ABSTRACT

OVEN, EMILY C. Broad and Fine-Scale Distributions of Macroparasitism and Ophidiomycosis in North American Snakes (Under the direction of Dr. Skylar Hopkins).

Parasites are a large component of global animal biodiversity, and they contribute to ecosystem structure and function. Additionally, some parasite species cause population declines in sensitive wildlife species and are targeted by disease control efforts. Despite their importance, patterns of parasitism and disease are poorly studied in many host taxa, making it difficult to conserve hosts and parasites. Data limitations for parasitism and disease are especially notable for snake hosts, which are cryptic and difficult to survey. Therefore, I provide the first broadscale biogeographical analysis of endoparasitic helminths and pentastomes in native snakes of the United States and Canada and the largest surveillance study to date of an emerging snake fungal disease (ophidiomycosis) in small-bodied snake species.

In Chapter 1, I conducted a systematic literature review of macroparasites that infect snakes using existing bibliographies for North America and additional database searches. During full-text analysis, I recorded information regarding snake hosts (e.g., species, size), parasites (e.g., species, prevalence of infection), and biogeography (e.g., region, habitat type). I determined that only 45% of native snake species in the U.S. and Canada have been included in parasitological studies, and snake species were more likely to have at least one parasitological study if they were relatively large and had a broad latitudinal range. Among studied snake species, the number of observed parasite species increased with the number of studies and the number of hosts sampled. After controlling for sampling effort and host phylogenetic relatedness, estimated parasite richness increased with snake size and latitudinal range and was higher for snakes with amphibian or fish diets. Overall, I documented at least 139 unique species of helminths, acanthocephalans, and pentastomes, but sampling the remaining snake host species

in the United States and Canada and increasing sampling effort for previously studied snake host species would greatly increase described parasite biodiversity.

In Chapter 2, I surveyed small-bodied snake species in natural areas and backyards in the North Carolina Piedmont for ophidiomycosis. Using a combination of area-constrained searches and opportunistic visual encounter surveys, I captured all encountered terrestrial colubrid snakes and collected skin swabs that were later tested for the presence of *Ophidiomyces ophidiicola* DNA using qPCR. I found that small-bodied snakes dominated terrestrial colubrid communities (96% of plots and 91% total), where Eastern worm snakes (*Carphophis amoenus*) were especially abundant. Among all 123 surveyed snakes, 8% were positive for *O. ophidiicola*, and all ten of those individuals showed clinical signs of ophidiomycosis. For the first time in the literature, I reported the clinical signs observed on *C. amoenus*, which included open ulcers and dry crusts that were usually located on the dorsal body. Apparent ophidiomycosis was more likely to be observed in *C. amoenus* in the spring (i.e., before mid-May) and in relatively large individuals. Finally, among sites where we encountered snakes, we detected ophidiomycosis in 5/11 parks and 0/5 backyards when including small-bodied and large-bodied snake species, whereas we only would have documented cases in 2/11 parks if we had limited our study to large-bodied snake species. Therefore, I suggest that while it requires substantial effort to survey and diagnose small-bodied snakes, these species may be useful targets for ophidiomycosis surveillance because they provide larger and more fine-scaled host sample sizes.

Overall, this work improves our understanding of the distribution of parasites and disease in North American snakes, providing critical baseline data for conservation efforts and pinpointing important gaps for future research.

Broad and Fine-Scale Distributions of Macroparasitism and Ophidiomycosis in North American Snakes

by
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DEDICATION

To my parents, who have supported me throughout my journey. Thank you for believing in me and trusting that no matter how far I am from you, I am okay.

To my partner, Jack, who has endured this journey with me. Thank you for your constant love and support. You are my rock, my joy, and my everything person.

BIOGRAPHY

Emily Oven was born and raised in the Pacific Northwest, which inherently comes with a love for wildlife and the outdoors. She spent her undergraduate years at the University of Washington where her love for fisheries ecology developed and grew, as she spent many terms studying Puget Sound fishes and many summers sampling salmon on the Oregon Coast. It was at the University of Washington that she was introduced to parasites by her undergraduate mentor Dr. Chelsea Wood, who helped open her eyes to a whole new wormy world inside of her beloved fish. Parasites have bridged her research interests in fisheries ecology and herpetology and led her to NC State University, where her knowledge of terrestrial ecology and love for snakes grew under the guidance of Dr. Hopkins.

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CHAPTER 1

Parasite richness is highest in large and aquatic snake species in the United States and Canada

Introduction

Parasites are ubiquitous, important, and vulnerable species in ecosystems, and their presence, richness, and abundance may be useful indicators of host and ecosystem health (Dobson & Hudson 1992, Lafferty et al. 2006, Lafferty 2008, Dunne et al. 2013, McQuaid & Britton 2015). However, most macroparasite species have not been discovered and described, and for most described macroparasites, little to nothing is known about their life cycles and ecology (Dobson et al. 2008, Wood & Johnson 2015). For example, an estimated 100,000–350,000 global helminth species infect vertebrates, and 80–95% of those species are still unknown to science (Carlson et al. 2020). These knowledge gaps are most prominent among reptile and amphibian hosts (Carlson et al. 2020), and there are no up-to-date, central repositories for known parasites of amphibians or reptiles. By describing what is currently known about parasite biogeography and pinpointing the knowledge gaps, we can pave the way for targeted studies to advance host and parasite conservation (Carlson & Hopkins et al. 2020). Therefore, we present the first broadscale biogeographical analysis of endoparasitic helminths, acanthocephalans, and pentastomes in native snakes of the United States and Canada.

There are ~175 species of snakes in the United States and Canada, which represent five snake families: Boidae, Colubridae, Elapidae, Leptotyphlopidae, and Viperidae (Crother et al. 2003, Ernst and Ernst 2006, Uetz et al. 2022: The Reptile Database). These snake species are spread across diverse habitats (e.g., deserts, alpine ecosystems, swamps) and have diverse prey and predators, and thus likely host diverse parasite species. However, snakes are cryptic and

difficult to research in adequate sample sizes, and relatively few studies have described parasite communities by surveying living snakes or snake feces (Colwell Jr. 1999, Davis et al. 2012, Uhrig et al. 2015, Yabsley et al. 2015, McAllister & Bursey 2016, McAllister et al. 2017). Many other studies have used vehicle-killed snakes to increase sample sizes (Foster et al. 2000, Davis et al. 2016, Miller 2017, Flowers & Beane 2021). Most of these papers were previously summarized in a bibliography that listed all known parasites of snakes in the United States and Canada (Ernst & Ernst 2006), which was updated in 2020 (Udchitz 2020). However, the lists were neither developed into accessible databases, nor were they used in any sort of ecological analysis of parasitism in snakes. In fact, snakes are one of the only vertebrate host groups that does not have a database of helminth parasites similar to FishPEST or the Global Mammal Database (Strona & Lafferty 2012, Stephens et al. 2017). Therefore, even the most basic questions about snake parasite biogeography, like which host species or regions have the highest parasite biodiversity, remain unanswered.

In studies of other vertebrate host taxa, study effort has been biased towards particular host species (Randhawa & Poulin 2019, Albery et al. 2022). For example, elasmobranch host species with larger latitudinal ranges had earlier average dates of discovery and description, and thus their tapeworms were also discovered earlier (Randhawa et al. 2015, Randhawa & Poulin 2019). More broadly, parasitological and ecological studies may be biased towards species that are widespread and abundant and thus relatively easy to sample in large numbers (e.g., rodents; Mihalca et al. 2012) or species that are large or otherwise charismatic (e.g., felines and cetaceans; Bjork et al. 2000, Romero et al. 2014). Therefore, we predicted that study effort would be highest for larger, more charismatic snake species and regions with high snake species richness (i.e., the Southwestern and Southeastern U.S.) and that study effort would

affect observed parasite richness–a widespread phenomenon in community ecology, including studies of host–parasite communities (Walther et al. 1995, Nunn et al. 2003).

After accounting for sampling effort, characteristics of parasites, hosts, and their environments may explain variation in estimated parasite richness among host species (Hechinger & Lafferty 2005, Ezenwa et al. 2006, Campião et al. 2015, Johnson et al. 2016). For example, larger host species may have more parasite species because they have larger ranges and encounter a broader suite of parasites (Lindenfors et al. 2007, Dáttilo et al. 2020); larger surface areas and greater potential parasite encounter rates (Morand 2000, Tsunoda & Tatsuzawa 2004); live longer and thus accumulate more parasite species (Pacala & Dobson 1988, Bell & Burt 1991); or consume more infected prey (Poulin 1995, Gregory et al. 1996, Kamiya et al. 2014). However, few studies have been able to disentangle the effects of size and other host traits that are correlated with size, such as geographical range, home range size, diet, and phylogenetic relatedness. In the United States and Canada, maximum snake snout–vent length ranges from 230 to 2950 mm and length-based mass estimates range from 2.81 to 7631 grams (Feldman et al. 2015), with many implications for snake ecology. Therefore, we predicted that size would be an important predictor of estimated parasite richness after accounting for phylogenetic relatedness and latitudinal range. To test these predictions, we systematically reviewed all published accounts of endoparasites in native snake species from the United States and Canada, extracting ecological data about the snakes, their parasites, and the locations where they were located.

Methods

We searched for published parasitological studies of snake species in the United States and Canada using two existing bibliographies and additional database searches. A total of 309 papers were sourced from Ernst and Ernst (2006) and 80 from Udchitz (2020) (Supplemental

Figure A.1). Additional Web of Science and Scopus database searches were conducted on 15 and 24 January 2022 with ~439 search terms (see supplemental methods, Appendix A) that were categorized by geography (e.g., "United States" OR "Canada"), snake taxonomy (e.g., "Colubrid* OR Viperid*"), and parasite taxonomy (e.g., "Endoparasit* OR Helminth*"). We screened all titles and abstracts to determine if records were about snake parasites and if they were duplicates, resulting in five additional relevant records that were not included in the bibliographies. Also, we looked for missed references by searching for each snake host species on the London Museum of Natural History Helminth Database (Gibson et al. 2005), which yielded an additional eight references.

These searches yielded a total of 402 potentially relevant references. We excluded 26 papers that were not written in English, for a total of 376 papers that were subjected to full-text analysis. During full-text analysis, we eliminated papers or records within papers based on the following criteria: (1) the snake hosts were collected outside of the United States or Canada or we could not confirm a United States or Canada origin (including captive snakes with unknown origins); (2) the snake species was unknown or not native to the U.S. or Canada; (3) the paper did not include information on any snake host species (e.g., other reptile hosts were examined); (4) the paper did not provide new information for the database because it was a duplicate paper (e.g., a paper and a thesis with the same information) or it was a secondary paper that contained information we had previously collected from primary sources; (5) the parasite records were incomplete, either because the parasite was not identified at least to genus, the parasite was not confirmed to infect the host (e.g., parasites were diet contents), or the parasite information was aggregated in such a way that it was unclear which host species a given parasite species used. We excluded any papers that only considered parasite groups outside of helminths,

acanthocephalans, and pentastomes (e.g., arthropods and apicomplexans). After eliminating studies that did not meet our inclusion criteria, we reviewed 183 papers and 1 book (Supplemental Figure A.1).

From each included reference, we extracted data pertaining to parasites, snakes, and spatial and temporal data. For parasites, we included information regarding taxonomy, host sample size, and infection prevalence. For snakes, we included information regarding snake species, collection method (e.g., roadkill, museum specimen), and sex. For spatial and temporal information, we recorded when (year, season, date) and where (country, state/province, county, spatial coordinates) the snakes were collected. After designing a data extraction protocol, pairs of evaluators tested the protocol on 15 papers each to ensure consistent methods among evaluators. After minor protocol revision, the remaining references were read in full by a single evaluator, and data were extracted from text, tables, and figures.

Before data analysis, the dataset was cleaned by removing any records where parasites were not identified to species (unless the unknown species was the only record for that genus for the given host species). Parasite taxonomy was compared against several text references (Byrd & Denton 1938, Petrochenko 1958, Schad 1962, Baker 1987, Anderson 2001, Gibson et al. 2002, Bray et al. 2008, Hodda 2022) and additional database searches to identify synonyms or name changes since publication, and the most recent valid names were used for analyses. Similarly, we updated snake taxonomy to use the most current synonym according to The Reptile Database (Uetz et al. 2022). Finally, before analysis, we removed parasite data from experimental infections or captive snakes, so the species richness observations and predictions reported here represent parasite biodiversity in native wild snakes.

We quantified existing biases towards studying particular snake host species using a logistic regression that described the probability that any given snake species had at least one existing parasitological study. We tested whether studies were more likely to exist for more charismatic host species (as indicated by maximum snout–vent length and snake family) or more widespread host species (as indicated by latitudinal range). Snake size data were sourced from Feldman et al. (2015), where we assumed that any snake species that were more recently recognized than Feldman et al. (2015) were approximately the same size as their most recent synonym (e.g., the Florida cottonmouth, *Agkistrodon piscivorus conanti*, is roughly the same size as the Northern cottonmouth, *Agkistrodon piscivorus*). Also, latitudinal range sizes were sourced from Feldman et al. (2015), but missing range data for species recognized more recently than Feldman et al. (2015) were sourced from distribution descriptions from The Reptile Database and converted to approximate minimum and maximum latitudes. All analyses were conducted in R version 4.2.2 (R Core Team 2022).

