



Detection of *Ophidiomyces ophiodiicola* at two mid-Atlantic natural areas in Anne Arundel County, Maryland and Fairfax County, Virginia, USA

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Abstract.—Since the early 2000s, ophidiomycosis has been reported with increasing frequency and associated with widespread morbidity in numerous North American snake species. *Ophidiomyces ophiodiicola* (*Oo*), the etiologic agent of ophidiomycosis, has been detected in over 30 species throughout most of the eastern United States, as well as in Europe and Australia; however, it is suspected that the distribution of this pathogen may be underestimated due to a lack of standardized inventories. To contribute to the existing but limited data on ophidiomycosis in the mid-Atlantic United States, snakes were sampled for *Oo* at two natural areas in this region—one in Anne Arundel County, Maryland and one in Fairfax County, Virginia. *Ophidiomyces ophiodiicola* was detected at both study sites. Thirty-four of 61 (55.7%) samples across eight species tested positive for the pathogen, with the highest detection rates occurring in *Nerodia sipedon* (73.1%) and *Pantherophis alleghaniensis* (70%). *Ophidiomyces ophiodiicola* was detected in snakes with (71.4%) and without (34.6%) clinical signs of ophidiomycosis. These results support the need for both increased *Oo* monitoring throughout the region, and implementation of more standardized and unbiased sampling protocols.

Keywords. Colubridae, ophidiomycosis, population decline, Reptilia, Serpentes, snake fungal disease

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Introduction

Ophidiomycosis has emerged as a growing threat to snakes throughout much of North America (Dolinski et al. 2014; Allender et al. 2015; Lorch et al. 2016; Paré and Sigler 2016) and has been associated with widespread morbidity in numerous species (Guthrie et al. 2016; Lorch et al. 2016; Stengle 2018). The disease is attributed to *Ophidiomyces ophiodiicola* (*Oo*), a mycotic pathogen that is only known to infect snakes (Allender et al. 2015; Lorch et al. 2016; Paré and Sigler 2016). Clinical manifestations of infection (see Fig. 1) typically include scabs, crusty scales, superficial pustules, subcutaneous nodules, and dysecydysis (Dolinski et al. 2014; McBride et al. 2015; Tetzlaff et al. 2015). Ophidiomycosis infections are generally chronic, but mild; however, severe infections with high mortality have been reported in several viperid species (Allender et al. 2013; Sigler et

al. 2013; Sleeman 2013; Lorch et al. 2015, 2016; Stengle et al. 2018). The precise mechanisms that influence lethal outcomes of the disease are still unclear, but are likely multifaceted (Lorch et al. 2015; Guthrie et al. 2016).

Since 2006, ophidiomycosis has been increasingly documented, with cases of infection reported in at least 20 states, including Maryland and Virginia (Allender et al. 2015; Guthrie et al. 2016; Tupper et al. 2015, 2018, 2019). Despite growing reports of ophidiomycosis throughout the mid-Atlantic, systematic studies designed to assess its prevalence using non-incident sampling methods are limited. The rising incidence of ophidiomycosis, coupled with habitat loss, pollution, and other anthropogenic stressors, poses an added challenge for snake conservation, underscoring the significance of ongoing disease monitoring (Franklinos et al. 2017; Kucherenko et al. 2018). The objective of this study was to assess the presence and prevalence of *Oo* at two mid-

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Fig. 1. The ventral scales of a symptomatic Northern Black Racer (*Coluber constrictor*) infected with *Ophidiomyces ophidiicola*. This 41 g male was captured and swabbed on 20 May 2018 at Huntley Meadows Park. Its total length was 123.2 cm and snout-to-vent length was 94.6 cm. Photo by Eva Lorentz.

Atlantic natural areas located in Maryland and Virginia, USA. The results obtained contribute toward an improved understanding of the distribution and prevalence of ophidiomycosis in the region.

