beaupre, S.J., Dunham, A.E., and Overall, K.L. 1993. The effects of consumption rate and temperature on apparent digestibility coefficient, urate production, metabolizable energy coefficient and passage time in Canyon lizards (*Sceloporus merriami*) from two populations. Functional Ecology 7:273—280.

Beng, K.C. and Corlett, R.T. 2020. Applications of environmental DNA (eDNA) in ecology and
conservation: Opportunities, challenges and prospects. Biodiversity and Conservation
29:2089—2121.

Bland, L.M. and Böhm, M. 2016. Overcoming data deficiency in reptiles. Biological
Conservation 204:16—22.

Böhm, M., Collen, B., Baillie, J.E.M., Bowles, P., Chanson, J., Cox, N., et al. 2013. The
conservation status of the world's reptiles. Biological Conservation 157:372—385.

Cagle, N.L. 2008. Snake species distributions and temperate grasslands: A case study from the
American tallgrass prairie. Biological Conservation 141:744—755.

Carbutt, C., Henwood, W.D., and Gilfedder, L.A. 2017. Global plight of native temperate
grasslands: Going, going, gone? Biodiversity and Conservation 26:2911—2932.

Chapin III, F.S., Zavaleta, E.S., Eviner, V.T., Rosamond, N.L., Vitousek, P.M., Reynolds, H.L.,
et al. 2000. Consequences of changing biodiversity. Nature 405:234—242.

Cobb, V.A. and Peterson, C.R. 2008. Thermal ecology of hibernation in a population of Great
Basin rattlesnakes. The Biology of Rattlesnakes (Hayes, W., Beaman, K., Cardwell, M.,
and Bush, S. eds). Loma Linda University Press, Loma Linda, California 11 p.

Corbett, E.A. 2004. A comparison of Illinois remnant prairies, 1976 to 1988. Proceedings of the
North American Prairie Conferences. University of Wisconsin-Madison, Madison,
Wisconsin 65:36—43.

COSEWIC. 2012. COSEWIC assessment and status report on the Massasauga *Sistrurus
catenatus* in Canada. Committee on the Status of Endangered Wildlife in Canada.
Ottawa, Canada 84 p.

Crawford, J.A., Dreslik, M.J., Baker, S.J., Phillips, C.A., and Peterman, W.E. 2020. Factors

affecting the detection of an imperiled and cryptic species. Diversity 12:177.

Danou, C.H. 1997. Habitat use and movement patterns of the Eastern Massasauga rattlesnake in Wisconsin. MSc Thesis. University of Wisconsin-Stevens Point, Stevens Point, Wisconsin 38 p.

Dawson, W.R. 1975. On the physiological significance of the preferred body temperatures of reptiles. Perspectives of Biophysical Ecology (Gates, D., Schmerl, R., eds). Springer, Heidelberg, Germany 30 p.

Dorcas, M.E., and Willson, J.D. 2009. Innovative methods for studies of snake ecology and conservation snakes. Snakes: Ecology and Conservation (Mullin, S.J., Seigel, R.A., eds). Cornell University Press, Ithaca, New York 32 p.

Dreslik, M.J. 2005. Ecology of the Eastern Massasauga (*Sistrurus catenatus catenatus*) from Carlyle Lake, Clinton County, Illinois. Ph.D. Dissertation. University of Illinois Urbana-Champaign, Urbana, Illinois 393 p.

Dreslik, M.J., Shepard, D.B., Jellen, B.C., and Phillips, C.A. 2017. Movement and home range of the Eastern Massasauga (*Sistrurus catenatus*) at its southern range limit. The Biology of Rattlesnakes II (Dreslik, M.J., Hayes, W.K., Beaupre, S.J., Mackessy, S.P., eds.). ECO Herpetological Publishing and Distribution, Rodeo, New Mexico 10 p.

Dreslik, M.J., Wylie, S.J., Davis, M.A., Wylie, D.B., and Phillips, C.A. 2011. Demography of the Eastern Massasauga (*Sistrurus c. catenatus*) at Carlyle Lake, Illinois. Illinois Natural History Survey, Champaign, Illinois 14:1—32.

Durbian, F.E., King, R.S., Crabill, T., Lambert-Doherty, H. and Seigel, R.A. 2008. Massasauga home range patterns in the Midwest. The Journal of Wildlife Management 72:754—759.

Gibbons, J.W., Scott, D.E., Ryan, T.J., Buhlmann, K.A., Tuberville, T.D., Metts, BS., et al.

2000. The global decline of reptiles, déjà vu amphibians: Reptile species are declining on

a global scale. Six significant threats to reptile populations are habitat loss and

degradation, introduced invasive species, environmental pollution, disease, unsustainable

use, and global climate change. BioScience 50:653—666.

Halstead, B.J., Wood, D.A., Bowen, L., Waters, S.C., Vandergast, A.G., Erson, J.S., et al. 2017.

An evaluation of the efficacy of using environmental DNA (eDNA) to detect giant

gartersnakes (*Thamnophis gigas*). U.S. Geological Survey Open-File Report, Reston,

Virginia p. 41.

Hamilton, B.T. and Nowak, E.M. 2009. Relationships between insolation and rattlesnake

hibernacula. Western North American Naturalist 69:319—328.

Harlow, H., Hillman, S., and Hoffman, M. 1976. The effect of temperature on digestive

efficiency in the herbivorous lizard, *Dipsosaurus dorsalis*. Journal of Comparative

Physiology 111:1—6.

Harvey, D.S. and Weatherhead, P.J. 2006a. Hibernation site selection by Eastern Massasauga

rattlesnakes (*Sistrurus catenatus catenatus*) near their northern range limit. Journal of

Herpetology 40:66—73.

Harvey, D.S. and Weatherhead, P.J. 2006b. A test of the hierarchical model of habitat selection

using Eastern Massasauga rattlesnakes (*Sistrurus c. catenatus*). Biological Conservation

130:206—216.

Henwood, W.D. 2010. Towards a strategy for the conservation and protection of the world's

temperate grasslands. Great Plains Research 20:121—134.

Hoekstra, J.M., Boucher, T.M., Ricketts, T.H. and Roberts, C. 2005. Confronting a biome crisis:

Global disparities of habitat loss and protection. Ecology Letters 8:23—29.

Huey, R.B. and Kingsolver, J.G. 1989. Evolution of thermal sensitivity of ectotherm

    performance. Trends in Ecology and Evolution 4:131—135.

Hunter, M.E., Ferrante, J.A., Meigs-Friend, G., and Ulmer, A. 2019. Improving eDNA yield

    and inhibitor reduction through increased water volumes and multi-filter isolation

    techniques. Scientific Reports 9:5259.

Jellen, B.C., Shepard, D.B., Dreslik, M.J., and Phillips, C.A. 2007. Male movement and body

    size affect mate acquisition in the Eastern Massasauga (*Sistrurus catenatus*). Journal of

    Herpetology 41:451—457.

Johnson, G.1995. Spatial ecology, habitat preference, and habitat management of the Eastern

    Massasauga, *Sistrurus c. catenatus* in a New York weakly-minerotrophic peatland. Ph.D.

    Dissertation. State University of New York College of Environmental Science and

    Forestry, Syracuse, New York 158 p.

Johnson, G., Kingsbury, B., King, R., Parent, C., Seigel, R., and Szymanski, J. 2000. The Eastern

    Massasauga rattlesnake: A handbook for land managers. U.S. Fish and Wildlife

    Service, Fort Snelling, Minnesota 52 p

Jones, P.C., King, R.B., Bailey, R.L., Bieser, N.D., Bissell, K. Campa III, H., et al. 2012.

    Range-wide analysis of Eastern Massasauga survivorship. Journal of Wildlife

    Management 76:1576—1586.

Katz, A.D., Harper, L.R., Sternhagen, E.C., Pearce, S.E., Melder, C.A., Sperry, J.H., et al. 2021.

    Environmental DNA is effective in detecting the federally threatened Louisiana

    Pinesnake (*Pituophis ruthveni*). Environmental DNA 3:409—425.

Kucherenko, A., Herman, J.E., Everham III, E.M. and Urakawa, H. 2018. Terrestrial snake

    environmental DNA accumulation and degradation dynamics and its environmental

application. Herpetologica 74:38—49.

Leempoel, K., Hebert, T., and Hadly, E.A. 2020. A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. Proceedings of the Royal Society B: Biological Sciences 287.

Lukoschek, V., Beger, M., Ceccarelli, D., Richards, Z., and Pratchett, M. 2013. Enigmatic declines of Australia's sea snakes from a biodiversity hotspot. Biological Conservation 166:191—202.

Markle, C.E., Moore, P.A., and Waddington, J.M. 2020. Primary drivers of reptile overwintering habitat suitability: Integrating wetland ecohydrology and spatial complexity. BioScience 70:597—609.

Marshall, J.C., Manning, J.V., and Kingsbury, B.A. 2006. Movement and macrohabitat selection of the Eastern Massasauga in a fen habitat. Herpetologica 62:141—15.

Martin, T.G., Camaclang, A.E., Possingham, H.P., Maguire, L.A., and Chadès, I. 2017. Timing of protection of critical habitat matters. Conservation Letters 10:308—316.

Mazerolle, M.J., Bailey, L.L., Kendall, W.L., Royle, A.J., Converse, S.J., and Nichols, J.D. 2007. Making great leaps forward: Accounting for detectability in herpetological field studies. Journal of Herpetology 41:672—689.

Merkling, J. 2019. Development of an environmental DNA assay for Eastern Massasauga. M.Sc. Thesis. Purdue University Graduate School, West Lafayette, Indiana 61 p.

Moore, J.A. and Gillingham, J.C. 2006. Spatial ecology and multi-scale habitat selection by a threatened rattlesnake: The Eastern Massasauga (*Sistrurus catenatus catenatus*). Copeia 4:742—751.

Nordberg, E.J. 2013. Thermal ecology and behavioral activity in hibernating Timber

rattlesnakes (*Crotalus horridus*). M.Sc Thesis. Middle Tennessee State University, Murfreesboro, Tennessee 53 p.

Piaggio A.J., Engeman R.M., Hopken, M.W., Humphrey J.S., Keacher K.L., Bruce W.E., et al. 2014. Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resource 14:374—80.

Pievani, T. 2014. The sixth mass extinction: Anthropocene and the human impact on biodiversity. Rendiconti Lincei. Scienze Fisiche e Naturali 25:85—93.

Pomara, L.Y., Ledee, O.E., Martin, K.J., and Zuckerberg, B. 2013. Demographic consequences of climate change and land cover help explain a history of extirpations and range contraction in a declining snake species. Global Change Biology 20:2087—2099.

Ratsch, R. 2019. Efficacy of using environmental DNA (eDNA) to detect Kirtland's snakes (*Clonophis kirtlandii*). M.Sc. Thesis. Purdue University Graduate School, West Lafayette, Indiana 54 p.

Ratsch, R., Kingsbury, B.A., and Jordan, M.A. 2020. Exploration of environmental DNA (eDNA) to detect Kirtland's snake (*Clonophis kirtlandii*). Animals: An Open Access Journal from MDPI 10:1057.

Reading, C.J., Luiselli, L.M., Akani, G.C., Bonnet, X., Amori, G., Ballouard, J.M., et al. 2010. Are snake populations in widespread decline? Biology Letters 6:777—780.

Reinert, H.K. and Kodrich, W.R. 1982. Movements and habitat utilization by the massasauga *Sistrurus catenatus catenatus*. Journal of Herpetology 16:162—171.

Rollings, N., Uhrig, E.J., Krohmer, R.W., Waye, H.L., Mason, R.T., Olsson, M., et al. 2017.

Age-related sex differences in body condition and telomere dynamics of red-sided garter snakes. Proceedings of the Royal Society B: Biological Sciences 284.

Root, T.L. and Schneider, S.H. 2002. Climate change: Overview and implications for wildlife. Wildlife Responses to Climate Change: North American Case Studies 10:765—766.

Seebacher, F. and Franklin, C.E. 2005. Physiological mechanisms of thermoregulation in reptiles: A review. Journal of Comparative Physiology 175:533—541.

Seigel, R.A. and Collins, J.T. 2001. Snakes: Ecology and behavior. Blackburn Press, Caldwell, New Jersey 414 p.

Shepard, D.B., Dreslik, M.J., Jellen, B.C., and Phillips, C.A. 2008. Reptile road mortality around an oasis in the Illinois corn desert with emphasis on the endangered Eastern Massasauga. Copeia 2:350—359.

Shine, R. 1978. Growth rates and sexual maturation in six species of Australian elapid snakes. Herpetologica 34:73—79.

Shine, R. and Charnov, E.L. 1992. Patterns of survival, growth, and maturation in snakes and lizards. The American Naturalist 139:1257—1269.

Shoemaker, K.T. 2007. Habitat manipulation as a viable strategy for the conservation of the massasauga rattlesnake in New York State. M.Sc Thesis. State University of New York. Albany, New York 109 p.

Smith, C.S. 2009. Hibernation of the Eastern Massasauga rattlesnake (*Sistrurus catenatus catenatus)* in northern Michigan. M.Sc. Thesis. Purdue University Graduate School. West Lafayette, Indiana 54 p.

Smolarz, A.G., Moore, P.A., Markle, C.E., and Waddington, J.M. 2018. Identifying resilient

Eastern Massasauga rattlesnake (*Sistrurus catenatus*) peatland hummock hibernacula. Canadian Journal of Zoology 96:1024—1031.

Suloway, L. and Hubbell, M. 1994. Wetland resources of Illinois: An analysis and atlas. Illinois Natural History Survey Special Publication 15, Champaign, Illinois 88 p.

Szymanski, J. 1998. Status assessment for Eastern Massasauga (*Sistrurus c. catenatus*). Report. U.S. Fish and Wildlife Service, Endangered Species Division, Fort Snelling, Minnesota 31 p.

Szymanksi, J., Pollack, C., Ragan, L., Redmer, M., Clemency, L., Voorhies, K., and Jaka, J. 2015. Species status assessment for the Eastern Massasauga rattlesnake (*Sistrurus catenatus*). U.S. Fish and Wildlife Service, Washington, D.C. 115 p.

U.S. Fish and Wildlife Service. 2016. Endangered and threatened wildlife and plants: Threatened species status for the Eastern Massasauga rattlesnake. Federal Register 81:67193—67214.

Valencia-Rodríguez, D., Jiménez-Segura, L., Rogéliz C.A., and Parra J.L. 2021. Ecological niche modeling as an effective tool to predict the distribution of freshwater organisms: The case of the Sabaleta *Brycon henni* (Eigenmann, 1913). PLOS ONE 16:e0247876.

Valentin, R.E., Fonseca, D.M., Gable, S., Kyle, K.E., Hamilton, G.C., Nielsen, A.L., et al. 2020. Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects. Molecular Ecology Resources 203:746—755.

Valentin, R.E., Kyle, K.E., Allen, M.C., Welbourne, D.J., and Lockwood, J.L. 2021. The state, transport, and fate of aboveground terrestrial arthropod eDNA. Environmental DNA 3:1081—1092.

Weatherhead, P.J. and Prior, K.A. 1992. Preliminary observations of habitat use and movements

of the Eastern Massasauga rattlesnake (*Sistrurus c. catenatus*). Journal of Herpetology 26:447—452.

White, R.P., Murray, S., and Rohweder, M. 2000. Pilot analysis of global ecosystems: Grassland ecosystems. World Resources Institute, Washington, D.C. 81 p.

Wood S.C. and Gonzales, R. 1996. Hypothermia in hypoxic animals: Mechanisms, mediators, and functional significance. Comparative Biochemistry and Physiology B: Biochemical and Molecular Biology 113:37—43.

Yagi, A.R., Planck, R.J., Yagi, K.T., and Tattersall, G.J. 2020. A long-term study on massasaugas (*Sistrurus catenatus*) inhabiting a partially mined peatland: A standardized method to characterize snake overwintering habitat. Journal of Herpetology 54:235—244

Zipkin, E.F., DiRenzo, G.V., Ray, J.M., Rossman, S., and Lips, K.R. 2020. Tropical snake diversity collapses after widespread amphibian loss. Science 367:814—816.

CHAPTER 2: OVERWINTER HABITAT MODELING FOR THE EASTERN

MASSASAUGA RATTLESNAKE IN A SOUTHWESTERN MESIC PRAIRIE

Introduction

Earth is experiencing extreme anthropogenic climate change and land conversion, resulting in the fragmentation, degradation, and destruction of important habitats. Such anthropogenic development and agricultural activities resulting in direct habitat loss have driven many species to extinction (Chapin et al., 2000; Hagen and Hodges, 2006; Gonclaves-Souza et al., 2020). Globally, 20.8% of all land areas have been converted for human use (Hoekstra et al., 2005), leading to an estimated loss of nearly 10% of all individual organisms (Banks-Leite et al., 2020). Researchers suggest the most severe regional extinction rates are closer to 75% (Gonclaves-Souza et al., 2020) in areas such as temperate grasslands and Mediterranean biomes, where habitat conversion exceeds habitat protection (Hoekstra et al., 2005). Implementing and extending land protection for vulnerable ecosystems is vital to curb extinction rates. To accurately identify important habitats, data on long-term species persistence, such as population dynamics, species behavior, and viability, should be included (Sidle, 1987; Hagen and Hodges, 2006; Camaclang et al., 2015; Martin et al., 2017). Collecting sufficient data can be difficult, especially for rare, cryptic, or hard-to-study species (Seigel & Collins, 2001; Mazerolle et al., 2007). In addition to low abundances, rare and endangered species often have reduced ranges, making it difficult to determine what habitat is truly unsuitable rather than simply unoccupied (Bartel and Sexton, 2009; Sousa-Silva et al., 2014). Recently, researchers have applied habitat suitability models to fill these data gaps and guide species conservation (Bartel & Sexton, 2009). ENMs are frequently used to identify potential habitats by connecting species presence to

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important environmental characteristics (Valencia- Rodriguez et al., 2021). A particularly useful form of ENMs is presence-only niche modeling, which allows the projection of potential habitats without the need for true absences, making it an invaluable tool for habitat protection for rare species. Despite advancements, data regarding habitat preferences and selection processes remains limited, meaning many species may not receive the land protection required for persistence (Martin et al., 2017).

Habitat loss is one of the largest contributing factors to the decline of endangered snake species globally and in the U.S. (Seigel et al., 1986). For species whose habitat choices are influenced by seasons or ontogeny, such as snakes, accurately evaluating habitat requirements is even more challenging (Mazerolle et al., 2007). Providing complete data on habitat selection through active, non-active, and, for females, gestational periods require time and resources that may not exist. Currently, most existing data is based disproportionately on habitats accessible to study, such as those used during active periods, when foraging, reproductive, and social behaviors are easily observable (Mazerolle et al., 2007). Despite a focus on active period habitat, overwintering habitat is equally crucial to survival at individual and population levels. Some snakes, especially those experiencing extreme seasonality in temperate or high-altitude environments, may spend half the year or more overwintering (Gienger & Beck, 2011). Depending on species and body condition, researchers estimate overwinter mortality rates range from ~11– 53% (Hirth, 1966; Gillingham & Carpenter, 1978; Kissner et al., 2005; Harvey & Weatherhead, 2006a). Environmental characteristics, such as weather and microhabitat features, have also been shown to affect survival, with cool temperatures, low humidity, and fluctuating water levels producing higher mortality rates (Costanzo, 1989; Kissner & Weatherhead, 2005). In addition to protection from harsh winter conditions, overwintering locations and

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characteristics provide opportunities following spring emergence, such as those associated with basking, reproduction, foraging, or dispersal (Gienger & Beck, 2011). Because of the high selectivity in site choice, appropriate overwintering habitat can be a limiting factor for many snake species (Rudolph et al., 2007).

One snake species threatened by habitat loss and degradation is the Eastern Massasauga rattlesnake, *Sistrurus cantenatus* (Szymanski, 1998; Dreslik, 2017), a thick-bodied, small rattlesnake distributed from eastern Iowa to western New York and southern Illinois to southern Ontario (Jones et al., 2012). Due to the effects of climate change, overgrowth of early successional habitat, and conversion of important mesic prairies, suitable habitat has been substantially reduced (Cagle, 2008; Pomara et al., 2013; Markle et al., 2020). As a result, the Eastern Massasauga was listed as threatened under the U.S. Endangered Species Act of 1973 and Canada's Federal Species at Risk Act (COSEWIC, 2012; USFWS, 2016). Current estimates suggest Eastern Massasauga populations have decreased by 38% range-wide, with western populations suffering the heaviest extirpations at a predicted rate of 97% over the next fifty years (USFWS, 2016). Protecting important habitats in the southwestern portion of the Eastern Massasauga's range is vital to maintaining stable populations but requires further research to understand habitat selection processes. Range-wide, Eastern Massasaugas preferentially use open, low-lying habitats with access to wetlands, often in the form of wet prairies, peatlands, and sedge meadows (Jones et al., 2016). Although they rarely use upland or heavily forested areas (Danou, 1997; Dreslik, 2005; Moore & Gillingham, 2006; Marshall et al., 2006; Durbian et al., 2007), landscapes with a canopy gradient provide important thermoregulatory opportunities and protection from predators (Szymanski, 1998). During the late spring, summer, and early fall, Eastern Massasaugas occupy more xeric habitats but return to mesic habitats in late fall for

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overwintering (Johnson et al., 2000; Bailey et al., 2012). Most frequently, the snakes use crayfish

or small mammal burrows within or near wetlands but sometimes occupy root systems, rock

crevices, or sphagnum hammocks in northern portions of their range (Weatherhead & Prior,

1992; Johnson, 1995; Johnson et al., 2000; Harvey & Weatherhead, 2006; Smolarz, 2018).

