

Estimating host and spatial variation in infection with the fungal pathogen that causes snake fungal disease

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Abstract

Emerging wildlife diseases represent a serious threat to conservation efforts. Impacts of emerging multi-host pathogens can vary greatly among species as well as geographically, and understanding which populations will be at greatest risk is essential for conserving biodiversity. Snake fungal disease (SFD), caused by the fungal pathogen *Ophidiomyces ophidiicola*, is responsible for lethal infections in snakes and has contributed to the decline of multiple North American snake populations. However, which species are most affected by this disease and how infections vary regionally remains unknown. Here we sampled 44 different species across 14 sites throughout the Southeastern and Mid-Atlantic United States. We found a strong effect of latitude on both pathogen prevalence and severity, with more severe infections at more northern latitudes. We also found high variability in pathogen prevalence and infection severity among species. There was a strong positive relationship between pathogen prevalence and disease severity, suggesting that SFD is not just highly prevalent in some populations but also highly virulent. More broadly, our results support that SFD likely has continued impacts on snake populations with some species experiencing greater disease than others attributed to spatial and host variation.

General Audience Abstract:

Conserving biodiversity is a significant challenge. Wildlife species are under multiple threats including habitat loss, changing climate, species introductions, pollution, and infectious diseases. Emerging wildlife diseases can pose a major problem for wildlife as they often go undetected until they cause substantial declines for the affected species, sometimes leading to population extirpations and extinction events. Snake fungal disease (SFD) is an emerging disease caused by the fungal pathogen *Ophidiomyces ophidiicola*, which has contributed to the decline of some North American snake populations. However, little is known about differences in infection, transmission, and host responses to SFD in a broader community context. To investigate the dynamics of this pathogen, we collected swab samples from 44 species from a total of 14 sites in New Jersey, Virginia, North Carolina, South Carolina, Georgia, Florida, and Louisiana. We sampled individual snakes to examine variation over a geographic gradient and among species. We found high variability among sites with more severe disease at northern sites. There was also high variability among species and some populations experienced both high pathogen prevalence and disease severity. Our results show that SFD is highly variable within snake communities and may still be causing population level effects.

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Introduction

Emerging infectious diseases pose a significant challenge for wildlife as they can cause population collapse, regional extirpation, and even the extinction of once common species (Berger et al., 1998; Fisher et al., 2012; Frick et al., 2010; Hawkins et al., 2006; Smith et al., 2006). Diseases can be more deadly for species with shared traits or behaviors, which can reduce the abundance of entire groups of organisms that serve a vital function within ecosystems (Anagnostakis, 1987; Langwig et al., 2012; Schultz et al., 2016). This can ultimately result in community collapse or alternative stable states in ecosystems (Holdo et al., 2009; Langwig et al., 2016; Skerratt et al., 2007). Estimating the impacts of emerging infectious diseases and determining which species are most at risk is vital to determining which conservation strategies should be used to minimize impacts (Langwig et al., 2015). However, capturing the initial impacts of an emerging pathogen is rare and the most obvious impacts to host communities are typically overlooked (Farrer et al., 2011; Jaric et al., 2019; McCallum, 2005; Riley et al., 2014; Weldon et al., 2004). Assessing host impacts after a pathogen becomes endemic in a population can be challenging, as population impacts become more cryptic. Ultimately, introduced pathogens can be particularly influential in filtering ecological communities, and are generally overlooked in wildlife (Brashares, 2010; Frick et al., 2015; LaDeau et al., 2007; Scheele et al., 2017).

Multi-host disease systems are a major conservation concern due to the increased risk of population extirpation and even extinction (LaDeau et al., 2007; Lips et al., 2006). Multi-host pathogens can result in sustained high exposure in susceptible species, where single host pathogens would otherwise have reduced R_0 (basic reproduction number for a pathogen) as host

density falls below the threshold for transmission (Anderson & May, 1979; Dobson, 2004; Hyman & Li, 2000; Lyles & Dobson, 1993). Species that may be able to tolerate or serve as reservoirs for a pathogen can maintain a high force of infection on the most susceptible species that share the same environments (Borzée et al., 2017; Brannelly et al., 2018; Scheele et al., 2017).

The impacts of an emerging pathogen depend on several factors, some of these include whether it can infect multiple hosts, the ability to persist in the environment, innate host susceptibility and geographical factors (Beldomenico & Begon, 2010; Cunningham et al., 2021; De Castro & Bolker, 2005; Gurr et al., 2011; Hoyt et al., 2020). Fungal pathogens have been of particular concern as they have caused catastrophic losses in North American bat populations affected by white-nose syndrome (*Pseudogymnoascus destructans*) and extinctions of frogs impacted by chytridiomycosis (*Batrochchytrium dendrobatidis*) (Berger et al., 1998; Frick et al., 2010; Langwig et al., 2012; Skerratt et al., 2007). For example, *Atelopus* is a frog genus highly susceptible to chytridiomycosis where 67% of the 110 species have been driven extinct, with population impacts varying due to differences in elevation, temperature, and host susceptibility (Flechas et al., 2012; Pounds et al., 2006). Fungal pathogens add additional challenges as they are typically able survive in the environment in the absence of hosts, sometimes remaining dormant for years, making eradication impractical (Hoyt et al., 2015; Johnson & Speare, 2003, 2005).

While investigating the dynamics of an emerging disease, infection prevalence and severity of infection are two fundamental factors to assess the impacts of a pathogen (Anderson & May, 1979). Prevalence provides information on how many hosts are infected and disease severity indicates how impacted host fitness is by the disease (Read, 1994; Seem, 1984).

Examining both factors across different populations allows us to determine which individuals may be important in transmission and which species might be at greatest risk from impacts.

Understanding the ecology of emerging wildlife diseases is crucial to developing effective conservation strategies. For example, the American Robin (*Turdus migratorius*), is moderately susceptible to West Nile virus but is less abundant than other bird species with equal susceptibility. However, they still play a predominant role in West Nile Virus infections because they are preferentially fed on over other species contributing more to the infected pool of mosquitoes and subsequent transmission (Kilpatrick et al., 2006). Simply targeting the most susceptible species, would divert limited resources to species that are less important in transmission. For many emerging pathogens, the complicated interactions that determine which species are most important in transmission remains unknown. An insufficient understanding of host dynamics can diminish the efficiency of conservation efforts, potentially leading to delayed or a complete lack of conservation interventions where they are needed.

Despite being described over a decade ago, snake fungal disease, caused by the fungal pathogen *Ophidiomyces ophidiicola*, has received little attention, and there has been limited research on this disease which constrains our understanding of host and spatial dynamics. Before it is possible to determine if and when conservation strategies are necessary, its essential to know which species may be facing impacts from this disease and how this varies across space.

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Chapter 1: Estimating host and spatial variation in infection with the fungal pathogen that causes snake fungal disease

Abstract

Emerging wildlife diseases represent a serious threat to conservation efforts. Impacts of emerging multi-host pathogens can vary greatly among species as well as geographically, and understanding which populations will be at greatest risk is essential for conserving biodiversity. Snake fungal disease (SFD), caused by the fungal pathogen *Ophidiomyces ophidiicola*, is responsible for lethal infections in snakes and has contributed to the decline of multiple North American snake populations. However, which species are most affected by this disease and how infections vary regionally remains unknown. Here we sampled 44 different species across 14 sites throughout the Southeastern and Mid-Atlantic United States. We found a strong effect of latitude on both pathogen prevalence and severity, with more severe infections at more northern latitudes. We also found high variability in pathogen prevalence and infection severity among species. There was a strong positive relationship between pathogen prevalence and disease severity, suggesting that SFD is not just highly prevalent in some populations but also highly virulent. More broadly, our results support that SFD likely has continued impacts on snake populations with some species experiencing greater disease than others attributed to spatial and host variation.

Introduction

Wildlife faces a wide variety of conservation challenges, including habitat loss, climate change, overexploitation, and infectious disease, which ultimately contributes to loss of biodiversity (Bay et al., 2018; Daszak et al., 2003; Eikelboom et al., 2020; Horváth et al., 2019; Mazor et al., 2018; Urban, 2015). Biodiversity provides society with substantial economic and environmental benefits (Cardinale et al., 2012; Myers, 1996; Pimentel et al., 1997). The current extinction rate is 100 – 1000 times greater than the predicted natural rate, resulting in the dire urgency to conserve biodiversity and understand the factors contributing to its decline (Pimm et al., 1995). Infectious diseases can reduce populations, spread into habitats beyond human intrusion, and combine with other factors causing species decline (D'Aoust-Messier et al., 2015; Lips et al., 2003; Oltean et al., 2014; Seimon et al., 2007; Smith et al., 2009; Thorne & Williams, 1988). Some wildlife diseases have been well studied (chytridiomycosis in amphibians; (Berger et al., 1998; Martel et al., 2013; Spitzen-van der Sluijs et al., 2013), however there are numerous others that remain poorly studied and require immediate attention.

