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Detection of *Ophidiomyces*, the Causative Agent of Snake Fungal Disease, in the Eastern Massasauga (*Sistrurus catenatus*) in Michigan, USA, 2014

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ABSTRACT: Snake fungal disease (SFD), caused by *Ophidiomyces ophiodiicola*, threatens free-ranging snake populations across the US. We assayed 112 swabs from 102 individual eastern massasaugas (*Sistrurus catenatus*) at three locations in Michigan in 2014 for *Ophidiomyces* using quantitative PCR (qPCR). We observed a 12.7% qPCR prevalence of skin lesions. Individuals at each site had lesions, and occurrence of skin lesions was not significantly different between sites. We detected *Ophidiomyces* DNA at each of the three sites in five individuals (4.9%). We found no difference in detection probabilities between sites; however, snakes with dermatitis had higher *Ophidiomyces* DNA detection probabilities ($P=0.15\pm 0.08$ SE) than snakes without dermatitis ($P=0.02\pm 0.01$ SE, $P=0.026$). The emergence of SFD mortalities has potentially serious consequences for the viability of the eastern massasauga in Michigan. Future work should track temporal patterns in vital rates and health parameters, link health data to body condition indices for individual snakes, and conduct a “hotspot” analysis to examine health on a landscape scale.

Key words: Disease, eastern massasauga, infection, *Ophidiomyces ophiodiicola*, reptile.

Assessing the overall wellness of wildlife populations will aid in forming conservation goals and developing recovery strategies to minimize population-level disease threats and enhance individual health. Monitoring pathogen prevalence has become paramount as the number of published case studies for disease outbreaks that cause population declines or extirpations is rapidly growing (Cunningham and Daszak 1998; Daszak et al. 2000; Schoegel et al. 2006). However, intervention and management strategies to mitigate the effects of disease outbreaks are hampered after a

pathogen becomes established in the environment. Consequently, continuous health monitoring and disease investigations are needed to provide insight into the overall ecological health of natural systems.

In the eastern and midwestern US, the eastern massasauga (*Sistrurus catenatus*), a candidate species for federal listing, has experienced ongoing mortalities from *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease (SFD; Allender et al. 2011, 2015b). The emergence of SFD from the skin, lung, muscle, and bone of several free-ranging and captive reptiles is alarming because of its broad geographic and taxonomic distributions (reviewed in Allender et al. 2015b).

Populations of eastern massasaugas in Michigan have been well studied with respect to habitat selection (Bailey et al. 2012) and spatial ecology (Moore and Gillingham 2006), but the presence of this pathogen has not been investigated. Our objectives were to determine whether *Ophidiomyces* was present at three sites in Michigan and, if present, estimate and characterize detection probabilities of *Ophidiomyces*.

During 2014, from spring egress (beginning in mid-April) through fall ingress (mid-October), we conducted visual encounter surveys of three wild eastern massasauga populations in Michigan: 1) Edward Lowe Foundation (ELF; 42°13'N, 83°44'W) in Cass County, 2) Pierce Cedar Creek Institute (PCCI; 42°39'N, 85°17'W) in Barry County, and 3) Camp Grayling (CG) in Kalkaska and Crawford counties (44°36'N, 84°54'W). We marked all individuals either by passive integrated tran-

sponder tagging or by painting their rattles. We identified sex by cloacal probing. Age classes were defined as juvenile: snout to vent length (SVL) <45 cm and mass <130 g; or adults: ≥ 45 cm SVL and ≥ 130 g. After processing, all snakes were returned to their initial points of capture. All procedures were approved by an Institutional Animal Care and Use Committee (Northern Illinois University: LA10-001; Indiana Purdue Fort Wayne: 1112000451; Grand Valley State University: 13-02-A).

We examined all animals for clinical signs consistent with SFD, (i.e., generalized dermatitis, skin lesions, facial swelling, or discharge; Allender et al. 2011; Clark et al. 2011). Using sterile techniques, cotton-tipped or nylon-flocked applicators were used to swab nasolabial pits on all animals and body lesions for individuals exhibiting skin pustules, nodules, or displaced scales. Samples were stored in 2-mL Eppendorf tubes and frozen at -20 C until analyzed. DNA extraction and quantitative PCR amplification (qPCR) were performed as reported (Allender et al. 2015a). Briefly, qPCR was performed in triplicate on an ABI 7500 real-time thermocycler (Applied Biosystems, Carlsbad, California, USA) targeting a 68-base pair segment of the internal transcribed spacer subunit 1 region between the 18S and 5.8S ribosomal RNA gene. Samples were considered positive if all three replicates had a lower cycle threshold (C_t) value than the lowest detected standard dilution.

