Research Article

Estimation of *Ophidiomyces* Prevalence to Evaluate Snake Fungal Disease Risk

ERIC T. HILEMAN,[1,2] *Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, USA*

MATTHEW C. ALLENDER, *Wildlife Epidemiology Lab, Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA*

DANIELLE R. BRADKE, *Biology Department, Grand Valley State University, Allendale, MI 49401, USA*

LISA J. FAUST, *Department of Conservation and Science, Lincoln Park Zoo, Chicago, IL 60614, USA*

JENNIFER A. MOORE, *Biology Department, Grand Valley State University, Allendale, MI 49401, USA*

MICHAEL J. RAVESI, *Camp Grayling Joint Maneuver Training Center, Grayling, MI 49739, USA*

SASHA J. TETZLAFF, *Department of Natural Resources and Environment Sciences, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA*

ABSTRACT Pathogenic fungi have become a global concern to wildlife populations over the last 2 decades. However, the threat of snake fungal disease (SFD; caused by *Ophidiomyces ophiodiicola*) to snake populations is still largely unknown. From 2014–2016, we monitored 3 disjunct populations of the federally threated eastern massasauga (*Sistrurus catenatus*) in Michigan, USA. We used clinical signs of SFD, quantitative TaqMan polymerase chain reaction (qPCR), repeated sampling of individuals and sites, and single-season occupancy models to estimate site-specific prevalence of *Ophidiomyces*. Point estimates of *Ophidiomyces* prevalence in 2016 were larger at the northernmost study site (0.17, 95% CI = 0.04–0.50), where 17 of 34 snakes were implanted with radio-transmitters, and smaller at southern sites (0.03, 95% CI = 0.00–0.19). However, *Ophidiomyces* prevalence was not different between snakes with transmitters and snakes without transmitters. Swabbing snakes with 1 applicator resulted in a high probability of failure in detecting *Ophidiomyces* DNA for individuals with clinical signs of SFD and the probability was even higher for individuals without clinical signs of SFD. Repeated sampling of individuals reduced the probability of obtaining a false-negative qPCR result by 72% for snakes with clinical signs and 12% for snakes without clinical signs when we swabbed individuals with 5 applicators. We recommend resampling individuals and sites as a sampling design for estimating fine-scale, site-specific *Ophidiomyces* prevalence and population-level responses to SFD. If clinical signs are used as a surrogate for SFD, we recommend researchers standardize diagnosis of clinical signs of SFD by providing technicians adequate field training and educational materials, and minimize the number of observers recording clinical signs. We discourage the use of radio-telemetry methods where SFD occurs unless sterile surgical, handling, and equipment protocols can be ensured and the benefits to the population from such activities outweigh the increased health risks to individuals. © 2017 The Wildlife Society.

KEY WORDS disease prevalence, false negatives, Michigan, occupancy models, *Ophidiomyces ophiodiicola*, qPCR, radio-telemetry, *Sistrurus catenatus*, snake fungal disease.

Over the past 2 decades, pathogenic fungi have become a global threat to wildlife populations (Fisher et al. 2012). Two striking examples are the widespread global amphibian declines from chytridiomycosis, caused by the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*; Berger et al. 1998, Lips et al. 2006, Kilpatrick et al. 2010), and declines in bat populations in the eastern United States from white-nose syndrome, caused by a psychrophilic fungus (*Pseudogymnoascus destructans*; Blehert et al. 2009, Frick et al. 2010, Lorch et al. 2011). Although numerous taxa in addition to amphibians and bats are affected by fungal pathogens, estimates of disease prevalence, pathogen prevalence on hosts, and population-level responses to these pathogens are lacking or poorly understood in many species (Fisher et al. 2012, Allender et al. 2016a).

A fungal pathogen causing growing concern is *Ophidiomyces ophiodiicola*, the species responsible for snake fungal disease (SFD; Lorch et al. 2015, Allender et al. 2015a). The earliest known record of SFD is from a museum specimen collected in

2000 from southern Illinois (Allender et al. 2016b). Snake fungal disease has now been recorded in 6 snake families representing over 30 species throughout most of the eastern half of the United States and captive snakes in parts of the United Kingdom, Germany, and Australia (Tetzlaff et al. 2015, Allender et al. 2015d, Lorch et al. 2016). Mortalities due to SFD have been reported for several snake species (Allender et al. 2013, Sigler et al. 2013, Sleeman 2013). However, a population-level response (decline) where SFD was suspected (along with other contributing factors such as inbreeding depression and high summer rainfall) has been documented only once, in a New Hampshire population of timber rattlesnakes (*Crotalus horridus*; Clark et al. 2011). Thus, the long-term effects of SFD on snake populations are largely unknown.

Knowledge of *O. ophiodiicola* ecology (Allender et al. 2015d, McCoy et al. 2017), geographic distribution (Allender et al. 2015d, Lorch et al. 2016), detection (Allender et al. 2016a), pathogenesis (Lorch et al. 2015, Allender et al. 2015a), and snake hematology (Allender et al. 2015c, 2016b) has increased over the last few years. However, unbiased estimates of *O. ophiodiicola* prevalence in host populations are still lacking. To date, investigators have relied on raw counts of *Ophidiomyces* presence or absence data as proxies for *Ophidiomyces* prevalence in host populations (Allender et al. 2016a). Although such data provide useful information, they are expected to underestimate *Ophidiomyces* prevalence within populations because the methods used to detect *Ophidiomyces* DNA (e.g., fungal cultures, polymerase chain reaction [PCR], quantitative PCR [qPCR]) yield false negatives (Lorch et al. 2016, Allender et al. 2016a). Unbiased estimation of *Ophidiomyces* prevalence is an important step to quantifying population-level responses to SFD.