Information regarding specific overwintering site characteristics remains limited, with winter

habitat-focused studies addressing population preferences and selection processes largely absent.

Rather, previous work has largely focused on macrohabitat characteristics. Although studies

investigating the effect of soil attributes (Smith, 2009), water table positions (Smolarz et al.,

2018), and environmental stochasticity (Yagi et al., 2020) on overwintering site selection exist,

there remains no replication or broader applications to confirm consistent patterns. Other work

identifying macrohabitat scale preferences has revealed populational differences, rather than

similarities, related to vegetative cover and proximity to groundwater (Reinert & Kodrich, 1982;

Weatherhead & Prior, 1992; Johnson et al., 2000; Harvey & Weatherhead, 2006; Moore &

Gillingham, 2006). More data is needed to identify important habitats and determine what

physical and biological features Eastern Massasaugas select for overwintering sites.

  To address current data deficiencies for the Eastern Massasauga, I developed an ENM

using spring emergence data from Eastern Massasauga populations in mesic prairie habitat at its

southern range limit. My objective was to develop a habitat suitability model which can

accurately and precisely predict Eastern Massasauga overwintering sites and provide information

regarding the importance of environmental variables in selection processes. Locally, the results

allow us to identify potential overwintering sites for additional monitoring, protection, or

restoration projects. Classifying environmental predictors useful in predicting Eastern

Massasauga presence at a broader scale could create guidelines for future use across other

overwintering sites. Specifically, researchers can tailor the methods and variables I described for other populations occupying similar habitats. A standard modeling framework for Eastern Massasauga overwintering sites provides a method to reduce data deficiencies regarding overwintering site use and selection and helps guide habitat protection and restoration. Understanding and documenting vital overwintering habitats is necessary to protect populations across the Eastern Massasauga range and ensure viability into the future.

Methods

Modeling Software

To predict suitable Eastern Massasauga overwintering habitat, I used a maximum entropy approach with Maxent 4.4.1 (Phillips, 2017). Maxent is a machine learning method that estimates habitat suitability using presence-only data. Known for its user-friendly interface and easy configuration capabilities (Morales et al., 2017), Maxent is also consistently accurate, often outperforming more traditional presence-absence modeling (Elith et al., 2006; Elith & Graham, 2009). Additionally, Maxent has been found to perform well with small sample sizes, making it a valuable tool for predicting cryptic or endangered species presence (Costa et al., 2010; Guillera-Arroita et al., 2014; Pearson et al., 2006).

Presence Data

I obtained occurrence data from 23 years of visual encounter surveys conducted by the Illinois Natural History Survey in Carlyle, Illinois. Between 1999 and 2023, spring emergence surveys (late February to early May) produced 1,349 Eastern Massasauga encounters across 26 overwintering sites. During egress, Eastern Massasaugas remain near their winter burrows,

periodically moving above and below ground as the weather fluctuates. Snakes disperse to active

period habitats once ground temperatures reach equilibrium with air temperatures (Kingsbury,

2002; Szymanski, 2016). Based on the timing and environmental conditions during surveys, I

expected most encounters to occur at or near occupied burrows. Encounter records contained

individually assigned snake numbers, encounter date, type of encounter (initial, between year

recapture, within year recapture, or sighting), common site name, and UTM coordinate locations

with a spatial accuracy of 2-3 m. I pulled records from sites exhibiting relatively stable

populations throughout the study, including Dam East and Dam West Recreation Area

(DERA/DWRA), South Shore State Park (SSSP), and Eldon Hazlet State Park (EHSP). I then

filtered the encounters to remove within-year recaptures and incomplete records. To confirm

spatial accuracy, I viewed the coordinate locations in ArcMap and removed obvious outliers,

resulting in 1071 unique presence points. I filtered the presence data to avoid sampling biases so

no nearest neighbor was closer than 2.5 m. I chose this distance for spatial thinning to ensure

each 5 x 5 m raster cell was represented by ~1 presence point, thereby reducing clustering in

high-density areas and preventing overrepresentation in the sample data. I used 859 presence

locations in the final model (Figure 2.1). Surveys were conducted primarily during spring

emergence at known overwintering sites, meaning that true absence data was unavailable for the

study extent.

Environmental Variables

Initially, I considered environmental variables previously described as important for Eastern

Massasauga survival and habitat selection (Bailey et al., 2012; Johnson, 1995; Johnson et al.,

2000; Moore & Gillingham, 2006; Reinert & Kodrich, 1982; Weatherhead & Prior, 1992).

Bioclimatic variables are relatively accurate and can predict indirect and direct effects on species distribution (Guisan & Zimmermann, 2000); however, differences in large-scale climatic variables would likely be undetectable in small areas of analysis. For this model, I chose a relatively small study extent (113 km^2), which covered current and historically occupied overwintering sites in the southern portion of Carlyle Lake and minimized the inclusion of inappropriate land cover, such as surrounding croplands. As a result, bioclimatic variables were not used in my model. Instead, I focused on identifying microhabitat characteristics that could be easily transferred across different landscapes. After consideration, I selected nine environmental predictors related to land and soil characteristics, four to vegetative structure, and two to water access (Table 2.1).

Under land characteristics, I included elevation, radiation area, and soil types. Eastern Massasaugas rely on groundwater to prevent freezing and desiccation during overwinter dormancy, meaning habitat with access to the water table is crucial for winter survival (Szymanski, 1998; Shoemaker, 2007; Markle et al., 2020). As a result, Eastern Massasauga overwintering sites often occur in areas with low elevation, such as wetlands and other poorly drained areas (Reinert & Kodrich, 1982; Moore & Gillingham, 2006; Szymanski et al., 2016). Thus, I hypothesized that low elevations and poorly drained soils would be strong predictors. Additionally, due to the importance of basking after winter dormancy (Gienger & Beck, 2011; Szymanski et al., 2016), I expected high solar radiation to be a good indicator of suitable overwintering habitat. To construct a digital elevation model (DEM), I used ground points classified by 2021 LiDAR data from the Illinois Geospatial Clearing House (Table 2.1). Using the DEM and the Area Solar Radiation tool in ArcMap, I produced a raster with the incident solar radiation during the typical spring emergence period (March 1st – April 30th), when basking

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is vital for successful egress (Gienger & Beck, 2011; Johnson et al., 2016; Robillard & Johnson, 2015). I obtained categorical polygon data from the Web Soil Survey for soil (Table 2.1) and created a categorical raster layer using the Polygon to Raster conversion tool. Although including continuous variables can increase model performance (Phillips & Dudik, 2008), obtaining such data can be difficult, especially in areas where large-scale ecosystem monitoring does not exist. Unfortunately, useful continuous data related to soil characteristics were unavailable for the Carlyle Lake region. However, one of the benefits of Maxent is its ability to consider both continuous and categorical data and identify the interactions between these variables (Phillips et al., 2006). Even in categorical form, incorporating soil variables offers important information about environmental preferences that can be expounded on for future models or used to provide broad management recommendations (Phillips, 2006).

Under vegetation, I included canopy height, canopy cover, distance to medium-height vegetation (1.5 – 6.0 m), and distance to high vegetation (> 6.0 m). Canopy structure and vegetation makeup are essential in maintaining habitat, and data regarding these factors should guide overwintering site management. In particular, understanding the role of vegetation in overwintering site selection provides insight into winter survival requirements and habitat preferences during ingress and egress (Johnson et al., 2000; Harvey & Weatherhead, 2006). Due to the Eastern Massasauga's preferences for open-canopied habitat with low grasses and sedges (Jones et al., 2016), I expected canopy density to be one of the most influential variables in my model. I also expected low habitat suitability as canopy height increased and distance to high vegetation decreased. I isolated all LiDAR points classified as vegetation (low, medium, and high) to find canopy height and calculated point statistics. The points were then used to create a raster using LAS Point Statistics as Raster tool in ArcMap. I calculated the proportion of first

returns classified as vegetation in each cell to find canopy cover, creating a raster with values ranging from 0% to 100%. For distances to medium-height and high vegetation, I isolated classified LiDAR cells and converted the points to a raster. I then used the rasters and the Euclidean distance tool to produce a raster where each cell's value is the distance to the nearest medium-height or high vegetation point.

For water characteristics, I included distance to the nearest water source and flood risk in my model. Carlyle Lake is an impounded reservoir created to manage flooding of the Kaskaskia River. Although the reservoir was built to maintain high water levels, periods of heavy rainfall can lead to sudden increases, resulting in flooding surrounding areas and alteration of important habitats (Tucker et al., 1995). Eastern Massasaugas in Carlyle, Illinois, use crayfish burrows for overwintering (Baker, 2016), making them particularly vulnerable to lake-level changes. Due to the reliance on groundwater and susceptibility to flooding, I expected proximity to water and 100-year floodplains to be important predictors of Eastern Massasauga habitat. Using the same method for medium-height and high vegetation distances, I calculated the distance to the nearest water source, which included Carlyle Lake, associated tributaries, and other inland water bodies. I obtained polygon data from FEMA's National Flood Hazard Layer (NFHL) Viewer for flood risk (Table 2.1). I then created a categorical raster using the Polygon to Raster tool. Areas were classified as one of the following: 100-year floodplain or areas with 1% of annual chance of flooding (A); 100-year floodplain or areas with 1% of annual chance of flooding with base flood elevation defined (AE); or minimal flood hazard or areas with 0.2% chance of annual flooding (X). All layers were projected to the same geographic coordinate system and resampled to a 5 m resolution. They were masked using a 113km^2 polygon layer with major bodies of water removed and finally converted to ASCII formats for inclusion in Maxent.

Another consideration when selecting environmental variables is collinearity, or the linear

dependence between environmental predictors, which can negatively impact model accuracy,

cause over-fitting, and reduce the model's transferability (Merow et al., 2013; Jarnevich &

Young, 2015; Feng et al., 2019). Collinearity is especially difficult to avoid in ecological

research because many widely used environmental predictors are inherently linked (Feng et al.,

2019). Consequently, variable contribution may be due to complex synergistic relationships

common in biological data rather than independent effects (Graham, 2003). True importance

may be indistinguishable in highly correlated predictors, increasing the risk of removing relevant

variables. Some researchers suggest retaining relevant variables regardless of potential

collinearity because inclusion ensures important variables are present, allows consideration of

hypothesized species requirements, and can improve prediction accuracy (Braunish et al., 2013).

Additionally, some work has shown collinearity has little impact on overall performance because

of Maxent's ability to regulate model complexity (Phillips & Dudik, 2008; Elith et al., 2011;

Feng et al., 2019). Identifying potential dependencies and deciding if and how they should be

managed in spatial modeling is necessary to achieve a reliable model. To address collinearity, I

compared performance between a model with all nine environmental variables versus a model

with correlated variables removed. Using methods described by Wang et al. (2017), I ran two

models sequentially, eliminating any variables with low contributions (<1%) after each was

complete. I then ran a model with only the retained variables and quantified discriminatory

abilities, fit, and accuracy for comparison. Due to low contributions, I considered canopy cover,

radiation area, and distance to high vegetation for removal from my model. However, after

comparing performance between models, I found no improvement in discriminatory ability, data

fit, or accuracy when the variables were removed. Specifically, I found the model with all

variables performed similarly to the model with variables removed, with an excellent AUC, very

good TSS and kappa, and a low omission rate. The omission rate was improved at 0.100, but

TSS and kappa were slightly lower at 0.847 and 0.790. Although rankings changed slightly, soil,

distance to medium vegetation, and distance to water remained the highest contributors,

indicating these variables were important independent of other variables. Based on these results,

I retained all variables for the final model.

Model procedure

The default settings in Maxent have previously shown high performance (Phillips & Dudik,

2008; Wang et al., 2017); however, I was concerned about overfitting because of the high

number of presence points and small study extent. Recent work suggests tuning parameters in

individual models can reduce over-complexity or over-simplicity, which may come from default

settings (Morales et al., 2017). Some parameters affect the model's fit within the study extent,

specifically, the regularization multiplier and the number of background points. The

regularization multiplier affects how focused or closely fit the predicted distribution is to the

original data (Merow et al., 2013). It generates an error bound, allowing the model to create more

generalized predictions and preventing exact fit (Yan et al., 2021). A smaller value results in a

localized model with a tight fit to the original data, whereas a larger value results in a more

distributional spread (Phillips, 2021). I reduced overfitting and complexity by tuning the

multiplier to the data (Morales et al., 2017). Background point selection or pseudo-absence

affects the area under the receiver operator curve (AUC) and prediction distribution by altering

model entropy (Phillips & Dudik, 2008). AUC is a threshold-independent measure that reflects

the discriminatory ability of the model, specifically whether the model can distinguish between

true presence and pseudo-absence (Radosavljevic & Anderson, 2014). As the number of background points increases, entropy increases, resulting in a wider spread and uniform probability distribution (Barbet-Massin et al., 2012). As a result, AUC scores also improve; however, a plateau occurs likely because presence points may be situated in pixels with highly unsuitable environmental conditions (Phillips & Dudik, 2008). Altering the background points allowed me to control probability distribution across the study extent and improve AUC.

Before the final habitat suitability model, I optimized feature configuration by testing three regularization multiplier settings (1, 2, and 5) and three background point values (1,000, 5,000, and 10,000) in nine individual models. I subset the presence points, 75% for training and 25% for testing, and applied a "10th percentile minimum training presence" threshold. The threshold is commonly used to achieve ecologically applicable results because it reduces outliers by omitting 10% of presence points with the lowest suitability from the training data (Escalante et al., 2013; Redon & Luque, 2010). All other parameters were left as default. I selected the best-performing model settings for the final product using accuracy, sensitivity, and specificity metrics.

The final model had nine environmental variables and 859 presence points (Figure 2.2). To produce more statistically reliable results, I ran ten cross-validated model replicates. Cross-validation divides presence data into k folds; each is used as testing data once, whereas the other folds (k -1) are used for training (Radosavljevic & Anderson, 2014). Models are iterated k times until each fold performed as training and testing data (Dorji et al., 2020). Such validation ensures all data is used while providing additional information about model predictability, such as range, standard error, and averages.

Model performance

AUC is one of Maxent model performance's most commonly used evaluation methods, providing a threshold-independent measure of a model's discriminatory ability (Merow et al., 2013). AUC ranges from 0-1.0, where 1.0 represents perfect discrimination, and less than 0.5 is random. For the model, I evaluated AUC as follows: excellent (0.9-1.0), very good (0.8-0.9), good (0.7-0.8), fair (0.6-0.7), and poor (0.5-0.6) (Duan et al., 2014). Despite its popularity, many studies warn against sole reliance on AUC (Buckland et al., 2022; Lobo et al., 2008; West et al., 2016) because it does not reflect actual suitability accuracy (Lobo et al., 2008). To fully evaluate model performance, it is best practice to incorporate other metrics, such as those associated with sensitivity and specificity (West et al., 2016).

In addition to AUC, I incorporated three threshold-dependent evaluation methods in my analysis: data omission rates, Cohen's kappa, and true skills statistics. Omissions can identify overfitting by comparing omission rates in testing data to the expected rate given a certain threshold (Phillips, 2006; Radosavljevic & Anderson, 2014; Burrows et al., 2022). At a 10th percentile threshold, I expected data omission rates to be at or near 10%. Higher omission rates imply overfitting, indicating the model sacrificed generality to fit the training data better (Radosavljevic & Anderson, 2014). As such, models with substantially higher test omission rates were unsuitable for the species. Alternatively, low omission rates may result in more potentially misclassified presences; however, this is preferred when searching for new populations because potentially suitable habitats will not be missed (Peterson, 2006). Models with lower omission rates were considered higher performing when compared to other models.

Another commonly used threshold-dependent metric of Maxent model performance is Cohen's kappa statistic, a chance-corrected measure of agreement between observed and

predicted data (Tarakesh & Jetschke, 2011). Kappa is derived from a confusion matrix that charts

predicted and observed presence/absence to identify omission (false absence) and commission

(false presence) errors (Li & Guo, 2013). Although kappa has been praised for its simplicity and

reliability, recent studies have questioned kappa's dependency on prevalence and the bias it

introduces to estimates of predictive accuracy (Allouche et al., 2006; Sahragard & Chahouki,

2015). To reduce potential errors in my analysis, I included the true skill statistic (TSS =

sensitivity + specificity - 1) (Allouche et al., 2006). Like kappa, TSS is a threshold-dependent

measure of discriminatory ability calculated using a confusion matrix and considers both

omission and commission errors (Merow et al., 2013; West et al., 2016); however, TSS corrects

for kappa's prevalence dependency, making it a better choice for predicting rare or endangered

species distributions (Allouche et al., 2006). Both kappa and TSS were calculated using a 10%

suitability threshold and methods described by Soultan (2012). Values range from 0 – 1, where 1

indicates perfect agreement. Evaluation criteria are as follows: excellent (0.85-1.0), very good

(0.7-0.85), good (0.55-0.7), fair (0.4-0.55), and fail (<0.4) (Duan et al., 2014).

Variable importance

Maxent software assesses variable importance through three functions: percent contribution,

permutation contribution, and the jackknife test. Percent contribution explains the importance of

environmental variables to the model as gain increases; however, because percent contribution is

measured during training, it depends on the specific path Maxent used to reach optimal results

(Phillips, 2006). A different algorithm could produce the same model with a different path.

Alternately, permutation contribution is calculated by randomly permuting the values of a

variable and measuring the resulting decrease in AUC (Kalle et al., 2013) and depends solely on

the final Maxent model rather than the path used (Phillips, 2006). Because of its independence

from the Maxent algorithm, permutation contribution is more reliable and informative than

percent contribution. Therefore, I only reported permutation values in my analysis. I also

included Maxent's jackknife of variable importance in my assessment. Jackknife tests measure

the environmental predictor's contribution by creating two models: one with each variable in

isolation and one with each variable excluded from all others. From these results, I evaluated the

variable's effect by comparing test gain and AUC scores for each model, where test gain

measures how well the predicted distribution fits the presence data. Environmental variables with

higher gain and accuracy provide the most useful information or are more important in model

construction (Phillips, 2006).

Overwintering habitat identification

To identify overwintering habitat, I first converted the average suitability map from Maxent into

a raster, using Arcmap's ASCII to Raster tool. I then selected habitats categorized as moderate

(>0.50) and high (>0.75) suitability and transformed these areas into single-part polygon

features. I buffered each area by 30 m and aggregated them so any suitable area within 100 m of

another suitable area would be connected. I chose this buffer distance because 30 m is a mean

estimate of how far Eastern Massasauga will move from their burrow during spring egress,

suggesting these might be important areas to search (Marshall et al., 2006). Additionally, some

research has suggested the Eastern Massasaugas will return to burrows within 100 m of their

previous year's overwintering sites (Harvey & Weatherhead, 2006). Habitat suitability may

change annually; thus, including nearby areas is important when discussing habitat protection or

restoration (Johnson et al., 2000).

<center>Results</center>

Model performance

My findings indicated tuning settings increased model performance, although only slightly. At

the default configuration, both AUC and TSS scores were high at 0.984 and 0.844, respectively,

and the test omission rate was within a reasonable range at 0.136, meaning the settings produced

accurate and suitable predictions (Table 2.2). However, when I altered the number of background

points and the regularization multiplier, differences in performance appeared. Specifically, I

found models with 10,000 background points had the highest AUC scores, but on average, they

had the lowest kappa values and higher omission rates. Alternatively, models with 5,000

background points had the highest on average TSS and kappa values and lowest omission rates

while maintaining excellent AUC scores, ranging from 0.909 to 0.920. Low omission rates

coupled with very good to excellent TSS and kappa scores suggest models with 5,000

background points may be a more reliable predictor of presence. In addition, a regularization

multiplier of 1 produced models with higher AUC, TSS, and kappa values than those with a

multiplier of 2 or 5, implying lowering the multiplier increased model and prediction accuracy.

When considering AUC and TSS values, I found the model with 5,000 background points and a

regularization multiplier of 1 performed, as well as the default configuration. However, due to a

lower omission rate at 0.117, a higher kappa value at 0.798, and a high AUC, I selected a

configuration of 5,000 background points and a regularization multiplier of 1.