We tested associations of sex and age class between site, SFD status, and clinical signs using a Fisher's exact and chi-square test. We tested differences in *Ophidiomyces* copy number between sites using a Mann-Whitney U -test. We calculated body condition index (BCI) for each snake based on previous studies in massasaugas (Allender et al. 2016), then tested differences in BCI between positive and negative snakes using an independent samples t -test. We used single-season occupancy models to estimate detection probabilities for *O. ophiodiicola* DNA. Using presence-absence data, these models estimate two parameters: *Occupancy* (Ψ_i), the probability that the species is present at site I ; and *detection probability* (P_{ij}), the probability that

TABLE 1. Descriptive statistics for *Ophidiomyces* surveillance in Michigan populations of eastern massasaugas using quantitative PCR (qPCR) of swabs, 2014.^a

	ELF	CG	PCCI
Sex, females/males	15/10	12/20	20/23
Age class, adults/juveniles	22/3	30/4	39/4
Skin lesions, n (%)	1 (4.0)	5 (14.7)	7 (16.3)
qPCR positive, n (%)	2 (8.0)	2 (5.9)	1 (2.3)
qPCR 95% CI	2.2–25.0	1.6–19.1	0.4–12.6

^a ELF = Edward Lowe Foundation; CG = Camp Grayling; PCCI = Pierce Cedar Creek Institute; CI = confidence interval.

the species is detected at time i at site j (MacKenzie et al. 2002). Detection probability as defined here is the product of two probabilities: 1) the probability of finding a snake that is harboring *Ophidiomyces* DNA and (2) the probability of detecting *Ophidiomyces* DNA on an animal that is harboring *Ophidiomyces* DNA. We considered two hypotheses: 1) *Ophidiomyces* DNA detection probabilities vary by site (i.e., a site classification factor with three levels), or (2) *Ophidiomyces* DNA detection probabilities vary by presence or absence of dermatitis (two-level classification factor). We used *Ophidiomyces* DNA constant detection probabilities as the null hypothesis and held Ψ constant in all models to reduce the number of parameters estimated. We used likelihood ratio tests to test for significant effects and profile likelihood confidence intervals for point estimates. We assessed statistical significance at $\alpha=0.05$ and conducted all statistical analyses using Program MARK version 8.0 (White and Burnham 1999) and IBM Statistics 22.0 (SPSS Inc., Chicago, Illinois, USA).

We collected 112 swabs from 102 eastern massasaugas from the three sites in Michigan. We sampled 25 individuals at ELF, 34 at CG, and 43 at PCCI. In adults, mean SVL (53.2 cm ± 5.3 SD) was not significantly different between sites ($P=0.449$). Each site sampled ratios of males and females ($P=0.140$) as well as adults and juveniles ($P=0.601$) equally, despite a trend of more females at ELF (Table 1). Two snakes at CG had no sex recorded. Body condition index of positive

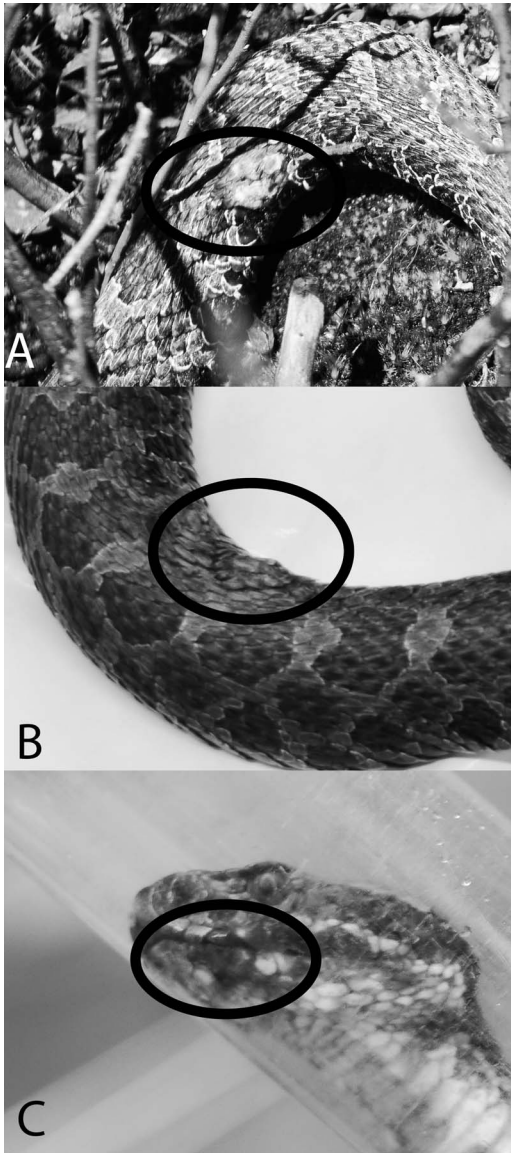


FIGURE 1. Eastern massasaugas (*Sistrurus catenatus*) with *Ophidiomyces*-positive skin lesions identified by PCR from Camp Grayling (A) and Pierce Cedar Creek Institute (B, C), Michigan, USA, 2014.

snakes (mean 15.1, SD 20.9) was not significantly lower than negative snakes (mean 33.5, SD 72.3; $P=0.574$).