We created a species accumulation curve to explore how observed parasite species richness increases with the number of host species sampled, and to predict how many parasite species could be discovered if the current level of sampling effort were applied to unsampled host species. To do this, we performed 1000 random simulations where we selected a number of sampled host species (from 1 to 79) and quantified the number of unique parasite species known from those host species across all papers. We fit a power function ($y = ax^b$) to the simulated data (Strona & Fattorini 2014) where parameters were estimated with three MCMC chains run for 5000 iterations and the first 1000 iterations were removed for burn-in (package 'R2jags'; Su & Yajima 2021). Both parameter priors were weakly informative, and chains were visually assessed for convergence using trace plots. We used the fitted power function to predict parasite

species richness when 175 unique snake host species were sampled, where 175 was based on species lists from Crother et al. (2003) and Ernst and Ernst (2006) that were updated with the most recent synonyms from The Reptile Database (excluding non-native species).

To quantify how observed species richness increased with sampling effort for any given snake host species, we also created rarefaction curves for eleven host species where more than 50 individuals had been sampled across all studies and there were at least 10 parasitological studies that included the species. For each study, we calculated the mean host sample size for that species per date and location and then summed across dates and locations. Alternatively, if no sample size data were included, we conservatively assumed the number of hosts sampled per date and location was the same as the number of reported infected individuals (if reported) or 1 (if no other information was available). We performed 400 random simulations where we selected a subset of papers and recorded how either the number of papers or the number of hosts sampled across those papers affected observed parasite species richness. We fit power functions using the same Bayesian methods described above for each combination of host species and method (number of papers vs. number of hosts).

Finally, we controlled for sampling effort (number of papers) by calculating estimated parasite species richness for each snake host species using Chao2, a nonparametric diversity metric (Chao 1984). Following Teitelbaum et al. (2020), Chao2 estimates were based on the ratio of the number of parasite species detected only in a single study for a given host species to the number of parasite species detected in at least two studies for a given host species. We quantified how Chao2 was correlated with snake size (maximum SVL, but mass produced the same qualitative results), latitudinal range, three diet categories (terrestrial vertebrate prey, invertebrate prey, fish or amphibian prey), and phylogenetic relatedness using a phylogenetic

generalized linear model (package 'phyr', Ives et al. 2020). We log-transformed the Chao2 response variable for this model to satisfy the homogeneity of variance assumption. Diet data were sourced from Ernst and Ernst (2006), where we assumed that any species recognized since Ernst and Ernst (2006) had the same prey types as their most recent synonym. We condensed our initial diet categories into three options because variance inflation factors indicated that some categories were correlated (e.g., snake species that eat mammals usually also eat birds). Snake phylogeny data were sourced from Burbrink et al. (2016), where the phylogeny was trimmed to the 74 snake species for which there were non-zero parasite data (package 'ape', Paradis & Schliep 2019).

Results

Out of 175 snake species in the U.S. and Canada, 79 (45%) have been examined in at least one parasitological study. Most papers focused on snakes in the United States and especially in the southeast and southwest, which are the regions of highest snake biodiversity (Figure 1.1). A few snake species were studied extensively, including *Coluber constrictor* (37 studies), *Thamnophis sirtalis* (33 studies), *Nerodia sipedon* (33), and *Agkistrodon piscivorus* (32), whereas all others occurred in fewer than 30 studies and most (76% of studied species) in five or fewer studies. The probability that a snake species had been examined in at least one parasitological study increased with snake size (coefficient ± SE = 0.0009 ± 0.0003; $p = 0.003$) and latitudinal range (coefficient ± SE = 0.049 ± 0.02; $p = 0.03$), and was not affected by snake family (Supplemental Table A.1).

For the 11 most sampled snake host species, the number of observed parasite species increased towards saturation with the number of parasitological studies that included that host species (Figure 1.2). However, because studies varied greatly in sample sizes (1–417 hosts per

species per study), individual studies represent different sampling efforts. When we assumed that the number of hosts sampled was the mean number sampled per host species per sampling location per date per study, the number of observed parasite species also increased towards saturation with the number of individual hosts sampled (Figure 1.2). However, the curves have several limitations: many studies did not report sample sizes (23%); it was sometimes impossible to accurately determine total sample sizes per host species per study given the breakdowns provided; and surveys in different locations likely represent different local parasite communities, rather than a single community sampled repeatedly in different studies. Therefore, although observed parasite richness increased with sampling effort, few rarefaction curves reached rarefaction, implying that even the best-studied snake host species have many undocumented parasite species.

After accounting for sampling effort (i.e., number of citations per host species), the log-transformed estimated parasite richness (Chao2) per snake species was positively correlated with host species size (coefficient ± SE = 0.001 ± 0.0003; $p < 0.0001$; Supplemental Table A.2). Host size was not correlated with the hosts' latitudinal range (Gaussian linear model; coefficient ± SE = 0.002 ± 0.001; $p = 0.11$; $R2 = 0.04$), and when both were included in the Chao2 phylogenetic regression, latitudinal range was still significantly correlated with log-transformed estimated parasite richness (coefficient ± SE = 0.046 ± 0.02; $p = 0.02$) (Figure 1.3). Among three prey item variables (terrestrial vertebrates, fish or amphibians, or invertebrates), only one was significantly correlated with estimated parasite richness, where snakes with amphibian or fish diets had higher average log-transformed Chao2 (coefficient ± SE = 0.84 ± 0.39; $p = 0.03$) (Figure 1.3). Within this model, phylogenetic relatedness only explained a small amount of variability in the log-transformed Chao2 across snake species (variance = 0.003, SD = 0.06).

Among all studied snake hosts, 139 unique endoparasites (helminths, acanthocephalans, and pentastomids) have been documented with species-level identification, representing 80 unique genera and 55 unique families. When unique genus-level identifications per host species were counted as unique parasite species (e.g., "*Echinostoma sp.*"), estimates increased to 179 unique parasite species. Helminths were the most reported parasite taxa in snakes (46% of species were trematodes, 35% were nematodes, and 12% were cestodes), whereas acanthocephalans (6%) and pentastomes (2%) were the least reported parasite taxa (Supplemental Figure A.2). Using a species accumulation curve (Figure 1.4), we predicted that future studies regarding the remaining 96 host species would yield at least 150 additional parasite species (329 total).

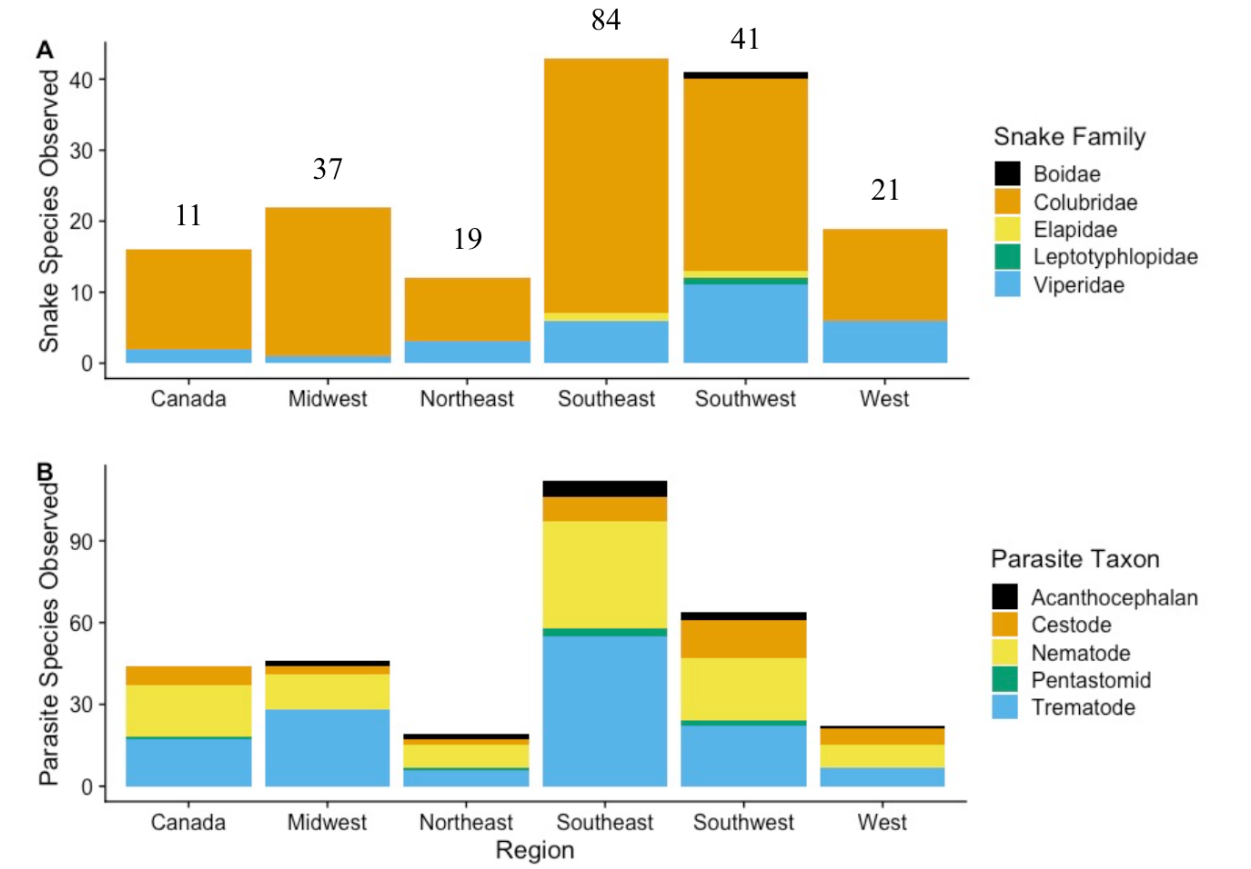

Figure 1.1 The snake species in each family and parasite species in each taxon examined from Canada and each region of the United States. The values above each bar represent the number of studies in our database from each region.

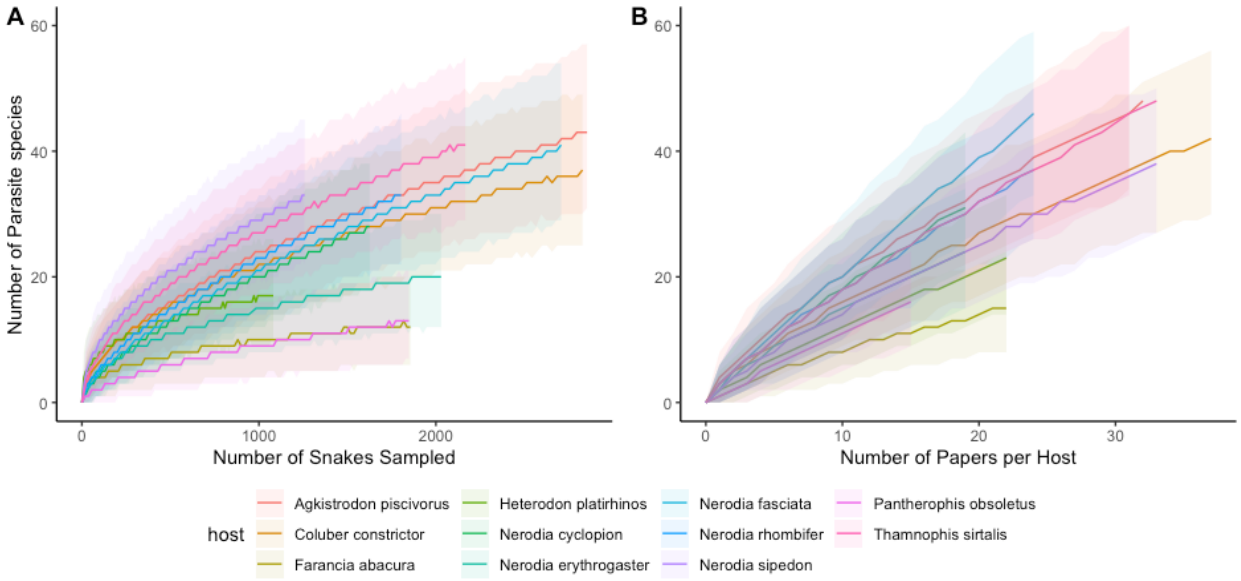

Figure 1.2. The number of unique parasite species observed in a given host species increases with **A)** the number of individual hosts sampled and **B)** the number of parasitological studies that include that host species. The curves were created from fitting a power function ($y=aX^b$) with the 400 random samples per host species. For each host species, the curves represent median fits based on 5,000 posterior distribution samples, and the shaded regions represent 95% credible intervals. The snake species shown here were the only species for which more than 50 individuals were sampled and more than 10 parasitological studies had been conducted.