Materials and Methods

Area-constrained visual encounter searches (Crump and Scott 1994) were used to sample for *Oo* in snakes from Huntley Meadows Park (HMP; 38°45'36.57" N, 77°05'44.13" W; Fig. 2) in Fairfax County, Virginia, and at the Smithsonian Environmental Research Center (SERC; 38°53'17.41"N, 76°33'15.52" W; Fig. 3) in Anne Arundel County, Maryland, between 22 April 2018 and 9 October 2018. Snakes were hand-captured (wearing sterile nitrile gloves) and visually inspected for clinical signs of ophidiomycosis (Allender et al. 2011; Clark et al. 2011). Then, using a modified protocol developed by Allender et al. (2016), snake skins were sampled with sterile dry swabs (no. MW113, Medical Wire and Equipment Company, Durham, North Carolina, USA) from all craniofacial scales and along the entire ventral length of the body separately, swabbing each region five times, taking care to swab any lesions, pustules, nodules, or displaced scales on snakes which showed signs of infection (Allender et al. 2011, 2016). Swabs were stored in sterile 1.5 mL microcentrifuge tubes and kept frozen until molecular analysis. Prior to release, each snake was measured, weighed, and photographed to help in differentiating conspecifics. Aseptic techniques were employed and appropriate biosecurity protocols were followed (see Rzadzowska et al. 2016; VHS 2016) to limit the transmission of *Oo*.

For the *Oo* assay, DNA was eluted from the swabs using the Purification of Total DNA from Animal Tissues Protocol (Qiagen®, Valencia, California, USA). To ensure samples were not contaminated during the

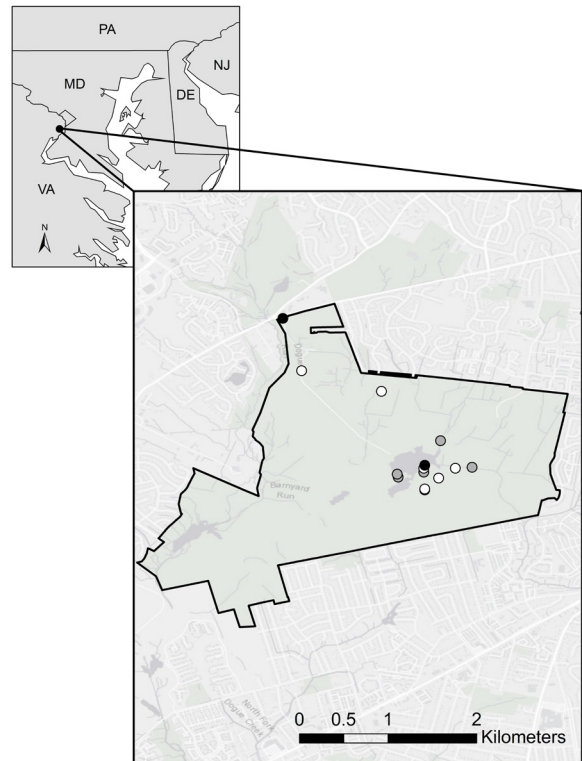


Fig. 2. Location of Huntley Meadows Park (HMP) snake capture locations. Black markers = all samples positive; white markers = all samples negative; gray markers = samples either positive or negative.

extraction process, a negative control was used, which included all elements of the extraction mixture other than DNA. Following methods described by Allender et al. (2015), 2.5 μ L of eluted DNA was combined with 12.5 μ L Sso Advanced™ universal probes supermix (Bio-Rad, Hercules, California, USA), 1.25 μ L of a combined target-specific primer (OphioITS-F and OphioITS-R)-probe, and water, creating a 25 μ L reaction mixture. The DNA was amplified via qPCR using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA), with the following cycling parameters: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec, followed by a final cycle at 72 °C for 10 min.

For each round of qPCR, a positive control was included by adding 2.5 μ L of a plasmid containing *Oo* (obtained from the Wildlife Epidemiology Laboratory at Illinois University at Urbana-Champaign, Illinois, USA) to a designated well containing the 22.5 μ L mixture of primer-probe, and water (as described above). A well was also included for the negative control, which contained only the 22.5 μ L mixture, but no DNA. These controls were used to determine whether the reaction mixture was prepared accurately, and to ensure that samples were not contaminated during qPCR preparation. Up to five rounds of qPCR were performed for each sample. A sample was considered positive if at least three rounds (per sample) had a lower cycle threshold (*C*) than the

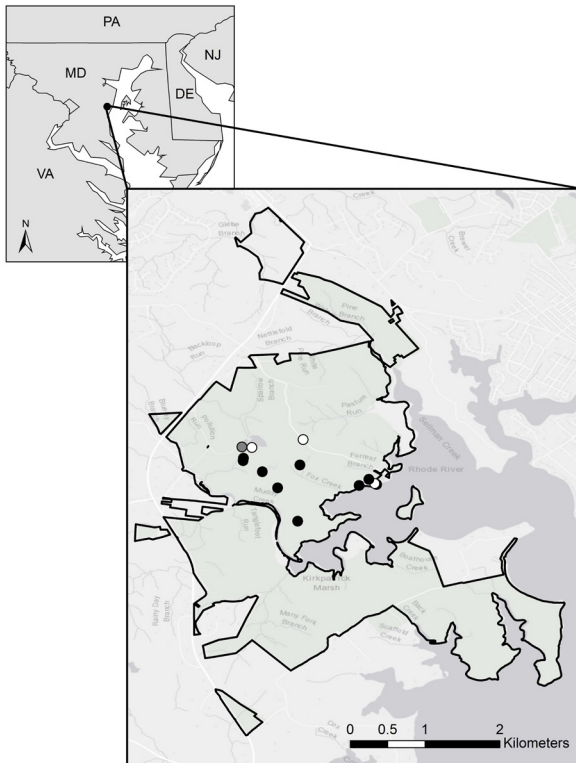


Fig. 3. Location of Smithsonian Environmental Research Center (SERC) snake capture locations. Black markers = all samples positive; white markers = all samples negative; gray markers = samples either positive or negative.

lowest detected standard dilution for the positive control (Allender et al. 2016).

Sampling sites within the study areas (indicating locations of positive and negative samples) were plotted with ESRI ArcMap (version 10.6). Snake nomenclature corresponds with Crother et al. (2017). Tables and descriptive statistics were completed with Microsoft Excel for Office 365 (Microsoft Corporation, Redmond, Washington, USA).

Results

Sixty snakes (35 from HMP and 25 from SERC) across nine species were captured and swabbed (Table 1). Northern Watersnake (*Nerodia sipedon*) comprised the largest proportion ($n = 26$; 43.3%) of the captures. Eastern Ratsnake (*Pantherophis alleghaniensis*; $n = 10$), Common Ribbonsnake (*Thamnophis sauritus*; $n = 9$), and Eastern Wormsnake (*Carphophis amoenus*; $n = 7$) were also well-represented, comprising 16.7%, 15%, and 11% of the total snake sample, respectively. Northern Black Racer (*Coluber constrictor*; $n = 2$), Eastern Kingsnake (*Lampropeltis getula*; $n = 1$), Northern Ring-necked Snake (*Diadophis punctatus*; $n = 1$), Eastern Gartersnake (*Thamnophis sirtalis*; $n = 2$), and Dekay’s Brownsnake (*Storeria dekayi*; $n = 2$) were all sparsely represented. *Ophidiomyces ophiodiicola* was detected in 33 snakes and in a shed skin of a Northern Black Racer, yielding an overall detection rate of 55.7%. More than half of the positive samples (55.9%) were from a single species—Northern Watersnake. Of the nine species sampled, Northern Watersnake had the highest detection rate (73.1%), followed closely by Eastern Ratsnake (70%). Northern Black Racer, Eastern Wormsnake, and Common Ribbonsnake were positive in 66.7%, 28.6%, and 11.1% of samples, respectively. Only one Eastern Kingsnake and one Northern Ring-necked snake were sampled, and both were positive. Dekay’s Brownsnake was positive in one of two samples and Eastern Gartersnake was the only species that did not test positive for *Oo*. Twenty-five of the 35 (71.4%) snakes showing clinical signs tested positive for *Oo*, and nine of the 26 (34.6%) without clinical signs were *Oo* positive (Table 1). Prevalence varied between study locations, with 34.6% of snakes testing positive at HMP and 84.6% at SERC. Of the 34 snakes testing positive, *Oo* was detected in both swabs in 18 snakes (52.9%) and in only one of two swabs (nine from the craniofacial swab only, seven from the body swab only) in 16 snakes (47.1%).

Table 1. Prevalence by species. S/+ = positive with clinical signs, A/+ = positive without clinical signs.

Species	N	Positive	Prevalence (%)			
			For species	Overall	S/+	A/+
Eastern Wormsnake (<i>Carphophis amoenus amoenus</i>)	7	2	28.6	5.9	0	2
Northern Black Racer (<i>Coluber constrictor constrictor</i>)	3	2	66.7	5.9	2	0
Northern Ring-Necked Snake (<i>Diadophis punctatus edwardsii</i>)	1	1	100	2.9	0	1
Eastern Kingsnake (<i>Lampropeltis getula</i>)	1	1	100	2.9	1	0
Northern Watersnake (<i>Nerodia sipedon sipedon</i>)	26	19	73.1	55.9	15	4
Eastern Ratsnake (<i>Pantherophis alleghaniensis</i>)	10	7	70	20.6	5	2
Dekay's Brownsnake (<i>Storeria dekayi</i>)	2	1	50	2.9	1	0
Common Ribbonsnake (<i>Thamnophis saurita saurita</i>)	9	1	11.1	2.9	1	0
Eastern Gartersnake (<i>Thamnophis sirtalis sirtalis</i>)	2	0	0	0	0	0
Total or overall prevalence	61	34	-	55.7	25	9

Discussion

Although *Oo* has previously been documented in Maryland and Virginia (Guthrie et al. 2016; Tupper et al. 2018), this work is one of only two studies (see Guthrie et al. 2016) to investigate *Oo* in these states. In Maryland, observations of fungal dermatitis have been reported from the Smithsonian Environmental Research Center (SERC) since 2014 (Tupper et al. 2015), with *Oo* recently being confirmed as the etiological agent of a dermal infection in Northern Watersnake (Tupper et al. 2018). These results add four new species (Eastern Wormsnake, Northern Black Racer, Northern Ring-necked Snake, and Eastern Ratsnake) to the documented host range of this pathogen in Maryland, which previously included only Northern Watersnake (Tupper et al. 2018) and Timber Rattlesnake (*Crotalus horridus*; Tupper et al. 2019). In eastern Virginia, Guthrie et al. (2016) documented *Oo* in four species (all with clinical signs): Northern Watersnake ($n = 3$), Rainbow Snake (*Farancia erythrogramma*; $n = 1$), Northern Black Racer ($n = 2$), and Brown Watersnake (*Nerodia taxispilota*; $n = 2$). This study adds four new hosts to the list of *Oo* positive species occurring in Virginia: Eastern Kingsnake, Eastern Ratsnake, Dekay's Brownsnake, and Common Ribbonsnake.

The overall detection rate of 57.4% is among the highest reported (except see McKenzie et al. 2018) across the eastern and midwestern United States (Smeenk et al. 2016; Allender et al. 2016). The prevalence of *Oo* throughout these regions appears to be highly variable, with detection rates as low as 0% and 4.9% in Ohio and Michigan, respectively (Smeenk et al. 2016; Allender et al. 2016), and up to nearly 62% in eastern Kentucky (McKenzie et al. 2018). We interpret these rates cautiously, however, taking into consideration the variation in species sampled between studies. It is still unclear how susceptibility and severity of infection differ between species (Grisnik et al. 2018), but the composition of species sampled in this study may partly explain the overall prevalence and the relatively high proportion of *Oo* positive snakes that did not show clinical signs of the disease.