Because of their secretive nature, eastern massasaugas (*Sistrurus catenatus*) and other snake species are often studied using radio-telemetry methods (Reinert and Cundall 1982, Webb and Shine 1997, Moore and Gillingham 2006). These methods typically involve intracoelomic implantation (Webb and Shine 1997), which disrupts the skin and body cavity and can therefore provide a portal for pathogenic fungus to enter the body. In addition, radio-telemetry surgery can induce post-surgery immune suppression due to infection (Lentini et al. 2011). Consequently, snakes implanted with radio-transmitters may be at higher risk of *Ophidiomyces* colonization than snakes without radio-transmitters.

Our objective was to estimate *Ophidiomyces* detection and occupancy (i.e., prevalence) probabilities for 3 eastern massasauga populations in Michigan, USA, a federally threatened rattlesnake species under the United States Endangered Species Act (U.S. Fish and Wildlife Service [USFWS] 2016). We hypothesized that *Ophidiomyces* occupancy probabilities would be largest at the site where naïve counts of individuals carrying *Ophidiomyces* DNA was highest, snakes with radio-transmitters would have higher *Ophidiomyces* occupancy probabilities than snakes without radio-transmitters, and repeated sampling of individuals would reduce the probability of obtaining a false-negative result. Based on previous work (Allender et al. 2016a), we also hypothesized that snakes with clinical signs of SFD would have higher *Ophidiomyces* DNA detection probabilities than snakes without clinical signs.

STUDY AREA

The study area included 3 disjunct eastern massasauga populations in Michigan (Fig. 1). These populations included the Edward Lowe Foundation (ELF) in Cass County, Pierce Cedar Creek Institute (PCCI) in Barry County, and Camp Grayling (CG) in Crawford and Kalkaska counties (Fig. 1). Michigan has a temperate continental climate with no dry season (Belda et al. 2014). Because of their proximity (Fig. 1), the ELF and PCCI sites share similar annual climate averages (air temp = 8.44, 8.78°C; precipitation = 950.47, 1,034.80 mm; frost-free days = 171, 175, respectively), whereas the more northern CG site is cooler (air temp = 5.78°C), drier (precipitation = 853.69 mm), and has fewer frost-free days (131; 1981–2010 normals; stations USC00202250, USC00203661, and USC00203391; National Oceanic and Atmospheric Administration [NOAA] 2017). Monthly average temperatures during the primary sampling periods for the study (Apr–Jun 2014–2016) ranged from 7.2–20.6°C (ELF), 6.8–20.2°C (PCCI), and 3.3–18.1°C (CG; stations USC00202250, USC00203661, and USC00203391; Midwestern Regional Climate Center [MRCC] 2017). Elevation is similar between ELF (244–280 m) and PCCI (259–266 m) and higher at CG (371 m; U.S. Geological Survey [USGS] 2017).

The ELF study area (∼64.3 ha) was nested within a privately owned and managed 1,052-ha parcel and consisted
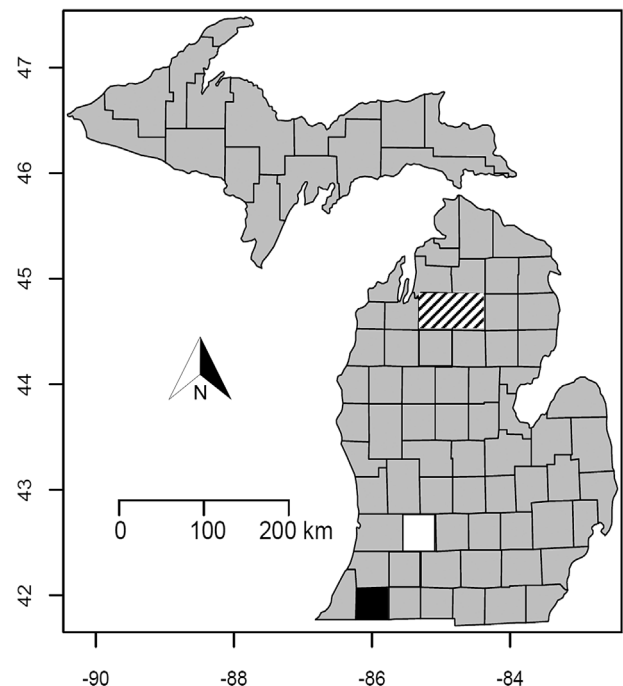

Figure 1. Counties of the eastern massasauga study localities, Michigan, USA, 2014–2016: Cass County (black), Barry County (white), and Crawford and Kalkaska counties (diagonal stripes).

of a composite of wetland (bisected by a creek), prairie, and woodland habitats surrounded by cropland and developed areas. Receding glaciers shaped the topography of the site, creating a valley system that retained a spring-fed kettle lake (∼4 ha) and multiple ponds, streams, and natural springs. The survey area was chiefly calcareous fen and marshes dominated by graminoids (e.g., *Carex* spp.), cattails (*Typha* spp.), common reed (*Phragmites* spp.), and woody species including tamarack (*Larix laricina*), willow (*Salix* spp.), cinquefoil (*Dasiphora fruticosa*), and poison sumac (*Toxicodendron vernix*). Fauna inventoried at the site included 31 reptile and amphibian species and >100 bird species.