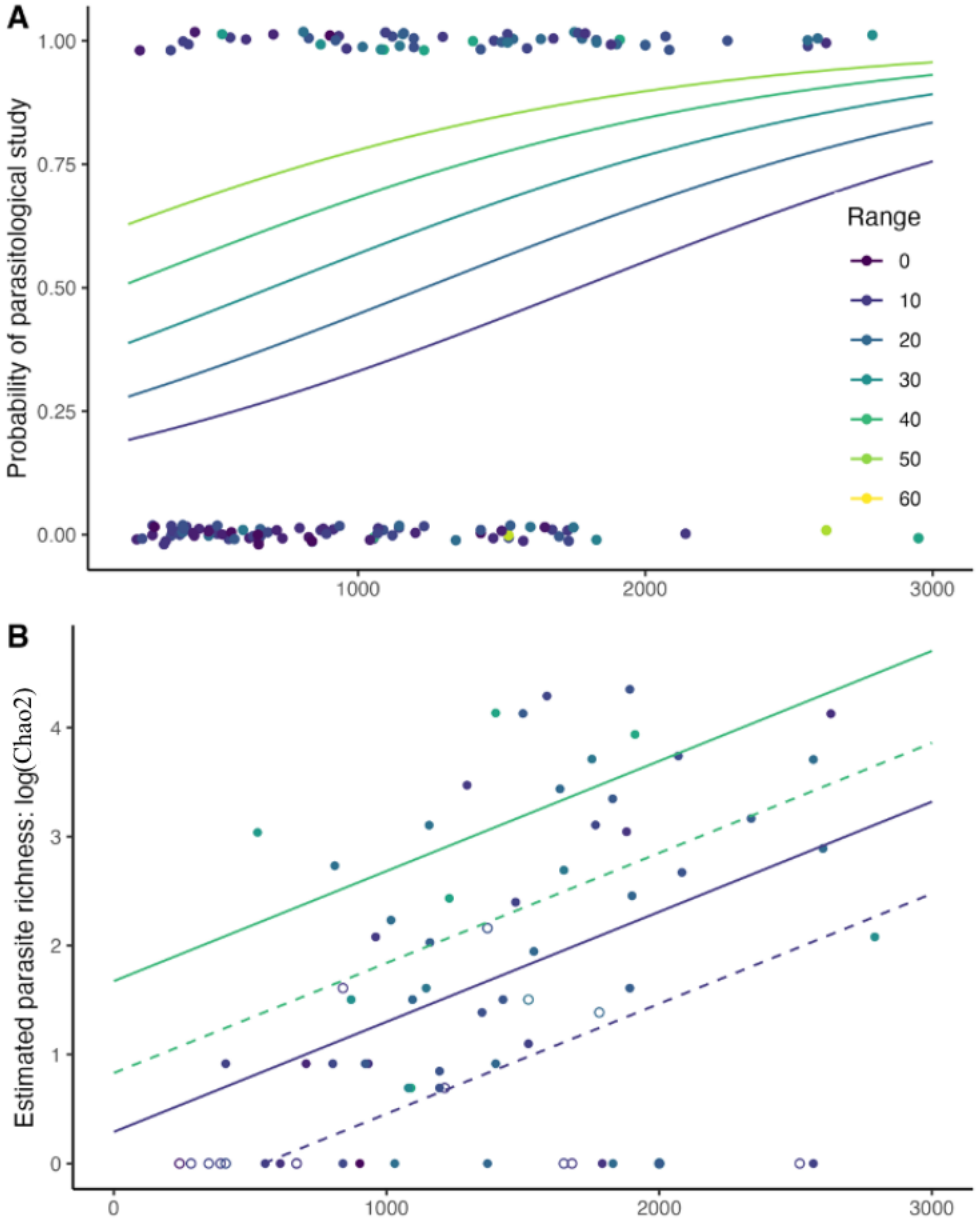

Figure 1.3. A) The probability that a given snake species occurred in at least one parasitological study increased with host size and latitudinal range (maximum - minimum latitude). The curves represent the predicted values from the best-fitting logistic model and points are jittered slightly on the Y axis to aid visualization. **B)** After controlling for sampling effort, estimated parasite species richness increased with host size and latitudinal range and was higher for snake species with fish or amphibian diets (solid lines and filled circles). The lines are the predicted values from the best-fitting phylogenetic linear model fit to the log-transformed Chao2 data.

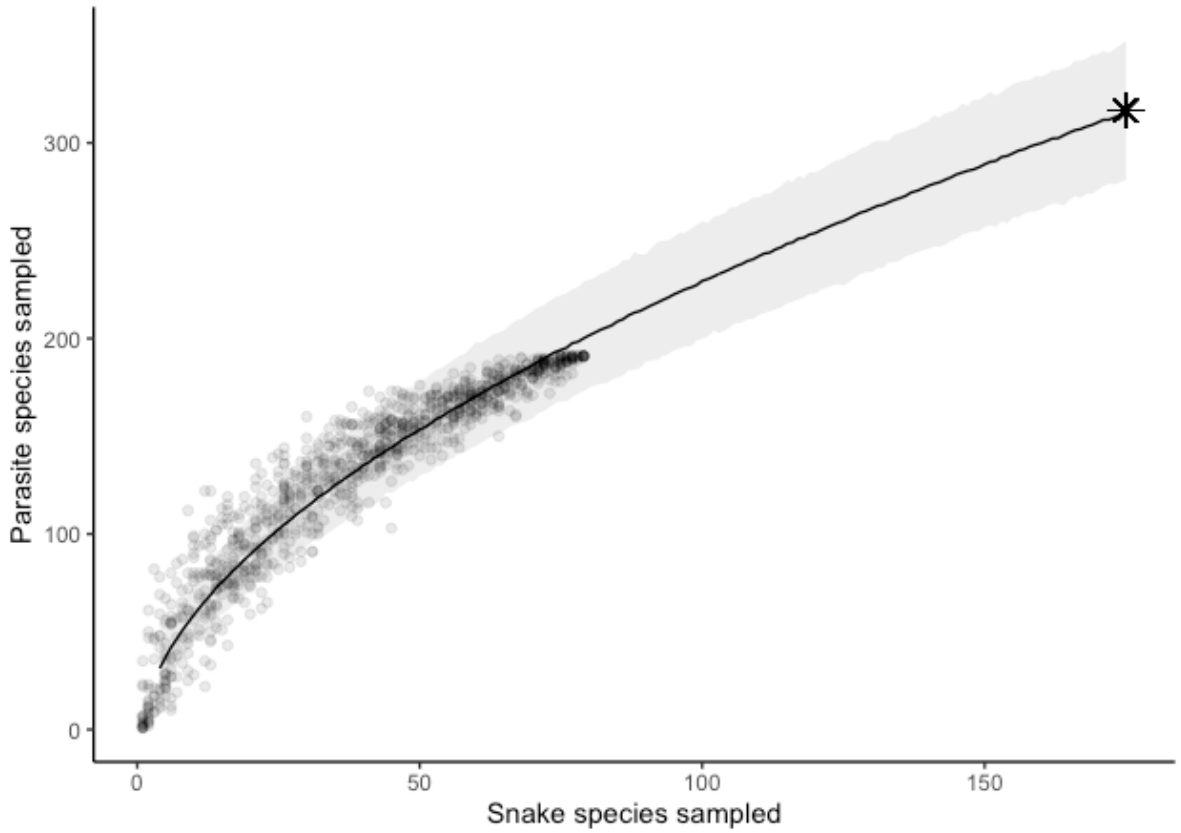

Figure 1.4. Species accumulation curve for parasite species detected in snakes in the United States and Canada. Points represent random samples of the host–parasite network, where a subset of host species were randomly selected, and the number of unique parasite species (or genera e.g., "*Echinostoma sp.*") in those host species were summed. The curve is the mean model fit for the power function ($y=aX^b$), and the shaded region represents the 95% credible interval. The asterisk represents all 175 snake species, which if sampled at present effort would lead to a predicted 329 described parasite species.

Discussion

In this first macroecological study of snake parasites, we determined that snakes in the United States and Canada host a diverse and understudied community of endoparasites. Parasitological studies were more likely to exist for snake species that were relatively large and/or had relatively large latitudinal ranges. Among the studied snake host species, there are at least 139 endoparasitic helminths, acanthocephalans, and pentastomes that have been described to the species level. Observed and estimated parasite richness in any given snake species increased with sampling effort, and few, if any, snake species have been sampled sufficiently to

document the full parasite community. After accounting for sampling effort, estimated parasite richness was highest in larger snake species, widespread snake species, and snake species with fish or amphibian diets. Host phylogenetic relatedness explained little variation in estimated parasite richness, which could be because the phylogenetic tree was heavily pruned by missing data; 55% (96/175) of snake species have never been surveyed for parasites anywhere in the United States and Canada, including 19 species that are considered threatened or endangered at the national level. The large gaps in our knowledge of snake ecology and parasite biodiversity likely hamper both snake and parasite conservation, and we highlight several research priorities for filling these gaps.

To describe parasite biodiversity more completely in snakes, we recommend sampling more snake species and more thoroughly sampling species that have previously been studied. We conservatively estimated that sampling the 96 unsampled snake host species would increase the number of known parasite species (including unique parasite genera per host species) from 179 to at least 329 (94% increase). Documented parasite biodiversity would increase further if we increased host sample sizes per study and across studies and/or collected higher quality data in any given study. For example, for this systematic literature review, we excluded many studies or parasite species where the parasite was not identified to species. In many cases, taxonomic resolution ended at family (e.g., “Oligacanthorhynchidae larvae”, “Diplostomidae mesocercariae”). This reflects a growing crisis in parasite taxonomy, where few people have the skills required for parasite identification (Blasco-Costa & Poulin 2017). However, genetic sequencing costs have decreased in recent years, which may make parasite identification more accessible to more researchers and conservation practitioners (Blasco-Costa & Poulin 2017, Doyle et al. 2019). While all 175 snake species need to be studied further, researchers hoping to

target undescribed parasite biodiversity may be most successful when targeting unstudied snake species that are large and have aquatic life histories (or diets that consist of amphibians and fish). Some examples of large snake species that have never been studied include the Mole kingsnake (*Lampropeltis rhombomaculata*) and the gray rat snake (*Pantherophis spiloides*), which is closely related to the abundant Eastern rat snake (*Pantherophis alleghaniensis*), a species that has surprisingly only been surveyed in two published parasitological studies. Of course, obtaining adequate sample sizes is always an issue in ecology and parasitology, so more coordinated efforts to collect vehicle-kill snakes for parasitological research could be a priority for snake and parasite conservation.

We determined that larger snake species had more observed and estimated parasite species because they were sampled more and because size has additional effects on true parasite species richness. This parallels studies in other host taxa, where parasite richness has often been determined to be a function of both sampling effort and host size (Ezenwa et al. 2006, Lindenfors et al. 2007, Campião et al. 2015, Albery et al. 2022). For example, larger carnivores live longer, consume more food, and often experience lower host mortality, which causes them to acquire more parasites over their lifespan (Gittleman 1993, Lindenfors et al. 2007). But few studies have been able to disentangle the effects of size and other host traits that are correlated with size, and in some cases, relationships between size and parasite richness completely disappear after accounting for host phylogenetic relationships (Poulin 1997, Nunn et al. 2003). Here, snake phylogeny explained little variation in estimated parasite richness, and after accounting for phylogenetic relatedness (and sampling effort), estimated parasite richness was still significantly correlated with host size, fish/amphibian diets, and host latitudinal range. Therefore, it appears that snake size impacts estimated parasite richness independently from latitudinal range, perhaps

because bigger snakes have larger surface area (Guégan & Hugueny 1994, Poulin 1995), live longer, and consume more prey and trophically-transmitted parasites (Pacala & Dobson 1988, Bell & Burt 1991).

Our new snake–parasite database could advance both parasite and snake conservation. Baseline data on parasites in snakes in the United States and Canada has already been critical for snake conservation. For example, past parasitological studies allowed researchers to determine that an invasive pentastome (*Raillietiella orientalis*) recently spilled over from Burmese pythons (*Python bivittatus*) to native snake species in Florida, with negative consequences for native species (Miller et al. 2018, 2020). However, these baseline data were difficult to access because individual published studies often require expensive subscriptions to obtain and existing bibliographies and species "check lists" are not sortable (Ernst & Ernst 2006, Udchitz 2020). Therefore, our snake–parasite database and associated ecological covariates will improve access and speed of access to baseline parasite data during future emerging infectious disease events in native snakes. Furthermore, these data will be useful more broadly in ongoing efforts to describe and conserve global parasite biodiversity, which has suffered from the lack of comprehensive, up-to-date data sources (Carlson et al. 2020). Future studies can continue to fill in the data gaps in our existing snake–parasite database by sampling more host species, increasing host sample sizes, and improving the quality of the ecological and parasitological data that are collected (e.g., taxonomic resolution, better reporting on sample sizes and study locations). These efforts will provide faster and more reliable information for conserving parasites and snakes in our rapidly changing ecosystems.

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CHAPTER 2

Surveillance of small-bodied snakes: apparent ophidiomycosis is more likely in the spring and in relatively large individuals

Introduction

Ophidiomycosis is a fungal disease that infects wild and captive snakes on several continents, and it may be an emerging or re-emerging infectious disease in North America (Lorch et al. 2016, Haynes & Allender 2021). In the United States, the fungus (*Ophidiomyces ophidiicola*) and fungal disease have been documented in at least 49 native snakes and 3 non-native snakes across 26 states (Haynes & Allender 2021). All snake species seem to be susceptible to this fungal pathogen, regardless of ecological traits or phylogeny (Burbrink et al. 2017). However, there is variation in the infection prevalence and disease severity among species both spatially and temporally (Grisnik et al. 2018, McKenzie et al. 2019, Fuchs et al. 2020, Haynes et al. 2020), and it is still unclear what causes this variability (Burbrink et al. 2017, Haynes & Allender 2021). Teasing apart these mechanisms is complicated by limited data availability for many species. Therefore, additional surveillance remains a critical priority for understanding and predicting the impacts that ophidiomycosis will have on snake populations (Lorch et al. 2016, Burbrink et al., 2017, Allender et al. 2020). Here, we focus on surveillance in small-bodied snake species (i.e., SVL < ~700 mm), in the hopes that insights learned from these species will be broadly beneficial to snake surveillance and conservation.