Snake fungal disease (SFD), also known as ophidiomycosis, is caused by the multi-host fungal pathogen *Ophidiomyces ophidiicola*, that has resulted in severe impacts to snake populations (Allender et al., 2011; Clark et al., 2011; Lorch et al., 2016). This disease has been largely understudied in comparison with other emerging wildlife diseases. SFD was first documented in 2006 in New Hampshire in a declining population of timber rattlesnakes (*Crotalus horridus*) exhibiting skin infections, and in 2008 it was tied to high mortality rates of Eastern massasauga rattlesnakes (*Sistrurus catenatus*) in Illinois (Allender et al., 2011; Clark et al., 2011). SFD is recognized as a potential threat to snake populations globally and has been detected in >62 species in the United States, Canada, Europe, Taiwan, China, and in soil samples

in Malaysia, and reports of affected species continue to rise (Ali et al., 2016; Blanvillain et al., 2022; Di Nicola et al., 2022; Franklinos et al., 2017; Lorch et al., 2016; Meier et al., 2018). Beyond wild populations, *O. ophidiicola* has been detected in captive snake colonies across the globe (Ladner et al., 2022). Isolated population declines due to *O. ophidiicola* have been reported in Eastern North America (Allender et al., 2011; Clark et al., 2011), with minimal documentation of severe infections for wild snakes in other parts of the world (Allain & Duffus, 2019; Blanvillain et al., 2022; Sun et al., 2021). These more frequent severe infections in North America may involve the multiple introductions of non-native strains of *O. ophidiicola* to the region (Ladner et al., 2022). Widespread assessments of population impact for most species are unknown, and documented impacts to snakes from SFD have largely occurred in vulnerable populations already under study (e.g., species listed regionally as endangered such as the timber rattlesnake, *C. horridus* or the federally threatened Eastern massasauga rattlesnake, *S. catenatus*) suggesting that the extent of SFD impacts to snake populations may be underestimated (Allender et al., 2011; Clark et al., 2011). Transmission of *O. ophidiicola* likely occurs through both direct and indirect contact, and variation has been observed among species (Campbell et al., 2021; Lorch et al., 2016; McKenzie et al., 2019). Sublethal effects of SFD on snakes may also contribute to a reduction in population abundance. Research has proposed that a reduction of sex steroids following infection, could result in reduced reproduction (Lind et al., 2019).

Ophidiomyces ophidiicola initially invades the snake's epidermal layer, creating lesions on the snake that can impair movement, and in some instances, feeding behavior. If the snake is unable to clear these infections through ecdysis or other immune responses, they can ultimately lead to death (Lorch et al., 2016). Recent research supports that snake fungal disease exhibits seasonal fluctuations (McCoy et al., 2017; McKenzie et al., 2019). The most severe infections

are associated with their brumation (period of inactivity during colder winter months), which begins in autumn and ends in spring (Lind et al., 2018; McCoy et al., 2017). During winter dormancy, the pathogen likely takes advantage of the snake's suppressed immune system (Wright & Cooper, 1981), the inability of snakes to shed their skin during winter, and the congregation of individuals which all likely contribute to the epizootics overwinter (Campbell et al., 2021; Lorch et al., 2015).

Ophidiomyces ophidiicola likely varies spatially among species and sites. However, limited studies have examined SFD across broad geographic ranges, with the majority of published studies being regionally focused (McCoy et al., 2017; Tetzlaff et al., 2017). This lack of breadth, limits extrapolations of the broader effects of SFD on species and geographic patterns. As season length and regional climate conditions vary across latitude, it can be assumed that pathogen dynamics should vary as well (Fecchio et al., 2020; Khatchikian et al., 2012). Reptiles are ectotherms, and while having an efficient metabolism, a cost is that they rely heavily on their environment to perform physiologically. These animals must engage in thermoregulation, photoregulation, and seasonal dormancy in Eastern North America (Conley & Lattanzio, 2022; Huey & Slatkin, 1976; Ladyman et al., 2003). This necessitates that snake's function and behavior is heavily dependent on seasonality. Other environmental factors also likely influence SFD infection as well. Species using aquatic environments have been thought to be more susceptible due to living in moist environments preferable for fungal growth, but little is known about specific abiotic conditions that influence this disease, and the species-specific differences in susceptibility and transmission among hosts (McKenzie et al., 2021).

Snake fungal disease and its impacts on snakes likely play an important role and are important to study because of their role in food webs (Willson & Winne, 2016). Snakes are a

diverse group of mesopredators, with 63 species in Eastern North America alone (Gibbons, 2017). Snakes are both specialists and generalists, with some snakes feeding exclusively on one group or taxa (e.g. eels or other snakes), whereas others consume prey varying from insects to small mammals (Grundler & Rabosky, 2021; Neill, 1964). The diverse array of prey species creates connections across trophic levels resulting in the cascading effects that could be influenced by the decline of snakes (Willson & Winne, 2016; Zipkin et al., 2020). Therefore, SFD remains an important system to understand which species are most impacted by disease and the factors contributing to differences in susceptibility.

Unusual snake mortality events have been recorded in southeast Virginia where snakes are found dead in their habitat with no apparent signs of predation or injury. This has been observed in the rainbow snake, *Farancia erytrogramma*, primarily through our research. Numerous *F. erytrogramma* have been found dead each spring, which now totals over 40 individuals in the last few years. Most of the *F. erytrogramma* found deceased have tested positive for *O. ophidiicola* and have clear histological signs indicative of SFD. This species is rarely observed, and these observations raise concern that the individuals being found constitute a small fraction of the total animals that suffer mortality from this disease annually. *F. erytrogramma* is a Tier IV Species of greatest conservation concern in Virginia's Wildlife Action Plan. This region has confirmed cases of SFD, with many of the snakes showing severe lesions characteristic of SFD and testing positive for the causative agent *O. ophidiicola* (Guthrie et al., 2015). Thus, SFD may be causing notable impacts on *F. erytrogramma* and other species emphasizing the need to understand what other species may be facing similar impacts.

Generally, this project will help inform which species are most susceptible to *O. ophidiicola*, including *F. erytrogramma*, allowing agencies to direct conservation focus to the

most vulnerable species. Most snake populations are cryptic and large-scale impacts similar to what has been observed in *F. erythrogramma* would likely go unnoticed, presenting an urgent need to understand which species are most susceptible to SFD for future monitoring and conservation. Investigating prevalence and severity among species is necessary to fully understand the impacts of SFD. Understanding which species are most susceptible to this disease will be essential for resource agencies and conservation organizations to make informed decisions about resource allocation. With an improved understanding, these organizations can implement population monitoring and conservation action for those at highest risk of extirpation or extinction.

Materials and Methods

We chose 14 sites distributed across eastern North America characterized by both high snake density and presence of *O. ophidiicola*, identified as pivotal factors influencing site selection. We distributed sites at the following locations: Pine Barrens, NJ; First Landing, VA; Back Bay, VA/NC; Alligator River, NC; Hyde County, NC; Francis Marion region, SC; Parris Island, SC; Savannah River, SC/GA; Southeastern GA; Jekyll Island, GA; Wakulla River drainage, FL; Eglin AFB; FL, Everglades National Park, FL; and Pearl River, LA (Fig. S2). Sites were chosen across the coastal plain to maintain similar habitats and elevation among sites. Sites were included a combination of coastal wetlands, mixed-hardwoods, and upland sandhill habitats to capture the snake species variety found in Southeastern and Mid-Atlantic regions of the United States.

We conducted surveys during the seasonal SFD epizootic period, beginning in January and ending in June when snakes first begin emerging from winter brumation and are most

frequently observed with severe SFD infections. Initial surveys were started at lower latitudes and progressed northwards coinciding with snake emergence from their winter dormancy. We continued sampling until early summer at northern sites (June) when the prevalence of *O. ophidiicola* has been shown to sharply decrease and snakes disperse on the landscape becoming less predictable (Hirth et al., 1969). We visited sites 2-3 times each spring with each capture period typically being over the course of 1-2 days, though we would occasionally shorten or lengthen visits depending on the number of individuals sampled (1-4 days). At each site we installed forty-five 1.5m x 1.22m x 1.22m plywood coverboards that were divided into three arrays deployed at each site in aquatic and upland habitats. Coverboards were chosen as a capture methodology, due to the ability to sample a wide range of species without necessitating daily monitoring (Grant et al. 1992). At Everglades National Park, we did not deploy coverboards due to the site having high recreational traffic and coverboards receiving less use from snakes with the more stable temperatures in the subtropical climate (Asad et al., 2022; Grant et al., 1992). We did not deploy board arrays at Parris Island, Jekyll Island, and New Jersey as animals were tracked using radio telemetry for separate research and cover objects were not necessary. For all sites, in addition to the coverboard arrays we used visual encounter surveys and driving transects in suitable habitat. We used these multiple capture methods to limit bias in infected vs non-infected individuals and to capture snakes in a varied range of their movements and habits). We captured non-venomous snakes by hand, which were held for processing in paper bags or disinfected cloth snake bags for larger individuals. We captured and restrained venomous snakes using clear, plastic Midwest Tongs 10-piece restraining tubes (Midwest Tongs, Greenwood, Missouri) and hooks. We differentiated species with similar morphology by geographic range with the most recent published taxonomic studies.

Pantherophis spilodes and *Pantherophis alleghaniensis* were separated at the Apalachicola River, Florida (Burbrink, 2001; Burbrink et al., 2000). *Agkistrodon piscivorus* and *Agkistrodon conanti* were separated at the coastal region of the Savannah River, Georgia, and Southeastern Alabama (Burbrink & Guiher, 2015).

For all captured snakes, we collected moistened (100 μ L of sterilized deionized H₂O) polyester sterile-tipped swabs rubbed in a standardized manner across the snake (Puritan®, Guilford, Maine, USA) to determine if the snake was infected and how much pathogen was present (Blanvillain et al., 2022). We performed standardized swabbing techniques among individuals by swabbing back and forth on the ventral and dorsal side of each animal five times each, as well as the face. We sexed snakes via stainless steel sexing probes that are thoroughly disinfected with ethanol and rinsed with water between individuals. Recently deceased snakes that were encountered were also swabbed, with most of these snakes resulting from vehicle strikes. These animals were swabbed within a few hours of their mortality; snakes that appeared dead more than 12 hours before encountering were not swabbed to avoid inaccurate pathogen collection from decomposition and ultraviolet light exposure.

For live captures, we individually marked each snake using a PIT tag (Passive Integrated Transponder; Biomark, Boise, ID) to allow for sampling the same individuals over time (see below for details). Snakes that were too small (<20cm) were excluded from being PIT tagged. To prevent exposing new snakes to *O. ophidiicola*, we used sterile nitrile gloves for each animal and changed between individual snakes, and all equipment used was thoroughly sterilized via a 10% bleach solution and 70% ethanol before processing a new animal. We retained snakes less than 20 minutes for the total processing time, and all were released where they were initially observed.

During 2020 and 2021, snake epidermal swabs were collected in duplicate. Upon analyzing samples via qPCR, we observed minimal differences in prevalence among the second and first swab. Therefore, during the 2022 and 2023 season we collected a single standardized swab. In addition, we collected morphometric measurements from each animal such as snout-vent length, tail length and circumference at 3 points on the snake. These measurements were used to calculate the surface for each snake by taking the average circumference of the anterior, mid-body, and posterior body and calculating the surface area of a cylinder ($r=2\pi/C$); ($A=2\pi r^2+2\pi rh$).

For individuals with skin lesions, we filled out a specific lesion datasheet, recording the number of lesions, location on the body, size, type, and color. For calculating total lesion severity, we photographed sampled snakes for subsequent lesion scoring. If severe necrotic lesions were present, we collected a small 2mm clipping of tissue for pathogen culturing. Based on our own experimental and field observations, we gave each lesion a score from 1-5 using the stage of lesion progression. Lesions with displaced scales and/or light inflammation would receive a score of 1. Lesions with crust, nodules, moderate inflammation, or puss would receive a score of 2. Lesions that had scabbed over ulcers, high inflammation, onsetting necrosis, crust penetrating beyond the outer epidermis would receive a score of 3. Lesions with exposed ulcers or severe necrosis received a score of 4. For the most severe lesions, we would score the highest ranking of 5, which would involve sloughing tissue, decomposing ulcers, critical necrosis, visual penetration beyond epidermis and into the muscle or body cavity (Fig. S1). These scores represent the progressive stages of infection and were then multiplied with the surface area for each individual lesion. For calculating the total lesion score, we combined these for each individual snake.

We analyzed a comprehensive total of 1938 swab samples from 1057 swabbed snakes using established qPCR protocols (Bohuski et al., 2015). Using 250uL Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA) in conjunction with 10mg of zirconium/silica beads, we extracted DNA from collected swabs. We subjected swab samples to a 45-second homogenization process facilitated by a homogenizer and lysis system (MP Biomedicals, Irvine, California, USA). We centrifuged samples for 30 seconds at 13000 rpm to concentrate material at the bottom of the tube. We homogenized and centrifuged the tubes, iterated the process, and then incubated the tubes at 100°C in a heat block for 10 minutes. Following heat treatment, we allowed samples to cool at room temperature for 2 minutes, followed by centrifugation at 13000 rpm for 3 minutes. For qPCR analysis, we pipetted 50µL to 100 µL of supernatant into a 96-well plate and preserve it at -80°C. To control for contamination, we prepared extraction negative controls using solely 250 µL of PrepMan® Ultra Sample Preparation Reagent and 10mg of zirconium/silica beads. Following Bohuski et al. (2015), we performed real-time qPCR with Quantifast mastermix by amplifying the internal transcribed spacer in between the 18S and 5.8S ribosomal RNA gene specific for *O. ophidiicola* using 5uL from the previously extracted DNA. To confirm the qPCR detection, we ran plates in duplicate to ensure accurate results. Prior to completing lab work, Qiagen changed their mastermix solutions. Therefore, we used Quantinova mastermix for many of the duplicate runs of plates. We compared and averaged the standardized results of Quantifast with Quantinova. We performed quantitative PCR using a real-time PCR QuantStudio 5 (Thermofisher Scientific, 181 Waltham, Massachusetts, USA). We used six standard curves of 500fg to 0.005fg of extracted DNA in triplicate, using plates in association with extractions from collected samples. This process allows us to calculate the amount of pathogen from each

swab and provide estimates of fungal load. Using Quantifast mastermix, the final reaction volume is 25 μ L, which includes 5 μ L of extracted DNA. Cycling conditions were: 95°C for 3 minutes, 95°C for 3 seconds, and finally 60°C for 30 seconds for a total of 40 cycles.

Quantinova mastermix had a final reaction volume of 20 μ L including the 5 μ L of extracted DNA. The Quantinova cycling conditions are listed as follows: 95°C for 2 minutes, 95°C for 5 seconds, and 60°C for 5 seconds, for a total of 40 cycles.

We used statistical models to examine the differences in *O. ophidiicola* prevalence and infection intensity. We defined prevalence as the average fraction of individuals with either a body swab or lesion swab testing positive for *O. ophidiicola*. To calculate difference in infection prevalence by species, we used a generalized linear mixed-effects model with a binomial distribution, where our response variable included whether a snake was either positive or negative (0|1) for *O. ophidiicola*, and species as our predictor. Sites (defined above, Fig. S2) were included as a random effect to control for variation observed among sites. We had a very limited number of recaptures (n=33) in the final dataset and examined relationships with and without these repeated measure for an individual. Analyses yielded qualitatively similar results and the final analyses included these 33 recaptures. We used a nearly identical model to above to examine differences in severity but using a linear mixed-effects model with a gaussian distribution and lesion score as our response. To examine difference in infection prevalence and severity by latitude, we used generalized linear model with binomial distribution and no random effects. We included whether a snake was positive or negative (0|1) for *O. ophidiicola* as our response variable and included latitude as an additional additive effect with species as our predictor variable. We also calculated the relationship between infection severity and latitude by using a similar model to above, except we used a linear model with a gaussian distribution and

total lesion score for an individual snake as our response variable. To assess the variability in behavior and infection prevalence, we used a generalized linear mixed effect model with behavior as our predictor variable, the presence of *O. ophidiicola* (0|1) as our response variable and species as a random effect. Our predictor variable (behavior) included five categories: “Basking in open” which included snakes found in a stationary position in sunlight; “Under artificial cover” for snakes that were found beneath coverboards; “Under natural cover”, for snakes that were found beneath logs or rocks; “On the crawl” for snakes found on the move and crossing roads; and “Ambush” which involved stationary snakes that were out of the sun and/or partially concealed.

We also examined the relationship between infection prevalence and lesion severity to determine whether *O. ophidiicola* was not just highly prevalent but also causing more severe disease in populations. We used linear mixed effects model with prevalence for an individual population as our predictor and average lesion severity for that population as our response variable. We included a random effect for species to account for the variation among species. Finally, to determine which species had generally higher susceptibility than would otherwise be expected by their exposure, we extracted the residuals from the relationship between prevalence and severity and calculated the median and 75% quartiles for each species.

Results

From 2020 to 2023, 1057 snakes were sampled for snake fungal disease, including 33 recaptures (Fig 1). Of these snakes 28.4% (n=300) were positive for *O. ophidiicola*, and 404 snakes had lesions. For snakes with lesions, ~58% (n=233) tested positive for *O. ophidiicola*. Prevalence of *O. ophidiicola* varied across species ranging from 0% to 77.8% (Fig. 2, Table 1).

Farancia erythrogramma had the highest prevalence 77.8% (65.1 - 86.8%), followed by *N. taxispilota* with 76.1% (66 - 84%) of individuals infected.

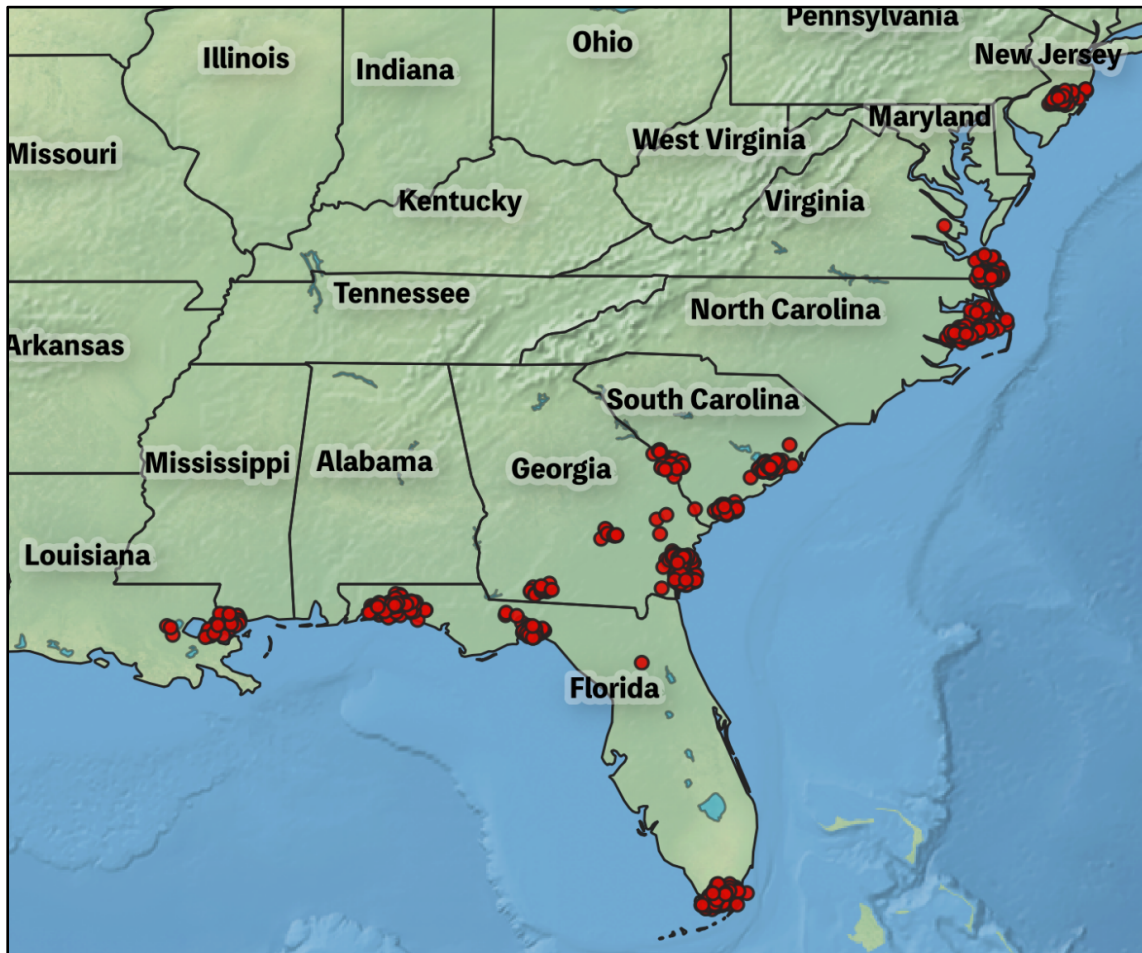


Figure 1: Spatial distribution of individual snakes sampled across the Southeast and Mid-Atlantic regions of the United States. Samples are jittered to obscure exact locations and to show overlapping points.

We also found high prevalence in several other species including *Pituophis melanoleucus*: 47.1% (26.2 - 69.1%), *Nerodia sipedon*: 54.5% (28 - 78.7%), and *N. erythrogaster*: 44% (26.7 - 62.9%). Two crotalid species also had moderate prevalence consistent with other studies (Allender et al., 2020; Haynes et al., 2020; Januszkiewicz et al., 2019), including *Crotalus horridus*: 37.5% (13.3

- 68.9%) and *C. adamanteus*: 36.4% (27.1 - 46.8%). Five species that had not been previously documented with *O. ophidiicola* were found to be positive in this study including, *Agkistrodon conanti*, *Haldea straitula*, *Lampropeltis holbrooki*, *Liodytes. rigida*, and *N. cyclopion*.

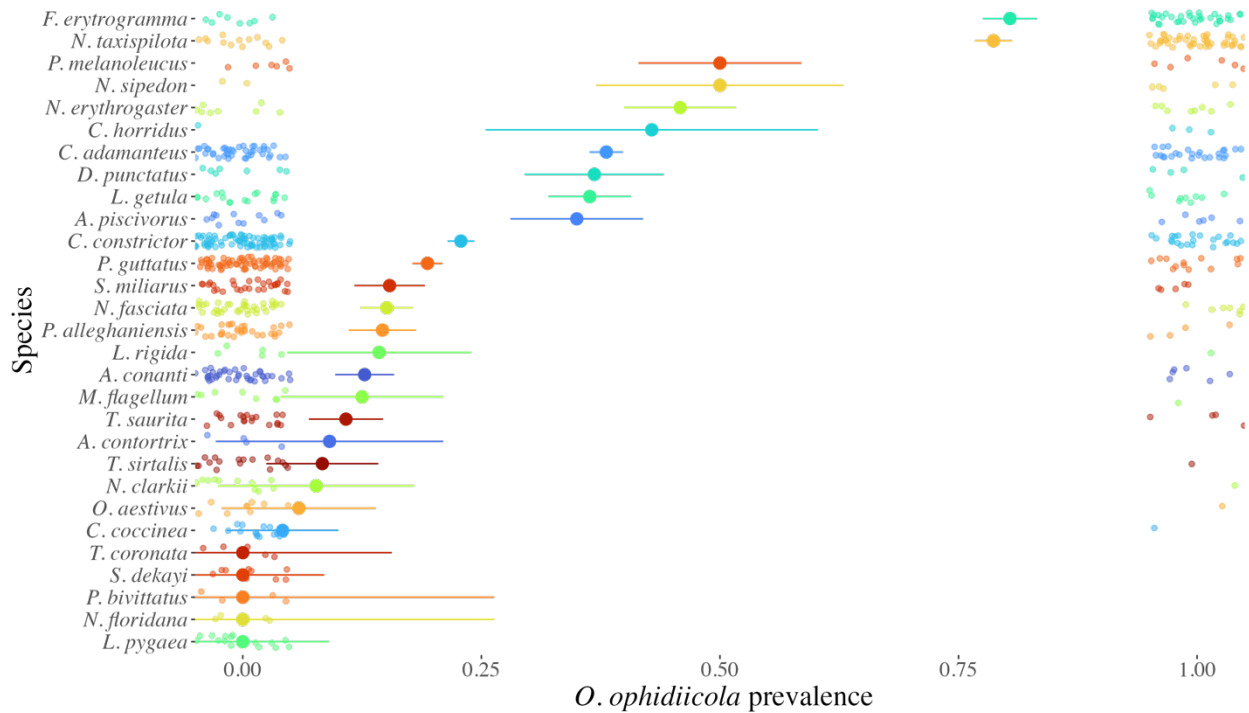


Figure 2: Prevalence of *O. ophidiicola* for different species of snakes across the Southeastern United States. On the x-axis, pathogen prevalence is displayed as a fraction and species is indicated on the y-axis. Each point represents whether an individual snake was positive or negative for *O. ophidiicola* and the larger points indicate the model fitted means to each species with the standard error. This figure is showing results from January to June, Fall sampling was omitted to display comparable results during the epizootic period.

Species	Number of swabbed captures	Prevalence
<i>Agkistrodon conanti</i>	47	12.8% (6 - 25.2%)
<i>Agkistrodon contortrix</i>	11	9% (1.6 - 37.6%)
<i>Agkistrodon piscivorus</i>	32	34.4% (20.4 - 51.7%)
<i>Carphophis amoenus</i>	4	50% (15 - 85%)
<i>Cemophora coccinea</i>	24	4.2% (1 - 20.2%)
<i>Coluber constrictor</i>	114	24.6% (17.6 - 33.3%)
<i>Crotalus adamanteus</i>	88	36.4% (27.1 - 46.8%)

<i>Crotalus horridus</i>	8	37.5% (13.3 - 68.9%)
<i>Diadophis punctatus</i>	19	36.8% (19.1 - 58.9%)
<i>Farancia abacura</i>	11	18.2% (5.2 - 47.7%)
<i>Farancia erytrogramma</i>	54	77.8% (65.1 - 86.8%)
<i>Haldea straitula</i>	7	28.6% (8.2 - 64.1%)
<i>Heterodon platirhinos</i>	2	0% (0 - 65.8%)
<i>Indotyphlops braminus</i>	5	0% (0 - 43.5%)
<i>Lampropeltis elapsoides</i>	6	33.3% (9.7 - 70%)
<i>Lampropeltis getula</i>	37	32.4% (19.6 - 48.5%)
<i>Lampropeltis holbrooki</i>	3	66.7% (20.8- 93.9%)
<i>Liodytes pygaea</i>	15	0% (0 - 20.4%)
<i>Liodytes rigida</i>	15	13.3% (3.7 - 37.8%)
<i>Masticophis flagellum</i>	17	11.7% (3.3 - 34.3%)
<i>Micrurus fulvius</i>	6	0% (0 - 39%)
<i>Nerodia clarkii</i>	13	7.7% (1.4 - 33.3%)
<i>Nerodia cyclopion</i>	2	50% (10% - 90.6%)
<i>Nerodia erythrogaster</i>	25	44% (26.7 - 62.9%)
<i>Nerodia fasciata</i>	53	15.1% (7.9 - 27.1%)
<i>Nerodia floridiana</i>	4	0% (0 - 49%)
<i>Nerodia rhombifer</i>	1	100% (20.7 - 100%)
<i>Nerodia sipedon</i>	11	54.5% (28 - 78.7%)
<i>Nerodia taxispilota</i>	84	76.1% (66 - 84%)
<i>Opheodrys aestivus</i>	21	4.8% (1 - 22.7%)
<i>Pantherophis alleghaniensis</i>	42	14.3% (6.7 - 27.9%)
<i>Pantherophis guttatus</i>	94	19.1% (12.4 - 28.2%)
<i>Pantherophis spiloides</i>	3	0% (0 - 56.2%)
<i>Pituophis melanoleucus</i>	17	47.1% (26.2 - 69.1%)
<i>Python bivittatus</i>	6	0% (0 - 39%)
<i>Sistrurus miliarius</i>	57	19.3% (11.1 - 31.3%)
<i>Storeria dekayi</i>	17	0% (0 - 18.4%)
<i>Storeria occipitomaculata</i>	5	20% (3.6 - 62.5%)
<i>Storeria victa</i>	2	0% (0 - 65.8%)
<i>Tantilla coronota</i>	8	0% (0 - 32.4%)
<i>Thamnophis proximus</i>	2	0% (0 - 65.8%)
<i>Thamnophis saurita</i>	38	10.5% (4.2 - 24.1%)
<i>Thamnophis sirtalis</i>	27	7.4% (2.1% - 23.4%)
Total species: 44	Total swabbed snakes: 1057	

Table 1: Total number of swabbed snakes and prevalence across the Southeastern United States from Spring and Fall sampling (n=1057), including recaptures (n=33). 95% binomial confidence intervals are shown with prevalence.

Analysis of total lesion severity revealed that *F. erytrogramma*, *C. horridus*, and *C. adamanteus* had the highest lesion severity (Fig. 3). Some species that had high infection prevalence had low lesion severity including *Diadophis punctatus* (prevalence = 36.8% (19.1 -

58.9%)); *Agkistrodon piscivorus* (prevalence = 34.4% (20.4 - 51.7%)), and *Pantherophis guttatus* (prevalence = 19.1% (12.4 - 28.2%)).

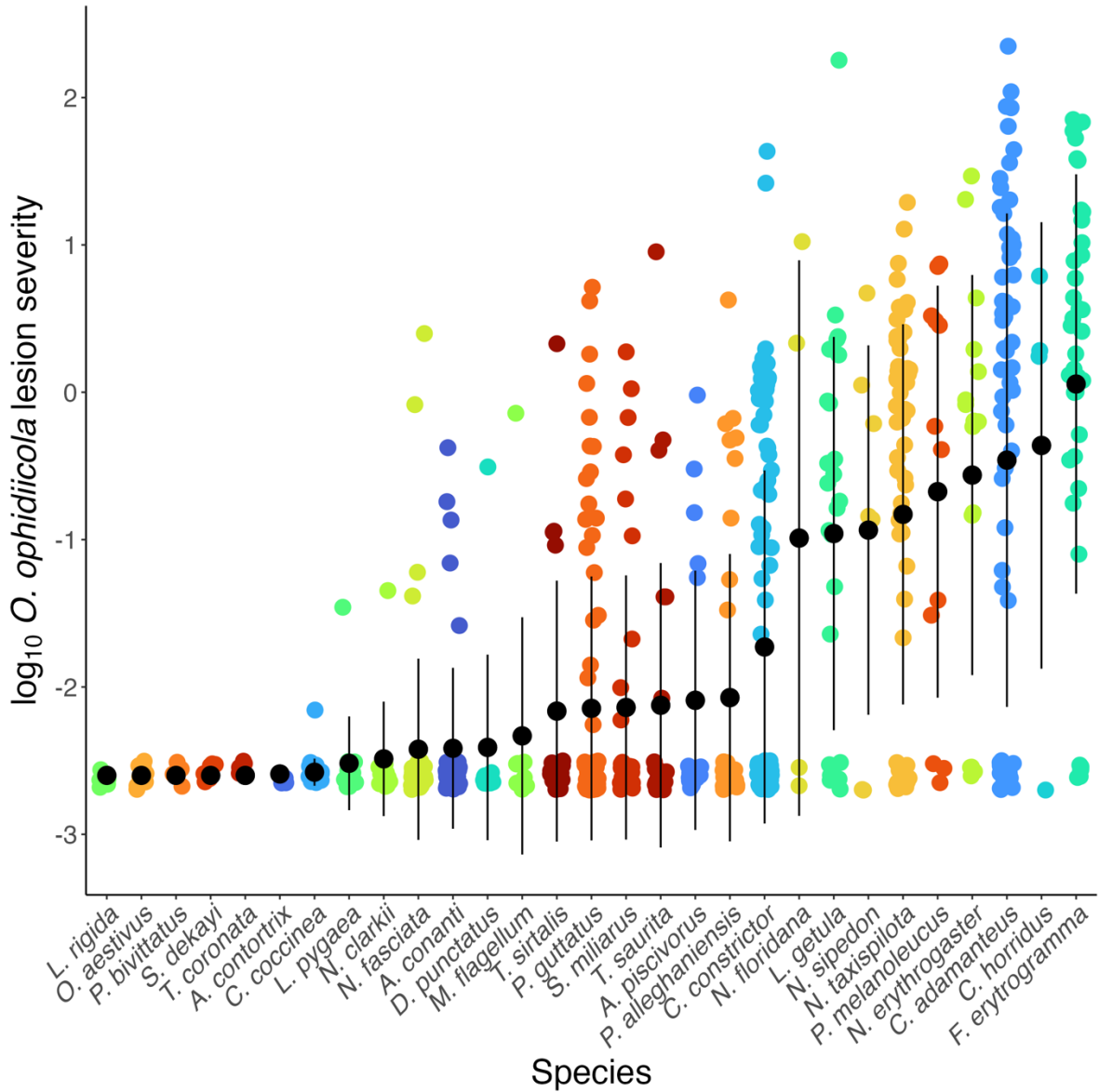


Figure 3: *Ophidiomyces ophidiicola* lesion severity across species. Lesions surface area was multiplied by the individual lesion score and summed for each individual snake. Mean lesion severity for species is indicated by the black circles with standard error bars. Colored points are individuals sampled; species are differentiated by color.

Examination of the relationship between infection prevalence and latitude revealed a strong positive relationship (coeff: 0.156 ± 0.03 , p-value < 0.001), such that species sampled

across the range of sites had higher prevalence at more northerly sites compared sites further south (Fig 4.). *Farancia erythrogramma* had a 15.1% decrease in prevalence from Back Bay, VA (n=31) to Southeastern Georgia (n=7). *Nerodia fasciata* experienced a 23.3% decline in prevalence from Francis Marion, SC (n=5) to the Everglades, FL (n=9). *Pantherophis guttatus* experienced a 23.1% decline in prevalence from the Pine Barrens, NJ (n=49), to the Everglades, FL (n=15). Back Bay, VA had some of the highest overall prevalence values with 65% of animals testing positive, followed by Parris Island, SC of 38.6%, and then Savannah River, GA at 32.6%. Everglades National Park had some of the lowest prevalence with only 6.1% of individuals infected.

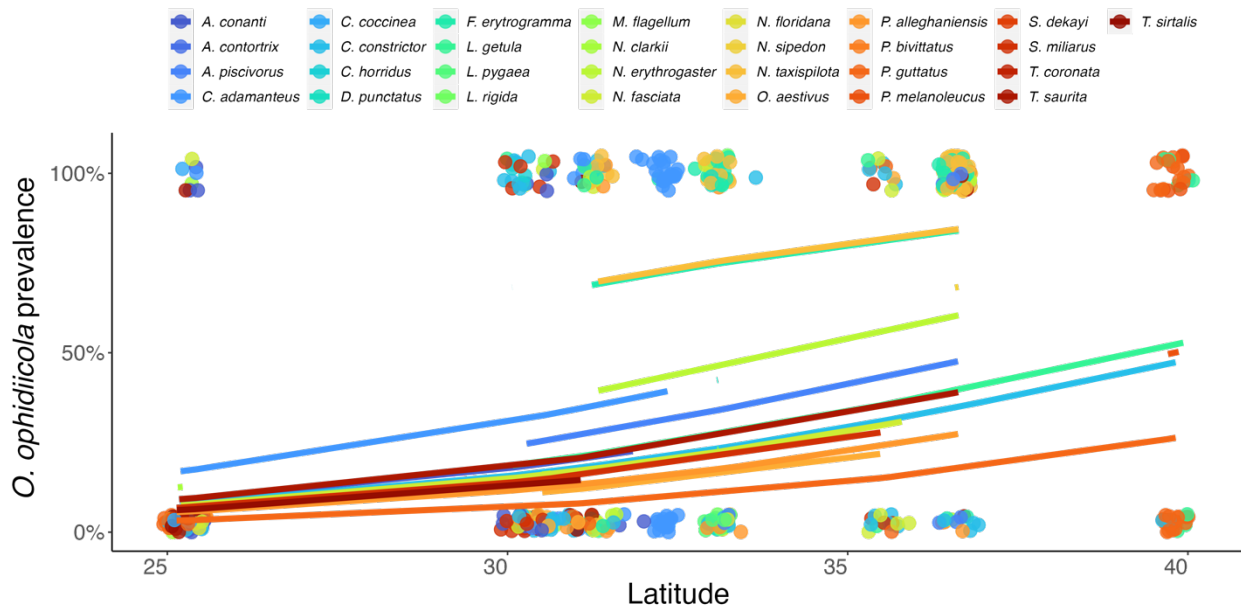


Figure 4: Relationship between pathogen prevalence and latitude. The x-axis shows individual snake latitude of capture (25: South Florida; 30: Florida Panhandle; 40: New Jersey). The y-axis is showing pathogen prevalence for the fraction of each species that was positive (100% positive, 0% negative). This data was restricted to samples collected in the spring, and sampling was adjusted to capture snakes just following winter emergence. Points on the graph are the raw data, indicating whether a snake was positive or negative. The lines are the predicted model fits and we have restricted the lines to encompass where we have sampled that species. Lines show output from a generalized linear model with binomial distribution (Latitude coeff: 0.15 ± 0.03 p-value < 0.001).

We found a similar relationship between \log_{10} lesion severity and latitude (Fig. S3), (coeff: 0.05 ± 0.01 , p-value <0.001). Finally, comparison of \log_{10} fungal loads measured as fg of DNA estimated using qPCR, revealed that *Lampropeltis getula* had the highest mean \log_{10} loads ($-0.522\text{fg}/\mu\text{L}$), followed by *P. guttatus* ($-1.021\text{ fg}/\mu\text{L}$), and *N. taxispilota* ($-1.227\text{ fg}/\mu\text{L}$) (Fig. 5).

Examination of the relationship between infection prevalence and severity revealed a positive relationship (Fig 6; coeff: 2.93 ± 0.36 , $p < 0.001$). To assess whether some individuals experienced higher or lower disease severity given their infection prevalence, we extracted the residuals of the relationship between prevalence and lesion severity (regression shown in Fig 6) for comparison among species. This analysis revealed that in some species, lesion severity is higher than expected based on their prevalence, possibly suggesting increased infection susceptibility in these species/populations, whereas in other species, severity was relatively low given their exposure rates (Fig 7). Species that had higher severity than would be otherwise predicted based on their exposure included *C. horridus*, *C. adamanteus*, and *L. getula*.

We used multiple surveying methods to get a more representative sample of snakes on the landscape, which included visual encounter surveys, driving transects, and the use of coverboards. Our capture methods were analyzed to assess for bias in infected and non-infected snakes. We found that snakes captured while basking had a significantly higher chance of being detected positive than when compared to snakes using artificial cover (Fig S4; coeff: 0.792 ± 0.47 , $p < 0.01$). While infection prevalence of snakes that were captured on the move was significantly lower (Fig S4; coeff: 0.506 ± 0.23 , $p < 0.05$.), we found no significant difference between snakes using artificial coverboards versus natural cover (Fig S4; coeff: 0.586 ± 0.38 , $p = 0.131$).

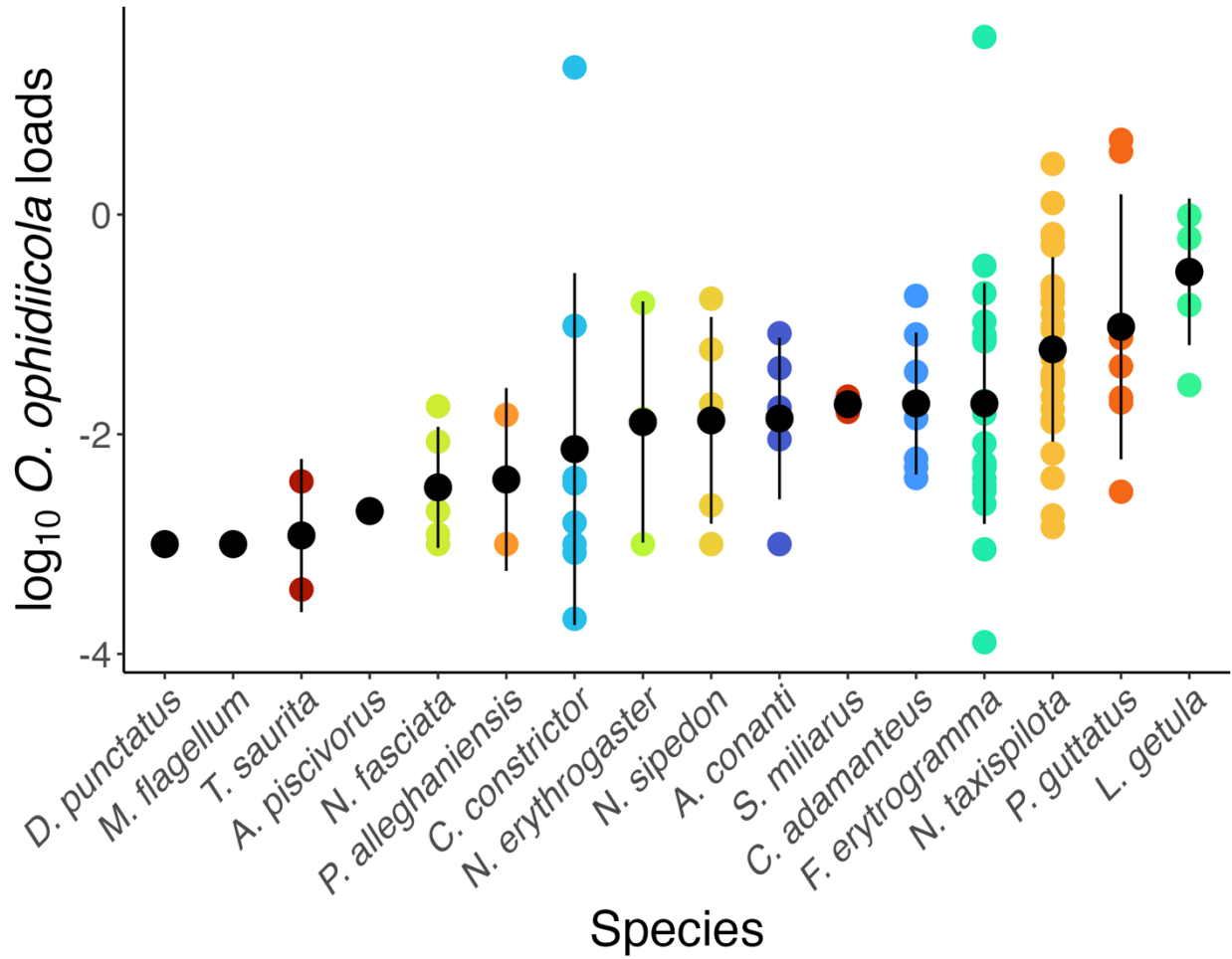


Figure 5: *Ophidiomyces ophidiicola* fungal loads across sampled species. Species is represented on the x-axis and log₁₀ fungal loads measured as fg/μL DNA using qPCR on our standardized swab sample on the y-axis. Colored points are showing individuals sampled, color differentiates species. Mean loads are indicated by the black circles with standard error bars. Species with no colored icons indicate species that had positive lesion swabs, but no positive body swabs.

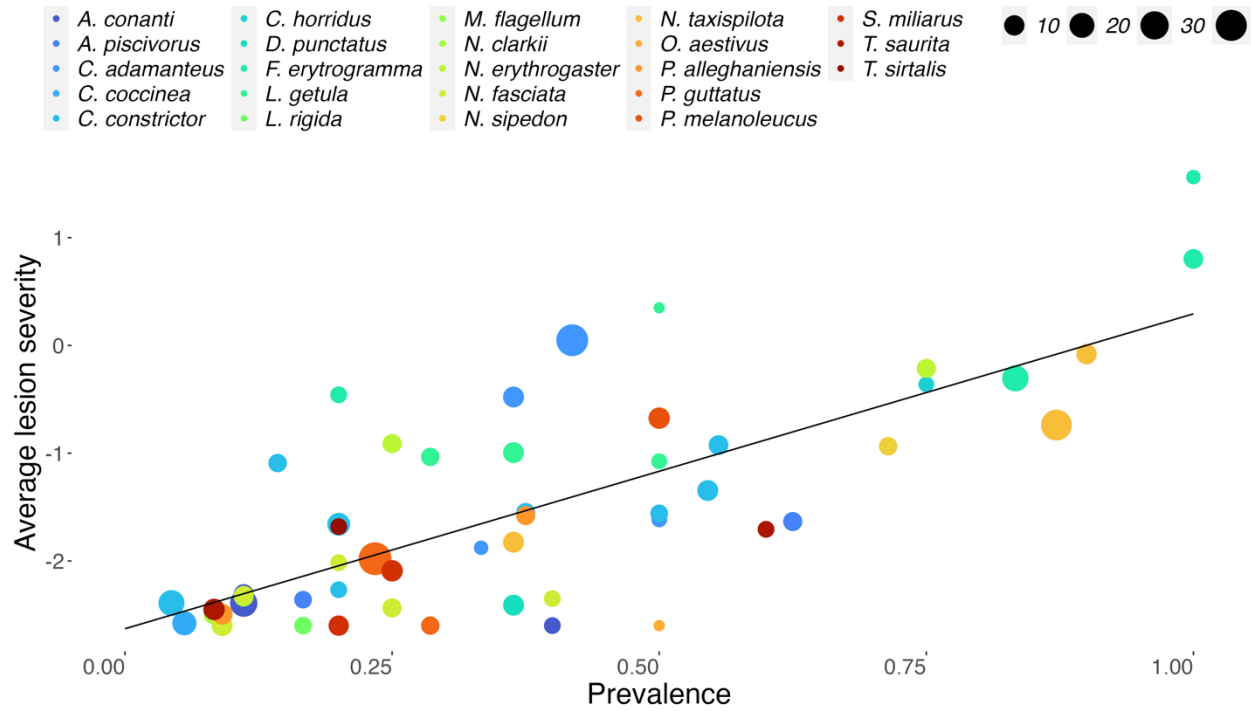


Figure 6: Linear relationship between prevalence percentage and lesion severity suggests that *O. ophidiicola* is not only highly prevalent in some populations but also causes higher pathology. Prevalence of *O. ophidiicola* is displayed on the x-axis and \log_{10} lesion severity on the y-axis. Scattered colored points indicate different species (Linear model, coefficient: 2.93 ± 0.36 $p < 0.001$).

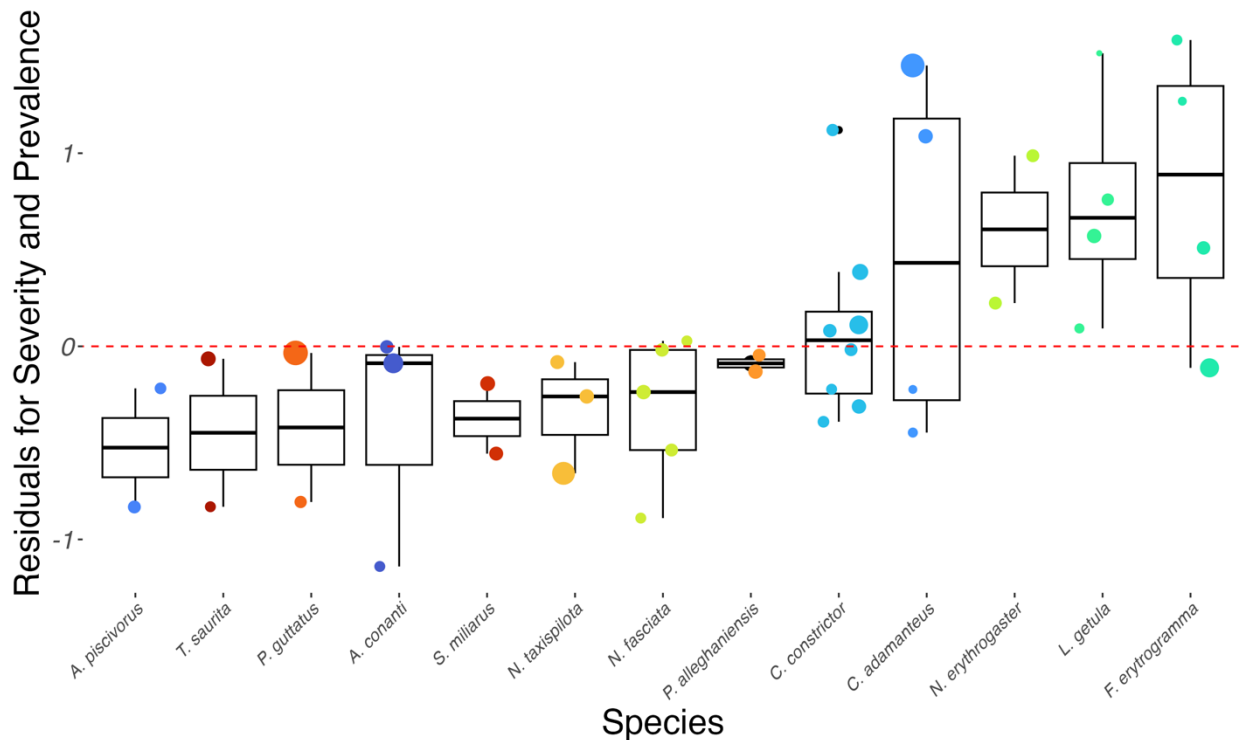


Figure 7: Residuals from the regression shown in Fig 7 comparing both severity and species prevalence. Each point is a separate population with color indicating species. Point size indicates sample size. The dashed horizontal line indicates a deviation from the regression of 0. For species above the x-intercept, severity is relatively high given their exposure/prevalence (e.g. given an equal exposure this indicates they may possess a higher susceptibility than other species).

Discussion

We found that a variety of snake species were infected with *O. ophidiicola* in the wild, and these infections vary among species as well as across space. We found several species with high prevalence (44 - 77.8%) and high severity. In addition, we found that within species infections were more prevalent and severe at more northern sites. Collectively, our results provide a comprehensive assessment of SFD across a broad community of snakes and indicate which species may be at greatest risk from the disease.

Species that had high pathogen prevalence and disease severity include, *F. erythrogramma*, *Nerodia sp.*, *P. melanoleucus*, *C. horridus*, and *C. adamanteus*. The elevated disease in these species could be a result of innate susceptibility, environmental factors, or behavioral differences. *Farancia erythrogramma* and *Nerodia* are aquatic species that spend a great portion of their life history in moist environments, which generally favors fungal growth (Lorch et al., 2016; McKenzie et al., 2019). *Nerodia taxispilota* has also been previously reported to have high prevalence of *O. ophidiicola* (Haskins et al., 2023; Haynes et al., 2020). This species frequently basks in the same areas as other species (Mills et al., 1995), creating potential hotspots for pathogen transmission. *Pituophis melanoleucus*, another highly infected species, and *F. erythrogramma* are both adapted to burrowing through sandy soils (Burger & Zappalorti, 1991; Richmond, 1945). This could result in repeated and numerous abrasions on their face and anterior regions, which has been shown to be essential to *O. ophidiicola* invading the epidermal tissue layer and establishing infections (Lorch et al., 2016). Rattlesnakes such as *C. horridus* and *C. adamanteus* are ambush predators that will sit and remain motionless for days until a potential prey item comes within striking distance. This stationary behavior requires the snake's ventral scales to be in contact with the ground for longer durations compared to other species, increasing environmental pathogen exposure which may exacerbate infection. *Crotalus adamanteus* will also take refuge in gopher tortoise burrows when available, that other species (i.e. *Drymarchon couperi*) with high *O. ophidiicola* prevalence also utilize (Chandler et al., 2019).

Notable disparities between fungal loads and severity were observed for a few species, such as *F. erythrogramma* that maintained relatively low fungal loads but the highest lesion severities. Increased ecdysis (skin molting) has been observed as a response to infections with *O.*

ophidiicola and could mean that some of the most infected individuals are repeatedly shedding their skin, reducing surface pathogen loads (Lorch et al., 2015). *Agkistrodon piscivorus* and *P. guttatus* had only moderate lesion severity but experienced high fungal loads. Which could be due to experience less severe pathology, as a species becomes infected but faces minimal physiological impacts. These differences could indicate that *A. piscivorus* and *P. guttatus* are more tolerant and thus may serve as amplifying hosts in the community as they may spread the pathogen, but do not succumb to severe infection. Other explanations of these results may include the ophiophagous (snake) diets of some species (*L. getula* and *A. piscivorus*), which would put them into contact with a high number of other snakes and incidentally result in high pathogen exposure (Godley et al., 2017; Vincent et al., 2004). Unlike *A. piscivorus*, *L. getula* had moderate lesion severity, which may indicate increased infection susceptibility in the latter species. Collectively, these results provide some of the first insights into species infection susceptibility, resistance, tolerance, and transmission of SFD, which should be clarified with future experimental work that seeks to understand the relative contributions of each factor to SFD ecology.

We found a strong latitudinal effect of prevalence and disease severity for SFD. Snakes at more northern sites were more likely to be infected with SFD and suffer from more severe infections. The latitudinal effect of higher prevalence and increased severity may be due to shifts in seasonal brumation behavior. For snakes at higher latitudes, winter dormancy duration is longer (Aleksiuk & Stewart, 1971). Longer brumation durations result in reduced ecdysis frequency and can limit immunological defenses in ectotherms (Harkewicz, 2001; Schilliger et al., 2023). Additionally, many snakes share their overwintering locations with other individuals and species which appears to be more pronounced in northern regions (Macartney et al., 1989;

Parker & Brown, 1973; Shine & Mason, 2004). These overwintering sites may be highly selective to snakes at more northern latitudes and snakes in these areas appear to frequently reuse the same den in consecutive years compared to snakes further south (Hirth, 1966). Therefore, increased snake densities at overwintering sites may serve as a source of annual environmental pathogen exposure. Elevational changes may also result in similar effects, resulting in potential higher disease severity. While in more southern latitudes, snakes have less selective pressure while choosing an overwinter hibernaculum and densities are lower per site (Rudolph et al., 2007). This could contribute to why we see lower prevalence in more southern latitudes. We generally observed *Nerodia* species with high to moderate prevalence, except for *N. clarkii* which had lower prevalence, 7.7% (1.4 - 33.3%). This may be due to their range being restricted to southern latitudes of Florida and the Gulf. Interestingly, much of the research on SFD has occurred at more southern latitudes, where snake diversity is higher, which may contribute to the general lack of consensus on whether SFD is threat to North American snake populations. Our results suggest increased monitoring for populations at northern latitudes and potentially high elevation is warranted. This may be particularly relevant for threatened montane endemic species such as *Crotalus pricei* and *Crotalus willardi spp.* (Davis et al., 2015).

We calculated lesion severity and found that *F. erytrogramma* had the most severe lesions followed by *C. horridus*, *C. adamanteus*, *N. erythrogaster*, *P. melanoleucus*, *N. taxispilota*, *N. sipedon*, and *L. getula*. Two introduced species were sampled during this study, the Burmese python (*Python bivittatus*) and the Brahminy blind snake (*Indotyphlops braminus*). Interestingly, neither of these species tested positive for *O. ophidiicola*, despite individuals having lesions.

We found a positive relationship between infection prevalence and severity which provides evidence that this pathogen is not only highly prevalent in some populations, but these populations also have more severe disease, suggesting that SFD is a threat to snake populations in Eastern North America. Further examination of the residuals from this relationship indicated that some species face more severe infections than might otherwise be predicted given their exposure (e.g. *L. getula*, *N. erythrogaster*, and *C. adamanteus*). Species that experience higher severity despite lower prevalence may be generally more susceptible to infection and thus could be vulnerable to population declines from *O. ophidiicola*.

To ensure the use of coverboards were not inadvertently increasing *O. ophidiicola* prevalence in captured snakes, we compared behavioral analysis and found no support that snakes were more likely be infected that were captured under these boards. We found that snakes were significantly less likely to be infected when using cover or on the crawl when compared to snakes basking in the open, with no significant difference between natural and artificial cover. This basking behavior may be attributed to behavioral fever, which has been suggested in other studies (McKenzie et al., 2021).

Our findings reveal that there is notable variation in snake fungal disease infection prevalence, fungal loads, and disease severity along a latitudinal gradient and across species. This understanding of spatial and species variability provides insights into which species may be facing the greatest impacts from this pathogen. Ultimately, this work can help to advance knowledge of the ecology and impacts of this disease contributing to the improvement of conservation efforts for snake populations.

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Conclusions

More broadly, our results highlight the importance of considering species variability when studying multi-host pathogens (Kilpatrick et al., 2006; Langwig et al., 2016; Woolhouse et al., 2001), as we found significant variation in responses among species. In addition, it is crucial to consider larger spatial scales as we found that latitude significantly influenced prevalence and severity. These findings necessitate more research on SFD in more northern latitudes as impacts to affected species is likely to be greatest in these regions. This research has the potential to contribute to future investigations on multi-host pathogens and establishes a foundation that advances our understanding of the ecological dynamics for this disease.

Our objective was to assess the impacts of SFD over a large geographic scale to capture spatial and host variation. We aimed to characterize the ecology of SFD by analyzing prevalence, severity of infection, and pathogen loads. By jointly considering these metrics, we can determine which species face the greatest risk from SFD infections.

By targeting species with the greatest threat of population decline, we can preserve biodiversity by allocating resources more effectively and reduce the probability of investing valuable resources to species that are low risk (Langwig et al., 2015). Creating a widespread treatment strategy or eradicating SFD is unrealistic, as it is often challenging and requires extensive resources with an established pathogen (Delahay et al., 2009; Langwig et al., 2015; Woodhams et al., 2011). Therefore, targeted strategies for species with elevated threats and/or managing other pressures species face is more feasible for species at higher risks of decline due to SFD (Bosch et al., 2015; Hoyt et al., 2023; Langwig et al., 2015).

We found that several species faced greater impacts from *O. ophidiicola* and likely the greatest risk of population decline. *Farancia erythrogramma*, *C. horridus*, *C. adamanteus*, *N.*

erythrogaster, *P. melanoleucus*, *N. taxispilota*, *N. sipedon*, and *L. getula* may face potential conservation threats from this disease given high pathogen prevalence and high disease severity. We also found that infection prevalence and severity varied among species and across latitudes. Most species had relatively low prevalence and populations at more northern latitudes had higher rates of infection. Additionally, we found variability in fungal loads that differed from prevalence and severity potentially indicating differing susceptibilities among species. We found that *F. erythrogramma*, *N. erythrogaster*, *L. getula* and *C. adamanteus* had high disease severity given their exposure rates, possibly suggesting high susceptibility.

Concerningly, *F. erythrogramma* has limited protection and knowledge of population trends, life history, and ecology. A related subspecies, the South Florida Rainbow snake, *F. erythrogramma seminola* (Neill, 1964), is now listed as extinct, which could suggest that *F. erythrogramma* may already be facing population pressures. Therefore, increased monitoring to determine population trends should be developed to understand whether this species is facing declines. For habitat specialists such as *F. erythrogramma*, *P. melanoleucus*, and *C. adamanteus*, special consideration should be taken to not destroy wetlands and pine forests these species depend on (Burger & Zappalorti, 1988; Miller et al., 2012; Neill, 1964; Waldron et al., 2006). Although more studied than *F. erythrogramma*, *P. melanoleucus* and *C. adamanteus* are both facing other threats and found to be of greater risk of decline from SFD (Burger & Zappalorti, 2016; Means, 2009; Waldron et al., 2006). *Pituophis melanoleucus* is a species of concern across their range, with the subspecies, *P. m. lodingi*, being federally threatened (Baxley & Qualls, 2009; Burger & Zappalorti, 2016). A similarly related species, *P. ruthveni* is also federally threatened and has experienced significant population declines (Rudolph et al., 2006). With

evidence of declines in this genus, its paramount to consider the added pressures that SFD may be contributing.

For future research, those should investigate the infection variation among species at different elevation as there is likely a similar relationship to what we observed with latitude. All our sites were near sea level but investigation of populations at different elevations will also be critical in determining risk to other species populations. It is also of importance to apply decontamination practices to reduce the risk of pathogen transfer to populations experiencing reduced infections, as novel strains could leave disastrous effects.

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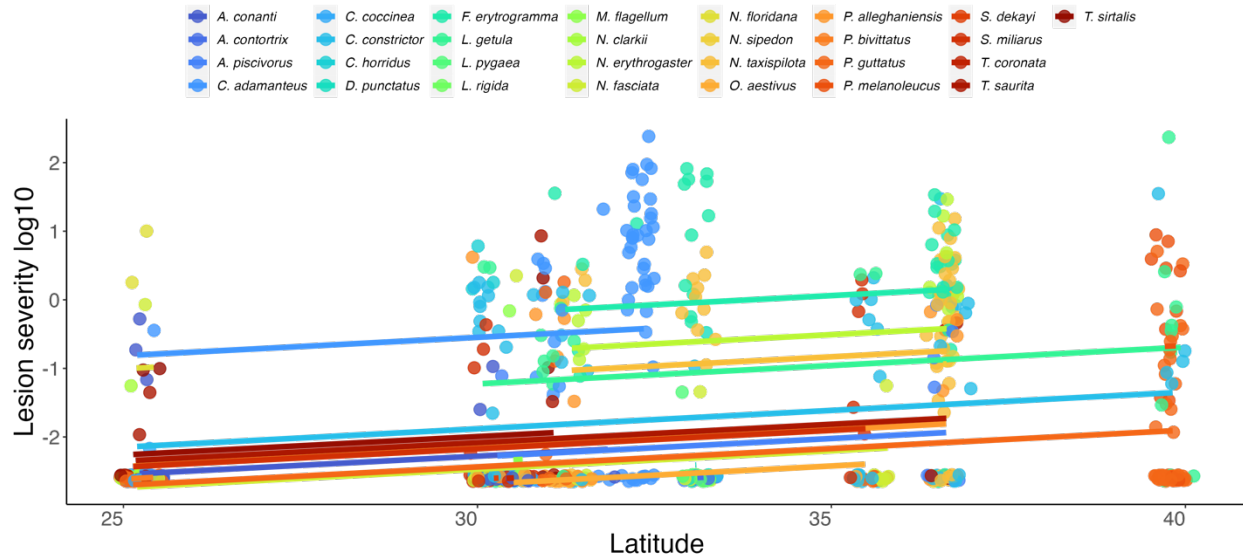
Supplemental Figures



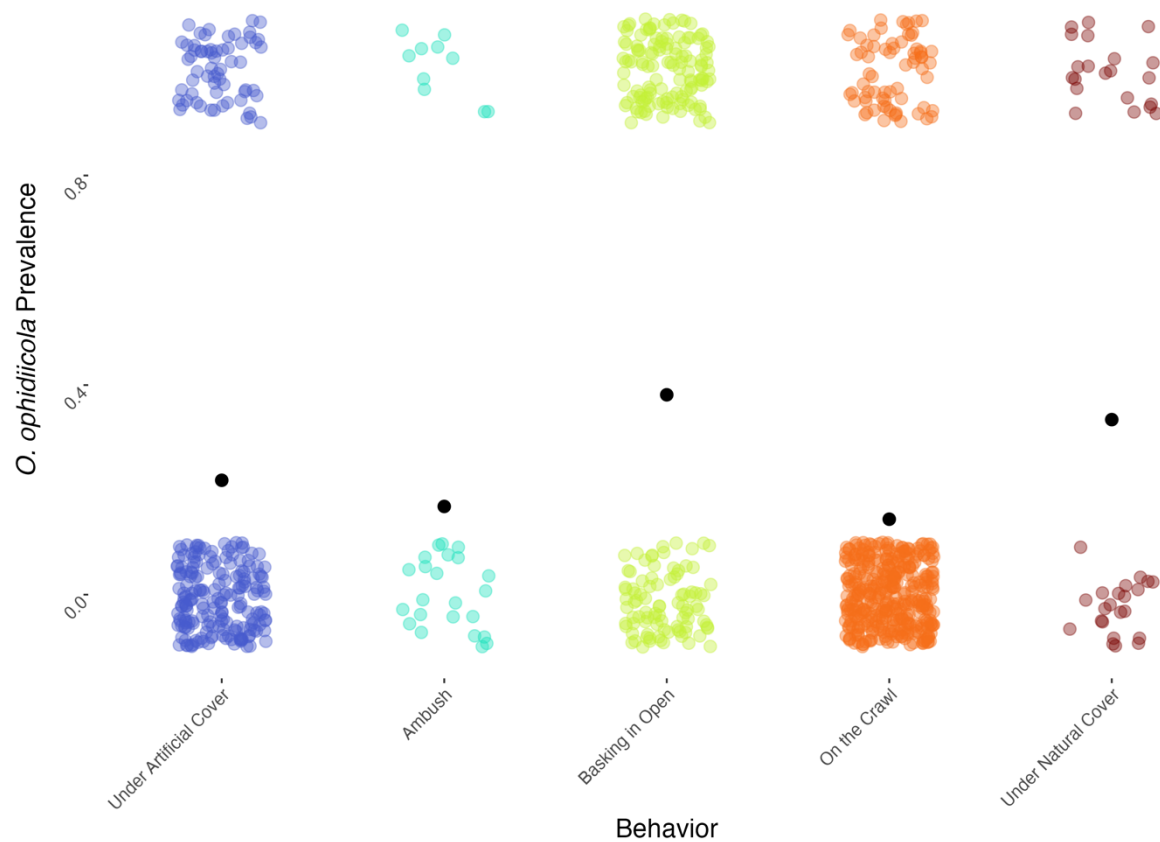
Supplemental Figure 1: Examples of lesion scoring variation, these images display the representation of lesion severity seen while sampling snakes in the field. Proceeding from left to right, top to bottom. Lesion 1) Light inflammation and disrupted scales on a *C. adamanteus*. Lesion 2) Crust and moderate inflammation on a *F. erythrogramma*. Lesion 3) Scabbed ulcer with onset necrosis on a *F. erythrogramma*. Lesion 4) Exposed ulcer on a *F. erythrogramma*. Lesion 5) Critical necrosis with infection penetrating beyond the epidermis on a *P. melanoleucus*, photo by Christopher Jeitner.



Supplemental Figure 2: Fourteen sampling sites spanning the Southeastern and Mid-Atlantic regions. Pine Barrens, NJ; First Landing, VA; Back Bay, VA/NC; Alligator River, NC; Hyde County, NC; Francis Marion region, SC; Parris Island, SC; Savannah River, SC/GA; Southeastern GA; Jekyll Island, GA; Wakulla River drainage, FL; Eglin AFB, FL, Everglades National Park, FL; Pearl River, LA. Data points do not convey sample site size.



Supplemental Figure 3: Relationship between latitude and lesion severity. Linear model relationship between latitude and lesion severity across 29 species. Species with sample size less than 3 individuals per site, had those sites omitted from this model (Latitude coeff: 0.05 ± 0.01 , $p < 0.005$).



Supplemental Figure 4: Prevalence of snakes captured during different behaviors. Black points show the predicted mean prevalence for observed snake behavior during capture. Deployed coverboards were categorized as “Under Artificial Cover. Using “Under Artificial Cover” as a reference, “Basking in Open” was significantly higher (Coeff: 0.792 ± 0.47 , $p < 0.01$) and “On the crawl” was significantly lower (Fig S4; coeff: 0.506 ± 0.23 , $p < 0.05$).