The PCCI study site was within a privately owned parcel of land (∼277 ha) primarily composed of wetlands, forest, prairie, old-field, and developed sites. Additionally, PCCI functioned as a biological research station, education center, and nature center with trails open to the public and was bisected by a public dirt road. The site's topography was formed by a retreating glacier and included low-lying wetlands bordering a kettle lake (∼5 ha) and third-order creek. The survey area (∼23.1 ha) was primarily prairie fen habitat and adjacent uplands dominated by graminoids, cattails, goldenrod (*Solidago* spp.), asters (*Aster* spp.), and woody species including tamarack, dogwood (*Cornus* spp.), willow, and poison sumac. The site was home to diverse fauna, with 16 reptile species, 14 amphibian species, 33 mammal species, and 131 bird species documented.

The CG site was located within the Camp Grayling Joint Maneuver Training Center (59,488 ha), the largest National Guard training facility in the United States. Local topography was shaped by the most recent glaciation and water bodies found on the landscape included glacial lakes, creeks, and rivers. Both the broader Camp Grayling training facility and the survey site was composed of coniferous and hardwood forests containing mainly spruce (*Picea* spp.), cedar (*Thuja* spp.), pine (*Pinus* spp.), maple (*Acer* spp.), oak (*Quercus* spp.), and aspen (*Populus* spp.) stands; barrens dominated by lichen and blueberry (*Vaccinium* spp.); and scrub-shrub wetlands comprised of graminoids (e.g., *Carex* spp., *Eleocharis* spp.), forbs and herbs (e.g., *Matteuccia struthiopteris*), and woody species such as willow and speckled alder (*Alnus incana*). Limited survey efforts indicated >103 bird species and 26 reptile and amphibian species inhabited the base.

METHODS
From 2014–2016, we conducted visual or radio-telemetry surveys at the 3 study sites. We surveyed sites during the species' active season from 6 May–6 August (2014), 30 April–29 August (2015), and 23 April–25 October (2016). We used visual surveys, cover objects, and drift fences with funnel traps and captured snakes with tongs using sterile handling and equipment protocols (Rzadkowska et al. 2016). We uniquely marked individuals via subcutaneous passive integrated transponders (PIT) or by painting rattle segments (Gibbons and Andrews 2004). We recorded snout-vent length (SVL, cm) and mass (g), and identified sex by cloacal probing (Schaefer 1934). We defined adults as SVL ≥45 cm and juveniles as SVL <45 cm (Allender et al. 2016a).

We inspected all animals for clinical signs consistent with SFD (e.g., presence of crusts, displaced scales, nodules, swelling, caseous discharge from skin pustules; Allender et al. 2015*d*). We recorded clinical signs as a binary variable: present (1) or absent (0). We used sterile cotton-tipped or flocked applicators to swab the epidermis of each animal. In 2014 and 2015, we swabbed the heat-pits of all individuals and active lesion sites (if present) so that all snakes were swabbed with ≥1 applicator. In 2016, we modified our sampling protocol to include multiple swab applicators (∼5), swabbing snakes at discrete locations on the body, including the dorsum, ventrum, flanks, and sites of lesions (including heat-pits). We released snakes at their site of capture within 24 hours. We stored samples in 2-ml Eppendorf tubes, frozen at −20°C, and sent batches to the Wildlife Epidemiology Laboratory at the University of Illinois (Urbana, IL, USA). We performed DNA extraction and quantitative TaqMan PCR amplification (qPCR) as previously reported (Allender et al. 2015*b*). Briefly, we ran each sample in triplicate on a plate with 7 serial positive dilutions and a non-template control as a negative control using a real-time thermocycler (ABI 7500, Life Technologies, Carlsbad, CA, USA). We evaluated the slope of each plate as previously described (Allender et al., 2015*b*) and considered samples positive if all 3 replicates had a lower cycle threshold (Ct) value than the lowest detected standard dilution. This research was approved under Michigan Scientific Collector's Permits and the following Institutional Animal Care and Use Committees: Northern Illinois University, DeKalb, Illinois (no. LA10-001); Indiana−Purdue University Fort Wayne, Fort Wayne, Indiana (no. 1112000451); Grand Valley State University, Allendale, Michigan (no.13-02-A); and Lincoln Park Zoo, Chicago, Illinois (no. 2015-013).

Single-Season, Single-Species Occupancy Models
We used single-season, single-species occupancy models to estimate *Ophidiomyces* DNA detection and occupancy probabilities (i.e., *Ophidiomyces* prevalence corrected for imperfect detection). These models use presence-absence data to estimate occupancy (ψ_i; the probability that the species is present at site i) and detection probability (p_{ij}; the probability that the species is detected at time i at site j, given the species is present at site j; MacKenzie et al. 2002). We defined species as *Ophidiomyces* DNA and site as an eastern massasauga from a given locality (ELF, PCCI, or CG) that had been swabbed with ≥1 applicator. For snakes that were repeatedly sampled, we collected all replicate swab applicators on a single date for a given individual.