Small-bodied snake species are relatively understudied in the ophidiomycosis literature. For example, in a recent review by Haynes and Allender (2021), only 10 papers considered small-bodied species (Lorch et al. 2016, Grisnik et al. 2018, Licitra et al. 2019, McKenzie et al. 2019, Fuchs et al. 2020, Haynes et al. 2020, Snyder et al. 2020, Lentz et al. 2021, Patterson et al.

2021, Gramhofer et al. 2022), which we here define as snake species with a maximum snout–vent length <700 mm (Feldman et al. 2015). Among the studies that did include small-bodied snake species, most had sample sizes <15 individual snakes, making it difficult to estimate pathogen and disease prevalence or observe variation among individuals. In several cases, it was unclear whether small-bodied snakes were targeted with equal effort to larger-bodied snakes, or whether the surveyed snakes were a biased sample of what might be present. Additionally, several studies that reported signs of ophidiomycosis in small-bodied snakes did not describe the signs, making it difficult to compare ophidiomycosis in small-bodied snakes to larger-bodied snake species. Given all these complications, it is difficult to interpret some of the high prevalences documented in small-bodied snake species. For example, previous studies with small sample sizes have reported high prevalence of the pathogen without clinical signs of disease in *Carphophis amoenus* (Eastern worm snake), such as 29% (1/4) in Virginia and Maryland (Fuchs et al. 2020); 25% (1/4) in Georgia (Patterson et al. 2021); and 64% (19/30) in Tennessee (Gramhofer et al. 2022). Do many *C. amoenus,* a common prey species for other snake species, carry the fungal pathogen without signs of disease? In contrast, a few studies with small and moderate sample sizes have reported high prevalence of the pathogen in the presence of clinical signs, such as 67% (4/6) in Kentucky (McKenzie et al. 2019) and 37% (11/30) in Tennessee (Gramhofer et al. 2022). Of course, we recognize that small and potentially biased sample sizes for small-bodied snake species reflect the challenges associated with locating and sampling snakes in general (Dorcas & Willson 2009, Durso et al. 2011, Steen et al. 2012), which might often lead to prioritizing species of greater conservation concern (e.g., larger species, rare species). Therefore, our objective was to determine whether extensive sampling of small-bodied

snake species yields additional information on ophidiomycosis prevalence compared to previous studies.

We propose that small-bodied snake species could be important surveillance targets for three main reasons. First, by surveilling small-bodied snake species, we will better understand disease dynamics and impacts on these species, which benefits small-bodied snake species and the ecosystems where they occur. Second, small-bodied snakes are often the most abundant snake species in ecosystems (Fitch 1975, Russell & Hanlin 1999, Rice et al. 2001, Willson & Dorcas 2004), which suggests they are the easiest to find and sample. Therefore, surveillance of small-bodied snake species may provide opportunities to detect *Ophidiomyces ophidiicola* (*Oo*) quickly and cheaply in ecosystems. And third, without comparable data from all snake species, it is difficult or even impossible to determine why some snake species seem to have higher prevalence or severity of infection (e.g., Burbrink et al. 2017), because existing relationships could be due to biased sampling effort. Therefore, by filling in the data gaps for small-bodied species, we might be better able to test hypotheses about the drivers of ophidiomycosis (e.g., host physiology, environmental correlates).

In this study, we surveyed wild terrestrial colubrid snakes for ophidiomycosis in the Piedmont ecoregion of North Carolina. Unlike many previous studies, we sampled snakes in proportion to the numerical abundance in which they were encountered, leading to large sample sizes of small-bodied snakes and especially *Carphophis amoenus* (n = 94). Among *C. amoenus*, we quantified how season, microclimates, and snake size affected the probability that an individual snake had ophidiomycosis and determined whether survey method (opportunistic visual encounter surveys vs. area-constrained searches) affected prevalence estimates. Also, we compared our prevalence estimates to those from other studies of *C. amoenus*. This was the

largest study of ophidiomycosis in small-bodied snakes to date, and we used these results to document several benefits and challenges for using small-bodied species as disease surveillance targets.

Methods

We sampled colubrid snakes from April to October 2022 in terrestrial habitats in the Piedmont ecoregion of North Carolina (Wake, Durham, and Orange Counties; Figure 2.1). These counties cover a gradient from urban to rural habitats, including a patchwork of housing developments, agricultural fields, and forested patches. Within this landscape, we surveyed 11 parks that varied in size, forest cover, human activity, and management, among other variables. We sampled two state parks (William B. Umstead State Park and Eno River State Park), two county parks (Historic Oak View County Park and Historic Yates Mill County Park), two University-owned research forests (G.W. Hill Forest and Carl Alwin Schenck Memorial Forest), four NGO-owned and publicly-accessed nature preserves (Brumley Nature Preserve, Johnston Mill Nature Preserve, Bailey and Sarah Williamson Nature Preserve, and Swift Creek Bluffs Nature Preserve), and one privately owned nature preserve (Brighton Forest). Also, we sampled 14 backyards from volunteer faculty and staff at North Carolina State University which varied in size, cover availability, tree cover, and other variables that were likely to affect microclimates and snake habitat availability.

We used two sampling methods to balance our efforts to estimate snake density and to achieve sufficient sample sizes needed to estimate ophidiomycosis prevalence in snake communities: area-constrained searches and opportunistic visual encounter surveys. For area-constrained searches in parks, we used satellite imagery from Google Earth Engine to randomly select sites within each park to sample on each visit. Randomly selected sites were

predominantly located in dry upland forest habitats dominated by Loblolly pine (29%) or oak/hickory (26%) (Supplemental Table B.1). At each randomly selected site, we delineated a 30 m^2 plot and checked under all cover items (rocks, logs, etc. that were liftable by two people) for snakes. We did not remove leaf litter to search for snakes. We sampled 1–4 plots per park per visit, and we never sampled the same location twice, to avoid repeat sampling of the same individual snakes. Though we visited large parks multiple times, our goal was to spend our effort sampling the broadest area, rather than repeatedly visiting only a few locations. In backyards, we did not randomly select points, but instead checked every available cover item in the yard and used ArcGIS Pro to quantify the area searched.

Area-constrained searches were important for estimating snake density, but they required high effort and yielded relatively low capture rates. Therefore, to increase our sample sizes, we also opportunistically searched for snakes under cover items as we walked to designated random plots within parks (Hutchens & DePerno 2009). Occasionally, snakes were encountered while crossing trails, on tree trunks, or basking. Most field surveys and thus snake captures occurred between 0800 and 1200 hours, and we tracked the number of miles covered and time spent searching (multiplied by the number of people searching) for all survey trips as metrics of search effort.

When we encountered a terrestrial colubrid snake, we captured it with gloved hands and performed a physical examination for clinical signs of ophidiomycosis: crusts, displaced scales, nodules, lesions, swelling, or caseous discharge from skin pustules (Allender et al. 2011, 2016). We used a 365 nm UV flashlight to scan the dorsal and ventral sides of the snake and noted any fluorescence that occurred (Turner et al. 2014, Vivirito et al. 2021). Whether any clinical signs were observed or not, at least one body swab was collected by passing a dry, sterile cotton swab

five times back and forth along the dorsal and ventral sides of the snake from head to tail tip (Allender et al. 2016). For larger snakes, we sometimes collected separate swabs for the dorsal and ventral body. If lesions were present, we collected separate lesion swabs, vigorously rubbing the lesion with the swab and collecting any displaced scales. Swabs were placed in 2 mL centrifuge tubes, stored on ice packs, and transported back to the lab and placed in a -4° C freezer. All skin swabs (150 in total) were sent to the Wildlife Epidemiology Lab at the University of Illinois at Urbana-Champaign to quantify *Ophidiomyces ophidiicola* DNA using TaqMan qPCR (sensu Allender et al. 2015). We did not quantify any other snake pathogens.

After swabbing, we used string and a tape measure to quantify the total length and tail length measurements for each snake. We collected microclimate measurements under every cover item where a snake was found, including soil temperature (Fluke 62 MAX IR laser thermometer) and soil moisture (%) and pH (Kelway soil and pH moisture meter).

After sampling, all snakes were released to their original point of collection within 10 minutes of capture. To prevent the spread of ophidiomycosis, clean gloves and supplies were used to swab and measure each snake and all gear was decontaminated between sampling trips. All work was covered under North Carolina State University IACUC protocol #22-161 and North Carolina wildlife collection permit #22-SC01472, as well as appropriate park-level permits.

We quantified the average encountered snake density in each park (encountered snakes per 30 m^2, averaged across plots) or yard (encountered snakes per searched area) on each sampling date and converted them to densities per hectare. We compared the average densities of all snakes and just *C. amoenus* (including multiple estimates for parks sampled on more than one date) between parks and backyards using Welch's t-tests. We compared apparent ophidiomycosis

prevalence from snakes encountered during area-constrained searches versus opportunistic visual encounter surveys using Wilson asymmetric binomial confidence intervals. We quantified whether the probability that a given *C. amoenus* had apparent ophidiomycosis was correlated with snake size (total length in mm), sample date, and microclimate variables (temperature, soil moisture, or pH where the snake was found), where we used AIC to compare models with different combinations of these variables. All models were logistic fixed effects models, where we decided not to use site or sampling date as random effects because these variables explained no variation in logistic mixed models, likely because most sites and sampling dates had zero snakes with apparent ophidiomycosis.

Results

From April 17th to October 18th 2022, we observed 136 snakes from ten species and swabbed 123 snakes from eight species (Table 2.1). Note that five snakes were purposely not captured because they were not terrestrial colubrids (one *Nerodia fasciata* and four *Agkistrodon contortrix*), whereas eight snakes were encountered but we failed to catch them (three *C. amoenus*, one *Coluber constrictor*, three *Storeria dekayi*, one *Haldea striatula*). Snake density was highest in the spring (Supplemental Figure B.1), which led to larger sample sizes and thus higher observed species richness (Figure 2.2). Whether we used area-constrained searches or opportunistic visual encounter surveys, most snakes were small bodied (96% of snakes in plots; 91% of all snakes observed), and *C. amoenus* was the most common species (83% of plots; 71% of all snakes observed; Figure 2.2). In our area-constrained searches (30 m^2 plots or entire yards), we estimated that average overall snake density was 137.3 snakes/ha (95% CI: 88.2 - 186.3) in parks and 6.25 snakes/ha (CI: 0.02 - 12.48) in backyards, whereas average *C. amoenus* density was 102.9 snakes/ha (CI 67.0 - 138.8) in parks and 1.48 snakes/ha (CI -0.70 - 3.65) in backyards.

Total snake density and *C. amoenus* density were both significantly lower in backyards than in parks (Welch's t-tests; total $t = -5.30$, $df = 68.7$, $p < 0.001$; *C. amoenus* $t = -5.63$, $df = 67.4$, $p < 0.001$; Supplemental Figure B.1).

Across all swabbed snakes and all sites, 8% (n = 10) were positive for *O. ophidiicola* DNA, and all ten (100%) of these individuals (representing four species) had clinical signs of ophidiomycosis (Table 2.1). The overall apparent ophidiomycosis prevalence estimate for all snakes encountered during opportunistic visual encounter surveys (95% Wilson CI: 2.8 - 12.5%) was not different from that of snakes encountered during area-constrained searches (95% Wilson CI: 7.0 - 37.1%), nor were they different when considering the prevalence in *C. amoenus* only (Figure 2.3). Among sites where we swabbed snakes, we detected apparent ophidiomycosis in 5/11 parks and 0/5 backyards. Sample sizes were too low at any given park or backyard to compare disease prevalence across sites; despite spending 547.5 human hours searching across all sites, the main determinant of whether apparent ophidiomycosis was present in a given site was still sample effort and sample size. Among the parks where we detected ophidiomycosis, 4/5 had at least one *C. amoenus* with apparent ophidiomycosis, and the other positive park had a *Storeria dekayi* with apparent ophidiomycosis. Conversely, we only detected large-bodied snakes with apparent ophidiomycosis at 2/5 parks.

The clinical signs exhibited by *C. amoenus* that tested positive were open ulcers and dry crusts that usually lacked blood or pustular discharge (Figure 2.4). In *C. amoenus* that tested positive, 4/7 had lesions only on the dorsal side of the body, 2/7 had lesions on both the dorsal and ventral sides, and 1/7 had crusts near the mouth and nostrils (Figure 2.4). None of the test-positive *C. amoenus* appeared lethargic or emaciated. Importantly, many *C. amoenus* that tested negative for *O. ophidiicola* had scale abnormalities and lesions (41/87 snakes; 47%). These may

have been injuries caused by predation attempts or something else and were not easily distinguishable from disease symptoms. For example, one *C. amoenus* had lost an eye and nearby scales were crusted over, but both the body swab and the eye swab tested negative for *O. ophidiicola*. Similarly, of the nine *Storeria dekayi* that we sampled, four had crusts and lesions without the presence of *O. ophidiicola* DNA (Table 2.1), and the one individual that tested positive for *O. ophidiicola* had several large and severely swollen ulcers on the body.

We attempted to improve diagnostic sensitivity by using a 365 nm UV flashlight, but that provided no additional insight: most of the PCR-negative *C. amoenus* that had scale abnormalities or lesions showed clear UV fluorescence at the lesions (35/41 snakes; 85%). Furthermore, many PCR-negative *C. amoenus* without lesions displayed some amount of UV fluorescence (27/46 snakes; 59%), especially if individuals were near ecdysis, where ecdysis was equally common in PCR-negative *C. amoenus* (10/87; 12%) as in PCR-positive *C. amoenus* (1/7; 14%). And three of the PCR-positive snakes displayed no UV fluorescence, despite having lesions (two *Carphophis amoenus* and 1 *Lampropeltis rhombomaculata*), suggesting the problem was that UV fluorescence was not a useful diagnostic tool, rather than that swabs and qPCR were not sensitive. We also tested 1–5 swabs per snake to improve detectability, where at least one swab covered the entire body and subsequent swabs focused on specific lesions, if they were present. For the ten snakes that tested positive, the first general body swab tested positive 90% of the time; one *C. amoenus* that had a negative body swab and a positive lesion swab. Likewise, when we took more than one swab, the lesion swabs tested positive for all snakes with positive body swabs, except one *C. amoenus* that had a positive body swab and only 50% (2/4) of the lesion swabs tested positive.

Carphophis amoenus with apparent ophidiomycosis were more likely to be detected early in the spring (coefficient ± SE = -0.12 ± 0.05; p = 0.02), where none were detected after May 10th, 2022 (Figure 2.5). Additionally, longer *C. amoenus* (total length) were more likely to have apparent ophidiomycosis (coefficient ± SE = -0.31 ± 0.15; p = 0.04). The most parsimonious model only included date and size (df = 3; AIC = 36.5), whereas models that included microsite temperature had AIC values ranging from 38.1 to 44.9 (Supplemental Table B.2) and microsite temperature was not a significant predictor of apparent ophidiomycosis in *C. amoenus* when date was included in the model. Similarly, neither soil moisture nor pH were significant predictors of apparent ophidiomycosis in *C. amoenus* (Supplemental Table B.2).

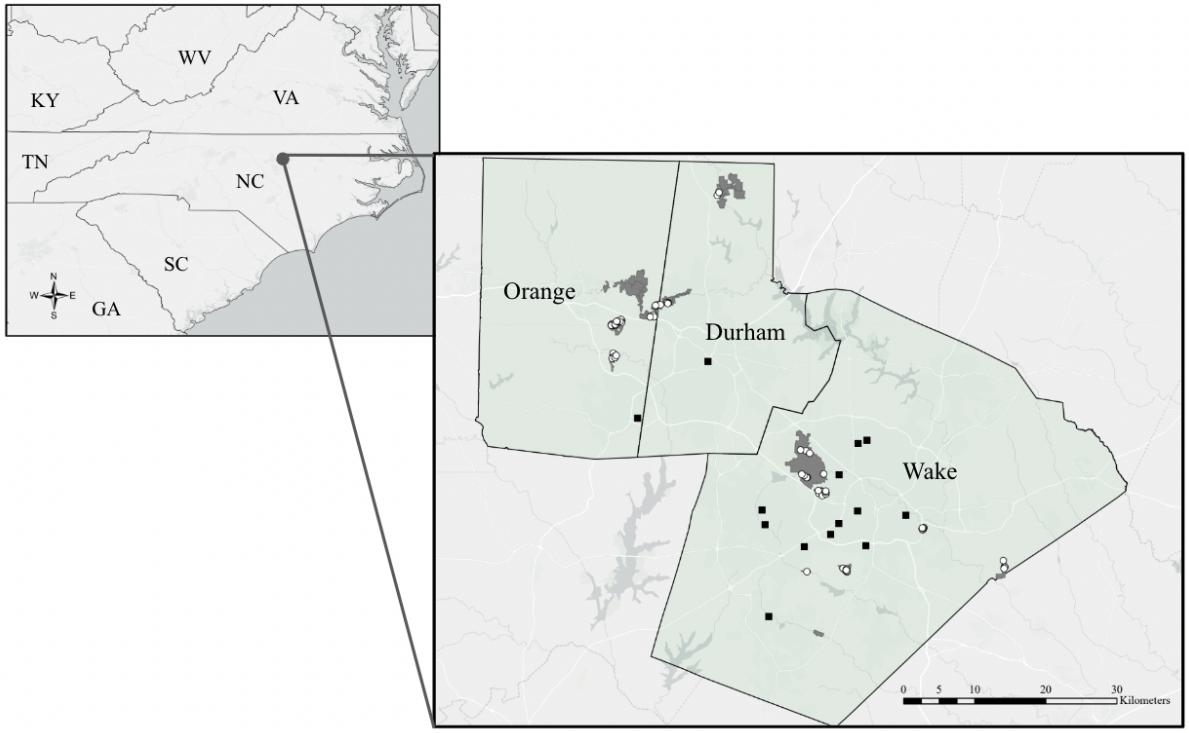

Figure 2.1. Location of sampling sites within Wake, Durham, and Orange Counties, North Carolina. Light gray polygons indicate water bodies, dark gray polygons represent park boundaries, white points are 30 m^2 plots sampled within parks, and black squares are yards sampled.

Table 2.1. Number of snakes sampled (and percent prevalence) across all study sites in Wake, Durham, and Orange Counties, North Carolina in 2022. The four categories of ophidiomycosis (following Gramhofer et al. 2021) are: (1) Negative (no clinical signs or qPCR detection of *O. ophidiicola* DNA); (2) *Ophidiomyces* present (qPCR detection in absence of clinical signs), (3) possible ophidiomycosis (presence of clinical signs in absence of qPCR detection), and (4) apparent ophidiomycosis (presence of clinical signs and qPCR detection).

Species	Negative (%)	*Ophidiomyces* present (%)	Possible ophidiomycosis (%)	Apparent ophidiomycosis (%)	Total snakes sampled
Eastern worm snake (*Carphophis amoenus*)	50 (47)	0	43% (40)	7.4 (7)	94
Ring-necked snake (*Diadophis punctatus*)	50 (6)	0	50 (6)	0	12
Dekay's brown snake (*Storeria dekayi*)	56 (5)	0	44 (4)	11 (1)	9
Eastern rat snake (*Pantherophis alleghaniensis*)	0	0	100 (3)	0	3
Black racer (*Coluber constrictor*)	0	0	50 (1)	50 (1)	2
Rough green snake (*Opheodrys aestivus*)	100 (1)	0	0	0	1
Rough earth snake (*Haldea striatula*)	0	0	100 (1)	0	1
Mole kingsnake (*Lampropeltis rhombomaculata*)	0	0	0	100 (1)	1

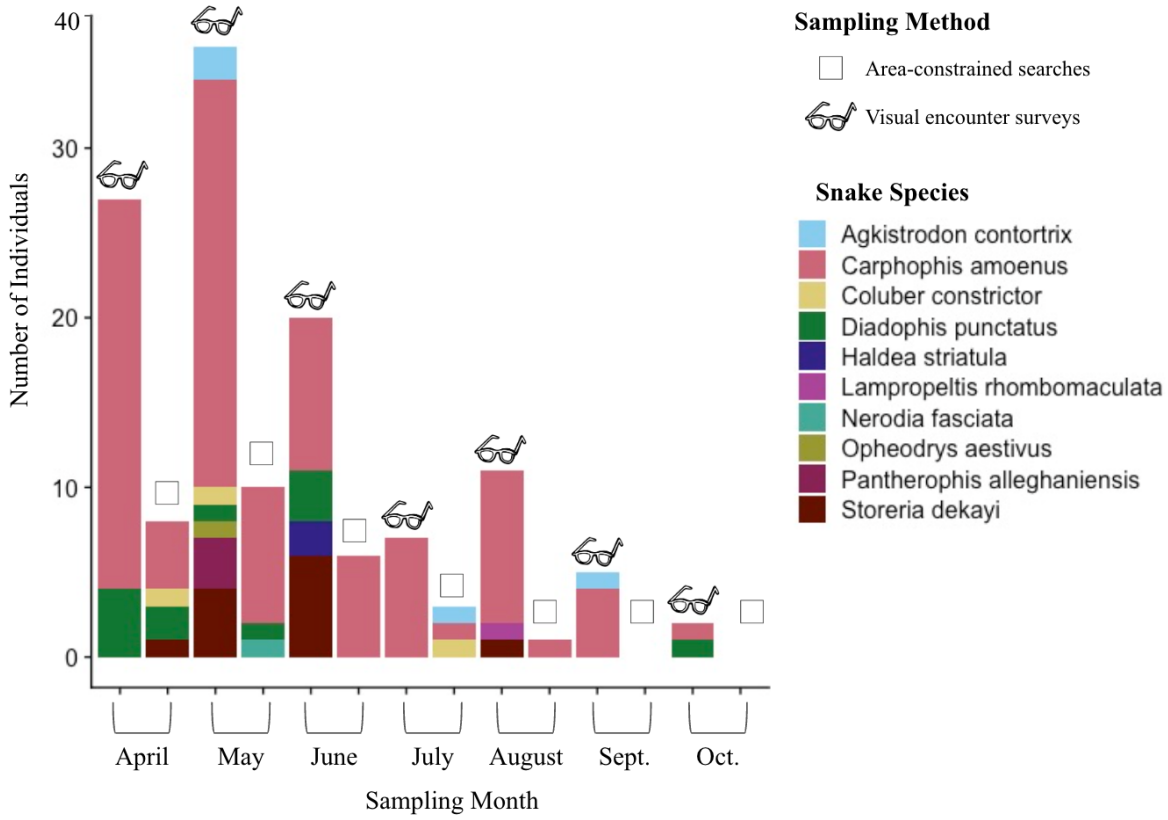

Figure 2.2. The relative abundance of snake species that were observed from April–October 2022, split by sampling method: area-constrained searches (entire yards or 30 m^2 plots in parks) and opportunistic visual encounter surveys. Note: area-constrained searches were conducted in September and October of 2022 but no snakes were found.

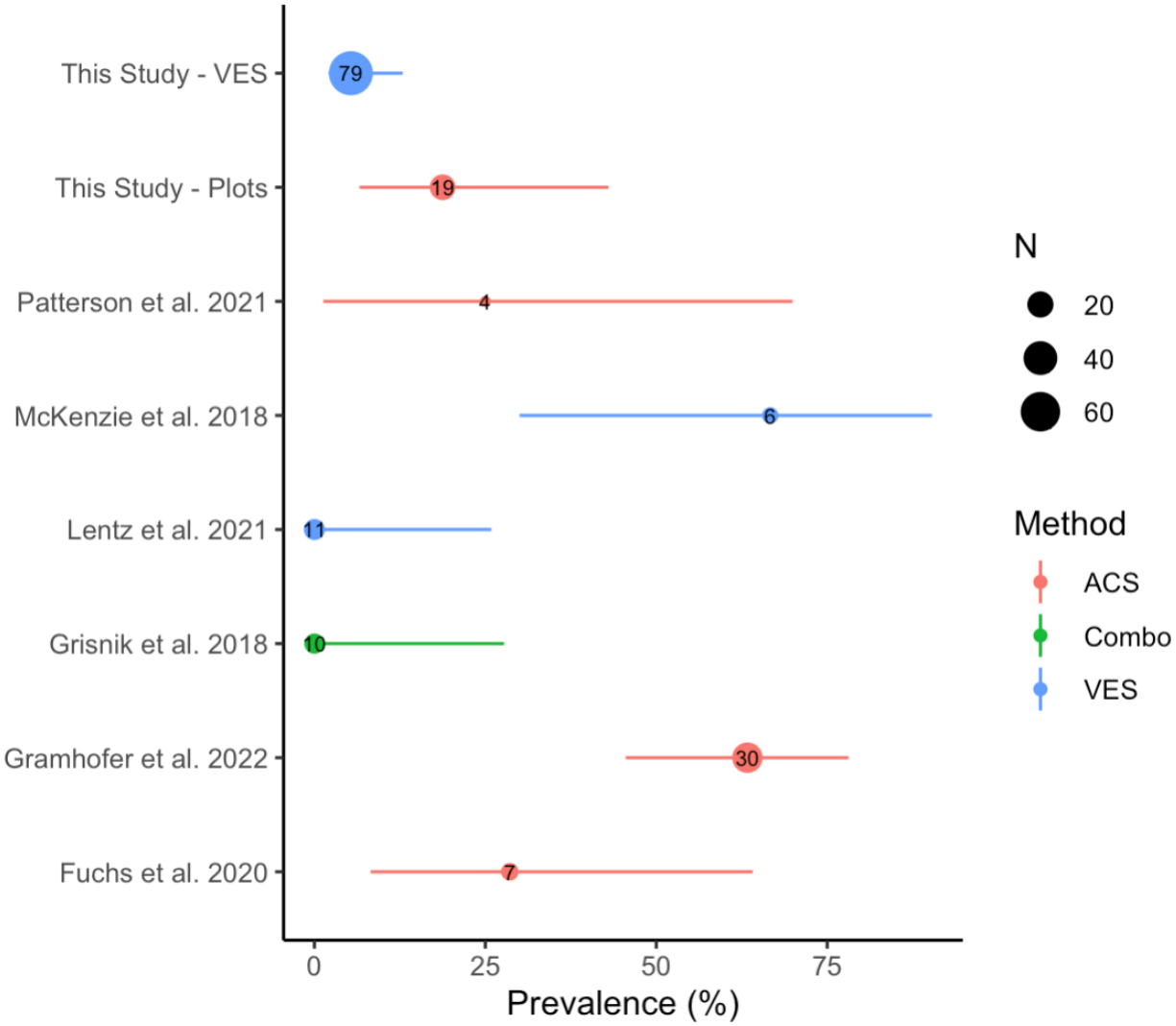

Figure 2.3. Prevalence of ophidiomycosis in *Carphophis amoenus* from our study and all other known studies of ophidiomycosis in *C. amoenus*. Colors represent various sampling methods (red: area-constrained searches, blue: opportunistic visual encounter surveys, or green: combination of area-constrained searches and visual encounter surveys) and circle sizes and numbers show sample sizes.

Figure 2.4. Clinical signs of ophidiomycosis observed in *Carphophis amoenus*. **A** and **B)** Ulcers and dry crusts observed on the body of a *C. amoenus* collected on 17 April 2022 from Eno River State Park, Durham County, NC. **C)** Dry crust observed on the snout of a *C. amoenus* collected on 5 May 2022 from Historic Yates Mill County Park, Wake County, NC.

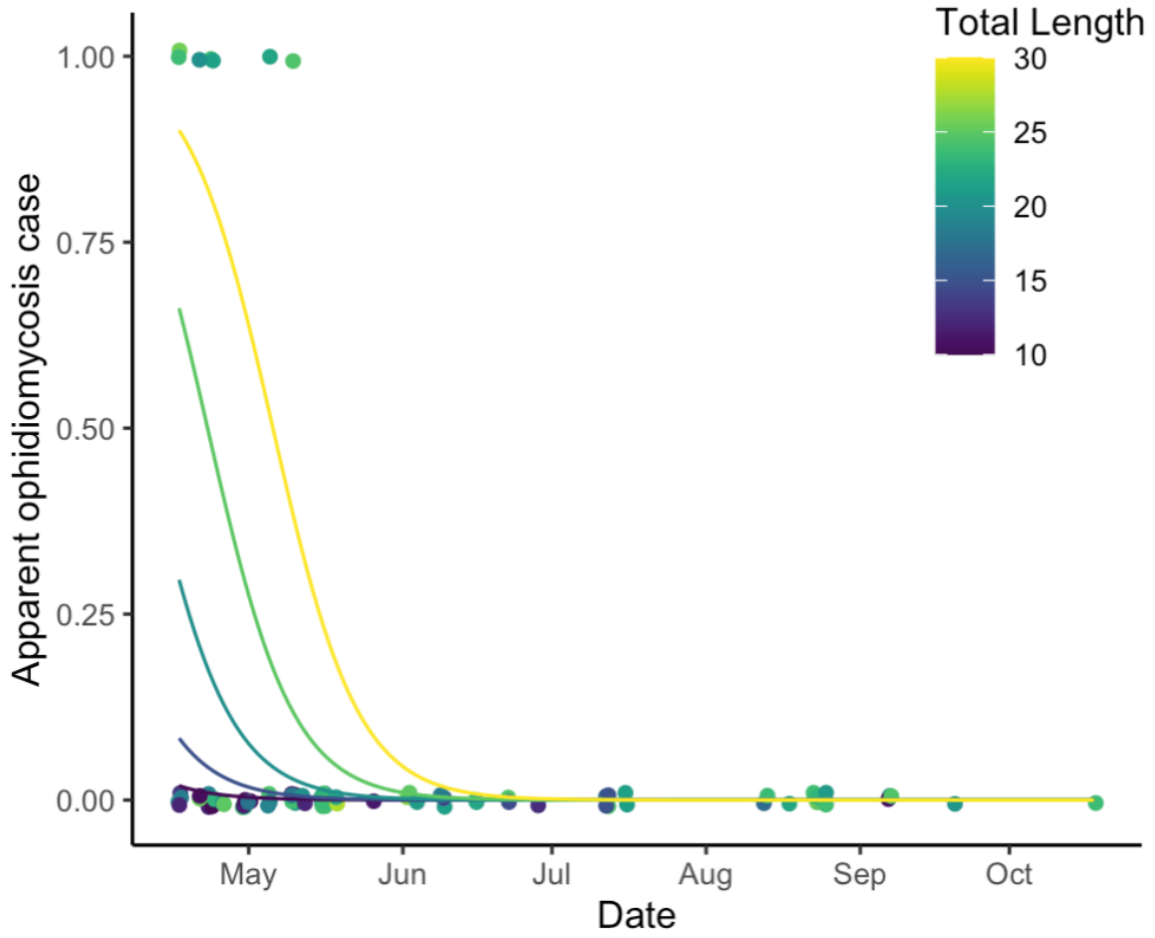

Figure 2.5. The probability that a given *Carphophis amoenus* had apparent ophidiomycosis declined with date and increased with total host length (mm). The curves show the fits of the most parsimonious logistic regression model and the points are jittered slightly on the Y axis to add visualization.

Discussion

Diurnal Piedmont snake communities were dominated by small-bodied species and most observed cases of apparent ophidiomycosis in these habitats were detected in small-bodied snakes (8/10 cases). In fact, we only encountered seven large bodied terrestrial colubrid snakes (representing three species and three parks), one of which was the first documented case of apparent ophidiomycosis in a mole kingsnake (*Lampropeltis rhombomaculata*; Oven et al., in press Herpetological Review). Among small-bodied snake species, *C. amoenus* was especially abundant in these snake communities (78% of individuals), and the probability that an individual *C. amoenus* had apparent ophidiomycosis varied among seasons and individuals; ophidiomycosis was more likely to be detected in the spring and in relatively large *C. amoenus*. Among all sampled snake species, apparent ophidiomycosis was only detected at some sites (5/11 parks and 0/5 backyards), which likely reflected achieved sample sizes and spatial variability in infectious processes. If we had only sampled large-bodied snake species, fewer sites would have documented cases of apparent ophidiomycosis (2/11 parks), suggesting that small-bodied species may add useful information for some forms of ophidiomycosis surveillance. However, small-bodied snakes still took extensive effort to find and diagnose.

Area-constrained searches and transects are time and personnel intensive, and because most transects/plots had no snakes (i.e., 72% of our $30m^2$ plots), it takes immense effort to generate snake sample sizes large enough to estimate disease prevalence. In contrast, visual encounter surveys require less effort to generate larger sample sizes, but they risk biasing disease surveillance efforts; for example, basking or shedding snakes may be more likely to be sampled, and snakes with ophidiomycosis may be more likely to bask or shed (McBride et al. 2015, Lorch et al. 2015, Lorch et al. 2016, Tetzlaff et al. 2017). However, there was no significant difference

between the prevalence estimated using the two methods for all snakes or only *C. amoenus* across all sampling areas and the entire sampling period. Additionally, our ophidiomycosis prevalence estimates from both methods were similar to other studies that included *C. amoenus* (Figure 2.3), but the confidence intervals from other studies tended to be wide due to low sample sizes. Future efforts to standardize surveillance methods and reporting would make studies easier to compare across space and time. For example, it is possible that our visual encounter surveys differed from those of other studies, because we attempted to catch and sample every terrestrial colubrid snake that we encountered and we analyzed all collected swabs, whereas other studies may have only sampled a subset of encountered snakes or processed only a subset of swabs. Therefore, we suggest that sampling all encountered snakes is important for avoiding biased prevalence estimates, and combining visual encounter surveys with transects or area-constrained searches is ideal for producing large enough sample sizes to accurately estimate incidence and prevalence.

Furthermore, the area-constrained searches (30 m^2 plots) that we used in this study created density and relative abundance estimates for small-bodied snakes that were comparable to other studies. We estimated that average *C. amoenus* density was 102.9 snakes/ha (CI 67.0 - 138.8) in parks and 1.48 snakes/ha (CI -0.70 - 3.65) in backyards. Backyards likely had lower snake density due to reduced cover item availability and habitat connectivity, as well as higher human disturbance (e.g., mowing, chemical use). Our park density estimates are similar to prior studies that detected 6.2 *C. amoenus* per 100m of drift fence in South Carolina (Russell & Hanlin 1999) and 108 C. *amoenus*/100 m transect/1 hour in Kentucky (Ernst & Barbour 1989), as well as a prior study that used funnel traps and detected 60–120 *C. vermis*/ha (Western worm snake) in Kansas (Clark 1970). In contrast, a four-year study in the western North Carolina Piedmont

that combined multiple survey methods (drift fences, pitfall traps, coverboards, and visual encounter surveys) estimated that there were only 1.32 *C. amoenus* per hectare (Willson & Dorcas 2004). Unfortunately, pitfall traps and drift fences are no longer usable methods in some places due to snake mortality (e.g., fire ants; Allen et al. 2016), including in North Carolina. Because we could not trap snakes, it is unclear if our estimates differed from Willson and Dorcas (2004) due to methodology or true population differences. However, Willson and Dorcas (2004) reported the highest *C. amoenus* abundances in dry upland forests, and our study included a greater proportion of dry upland forest sites than Willson and Dorcas (2004). When possible, combining multiple sampling efforts provides the best description of snake communities (Hutchens & DePerno 2009). At minimum, we suggest that at least one density-explicit ophidiomycosis surveillance method be used to complement visual encounter surveys, because host density often influences infectious disease processes (Anderson & May 1979, Grenfell & Dobson 1995, Krkošek 2010) and yet nothing is known about how host density affects ophidiomycosis dynamics.

While surveying for ophidiomycosis in small-bodied snakes, we encountered several challenges that were not discussed in previous papers. Most importantly, it was not easy to distinguish diseased *C. amoenus* in the field from uninfected or asymptomatic individuals. Because the snakes were small, their scale abnormalities and lesions were also small, which could easily be confused with damage from failed predation events or other injuries; many (47%) *C. amoenus* that tested negative for *Oo* DNA had at least one notable skin abnormality. Furthermore, most of these skin abnormalities in PCR-negative snakes fluoresced under 365 nm UV light, which either suggested that UV fluorescence was not a useful diagnostic tool for these snakes (in contrast to *Nerodia*; Vivirito et al. 2021) or that qPCR missed many fungus-positive

snakes. We expect the swabs and the standard qPCR test (Allender et al. 2015) were sensitive, because we usually used multiple swabs per snake to increase detectability (Hileman et al. 2018), and when one swab tested positive, most other swabs also tested positive; therefore, we suggest that one body swab and one lesion swab may be sufficient to detect *Oo* in most small-bodied snakes. Among the *C. amoenus* that tested positive by qPCR, 100% had clinical signs of ophidiomycosis in the form of lesions and dry crusts that lacked blood or pustular discharge, which were usually located on the body but sometimes occurred on the head near the mouth (see Table 2.1, Figure 2.4). To our knowledge, our study is the first to describe and photograph these symptoms, although other studies have noted that clinical signs were present (McKenzie et al. 2019, Gramhofer et al. 2022). Unfortunately, skin biopsies would be too invasive for these small-bodied snakes, so we could not confirm ophidiomycosis using histology (Baker et al. 2019) which remains an important avenue for future research with *C. amoenus*, potentially using vehicle-killed snakes. Overall, we conclude that ophidiomycosis surveillance with small-bodied snakes faced many of the same challenges as that with large-bodied snakes, and diagnostic clarity could be improved through additional research.

Although this was the largest study of ophidiomycosis in small-bodied snake species to date, this study was limited to terrestrial habitats in three counties and a single survey year. Longer-term and broader-scale studies are needed to fully understand ophidiomycosis dynamics. For example, none of the microclimate variables included in our study were predictors of apparent ophidiomycosis in *C. amoenus*, but we only observed eight *C. amoenus* with apparent ophidiomycosis and 2022 was an especially dry year. Sampling across multiple years, and especially across multiple springs (when disease is more likely), might provide a broader gradient of environmental conditions and disease outcomes. Furthermore, more research is

needed to understand how ophidiomycosis prevalence and severity vary across ecosystems and ecoregions in North Carolina (e.g., in the Appalachian Mountains and Coastal Plain). Future studies that track individual survival or population dynamics of small-bodied snakes with ophidiomycosis across space and time will be especially useful for understanding the current impacts of this disease and whether it will be exacerbated by future global change.

Most ophidiomycosis monitoring studies have focused on large-bodied and often threatened snake species, and data gaps make it difficult to determine whether small-bodied snakes experience similar disease prevalence, severity, and population impacts. Here, we show that because of their high relative abundance, small-bodied snakes are relatively easy to sample in large numbers and may be useful indicators of ophidiomycosis presence on the landscape. Also, we demonstrated that surveillance in small-bodied snakes can parallel relationships detected in larger-bodied snakes; for example, *C. amoenus* were more likely to have ophidiomycosis in the spring, which has been documented in many other studies where large-bodied snakes are more likely to have disease symptoms during and just after brumation (McCoy et al. 2017, Lind & Moore et al. 2018, Lind et al. 2022). While snake surveillance will always be time consuming and ophidiomycosis diagnostics will be costly, focusing on small-bodied snakes may provide relatively high-resolution data for relatively low effort, depending on the specific surveillance goals.