Variable importance

The three environmental variables with the highest permutation importance accounted for 82.7%

of the model's accuracy (Table 2.3). Distance to medium vegetation had the greatest influence at

42.4%, followed by distance to water at 20.5% and soil at 19.8%. Habitat suitability was highest

at 0.5 m for distance to medium-height vegetation but rapidly declined as distance increased

(Figure 2.3); however, our presence data had spatial accuracy of 2-3 m, meaning the actual

distance could be slightly higher. Similarly, the three most influential variables had the greatest

average test gains when used in isolation in jackknife tests (Table 2.3). Soil received the highest

scores with an average test gain of 0.954 and AUC of 0.852, indicating it is better at predicting

suitable habitat when compared to other variables. The most influential soil types, specifically

those displaying higher than average suitability (>0.211), were poorly drained silt loams,

primarily near significant water bodies, such as Carlyle Lake and associated rivers and streams.

Habitat suitability peaked at 742.7 m for distance to water and rapidly declined as distance

increased. Elevation had a higher average AUC score than the distance to medium vegetation

and water; however, permutation importance and test gain were low at 5.7% and 0.141,

respectively, meaning elevation may be an accurate predictor but is less important than other

variables. The environmental variables with the lowest permutation importance were radiation

area at 0.5%, canopy cover at 0.1%, and flood zones at 2.5%. Similarly, canopy cover and

radiation area had low average test gains and AUC scores. Canopy height had the second lowest

test gain of 0.122 ($SE = 0.024$) and the lowest AUC scores at 0.599 ($SE = 0.011$); interestingly,

the variable had the fifth highest permutation importance. Results may indicate canopy height is

an important variable for model building but not an accurate predictor of suitable habitat.

Overwintering habitat

Using the highest-performing settings, I created an average suitability model composed of 10

cross-validated replicates. From the model, I identified 0.498 km^2 of highly suitable habitat

(>0.75) and 6.230 km^2 of moderately suitable habitat (>0.50) (Figure 2.4). Most suitable habitats fell within previously known overwintering sites; however, there were some areas where records did not exist. Furthermore, I identified several historical sites as suitable, places where Eastern Massasaugas had not been observed in several years. My final model had an excellent test AUC of 0.916 ($SE = 0.003$), TSS was excellent at 0.852 ($SE = 0.001$), and kappa was very good at 0.792 ($SE = 0.002$). Across 10 replicates, the average logistic threshold for the 10th percentile omission rate was 0.321 ($SE = 0.003$). The average test omission rate was 0.115 ($SE = 0.014$), slightly higher than our predicted rate of 0.10, but it was still within a reasonable range.

Discussion

Like many species, the Eastern Massasauga faces range-wide declines due to habitat fragmentation, degradation, and destruction (Cagle, 2008; Pomara et al., 2013; USFWS, 2016; Markle et al., 2020). Although protecting and restoring important habitat is crucial for long-term conservation plans, collecting the appropriate data is often difficult for this rare, endangered species (Seigel & Collins, 2001; Mazerolle et al., 2007). In particular, data regarding overwintering site choice and selection processes is limited. To fill these gaps in knowledge, I developed a Maxent presence-only model using spring emergence data from Carlyle, Illinois, and nine environmental variables. Distance to medium-height vegetation and water and soil characteristics had the highest importance in the model; specifically, overwintering habitat suitability was highest in areas of poorly drained silt loams near water and 1.5 m to 6.0 m tall vegetation. My results support documented preferences for low-lying habitats with access to wetlands (Jones et al., 2016) but provide more robust information about vegetation preferences. Environmental predictors related to medium-height vegetation, distance to water, and soil

characteristics should be included in future models to predict overwintering habitat in other

southwestern mesic prairies accurately. Based on the presence points and environmental

variables, I identified 0.498 km^2 of highly suitable overwintering habitat and 6.230 km^2 of

moderately suitable habitat, most of which was predicted in current known overwintering sites.

Suitable habitat was also identified in historically known sites, questioning whether habitat

degradation and fragmentation were the true cause of site extirpation. Some new areas were

identified along the lake's eastern shore and southern portion, providing opportunities for further

surveying and potential restoration sites.

Environmental variable importance

Model performance did not improve when predictors with potential collinearity were removed,

leading to retaining all variables in my final model. Collinearity can make discerning predictor

relationships difficult (Graham, 2003; Feng et al., 2019), but by linking Maxent results to

previously known knowledge of Eastern Massasauga life histories, I could broadly analyze

environmental characteristics and their importance in the study extent. Additionally, by including

all variables in the model, I ensured high contributors were not lost, which can result in reduced

prediction accuracy (Braunish et al., 2013). Overall, including a full set of environmental

predictors in my models provides a broader framework for application in other southwestern

mesic prairies that can be customized for differences in landscape or population preferences.

Distance to medium-height vegetation was one of the most important environmental

predictors, contributing to model accuracy and fit of presence data. Habitat suitability was

highest when values were at or near 0.5 m and rapidly decreased as distance from medium-height

vegetation increased, indicating overwintering sites near areas of medium-height vegetation were

selected. Eastern Massasaugas tend to avoid heavily wooded areas (Danou, 1997; Dreslik, 2005;

Moore & Gillingham, 2006; Marshall et al., 2006; Durbian et al., 2007) but often select sites near

small trees and shrubs (Bielema, 1973; Seigel, 1986), which provide important thermoregulatory

opportunities and protection from aerial predators during ingress and egress (Johnson et al.,

2000; Harvey & Weatherhead, 2006). However, because Eastern Massasaugas preferentially use

open, low-lying habitat (Jones et al., 2016), land management efforts prioritize inhibiting

succession and maintaining ground cover composed of grasses or sedges (Johnson et al., 2000;

Kingsbury, 2002; USFWS, 2016). Management recommendations may note the importance of a

canopy gradient (Szymanski, 1998; Johnson et al., 2000; Moore and Gillingham, 2006) but do

not provide details on vegetation types (herbaceous versus woody) or canopy structure. My

results indicate medium-height vegetation should be incorporated into Eastern Massasauga

overwintering habitat management; however, further research is necessary to identify specific

preferences in this vegetation category.

Interestingly, other variables relevant to vegetation were not strong predictors compared

to the distance to medium-height vegetation. In particular, the least important variables in

constructing the final model were canopy cover and distance to high vegetation. However, low

importance scores may not be a true sign of insignificance in the model; rather, these results

could be due to correlations between variables. When testing the effects of collinearity, I found

the distance to high vegetation and canopy cover had low contributions to the model, indicating

they may be non-independent. Additionally, Maxent response curves showed including other

variables in the model reduced the effect of distance to high vegetation and canopy cover.

Medium-height vegetation showed minimal changes in effect between response curves and had

high permutation importance and test gain, showing the variable contributed more to model

accuracy. Further, when the distance to high vegetation and canopy cover were removed from the model, distance to medium-height vegetation was still the second greater contributor, indicating its importance was independent of other environmental variables. Reduced effects of distance to high vegetation and canopy could result from landscape saturation by agriculture, which comprises most of the Carlyle Lake region (SIMAPC, 2021). Because of the prevalence of agriculture in this study extent, correcting for the effect of croplands on variable performance may be necessary. The methods I used to develop the medium-height and high vegetation and canopy cover do not discern between native vegetation and crops. Additionally, I found the inclusion of land use data resulted in the misclassification of several habitats, so this was not included. Removing agricultural land from the study extent could increase model accuracy but may require manual identification by aerial imaging or ground truthing.

I found the distance to water was a major predictor of overwintering habitat, with habitat suitability peaking at ~750 m before rapidly declining. Because of their survival requirements for winter periods, Eastern Massasaugas preferentially select habitats near water (WDNR, 2013; Cross et al., 2015). Overwintering in an aquatic system, such as crayfish burrows, prevents freezing by maintaining a resilience zone with temperatures above 0 °C (Johnson et al., 2000; Markle et al., 2020). However, using crayfish burrows as refugia makes them more susceptible to environmental changes, such as drought and flooding (Yagi et al., 2020). Dropping water tables during winter, caused by freezing soil preventing recharge, can have devastating effects, such as increased susceptibility to desiccation, depletion of lipid stores, and mortality (Johnson et al., 2000). Additionally, while increasing water levels is not necessarily detrimental during overwintering, sudden and extreme flooding during ingress and egress can lead to mass mortality events (WDNR, 2013).

Soil was a major contributor to our model and had the highest permutation importance

and test gain when run alone. The most important soil types were poorly drained silt loams.

Within Illinois, silt loams are often associated with medium-texture tills (Fehrenbacher et al.,

1984), comprising >50% silt and 12-27% clay or 50-80% silt and <12% clay (Cooperative

Extension of Suffolk County, 2013). Silt loams are good soil parent material and are ideal for

highly productive crop growth and thus are considered prime farmland (Fehrenbacher et al.,

1984). Largescale draining and conversion to agriculture have removed much of the native flora;

however, what remains are typically grass/herbaceous cover, prairie grass, hardwood forest, or

deciduous forests, mainly oak and hickory species (NCSS, 2022). Likewise, poorly drained soils

indicate these areas can support crayfish and maintain a suitable water table (Richardson, 2007),

providing appropriate overwintering refuge for Eastern Massasauga populations. Soil

characteristics provide information about burrow availability, groundwater levels, and naturally

occurring vegetation that may not be easily attainable otherwise. As a strong indicator of

overwintering habitat, soil types should be included in future models.

It is important to note Eastern Massasaugas are found in diverse habitats across their

range and use a variety of overwintering refugia, such as small animal burrows, rock crevices, or

sphagnum hummocks (Weatherhead & Prior, 1992; Johnson, 1995; Johnson, 2000; Harvey &

Weatherhead, 2006; Smolarz, 2018). My model is designed to fit mesic prairie habitats and

emphasizes using crayfish burrows for overwintering, meaning it may have low transferability

across other landscapes (Torres et al., 2015). As a result, the variables identified may not be

useful indicators of suitable overwintering sites for other populations. For example, researchers

in Ontario, Canada found that Eastern Massasaugas frequently selected unflooded peatlands for

overwintering and, unlike Carlyle Lake's snakes, sites were associated with the presence of tall

trees, such as maple and white pine and hummock height (Markle et al., 2020a; Markle et al., 2020b). Researchers should customize the model to the specific habitat in populations with vastly different survival requirements and landscape preferences. Specifically, I suggest selecting additional environmental variables most relevant to the landscape and testing their importance before building a final model. Taking this step will help reduce overfitting and produce more accurate results.

Overwintering habitat

With a maximum suitability of ~90%, the model identified a limited amount of high-probability habitat within the study extent, indicating Eastern Massasauga preferences restrict potential overwintering sites. The predicted areas were also mainly confined to previously known sites in EHSP, DEWA/DRWA, and SSPP, where environmental conditions are considered ideal. Some suitable land was identified along the lake's eastern shore and southern part of the extent; however, the areas were small, ranging from only 0.03 km^2 to 0.07 km^2. What remained was highly fragmented by distance and human development, such as roads or buildings, effectively preventing movement between patches. In contrast, moderately suitable habitat was more abundant, extending beyond the parks into southern, northwestern, and eastern areas around the lake. The model also identified areas connected by potentially less appropriate habitats, such as farmland or roads, which could be useful for future search efforts or developing wildlife corridors. One undesirable outcome observed when relaxing suitability restrictions was more suitable areas were predicted near development, particularly in the southern and western extent. Synergistic effects of habitat loss, such as road mortality and human-wildlife conflict, are leading causes of Eastern Massasauga declines across their range (Szymanski, 1998; Durbian et al.,

2008; Shepard et al., 2008; Baker, 2016), so reducing such interactions is a vital consideration when selecting habitat for restoration or protection.

I also found suitable habitats where Eastern Massasauga populations historically occurred. Specifically, potential overwintering sites overlapped with two known sites on the eastern side of the study extent, where surveyors observed snakes until 2002. The absence of snakes despite suitable habitat suggests something other than overwintering habitat availability drove site extirpation. Additional threats may be influencing population survival within the Carlyle Lake region. For example, research shows living in disturbed habitats can lead to poor body condition, increasing susceptibility to disease, decreased fecundity, and mortality (Jenkins et al., 2009). *Ophidiomyces,* also known as Snake fungal disease, is known to lead to high morbidity and mortality in snakes, including the Eastern Massasauga. Some research suggests a 40% mortality rate after inoculation (Allender et al., 2015). Road mortality is another significant threat within the highly fragmented Carlyle Lake region, especially when snakes search for mates, foraging opportunities, or overwintering sites (Shepard et al., 2008; Shepard et al., 2008). Additionally, increased edge habitat is correlated to mesopredator presence, leading to high levels of predation (Baker et al., 2016). Consequently, determining the causes of decline in these areas must be a priority in long-term conservation. While protecting and maintaining the integrity of potential overwintering habitat is important, doing so is futile without removing other major threats.

Conservation implications

Suitable overwintering refugia may be a limiting factor for snake populations (Harvey & Weatherhead, 2006), emphasizing the importance of site identification and protection for species

survival (Johnson et al., 2000). I have identified overwintering habitat for the Eastern Massasauga rattlesnake in the Carlyle Lake region, Clinton Country, Illinois, and also described important environmental predictors. I found distance to medium-height vegetation, distance to water, and soil type were the most important model variables, consistent with previously known Eastern Massasauga overwintering site requirements. The inclusion of these environmental predictors is recommended for future models.

Interestingly, distance to water and soil type were the most static, meaning they cannot be changed or managed; rather, they would need to be maintained in their current state. Consequently, restoration projects may be less effective when expanding or restoring habitat. Moving forward, preserving all existing suitable overwintering sites demarcated should be the priority for Eastern Massasauga populations in the Carlyle Lake region. In particular, conserving habitat with substantial vegetation between 1.5 m and 6.0 m tall, including grasses, native shrubs and small trees, may be crucial. Additionally, these areas should have poorly drained silt loam soils located at most ~750 m from a water source. Given appropriate overwintering refugia are limited and identifying them all is likely unachievable, any potential overwintering sites should be protected (Johnson et al., 2000), especially true for small, fragmented populations like Carlyle Lake's Eastern Massasauga, which are likely to be extirpated without human intervention.

Table 2.1. Environmental variables with data sources used in the Maxent presence-only habitat suitability model of Eastern Massasauga overwintering habitat in the southern Carlyle Lake region, Clinton County, Illinois. Data sources include Illinois Height Modernization (ILHMP): LiDAR Data, FEMA's National Flood Hazard Layer (NFHL) Viewer, and Soil Survey Geographic Database (SSURGO).

Group	Variable	Description	Units	Source
Land	Elevation	Distance of the land above sea level	Meters	ILHMP
	Solar radiation	Total incoming solar (direct and diffuse) insolation calculated for the input surface	W.H./m^2	ILHMP
	Soil characteristics	Polygon describing 37 kinds and distributions of soils with study area, as well as some hydrography data	Description of soil attributes	SSURGO
Vegetation	Canopy height	Height of all vegetation	Meters	ILHMP
	Canopy cover	Density of low, medium, and high vegetation within each cell	Number of classified vegetation points per cell	ILHMP
	Distance to high vegetation	Distance to nearest high vegetation point	Meters	ILHMP
	Distance to medium vegetation	Distance to nearest medium vegetation point	Meters	ILHMP
Water	Distance to water	Distance to nearest water point	Meters	ILHMP
	Flood risk	Polygon containing information about current flood hazards	Description of potential flood hazard	NFHL

Table 2.2. Feature configurations (bp – background points, rm - regularization multiplier) and quantitative evaluation metrics (AUC - Area Under the Curve, omission rate, TSS - true skill statistic, and Kappa) of Maxent habitat suitability models created with different feature configurations for Eastern Massasauga overwintering habitat in the southern Carlyle Lake region, Clinton County, Illinois. Omission rate, TSS, and Kappa were calculated using a 10% training omission threshold.

Feature Configuration		Quantitative Evaluation			
bp	Rm	AUC	Omission Rate	TSS	Kappa
10,000	1	0.948	0.136	0.844	0.697
	2	0.945	0.136	0.838	0.672
	5	0.935	0.112	0.818	0.591
5,000	1	0.920	0.117	0.852	0.798
	2	0.918	0.126	0.836	0.762
	5	0.909	0.121	0.824	0.723
1,000	1	0.769	0.136	0.839	0.842
	2	0.772	0.117	0.826	0.827
	5	0.768	0.145	0.787	0.787

Table 2.3. Maxent permutation importance, test gain, and jackknife of AUC values of environmental variables when included in a habitat suitability model created for Eastern Massasauga overwintering habitat in the southern Carlyle Lake region, Clinton County, Illinois.

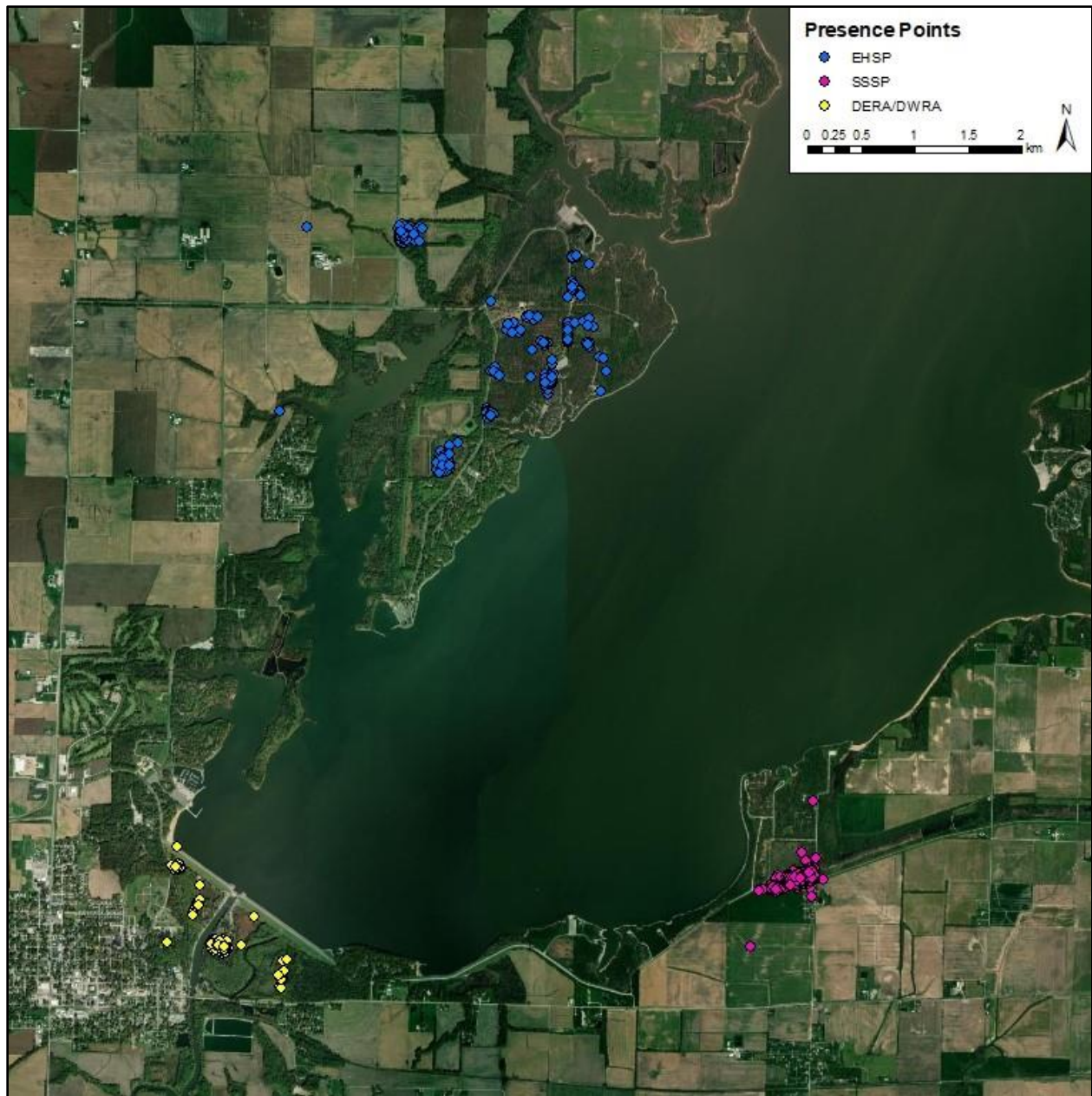| Variable	Permutation importance	Test Gain	Jackknife of AUC
Distance to medium vegetation	42.4	0.608	0.775
Distance to water	20.5	0.835	0.748
Soil	19.8	0.954	0.852
DEM	5.7	0.141	0.790
Canopy height	5.1	0.122	0.599
Distance to high vegetation	3.4	0.477	0.755
Flood zone	2.5	0.293	0.694
Radiation	0.5	0.077	0.615
Canopy cover	0.1	0.145	0.606

Figure 2.1. Presence points within the study extent of Eastern Massasaugas in Carlyle, Illinois, across

three main overwintering sites, including Eldon Hazlet State Park (EHSP), South Shore State Park

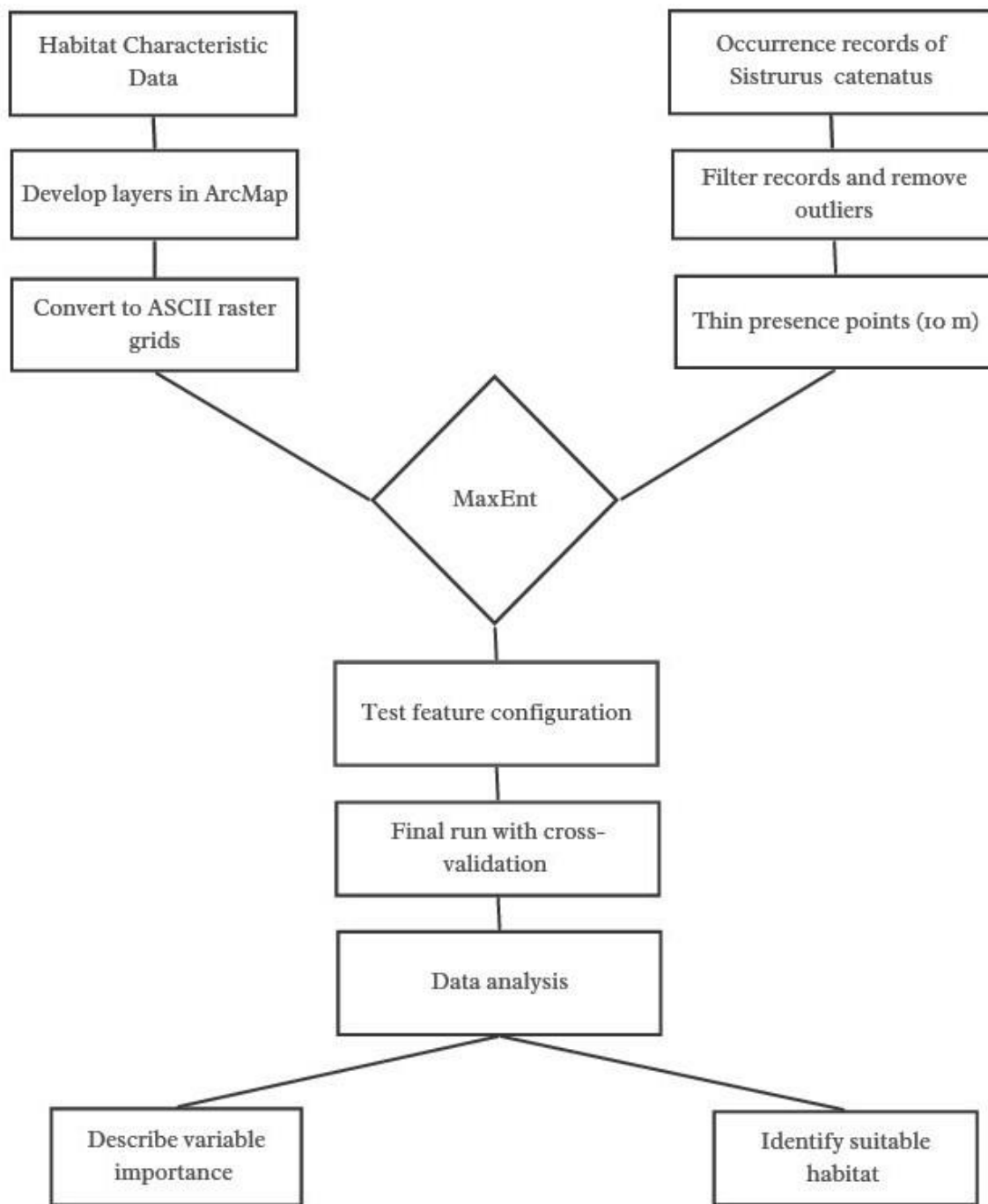(SSSP), Dam East/Dam West Recreation Area (DERA/DWRA).

Figure 2.2. Workflow of Maxent presence-only habitat suitability model development for Eastern

Massasauga overwintering habitat in the southern Carlyle Lake region, Clinton County, Illinois.
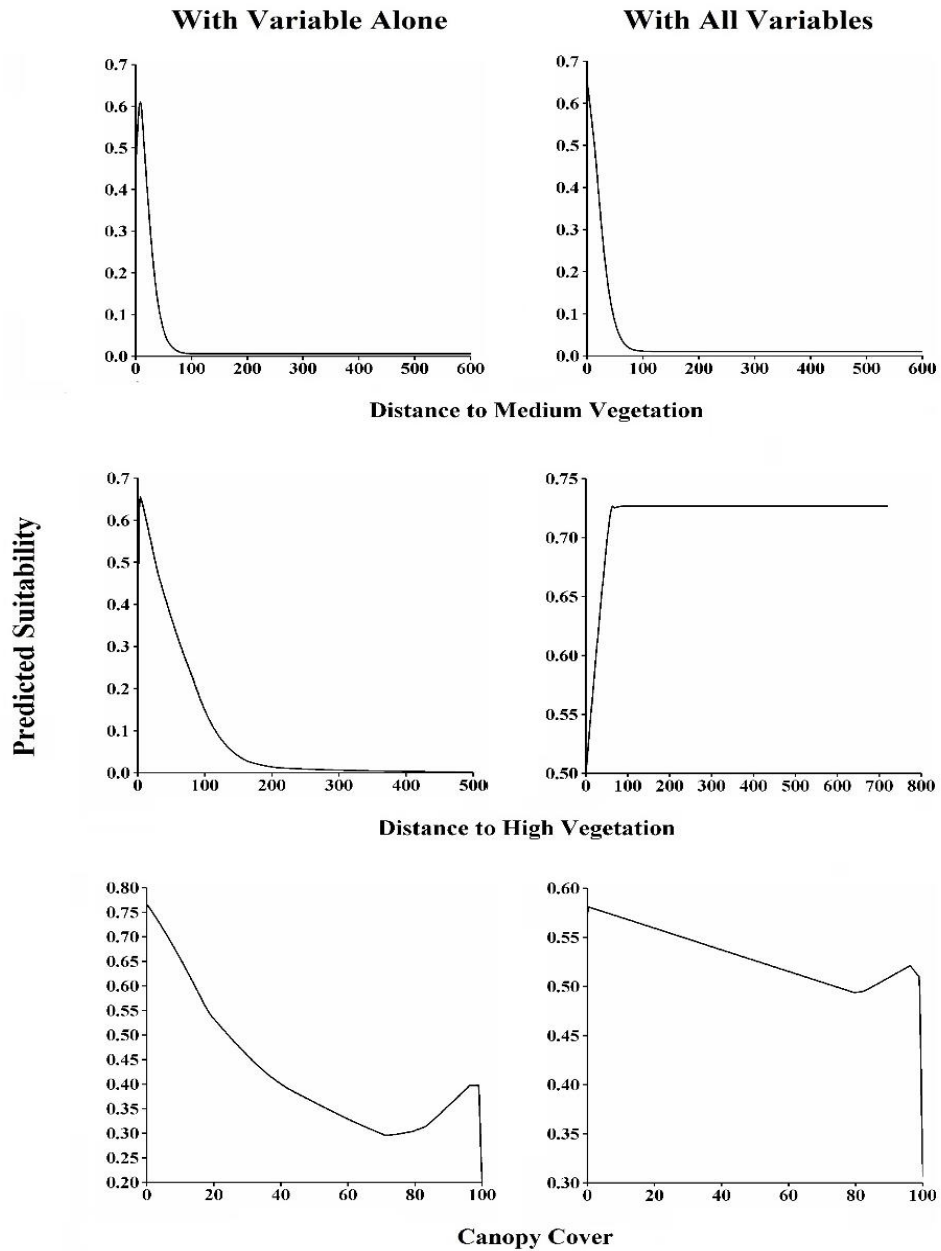
Figure 2.3. Multivariate and univariate response curves showing the effect of possible collinearity on predicted suitability for distance to medium vegetation, distance to high vegetation, and canopy cover of a habitat suitability model created for Eastern Massasauga overwintering habitat in the southern Carlyle Lake region, Clinton County, Illinois.
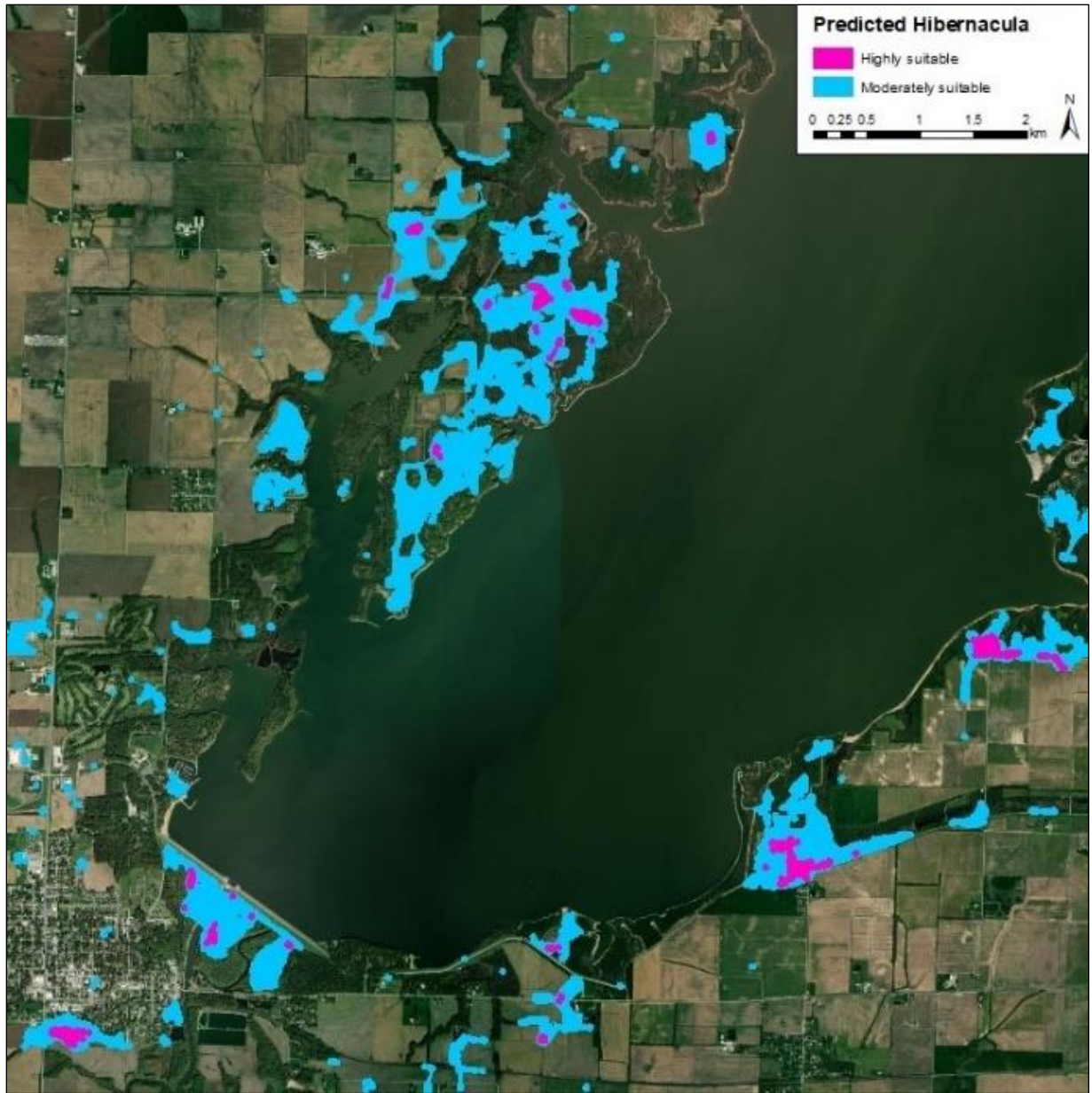
Figure 2.4. Highly suitable (>0.75) and moderately suitable (>0.50) overwintering habitat for Eastern

Massasaugas in the southern Carlyle Lake region, Clinton County, Illinois.

Literature Cited

Allender, M.C., Baker, S., Wylie, D, Loper, D., Dreslik, M.J., Phillips, C.A., et al. 2015.

Development of snake fungal disease after experimental challenge with *Ophidiomyces ophiodiicola* in cottonmouths (*Agkistrodon piscivorous*). PLOS ONE 10:e0140193.

Allouche, O., Tsoar, A., and Kadmon, R. 2006. Assessing the accuracy of species distribution models: prevalence, kappa and the true skill statistic (TSS). Journal of Applied Ecology 43:1223—1232.

Bailey, R.L., Campa III, H., Bissell, K.M., and Harrison, T.M. 2012. Resource selection by the Eastern Massasauga rattlesnake on managed land in southwestern Michigan. The Journal of Wildlife Management 76:414—421.

Baker, S.J. 2016. Life and death in a corn desert oasis: reproduction, mortality, genetic diversity, and viability of Illinois' last Eastern Massasauga population. Ph.D. Dissertation. University of Illinois at Urbana Champaign 131 p.

Banks-Leite, C., Ewers, M., Folkard-Tapp, H., and Fraser, A. 2020. Countering the effects of habitat loss, fragmentation, and degradation through habitat restoration. One Earth 3:672—676.

Barbet-Massin, M., Jiguet, F., Albert, C.H. and Thuiller, W. 2012. Selecting pseudo-absences for species distribution models: How, where and how many? Methods in Ecology and Evolution 3:327—338.

Bartel, R.A. and Sexton, J.O. 2009. Monitoring habitat dynamics for rare and endangered species using satellite images and niche-based models. Ecography 32:888—896.

Bielema, B.J. 1973. The Eastern Massasauga (*Sistrurus catenatus*) in West-Central Illinois. M.Sc. Thesis. Western Illinois University, Macomb, Illinois 80 p.

Braunisch, V., Coppes, J., Arlettaz, R., Suchant, R., Schmid, H., and Bollmann, K. 2013.

Selecting from correlated climate variables: A major source of uncertainty for predicting species distributions under climate change. Ecography 36:971—983.

Buckland, C.E., Smith, A.J.A.C, and Thomas, D.S.G. 2022. A comparison in species distribution model performances of succulents using keys species and subsets of environmental predictors. Ecology and Evolution 12:e8981.

Burrows, H., Slatculescu, A.M., Feng, C.X., Clow, K.M., Guillot, C., Jardine, C.M., et al. 2022. The utility of a maximum entropy species distribution model for *Ixodes scapularis* in predicting the public health risk of Lyme disease in Ontario, Canada. Ticks and Tick-borne Diseases 13:101969.

Cagle, N.L. 2008. Snake species distributions and temperate grasslands: A case study from the American tallgrass prairie. Biological Conservation 141:744—755.

Camaclang, A.E., Maron, M., Martin, T.G., and Possingham, H.P. 2015. Current practices in the identification of critical habitat for threatened species. Conservation Biology 29:482–492.

Chapin III, F.S., Zavaleta, E.S., Eviner, V.T., Rosamond, N.L., Vitousek, P.M., Reynolds, H.L., et al. 2000. Consequences of changing biodiversity. Nature 405:234—242.

Committee on the Status of Endangered Wildlife in Canada (COSEWIC). 2012. COSEWIC assessment and status report on the Massasauga *Sistrurus catenatus* in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa, Canada 84 pp.

Costa, G.C., Nogueira, C., Machado, R.B., and Colli, G.R. 2010. Sampling bias and the use of ecological niche modeling in conservation planning: A field evaluation in a biodiversity hotspot. Biodiversity and Conservation 19:883—899.

Costanzo, J.P. 1989. Effects of humidity, temperature, and submergence behavior on

survivorship and energy use in hibernating garter snakes, *Thamnophis sirtalis*. Canadian
Journal of Zoology 67:2486—2492.

Crawford, J.A., Dreslik, M.J., Baker, S.J., Phillips, C.A., and Peterman, W.E. 2020. Factors
affecting the detection of an imperiled and cryptic species. Diversity 12:177.

Cross, M.D., Root, K.V., Mehne, C.J., McGowan-Stinski, J., Pearsall, D., and Gillingham, J.C.
2015. Multi-scale responses of Eastern Massasauga rattlesnakes (*Sistrurus catenatus)* to
prescribed fire. The American Midland Naturalist 173:346—362.

Danou, C.H. 1997. Habitat use and movement patterns of the Eastern Massasauga rattlesnake in
Wisconsin. MSc Thesis. University of Wisconsin-Stevens Point, Stevens Point,
Wisconsin 38 p.

Duan, R.Y., Kong, X.Q., Huang, M.Y., Fan, W.Y., and Wang Z.G. 2014. The predictive
performance and stability of six species distribution models. PLOS ONE 9:e112764.

Dorji, T., Linke, S., and Sheldon, F. 2020. Optimal model selection for Maxent: A case of
freshwater species distribution modeling in Bhutan, a data poor country. Preprint.

Dreslik, M.J. 2005. Ecology of the Eastern Massasauga (*Sistrurus catenatus catenatus*) from
Carlyle Lake, Clinton County, Illinois. Ph.D. Dissertation. University of Illinois Urbana-
Champaign, Urbana, Illinois 393 p.

Dreslik, M.J. 2017. Conservation genetics of the Eastern Massasauga (*Sistrurus cantenatus*) in
Illinois. Unpublished Report. University of Illinois Urbana-Champaign 16 p.

Dreslik, M.J., Shepard, D.B., Jellen, B.C., and Phillips, C.A. 2017. Movement and home range of
the Eastern Massasauga (*Sistrurus catenatus*) at its southern range limit. The Biology of
Rattlesnakes II (Dreslik, M.J., Hayes, W.K., Beaupre, S.J., Mackessy, S.P., eds.). ECO
Herpetological Publishing and Distribution, Rodeo, New Mexico 10 p.

Durbian, F.E., King, R.S., Crabill, T., Lambert-Doherty, H. and Seigel, R.A. 2008. Massasauga
    home range patterns in the Midwest. The Journal of Wildlife Management 72:754—
    759.

Elith, J. and Graham, C.H. 2009. Do they? How do they? WHY do they? On finding reasons for
    differing performances of species distribution models. Ecography 32:66—77.

Elith, J., Graham, C.H., Anderson, R.., Dudík, M., Ferrier, S., Guisan, A., et al. 2006. Novel
    methods improve prediction of species' distributions from occurrence data. Ecography
    29:129—151.

Escalante, T., Rodríguez-Tapia, G., Linaje, M., Illodi-Rangel, P., and Gonzalez-Lopez, R. 2013.
    Identification of areas of endemism from species distribution models: Threshold selection
    and Nearctic mammals. Revista Especializada en Ciencias Quimico-Biologicas 16:5—17.

Fehrenbacher, J.B., Alexander, J.D., Jansen, I.J., Darmody, R.G., Pope, R.A., and Flock, M.A.
    1984. Soils of Illinois. University of Urbana-Champaign College of Agriculture, Illinois,
    USA.

Feng, X., Park, D.S., Liang, Y., Pandey, R., and Papes, M. 2019. Collinearity in ecological niche
    modeling: Confusions and challenges. Ecology and Evolution 9:10365—10376.

Gienger, C.M. and Beck, D.D. 2011. Northern Pacific rattlesnakes (*Crotalus oreganus*) use
    thermal and structural cues to choose overwintering hibernacula. Canadian Journal of
    Zoology 89:1084—1090.

Gillingham, J.C. and Carpenter, C.C. 1978. Snake hibernation: Construction of and observations
    on a man-made hibernaculum (Reptilia, Serpentes). Journal of Herpetology 12:495—498.

Goncalves-Souza, D., Verburg, P.H., and Dobrovolski, R. 2020. Habitat loss, extinction

predictability and conservation efforts in the terrestrial ecosystems. Biological

Conservation 246:108579.

Graham, M.H. 2003. Confronting multicollinearity in ecological regression. Ecology

84(11):2809—2815.

Guillera-Arroita, G., Lahoz-Monfort, J.J. and Elith, J. 2014. Maxent is not a presence-absence

method: A comment on Thibaud et al. Methods in Ecology and Evolution 5(11):1192—

1197.

Guisan, A. and Zimmermann, N.E. 2000. Predictive habitat distribution models in ecology.

Ecological Modelling, 135:147—186.

Hagen, A.N. and Hodges, K.E. 2006. Resolving critical habitat designation failures:

Reconciling law, policy, and biology. Conservation Biology 20:399—407.

Harvey, D.S. and Weatherhead, P.J. 2006a. A test of the hierarchical model of habitat selection

using Eastern Massasauga rattlesnakes (*Sistrurus c. catenatus*). Biological Conservation

130:206—216.

Harvey, D.S. and Weatherhead, P.J. 2006b. Hibernation site selection by Eastern Massasauga

rattlesnakes (*Sistrurus catenatus catenatus*) near their northern range limit. Journal of

Herpetology 40:66—73.

Hirth, H.F. 1966. Weight changes and mortality of species of snakes during hibernation.

Herpetological 22:8—12.

Hoekstra, J.M., Boucher, T.M., Ricketts, T.H. and Roberts, C. 2005. Confronting a biome crisis:

Global disparities of habitat loss and protection. Ecology Letters 8:23—29.

Jarnevich, C.S. and Young, N.E. 2015. Using MAXENT program for species distribution

modelling to assess invasion risk. Pest risk modeling and mapping for invasive alien species. USGS Publications Warehouse, Reston, Virginia 16 p.

Jenkins, C.L., Peterson, C.R., Doering, S.C., and Cobb, V.A. 2009. Microgeographic variation in reproductive characteristics among Western rattlesnake (*Crotalus oreganus*) populations. Copeia 2009:774—780.

Johnson, G.1995. Spatial ecology, habitat preference, and habitat management of the Eastern massasauga, *Sistrurus c. catenatus* in a New York weakly-minerotrophic peatland. Ph.D. Dissertation. State University of New York College of Environmental Science and Forestry, Syracuse, NY 158 p.

Johnson, G. 2000. Spatial ecology of the Eastern Massasauga (*Sistrurus c. catenatus*) in a New York peatland. Journal of Herpetology 34:186—192.

Johnson, B.D., Gibbs, J.P., Bell, T.A. Jr., and Shoemaker, K.T. 2016. Manipulation of basking sites for endangered Eastern Massasauga rattlesnakes. Journal of Wildlife Management 80:803—811.

Johnson, G., Kingsbury, B., King, R., Parent, C., Seigel, R., and Szymanski, J. 2000. The Eastern Massasauga rattlesnake: A handbook for land managers. U.S. Fish and Wildlife Service, Fort Snelling, Minnesota 52 p.

Jones, P.C., King, R.B., Bailey, R.L., Bieser, N.D., Bissell, K. Campa III, H., et al. 2012. Range-wide analysis of Eastern Massasauga survivorship. Journal of Wildlife Management 76:1576—1586.

Kalle, R., Ramesh, T., Qureshi, Q., and Sankar, K. 2013. Predicting the distribution pattern of small carnivores in response to environmental factors in the Western Ghats. PLOS One 8:e79295.

Kingsbury, B.A. 2002. Conservation approach for Eastern Massasauga (*Sistrurus c. cantenatus*). Center for Reptile and Amphibian Conservation and Management, Fort Wayne, Indiana 54 pp.

Kissner, K.J. and Weatherhead, P.J. 2005. Phenotypic effects on survival of neonatal northern watersnakes *Nerodia sipedon*. Journal of Animal Ecology 74:259—265.

Lobo, J.M., Jiménez-Valverde, A. and Real, R. 2008. AUC: A misleading measure of the performance of predictive distribution models. Global Ecology and Biogeography 17: 145—151.

Markle, C.E., Moore, P.A., and Waddington, J.M. 2020a. Temporal variability of overwintering conditions for a species-at-risk snake: Implications for climate change and habitat. Global Ecology and Conservation 22:e00923.

Markle, C.E., North, T.D., Harris, L.I. Moore, P.A., and Waddington, J.M. 2020b. Spatial heterogeneity of surface topography in peatlands: Assessing overwintering habitat availability for the Eastern Massasauga rattlesnake. Wetlands 40:2337—2349.

Marshall, J.C., Manning, J.V., and Kingsbury, B.A. 2006. Movement and macrohabitat selection of the Eastern Massasauga in a fen habitat. Herpetologica 62:141—15.

Mazerolle, M.J., Bailey, L.L., Kendall, W.L., Royle, A.J., Converse, S.J., and Nichols, J.D. 2007. Making great leaps forward: Accounting for detectability in herpetological field studies. Journal of Herpetology 41:672—689.

Merow, C., Smith, M.J., and Silander, J.A. 2013. A practical guide to MaxEnt for modeling species' distributions: What it does, and why inputs and settings matter. Ecography 36:1058—1069.

Moore, J.A. and Gillingham, J.C. 2006. Spatial ecology and multi-scale habitat selection by a

threatened rattlesnake: The Eastern Massasauga (*Sistrurus catenatus catenatus*). Copeia 2006:742—751.

Morales, N.S., Fernandez, I.C., and Baca-González, V. 2017. MaxEnt's parameter configuration and small samples: Are we paying attention to recommendations? A systematic review. PeerJ 5:e3093.

National Cooperative Soil Survey (NCSS). 2022. Official Soil Series Description. Natural Resources Conservation Service, Washington, D.C., USA.

Pearson, R.G., Raxworthy, C.J., Nakamura, M., and Peterson, A.T. 2006. Predicting species distributions from small numbers of occurrence records: A test case using cryptic geckos in Madagascar. Journal of Biogeography 34:102—117.

Peterson, A.T. 2006. Uses and requirements of ecological niche models and related distributional models. Biodiversity Informatics 3:59—72.

Perzanowski, K., Januszczak, M., and Łopucki, R. 2019. Historical changes in land use influence current habitat preferences of large herbivores. Landscape Ecology 34:2251—2259.

Phillips, S.J. 2005. A brief tutorial on Maxent. AT&T Research 190:231—259.

Phillips, S.J. and Dudik, M. 2008. Modeling of species distributions with Maxent: New extensions and a comprehensive evaluation. Ecography 31:161—175.

Phillips, S.J., Anderson, R.P., and Schapire, R.E. 2006. Maximum entropy modeling of species geographic distributions. Ecological Monitoring 190:231—259.

Pomara, L.Y., Ledee, O.E., Martin, K.J., and Zuckerberg, B. 2013. Demographic consequences of climate change and land cover help explain a history of extirpations and range contraction in a declining snake species. Global Change Biology 20:2087—99.

Radosavljevic, A. and Anderson, R.P. 2014. Making better Maxent models of species

distributions: Complexity, overfitting and evaluation. Journal of Biogeography 41:629—
643.

Redon, M. and Luque, S. 2010. Presence-only modelling for indicator species distribution:
Biodiversity monitoring in the French Alps. Proceedings of the 6th Spatial Analysis and
Geomatics International Conference, Toulouse, France 13 p.

Reinert, H.K. and Kodrich, W.R. 1982. Movements and habitat utilization by the Massasauga,
Sistrurus catenatus catenatus. Journal of Herpetology 16:162—171.

Richardson, A.M.M. 2007. Behavioral ecology of semiterrestrial crayfish. Evolutionary Ecology
of Social and Sexual systems: Crustaceans as Model Organisms (Duffy, J.E., Thiel, M.,
eds). Oxford University Press, New York, New York 19 p.

Robillard, A. and Johnson, B. 2015. Edge effects on Eastern Massasauga rattlesnakes basking
in managed habitat. Northeastern Naturalist 22:200—208.

Rudolph, D.C., Schaefer, R.R., Burgdorf, S.J., Duran, M., and Conner, R.N. 2007. Pine snake
(*Pituophis ruthveni* and *Pituophis melanolecucus lodingi*) hibernacula. Journal of
Herpetology 41:560—565.

Sahragard, H.P. and Chahouki, M.A. 2015. An evaluation of predictive habitat models
performance of plant species in Hoze sultan rangelands of Qom province. Ecological
Modelling 309:64—71

Seigel, R.A. 1986. Ecology and conservation of an endangered rattlesnake, *Sistrurus catenatus*,
in Missouri, USA. Biological Conservation 35:333—346.

Seigel, R.A. and Collins, J.T. 2001. Snakes: Ecology and behavior. Blackburn Press, New
Jersey, U.S.A. 414 p.

Shepard, D.B., Dreslik, M.J., Jellen, B.C., and Phillips, C.A. 2008a. Reptile road mortality

around an oasis in the Illinois corn desert with emphasis on the endangered Eastern Massasauga. Copeia 2:350—359.

Shepard, D.B., Kuhns, A.R., Dreslik, M.J., and Phillips, C.A. 2008b. Roads as barriers to animal movement in fragmented landscapes. Animal Conservation 11:288—296.

Shoemaker, K.T. 2007. Habitat manipulation as a viable strategy for the conservation of the massasauga rattlesnake in New York State. M.Sc Thesis. State University of New York, Albany, New York 109 p.

Sidle, J.G. 1987. Critical habitat designation: Is it prudent? Environmental Management 11:429—437.

Smith, C.S. 2009. Hibernation of the Eastern Massasauga rattlesnake (*Sistrurus catenatus catenatus*) in northern Michigan. M.Sc. Thesis. Purdue University Graduate School, West Lafayette, Indiana 54 p.

Smolarz, A.G., Moore, P.A., Markle, C.E., and Waddington, J.M. 2018. Identifying resilient Eastern Massasauga rattlesnake (*Sistrurus catenatus*) peatland hummock hibernacula. Canadian Journal of Zoology 96:1024—1031.

Soultan, A. 2012. TSS excel sheet and RStudio code. St. Katherine Protectorate, Sinai, Egypt.

Sousa-Silva, R., Alves, P., Honrado, J., and Lomba, Â. 2014. Improving the assessment and reporting on rare and endangered species through species distribution models. Global Ecology and Conservation 2:226—237.

Southwestern Illinois Metropolitan and Regional Planning Commission (SIMPAC). 2021. Clinton County Comprehensive Plan 2021 Update. County Board, Clinton Country, Illinois 76 pp.

Szymanski, J. 1998. Status assessment for Eastern Massasauga (*Sistrurus c. catenatus*). Report.

U.S. Fish and Wildlife Service, Endangered Species Division, Fort Snelling, Minnesota 31 p.

Szymanski, J., Pollack, C., Ragan, L., Redmer, M., Clemency, L., Voorhies, K., and Jaka, J. 2015. Species status assessment for the Eastern Massasauga rattlesnake (*Sistrurus catenatus*). U.S. Fish and Wildlife Service, Washington, D.C. 115 p.

Tarkesh, M. and Jetschke, G. 2012. Comparison of six correlative models in predictive vegetation mapping on a local scale. Environmental and Ecological Statistics 19:437—457.

Torres, L.G., Sutton, P.J.H., Thompson, D.R., Delord, K., Weimerskirch, H., Sagar, P.M., et al. 2015. Poor transferability of species distribution models for a pelagic predator, the grey petrel, indicates contrasting habitat preferences across ocean basins. PLOS ONE 10:e0120014.

Tucker, J.K, Soergel, D.W., and Hatcher, J.B. 1995. Flood-associated activities of some reptiles and amphibians at Carlyle Lake, Fayette County, Illinois. Transactions of the Illinois State Academy of Science 88:73—81.

U.S. Fish and Wildlife Service. 2016. Endangered and threatened wildlife and plants: Threatened species status for the Eastern Massasauga rattlesnake. Federal Register 81: 67193—67214.

U.S. Fish and Wildlife Service. 2017. Critical habitat: What is it? U.S. Fish and Wildlife Service: Endangered Species Programs, Fort Snelling, Minnesota 2 p.

Valencia-Rodríguez, D., Jiménez-Segura, L., Rogéliz, C.A. and Parra, J.L. 2021. Ecological niche modeling as an effective tool to predict the distribution of freshwater organisms: The case of the Sabaleta *Brycon henni* (Eigenmann, 1913). PLOS ONE 16:e0247876.

Wang, B., Xu, Y., and Ran, J. 2017. Predicting suitable habitat of the Chinese monal (*Lophophorus lhuysii*) using ecological niche modeling in the Qionglai Mountains, China. PeerJ 5:e3477.

Weatherhead, P.J. and Prior, K.A. 1992. Preliminary observations of habitat use and movements of the Eastern Massasauga rattlesnake (*Sistrurus c. catenatus*). Journal of Herpetology 26:447—452.

West, A.M., Kumar, S., Brown, C.S., Stohlgren, T.J., and Bromberg, J. 2016. Field validation of an invasive species Maxent model. Ecological Informatics 36:126—134.

Wisconsin Department of Natural Resources (WDNR). 2013. Wisconsin Eastern Massasauga species guidance. Bureau of Natural Heritage Conservation, Wisconsin Department of Natural Resources, Madison, Wisconsin PUB-ER-713

Yagi, A.R., Planck, R.J., Yagi, K.T., and Tattersall, G.J. 2020. A long-term study on massasaugas (*Sistrurus catenatus*) inhabiting a partially mined peatland: A standardized method to characterize snake overwintering habitat. Journal of Herpetology 54:235—244.

Yan, H., He, J., Xu, X., Yao, X., Wang, G., Tang, L., et al. 2021. Prediction of potentially suitable distributions of *Codonopsis pilosula* in China based on an optimized MaxEnt model. Frontiers in Ecology and Evolution 9:773396.

CHAPTER 3: OPTIMIZING AN eDNA ASSAY FOR EASTERN MASSASAUGA RATTLESNAKE (*SISTRURUS CATENATUS*) DETECTION

Introduction

A popular detection method among conservationists is environmental DNA (eDNA), genetic material left in water, soil, or sediment, which can be extracted and identified using qPCR (Barnes & Turner, 2015; Beng & Corlett, 2019). It became increasingly popular due to its fast, relatively inexpensive methodology, non-invasive nature, and propensity to yield high detection probabilities (Beng & Corlett, 2019). Thus, the method has been valuable to traditional monitoring for difficult-to-detect species (Ma et al., 2016; Pfleger et al., 2016; Lor et al., 2019; Wood et al., 2020). Using eDNA could be a more practical choice when monitoring large-scale population trends and identifying extinction events, information necessary for effective conservation strategies. In particular, endangered species benefit from eDNA because the method can improve detection rates in low-density populations and reduce direct interactions with already rare organisms (Corlett, 2017). As a historically difficult group to study, snake studies could be improved using eDNA methods by providing scientists with a new, potentially more efficient detection method. Snakes represent one of the highest data-deficient groups due to their cryptic nature, limited activity patterns, and often inaccessible habitat (Mazerolle et al., 2007). Traditional monitoring methods are costly and time-consuming. Even with proper monitoring, detecting population-level changes in rare or cryptic species is challenging (Durso et al., 2011), as declines could be attributed to poor sampling methods or random chance (Kery, 2002). Due to the magnitude of data-deficient species, it is unlikely the time and resources necessary to collect long-term data are or are readily available (Baker, 2016). Alternate detection

methods could provide an efficient and cost-effective addition to traditional monitoring when determining presence or mapping distribution, especially for rare or cryptic species (Bohman et al., 2014).

Successful eDNA monitoring could benefit the Eastern Massasauga (*Sistrurus catenatus*). Only one cluster of populations within Illinois remains in the Carlyle Lake, Clinton County region; however, twenty-three years of capture-mark-recapture data provides managers with robust datasets to monitor population demographics and distribution. Presence data is readily available for this region, presenting an excellent opportunity to test and compare other detection methods, such as eDNA, for broad application across the Eastern Massasauga's range. Population monitoring in Carlyle occurs during spring emergence when individuals are easier to detect in areas of clustered overwintering refugia, are found at increased densities, and exhibit thermoregulatory behavior. In Illinois, Eastern Massasaugas primarily overwinter in crayfish burrows reaching the water table, allowing them to submerge during long periods of sub-freezing temperatures (Smith, 2009). During spring, we presume burrows used have high levels of detectable DNA due to long periods of occupation and a relatively cool and UV-free environment. However, the few studies evaluating eDNA detection for Eastern Massasaugas had little success, with one study detecting DNA in two of 100 burrow samples and the other detecting only one in 60 (Baker et al., 2018; Merkling, 2019). Low detection rates could be a result of collection and filtration methods. Baker et al. (2018) centrifuged burrow samples to separate water from sediment but did not use the sediment samples for further testing, potentially reducing DNA concentrations in the water, and Merkling (2019) did not use sample replicates to increase detection in individual burrows. Researchers also suggest environmental conditions, such as temperature, rainfall, pH, and dissolved oxygen, could affect degradation rates or

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produce inhibition (Baker et al., 2018; Merkling, 2019). Additionally, species behavior and physiology, such as reduced shedding during periods of inactivity, could impact the effectiveness of eDNA detection (Merkling, 2019; Katz et al., 2021).

Some results suggest eDNA may not be viable for small, semi-aquatic, or terrestrial snake species (Halstead et al., 2017; Rose et al., 2019; Ratsch et al., 2019). Ratsch et al. (2020) found eDNA ineffective for the Kirkland's snake (*Clonophis kirtlandii*), a species with an overlapping range and similar habitat use to the Eastern Massasauga. They attributed their results to the species' low eDNA production, possibly caused by the snake's keratinized scales, small size, or propensity to defecate and shed away from their burrow (Ratsch, 2019; Ratsch et al., 2020). However, this is in direct opposition to the successful eDNA work conducted in Florida with the invasive Burmese python (*Python molurus bivittatus*), a large semi-aquatic species with nearly year-round activity (Piaggio et al., 2014; Hunter et al., 2015; Hunter et al., 2019). Future work should also consider the impact of environmental conditions on degradation rates. Degradation in freshwater systems can begin anywhere from 1 to 60 days after a snake is present, depending on UV-B, temperature, pH, and other external variables (Piaggio et al., 2014; Barnes et al., 2014; Strickler et al., 2015; Thomsen, 2021). Continued investigation of environmental effects on eDNA monitoring could increase success in difficult-to-detect species. Although further work is needed to develop an effective assay for the Eastern Massasauga, the potential of eDNA monitoring has broad implications for conservation across its range.

In this chapter, I validated and optimized an eDNA assay for the Eastern Massasauga, whereby I tested its effectiveness on samples collected from crayfish burrows from Carlyle Lake, IL, a known overwintering site. Using a primer/probe previously described by Baker et al. (2018), I improved on previous studies by improving sensitivity, incorporating sediment and

water samples, and increasing sample replicates. My research broadly benefits Eastern

Massasauga conservation within Illinois and across its range. Specifically, my study generates an

additional monitoring methodology for agencies hoping to conduct preliminary searches at a new

site or those unable to conduct formal capture-recapture studies because of constrained logistical,

time, or financial resources and increases our overall knowledge of range-wide population

trends. Additionally, developing an efficient technique for determining presence provides two

major benefits. First, land management regimes where populations are declared extirpated can

shift (i.e., later spring burns, fall burns, mechanical clearing, etc.). Second, updating Eastern

Massasauga status demonstrates the severity of declines across its range and influences species

protection, funding, and conservation efforts.

Methods

Sample Collection and Filtration

Samples were collected at South Shore State Park (SSSP), one of the largest known

subpopulations in the Carlyle Lake region, Clinton County, Illinois. I took eDNA samples from

50 crayfish burrows during spring emergence (March and April) for the 2021 and 2022 field

seasons, totaling 100 individual burrows. Burrows were chosen for ease of sampling (i.e., open

chimney and visible water) and at a distance of at least 2 m from other sites to avoid overlap of

burrow complexes. Using 10mL pipettes, pipette bulbs, and silicon tubing, I pumped water from

each crayfish burrow into three 50 mL falcon tubes as needed. I also carried a 50 mL "field

blank" tube of distilled water into the field and extracted it with the other samples. After

collection, I froze one tube for future sediment separation and filtered the remaining two within

12 hr. of collection, using a hand-made multi-sample filter station and vacuum pump. I then

placed the 0.2 um filters into 2mL tubes with 900uL of cationic detergent

cetyltrimethylammonium bromide (CTAB) lysis buffer to preserve DNA. All filtering was

conducted at the Carlyle Lake field station in a clean room without recent Eastern Massasauga

presence. The samples were stored at room temperature until extraction, and the sediment

samples remained frozen until extraction.

DNA Extraction

Extractions were performed on the University of Illinois, Urbana-Champaign campus in a clean

room where no Eastern Massasaugas had ever been present. I extracted water samples using a

modified chloroform extraction method described by Renshaw et al. (2015). Frozen samples

were placed in a 65°C hot water bath for 1 hr., after which ~900uL of liquid from the sample was

transferred to a 2.0mL centrifuge tube, leaving the filter behind. Next, I added 900uL of

Chloroform-Isoamyl Alcohol (24:1), vortexed the mixture for 5 sec, and centrifuged for 5 min at

15,000xg. I transferred ~700uL of the aqueous layer to a new centrifuge tube, followed by 700uL

of ice-cold isopropanol and 350uL of 5x NaC. Samples precipitated in the freezer for at least 1

hr. but were left overnight if needed. Following precipitation, I centrifuged the sample for 10 min

at 15,000xg and gently poured off the supernatant, leaving the pellet. The pellet was washed

twice by adding 150uL of 70% ethanol, centrifuging for 5 min at 15,000xg, and decanting the

liquid. After this, I allowed the tubes to dry completely before adding 200uL of lX TE Buffer

and Low EDTA to rehydrate the pellet. Samples were frozen until needed for qPCR.

Soil extractions followed a similar protocol listed above. I incubated samples at 65°C for

1 hr. before adding 10mL Chloroform-Isoamyl alcohol (24:1), vortexing the solution for 5 min

and centrifuging it for 15 min at 3,000xg to separate phases. I then transferred the supernatant to

a new 50mL tube and added equal volumes of ice-cold isopropanol (~10ml) and half of 5x NaCl (~5ml). The sample was left to precipitate in the freezer for at least 1 hr. or overnight. After precipitation, I centrifuged the sample for 15 min at 3,000xg and poured off the supernatant. The pellet was washed with 2mL of 70% ethanol and centrifuged for 2 min at 3000xg. I then poured off the ethanol and allowed the tube to dry completely before adding 1mL of low TE buffer. The sample was heated in a 45°C water bath to resuspend the DNA and then transferred to a 2mL centrifuge tube. Samples were frozen until needed for qPCR.

I extracted blood samples for Eastern Massasaugas and allospecific snake species found in Carlyle, IL using a DNeasy Blood and Tissue Kit to ensure assay specificity. Due to high DNA concentrations, I diluted the samples in a 10-fold dilution series until reaching 1:10,000. I also ran blood samples through a Thermo Scientific NanoDrop One to ensure the smallest dilution was between 0.1 and 0.2 ng/μL, a range predicted to be similar to environmental concentrations (Ratsch et al., 2020). All extractions were performed with a corresponding field blank to ensure contamination was not introduced during the process.

qPCR

I identified DNA presence through qPCR using a primer/probe assay previously described by Baker et al. (2018). Researchers developed a 137-bp fragment primer (Table 3.1) using a sequence library for the mitochondrial cytochrome oxidase subunit 1 (CO1) and confirmed for specificity using the Primer-BLAST tool; the probe was labeled with fluorescent dyes 6-FAM and a TAMARA quencher at the 5' and 3' ends, respectively (Baker et al., 2018). Using the primer/probe, I ran 20uL reaction volumes on a 96-well plate. Each reaction contained 10.0 uL of TaqMan environmental master mix, 5.55 uL of dH$_2$O, 0.25 uL of the primer/probe, 1.0 uL of

10x exogenous control, 0.2 uL of 50x exogenous control, and 3 uL of the extracted sample. I

performed qPCR on a ThermoFisher Scientific QuantStudio3 Real-Time PCR System. The

thermal cycling profile comprised 95°C for 10 min, 50 cycles of 95°C for 15 s, and 58°C for 1

min. I verified assay specificity by first running blood samples of Eastern Massasaugas and non-

target species, which had documented presence in the Carlyle Lake region (*Thamnophis sirtalis*,

Lampropeltis calligaster, *Coluber constrictor*, and *Nerodia sipedon*). Then I tested the field

samples, field blanks, and qPCR negatives run in triplicate. All samples were tested against a

positive standard curve created using a 1×10^5 to 1×10^0 serial dilution of an Eastern Massasauga

product of known concentration. A 10X and 50X TaqMan Exogenous Internal Positive Control

(EXO IPC) was included in the reaction volume to distinguish true negatives from false

negatives, which may occur in the case of PCR inhibition. Unexpectedly late Ct values identified

either inhibition, lack of amplification in the endogenous control target, or high efficiency

(Sidstedt et al., 2020).

Analysis

I tested efficiency and sensitivity to increase detection within the qPCR assay. I determined the

limit of detection (LOD), defined as the minimum concentration needed for a 95% detection of

positive samples, and the limit of quantitation (LOQ), defined as the minimum concentration

needed to determine presence quantitatively (Bustin et al., 2009; Klymus et al., 2019). I ran a

1×10^6 to 1×10^{-4} serial dilution of an Eastern Massasauga product of known concentration with a

minimum of eight replicate wells to determine the PCR cycle in the sample curve intersects the

threshold line (C_q). I then used code *LoD-calculator.R* developed by Kylmus et al. (2019), along

with the C_q values and expected concentrations of each dilution to calculate LOD and LOQ. I set

a detection probability of 95% for LOD and a 35% coefficient of variation (C_v) threshold for LOQ. Next, I used the same reactions to find LOD to determine PCR efficiency by creating a calibration curve in QuantStudio Design and Analysis Software using the equation PCR efficiency $= 10^{-1/slope} - 1$ described by Bustin et al. (2009). Target and non-target snake species samples were also tested to assess the assay's specificity. For any positive detections in the non-target species samples, I compared sample concentrations to those typically found in the environment to determine the likelihood of amplifying non-target DNA (Klymus et al., 2020). After LOD, LOQ, and specificity were confirmed, I processed samples. I estimated DNA concentrations by plotting Cq values on the cumulative standard curve of qPCR standards. Because water collections typically have low eDNA concentrations, samples with any amplifications were considered for further testing (Pfleger et al., 2016). I repeated qPCR to confirm amplifications for samples with only 1 positive replicate of 3.

Response to lost samples

After running qPCR, I prepared positive samples for MiSeq sequencing at the Functional Genomic Unit at the University of Illinois, Urbana-Champaign. Unfortunately, my extracted samples were exposed to potentially detrimental temperatures for at least 48 hrs. due to a freezer failure. Most protocols recommend storing samples at -20 °C or -80 °C to maintain long-term sample integrity (Smith et al., 2005; Allison et al., 2021; Zizka et al., 2022). DNA samples stored at room temperature may be relatively stable for long periods, depending on storage technique and original concentrations. Some research suggests samples stored at room temperature experience degradation after six months and total evaporation at nine months (Wu et al., 2009). However, very high temperatures (>50 °C) cause denaturation and degradation at accelerated

rates (Strickler et al., 2015; Qian et al., 2022). Because the freezer was hot when discovered and eDNA is already fragmented and found in low concentrations, I was concerned the samples would not be viable for sequencing. According to the Functional Genomics Unit, DNA concentrations should ideally be between 10 and 50 ng/uL for sequencing but could be used at lower concentrations. I tested the samples through two means to determine whether enough DNA was present for further analysis. First, I reran my positive samples through qPCR using the same assay I described. Second, I measured concentrations using a Qubit 4 Fluorometer. Following protocol, I prepared 200uL of working solution for each sample and standard by adding 10uL of Qubit reagent to 190uL of Qubit buffer. I then combined 10uL of the sample or standards and 190uL of the working solution into a new 1 ml tube. After vortexing and incubating samples at room temperature for 2 min, I calibrated the fluorometer with the two standards and took readings for each sample. Using this data, I assessed whether sequencing was still a viable option.

<div align="center">Results</div>

Assay Sensitivity and Specificity

To ensure the specificity of my assay, I tested DNA blood samples collected from Eastern Massasaugas and allospecific snake species found in Carlyle, IL. I found samples taken from the Common Gartersnake (*T. sirtalis*) resulted in two replicate amplifications; however, concentration estimates were low at ~0.234 copies/uL even with DNA-dense blood samples, suggesting large amounts of genetically material must be present to bind to the primer. Using the curve-fitting code created by Klymus et al. (2019), I determined LOD and LOQ for my assay at a 95% confidence interval. With an r^2 value of 0.961, LOD was 10 copies of target DNA/reaction,

and LOQ was 67 copies of target DNA/reaction (Figure 3.1). Using the slope of my standard curve, I calculated an efficiency of 125.37%, higher than the recommended values of 90%-110% (Klymus et al., 2020). There were no amplifications in the no template control (NTC) wells, and positive controls amplified in the expected range.

Amplifications

I detected eDNA in 27 water samples; however, due to a positive detection in a field blank, I was forced to discard nine corresponding samples. Of the remaining 18, there were five sets of duplicates, meaning two water samples from the same burrow returned positive results. I found only six samples had positive detections in all three replicates, while nine had two positive detections and two had one positive detection. Sample C_q values ranged from 30.932 to 38.098, with corresponding DNA concentrations between 0.103 copies/uL to 26.643 copies/uL. Most water samples showed low concentrations, with 13 out of the 18 positive samples returning less than 1.00 copy/uL. No DNA was detected in the sediment samples; however, due to signs of inhibition during the original qPCR procedure, including unexpectedly late Ct values and lack of amplification in the endogenous control target, I reran 13 samples. First, I cleaned the samples using a OneStep PCR Inhibitor Removal Kit, which removes inhibitors such as polyphenolic compounds, humic/fulvic acids, and tannins. I then reran the samples through qPCR but found inhibition remained in nine samples. Unfortunately, I could not clean the samples again due to the freezer failure. Of the remaining sediment samples, I found no DNA. NTC samples had no amplifications for all assays, and positive controls amplified in the expected range.

Freezer failure

To test the viability of my samples after heat exposure, I reran any previously noted positive samples through qPCR. I found that 13 out of 18 samples still had positive detections, with C_q values between 30.214 and 41.561 and estimated concentrations between 0.027 copies/uL and 76.518 copies/uL. I tested each sample in the Qubit Fluorometer for more accurate concentration measurements. Despite showing up on qPCR, concentrations were extremely low for most samples. Eight samples had undetectable DNA levels, and five had concentrations under 0.60 ng/uL. Only one sample could be considered for further testing with a concentration of 8.14 ng/uL, which was still below the ideal conditions for MiSeq sequencing. Due to these results, I did not move forward with sequencing.

Discussion

Assays specificity and sensitivity

I developed a highly specific and sensitive assay targeting the cryptic and endangered Eastern Massasauga—positive controls amplified in all assays and specific blood samples amplified at high starting concentrations. I found that Common Garter snake blood samples amplified in my assay; however, given the highly degradable nature of eDNA and the limited shedding of genetic material by snakes (Halstead et al., 2017; Kucherenko et al., 2018; Ratsch et al., 2020), the DNA concentrations needed to amplify for this species are unlikely to exist naturally in the environment. Thus, I concluded the other species did not cause positive detections in my final assays. I found the LOD was 10 copies/uL and LOQ was 67 copies/uL, which were normal compared to LODs in other semi-aquatic snake eDNA detection assays (1 copy/uL–100 copies/uL) (Merkling, 2009; Hunter et al., 2019; Katz et al., 2021; Ratsch et al., 2020). However,

I did find that the r^2 value and efficiency fell outside the ranges recommended by researchers. Klymus et al. (2020) suggested the r^2 value should be above 0.98, with ideal results approaching 1.0, and efficiency should fall between 90%-110%, with ideal values near 100%. A low r^2 value (0.961) and high efficiency (125.37%) indicate problems with the assay. High efficiencies can originate from contamination on the plate, pipetting errors, inhibitors, or primer dimers (Cepin, 2017). However, because I included internal inhibitor controls, found no contamination in the no template control (NTC) wells, and used a probe-based method, I believe that inaccurate standard concentrations were the primary cause of the high efficiency of my assay. I planned to sequence any positive samples to confirm the target DNA and compare concentration estimates to verify accuracy.

Soil Detection

Given previous success (Leempoel et al., 2020; Valentin et al., 2020; Allen et al., 2021), I expected to detect Eastern Massasauga eDNA in my sediment samples. Research shows detection rates are often higher in sediment than in water samples (Bairoliya et al., 2022), largely due to the ability of DNA to bind to particles in the soils. DNA is protected from degradation by binding to mineral and humic products, leading to longer environmental persistence and higher detection rates (Sakata et al., 2020). Surprisingly, though, I found no positive detections in my sediment samples. Although a lack of detection could be due to issues with the extraction protocol or inhibition, I believe environmental factors, including the sediment, partially caused the results. Research shows loamy soils, like those found at my field site, result in lower detections because DNA is less likely to bind to fine particles, leading to faster degradation (Buxton et al., 2018). Similarly, moist soils resulted in accelerated decay rates and reduced

stabilization compared to dry soils (Bairoliya et al., 2022), and certain soils can also introduce different types or quantities of inhibitors (Buxton et al., 2017). I performed a χ^2 Goodness of Fit Test to determine whether there was a significant relationship between sample type and inhibition rate and found that sediment samples were more likely than water samples to show inhibition ($\chi^2_{1, 300} = 27.18$, p << 0.0001). The presence of inhibitors requires additional clean-up during or post-extraction, which may dilute samples, leading to even lower detection rates (Matthias et al., 2021). Given my results and the impact of environmental characteristics on eDNA sediment detection, I believe soils present at my field site caused high levels of degradation and inhibition in qPCR, resulting in no positive detections. I recommend future work to assess the use of sediment in Eastern Massasauga eDNA detection by investigating degradation rates in various soils and testing extraction kits to improve detection and reduce inhibition. Additionally, because crayfish burrows are complex and little is known about snake movement within them (Katz et al., 2021), sampling protocols should be reviewed to increase the likelihood of capturing eDNA. Collecting samples deeper in burrows or timing collections when snakes are more likely to be near the surface could improve detection.

Water detection

I detected DNA in 27 of my water samples, nine of which were removed due to contamination of the associated field blank. The remaining 18 positive samples had concentrations ranging from 0.103 copies/uL to 26.643 copies/uL, with five samples falling below the LOD. Although samples below the calculated LOD cannot be quantified, the qPCR specificity means there is no lower limit for true positives (Klymus et al., 2020). Therefore, all samples were considered for further analysis and prepared for sequencing. Based on past results, I expected to see low

detection rates. Previously, Merkling (2019) identified Eastern Massasauga DNA in six out of 60

samples, while Baker et al. (2018) identified just two out of 100 samples. Similarly, Ratsch et al.

(2020) found Kirkland's snake DNA in six out of 380 samples. Because I had to discard samples

due to contamination, I only had samples from 90 burrows; however, I still had a better detection

rate than in previous work, with positive detection in 13 out of 90 burrows. Unfortunately, I

could not confirm whether any positive detections were from Eastern Massasaugas. Due to

significant heat exposure during the freezer failure, sample DNA concentrations did not meet the

threshold needed for MiSeq sequencing. Because this form of sequencing is costly, and I was

unlikely to receive conclusive results, I did not proceed with the procedure.

Future use

Past work regarding Eastern Massasauga eDNA reported low detection rates even when snake

and DNA presence were confirmed (Merkling, 2019; Baker et al., 2020). DNA deposition,

degradation rates, and environmental factors heavily influence the effectiveness of eDNA

detection (Merkling, 2019; Katz et al., 2021; Matthias et al., 2021). Crayfish burrows are

occupied by Eastern Massasaugas during winter, when activity and metabolic functions are low

(Cobb & Peterson, 2008). During this period, snakes may not shed their skin or defecate for

several months, meaning minimal genetic material is deposited into the environment (Ratsch et

al., 2019). Additionally, previous work shows that DNA can persist in nature from 14 to 60 days

(Santas et al., 2013; Goldberg et al., 2015); however, some studies found snake DNA began

degrading significantly in soil within four to ten days and in water within two days (Piaggio et

al., 2014; Kucherenko et al., 2018). Environmental conditions, such as temperature, rainfall, pH,

and dissolved oxygen, can also increase degradation (Baker et al., 2018; Merkling, 2019),

meaning collections may need to be tailored to ideal conditions. Overall, lack of shedding and high degradation rates will likely reduce the amount of detectable DNA and, thus, the effectiveness of eDNA monitoring. Research to understand degradation in sediment, to evaluate genetic material deposition rates by snakes during overwintering periods, and to optimize sample collection should precede any future eDNA work for the Eastern Massasauga.

Despite low detection rates, all previous Eastern Massasauga and Kirtland's snake eDNA studies were able to confirm presence at their respective study site (Baker et al., 2018; Merkling, 2019; Ratsch et al., 2019), and I was able to detect Eastern Massasauga DNA in 13 burrows during two field seasons. While it may not be an effective alternative to traditional monitoring, eDNA could provide a prescreening or synchronous survey tool. eDNA has been broadly used to monitor low-density or difficult-to-detect species in known habitats (Barnes & Turner, 2016; Corlett, 2017; Beng & Corlett, 2020), but it can also be beneficial for initial detection or identification of species in the early stages of research (Darling, 2020). eDNA has been particularly useful in the management of invasive species, which can be used to detect introductions, track their spread, and monitor for survivors after eradication efforts (NISC, 2022). Additionally, eDNA could be used as a compliment to traditional survey methods. Other monitoring methods provide information about abundance and population dynamics that eDNA, as a presence indicator, cannot achieve; however, under certain conditions, eDNA may be more sensitive for detecting target species (McKee et al., 2015). For the Eastern Massasauga, eDNA may provide an additional tool to assess potentially occupied habitats and identify presence in known sites.

Table 3.1. Primers and probes developed by Baker et al. (2018) used to detect Eastern

Massasauga eDNA.

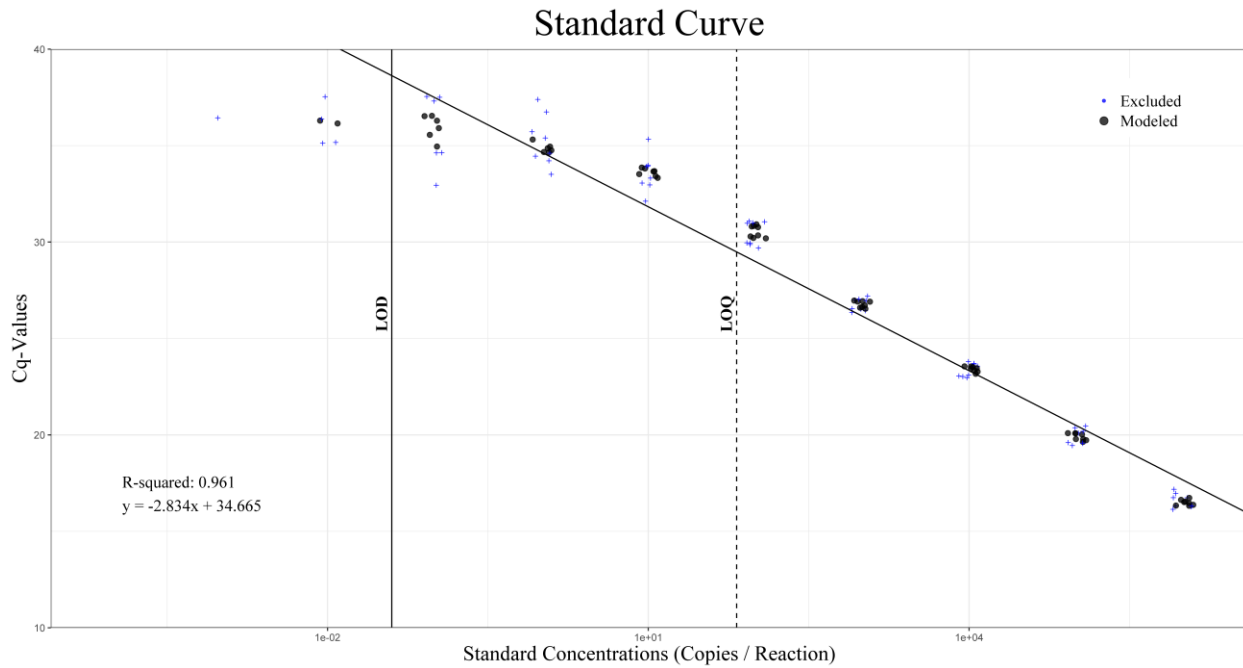| Contains	nmoles	Sequence
Forward	5	5′-CACTACTTCTCCTACTCTCCTC-3′
Reverse	5	5′-GGCCAAATGAAGGGAGAAA-3′
Probe	2.5	5′-/ 6-FAM/AACAGTTCATCCTGTCCCTGCGCC/36TAMSp/-3′

Figure 3.1. Standard curve plot with Cq values and standard concentrations, displaying the limit of detection (LOD) and limit of quantification (LOQ). Black points are those in the middle 2 quartiles of standards with ≥50% detection, and blue points are those outside the middle 2 quartiles or for standards with <50% detection.

Literature Cited

Allen, M.C., Nielsen, A.L., Peterson, D.L., and Lockwood, J.L. 2021. Terrestrial eDNA survey
outperforms conventional approach for detecting an invasive pest insect within an
agricultural ecosystem. Environmental DNA 3:1102—1112.

Allison, M.J., Round, J.M., Bergman, L.C., Mirabzadeh, A., Allen, H., Weir, A., et al. The effect
of silica desiccation under different storage conditions on filter-immobilized
environmental DNA. BMC Research Notes 14:106.

Bairoliya, S., Koh Zhi Xiang, J., and Cao, B. 2022. Extracellular DNA in environmental
samples: Occurrence, extraction, quantification, and impact on microbial biodiversity
assessment. Applied Environmental Microbiology 88:e0184521.

Baker, S.J., Anthonysamy, W.J.B., Davis, M.A., Dreslik, M.J., Douglas, M.R., Douglas, M.E., et
al. 2018. Temporal patterns of genetic diversity in an imperiled population of the Eastern
Massasauga rattlesnake (*Sistrurus catenatus*). Copeia 106:414—420.