We observed a 12.7% ($n=13$) overall prevalence of skin lesions (Fig. 1). Individuals at each site had lesions, which was not significantly different between sites ($P=0.274$; Table 1). We detected *Ophidiomyces* DNA at

each of the three sites in five individuals (two adult females, and three adult males; Table 1). Of the five positive snakes, three and two animals had *Ophidiomyces* detected in skin lesions and in nasolabial pits, respectively. We found no evidence for variation in detection probabilities of *Ophidiomyces* DNA between sites ($P=0.264$). However, snakes with dermatitis had significantly higher *Ophidiomyces* DNA detection probabilities ($P=0.15\pm 0.08$ SE; 95% confidence interval 0.04–0.34) than snakes without dermatitis ($P=0.02\pm 0.01$ SE; 95% confidence interval 0.00–0.06; $P=0.026$). Fungal copy numbers in the five positive snakes ranged from 11 to 308 copies/qPCR reaction, with no significant difference between sites ($P=0.121$).

The emergence of SFD mortalities has potentially serious consequences for the viability of the eastern massasauga in Michigan. Reported survival from SFD is low, yet variable, depending on species and presentation (Allender et al. 2011; Clark et al. 2011; McBride et al. 2015; Tetzlaff et al. 2015). In 2013, two cases of SFD were confirmed at the CG site through diagnostic testing of clinically ill snakes and resulted in death for both individuals (Tetzlaff et al. 2015). In 2014, SFD was present at the two additional Michigan sites sampled here; however, we did not find a difference in detection probabilities of *Ophidiomyces* DNA between sites. The fate of positive snakes is unknown and increased surveillance at these sites might reveal both site differences and mortality rates as statistical power increases.

The SFD lesions in eastern massasaugas have been associated exclusively with lesions of the skin (Allender et al. 2015b). Skin lesions (dermatitis) were not uncommon in this study (12.7% of sampled snakes), and three of the five positive snakes had associated clinical signs that were detected with *Ophidiomyces* DNA. We demonstrated that clinical signs are significantly associated with higher detection probabilities of *Ophidiomyces* DNA. However, the lack of clinical signs does not preclude infection. The two positive snakes with no clinical signs in this study also had the lowest numbers of fungal copies. It is

possible that these snakes had early infections that had not yet developed clinical signs. Additionally, 10 individuals had dermatitis, but were qPCR-negative. It is unknown whether these individuals were truly negative (i.e., *Ophidiomyces* DNA is absent) or if they represent false negatives and are a consequence of detection rates that are <1 . This has been proposed previously for this fungus because of its invasion of deeper tissue, thus requiring aggressive swabs or biopsies to confirm diagnosis (Allender et al. 2013). The proportion of false negatives can be estimated using the methods described here if a larger sample size of repeated measures is used. Thus, if the proportion of false negatives and other sources of heterogeneity are accounted for, unbiased estimates of occupancy are possible.

Michigan is the only state or Canadian province that does not currently list the eastern massasauga as threatened or endangered. Therefore, protecting and reducing disease effect in a relatively robust population may be more likely to succeed in Michigan compared with other parts of their range. The approach to wildlife disease epidemics has historically been descriptive. It is critical for land managers to document the extent of pathogens across the landscape so that future efforts may focus on environmental control. Furthermore, sick or dead animals with associated clinical signs should be evaluated and tested for this pathogen. We stress the importance of continuing annual monitoring programs to document both population vital rates (λ) and disease prevalence. Marked decreases in λ or annual survival rates may be strong indicators of compromised population health (Williams et al. 2002). Future work should include linking health data to temporal patterns in vital rates and body condition indices for individual snakes. The presence of abnormalities in 1 yr gives a snapshot indication of individual health at one time point; however, monitoring populations through time may allow for the early detection of deteriorating population health and identification of possible mechanisms for the emergence of SFD. This should be multifac-

eted, but can be initiated with environmental sampling (through environmental DNA), radiotelemetry or capture-recapture studies of known populations with *Ophidiomyces* infections, habitat quality, and composition assessments of known *Ophidiomyces* areas for this species and others across the pathogen's range.

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