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APPENDICES

Appendix A: Chapter 1

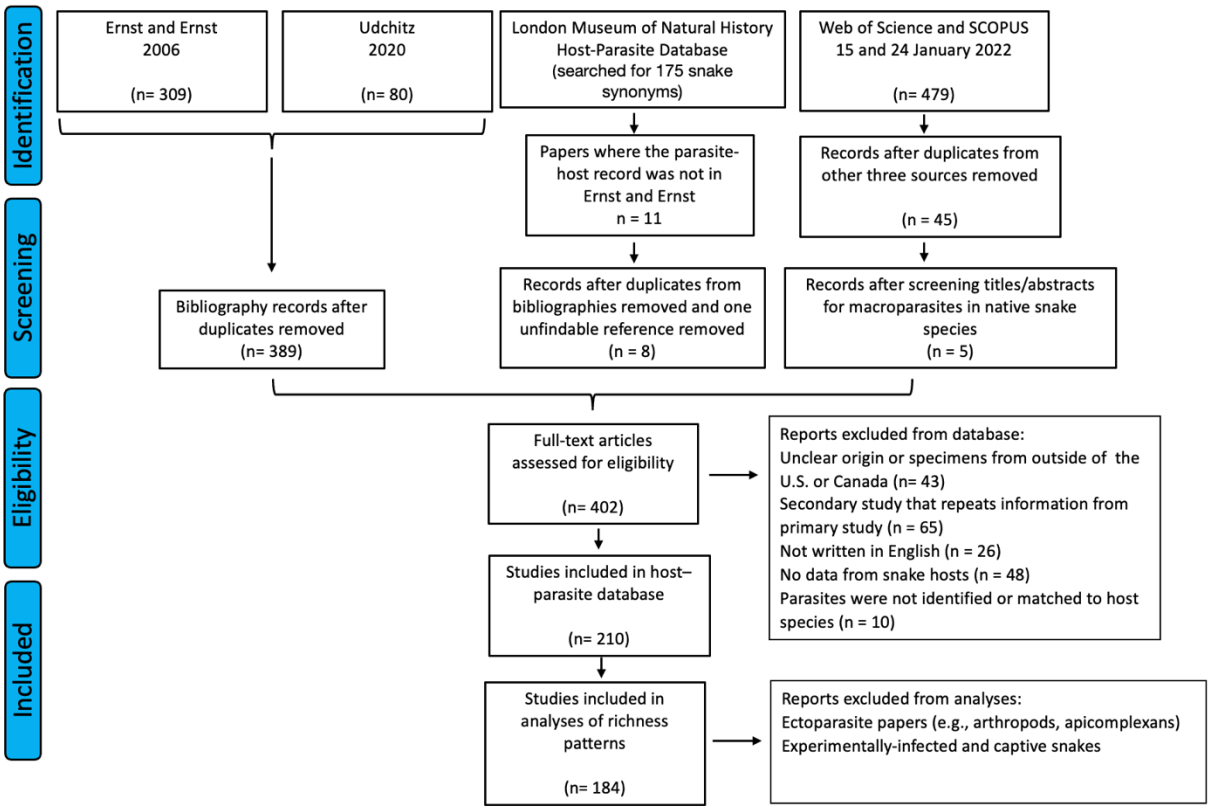
Supplementary Table A.1. Logistic regression for the probability that a given snake species from the United States or Canada had been surveyed in at least one parasitological study. Parameter estimates are on the logit scale and can be back transformed as exp(x)/(1+exp(x)).

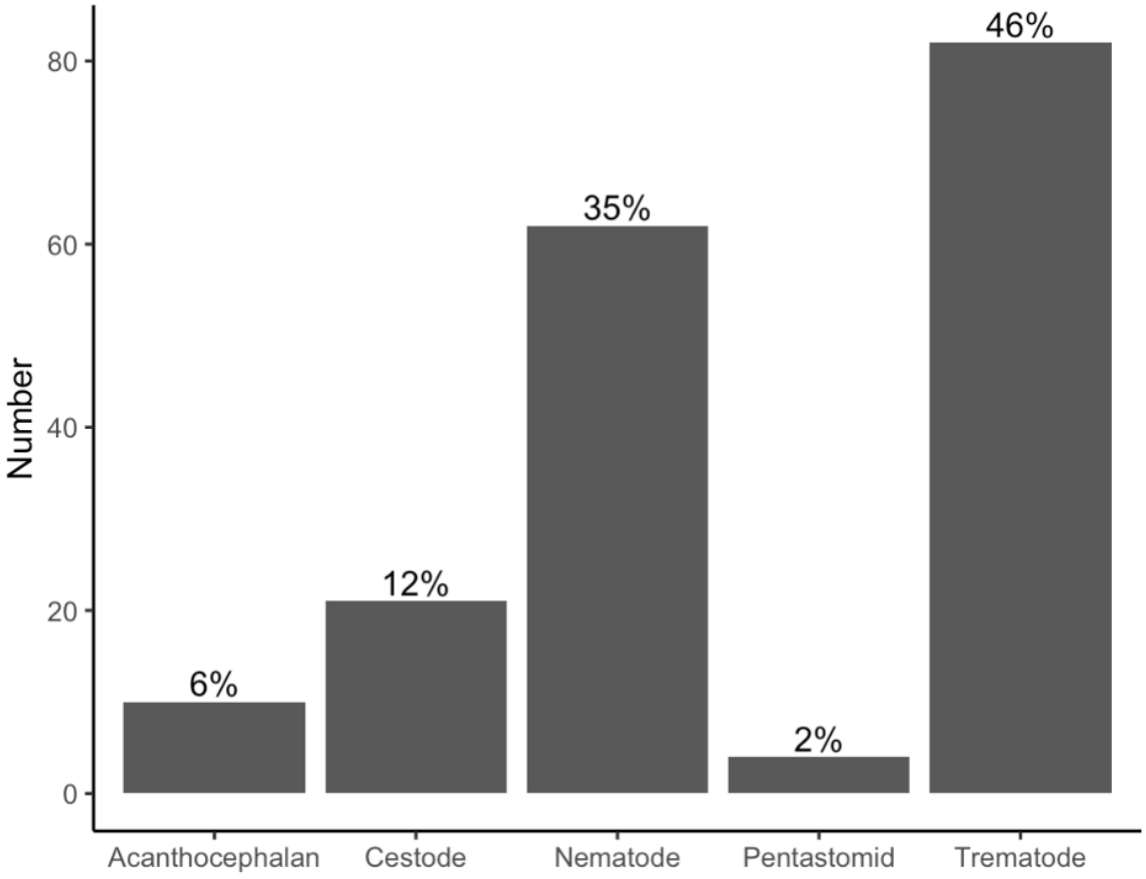
Parameter	Coefficient estimate	Standard Error	P value
Intercept (colubrids)	-2.11	0.45	<0.001
Maximum SVL (mm)	0.0009	0.0003	0.003
Latitudinal range	0.05	0.02	0.03
"Other" snake family	1.34	0.96	0.16
Viperidae	0.68	0.45	0.12

Supplemental Table A.2. Phylogenetic linear model for estimated parasite species richness in snake species from the United States or Canada that were included in at least one parasitological study. The phylogenetic random effects variance was 0.003, with a standard deviation of 0.06, indicating that species relatedness only explained a small amount of variation in estimated parasite richness.

Parameter	Coefficient estimate	Standard Error	P value
Intercept	-1.01	0.72	0.16
Maximum SVL (mm)	0.001	0.0003	<0.001
Latitudinal range	0.05	0.02	0.02
Amphibian or fish prey	0.84	0.39	0.03
Terrestrial vertebrate prey	-0.58	0.37	0.12
Invertebrate prey	-0.05	0.39	0.90

Supplemental Figure A.1. Systematic literature Review PRISMA diagram.





Supplemental Figure A.2. The number of parasite species observed for each parasite taxon in snakes sampled from the United States and Canada. Helminths, especially trematodes and nematodes, were the most reported parasites taxa in snakes. Percentages represent the number of parasite species in one taxon compared to the total observed number of species across taxa.

Supplemental Methods

Web of Science and Scopus database searches were conducted on 15 and 24 January 2022 with ~439 search terms that were categorized by geography, snake taxonomy, and parasite taxonomy. Search strings took the form of [geographical terms] AND [snake taxonomy terms] AND [parasite taxonomy terms]. The specific terms are listed for each category below.

Geographical Terms

"United States" OR U.S.A. OR Canada OR Alabama OR Alaska OR Arizona OR Arkansas OR California OR Colorado OR Connecticut OR Delaware OR Florida OR Georgia OR Hawaii OR Idaho OR Illinois OR Indiana OR Iowa OR Kansas OR Kentucky OR Louisiana OR Maine OR Maryland OR Massachusetts OR Michigan OR Minnesota OR Mississippi OR Missouri OR Montana OR Nebraska OR Nevada OR "New Hampshire" OR "New Jersey" OR "New Mexico" OR "New York" OR "North Carolina" OR "North Dakota" OR Ohio OR Oklahoma OR Oregon OR Pennsylvania OR "Rhode Island" OR "South Carolina" OR "South Dakota" OR Tennessee OR Texas OR Utah OR Vermont OR Virginia OR Washington OR "West Virginia" OR Wisconsin OR Wyoming OR Alberta OR "British Columbia" OR Manitoba OR "New Brunswick"

Newfoundland OR Labrador OR “Northwest Territories” OR “Nova Scotia” OR Nunavut OR Ontario OR “Prince Edward Island” OR Quebec OR Saskatchewan OR Yukon

Parasite terms
“Parasitic worm” OR Endoparasit* OR Helminth* OR Platyhelminth* OR Nematod* OR Trematod* OR Cestod* OR Acanthocephala* OR Tapeworm* OR Fluke* OR Roundworm* OR Pentastom*

Snake terms
Leptotyphlopid* OR Typhlopid* OR Boidae OR Colubrid* OR Viperid* OR Elapid* OR "Agkistrodon conanti" OR "Agkistrodon contortrix" OR "Agkistrodon laticinctus" OR "Agkistrodon piscivorus" OR "Arizona elegans" OR "Arizona elegans arenicola" OR "Arizona elegans candida" OR "Arizona elegans eburnata" OR "Arizona elegans elegans" OR "Arizona elegans noctivaga" OR "Arizona elegans occidentalis" OR "Arizona elegans philipi" OR "Bogertophis rosaliae" OR "Bogertophis subocularis" OR "Bogertophis subocularis subocularis" OR "Carphophis amoenus" OR "Carphophis amoenus amoenus" OR "Carphophis amoenus helenae" OR "Carphophis vermis" OR "Cemophora coccinea" OR "Cemophora coccinea coccinea" OR "Cemophora coccinea copei" OR "Cemophora lineri" OR "Charina bottae" OR "Charina umbratica" OR "Charina trivirgata" OR "Chilomeniscus stramineus" OR "Chilomeniscus cinctus" OR "Chionactis annulata" OR "Chionactis annulata annulata" OR "Chionactis annulata klauberi" OR "Chionactis occipitalis" OR "Chionactis palarostris" OR "Chionactis palarostris organica" OR "Clonophis kirtlandii" OR "Coluber bilineatus" OR "Coluber constrictor" OR "Coluber constrictor anthicus" OR "Coluber constrictor constrictor" OR "Coluber constrictor etheridgei" OR "Coluber constrictor flaviventris" OR "Coluber constrictor foxii" OR "Coluber constrictor helvigularis" OR "Coluber constrictor latrunculus" OR "Coluber constrictor mormon" OR "Coluber constrictor oaxaca" OR "Coluber constrictor paludicola" OR
"Coluber constrictor priapus" OR "Coluber fuliginosus" OR "Coluber lateralis" OR "Coluber lateralis euryxanthus" OR "Coluber lateralis lateralis" OR "Coluber schotti" OR "Coluber schotti ruthveni" OR "Coluber schotti schotti" OR "Coluber taeniatus" OR "Coluber taeniatus girardi" OR "Coluber taeniatus taeniatus" OR "Coniophanes imperialis" OR "Coniophanes imperialis imperialis" OR "Contia longicauda" OR "Contia tenuis" OR "Crotalus adamanteus" OR "Crotalus atrox" OR "Crotalus cerastes" OR "Crotalus cerastes cerastes" OR "Crotalus cerastes cercobombus" OR "Crotalus cerastes laterorepens" OR "Crotalus cerberus" OR "Crotalus horridus" OR "Crotalus lepidus" OR "Crotalus lepidus klauberi" OR "Crotalus lepidus lepidus" OR "Crotalus mitchellii" OR "Crotalus molossus" OR "Crotalus oreganus" OR "Crotalus oreganus abyssus" OR "Crotalus oreganus concolor" OR "Crotalus oreganus helleri" OR "Crotalus oreganus lutosus" OR "Crotalus oreganus oreganus" OR "Crotalus ornatus" OR "Crotalus pricei" OR "Crotalus pricei pricei" OR "Crotalus pyrrhus" OR "Crotalus ruber" OR "Crotalus scutulatus" OR "Crotalus scutulatus scutulatus" OR "Crotalus stephensi" OR "Crotalus tigris" OR "Crotalus viridis" OR "Crotalus willardi" OR "Crotalus willardi obscurus" OR "Crotalus willardi willardi" OR "Diadophis punctatus" OR "Diadophis punctatus acricus" OR "Diadophis punctatus amabilis" OR "Diadophis punctatus arnyi" OR "Diadophis punctatus edwardsii" OR
"Diadophis punctatus modestus" OR "Diadophis punctatus occidentalis" OR "Diadophis punctatus pulchellus" OR "Diadophis punctatus punctatus" OR "Diadophis punctatus regalis"