Although clinical signs of SFD are positively associated with *Ophidiomyces* DNA detection probabilities (Allender et al. 2016*a*), SFD diagnosis based on qPCR results and descriptive characteristics includes some uncertainty, especially for animals that are positive for *Ophidiomyces* DNA but do not present clinical signs of SFD. Therefore, we modeled *Ophidiomyces* prevalence rather than directly modeling SFD prevalence. This required interpretation of the various combinations of *Ophidiomyces* DNA presence-absence and clinical signs of SFD presence-absence (Fig. 2). *Ophidiomyces*
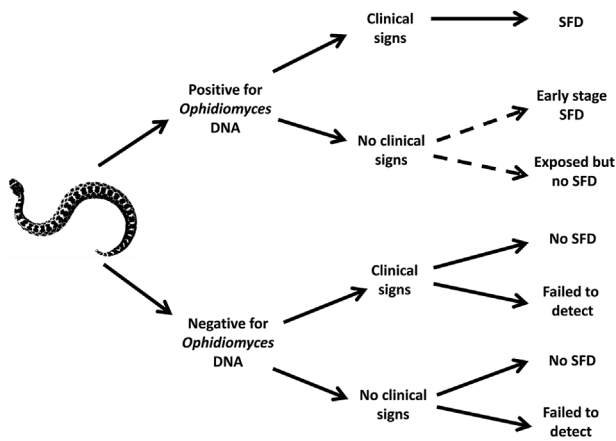
Figure 2. Combinations of *Ophidiomyces* DNA presence-absence and clinical signs of snake fungal disease (SFD) presence-absence in eastern massasaugas, Michigan, USA, 2014–2016. The dashed arrows indicate pathways that cannot be decoupled using the single-season, single-species occupancy model. Thus, an individual positive for *Ophidiomyces* DNA but lacking clinical signs is interpreted as either having early stage SFD or being SFD negative but exposed to the disease causing pathogen.

prevalence refers to the fraction of the population comprised of individuals that have SFD and individuals that do not have SFD but are positive for *Ophidiomyces* (Fig. 2).

We used an information-theoretic approach and multi-model inference to evaluate multiple competing hypotheses that explained detection and occupancy probabilities of *Ophidiomyces* (Burnham and Anderson 2002, Anderson 2008). For model selection, we used Akaike's Information Criterion adjusted for small sample size (AIC_c; Akaike 1973). Unless the top-ranked model received ≥90% of the AIC_c weight, we used model averaging and unconditional standard errors to account for model and parameter estimate uncertainty (Burnham and Anderson 2002). We used profile likelihood confidence intervals for model-averaged point estimates (White and Burnham 1999) and analysis of deviance (ANODEV) to assess model fit. Analysis of deviance is analogous to r^2 for models using maximum likelihood methods (Harris et al. 2005). To ensure that we sampled comparably across populations and years, we used analysis of covariance to test if SVL of swabbed individuals differed between years and populations. For frequentist statistics, we set $a = 0.05$. We conducted all statistical analyses using Program MARK version 8.1 (www.phidot. org/software/mark/downloads/index.html, accessed 1 Jul 2016; White and Burnham 1999) and SPSS Version 18.0 (SPSS, Chicago, IL, USA). We used the ggplot2 package (Wickham 2009) in R version 3.3.2 (www.r-project.org, accessed 1 Nov 2016) to graphically depict data.

For our occupancy analysis, we first constructed capture histories for all swabbed snakes, where 1 indicated a snake tested positive for *Ophidiomyces* DNA at time i (i.e., applicator i), 0 indicated a snake tested negative for *Ophidiomyces* DNA with applicator i, and a dot (.) indicated that a snake was not swabbed with applicator i. We treated year as a classification factor with 3 levels so the dataset spanning 2014–2016 could be included and analyzed

simultaneously in a single-season framework. Furthermore, we truncated the 2016 data to include only swab applicators collected from 23 April–18 June so the assumption of closure for each population could be reasonably approximated. In other words, each snake was sampled during a single date, but we could not sample all snakes on the same date. Therefore, we assumed that snakes did not change in *Ophidiomyces* status from 23 April–18 June. Because recaptures of individual snakes (i.e., sites) were rare and non-telemetered individuals at CG were not uniquely identified between years, multi-season occupancy modeling was not possible. Our sampling methods were consistent between 2014 and 2015, but were sparse for repeated samples of individuals, whereas sampling methods in 2016 were more intensive (replicate swab applicators for individuals increased) and comprehensive (additional areas of the body were sampled) than the previous 2 years. Therefore, we attempted to estimate occupancy only for 2016. To model the disparate sampling years (2014 and 2015 vs. 2016) and to address data sparseness in the first 2 years, we considered a reduced time model that treated *Ophidiomyces* detection probability as constant (i.e., equal) between 2014 and 2015 but allowed detection probability to differ in 2016. In addition, Allender et al. (2016a) previously demonstrated that clinical signs of SFD are a reasonable predictor of *Ophidiomyces* DNA detection. Thus, we also included clinical signs (CS) as a covariate to explain detection probability. This resulted in 4 detection probability models (Table 1).

For 2014 and 2015, we treated occupancy (ψ) probabilities as a single nuisance parameter and held it constant across localities for these years to reduce the number of estimated parameters. This allowed us to estimate detection probabilities for all 3 years and occupancy in 2016, where data were most abundant. For 2016 ψ probabilities, we considered a population locality effect. Because of sparseness of *Ophidiomyces* DNA presence data at ELF and PCCI, we treated

Table 1. *Ophidiomyces* DNA detection (p) and occupancy (ψ) probability models considered using 2014–2016 data collected from eastern massasaugas at Camp Graying (CG), the Edward Lowe Foundation (ELF), and Pierce Cedar Creek Institute (PCCI), Michigan, USA. Detection is modeled by period as a function of clinical signs (CS) or as a constant (.). Occupancy is modeled for 2016 only.