In this study, *Oo* was detected in eight of the nine species sampled, which was not surprising given that each of these species has previously tested positive for the pathogen in the eastern and mid-western United States (Lorch et al. 2016; Persons et al. 2017; Grisnik et al. 2018; McKenzie et al. 2018). However, the small sample sizes in certain species made it impossible to assess how each of these species actually influence the overall detection rate. *Ophidiomyces ophiodiicola* was found to be most prevalent in Northern Watersnake, with a detection rate of 73%. This species represented nearly 43% of the total sample and thus had a strong influence on overall prevalence (55.7%). Prior studies with similar proportions of aquatic species have also demonstrated relatively high *Oo* detection rates among Northern

Watersnakes and other species with aquatic affiliations. However, this trend in detection may partially reflect the habitat preferences of the pathogen (Lorch et al. 2016; McKenzie et al. 2018), rather than an inherent biological susceptibility to the pathogen. Additional work is needed to better understand susceptibility to the disease.

Variability in sampling methods between studies should also be considered when interpreting results (McCoy et al. 2017; Grisnik et al. 2018; Hileman et al. 2018; McKenzie et al. 2018). For instance, the number of sterile dry swab applicators used per snake has been shown to influence detectability of *Oo*, with the use of only one applicator greatly increasing the probability of obtaining false-negatives (Hileman et al. 2018). The results obtained here support this concept, with 47.1% of snakes testing positive for *Oo* in only one of two swabs.

Underestimation of the prevalence of *Oo* may also occur when diagnostic tests are limited only to snakes that present clinical manifestation of infection (see Guthrie et al. 2016). While clinical signs have been associated with a higher probability of PCR-positive results (Allender et al. 2016), studies have also demonstrated that anywhere from 6% (Bohuski et al. 2015) to 38% (Hileman et al. 2018) of snakes without clinical signs test positive for *Oo*. The data reported here support these studies, with 26.5% of *Oo* positive snakes in this sample showing no signs of infection. One possible explanation is that clinical signs may be subtle and overlooked during inspection, because a snake is either in the early stages of infection or effectively clearing the infection through repeated sheds (Lorch et al. 2016; Grisnik et al. 2018; Hileman et al. 2018). Detection without clinical signs may also reflect the absence of infection in a specimen altogether. *Ophidiomyces ophiodiicola* can persist as a saprobe in the soil, which can facilitate transmission and increase the likelihood of a snake encountering *Oo* (Allender et al. 2015; Lorch et al. 2016). The presence of *Oo* on the skin, however, does not necessarily indicate infection. Therefore, while swabbing can be an effective, low-cost, and minimally invasive method for detecting the pathogen, it cannot be used to infer or imply infection status.

Results from this study confirm that *Oo* is present and relatively prevalent in both Maryland and Virginia, and that the presence of *Oo* is more often accompanied by clinical manifestations consistent with ophidiomycosis than not. The geographic distribution and host range of the pathogen are still largely unknown (Burbrink et al. 2017), and ophidiomycosis may be more widely distributed than documented cases suggest (USGS 2018). Some have proposed that biased sampling methods may result in underestimations of prevalence within a population (Grisnik et al. 2018; Hileman et al. 2018). This potential for inaccurate assessments highlights the need for more standardized sampling efforts and diagnostic protocols. Based on the increasing number of reports of ophidiomycosis throughout the eastern United States, we suggest increased efforts to identify and monitor

Oo throughout the mid-Atlantic region. Additionally, enhanced biosecurity protocols should be implemented to limit disease transmission throughout the region.

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Ophidiomycosis in the mid-Atlantic USA



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