Revisiting the epidemiology of the Snake Fungal Disease: Molecular and serological investigation in Switzerland

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Abstract

Emerging infectious diseases (EID) are a major threat to wildlife and biodiversity. Amongst them, fungal diseases have recently had a considerable impact on animal populations in short durations, as chytridiomycosis, induced by *Batrachochytrium sp.* in amphibian species worldwide. In snakes, the recently discovered fungal pathogen *Ophidiomyces ophidiiocola* (*Oo*), causing Ophidiomycosis, also called Snake Fungal Disease, is responsible for the massive decline of snake's populations in North America. This pathogenic agent was recently found in Europe (Great Britain, Czech Republic and Switzerland), but the ecology, repartition and the lineages of *Oo* in Europe are still to be determined. In this study, we screened and collected swabs and blood samples from 271 individuals in Switzerland to determine the presence, the incidence, and the ecological and serological features of SFD. Using conventional PCR, we found and overall relatively high incidence of SFD in Switzerland (21.9%), with its presence in most of the study sites. We detected the two actual lineages

of *Oo* (European and American), plus a putative third independent lineage. Regarding the ecological aspect, we found that aquatic snakes were more likely to be affected by SFD, and we discovered that the human attendance, defined as the level of displacement, capture and release by humans, was also correlated with SFD incidence. Sites with strong human attendance were more likely to host snakes positive for SFD. On the contrary, neither seasonality, body condition nor species introduction were significantly correlated with SFD incidence in our study. Additionally, we developed the first ELISA test for the detection of anti *Oo* immunoglobulins in colubrid snakes. The absence of correlation between the infection status of the snake and the OD reading of the ELISA is consistent with a possible lack of an adaptive humoral response following *Oo* infection, but further study is needed to confirm this hypothesis.

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Introduction

The last decades have been marked by the emergence and re-emergence of emergent infectious agents having major impacts on wildlife and biodiversity (Daszak et al., 2000). Emerging infectious diseases (EIDs) are favoured by biodiversity loss (Keesing et al., 2010) and the international pet trade market is a major contributing factor of emergent infectious agent translocation (Fisher & Garner, 2007; Ashley et al., 2014; Ladner et al., 2022). This highlights the importance of understanding factors promoting and instrumental to the occurrence of EIDs, to adapt conservation measures, reduce habitat pressure and biodiversity loss, as well as have a better control on local or international animal displacement.

Amongst EIDs, fungal diseases have been known to be major threats to animal populations and act in short period of time, such as *Geomyces destructans,* the fungal causative agent of the "White nose syndrome" in bats, responsible of massive population reduction up to 75% in hibernacula in few years (Blehert et al., 2009). In amphibians, recent crises were caused by emerging fungal agents, such as *Batrachochytrium dendrobatidis*, a known generalist pathogen causing worldwide amphibian decline (Skerratt et al., 2007), or *B. salamandrivorans* which causes lethal chytridiomycosis in Salamanders in north-western Europe (Martel et al., 2013). These two emerging pathogens have been shown to play an important role in the decline of over 400 amphibian species worldwide and led to the presumed extinction of other 90 (Scheele et al., 2019).

In wild and captive snakes, a recent emerging disease called Ophidiomycosis or Snake Fungal Disease (SFD), caused by the ascomycete fungus *Ophidiomyces ophidiiocola* (*Oo*; Lorch et al., 2015; Allender et al., 2015) is raising concerns in wildlife conservation in North American and in Europe, and within snake breeders all around the world. The clinical signs associated with SFD are a multifocal dermatitis, which can be followed by "myositis, osteomyelitis, and pneumonia" (Allender et al., 2015), leading to high morbidity and eventually mortality, in the wild (Clark et al., 2011), as in experimental conditions (Lorch et al., 2015; McKenzie et al., 2020). SFD was first observed in the eastern USA in the second half of the 2010's where it caused deaths amongst pitviper populations (Clark et al., 2011, Allender et al., 2011).

An older case of mycotic infection outbreak could suggest SFD presence in Florida in the late 1990's, but *Oo* was not identified by fungal isolation (Cheatw*Oo*d et al., 2003; Allender et al., 2015). SFD was then diagnosed or suspected in several population of snakes in the eastern USA (listed in Allender et al., 2015) and in a broad range of snake species (wild and captive; Lorch et al., 2016).

The first evidence of SFD caused by *Oo* outside of North America in wild snakes was found by Franklinos et al. (2017). Sixteen moulted skin sheds and 8 carcasses from Great Britain and one skin shed from Czech Republic were detected positive to *Oo* with PCR after screening of 336 samples (335 from Great Britain and 1 from Czech Republic) from 2010 to 2016 (Franklinos et al., 2017). Phylogenetic analyses suggested that the strains of *Oo* found in those samples differ from the American one and represent a distinct "European" clade (Franklinos et al., 2017). A recent study examining museum specimens demonstrated *Oo* presence in North America since 1945 (Lorch et al., 2021), highlighting an older origin of *Oo* in the USA than previously thought. A novel genetic analysis study indicated that the two clades (American and European) diverged too recently to explain the presence on both continents by natural snake movements by the Beringia (Ladner et al., 2022). The results indicated multiple *Oo* introductions to the USA including some within the last hundreds of years, and a recent expansion of American strains, estimated between 1985 and 2007 (Ladner et al., 2022). More recently, 3 cases of Ophidiomycosis were found on snakes imported from Indonesia shortly after their arrival (Ovchinnikov et al., 2021), on one wild Burmese python in China (Grioni et al., 2021), and on two wild snakes (one Red-Banded snake and one Chinese cobra) in Taiwan (Sun et al., 2022), extending the range of *Oo* repartition in wild populations.