Baker, S.J., Niemiller, M.L., Stites, A.J., Ash, K.T., Davis, M.A., Dreslik, M.J., et al. 2020.
Evaluation of environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces
ophiodiicola* in crayfish burrows. Conservation Genetics Resources 12:13—15.

Barnes, M.A. and Turner, C.R. 2016. The ecology of environmental DNA and implications for
conservation genetics. Conservation Genetics 17:1—17.

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L., and Lodge., D.M.
2014. Environmental conditions influence eDNA persistence in aquatic systems.
Environmental Science and Technology 48:1819—1827.

Beng, K.C. and Corlett, R.T. 2020. Applications of environmental DNA (eDNA) in ecology and
conservation: Opportunities, challenges and prospects. Biodiversity and Conservation

29:2089—2121.

Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C., et al. 1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Research 25:3235—3241.

Bustin, S.A., Benes V., Garson J.A., Hellemans, J., Huggett J., Kubista M., et al. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55:611—22.

Buxton, A.S., Groombridge, J.J., and Griffiths, R.A. 2017. Is the detection of aquatic environmental DNA influenced by substrate type? PLOS ONE 12:e0183371.

Buxton, A.S., Groombridge, J.J, and Griffiths, R.A. 2018. Seasonal variation in environmental DNA detection in sediment and water samples. PLOS ONE 13:e0191737.

Cepin, U. 2017. Understanding qPCR efficiency and why it can exceed 100%. BioSistemika.

Cobb, V.A. and Peterson, C.R. 2008. Thermal ecology of hibernation in a population of Great Basin rattlesnakes. The Biology of Rattlesnakes (Hayes, W., Beaman, K., Cardwell, M., and Bush, S. eds). Loma Linda University Press, Loma Linda, California 11 p.

Corlett, R.T. 2017. A bigger toolbox: Biotechnology in biodiversity conservation. Trends in Biotechnology 35:55—65.

Crawford, J.A., Dreslik, M.J., Baker, S.J., Phillips, C.A., and Peterman, W.E. 2020. Factors affecting the detection of an imperiled and cryptic species. Diversity 12:177.

Darling, J.A. How to learn to stop worrying and love environmental DNA monitoring. 2020. Aquatic Ecosystems Health and Management 22:440–451.

Das, S., Mohapatra, S.C., and Hsu, J.T. 1999. Studies on primer-dimer formation in polymerase chain reaction (PCR). Biotechnology Techniques 13:129—134.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., and Minamoto, T. 2015. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PLOS ONE 10:e0122763.

Durso, A.M., Willson, J.D., and Winne, C.T. 2011. Needles in haystacks: Estimating detection probability and occupancy of rare and cryptic snakes. Biological Conservation 144:1508—1515.

Goldberg, C.S., Strickler, K.M., and Pilliod, D.S. 2015. Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. Biological Conservation 183:1—3.

Halstead, B.J., Wood, D.A., Bowen, L., Waters, S.C., Vandergast, A.G., Erson, J.S., et al. 2017. An evaluation of the efficacy of using environmental DNA (eDNA) to detect giant gartersnakes (*Thamnophis gigas*). U.S. Geological Survey Open-File Report, Reston, Virginia p. 41.

Hunter, M.E., Meigs-Friend, G., Ferrante, J.A., Smith, B.J., and Hart, K.M. 2019. Efficacy of eDNA as an early detection indicator for Burmese pythons in the ARM Loxahatchee National Wildlife Refuge in the greater Everglades ecosystem. Ecological Indicators 102:617—622

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J.A., Smith, B.J., Hunter, C.T., et al. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. PLOS One 10:e0121655.

Katz, A.D., Harper, L.R., Sternhagen, E.C., Pearce, S.E., Melder, C.A., Sperry, J.H., et al. 2021. Environmental DNA is effective in detecting the federally threatened Louisiana Pinesnake (*Pituophis ruthveni*). Environmental DNA 3:409—425.

Kery, M. 2002. Inferring the absence of a species: A case study of snakes. The Journal of
Wildlife Management 66:330—338.

Klymus, K.E., Merkes, C.M., Allison, M.J., Goldberg, C.S., Helbing, C.C., Hunter, M.E., et al.
2019. Reporting the limits of detection and quantification for environmental DNA assays.
Environmental DNA 2:271—282.

Klymus, K.E., Ruiz-Ramos, D.V., Thompson, N.L., and Richter, C.A. 2020. Development and
testing of species-specific quantitative PCR assays for environmental DNA applications.
Journal of Visualized Experiments 165:e61825.

Kucherenko, A., Herman, J.E., Everham III, E.M. and Urakawa, H. 2018. Terrestrial snake
environmental DNA accumulation and degradation dynamics and its environmental
application. Herpetologica 74:38—49.

Leempoel, K., Hebert, T., and Hadley, E.A. 2020. A comparison of eDNA to camera trapping for
assessment of terrestrial mammal diversity. Proceedings of the Royal Society B 287:
20192353.

Lor, Y., Schreier, T.M., Waller, D.L., and Merkes, C.M. 2020. Using environmental DNA
(eDNA) to detect the endangered Spectaclecase mussel (*Margaritifera monodonta*).
Freshwater Science 39:837—847.

Ma, H., Stewart, K., Lougheed, S., Zheng, J., Wang, Y., and Zhao, J. 2016. Characterization,
optimization, and validation of environmental DNA (eDNA) markers to detect an
endangered aquatic mammal. Conservation Genetics Resources 8:561—568.

Matthias, L., Allison, M.J., Maslovat, C.Y., Hobbs, J., and Helbing, C.C. 2021. Improving
ecological surveys for the detection of cryptic, fossorial snakes using eDNA on and under
artificial cover objects. Ecological Indicators 131:108187.

Mazerolle, M.J., Bailey, L.L., Kendall, W.L., Royle, A.R., Converse, S.J., and Nichols, J.D.

    2009. Making great leaps forward: Accounting for detectability in herpetological field

    studies. Journal of Herpetology 41:672—689.

McKee, A.M., Calhoun, D.L., Barichivich, W.J., Spear, S.F., Goldberg, C.S., and Glenn, T.C.

    2015. Assessment of environmental DNA for detecting presence of imperiled aquatic

    amphibian species in isolated wetlands. Journal of Fish and Wildlife Management 2015

    6:498–510.

Merkling, J. 2019. Development of an Environmental DNA Assay for Eastern Massasauga.

    M.Sc. Thesis. Purdue University Graduate School, West Lafayette, Indiana 61 p.

National Invasive Species Council (NISC). 2022. Environmental DNA as a tool for invasive

    species detection and management. U.S. Department of the Interior, Washington, D.C.

    7 p.

Pfleger, M.O., Rider, S.J., Johnston, C.E., and Janosik, A.M. 2016. Saving the doomed: Using

    eDNA to aid in detection of rare sturgeon for conservation (*Acipenseridae*). Global

    Ecology and Conservation 8:99—107.

Piaggio A.J., Engeman R.M., Hopken, M.W., Humphrey J.S., Keacher K.L., Bruce W.E., et al.

    2014. Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python

    in Florida waters and an assessment of persistence of environmental DNA. Molecular

    Ecology Resource 14:374—80.

Qian, T., Shan, X., Wang, W., and Jin, X. 2022. Effects of temperature on the timeliness of

    eDNA/eRNA: A case study of *Fenneropenaeus chinensis*. Water 14:1155—1567.

Ratsch, R. 2019. Efficacy of using environmental DNA (eDNA) to detect Kirtland's snakes

(*Clonophis kirtlandii*). M.Sc Thesis. Purdue University Graduate School, West Lafayette, Indiana 54 p.

Ratsch, R., Kingsbury, B.A., and Jordan, M.A. 2020. Exploration of environmental DNA (eDNA) to detect Kirtland's snake (*Clonophis kirtlandii*). Animals: An Open Access Journal from MDPI 10:1057.

Rose, J.P., Wademan, C., Weir, S., Wood, J.S., and Todd, B.D. 2019. Traditional trapping methods outperform eDNA sampling for introduced semi-aquatic snakes. PLOS ONE 14:e0219244.

Sakata, M.K., Yamamoto, S., Gotoh, R.O., Miya, M., Yamanaka, H., and Minamoto, T. 2020. Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. Environmental DNA 2:505—518.

Santas, A.J., Persaud, T., Wolfe, B.A., and Bauman, J.M. 2013. Noninvasive method for a statewide survey of eastern hellbenders *Cryptobranchus alleganiensis* using environmental DNA. International Journal of Zoology 2013:1—6.

Sidstedt, M., Radstrom, P., and Hedman, J. 2020. PCR inhibition in qPCR, dPCR, and MPS-mechanisms and solutions. Analytical and Bioanalytical Chemistry 412: 2009–2023.

Smith, C.S. 2009. Hibernation of the Eastern Massasauga rattlesnake (*Sistrurus catenatus catenatus*) in northern Michigan. M.Sc. Thesis. Purdue University Graduate School, West Lafayette, Indiana 54 p.

Smith, S. and Morin, P.A. 2005. Optimal storage conditions for highly dilute DNA samples: A role for trehalose as a preserving agent. Journal of Forensic Science 50:426—534.

Strickler, K.M., Fremier, A.K., and Goldberg, C.S. 2015. Quantifying effects of UV-B,

temperature, and pH on eDNA degradation in aquatic microcosms. Biological

Conservation 183:85—92.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Ramussen, M., Gilbert, M.T.P., et al. 2012.

Monitoring endangered freshwater biodiversity using environmental DNA. Molecular

Ecology 21:2565—2573.

Valentin, R.E., Fonseca, D.M., Gable, S., Kyle, K.E., Hamilton, G.C., Nielsen, A.L., et al. 2020.

Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect

invasive forest insects. Molecular Ecology Resources 20:746—755.

Wood, Z.T., Erdman, B.F., York, G., Trial, J.G., and Kinnison, M.T. 2020. Experimental

assessment of optimal lotic eDNA sampling and assay multiplexing for a critically

endangered fish. Environmental DNA 2:407—417.

Wu, J., Cunanan, J., Kim, L., Kulatunga, T., Huang, C., and Anekella, B. 2009. Stability of

genomic DNA at various storage conditions. Poster. International Society for Biological

and Environmental Repositories 2009 Annual Meeting, Portland, Oregon.

Zizka, V., Koschorreck, J., Khan, C.C., and Astrin, J.J. 2022. Long-term archival of

environmental samples empowers biodiversity monitoring and ecological research.

Environmental Sciences Europe 34:40.

CHAPTER 4: SUMMARY AND CONSERVATION IMPLICATIONS

Environmental variable selection

Selecting environmental predictors is critical in developing successful and transferable habitat

suitability models (Regos et al., 2019). Although climate attributes are popular for their accuracy

and predictive power (Guisan & Zimmermann, 2000), these variables are unsuitable for all

projects due to differences in extent, size, and resolution. Spatial scales often determine the

importance of different processes and biotic interactions in spatial modeling (Pearson & Dawson,

2003). For example, on a continental scale, climate is typically the dominant factor; however, as

geographic resolution is reduced, land cover and topography become increasingly important

(Phillips et al., 2006). Spatial scales also affect a model's transferability or ability to predict

species distribution in unsampled regions (Randin et al., 2006). Not only is transferability an

important component of model evaluation, but as a conservation biology and wildlife

management tool, models with high transferability are valuable for extrapolation into new areas

and predictions of climate-change responses (Randin et al., 2006). However, transferability can

be difficult to achieve and requires selecting a small set of direct and meaningful environmental

predictors (Regos et al., 2019).

One of my objectives for Chapter 2 was to provide methods for future habitat modeling

by identifying important environmental characteristics for Eastern Massasauga overwintering

sites in Carlyle Lake, Clinton County, Illinois. To do this, I selected variables directly related to

the species' survival, logical for the small resolution of my study extent, and relevant to other

southwestern mesic prairie habitats. For the final model, I included three variables related to land

characteristics, four to vegetation and two to water access. Distance to medium-height vegetation

(1.5 – 6.0 m), distance to water, and soil characteristics made the greatest model contributions, indicating the variables are important for Eastern Massasaugas overwintering sites. Elevation (DEM) and canopy height showed moderate importance, whereas the distance to high vegetation and flood zone contributed marginally to predictive accuracy. Based on my results, I suggest including distance to water and soil characteristics in future models. For Eastern Massasauga populations relying on crayfish burrows for overwintering, access to the water table is crucial for winter survival, making proximity to water and poorly drained soils, such as silt loam, good indicators of appropriate habitat. I recommend including elevation and associated variables, such as topographic position or wetness, because of their moderate contribution to the model. I hypothesized elevation would be an important variable for Eastern Massasauga overwintering sites because of its effect on groundwater access; however, the contribution of DEM was lower than expected, likely caused by smooth topography within my study extent. DEM may be a significant predictor in areas with increased elevation variation as more areas are less appropriate for Eastern Massasauga overwinter survival.

As the highest performing variable, I would include distance to medium vegetation; however, because of differences in land cover and plant communities across sites, I would also consider further research to determine what vegetation types and heights are most important to the specific study extent. For example, some sites may have much higher levels of succession and, thus, a denser canopy. In this situation, open canopy areas may be more significant in predicting overwintering sites than canopy height or distance to certain vegetation types. One method to determine what variables should be included is running a model with all vegetation variables, removing those with a contribution of <1% (Wang et al., 2017), and retaining only the highest-performing variables for the final model. While this additional step could improve model

fit, retaining correlated vegetation variables may not affect overall performance (Phillips &

Dudik, 2008; Elith et al., 2011; Feng et al., 2019). Given performance did not improve when I

removed potential collinearity and aimed to provide a framework for broader use, I retained all

nine environmental variables. To guide variable inclusion, future users should determine whether

accuracy or predictive capabilities are affected by low-performing variables and whether

removing or retaining them impacts the overall objective of the study.

<u>Overwintering site management</u>

Researchers estimate overwinter mortality rates range from ~11–53% for snakes due to harsh

winter conditions, such as freezing temperatures, low humidity, and fluctuating water levels

(Hirth, 1966; Gillingham & Carpenter, 1978; Costanzo, 1989; Kissner et al., 2005; Harvey &

Weatherhead, 2006). Because of specific survival requirements and diminishing habitats,

suitable overwintering refugia may be a limiting factor for snake populations (Harvey &

Weatherhead, 2006). Additionally, fragmentation and landscape degradation may impair a

species' ability to overwinter successfully and engage in crucial activities associated with ingress

and egress, such as basking and foraging (Markle et al., 2018). Protecting and managing

important overwintering habitats should be prioritized to ensure endangered species' survival.

Habitat protection is especially important for the Eastern Massasauga, whose western

populations are predicted to suffer 97% extirpations in the next fifty years (USFWS, 2016).

Within Illinois, populations have already declined by 96% (Szymanski et al., 2015) due to the

significant loss of important tall grass prairies and wetland habitats (Corbett, 2004).

Using my model, I identified 17 state or federally-protected sites containing highly

suitable habitat. Most locations I identified are known overwintering sites in EHSP, SSSP, and

DERA/DWRA, 14 of which had at least one visual encounter in the last two years of surveys (Figure 4.1). Although these habitats are spread broadly across the study extent, most are connected by moderately suitable habitat or are close to at least one other site (12 m to 226 m). Connectivity between sites provides important dispersal space during the summer and fall, which can increase prey availability and encourage genetic diversity (Taylor et al., 1993; Lowe & Allendorf, 2010; Zeller et al., 2020). Because Eastern Massasaugas have persisted at these sites and high-quality habitat is present, these areas should be prioritized for management. Specifically, I recommend restoring the 14 sites to their original condition to provide high-quality overwintering habitat.

Invasive species are the primary threat to tall-prairie grass restoration (Rowe, 2010), and thus to combat invasive vegetation and woody encroachment, management should maintain regular disturbance regimes in two- to three-year cycles (Peterson & Reich, 2008). Burning and mowing are already used in the Carlyle Lake region, but additional methods, such as manual removal or herbicide application, may be required to control previously established or persistent woody vegetation. I also suggest increasing plant diversity by including a broader range of native species, which can suppress exotic plant density (Middleton et al., 2009). Research suggests introducing a mix of grasses and forbes through broad cast and drill seeding and seedling plantings to achieve maximum diversity (Biondini, 2007; Wold et al., 2011; Harmon-Threatt & Chin, 2016). Based on my results, I suggest incorporating medium-height vegetation into plant management regimes. I found habitat suitability was highest at 0.5 m for distance to medium-height vegetation; however, retaining this level of vegetation would reduce open-canopy availability. To provide appropriate cover and maintain vital open canopies, I suggest managing overwintering sites so every possible refugium is 30 m away from medium-height vegetation.

Previous work estimates Eastern Massasaugas will move an average of 30 m from their burrow during spring egress, meaning cover within this distance should be accessible (Marshall et al., 2006). For smaller sites with pre-existing vegetation, efforts should focus on preserving shrubs and trees between 1.5 and 6.0 m tall, while larger sites may require plantings. Before starting any large-scale restoration project, I recommend further research to create specific guidelines for medium-height vegetation management. Specifically, future work should distinguish between woody and herbaceous vegetation use, identify important species, and quantify density requirements. Other considerations for restoration include reducing edge effects by maintaining buffer zones around sites and creating habitat gradients for seasonal dispersion (Fischer & Lindenmayer, 2007).

The remaining three sites containing highly suitable habitats were Admin East, North Allen Branch Public Hunting Area, and Allen Branch Public Boat Launch, which have no recent encounters (Table 4.1). Admin East is located near the admin building, extending ~390 m east towards the road and ~150 m north into a wooded portion of the park. Although near Admin South, Admin East has not been prioritized for surveys, with only four searches documented between 2002 and 2022 and the most recent encounter occurring in 2000. Interestingly, both sites are connected by highly suitable habitat, most of which falls into Admin East's boundaries. Because of the recent encounter in Admin South and the connectivity between sites, I suggest including Admin East in future searches, with special attention paid to the suitable habitat between the two sites. This area may be an ideal corridor for dispersal. North Allen Branch is a 15 km^2 park north of the Allen Branch inlet, which divides the public hunting area from the rest of EHSP. Preliminary ground truthing revealed that sizable portions of the area were cleared of most vegetation, likely for turkey and pheasant hunting in the spring. Although North Allen

Branch is within a public hunting area and access may be restricted during certain periods, management should consider assessing and subsetting the highly suitable habitat for restoration. I recommend conducting habitat and wildlife surveys to determine habitat quality, prey abundance, and Eastern Massasauga presence. Allen Branch Public Boat launch is on the northern tip of EHSP and contains 0.003 km^2 of highly suitable habitat, which falls directly in the middle of a parking lot. Construction of this parking lot may have pushed the snakes outwards, so I suggest searching the boundaries to determine the presence status. Specifically, there is an open-canopied area south of the boat launch with important silt loam soils and proximity to water, indicating it may be quality habitat.

All other known sites had moderately suitable habitat except four: James Hawn Dove Field, Massasauga Parking Lot, Pump Station Wetland, and Fish Ponds. Massasauga Parking Lot has had eight encounters up until 2009, while James Hawn has only one encounter from 2000. There are no encounters listed within the defined boundaries of Pump Station Wetland. All three sites are highly isolated from EHSP, SSSP, and DERA/DWRA, meaning dispersal to other suitable habitats is impossible. Searching has resumed at these sites to determine extirpation; however, due to the minimal historical presence, unsuitable habitats, and fragmentation from other sites, efforts should not continue beyond these final surveys. Fish Ponds displays no suitability in the current survey areas; however, there have been two encounters in the last four years, one in 2019 and one in 2023. These encounters are likely due to dispersal from nearby moderately suitable habitats along the southeast, northwest, and northeast boundaries. Based on these results, I recommend extending searches into the surrounding area rather than continuing to search the path directly around the ponds.

Other sites of interest include Coles Creek and Gerdes Cemetery, along the eastern shore just north of SSSP and containing moderately suitable habitat. Both sites have documented encounters until 2002, but Gerdes Cemetery had only one snake recorded, whereas Coles Creek had six. Because of previous encounters and suitable habitat, I suggest adding searches at these sites to determine Eastern Massasauga presence. More effort should be directed to Coles Creek because of the history of encounters, and searches should be extended past the specified boundaries into the east and south.

Most of the potential overwintering habitat I identified is concentrated in the three primary study areas. EHSP contains the most suitable habitat at ~2.48 km^2, SSSP at ~0.68 km2, and DERA/DWRA at ~0.55 km^2. Historically, Carlyle, Illinois, was a mosaic of tall-grass prairie and wetland habitat (Corbett, 2004), but urban development (i.e., campgrounds, parking lots, or roads) and late successional habitat have caused persistent fragmentation, leading to isolation of important Eastern Massasauga overwintering sites. Despite the fragmentation, occupied sites are often connected by moderately suitable habitats, meaning aggregating habitat patches may be possible. To improve connectivity, I suggest creating open-canopied grassland corridors between patches, allowing dispersal between sites and increasing genetic exchange.

