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Unravelling the disease ecology of snake fungal disease: high genetic variability and ecological features of *Ophidiomyces ophidiicola* in Switzerland

Nicolas Joudrier[1,2,3,*], Gaelle Blanvillain[4], Gregoire Meier[5], Joseph Hoyt[4], Maxime Chèvre[1], Sylvain Dubey[6,7], Francesco C. Origgi[2,8,**], Sylvain Ursenbacher[1,9,10,**]

1 - info fauna, 2000 Neuchâtel, Switzerland
2 - Institute of Animal Pathology (ITPA), University of Bern, 3012 Bern, Switzerland
3 - Institute of Biology, University of Neuchâtel, 2000 Neuchatel, Switzerland
4 - Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA
5 - Via degli Orti 3, 6809 Medeglia, Switzerland
6 - Department of Ecology and Evolution, HW Romandie SA, 1820 Montreux, Switzerland
7 - Department of Ecology and Evolution, University of Lausanne, 1015 Lausanne, Switzerland
8 - Department of Veterinary Sciences, University of Messina, 98168 Messina, Italy
9 - Department of Environmental Sciences, University of Basel, 4056 Basel, Switzerland
10 - Balaton Limnological Research Institute, 8237 Tihany, Hungary
*Corresponding author; e-mail: nicolas.joudrier@infofauna.ch
**These authors equally contributed to this work
ORCID iDs: Joudrier: 0000-0001-8950-3525; Blanvillain: 0000-0002-2486-209X; Hoyt: 0000-0003-0398-8264; Dubey: 0000-0003-4342-9432; Origgi: 0000-0002-2874-6563; Ursenbacher: 0000-0001-5093-6403

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Abstract. The discovery of the fungal pathogen *Ophidiomyces ophidiicola* (*Oo*), the aetiologic agent of Snake Fungal Disease (SFD), has raised a growing interest in the North American and European scientific communities, in particular toward conservation. This pathogen is known or suspected to be associated with the declines of some snake populations in North America and was detected later in Europe. Its ecology, distribution and phylogeography still remain largely unknown. In this study, we collected skin swabs from 271 free-ranging snakes in Switzerland across 8 different species and 13 sites. The overall pathogen prevalence was at least 28% with sequences consistent with both the European and the North American lineages (respectively Clade I and II) of *Oo*. Semi-aquatic snakes were more likely to be infected by *Oo*, and high human disturbance (human frequentation and direct impact on snakes) was associated with a higher *Oo* prevalence, whereas season, body condition and snake species introduction was not. This study suggests that Switzerland might represent a region characterised by high genetic variability in *Oo*, and where long-term monitoring might be particularly important to follow the evolution of the disease in free-ranging snakes.

Keywords: conservation, epidemiology, fungus, habitat, human impact, lineages, reptiles, seasonality.

Introduction

Fungal pathogens have been found to be a major threat to a large number of host species and proven aetiologic agents of several recent emerging diseases in wildlife. Among others, *Pseudogymnoascus destructans*, the aetiologic agent of white-nose syndrome in bats, has caused > 90% declines in bat populations over just a few years (Hoyt, Kilpatrick and Lang-

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wig, 2021). In amphibians, *B. dendrobatidis* and *B. salamandrivorans* have been shown to play an important role in the decline of over 400 amphibian species worldwide and led to the presumed extinction of about 90 of them (Scheele et al., 2019).

An emerging disease affecting wild and captive snakes, ophidiomycosis, also known as Snake Fungal Disease (SFD) is caused by the ascomycete fungus *Ophidiomyces ophidiicola* (*Oo*). There is a growing concern over its potential impact to free-ranging snake populations in North America and Europe, and captive snake collections around the world (Allender et al., 2015b; Lorch et al., 2015). An overview of specific findings and circumstantial evidences concerning the decline associated with *Oo* are summarized in Allender et al., 2015b; Lorch et al., 2016; Di Nicola et al., 2022. The classic clinical sign associated with SFD is a variable extensive to multifocal dermatitis (see examples in supplementary fig. S1A), which can be complicated by a multisystemic disease characterised by myositis, osteomyelitis, and pneumonia (Allender et al., 2015a), leading eventually to death, at least in experimental conditions (McKenzie et al., 2020).

The first evidence of SFD outside North America in wild snakes is relatively recent. Positive samples from carcasses and moulted skin sheds of free-ranging snakes were detected in Great Britain and the Czech Republic (Franklinos et al., 2017). Phylogenetic analyses suggested that the strains of *Oo* found in those samples differed from the North American isolates and represented a distinct "European" clade (Franklinos et al., 2017). A study investigating museum specimens revealed the presence of *Oo* in North America since 1945 (Lorch et al., 2021), consistent with an earlier presence of *Oo* in the USA than previously thought. In another study the authors concluded that multiple *Oo* introductions have likely occurred into the USA, and might have been facilitated by the international pet trade (pathogen pollution). Furthermore, sequential *Oo* introductions would have occurred within the last few hundreds of years in wild populations, followed by an expansion of some of the North American clonal lineages, with the most recent one estimated between 1985 and 2007 (Ladner et al., 2022).

The first case of apparent ophidiomycosis in Switzerland was documented in a wild grass snake, *Natrix helvetica*, in Canton Ticino (southern Switzerland) (Meier et al., 2018). However, several observations of snakes presenting lesions consistent with SFD were reported in the surrounding areas, increasing the suspicion that a wider presence of *Oo* was occurring in Switzerland and in the bordering countries (Meier et al., 2018). Additionally, two of us (FCO, SU) investigated the presence of *Oo* in museum samples and five cases (over 1100 snakes examined) were confirmed positive by PCR and histopathology, including three samples from Switzerland and two from Italy, with the oldest sample dating back to 1959 (Origgi et al., 2022). Moreover, strains clustering either with the North American (Clade II) or the European (Clade I) lineages have been found among these museum samples, which come from the same geographical area, indicating the oldest record of a strain clustering with the Clade II, detected outside of the USA (Origgi et al., 2022).

The surprising findings of SFD in historic samples from Switzerland sparked an interest in the current status of *Oo* in free-ranging snakes, in particular concerning the actual distribution of the fungus in Switzerland and if both the proposed Clade II and Clade I were present in the current wild snake population. This question was the driver of the current study. Furthermore, we aimed to better characterise the ecology of *Oo* by investigating the potential contributing factors to the occurrence of the pathogen including (1) habitat preference by assessing if snakes living in aquatic habitats might have a higher probability to carry *Oo* (McKenzie et al., 2019) and are overall more subject to fungal infections (Schumacher, 2003); (2) the seasonality by

assessing if the prevalence of *Oo* and the severity of clinical signs of SFD may vary with seasons (McCoy, Lind and Farrell, 2017; McKenzie et al., 2019; Dillon et al., 2022; Lind et al., 2023a); (3) human disturbance by assessing if the anthropic impact (including, but not limited to snake captures – by professionals or hobbyists – and overall human frequentation-walking near snakes, pets, landscaping projects) could have a role in the spread of the fungal agent between sites and within the sites, and reduce the ability of snakes to actively fight the disease (stress including disturbance during basking); (4) whether introduced snake species (as possible vector of *Oo* transmission) to the capture location could explain part of the *Oo* spread pattern across regions; and (5) the body condition by assessing if the snakes that were qPCR positive would have a lower body condition than the overall population (McCoy, Lind and Farrell, 2017; Chandler et al., 2019).

Material and methods

Sample collection

This investigation was part of an international collaboration between European (University of Neuchatel and University of Bern, Switzerland) and North American (Virginia Tech, USA) institutions. Sampling resulted in a total of four different batches of samples listed in the table 1.

Sample collection for *Oo* detection was carried out by full-body swabs from each snake in two replicates (six passages including ventral and dorsal surfaces of the head, the body, and the cloaca). Additionally, after visual examination, snakes presenting skin lesions or deeper wounds were swabbed in duplicate specifically on the lesions themselves, and lesion scales were sampled when possible. Sheds, when found in the field, were collected and tested as well. All the swabs, scales and sheds were put in sterile 1.5 ml Eppendorf tubes and stored at −20°C as soon as delivered to the collection sites, before final storage at −80°C once at the laboratory.

The two replicates of full-body swabs from batches 1 and 3 (see table 1) and one replicate of swabbed lesions (when present) from batches 1 and 3 were sent to one of the co-authors (GB) for detection of *Oo* by qPCR (see protocol in Blanvillain et al., 2022). The other replicate of the swabbed lesions from batch 3, along with the swabs from batch 2, lesioned scales and 12 sheds from batch 4, were analysed by another co-author (NJ) at the Institute of Animal Pathology (ITPA), Vetsuisse faculty, University of Bern according to the protocol listed in this article.

Study sites were chosen across seven different cantons of Switzerland (BE, NE, NW, OW, SZ, TI, VD) in order to represent Swiss geography, species diversity and according to different criteria such as: (1) ease of accessibility; (2) known presence of snake populations; and (3) a high snake population density. Moreover, we specifically selected places with known introduced snake populations, i.e., species outside of their natural distribution range resulting of accidental or illegal displacements, events that are unfortunately frequent in Switzerland. We expected this variable to correlate to pathogen introduction (via displacement from an infected population to a naïve one). Sampling sites included areas either adjacent or disconnected from a water source (pond, lake, or river) to test for the influence of habitat specialisation (semi-aquatic versus terrestrial species) on *Oo* presence. Snakes were caught during visual surveys regardless of their size or behavioural activity and kept in paper bags in the field, until sampling. They were then released at their exact capture location. For each snake, several morphological measures including snout-vent length, tail length, body mass and sex were recorded to test for potential SFD associated or contributing factors. Individual identification pictures of the ventral scale pattern (for *Natrix* species) of the head (for other species) were taken to rule out the potential recapture of an individual within a site.

A number of precautions were taken to avoid contamination between individuals and between sites (for every site, we used of different pairs of gloves and paper bags,

Table 1. List of the different sample batches collected during this investigation and associated collection strategies.

Batch of samples	Number of samples	Sample collector	Lacking information	Main laboratory investigator and sampling strategy
1	41	Sylvain Ursenbacher	None	Gaelle Blanvillain
2	17	Gregoire Meier	Morphological data	Nicolas Joudrier
3	201	Nicolas Joudrier	None	Nicolas Joudrier (one replicate of swabbed lesions when present) Gaelle Blanvillain (two replicates of full body swabs + one replicate of swabbed lesions when present)
4	12 (sheds)	Nicolas Joudrier	Morphological data	Nicolas Joudrier

every snake was manipulated wearing disposable latex plastic gloves, and all material was disinfected following established protocols (Rzadkowska et al., 2016)). All the necessary authorisation from regional and federal authorities were obtained before any capture (details in Authorisation section).

Detection of Ophidiomyces ophidiicola

Specific batches of samples (full body swabs in duplicate and lesion swab when present; see Table 1) were analysed to test for *Oo* presence according to the protocol of (Blanvillain et al., 2022), consisting in real-time PCR targeting the internal transcribed spacer region (ITS). The others were analysed for the same purpose following a multi-target qualitative PCR protocol, described below. Given the known lower sensitivity of qualitative PCR protocols versus quantitative ones and the difference in swab replicates, we estimated the false negative rate of the qualitative protocol conducted on one swab, by comparing it to the quantitative one on 3 swabs as an arbitrary gold standard. Briefly, this was carried out by dividing the numbers of samples, which were negative by conventional PCR but positive by qPCR, by the total number of samples analysed with both methods. The aim of this simple calculation is to give us an estimation of how many false negatives we obtained due to our simplified protocol and the resulting bias in the analyses.

DNA amplification

Molecular tests (qualitative/conventional PCR) were performed on swabs collected from skin lesions, on scales with lesions and sheds to detect the presence of *Oo*. For sheds, approximatively 3 cm^2 of tissue showing obvious pathological changes (thickened or/and folded tissue) was cut out and put in microtubes for DNA extraction. Total DNA extraction was carried out using the DNeasy Qiagen extraction kit (Qiagen, Hobrechtikon, CH) according to the manufacturer's instructions for all the sample types. The final DNA concentration was assessed by spectrophotometry using a Nanodrop ND-1000 device (Thermo Scientific, Witec AG, Littau, CH). Conventional qualitative PCRs protocols were carried out to detect specific *Oo* genomic sequences following the protocol used in Origgi et al., 2022 (supplementary text S1). Specifically, the protocols included one targeting the partial sequence of the 5.8-28 s RNA internal transcribed spacer 2 (ITS), another the partial sequence of the actin gene (ACT) and the third, the partial sequence of the transcription elongation factor (TEF; supplementary text S1). An additional PCR protocol was carried out on *Oo* negative samples with broadly reacting primers against conserved fungal genomic sequences (panfungal PCR; D1-D2 region) (Borman et al., 2006), in order to identify the most abundant fungal agent in the sample, which might have been associated with the detected lesions when negative for *Oo*.

Phylogenetic analysis

Sequences with ambiguous but clear chromatogram peaks were manually edited with Chromas v2.6.6 (Technelysium Pty Ltd), resolving them when possible. All the sequences obtained from the positive amplicons (supplementary text S2) along with that of *Pseudoamauroascus australiensis* strain FMR 5482 (outgroup) homologous to the specific genomic targets and available in Genbank, were used to build up a maximum-likelihood phylogenetic tree for each of the targeted *Oo* genomic regions using the software MEGA7 (Kumar, Stecher and Tamura, 2016) using standard settings (Tamura-Nei model) and 10 000 bootstrap replicates.

Statistical analyses

We used a generalised linear mixed effects model using Template Model Builder (glmmTMB) with a binomial distribution and the site of capture as a random effect. We used this model to test for the significance of three different explanatory variables in explaining the prevalence of *Oo* (0|1) on individual snakes. First, we assigned an ecological trait to every snake species of the study (semi-aquatic: *Natrix tessellata*, *N. helvetica*, *N. maura* or terrestrial: *Hierophis viridiflavus*, *Coronella austriaca*, *Vipera aspis*, *V. berus*, *Zamenis longissimus*). We also assigned a variable "introduction" to each observation based on whether the species was introduced or native to a specific site. Finally, we assigned a variable "human impact" (limited, medium, or strong) as an estimation by the Swiss coordinator for the reptile conservation and the local specialists of the aspects defined in the introduction and further cross-validated by the other Swiss co-authors. Eight different models were constructed from the most to least complex, and compared with the Akaike information criterion (AIC-total number of data = 271, see supplementary table S1).

The effect of seasonality on *Oo* was addressed on a subset of the dataset, including samples collected for six consecutive months (from April to September) at the site NE Lake North (total number of samples = 85), where samples were more regularly collected compared to the other sites. We examined the correlation between *Oo* prevalence and seasonality using a glmmTMB with a binomial distribution and location as random effect compared with a null model ($Oo \sim 1$).

Finally, to determine if *Oo* had an impact on snake body condition, we calculated a Body Condition Index (BCI) normalised for both sexes and each snake species of the three main infected ones (*N. helvetica*, *N. tessellata*, *H. viridiflavus*) (total n = 211). Females showing obvious signs of gravidity as well as individuals who recently fed (prey visible in the stomach) were excluded from this analysis. BCI was calculated as the residuals of the linear regression of mass on snout-vent length (SVL), as in previous study (Gimmel, Öfner and Liesegang, 2021). The correlation of BCI with the probability of an infected snake was tested using and the same approach used for seasonality but with both location and species as random effects.

All statistical analyses were performed using the software R, version 4.0.2. (R Core Team, 2020).

Results

Detection of Ophidiomyces ophidiicola

After combination of the results from the 2 protocols, a total of 76 samples out of 271 individuals (28%; 68 live snakes out of 258 and 8 sheds out of 13) were positive for *Oo* by PCR (at least one swab positive) and confirmed by sequencing (for qualitative PCR samples only, which were conclusively considered positive if at least one of the three targets could be amplified and successfully sequenced). *Oo* was found at different locations in all Swiss cantons tested in the study. In Central-Switzerland (cantons BE, OW, NW, SZ), the prevalence of *Oo* was the highest with 45% (30 out of 66). The lowest *Oo* incidence was observed in Romandy (western Switzerland, cantons NE and VD; see fig. 1A, B) with 21% of the samples testing positive (29 out of 141). Finally, in Ticino (Swiss Italian region, Southern Switzerland), the prevalence of *Oo* was 27% (17 out of 64). The difference in *Oo* prevalence between the three regions was statistically significant (Pearson's Chi-squared test, $\chi^2 = 13.89$, $Df = 2$, p-value < 0.005), even when considering only semi-aquatic species (Pearson's Chi-squared test, $\chi^2 = 10.83$, $Df = 2$, p-value < 0.005).

Lineage

We obtained relevant *Oo* sequences for 44 samples from the positive ITS-PCR (n = 48), 33 from the positive TEF-PCR (n = 43) and 45 from the positive ACT-PCR (n = 48), allowing us to determine the clade positioning of 46 distinct samples. Phylogenetic analyses of the strains revealed the presence of sequences clustering consistently either with the Clade II (former North American clade) or Clade I (former European clade) (Franklinos et al., 2017) in Switzerland. The two clades of *Oo* were not distributed equally across the 3 regions (Romandy (n = 14), Central-Switzerland (n = 21), and Ticino (n = 11)). Indeed, in Romandy, all 14 samples sequenced clustered together and are consistent with the Clade I, whereas the 11 samples sequenced in Ticino formed a cluster consistent with the Clade II. Additionally, the Central-Switzerland region included 10 samples with a genotype consistent with the Clade II (sites "Alpnachersee" and "Brienz"; see fig. 1A), 3 samples consistent with the Clade I (site "Ingenbohl") and 8 other samples clustering consistently with a different sublineage of the Clade II revealed with the internal Transcribed Spacer 2 (ITS) tree (lineage II-F (Blanvillain et al., 2022; Ladner et al., 2022); site "Brienz").

The separation between the two clades was confirmed for all the molecular targets used for the phylogenetic study (the actin genes (ACT), the transcription elongation factor (TEF) and the ITS), with a higher resolution in the ITS tree, displaying two sublineages within the Clade II (see supplementary fig. S2).

Environmental and host traits

The best model based on the AIC included an additive effect between the variables "Ecological trait" and "human impact" but was not statistically different from the model including "Ecological trait" only (see supplementary table S1). This simpler model with "Ecological trait" as the only response variable was retained for the analyses. Semi-aquatic snakes represented 75% of all snakes sampled (see supplementary table S2) and were more likely to be found with *Oo* (n = 202, average mean of *Oo* detection = 35%) than terrestrial snakes (n = 69, average mean of *Oo* detection = 7.2%; see fig. 2A; $\chi^2 = 11.16$, $Df = 1$, $p = 0.0008$). Additionally, sites with a strong human impact had a higher *Oo* prevalence (see fig. 2B), although this trend was only closely approaching the statistical significance ($\chi^2 = 5.66$, $Df = 2$, $p = 0.059$). No significant effect on *Oo* detection ($\chi^2 = 0.99$, $Df = 1$, $p = 0.32$) was associated with the origin of the snakes (either native or introduced to the site; see fig. 2C). We also found no effect of seasonality on the probability of *Oo* detection ($\chi^2 = 4.87$, $Df = 5$, $p = 0.43$, see supplementary

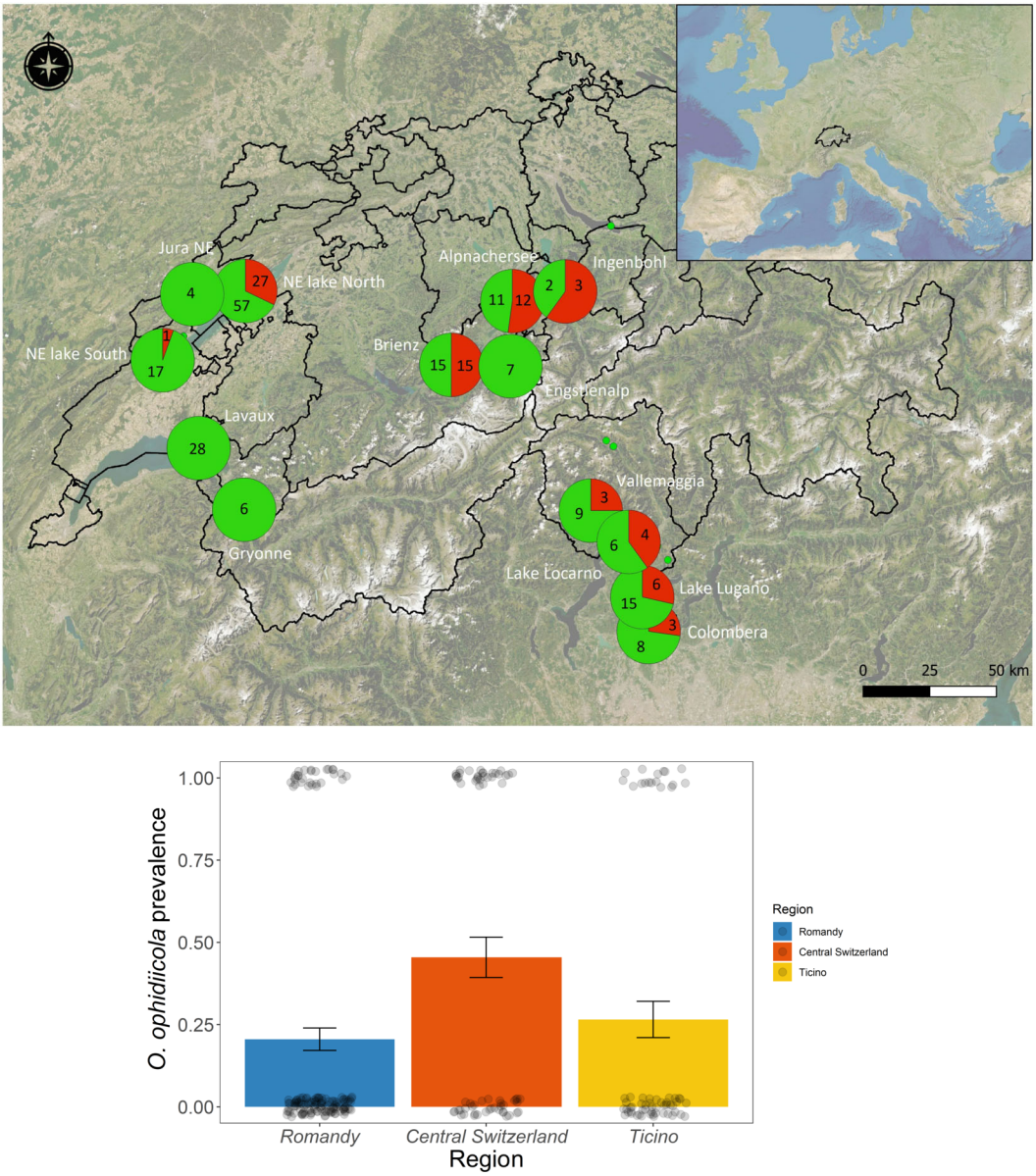

Figure 1. (A) Mapping of the frequency (occurrence) of *Ophidiomyces ophidiicola* in Switzerland. Pie charts represent the proportion of infected snakes tested by PCR (green = negative; red = positive). The number of positive and negative samples are shown for each region. Black lines correspond to the Swiss canton borders. Made with QGIS 3.22. (B) Graphical representation of *Oo* prevalence in the three different regions of sampling (Romandy, Central-Switzerland and Ticino). The prevalence of *Oo* was significantly higher in the Central-Switzerland than in the two other regions.

table S3 and supplementary fig. S3). Finally, no significant correlation between snake BCI and the probability of *Oo* detection was observed ($\chi^2 = 0.04$, $Df = 1$, $p = 0.84$, see supplementary table S4).

Discussion

False negatives estimation

As mentioned in the methods, different batches of samples (swabs, scales and/or sheds) were

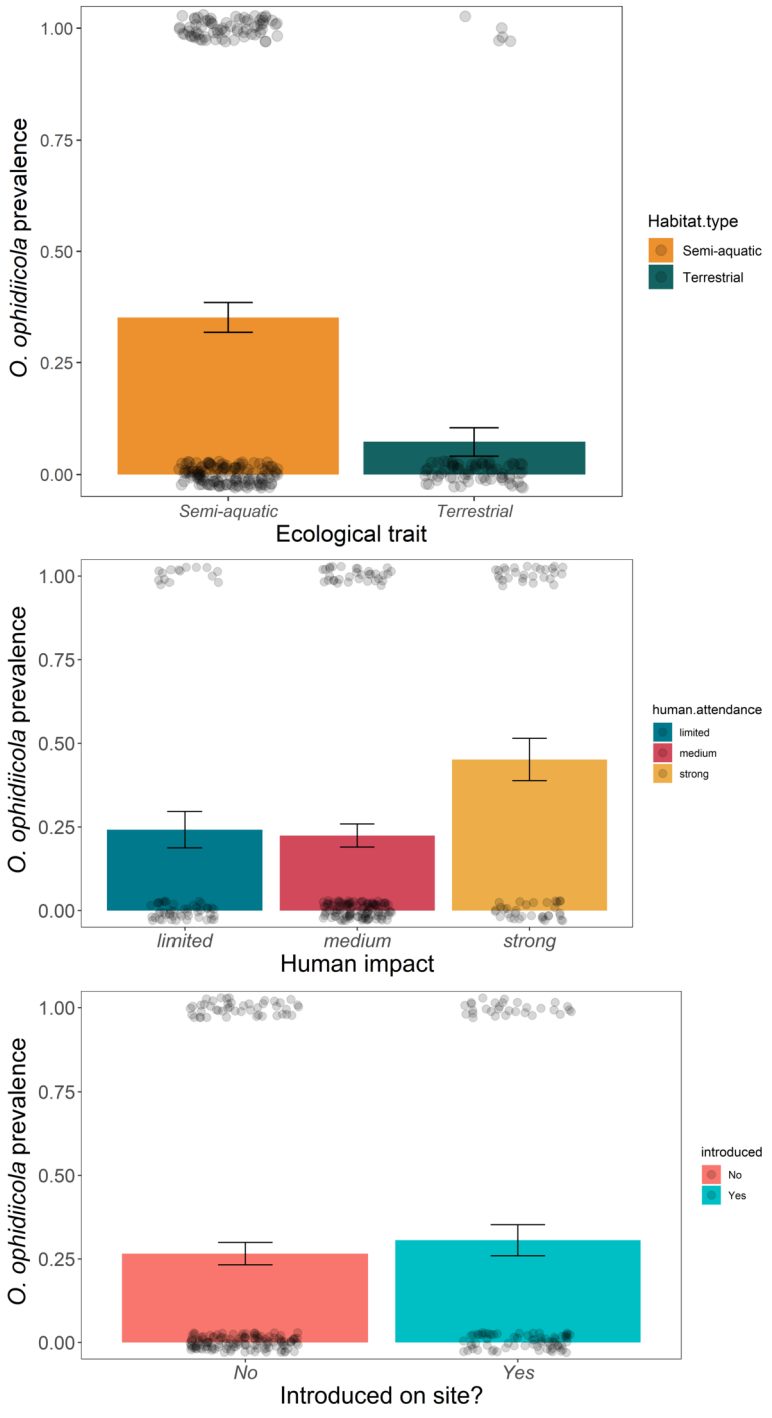

Figure 2. Graphical representation of the variability of the detection of *Ophidiomyces ophidiicola* by PCR in (A) semi-aquatic and terrestrial species, (B) populations with different levels of human impact and according to the (C) introduction status of the species on the site of capture. Semi-aquatic snakes (*Natrix sp.*) were significantly more affected by *Oo* than terrestrial snakes (genera *Vipera*, *Hierophis*, *Zamenis* and *Coronella*) (A). Sites with strong human frequentation and direct impact show a higher *Oo* prevalence on the captured snakes (B). The introduction of new species on sites did not seem to correlate with *Oo* prevalence in our study (C).

analysed following two different protocols: conventional PCR, such as the protocol used in the present study, is known to be normally less sensitive than qPCR, such as that used by our collaborator GB. Furthermore, the sampling carried out for this study included one swab per animal, whereas three were collected from each individual snake with the other (GB) protocol. The choice of the conventional protocol used in this study was based on the possibility of exploring three different targets and to provide a broader phylogenetic characterisation than that possible on a single target. Accordingly, a higher number of false negative samples than that obtained by qPCR was expected. To quantify the bias resulting from these differences, we estimated the percentage of false negative samples induced by both the different sampling and laboratory protocols as 13% (calculated by dividing the numbers of samples tested negative by conventional PCR but positive by qPCR by the total of samples analysed with both: 8/62). Accordingly, within the samples that were analysed only with our protocol (n = 29, batches 2 and 4, table 1) we can estimate that we missed around 4 true positives (0.13*29), which induces only a small underestimation of the prevalence of *Oo* in this study. On the other side, false negative rate is known to be relatively high in qPCR carried out on DNA from swabs (Hileman et al., 2018). Accordingly, we cannot rule out a possible additional number of false negatives samples secondary to this sampling strategy. However, snakes with lesions were swabbed in four replicates, reducing the chance of a false negative result.

Clades I and II lineages are present in Switzerland

The overall *Oo* prevalence in Switzerland was particularly high (28%, n = 76) compared to the previous study conducted in Europe which found an *Oo* prevalence of 8.6% (Franklinos et al., 2017). Differently, it is closer to the *Oo* prevalence found in some North American regions, such as in Kentucky, where it ranged from 20.5% to 37.9% for two different species (McKenzie et al., 2021). According to our study sites, the most affected region was Central-Switzerland, with a prevalence reaching 45% among the 66 tested snakes. When only the semi-aquatic species are considered, the *Oo* prevalence peaks at 53% over 53 individuals, almost twice as much of the two other regions. The presence of different genotypes consistent with both Clade I and Clade II lineages in Switzerland was revealed, corroborating a previous publication where both clades were found in the same geographical area (Switzerland and Northern Italy, less than 50 km away; Origgi et al., 2022). Interestingly, the distribution of the various genotypes is not homogeneous and while both Clades I and II are present in Central-Switzerland, differently, a single clade was detected in each site of the western and eastern regions, respectively. Curiously, a genotype similar to the sublineage of the Clade II (lineage II-F reported in previous studies) (Blanvillain et al., 2022; Ladner et al., 2022) was observed in Central-Switzerland as well, but at a single site. The detection of strains clustering either with Clades I and II lineages in Switzerland is intriguing and needs to be further explored because it could be a critical aspect to understand the evolution of this fungus and its natural history including possible dispersal and introductions (Origgi et al., 2022). Furthermore, the presence of strains clustering with sublineage II-F in Switzerland, which has been detected only in the USA so far, presents an important outstanding question. The parallel and independent evolution of this sublineage both in Europe and in North America is possible, but unlikely. Our data together with the findings of a previous publication (Ladner et al., 2022) would complement the hypothesis of a possible introduction of *Oo* from Europe to the USA. Interestingly, the species infected with strains belonging to the sublineage II-F was only *N. tessellata*. This species is known to have been introduced in this area from another

region of Switzerland, where the current sampling did not reveal the presence of this strain, adding a further level of complexity to the natural history of this pathogenic fungus and its origin. However, these results are to be considered carefully due to the short sequences of the three genes used for the phylogenetic analysis.

Semi-aquatic species are more likely to become infected with Oo

A strong significant difference between the prevalence of *Oo* in semi-aquatic and terrestrial species was observed in this study. The semi-aquatic species (*N. maura*, *N. helvetica*, *N. tessellata*) were more likely to be detected with *Oo* than terrestrial species, and the only terrestrial species tested positive was *Hierophis viridiflavus* with 11% of *Oo* prevalence. This result is consistent with previous studies describing a higher occurrence of apparent ophidiomycosis in aquatic snakes (McKenzie et al., 2019), the observation of skin infections following abnormally wet years (Clark et al., 2011), and the growth of fungal pathogens favoured by humid conditions (Schumacher, 2003). The significance of these findings within the context of the ecology of *Oo* requires further investigation. A thorough survey of humid habitats, along lakes and rivers when studying SFD in Europe might provide interesting insights in this direction. Similarly, of interest could be to determine the latitude intervals which are permissive for the growth of *Oo*. The recent report of three positive specimens exported from Indonesia (Ovchinnikov et al., 2021), additionally to the detection of *Oo* in Florida (Lind, McCoy, and Farrell, 2018; Glorioso, Bartoszek and Lorch, 2020) might be suggestive of the presence of *Oo* outside temperate regions.

Strong human impact is associated with higher Oo *prevalence*

Our data are suggestive of human disturbance having a potential impact on the prevalence of *Oo*. Sites with a strong human impact seemed to have higher *Oo* prevalence compared to those with medium and low impact (respectively 0.45+/−0.06; 0.22+/−0.03; 0.24+/−0.05). Consequently, snakes present at sites associated with strong human disturbance were more likely to be infected with *Oo*, although the subjective classification of the sites based on current knowledge, and the difficulty to assess the causation of this factor are important *caveat* to consider. However, interestingly, in Agazzi's desert tortoises (*Gopherus agassizii*), the prevalence of *Mycoplasma agassizii* and *M. testudineum* were found to be higher in areas closer to human populations (Berry et al., 2015).

The status of either introduced or native species at a specific site did not correlate with the probability of detecting *Oo*, contrary to what we originally hypothesised. This result could be explained by the presence of two important sampling sites that could have biased the results (one with no introduced species but with a high *Oo* prevalence, and one site with several introduced species without any *Oo* positive snake).

Seasonality does not appear to impact Oo *prevalence in snakes in Switzerland*

Interestingly, contrary to previous studies (McCoy, Lind and Farrell, 2017; McKenzie et al., 2019; Dillon et al., 2022; Lind et al., 2023a), we did not observe any significant effect of seasonality on *Oo* prevalence. According to the current literature, SFD seemed to exhibit seasonal variation in both prevalence and infection severity (McCoy, Lind and Farrell, 2017; McKenzie et al., 2019; Lind et al., 2023a), with higher prevalence of infection in Spring and Summer possibly secondary to the dynamics associated with hibernation (McKenzie et al., 2019). The apparent inconsistency of our data in comparison with the consolidated knowledge could be due to the relatively low and not homogeneous sample sizes when divided into 6 months. Seasonal patterns could be secondary to environmental conditions, but also individual

physiological conditions, including the shedding occurrence. This latter might contribute to clearing the skin lesions and reduce fungal load following seasonal patterns, as found in Lind et al. (2023a). Ecdysis (shedding of skin) has been found to be an important innate mechanism to respond to skin infections, and snakes seem to be able to clear superficial lesions caused by SFD by shedding (Lorch et al., 2015). In addition, direct field observations during the present study were made on snakes resolving clinical signs of infection by moulting during sampling. Indeed, snakes in the process of shedding, which initially presented skin lesions seemed to be clear of detectable lesions after shedding, suggesting a remarkable efficiency in clearing, at least visually, SFD-like-associated skin lesions.

Body condition is not significantly associated with Oo *prevalence in Switzerland*

The body condition of hosts, calculated with the residuals of the regression of SVL against mass, did not significantly correlate with *Oo* prevalence. We hypothesised to find a negative correlation between *Oo* prevalence and BCI. This was based on two alternative assumptions, namely: (1) snakes with poor body condition are likely to have impaired immune functions as part of an overall loss of general conditions (Palacios, Cunnick, and Bronikowski, 2013) and accordingly are more sensitive to infection and more likely to develop SFD; (2) *Oo* has a negative impact on the health of the snakes leading to poor body conditions, via reducing (either directly or indirectly) for example food intake (anorexia) (Lorch et al., 2015). Our findings are in contrast with those of previous study which found that snakes developing SFD had poorer body condition compared to that of the overall population (McCoy, Lind and Farrell, 2017), but consistent with another one where BCI did not correlate with *Oo* infection status (Chandler et al., 2019). These discrepancies could rely on the specific stage of the disease at the timing of capture, which might play an important role. Positive individuals might have their skin recently colonised by *Oo*, without having yet developed clinical signs (i.e., lesions), and consequently not have a reduced BCI. The females that were carrying eggs but for which this condition was not detected, as well as all individuals who fed on small and not visible preys prior to the capture, could induce a bias in the formula of the BCI. Finally, the methods to calculate BCI can also vary between studies (Falk, Snow and Reed, 2017). Furthermore, ophidiomycosis has been reported to impact some non-grossly assessable physiological contributors to snake's fitness, including, but not limited to levels of hormones or metabolites implicated in acute stress response (Lind et al., 2023b). Further investigations are required to evaluate the impact of SFD on the health and the ecology of snakes globally.