OR "Diadophis punctatus similis" OR "Diadophis punctatus stictogenys" OR "Diadophis punctatus vandenburgii" OR "Drymarchon corais" OR "Drymarchon couperi" OR "Drymarchon kolpobasileus" OR "Drymarchon melanurus" OR "Drymarchon melanurus erebennus" OR "Drymobius margaritiferus" OR "Drymobius margaritiferus margaritiferus" OR "Elaphe bairdi" OR "Elaphe emoryi" OR "Elaphe guttata" OR "Elaphe obsoleta" OR "Elaphe vulpina" OR "Farancia abacura" OR "Farancia abacura abacura" OR "Farancia abacura reinwardtii" OR "Farancia erytrogramma" OR "Farancia erytrogramma erytrogramma" OR "Farancia erytrogramma seminola" OR "Ficimia streckeri" OR "Gyalopion canum" OR "Gyalopion quadrangulare" OR "Haldea striatula" OR "Heterodon gloydi" OR "Heterodon kennerlyi" OR "Heterodon nasicus" OR "Heterodon platirhinos" OR "Heterodon simus" OR "Hydrophis platurus" OR "Hypsiglena chlorophaea" OR "Hypsiglena chlorophaea deserticola" OR "Hypsiglena chlorophaea loreala" OR "Hypsiglena chlorophaea chlorophaea" OR "Hypsiglena jani" OR "Hypsiglena jani texana" OR "Hypsiglena ochrorhyncha" OR "Hypsiglena ochrorhyncha nuchalata" OR "Hypsiglena ochrorhyncha klauberi" OR "Hypsiglena torquata" OR "Lampropeltis alterna" OR "Lampropeltis annulata" OR "Lampropeltis californiae" OR "Lampropeltis calligaster" OR "Lampropeltis elapsoides" OR "Lampropeltis extenuata" OR "Lampropeltis floridana" OR "Lampropeltis gentilis" OR "Lampropeltis getula" OR "Lampropeltis holbrooki" OR "Lampropeltis knoblochi" OR "Lampropeltis meansi" OR "Lampropeltis multifasciata" OR "Lampropeltis nigra" OR "Lampropeltis nigrita" OR "Lampropeltis occipitolineata" OR "Lampropeltis pyromelana" OR "Lampropeltis rhombomaculata" OR "Lampropeltis splendida" OR "Lampropeltis triangulum" OR "Lampropeltis zonata" OR "Leptodeira septentrionalis" OR "Leptotyphlops dulcis" OR "Leptotyphlops humilus" OR "Lichanura orcutti" OR "Lichanura trivirgata" OR "Liochlorophis vernalis" OR "Liodytes alleni" OR "Liodytes pygaea" OR "Liodytes pygaea cyclas" OR "Liodytes pygaea paludis" OR "Liodytes pygaea pygaea" OR "Liodytes rigida" OR "Liodytes rigida deltae" OR "Liodytes rigida rigida" OR "Liodytes rigida sinicola" OR "Masticophis bilineatus" OR "Masticophis flagellum" OR "Masticophis lateralus" OR "Masticophis schotti" OR "Masticophis taeniatus" OR "Micruroides euryxanthus" OR "Micruroides euryxanthus euryxanthus" OR "Micrurus fulvius" OR "Micrurus tener" OR "Micrurus tener tener" OR "Nerodia clarkii" OR "Nerodia clarkii clarkii" OR "Nerodia clarkii compressicauda" OR "Nerodia clarkii taeniata" OR "Nerodia cyclopion" OR "Nerodia erythrogaster" OR "Nerodia fasciata" OR "Nerodia fasciata confluens" OR "Nerodia fasciata fasciata" OR "Nerodia fasciata pictiventris" OR "Nerodia floridana" OR "Nerodia harteri" OR "Nerodia paucimaculata" OR "Nerodia rhombifer" OR "Nerodia rhombifer rhombifer" OR "Nerodia sipedon" OR "Nerodia sipedon insularum" OR "Nerodia sipedon pleuralis" OR "Nerodia sipedon sipedon" OR "Nerodia sipedon williamengelsi" OR "Nerodia taxispilota" OR "Opheodrys aestivus" OR "Opheodrys aestivus aestivus" OR "Opheodrys aestivus carinatus" OR "Opheodrys vernalis" OR "Oxybelis aeneus" OR "Pantherophis alleghaniensis" OR "Pantherophis bairdi" OR "Pantherophis emoryi" OR "Pantherophis guttatus" OR "Pantherophis obsoletus" OR "Pantherophis ramspotti" OR "Pantherophis slowinskii" OR "Pantherophis spiloides" OR "Pantherophis vulpinus" OR "Pelamis platurus" OR "Phyllorhynchus browni" OR "Phyllorhynchus decurtatus" OR "Pituophis catenifer" OR "Pituophis catenifer affinis" OR "Pituophis catenifer annectens" OR "Pituophis catenifer catenifer" OR "Pituophis catenifer deserticolor" OR "Pituophis catenifer pumilus" OR "Pituophis catenifer sayi" OR "Pituophis melanoleucus" OR "Pituophis melanoleucus lodingi" OR "Pituophis melanoleucus melanoleucus" OR "Pituophis melanoleucus mugitus" OR "Regina grahamii" OR "Regina septemvittata" OR "Regina alleni" OR "Regina rigida" OR "Rena

dissecta" OR "Rena dulcis" OR "Rena dulcis dulcis" OR "Rena dulcis rubella" OR "Rena humilis" OR "Rena humilis cahuilae" OR "Rena humilis humilis" OR "Rena humilis segrega" OR "Rena humilis utahensis" OR "Rhadinaea flavilata" OR "Rhinocheilus lecontei" OR "Salvadora deserticola" OR "Salvadora grahamiae" OR "Salvadora grahamiae grahamiae" OR "Salvadora grahamiae lineata" OR "Salvadora hexalepis" OR "Salvadora hexalepis deserticola" OR "Salvadora hexalepis hexalepis" OR "Salvadora hexalepis mojavensis" OR "Salvadora hexalepis virgultea" OR "Seminatrix pygaea" OR "Senticolis triaspis" OR "Senticolis triaspis intermedia" OR "Sistrurus catenatus" OR "Sistrurus miliarius" OR "Sistrurus miliarius barbouri" OR "Sistrurus miliarius miliarius" OR "Sistrurus miliarius streckeri" OR "Sistrurus tergeminus" OR "Sistrurus tergeminus tergeminus" OR "Sistrurus tergeminus edwardsii" OR "Sonora semiannulata" OR "Sonora semiannulata semiannulata" OR "Sonora semiannulata taylori" OR "Stilosoma extenuatum" OR "Storeria dekayi" OR "Storeria occipitomaculata" OR "Storeria victa" OR "Tantilla atriceps" OR "Tantilla coronata" OR "Tantilla cucullata" OR "Tantilla gracilis" OR "Tantilla hobartsmithi" OR "Tantilla nigriceps" OR "Tantilla oolitica" OR "Tantilla planiceps" OR "Tantilla relicta" OR "Tantilla relicta neilli" OR "Tantilla relicta pamlica" OR "Tantilla relicta relicta" OR "Tantilla wilcoxi" OR "Tantilla yaquia" OR "Thamnophis atratus" OR "Thamnophis atratus atratus" OR "Thamnophis atratus hydrophilus" OR "Thamnophis atratus zaxanthus" OR "Thamnophis brachystoma" OR "Thamnophis butleri" OR "Thamnophis couchii" OR "Thamnophis cyrtopsis" OR "Thamnophis cyrtopsis cyrtopsis" OR "Thamnophis cyrtopsis ocellatus" OR "Thamnophis elegans" OR "Thamnophis elegans elegans" OR "Thamnophis elegans terrestris" OR "Thamnophis elegans vagrans" OR "Thamnophis eques" OR "Thamnophis eques megalops" OR "Thamnophis gigas" OR "Thamnophis hammondii" OR "Thamnophis marcianus" OR "Thamnophis marcianus marcianus" OR "Thamnophis ordinoides" OR "Thamnophis proximus" OR "Thamnophis proximus diabolicus" OR "Thamnophis proximus orarius" OR "Thamnophis proximus proximus" OR "Thamnophis proximus rubrilineatus" OR "Thamnophis radix" OR "Thamnophis rufipunctatus" OR "Thamnophis saurita" OR "Thamnophis saurita nitae" OR "Thamnophis saurita sackenii" OR "Thamnophis saurita saurita" OR "Thamnophis sauritus" OR
"Thamnophis saurita septentrionalis" OR "Thamnophis sirtalis" OR "Thamnophis sirtalis annectens" OR "Thamnophis sirtalis concinnus" OR "Thamnophis sirtalis dorsalis" OR "Thamnophis sirtalis fitchi" OR "Thamnophis sirtalis infernalis" OR "Thamnophis sirtalis pallidulus" OR "Thamnophis sirtalis parietalis" OR "Thamnophis sirtalis pickeringii" OR "Thamnophis sirtalis semifasciatus" OR "Thamnophis sirtalis similis" OR "Thamnophis sirtalis sirtalis" OR "Thamnophis sirtalis tetrataenia" OR "Trimorphodon biscutatus" OR "Trimorphodon lambda" OR "Trimorphodon lyrophanes" OR "Trimorphodon vilkinsonii" OR "Tropidoclonion lineatum" OR "Virginia striatula" OR "Virginia valeriae" OR "Virginia valeriae elegans" OR "Virginia valeriae valeriae" OR "Virginia valeriae pulchra"

Appendix B: Chapter 2

Supplemental Table B.1. Habitats that were sampled in each of our plots across all 11 parks in Wake, Durham, and Orange Counties, North Carolina in 2022. Habitats that were described as "Other" included areas sampled that were a mix of habitats (e.g., dry pine forest and flood plain). Note: dry pine and oak/hickory are upland habitats.

Habitat	Percent of plots (%)
Dry Pine	29 (23/80)
Edge Habitat	1 (1/80)
Field/Clearing	18 (14/80)
Flood Plain	8 (6/80)
Mesic Upland	15 (12/80)
Oak/Hickory	26 (21/80)
Other	3 (2/80)
Wetland	1 (1/80)

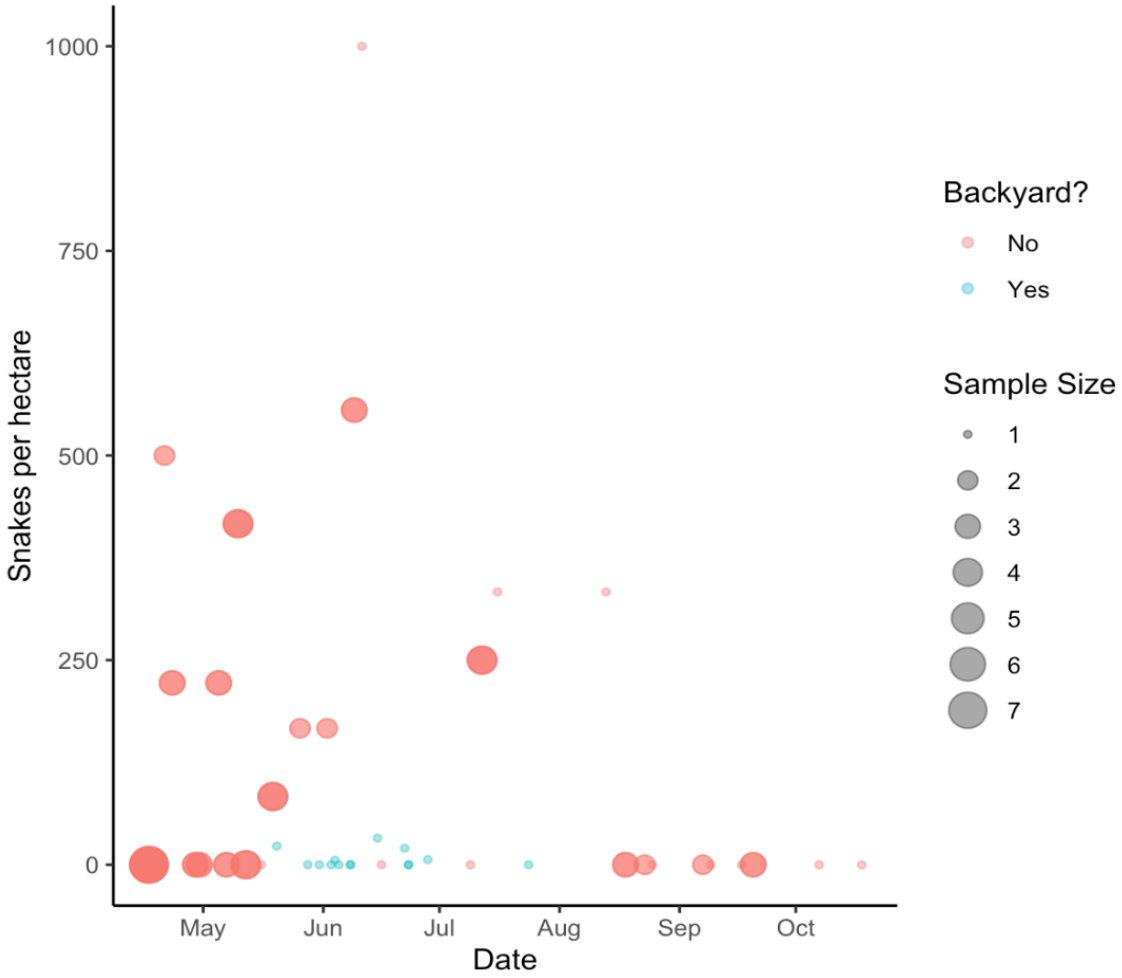
Supplemental Table B.2. Model output and model selection criteria for five binomial generalized linear models for describing how the probability that an individual sampled *C. amoenus* had apparent ophidiomycosis varied with snake size, season, and/or microclimate conditions. Size is the total length (snout to tail tip) in mm, temperature is the soil temperature where the snake was found (°C), and soil moisture (%) and pH are the micro soil conditions where the snake was found.

Model with lowest AIC

Parameter	**Parameter estimate (SE)**	**P value**
Intercept	2224.7 (936.9)	0.02
Date	-0.12 (0.05)	0.02
Size	0.31 (0.15)	0.04

Model comparisons:

Predictors	**df**	**AIC**
Date + Size	3	36.5
Date + Temperature + Size	4	38.1
Date + Temperature + Size + Soil moisture	4	38.1
Date + Temperature + Size + pH	4	38.3
Temp + Size	3	44.9



Supplemental Figure B.1. Estimated snake density based on area-constrained searches, where each circle represents a given backyard or park on a given survey date. The size of the circle corresponds to the sample size of snakes.