Probability model	Model interpretation
Detection	
p [(CS)$^{2014, 2015}$, (CS)^{2016}]	Detection varies between years and between individuals based on the presence or absence of clinical signs.
p [(.)$^{2014, 2015}$, (CS)^{2016}]	Detection varies between years and between individuals based on the presence or absence of clinical signs (2016 only).
p [(CS)$^{2014, 2015}$, (.)^{2016}]	Detection varies between years and between individuals based on the presence or absence of clinical signs (2014 and 2015 only).
p [(.)$^{2014, 2015}$, (.)^{2016}]	Detection varies between years only.
Occupancy	
ψ (CG ≠ ELF = PCCI)	Occupancy at CG is different than occupancy at ELF and PCCI.
ψ (.)	Occupancy is constant across localities.

these localities as having equal ψ but allowed CG ψ to vary from the other 2 localities because naïve estimates (i.e., the ratio of positive animals/total animals sampled using uncorrected count data) of *Ophidiomyces* prevalence based on qPCR results were largest at CG. This resulted in 2 models that explained occupancy in 2016 (Table 1). Considering all iterations of these detection and occupancy parameterizations resulted in 8 candidate models (Table 2).

The Probability of Obtaining a False Negative $(1-p)$

False negatives can occur when a swab fails to capture *Ophidiomyces* DNA (when it actually is present on the animal), when *Ophidiomyces* DNA is not extracted from a positive sample, or when DNA fails to amplify during qPCR. To estimate the probability of acquiring a false-negative result (i.e., $1-p$), we used the ψ parameterization of the top ranked model from the previous analysis. For the detection p parameter, we used clinical signs as a covariate to explain *Ophidiomyces* detection probability and treated time as constant across years so that estimates of false negatives would be generalized rather than applicable to a single year. Because our interest was in estimating false-negative probabilities rather than occupancy, we used the complete dataset and treated occupancy as a nuisance parameter. Using a likelihood ratio test, we compared the clinical signs detection model with a null model that treated *Ophidiomyces* detection as constant among snakes. Based on the favored model from the likelihood ratio test, we calculated the cumulative probability of obtaining a false-negative result from using (T) surveys: $(1-p)^T$, where p is the single survey detection probability and T is the number of applicator swabs analyzed (Kéry 2002, Halstead et al. 2011). We approximated the variance using the delta method (Seber 1982).

Radio-Transmitter Effects on *Ophidiomyces* Prevalence

Using only the CG locality 2014–2016 data, the locality where all but 3 snakes implanted with radio-transmitters were from ($n = 25$), we considered a single-season occupancy model that included a radio-transmitter effect as a way to explain *Ophidiomyces* prevalence and treated detection as constant across years. We modeled the radio-transmitter effect as a binary covariate (present or absent). We assigned snakes swabbed prior to radio-transmitter surgery a 0 for no radio-transmitter effect, and assigned snakes swabbed the season after radio-transmitter surgery a 1 for a radio-transmitter effect. We compared the radio-transmitter effect model with a null model that treated *Ophidiomyces* prevalence as constant between snakes with and without radio-transmitters using a likelihood ratio test.

RESULTS

Across localities, we collected 118 swab applicators from 100 snakes in 2014, 117 applicators from 92 snakes in 2015, and 535 applicators from 105 snakes in 2016 (Table 3). Thirteen individuals (19 applicators) tested positive for *Ophidiomyces*. Eight of these individuals (13 applicators) had clinical signs of SFD. Using locality as a fixed factor and year as a random factor, mean SVL (52.6 ± 6.5 [SD]) did not differ across localities ($F_{2, 4} = 0.358$, $P = 0.718$), years ($F_{2, 4} = 0.275$, $P = 0.772$), or by an interaction between these 2 variables ($F_{4, 280} = 1.105$, $P = 0.354$). The percentage of sampled snakes with documented skin lesions ranged from 0 to 61.1% across years and localities (Table 3). The percentage of sampled snakes with qPCR-positive results ranged from 0 to 13.3% across years and localities (Table 3).

Single-Season, Single-Species Occupancy Models

In 2016, we sampled 34 individuals (184 applicators) at CG, 35 individuals (171 applicators) at ELF, and 36 individuals (180 applicators) at PCCI. At CG, we usually swabbed each snake with 5 applicators, with sample size ranging from 1–9/individual. At ELF, we swabbed 34 snakes with 5 applicators each, and swabbed 1 snake with a lesion once. At PCCI, we swabbed 36 snakes with 5 applicators each. We excluded 13 individuals from CG, including 1 that tested positive for *Ophidiomyces*, from the occupancy analysis when we truncated the data to meet the assumption of closure. Despite reasonable sample sizes, the data were temporally insufficient to model seasonal effects of *Ophidiomyces*.

Of the 8 models considered in our occupancy analysis, only the top 4 models garnered support based on AIC_c weight (w_i; Table 2). The top-ranked model (model 1) received 0.55

Table 2. Candidate single-season occupancy models for eastern massasaugas considered using 2014–2016 data collected from Camp Graying (CG), the Edward Lowe Foundation (ELF), and Pierce Cedar Creek Institute (PCCI), Michigan, USA. The single nuisance occupancy parameter for 2014–2015 (not shown) contributes 1 parameter (K) to each model below. We modeled detection probability (p) by period as a function of clinical signs (CS) or as a constant (.) and modeled occupancy (ψ) for 2016 only.

	Model	AIC_c[a]	ΔAIC_c[b]	w_i[c]	K	Deviance[d]	ANODEV[e]
1)	$p\,[(CS)^{2014,\ 2015}, (.)^{2016}]\psi\ (CG \neq ELF = PCCI)$	133.44	0.00	0.55	6	−74.01	0.97
2)	$p\,[(CS)^{2014,\ 2015}, (CS)^{2016}]\ \psi\ (CG \neq ELF = PCCI)$	134.89	1.45	0.26	7	−74.67	1.00
3)	$p\,[(CS)^{2014,\ 2015}, (.)^{2016}]\psi\ (.)$	136.34	2.90	0.13	5	−69.03	0.75
4)	$p\,[(CS)^{2014,\ 2015}, (CS)^{2016}]\ \psi\ (.)$	137.85	4.41	0.06	6	−69.60	0.78
5)	$p\,[(.)^{2014,\ 2015}, (.)^{2016}]\psi\ (CG \neq ELF = PCCI)$	148.31	14.87	0.00	5	−57.05	0.22
6)	$p\,[(.)^{2014,\ 2015}, (CS)^{2016}]\psi\ (CG \neq ELF = PCCI)$	149.75	16.30	0.00	6	−57.71	0.25
7)	$p\,[(.)^{2014,\ 2015}, (.)^{2016}]\psi\ (.)$	151.23	17.78	0.00	4	−52.07	0.00
8)	$p\,[(.)^{2014,\ 2015}, (CS)^{2016}]\psi\ (.)$	152.72	19.28	0.00	5	−52.64	0.03

[a] Akaike's Information Criterion corrected for sample size $= -2\log(\mathcal{L}(\theta | x) + 2K + 2(K(K+1))/(n - K - 1)$, where n is the sample size and K is the number of parameters in the model.

[b] AIC_c difference between model i and the top-ranked model.

[c] Probability that model i is the best model given the data and alternative models in the candidate set.

[d] Adjusted difference in $-2\log(\mathcal{L})$ of the current model and $-2\log(\mathcal{L})$ of the saturated model.

[e] Analysis of deviance explains model fit relative to the global model.

Table 3. Descriptive statistics for *Ophidiomyces* surveillance in 3 populations of eastern massasaugas using quantitative polymerase chain reaction (qPCR) of swab applicators. Samples are from Camp Graying (CG), the Edward Lowe Foundation (ELF), and Pierce Cedar Creek Institute (PCCI), Michigan, USA. Values in parentheses for sex and age class indicate number of individuals with unknown status.

		CG	ELF	PCCI
2014				
Sex	F/M	12/20 (2)	15/10	20/23
Age class	Adult/juveniles	30/4	22/3	38/5
Skin lesions	*n* (%)	5 (14.7)	1 (4.0)	7 (16.3)
qPCR positive	*n* (%)	2 (5.9)	2 (8.0)	1 (2.3)
qPCR positive and skin lesions	*n* (%)	2 (5.9)	0 (0)	1 (2.3)
2015				
Sex	F/M	7/8	6/10	36/25
Age class	Adult/Juveniles	15/0	15/1	50/11
Skin lesions	*n* (%)	2 (13.3)	0 (0)	6 (9.8)
qPCR positive	*n* (%)	2 (13.3)	0 (0)	1 (1.6)
qPCR positive and skin lesions	*n* (%)	2 (13.3)	0 (0)	1 (1.6)
2016				
Sex	F/M	15/17 (2)	23/12	20/16
Age class	Adult/Juveniles	26/1 (7)	32/3	36/0
Skin lesions	*n* (%)	6 (17.6)	1 (2.9)	22 (61.1)
qPCR positive	*n* (%)	4 (11.8)	0 (0)	1 (2.8)
qPCR positive and skin lesions	*n* (%)	2 (5.9)	0 (0)	0 (0)

AIC_c weight and explained 0.97 of the model deviance (ANODEV), using 1 fewer parameter than the global model (Table 2). Model 1 included clinical signs of SFD to explain *Ophidiomyces* DNA detection probabilities in 2014 and 2015 but held detection probabilities constant between individuals with and without clinical signs in 2016. Occupancy for model 1 also included a locality effect that treated occupancy probabilities at ELF and PCCI as equal but allowed CG to differ in occupancy from the other localities. Models 2 and 4 were identical to models 1 and 3, respectively, but included clinical signs of SFD as an explanatory variable for *Ophidiomyces* DNA detection probabilities for 2016. We considered clinical signs as an explanatory variable for 2016 to be uninformative for models 2 and 4 because the deviance for each model decreased by fewer than 2 units when compared to their nested counterparts (i.e., models 1 and 3; Anderson 2008). Thus, model 2 absorbed 0.26 w_i that would have otherwise gone to model 1. Model 3 received little support ($w_i = 0.13$) and was identical to model 1 in detection probabilities but treated occupancy as constant across localities. Model 4 was a generalization of model 3, but as mentioned above, included the uninformative variable clinical signs for 2016. Thus, the marginal support ($w_i = 0.06$) model 4 received would have been allocated to model 3 if not for the presence of this pretending variable (Anderson 2008, Arnold 2010).

Model averaging across the candidate models (excluding models 2 and 4, the uninformative embellishments of models 1 and 3) resulted in *Ophidiomyces* DNA detection probabilities that were higher for snakes with clinical signs of SFD (0.23, 95% CI = 0.08–0.51) than for snakes without clinical signs of SFD (0.01, 95% CI = 0.00–0.09) for 2014 and 2015. However, detection probabilities in 2016 were indistinguishable between snakes with clinical signs of SFD and snakes without clinical signs of SFD (0.22, 95% CI = 0.08–0.48). Point estimates of *Ophidiomyces* prevalence in 2016 were larger at CG (0.17, 95% CI = 0.04–0.50) than at ELF and PCCI (0.03, 95% CI = 0.00–0.19).