In Switzerland, the first and only known case of live wild snake diagnosed with SFD was a *Natrix helvetica* in Ticino (southern Switzerland; Meier et al., 2018). However, several observations of snakes presenting lesions similar to that of SFD were made in the surrounding area and increased suspicion of broader *Oo* repartition in Switzerland and surrounding countries (Meier et al. 2018). In addition to this, a recent study screened specimens from Swiss museums and 5 cases of SFD were confirmed by PCR and histopathology, including 3 specimens in Switzerland and 2 specimens in Italy, with the oldest

sample dating from 1959 (Origgi et al. EID, in press). Moreover, both the American and the European lineages have been found among these Swiss samples, indicating an older hypothetical divergence of the two lineages than previously thought (Origgi et al. EID, in press).

In Europe, the results are very preliminary and the knowledge about the distribution of SFD in free-living snakes is very limited. More specifically, we wondered if in Switzerland, the SFD occurs only in Ticino or if the occurrence is more widespread in Switzerland.

We screened 271 individuals to investigate the occurrence of SFD in Switzerland, and if present, the presence of both clades, along with its associated ecological features, directed to improve conservation measures. We hypothesized that the case of SFD in Ticino (Meier et al. 2018) was not an isolated case and that the prevalence and distribution of SFD in Switzerland is significant. Following previous studies, we also hypothesized that aquatic snakes would be more affected by SFD (McKenzie et al., 2019), which would be consistent with the fact that mycotic pathogenic agents of reptiles diseases grow better in humid conditions (Schumacher, 2003). As seasonality was found to play a role in the incidence (McCoy et al., 2017; McKenzie et al., 2019), we assessed the effect of seasons on SFD incidence. Moreover, we tested the impact of human disturbance on SFD incidence by assessing the significance of the human attendance (displacement, capture/release) and if the species is introduced or native to the site. We hypothesised that strong human attendance and introduced species would increase the probability of PCR positive to *Oo*, which could explain the spread of *Oo* in isolated populations. We finally quantified the effect of the body condition on the incidence of SFD, expecting to find significantly positive correlation between lower body condition and SFD incidence, meaning that infection by *Oo* might have a remarkable negative ecological impact on the snakes and would be critical for conservation measures.

On the other hand, another aim of the study was to assess the presence of an adaptive immunological response to SFD in snakes via a serological study. Indeed, although a number of investigations have been carried out on the serological immune response of snakes to infectious agents (Korzyukov et al., 2016; Neul et al., 2017), there are virtually no information concerning their response

to fungal agents. Accordingly, we expected to find a serological immune response of free-ranging wild snakes to *Oo* to contribute to close this scientific gap. In addition, a reliable serological test is necessary to assess snake's infection by *Oo* in the field and in captive colonies. This would provide a critical tool to prevent the spread of the infectious agent in subclinically infected animals. Furthermore, in case of non-clinical animals, which have either cleared or partially cleared the infection, the conventional PCR result is expected to be negative, whereas a serological test would still be able to detect the antibodies (if actually produced) against the fungus. Only this approach would allow to determine in wild animals if superficial lesions had been cleared by ecdysis, as currently suspected (Allenderet al., 2015; Lorch et al., 2015, 2016; McKenzie et al., 2020). For this combination of reasons, we developed an indirect ELISA test to assess the occurrence of an adaptive humoral response to *Oo* in exposed snakes. We hypothesized that infection status to *Oo* would positively correlate with immunoglobulin levels (using the ELISA OD reading as a proxy) and we accordingly used the PCR results as gold standard to evaluate the ELISA results, fully aware of the caveats associated with it.

Material and Methods

Sample collection

The project was conducted in collaboration with different people (Gaelle Blanvillain, a PhD student from Virginia Tech University, USA; Gregoire Meier). Consequently, the sampling was not always conducted in the same way and could be classified in 4 different batches (see Table 1) accordingly to the field responsible and the main laboratory investigator.

Study sites were chosen across 7 different cantons of Switzerland (BE, NE, NW, OW, SZ, TI, VD) in order to represent Swiss diversity and according to different criteria: they must have an easy access, known populations of snakes, high snake population density. Moreover, we also specifically selected places with introduced species as more susceptible to human interaction. Most of the sites were located near a water source (pond, lake, or river) given the SFD is known to be more commonly present in aquatic

species (McKenzie et al., 2019). Snakes were caught in visual surveys regardless of their size or behavioural activity and kept in paper bags before sampled on the field. They were then released at the exact location where they had been captured. For each snake, several morphological measures, as size, body mass or sex were recorded to test for potential SFD associated or contributing factors. Individual identification pictures of the ventral scales were taken to rule out potential recaptures within a site.

To collect DNA material for *Oo* detection, full-body swabs were collected from each snake in two replicates. Every part of the snake, including ventral and dorsal surfaces of the head, the body, and the cloaca, were swabbed with six passages. