Hotspots for snake fungal disease across Europe are maintained by host and pathogen identity

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Abstract

Infectious diseases are influenced by interactions between host and pathogens in variable environments and are rarely homogenous across the landscape. Areas with elevated pathogen burden and transmission may indicate a disruption to steady-state disease dynamics. However, isolating processes that result in increases in infection prevalence and intensity remains a challenge. Here we elucidate the contribution of host species, and pathogen clade in disease hotspots. We examined broad-scale patterns of infection of *O. ophidiicola,* the pathogen that causes snake fungal disease, in 21 species of snakes across 10 countries in Europe. Disease hotspots were evident across several regions in Europe, and our analyses revealed significant differences in infection based on host species and pathogen clade. Over 80% of positive detections were from host species in the *Natrix* genus, indicating potential higher susceptibility in this group. The presence of *O. ophidiicola* genotypes that have been associated with more severe disease in North America, also resulted in high rates of infection compared to genotypes only described from Europe. Elevated infection prevalence was best explained by an interaction between host and pathogen identity which was not uniform across all species. More broadly, these findings present important mechanisms underlying disease hotspots across a disease endemic region.

1. Introduction

Infectious diseases can shape ecological communities by altering host abundance and distributions across the landscape [1-3]. Disease outcomes are determined by host-pathogen interactions which are multifaceted and can interact with environmental conditions, creating a mosaic of disease hotspots across broad spatial scales [4-7]. For diseases that have been established for long periods of time in host communities, hotspots of high pathogen burden are locations where disease dynamics may no longer be in steady state [8], and thus represent potential areas of continued impacts to host populations.

Heterogeneity in species innate susceptibility is recognized as a strong force influencing pathogen transmission and disease impacts for multi-host pathogens [1, 9-11]. The distribution of highly susceptible species can determine areas of high prevalence if they are critical in pathogen maintenance [12, 13]. However, the disproportionate contribution of a particular species may be modified by differences in community structure, environmental conditions among patches, and variation in pathogen virulence [5, 14]. Pathogen virulence can also differ across the landscape, producing additional variation in disease prevalence [15, 16]. Areas can maintain highly virulent strains due to multiple factors, including the introduction of novel strains to new locations or hosts [17, 18], ease or independence of transmission from affected hosts [19, 20], and the development of novel mutations or adaptations that facilitate the escape from host resistance [21]. Although the interaction between host and pathogen identity is rarely examined, theory suggests that presence of highly resistant species could modify the effects of pathogen virulence creating cold spots of transmission across the landscape [22]. Conversely, highly virulent pathogen strains in the presence of more moderately affected species could drive hotspots of infection [23-25].

The fungal pathogen *Ophidiomyces ophidiicola,* that causes snake fungal disease (SFD), has been documented in over 42 species of wild snakes across three continents [26-33], and is considered a serious threat to the conservation of snake populations. Infections caused by *O. ophidiicola* can range from mild skin lesions, from which snakes can recover, to severe infections that impair movement, disrupt feeding behavior, and can ultimately lead to death [26]. Although population declines associated with SFD have been documented in some species of North American snakes [26], no such declines have been reported in species across Europe, where *O. ophidiicola* has most likely coexisted with snakes for longer periods of time [34].

However, only limited information is available on *O. ophidiicola* infections across Europe, with just a few individual snakes confirmed to be infected with this disease from mainland Europe [28, 29, 35].

Little is known of the origin of *O. ophidiicola*. However, there have been three distinct clades of *O. ophidiicola* described to date: clade I has been found exclusively in wild snakes in Europe; clade II has been reported in wild snakes in North America and Taiwan, as well as in captive snakes on multiple continents; and clade III, which has only been found in captive snakes [34]. The estimation of the most recent common ancestor between clade I and II (around 2000 years ago), as well as a lack of nonrecombinant intermediates in North America strongly indicate that *O. ophidiicola* was introduced to North America, potentially through multiple introduction events [34]. More recently, limited genotyping indicated the presence of both clades I and II in Switzerland dating back to at least 1959 [35]. In addition, slower growth rates have been reported for clade I strains of *O. ophidiicola*, suggesting that they may be less virulent than clade II strains [28]. However, prevalence and severity associated with the two strains has not been directly compared.

To investigate the macroecological patterns of SFD across Europe we examined host and pathogen factors that may influence variable intensity of infection across the landscape. We assessed the relative importance of geographic hotspots of infection, differences in susceptibility among hosts (21 species belonging to six different genera), and the effect of *O. ophidiicola* clade on infection prevalence and severity. By combining host species and pathogen clade, our study is the first to investigate factors that may lead to disruption of steady state disease dynamics and result in hotspots of infection across the landscape in Europe.

2. Methods

(a) Location and host species considered

Free-ranging snakes were captured from March 2020 to June 2022 across 10 countries: Portugal, Spain, France, Switzerland, Germany, Austria, Czech Republic, Hungary, Poland, and Ukraine. The number of sites where snakes were collected ranged from one to nine per country, for a total of 45 sites. A total of 21 species belonging to 6 genera were sampled representing important variation in behavior and ecology.

(b) Capture and sampling

Snakes were located by visual encounter surveys, an approach frequently used for sampling snakes [36]. Surveys were guided by pre-existing knowledge of snake presence or prediction of suitable habitats to achieve sufficient sample sizes. Snakes were captured by hand, placed in individual cloth bags for temporary holding during processing and sampling, and released at their capture location. Sterile handling procedures, including frequent glove changes and decontamination of gear between each snake were followed during sample collection to avoid cross-contamination. Snakes were individually identified using photo-identification or marking. For each snake captured, location and morphometric data were collected including latitude/longitude, species, sex, snout-vent-length, tail length, and weight.