The Probability of Obtaining a False Negative (1−*p*)

Snakes with clinical signs that were swabbed with only 1 applicator had a high probability of *Ophidiomyces* DNA being missed (0.73, 95% CI = 0.53–0.86), whereas for snakes without clinical signs, the probability of obtaining a false negative was even higher (0.97, 95% CI = 0.91–0.99; likelihood ratio test, $\chi_1^2 = 14.804$, $P < 0.001$; Fig. 3). Increasing the number of swab applicators collected per snake from 1 to 5 reduced the probability of obtaining a false negative by 72% (0.20, 95% CI = 0.06–0.53) for snakes with clinical signs and only by 12% (0.85, 95% CI = 0.63–0.95) for snakes without clinical signs (Fig. 3).

Radio-Transmitter Effects on *Ophidiomyces* Prevalence

Uncorrected or naive estimates of *Ophidiomyces* prevalence at the CG locality were 0.16 (4 positive, 21 negative) for snakes
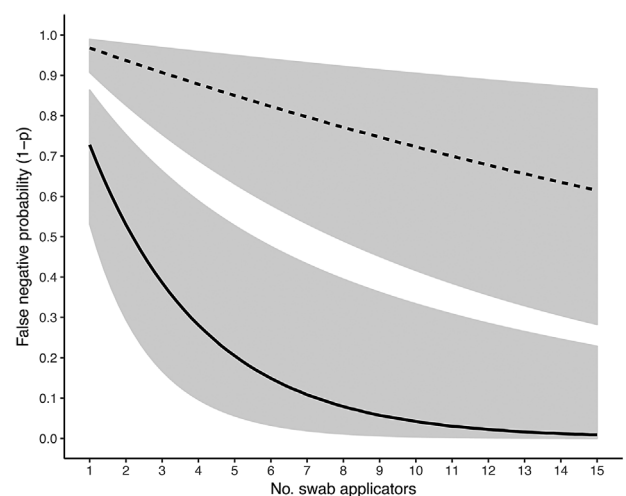

Figure 3. Relationship between the number of swab applicators collected per eastern massasauga and the probability of obtaining an *Ophidiomyces* DNA negative result when the pathogen is actually present on a snake with clinical signs (solid line) or a snake without clinical signs (dashed line), Michigan, USA, 2014–2016. The shaded areas represent 95% confidence intervals.

with radio-transmitters compared to 0.07 (4 positive, 54 negative) for snakes without radio-transmitters. Adjusted point estimates of *Ophidiomyces* prevalence resulting from the occupancy model were 0.35 (95% CI = 0.11–0.70) for snakes with radio-transmitters and 0.13 (95% CI = 0.04–0.32) for snakes without radio-transmitters (likelihood ratio test, $\chi_1^2 = 2.213$, $P = 0.137$).

DISCUSSION

Snake fungal disease caused by *Ophidiomyces ophiodiicola* has been proposed as a threat to wild snake populations, and was included in the final determination listing the eastern massasauga as federally threatened (USFWS 2016). However, estimating *Ophidiomyces* prevalence and documenting population-level effects of this pathogen has been elusive because of the occurrence of false negatives from available diagnostic tests (Allender et al. 2016a, Lorch et al. 2016). Our study is the first to account for false negatives, estimate false-negative probabilities, and provide detection-corrected estimates of Ophidiomyces prevalence for any wild snake population. Using novel sampling protocols, single-season occupancy models, and existing diagnostic tests, we provide a framework for using non-invasive swabbing to estimate Ophidiomyces prevalence and quantify population responses to snake fungal disease.

We demonstrated that *Ophidiomyces* prevalence varies across eastern massasauga populations, with *Ophidiomyces* prevalence being higher at CG (17%) than at the other 2 localities (3%, ELF and PCCI) we investigated in Michigan. Restricting our occupancy analysis to CG, point estimates of *Ophidiomyces* prevalence were higher for snakes implanted with radio-transmitters (35%) than for snakes without radio-transmitters (13%). Although these differences were not statistically different, the lack of significance may be due to small sample size and should not be interpreted to mean that surgical implantation of radio-transmitters has no effect on *Ophidiomyces* prevalence. Radio-implantation may increase the incidence of *Ophidiomyces* if introduced through non-sterile surgical techniques (iatrogenic), through creation of a skin defect allowing *Ophidiomyces* already present to colonize, or through immune suppression post-surgery (Lentini et al. 2011). Whether *Ophidiomyces* is part of the normal fungal flora of eastern massasauga skin, as has been observed with other similar fungal species on the skin of reptiles, remains unknown (Pare et al. 2003). Resolution of this question is necessary to assess the associated risks of surgical procedures, including diagnostic biopsy.

The larger point estimates of *Ophidiomyces* prevalence at CG may be related to sampling bias (17 of 34 snakes at CG in 2016 had radio-transmitters), local climate, or environmental differences among localities. For example, the average minimum temperature near CG was lower (3.8°C, station USC00203391) than at weather stations near ELF (8.2°C, station USC00202250), or PCCI (10.9°C, station USC00203661) for the sampling months we estimated prevalence (Apr–Jun 2016; MRCC 2017). Clinical signs of SFD were inversely related to mean monthly surface temperature in a Florida population of pigmy rattlesnakes (*Sistrurus miliarius*), with lower temperatures resulting in clinically more severe SFD symptoms (McCoy et al. 2017). Our data were not adequately abundant (temporally) to investigate if this pattern holds for eastern massasaugas. Future research efforts should incorporate testing environmental samples for *Ophidiomyces* using environmental DNA methods to assess what environmental factors (e.g., soil, water) contribute to *Ophidiomyces* occupancy across localities. Linking *Ophidiomyces* prevalence with environmental factors may be important for establishing management efforts to reduce disease occurrence.

We provided evidence that the probability of obtaining a false-negative result for *Ophidiomyces* DNA using current diagnostic methods (i.e., swabbing methods and qPCR), is exceptionally high for snakes with clinical signs (73%) and snakes without clinical signs (97%) if only one swab applicator is collected per animal (Fig. 3). However, false-negative results were reduced by 72% when snakes with clinical signs were resampled using 5 applicators (Fig. 3). The probability of obtaining a false negative result reduced to <5% when snakes with clinical signs were resampled with 10 applicators, whereas snakes without clinical signs needed to be resampled 93 times to reach this same threshold.

The false negatives detected in our study are likely due primarily to swabbing methods and the stage of infection rather than qPCR techniques. The qPCR methods we used are highly sensitive to *Ophidiomyces*, detecting the fungal pathogen with as few as 10 DNA copies present per assay (Allender et al. 2015b). Still, qPCR methods could have failed to detect very low concentrations of *Ophidiomyces* DNA, or failed during extraction or amplification. Fortunately, occupancy models account for false negatives regardless of what stage (e.g., swabbing, extraction, amplification) in the process they occur. If most false negatives occurred during swabbing as suspected, then future researchers should work to refine swabbing techniques so that *Ophidiomyces* DNA is acquired more consistently. If *Ophidiomyces* is only present in the deeper tissues of some animals, swabbing the skin surface will result in a false negative regardless of technique or the number of applicators used. In this scenario, prevalence will be underestimated because this group of snakes will have a zero probability of *Ophidiomyces* being detected.

Using 1 year of data (2014), Allender et al. (2016a) reported that snakes with clinical signs of SFD (e.g., dermatitis) had higher *Ophidiomyces* DNA detection probabilities (0.15, 95% CI = 0.05–0.38) than snakes without clinical signs of SFD (0.02, 95% CI = 0.00–0.06). Adding an additional year of data (2015 samples) did not change these original findings; snakes with clinical signs of SFD still had higher *Ophidiomyces* DNA detection probabilities (0.23, 95% CI = 0.08–0.51) than snakes without clinical signs of SFD (0.01, 95% CI = 0.00–0.09). However, we found no support for differences in *Ophidiomyces* DNA detection probabilities in 2016 between snakes with clinical signs of SFD and snakes without clinical signs of SFD (0.22, 95% CI = 0.08–0.48). This lack of support may be methodological rather than biological because the ability

to identify clinical signs likely increased with researcher experience over time. In other words, subtler symptoms may have been included in 2016 than in previous years, resulting in clinical signs having weaker explanatory power.

Natural systems are complex and are influenced by many factors including weather, community structure and composition, changes in land use, and climate. Thus, elucidating the causal factors of a fungal pathogen like *Ophidiomyces* will require increased spatial and temporal sampling efforts paired with simultaneous animal and environmental sampling. Continued monitoring using the methods described here will allow for future assessment of the impact of *Ophidiomyces* on eastern massasauga and other snake populations.

MANAGEMENT IMPLICATIONS

Our results demonstrate that *Ophidiomyces* prevalence differs among eastern massasauga populations and that the probability of obtaining an *Ophidiomyces* DNA false-negative result using swabbing methods is exceedingly high if only 1 swab is collected per animal. To overcome this obstacle, we recommend future monitoring efforts include repeated sampling (≥5 applicator swabs/snake) and increased sample sizes (≥40 individuals/locality) as a starting point to estimate locality-specific *Ophidiomyces* DNA prevalence. After initial detection and prevalence parameters are estimated, this sampling regime should be reassessed and calibrated to obtain the desired estimates of error (MacKenzie and Royle 2005). Alternatively, until the cost of qPCR is reduced, a more practical and cost effective approach may be to use clinical signs as a surrogate for SFD. If clinical signs are used as a proxy for SFD, researchers should standardize diagnosis of positive and negative clinical signs of SFD by providing technicians field training and educational materials including detailed descriptions, photographs, and representative specimens (when possible), and minimize the number of observers recording clinical signs so documentation remains consistent across years and study sites.

Another strategy would be to use qPCR and target only animals with obvious clinical signs. A major drawback to targeted sampling is that it precludes estimation of site-specific population-level responses, limiting inference to individual health or estimation of *Ophidiomyces* prevalence for the proportion of the population with clinical signs. However, targeted sampling of individuals could be used to assess range-wide responses over time (e.g., changes in *Ophidiomyces* occupancy [presence-absence] across populations) without introducing sampling bias. To get at this coarser grain question, we recommend the use of occupancy models, targeted sampling, locality-specific covariates, and a range-wide expansion of sampling efforts to estimate *Ophidiomyces* occupancy throughout the eastern massasauga's distribution and to identify predictive variables (e.g., temperature, precipitation) important for explaining *Ophidiomyces* occupancy. We recommend minimizing or avoiding the use of radio-telemetry methods where SFD occurs unless sterile surgical, handling, and equipment protocols can be ensured and the benefits to the population from such activities outweigh the increased health risks to individuals. If these conditions are met, judicious use of radio-telemetry may be justified.

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Associate Editor: Robin Russell.