Research has shown appropriate winter refugia can be a limiting factor for many snake species and identifying them all is likely unachievable (Johnson et al., 2000; Rudolph et al., 2007). Based on my findings, I recommend restoring and increasing the connectivity of all overwintering sites in EHSP, SSSP, and DERA/DWRA. Sites with high suitability and present occupation should be prioritized for restoration, while those with unknown presence status, including North Allen Branch Public Hunting Area, Allen Branch Boat Launch, Gerdes Cemetery, and Coles Creek, should be searched to determine whether snakes are present.

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Because of the limited amount of suitable and protected habitat in the Carlyle Lake region, it may be necessary to purchase or enroll into easements additional habitat for restoration. Research shows more area generally means more resources, environmental variation, and individuals (Hodgson et al., 2009), so extending important habitat could improve Eastern Massasauga survival. Land purchased or enrolled in easements should undergo rigorous evaluations to ensure appropriate environmental characteristics are present. Specifically, lands should be within ~750 m of a water source and contain silt loam soils capable of supporting crayfish populations. For small populations with high extirpation risks, like Carlyle Lake's Eastern Massasaugas, overwintering site availability could impact survival, meaning protecting any suitable habitat is crucial in long-term conservation plans.

<u>eDNA survey method</u>

Environmental DNA (eDNA) is a popular detection method (Barnes & Turner, 2015; Beng & Corlett, 2019). By extracting genetic material left in water, soil, or sediment, researchers can conduct quick, non-invasive, relatively inexpensive surveys compared to traditional monitoring (Beng & Corlett, 2019). Additionally, eDNA can yield high detection probabilities for difficult-to-detect, low-density, or endangered species (Ma et al., 2016; Pfleger et al., 2016; Lor et al., 2019; Wood et al., 2020). Previous work involving snake eDNA detection had mixed results, with projects surrounding small, semi-aquatic, or terrestrial snake species showing low detection rates (Halstead et al., 2017; Rose et al., 2019; Ratsch et al., 2019). Attempts to develop an efficient eDNA detection protocol for the Eastern Massasauga have also shown little success (Baker et al., 2018; Merkling, 2019). Research shows that DNA deposition, degradation rates, and environmental factors heavily influence the effectiveness of eDNA detection (Merkling,

2019; Katz et al., 2021; Matthias et al., 2021). Crayfish burrows are occupied by Eastern Massasaugas during winter, when activity and metabolic functions are low (Cobb & Peterson, 2008), indicating that minimal amounts of genetic material are being deposited into the environment (Ratsch et al., 2019). Keratinized scales, small size, and propensity to defecate and shed away from their burrow also reduce DNA deposition (Ratsch, 2019; Ratsch et al., 2020).

Although I did not find Eastern Massasauga DNA in my sediment samples, I found a higher detection rate in water compared to other studies, with detections in 13 out of 100 crayfish burrows. Lack of detection in sediment is likely a result of accelerated decay rates and the introduction of inhibitors from the loamy, moist soils found at my field site (Buxton et al., 2018; Bairoliya et al., 2022). I could not confirm whether positive water detections were Eastern Massasauga due to the loss of samples caused by a freezer failure. However, based on my preliminary results, I suggest eDNA be considered a prescreening or synchronous tool to traditional monitoring methods for Eastern Massasauga management. Despite low detection rates, all research regarding Eastern Massasauga or Kirtland's snake detection was able to confirm presence at their study sites (Baker et al., 2018; Merkling, 2019; Ratsch et al., 2020), suggesting that eDNA is effective at detecting the species. However, given the overall low detection rates and lack of amplifications in sediment samples, further research should be done into sampling protocols. Specifically, improving our understanding of how eDNA behaves in crayfish burrows could increase detection rates. I suggest investigating degradation rates in sediment and testing collection methods to account for environmental conditions, the shedding of genetic material, and the location of DNA within the burrow. Additionally, introducing new extraction and inhibition cleaning methods could reduce inhibition in the sediment samples and improve overall detection. Optimizing a specific and sensitive eDNA assay improves

conservation efforts by providing a method to detect Eastern Massasaugas in new habitats,

identify extirpated sites, and confirm presence.

Table 4.1. Overwintering sites with highly suitable habitat in the Carlyle Lake region, Clinton County, Illinois, with the total suitable area and last documented snake encounter.

| Site name	Highly Suitable Area	Last Snake Encounter
Admin East	0.036 km^2	2000
Admin South	0.006 km^2	2022
Allen Branch Boat Launch	0.003 km^2	N/A
Apache Boat Ramp	0.013 km^2	2023
Archery	0.008 km^2	2022
Combat Field	0.006 km^2	2023
Dam East	0.027 km^2	2023
Dam West	0.018 km^2	2023
Dam East Old Channel	0.005 km^2	2023
Dam East Boat Ramp	0.010 km^2	2010
Hazlet Field #3	0.016 km^2	2023
Hazlet Field #6	0.035 km^2	2022
HR3E	0.085 km^2	2023
HR3W	0.023 km^2	2023
Illini Trail	0.011 km^2	2023
North Allen Branch Hunting Areas	0.14 km^2	N/A
Restoration Roadside	0.003 km^2	2022
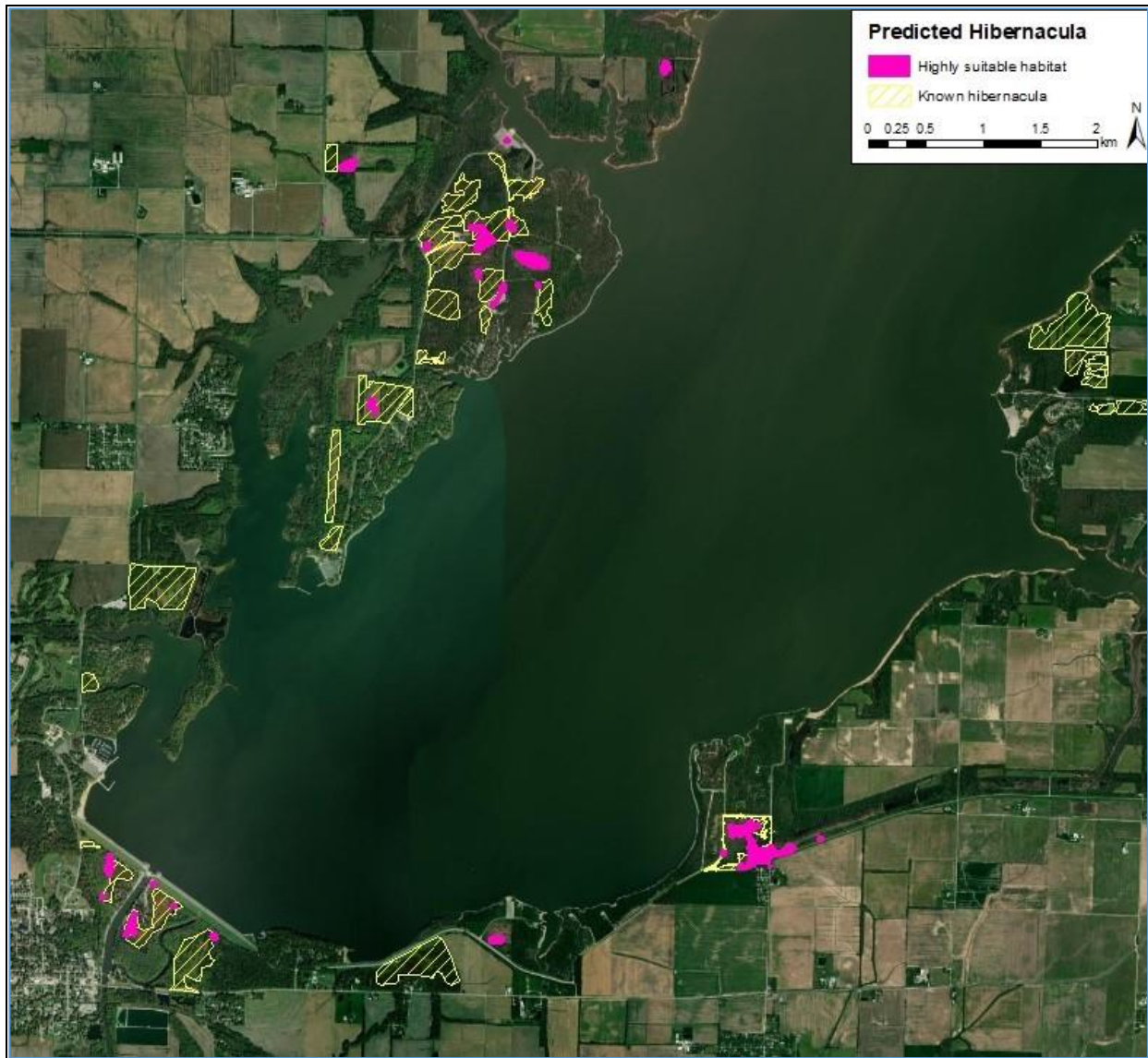
Figure 4.1. Site boundaries and highly suitable (>0.75) overwintering habitat for Eastern

Massasaugas in the southern Carlyle Lake region, Clinton County, Illinois.

Literature Cited

Abd-Elsalam, K.A. 2003. Bioinformatic tools and guideline for PCR primer design. African

    Journal of Biotechnology 2:91—95.

Bairoliya, S., Koh Zhi Xiang, J., and Cao, B. 2022. Extracellular DNA in environmental

    samples: Occurrence, extraction, quantification, and impact on microbial biodiversity

    assessment. Applied Environmental Microbiology 88:e0184521.

Baker, S.J., Niemiller, M.L., Stites, A.J., Ash, K.T., Davis, M.A., Dreslik, M.J., et al. 2020.

    Evaluation of environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces*

    ophiodiicola in crayfish burrows. Conservation Genetics Resources 12:13—15.

Barnes, M.A. and Turner, C.R. 2016. The ecology of environmental DNA and implications for

    conservation genetics. Conservation Genetics 17:1—17.

Beng, K.C. and Corlett, R.T. 2020. Applications of environmental DNA (eDNA) in ecology and

    conservation: Opportunities, challenges and prospects. Biodiversity and Conservation

    29:2089—2121.

Biondini, M. 2007. Plant diversity, production, stability, and susceptibility to invasion in restored

    northern tall grass prairies (United States). Restoration Ecology 15:77—87.

Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C., and Little, S.

    1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Research

    25:3235—3241.

Buxton, A.S., Groombridge, J.J., and Griffiths, R.A. 2018. Seasonal variation in environmental

    DNA detection in sediment and water samples. PLOS ONE 13:e0191737.

Cobb, V.A. and Peterson, C.R. 2008. Thermal ecology of hibernation in a population of Great

Basin rattlesnakes. The Biology of Rattlesnakes (Hayes, W., Beaman, K., Cardwell, M., and Bush, S. eds). Loma Linda University Press, Loma Linda, California 11 p.

Costanzo, J.P. 1989. Effects of humidity, temperature, and submergence behavior on survivorship and energy use in hibernating garter snakes, *Thamnophis sirtalis*. Canadian Journal of Zoology 67:2486—2492.

Corbett, E.A. 2004. A comparison of Illinois remnant prairies, 1976 to 1988. Proceedings of the North American Prairie Conferences, University of Wisconsin-Madison, Madison, Wisconsin 8 p.

Das, S., Mohapatra, S.C., and Hsu, J.T. 1999. Studies on primer-dimer formation in polymerase chain reaction (PCR). Biotechnology Techniques 13:129—134.

Elith, J., Phillips, S.J., Hastie, T., Dudík, M., Chee, Y.E., and Yates, C.J. 2011. A statistical explanation of MaxEnt for ecologists. Diversity and Distributions 17:43—57.

Feng, X., Park, D.S., Liang, Y., Pandey, R., and Papes, M. 2019. Collinearity in ecological niche modeling: Confusions and challenges. Ecology and Evolution 9:10365—10376.

Fischer, J. and Lindenmayer, D.B. 2007. Landscape modification and habitat fragmentation: A synthesis. Global Ecology and Biogeography 16:265—280.

Gillingham, J.C. and Carpenter, C.C. 1978. Snake hibernation: Construction of and observations on a man-made hibernaculum (Reptilia, Serpentes). Journal of Herpetology 12:495—498.

Guisan, A. and Zimmermann, N.E. 2000. Predictive habitat distribution models in ecology, Ecological Modelling, 135:147—186.

Halstead, B.J., Wood, D.A., Bowen, L., Waters, S.C., Vandergast, A.G., Erson, J.S., et al. 2017.

An evaluation of the efficacy of using environmental DNA (eDNA) to detect giant gartersnakes (*Thamnophis gigas*). U.S. Geological Survey Open-File Report, Reston, Virginia p. 41.

Harmon-Threatt, A. and Chin, K. 2016. Common methods for tallgrass prairie restoration and their potential effects on bee diversity. Natural Areas Journal 36:400—411.

Harvey, D.S. and Weatherhead, P.J. 2006. Hibernation site selection by Eastern Massasauga rattlesnakes (*Sistrurus catenatus catenatus*) near their northern range limit. Journal of Herpetology 40:66—73.

Hirth, H.F. 1966. Weight changes and mortality of species of snakes during hibernation. Herpetological 22:8—12.

Hodgson, J.A., Thomas, C.D., Wintle, B.A., and Moilanen, A. 2009. Climate change, connectivity and conservation decision making: Back to basics. Journal of Applied Ecology 46:964—969.

Hunter, M.E., Meigs-Friend, G., Ferrante, J.A., Smith, B.J., and Hart, K.M. 2019. Efficacy of eDNA as an early detection indicator for Burmese pythons in the ARM Loxahatchee National Wildlife Refuge in the greater Everglades ecosystem. Ecological Indicators 102:617—622

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J.A., Smith, B.J., Hunter, C.T., et al. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. PLOS One 10:e0121655.

Johnson, G. 2000. Spatial ecology of the Eastern Massasauga (*Sistrurus c. catenatus*) in a New York peatland. Journal of Herpetology 34:186—192.

Johnson, G., Kingsbury, B., King, R., Parent, C., Seigel, R., and Szymanski, J. 2000. The Eastern

Massasauga rattlesnake: A handbook for land managers. U.S. Fish and Wildlife Service. Fort Snelling, Minnesota 52 p.

Katz, A.D., Harper, L.R., Sternhagen, E.C., Pearce, S.E., Melder, C.A., Sperry, J.H., et al. 2021. Environmental DNA is effective in detecting the federally threatened Louisiana Pinesnake (*Pituophis ruthveni*). Environmental DNA 3:409—425.

Kissner, K.J. and Weatherhead, P.J. 2005. Phenotypic effects on survival of neonatal northern watersnakes *Nerodia sipedon*. Journal of Animal Ecology 74:259—265.

Lor, Y., Schreier, T., Waller, D., and Merkes, C.M. 2020. Using environmental DNA (eDNA) to detect the endangered Spectaclecase mussel (*Margaritifera monodonta*). Freshwater Science 39:837—847.

Lowe, W.H. and Allendorf, F.W. 2010. What can genetics tell us about population connectivity? Molecular Ecology 19:3038—3051.

Ma, H., Stewart, K., Lougheed, S., Zheng, J., Wang, Y., and Zhao, J. 2016. Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal. Conservation Genetics Resources 8:561—568.

Markle, C.E., Chow-Fraser, G., and Chow-Fraser, P. 2018. Long-Term habitat changes in a protected area: Implications for herpetofauna habitat management and restoration. PLOS ONE 13:e0192134.

Marshall, J.C., Manning, J.V., and Kingsbury, B.A. 2006. Movement and macrohabitat selection of the Eastern Massasauga in a fen habitat. Herpetologica 62:141—15.

Matthias, L., Allison, M.J., Maslovat, C.Y., Hobbs, J., and Helbing, C.C. 2021. Improving ecological surveys for the detection of cryptic, fossorial snakes using eDNA on and under artificial cover objects. Ecological Indicators 131:108187.

Merkling, J. 2019. Development of an Environmental DNA Assay for Eastern Massasauga. M.Sc. Thesis. Purdue University Graduate School, West Lafayette, Indiana 61 p.

Middleton, E.L., Bever, J.D., and Schultz, P.A. 2009. The effect of restoration methods on the quality of the restoration and resistance to invasion by exotics. Restoration Ecology 18:181—187.

Pearson, R.G. and Dawson, T.P. 2003. Predicting the impacts of climate change on the distribution of species: Are bioclimate envelope models useful? Global Ecology and Biogeography 12:361—371.

Peterson, D.W. and Reich, P.B. 2007. Fire frequency and tree canopy structure influence plant species diversity in a forest-grassland ecotone. Plant Ecology 194:5—16.

Pfleger, M.O., Rider, S.J., Johnston, C.E., and Janosik, A.M. 2016. Saving the doomed: Using eDNA to aid in detection of rare sturgeon for conservation (*Acipenseridae*). Global Ecology and Conservation 8:99—107.

Phillips, S.J., Anderson, R.P., and Schapire, R.E. 2006. Maximum entropy modeling of species geographic distributions. Ecological Monitoring 190:231—259.

Phillips, S.J. and Dudik, M. 2008. Modeling of species distributions with Maxent: New extensions and a comprehensive evaluation. Ecography 31:161—175.

Piaggio A.J., Engeman R.M., Hopken, M.W., Humphrey J.S., Keacher K.L., Bruce W.E., et al. 2014. Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resource 14:374—80.

Randin, C.F., Dirnbock, T., Dullinger, S., Zimmermann, N.E., Zappa, M., and Guisan, A. 2006.

Are niche-based species distribution models transferable in space? Journal of
Biogeography 33:1689—1703.

Ratsch, R. 2019. Efficacy of using environmental DNA (eDNA) to detect Kirtland's snakes
(*Clonophis kirtlandii*). M.Sc Thesis. Purdue University Graduate School, West Lafayette,
Indiana 54 p.

Ratsch, R., Kingsbury, B.A., and Jordan, M.A. 2020. Exploration of environmental DNA
(eDNA) to detect Kirtland's snake (*Clonophis kirtlandii*). Animals: An Open Access
Journal from MDPI 10:1057.

Regos, A., Gagne, L., Alcaraz-Segura, D., Honrado, J.P., and Dominguez, J. 2019. Effects of
species traits and environmental predictors on performance and transferability of
ecological niche models. Scientific Reports 9:4221.

Rose, J.P., Wademan, C., Weir, S., Wood, J.S., and Todd, B.D. 2019. Traditional trapping
methods outperform eDNA sampling for introduced semi-aquatic snakes. PLOS ONE
14:e0219244.

Rowe, H.I. 2010. Tricks of the trade: Techniques and opinions from 38 experts in tallgrass
prairie restoration. Restoration Ecology 18:253—262.

Rudolph, D.C., Schaefer, R.R., Burgdorf, S.J., Duran, M., and Conner, R.N. 2007. Pine snake
(*Pituophis ruthveni* and *Pituophiss melanolecucus lodingi*) hibernacula. Journal of
Herpetology 41:560—565.

Szymanksi, J., Pollack, C., Ragan, L., Redmer, M., Clemency, L., Voorhies, K., et al. 2015.
Species status assessment for the Eastern Massasauga rattlesnake (*Sistrurus catenatus*).
U.S. Fish and Wildlife Service, Washington, D.C. 115 p.

Taylor, P.D., Fahrig, L., Henein, K., and Merriam, G. 1993. Connectivity is a vital element of

landscape structure. Oikos 68:571–573.

Templeton, A.R., Shaw, K., Routman, E., and Davis, S.K. 1990. The genetic consequences of habitat fragmentation. Annals of the Missouri Botanical Garden 77:13–27.

U.S. Fish and Wildlife Service. 2016. Endangered and threatened wildlife and plants: Threatened species status for the Eastern Massasauga rattlesnake. Federal Register 81:67193—67214.

Wang, B., Xu, Y., and Ran, J. 2017. Predicting suitable habitat of the Chinese monal (*Lophophorus lhuysii*) using ecological niche modeling in the Qionglai Mountains, China. PeerJ 5:e3477.

Wold, E.N., Jancaitis, J.E., Taylor, T.H., and Steeck, D.M. 2011. Restoration of agricultural fields to diverse wet prairie plant communities in the Willamette Valley, Oregon. Northwest Science 85:269—287.

Wood, Z.T., Erdman, B.F., York, G., Trial, J.G., and Kinnison, M.T. 2020. Experimental assessment of optimal lotic eDNA sampling and assay multiplexing for a critically endangered fish. Environmental DNA 2:407—417.

Zeller, K.A., Lewison, R., Fletcher, R.J., Tulbure, M.G., and Jennings, M.K. 2020. Understanding the importance of dynamic landscape connectivity. Land 9:303.