Snakes were swabbed in duplicate using a pre-moistened, sterile polyester-tipped applicator (Puritan®, Guilford, Maine, USA) by running the swab five times (back and forth counting as a single pass) on the ventral and dorsal areas (from the neck down to the vent), and two times on the face of the snake. If a skin lesion was observed, a separate swab was used to specifically swab the lesion and skin immediately adjacent to it by forcefully rubbing the swab over the affected skin. In addition, for all snakes that had visible lesions, we collected photos for later quantification of infection severity (see below). Swab tips were individually stored in a 2 mL sterile tube in a cooler with ice while in the field, and later stored frozen at -20°C until analysis.

(c) Sample extraction and qPCR

DNA was extracted from swabs using 250 µL of PrepMan® Ultra Sample Preparation Reagent (Life Technologies, Carlsbad, California, USA) with 100 mg of zirconium/silica beads, following previously published protocol [37]. Briefly, samples were homogenized for 45 sec in a bead beating grinder and lysis system (MP Biomedicals, Irvine, California, USA) and centrifuged for 30 sec at 13000 g to settle all material to the bottom of the tube. Homogenization and centrifugation steps were repeated, and tubes were incubated at 100°C in a heat block for 10 min. Tubes were then cooled at room temperature for 2 min, then centrifuged 3 min at 13000 g. Fifty to 100 µL of supernatant was recovered and stored at -80°C. Extraction blanks (negative controls) were prepared using 250 µL of PrepMan® Ultra Sample Preparation Reagent and 10 mg of zirconium/silica beads only.

Quantitative PCR targeting the internal transcribed spacer region (ITS) specific to *O. ophiodiicola* was performed on a real-time PCR QuantStudio 5 (Thermofisher Scientific, Waltham, Massachusetts, USA) [38]. QuantiFast Master Mix (QuantiFast Probe PCR + ROX vial kit, Qiagen, Germantown, USA) was prepared according to manufacter's recommendations for a final reaction volume of 25 µL, which included 5 µL of extracted DNA. Cycling conditions were as followed: 95°C for 3 min, then 95°C for 3 sec and 60°C for 30 sec for a total of 40 cycles. For each plate run, a negative control (water added instead of extracted DNA) and a 6-point (each point run in triplicate) standard curve using synthetic double-stranded DNA (gBlock, Integrated DNA Technologies, Coralville, Iowa) of the target region (1.0×10^{2}, 1.0×10^{1}, 1.0×10^{0}, 1.0×10^{-1}, 1.0×10^{-2}, 1.0×10^{-3} fg/µL) were included. Samples were analyzed in duplicate, and a snake was determined to be positive if any swab associated with that snake was positive by qPCR.

(d) Sequencing and genotyping

Samples in which *O. ophidiicola* was detected by qPCR were subjected to follow up genotyping analysis. We targeted a portion of the internal transcribed spacer 2 (ITS2) for this analysis because the ITS2 exhibits variability between previously described clades of *O. ophidiicola* and because ITS2 is a multicopy gene that can be amplified from samples containing very small amounts of *O. ophidiicola* DNA (it is also the target of the qPCR assay). We used a nested PCR protocol that consisted of first amplifying the entire ITS2 region with the panfungal primer ITS3 and ITS4 [39]. The first reaction consisted of 10 µL of 2x QuantiNova probe PCR master mix (Qiagen, Venlo, Netherlands), 3.9 µL of molecular grade water, 0.5 µL of each primer (20 µM each), 0.1 µL of 20µg/µL bovine serum albumin, and 5 µL of DNA extracted with the PrepMan procedure described above. Cycling conditions were as follows: 95˚C for 3 min; 40 cycles of 95˚C for 10 sec, 56˚C for 30 sec, and 72˚C for 30 sec; final extension at 72˚C for 5 min. For the second reaction, primers ITS3 and Oo-rt-ITS-R [38] were used. Each reaction consisted of 0.5 µL of the PCR product from the first reaction added to 13.375 µL molecular grade water, 5 µL of GoTaq Flexi buffer (Promega Corporation, Madison, Wisconsin, USA), 2 µL of dNTPs (2.5 mM each), 1.5 µL of 25 mM $MgCl_2$, 1.25 µL of each primer (20 µM each), and 0.25 µL of GoTaq polymerase. Cycling conditions for the second PCR were: 95˚C for 10 min; 45 cycles of 95˚C for 30 sec, 56˚C for 30 sec, and 72˚C for 1 min; final extension at 72˚C

for 5 min. Products from the second PCR were visualized on an agarose gel, and those containing bands were sequenced in both directions using primers ITS3 and Oo-rt-ITS-R.

Samples that generated messy chromatograms or appeared to contain single nucleotide polymorphisms (SNPs) indicative of multiple *O. ophidiicola* genotypes were re-amplified with the second PCR using a proofreading polymerase (15.75 µL of molecular grade water, 5 µL of 5x SuperFi buffer [Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA], 2 µL of dNTPs (2.5 mM each), 0.625 µL of 20 µM each primer, 0.5 µL of Platinum SuperFi DNA polymerase [2U/µL], and 0.5 µL of product from the first PCR; cycling conditions were the same as described for the second reaction above), the resulting amplicons cloned using the Invitrogen Zero Blunt TOPO PCR cloning kit for sequencing (Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA), and individual transformants were sequenced.

Sequences were assigned to genotypes based on existing sequence data available in GenBank for the ITS2 region of *O. ophidiicola* and to one of the three major clades of *O. ophidiicola* [34].

(e) Quantification of infection severity

Infection severity was measured by calculating the surface area of each snake covered by lesions. Using the image processing program ImageJ [40] and the photos of the snakes taken in the field, we measured each lesion five times and recorded the mean length and width. Each surface area of a snake's lesion was calculated, and all surface areas were added to get a total lesion surface area. Using the morphometric measurements collected in the field, we also calculated each snake's total surface area from the snout to the tail tip. We then calculated the percentage of total surface area covered by lesions. We also quantified infection severity following a previously described SFD scoring system [41] using a combination of lesion type, location, number, and coverage. Scores ranged from 4 (mild) to 12 (most severe). We compared the relationship between lesion score and lesion surface area (Fig. S1) and used lesion surface area as a more quantitative measure of severity as opposed to the ordinal metric in the final analyses to describe infection severity.

(f) Statistical analyses

Data were analyzed using Bayesian hierarchical models and we assessed statistical support for effects of genus, species, lesion presence/severity and pathogen clade using credible intervals that do not overlap zero. We fit all models (unless otherwise noted) using the No-U-Turn Sampler (NUTS), an extension of Hamiltonian Markov chain Monte Carlo (HMCMC). All Bayesian models were created in the Stan computational framework (http://mc-stan.org/) accessed with the "brms" package [42]. To improve convergence and avoid over-fitting, we specified weakly informative priors (a normal distribution with mean of zero and standard deviation of 10), unless otherwise noted. Models were run with a total of 4 chains for 2000 iterations each, with a burn-in period of 1000 iterations per chain resulting in 4000 posterior samples, which, given the more efficient NUTS sampler, was sufficient to achieve adequate mixing and convergence. All $\hat{R}$ values were less than or equal to 1.01, indicating model convergence. All statistical analyses were performed in R software version 4.2.0 [43].

To examine hotspots (i.e., relative disease risk) of the pathogen *O. ophidiicola* across Europe, we used the Getis-Ord Gi* analysis with Local Indicators of Spatial Association (LISA) statistics in the open-source Geographic Information System QGIS (version 3.22.10). A grid map of 100 km x 100 km was created across the landscape to aggregate point data and look at significant spatial clustering of neighboring features (each square) using the resultant z-scores and p-values. A heatmap was created to visualize the *O. ophidiicola* infection risk zone using a kernel density estimation with a quartic kernel shape and kernel radius of 100 km in QGIS. To investigate the effect of species on pathogen prevalence, we used a Bayesian multilevel model with a Bernoulli distribution, pathogen detection as our response variable (0|1), species as our predictor variables, and a group-level effect of site. We excluded any species that had been sampled fewer than four times. We ran a similar model as described above with genus instead of species and included species as a group-level effect.

To investigate differences in lesion prevalence and severity in snakes that were qPCR positive, we first ran a multilevel model with a Bernoulli distribution, detection of lesions on a snake in the field (0|1) as our response variable, species as our predictor variable, and a group-level effect of site. To examine differences in lesion severity for the snakes that were positive for *O. ophidiicola*, our response variable was each square millimeter of surface area of the snake, which were treated as Bernoulli trials in a binomial sample (1 = lesion present or 0 = no lesion), and our group-level effects were individual snake and site. We performed a comparison between

our lesions severity metric (percent of body covered in lesion) and previously described lesion scoring system [41], using a hierarchical model with the scoring system as our response variable, and the fraction of skin covered in lesions as our predictor with a Poisson distribution.

We examined the best model that explained *O. ophidiicola* prevalence across the landscape using leave-one-out cross-validation (LOO). The multilevel models included the population level effects of just species, just pathogen clade, an additive model of species and clade, and an interactive model of species and clade, with a Bernoulli distribution, pathogen detection as our response variable (0|1), and with site as a group-level effect for all four models. Models were run with a total of 4 chains for 6000 iterations each, with a burn-in period of 1500 iterations per chain resulting in 18000 posterior samples. We used clade as a variable instead of specific genotypes because the four genotype sample sizes (i.e., I-A, I-B, II-D/E and II-F) were too small to discern plausible data patterns. The clade variable consisted of either clade I (sites where only snakes infected with clade I were detected) or both clade I and II (sites where snakes were infected with either clade I or clade II, as there were only few locations with detections of only clade II). Sites with no clade confirmation were assigned to clade I or clade I and II when they were less than 100-km from the nearest sample of known clade. All sites outside this 100 km radius that could not be assigned a clade were dropped from the final analysis. The final clade dataset used for this analysis included 21 sites and 787 observations. Prevalence by pathogen clade comparisons are only reported for four species (*N. natrix*, *N. tessellata*, *N. helvetica*, and *Z. longissimus*) for which there was sufficient data. To determine how pathogen clade influenced infection severity, we performed an analysis similar to the one described above, where our response variable was each square millimeter of surface area of the snake, which was treated as a Bernoulli trial in a binomial sample and our predictor was pathogen clade, with a group-level effect of individual snake and species.

3. Results

We captured 1254 individual snakes representing 21 species (Fig. 1, Table S1). A total of 2628 swabs were collected, including 2357 full body skin swabs and 271 lesion swabs. Overall, *O. ophidiicola* prevalence confirmed by qPCR was 8.7% (n = 109 positive snakes) and prevalence was highly variable across the landscape. The hotspot analysis confirmed that Switzerland and the immediately surrounding areas were hotspots for pathogen presence (99%

confidence interval, Z-score ≥ 2.58; $p \leq 0.01$, Fig. 1 and S2), as well as sites sampled in southern Germany, eastern Austria, and eastern Ukraine, but to a lesser extent (95% confidence interval, $1.96 \leq Z$ score < 2.58; $0.01 < p \leq 0.05$). Pathogen prevalence was highest in Switzerland (26.7%, Fig. 1, S3), followed by Germany (12.5%, Fig. S3) and Ukraine (12.1%, Fig. S3). Poland and the Iberian Peninsula (Spain and Portugal) had the lowest prevalence at 2.7%, 0.0%, and 0.0% respectively, despite comparable sample sizes to other locations (Table S1).

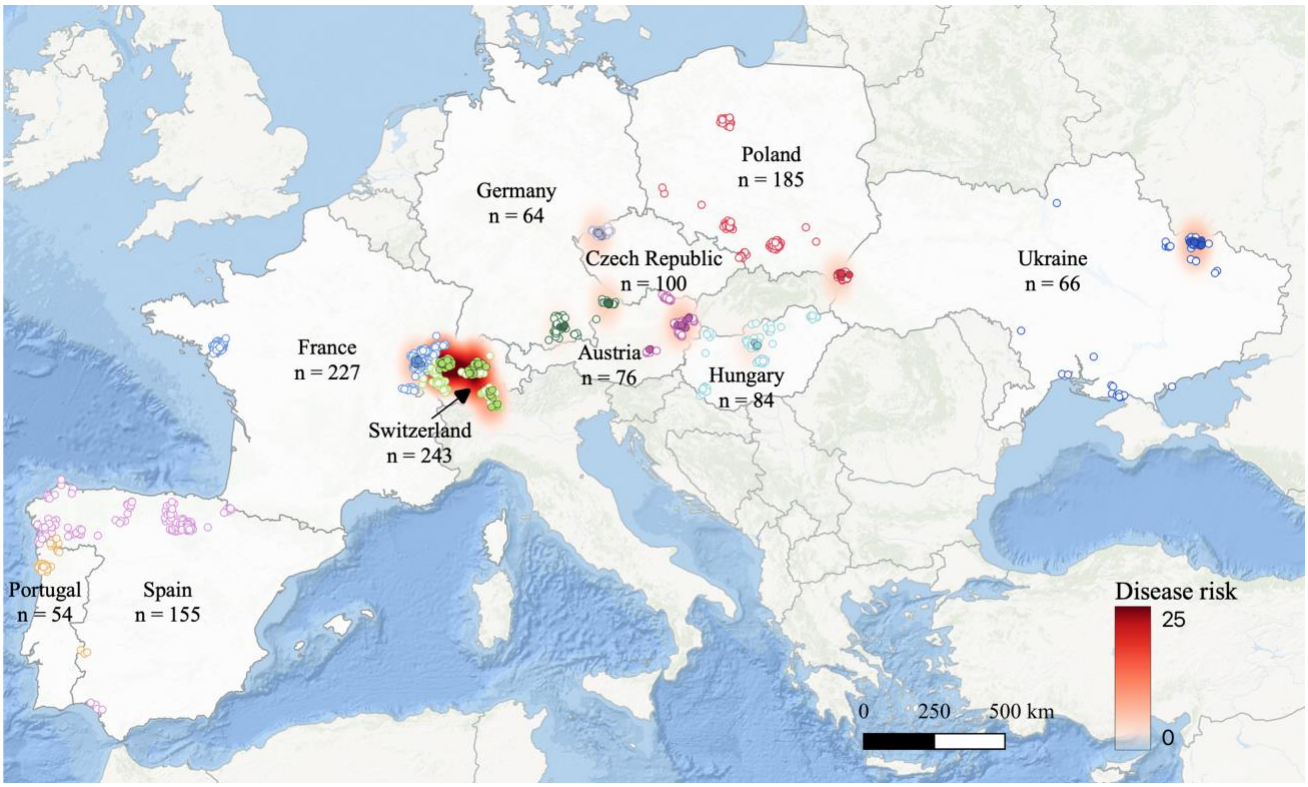

Figure 1. Spatial distribution of snake captures and detections of *O. ophidiicola* across Europe. Each circle represents an individual snake capture and overlapping points were slightly jittered for visualization. Different colors are used to distinguish countries, filled points indicate snakes that were qPCR positive, and outlined points are qPCR negative snakes. Underlying density heatmap shows spatial distribution of *O. ophidiicola* infection risk based on qPCR positive detections using a kernel density estimation algorithm for visualization. We used a 100-km radius around each positive point and the scale bar indicates point density (i.e. relative disease risk) across each region.

In addition to geographic variation, differences in infections were considerable among hosts. We found statistical support (results formatted as coefficient ± standard error (95% credible intervals)) that the *Natrix* genus had higher prevalence (8.2%; intercept: -2.51 ± 0.50 (-3.62, -1.67)) than other genera sampled (0.7%, *Coronella* coeff: -3.13 ± 1.23 (-6.07, -1.24); 0.3%, *Dolichophis* coeff: -8.64 ± 5.60 (-22.53, -0.99); 0.2%, *Vipera* coeff: -4.05 ± 0.87 (-5.95, -2.53)), except for *Hierophis* and *Zamenis* (4.2% and 5.9 %, coeffs: -0.81 ± 0.48 (-1.79, 0.10) and -0.41 ± 0.43 (-1.27, 0.39), respectively), indicating that species in these genera may be more susceptible to *O. ophidiicola* infections. There was also large variation in pathogen prevalence among congeneric species. *Natrix tessellata* had higher *O. ophidiicola* prevalence (model prediction: 15.8 ± 6.2%; Fig. 2 and Table S2) compared to several other members of the *Natrix* genus including *Natrix astreptophora* (0.6%, coeff: -8.5 ± 5.8 (-21.77, -0.39)), and *Natrix maura* (2.0%, coeff: -2.66 ± 1.31 (-5.7, -0.56), but there was no statistical support for differences from *Natrix helvetica* (13.7%, coeff: -0.16 ± 0.38 (-0.9, 0.58)) or *Natrix natrix* (7.6%, coeff: -0.75 ± 0.49 (-1.73, 0.23)); Fig. 2 and Table S3). There was also support that a third species with higher prevalence, *Zamenis longissimus*, that did not differ from *N. tessellata* (6.8%, coeff: -0.94 ± 0.51 (-1.98, 0.07)).

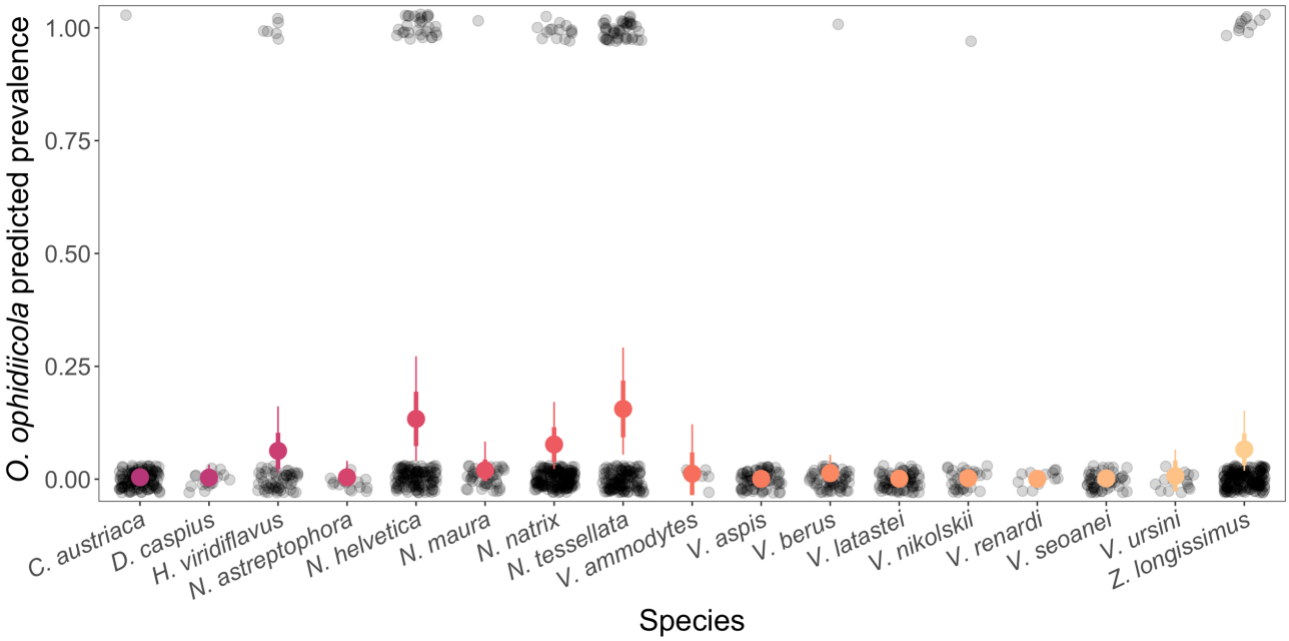

Figure 2. Prevalence of *O. ophidiicola* in different species of snakes across Europe. Each black circle represents a single snake as being either negative or positive. Larger circles and whiskers show the model predicted posterior mean ± standard error (thick lines), and 95%

credible intervals (thin lines) for each species across all countries. Colors indicate different species.

Overall, skin lesions were observed in 187 snakes from 15 species across all countries, but only 46.5% of those tested positive by qPCR (n = 87). Of all the snakes that tested positive by qPCR (n = 109), 80% of those had skin lesions that were consistent with *O. ophidiicola* infection, while the other 20% had no visual signs of infection (n = 22) (Fig. 3A, Fig. S4). We found a high probability of finding lesions on a snake if they tested positive for *O. ophidiicola* (range 46.2% – 92.8%, except for two viper species which had no visual sign of infection, Fig. 3A). We observed variation in infection severity but no statistical support for differences among species that were confirmed qPCR positive (Fig. 3B, Table S3).

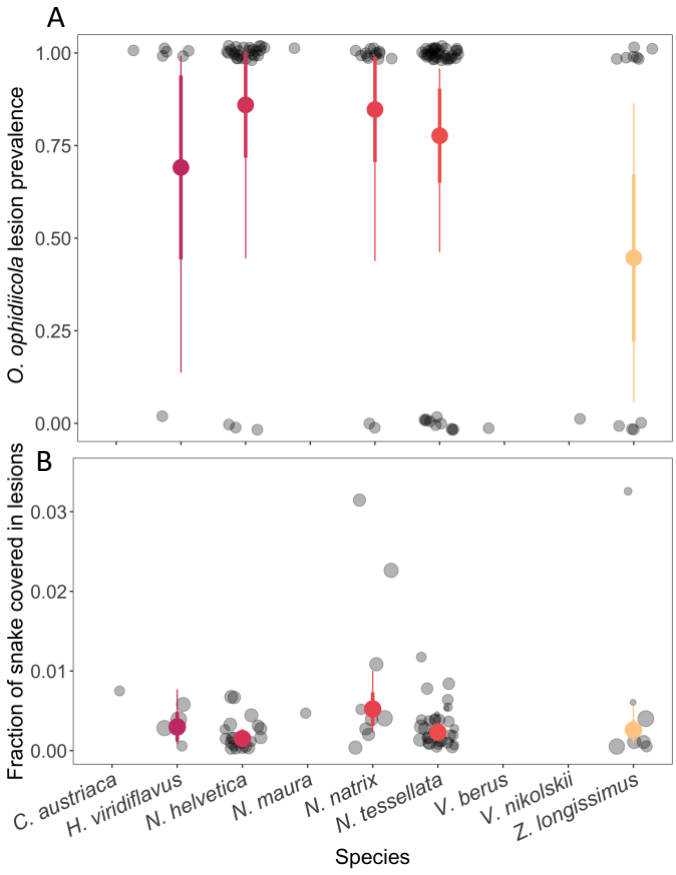

Figure 3. Lesion prevalence (A) and severity of infection (B) in *O. ophidiicola*-positive snakes across different species. Color circles and whiskers show the model predicted posterior mean, ± standard error (thick lines), and 95% credible intervals (thin lines) for different species

across all countries. (A) Each black circle represents a single snake as being either negative or positive for presence of lesion. (B) Each black circle represents the fraction of the body of a single snake covered in lesions and the size of the circle is proportional to the total surface area of the snake (scale ranges 250 – 1,000 cm^2).