Other fungal agents are associated with lesions similar to those characteristics of SFD

Thirty-seven percent (n = 41) of the snakes presenting skin lesions consistent with SFD were tested negative to *Oo* by PCR (supplementary fig. S1B). In addition to the possible false negatives of *Oo* presence as discussed previously, it is known that SFD associate lesions are by definition not pathognomonic and could be caused by a number of infectious agents including bacteria, viruses and other fungal agents. More specifically, we found eight different fungal organisms associated with these lesions using a broad-spectrum PCR targeting fungal agents (see supplementary table S5). *Cladosporium sp*., a potentially clinically relevant fungus in reptiles (Jacobson, Cheatwood and Maxwell, 2000) was the predominant taxon. The actual clinical significance of all these fungal agents needs to be investigated by histopathology, a fundamental discriminatory diagnostic tool to draw a conclusive assessment on their role as potential pathogens. Interestingly, in a previous study, the investigation of a Swiss snake from Romandy presenting several macroscopic

lesions, revealed the presence of up to 18 distinct species of fungi in the lesions, including *Cladosporium sp.* (Dubey et al., 2022). However, *Cladopsorium sp*. was reported as contaminant in a number of studies carried out to investigate the presence of *Oo* (McBride et al., 2015; Marini et al., 2023). In our study, in absence of a supportive histopathology examination, the role of this fungus remains unclear. However, it is important to keep exploring the possible presence and role of other fungal organisms, other than *Oo* in free-ranging snakes, given that a number of unexplored and unknown potential pathogens are highly likely to be present in the environment and eventually potentiated by the climate change (Garcia-Solache and Casadevall, 2010). In addition to this, a site along the Geneva Lake in Romandy had a prevalence of *Oo* of 0%, although 16 snakes out of 28 had lesions consistent with SFD (see supplementary fig. S1B) and were tested several times each. These findings call for attention to a proper differential diagnosis scheme to follow when investigating SFD consistent lesions in snakes.

Ophidiomyces ophidiicola *detection in snakes in the absence of visible skin lesions*

Five out of 159 snakes without lesions were tested positive for *Oo* consistently with previous studies (Hileman et al., 2018; Chandler et al., 2019; Lizarraga et al., 2023), possibly suggesting that: (1) the snakes were in early stages of infection; (2) direct contact between infected and not infected snakes might have allowed a superficial load of *Oo* to be transferred to the uninfected individual and to be sufficient to give a positivity by PCR; (3) the efficiency of clearing the superficial infection by shedding was not complete and left residual *Oo*; (4) snakes would have been in contact of *Oo* that was present in the environment but would have not developed clinical signs yet (Allender et al., 2015b); and (5) *Oo* growth might have been inhibited by soil microbial communities (Campbell et al., 2021). All these hypotheses are not necessarily mutually exclusive. More investigation on the infection, transmission of *Oo*, and development of SFD in snakes and how/if it can be cleared in European snake populations along with its impact on their ecology is necessary.

Conservation implications

Our results suggest that high human disturbance may correlate with higher *Oo* prevalence. We recommend to every individual requiring wild snakes' manipulation to wear disposable gloves and to strictly follow the rule of one pair of catching gloves per site, more particularly in humid habitats. Additionally, we strongly recommend disinfecting hands and all material that came in contact with the snakes using 70% ethanol or 3% bleach solutions for at least 2 min (Rzadkowska et al., 2016). Furthermore, when translocating wild animals, we suggest establishing a quarantine protocol and testing them by PCR to confirm the presence/absence of *Oo*. Finally, more closely monitoring and biosafety implementation concerning the local and international animal trade and movement are warranted. The impact of ophidiomycosis in wild populations is yet to be conclusively determined and is being actively investigated by several different studies across the world, including one carried out here in Switzerland. Estimation of survival rates, assessed by different approached such as Capture-Mark-Recapture might help us understand the impact of *Oo* in European free-ranging snake populations.

Conclusion

This investigation is one of the most extensive studies to date carried out on *Oo* in a single European country, to the best of our knowledge, and is the broadest investigation concerning the presence, distribution, and ecology of *Oo* and its associated disease in Switzerland. The results presented here are supported by a large ($n = 271$) and diverse sample size composed of different snake species from seven Swiss Cantons.

Our results showed that *Oo* is widely distributed in Switzerland with a high overall prevalence and the presence of both the North American (Clade II) and the European (Clade I) lineages and relevant for the reconstruction of the natural history of this fungus. Finally, we provided evidence that semi-aquatic species are more likely to be infected by *Oo*, and that strong human impact could be instrumental to the introduction, spread and development of *Oo* in wild populations of snakes.

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