Genotyping analyses were successful for 85.3% of positives swabs (93 total swab samples) that were qPCR positive for *O. ophidiicola*. A total of four unique genotypes were observed, belonging to two clades (clade I & II). Two of these genotypes (designated here as I-A and I-B) resided within clade I (i.e., the "European clade") with genotypes I-A and I-B having an ITS2 region sequence identical to strains previously isolated from Great Britain and Czechia, respectively [28]. The remaining two genotypes resided within clade II (i.e., the "North American clade") and were identical to ITS2 region sequences of clonal lineages II-D/E (lineages D and E have identical sequences in the ITS2 region) and II-F [34]. Here we refer to these genotypes as II-D/E and II-F, although strains detected in this study may not be true representatives of the clonal lineages reported from North America. We found that both clade I and clade II are present across much of continental Europe, ranging from eastern France to eastern Ukraine; however, both clades were not present in all locations where *O. ophidiicola* was detected. Genotype I-A was detected primarily in western Europe (Switzerland, Germany, and Austria), whereas genotype I-B was detected in eastern Europe (Czechia, Austria, Hungary, Poland, and Ukraine) (Fig. 4). Genotype II-D/E was more widely distributed across Europe, whereas genotype II-F was only found on a single lake in Switzerland (Fig. 4). On two occasions, snakes were found to be infected with multiple genotypes of *O. ophidiicola*: a snake from Switzerland from which genotypes I-A and II-D/E were detected and a snake from Hungary from which genotypes I-A and I-B were detected.

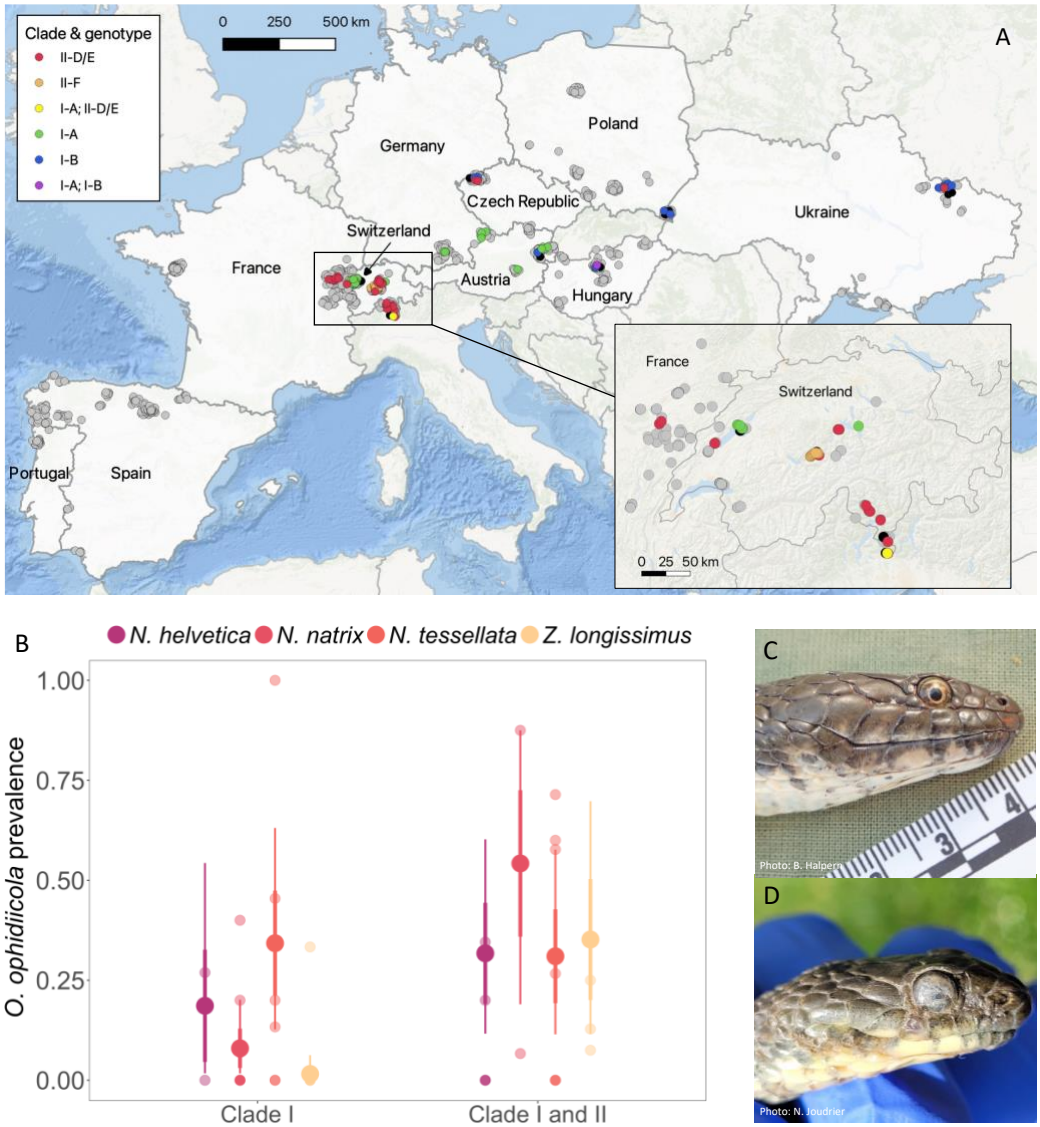

Figure 4. Distribution of *O. ophidiicola* clades across Europe. (A) *O. ophidiicola* clades and lineages from positive detections across the landscape in Europe. Points are slightly jittered for better visual representation of the sampling range. Color of the point indicates the clades and genotypes, samples that were qPCR negative are represented as grey points, and samples that failed to amplify with the genotyping PCR are represented as black points. Yellow and purple points represent simultaneous detections of genotypes I-A and II-D/E and genotypes I-A and I-B, respectively, from the same swab sample (i.e., snakes infected with multiple genotypes). The enlarged map (inset) shows better resolution of detections in Switzerland. (B) *O. ophidiicola* prevalence across snake species based on *O. ophidiicola* clade presence at the different sites.

Small color points are mean prevalence at a site for a given species and clade. Large color points and whiskers show the model predicted posterior mean, ± standard error (thick lines), and 95% credible intervals (thin lines) across different species. (C) Photo of a *N. tessellata* from Hungary infected with *O. ophidiicola* from clade I-B. (D) Photo of a *N. tessellata* from Switzerland infected with *O. ophidiicola* from clade II-F showing facial infection.

The top two models, as determined through LOO, that explained *O. ophidiicola* prevalence across the landscape included both host species and pathogen clade as predictor variables, with the best fit model including an interaction between these two variables (Table S4). This indicated that variation in prevalence was best explained by both species present at a site and *O. ophidiicola* clade. There was statistical support that *N. natrix* and *Z. longissimus* have higher probabilities of being infected when clade II was detected at a site compared to just clade I (*N. natrix* coeff: 2.95 ± 1.15 (0.76, 5.10), *Z. longissimus* coeff: 4.15 ± 1.32 (1.75, 6.94)). A third species, *N. helvetica*, was associated with higher probability of infection when clade II was detected at a site, but the credible intervals included zero (coeff: 1.30 ± 1.26 (-1.04, 3.92)). Conversely, we found that the species with the highest pathogen prevalence, *N. tessellata*, had no differences in infection probability based on clade (Fig. 4B, Table S5). Snakes that were infected by a strain of *O. ophidiicola* belonging to clade I generally had lower levels of infection when compared to snakes infected with a strain from clade II, although the credible intervals overlapped zero (clade I coeff: -0.21 ± 0.29 (-0.78, 0.36), Fig. S5).

4. Discussion

Disruption to the relationship between host, pathogen, and environment can lead to alteration of steady-state dynamics and local or regional disease outbreaks. Our results support the presence of hotspots for SFD across Europe and the potential mechanisms underlying areas of higher infection prevalence. *O. ophidiicola* was detected in all countries studied except for those on the Iberian Peninsula (Spain and Portugal), which could be attributed to geographical barriers (Pyrenees mountains) or to environmental conditions that may be unsuitable for persistence and growth of *O. ophidiicola*. Our surveys establish four regions with elevated prevalence of *O. ophidiicola* in Europe, with the largest in Switzerland. However, overall, prevalence was relatively low (8.7%) when compared to studies conducted in North America.

Samples collected from 31 states in the U.S. and Puerto Rico detected *O. ophidiicola* in 17.2% of wild snakes, and a meta-analysis of 33 studies reported overall *O. ophidiicola* infection in 18.5% of free-ranging snakes in the USA and Canada, with 9.8% observed mortality [30, 33].

Snake fungal disease has garnered much attention over the last few decades, as this disease has negatively affected snake populations [26, 44]. Despite this, few studies have systematically examined differences in host infection across species. We found high variability of infection prevalence among snake species, irrespective of the sampling location. Generally, snakes in the *Natrix* genus had a higher probability of infection compared to other genera and were important for elevated disease incidence in a region. Underlying host characteristics such as dependence on aquatic habitats, have previously been found to be associated with higher *O. ophidiicola* infection prevalence [45], which could partly explain increased infection in this genus. The species *N. tessellata* had the highest pathogen prevalence, followed by *N. helvetica* and *N. natrix*, with all three species being either semiaquatic or living near water (with *N. tessellata* being more piscivorous). Interestingly, only two vipers (out of a total of 341 samples) tested positive for *O. ophidiicola*, and both snakes had no visual signs of infection (i.e. no lesions present). This indicates that viperids may not be competent hosts for *O. ophidiicola*, possibly due to environmental associations or behavioral and physiological mechanisms. Contrary to this, North American pit vipers, such as the massassauga rattlesnake (*Sistrurus catenatus*), have been reported to develop severe SFD symptoms [46]. This could be attributed to a sampling bias from more intensive monitoring of threatened rattlesnake species, or rattlesnakes may have increased susceptibility to SFD due to their habitat requirements (massassauga rattlesnakes are found near swamps and marshes) compared to European vipers, which are generally associated with drier environments (except for *V. berus* which can be associated with cool and humid habitats).

In our study, no mortality was reported, and snakes generally appeared healthy except in a few cases where infection were severe and had spread to the face with possible associated disruptive foraging behavior (Fig. S4). The reduction in disease severity in Europe could be the result of increased host resistance or generally lower pathogen virulence. We also found that only 46% of snakes with lesions tested positive for *O. ophidiicola*, which has also been observed in North America [47, 48]. The lesions that could not be attributed to *O. ophidiicola* infection looked similar to SFD skin lesions and may be fungal or bacterial in origin [49]. Further research

should investigate other sublethal effects of SFD infection and the interaction between *O. ophidiicola* and other unknown infections.

We found that models accounting for both host species and pathogen clade best explained the variation in pathogen prevalence across the landscape. Importantly, the top model included an interaction between host species and pathogen clade, indicating that the effect of clade is not the same across species. Clade II was found in two of the four pathogen hotspots across Europe, and generally when clade II was present in an area, we found support that the probability of detecting *O. ophidiicola* was higher for three (*N. natrix*, *Z. longissimus*, and *N. helvetica)* of the four species with the highest prevalence. Hotspots in Switzerland were primarily driven by *N. tessellata*, which showed no difference in detection based on whether just clade I or both clades I and II were present. This indicates that *N. tessellata* may be equivalently susceptible to both clades I and II or that other factors may be contributing to infection probability in this species.

The history and origin of *O. ophidiicola* in Europe is unclear. Sampling of museum specimens has revealed that strains of *O. ophidiicola* belonging to clades I and II were present in Switzerland as early as 1959 [35]. It is estimated that clade I shared a common ancestor within the last 100 to 500 years, but that analysis only included four clade I strains and may greatly underestimate the time that *O. ophidiicola* has been present in Europe [34]. Despite the designation of clade II as the “North American” clade, it is believed that *O. ophidiicola* is not native to that continent and that multiple introductions (most likely from Eurasia) have occurred over the last century [34]. Thus, it is plausible that either or both clades I and II are native to Europe. Strains of *O. ophidiicola* isolated from wild snakes in Taiwan reside within clade II [32, 34], which could also indicate a southeast Asian origin for that clade, raising the possibility that clade II is also not native to Europe. Clade II has been detected on captive snake populations in Europe [34], which could facilitate transmission into wild populations. An introduction of clade II into Europe sometime before 1960 and subsequent spread could explain the wide distribution of this clade as detected in our sampling. We detected two genotypes within clade II. One of these (II-D/E) was widely distributed, whereas the other (II-F) was detected in a single snake community around a lake in Switzerland. That snake community includes an introduced population of *N. tessellata*. Taken together, this could indicate that genotype II-F was more recently introduced to Europe, perhaps through the release of snakes originating in captivity.

However, determining the genetic diversity and origin of the various lineages of *O. ophidiicola* in Europe would require more in-depth studies.

We find that disease hotspots can at least partly be explained by host species and the presence of certain pathogen clades. The shape of this relationship varied among areas with higher disease prevalence, and in some cases, host species had higher infections regardless of pathogen genotypes, and others were likely attributed to the presence of specific susceptible hosts infected with a pathogen clade that may be more transmissible. Although virulence is recognized as an important factor in the effects of disease of host populations, the general lack of landscape level data on pathogen lineage distribution and association with disease has likely limited our ability to determine its importance for other disease hotspots.

Ethics

Handling of snakes was reviewed and approved by Virginia Tech Institute for Animal Care and Use Committee protocol 20-055. Permits to conduct our field study were pulled when necessary and were granted by the Regional office of the Karlovarian region, Department of Environment and Agriculture in Czech Republic (permit # KK/1098/ZZ/20-4), the General and Regional Directorates of Nature Conservation in Poland (permit # DZP- WG.6401.91.2020.TŁ; DZP-WG.6401.91.2020.TŁ.2, WPN.6401.270.2019.MF, WPN.6401.17.2020.KW.2, WPN.6401.9.2021.KW.2), Portuguese and Spanish wildlife legislation (permits # 295/2020/CAPT, 146/2021/CAPT, 201999902471003/IRM/MDCG/mes, EB-018/2020, EB-015/2021, AUES/CYL/192/2020, AUES/CYL/54/2021, A/2021/036, 0001-0261-2021-000003), the Direction régionale de l'environnement of Franche-Comte (ONAGRE 2020-01-17-00122; 25-2022-02-17-00001) and Loire-Atlantique (Cerfa 13616, permit # 64/2016) in France, the Museum of Natural History in Budapest, Hungary (permit # PE-KTFO/1568-18/2020), Austrian legislations (permits # MA22 – 1089768-2020, RU5-BE-64/023-2022, RU5-BE-64//022-2021, ABT13-53W-50/2018-2), the Federal Office for the Environment (FOEN) in Switzerland with veterinary authorization (VD3718, ID 33612), and cantonal authorizations (Neuchatel: FS-08/2021, Grisons: AV-2022-338, Vaud: 2021-3537).

Data, code, and materials

The datasets and code generated for this study will be deposited in Dryad Digital Repository upon final submission.

Author contributions

GB: conceptualization, data curation, investigation, formal analysis, visualization, methodology, writing—original draft, writing—review and editing; JML: methodology, investigation, writing—review and editing; NJ: methodology, resources, investigation, writing—review and editing; SB: investigation, resources, writing—review and editing; TC: methodology, investigation, writing—review and editing; MF: investigation, writing—review and editing; FM-F: investigation, writing—review and editing; GG: investigation, writing—review and editing; BH: investigation, writing—review and editing; AK: investigation, writing—review and editing; KK: investigation, writing—review and editing; OL: investigation, writing—review and editing; AM: methodology, investigation, writing—review and editing; RM: investigation, writing—review and editing; SS: investigation, review and editing; BS: investigation, writing—review and editing; SU: methodology, resources, investigation, writing—review and editing; OZ: investigation, writing—review and editing; JRH: conceptualization, resources, supervision, funding acquisition, writing—review and editing.

Conflict of interest declaration

The authors declare no competing interests.

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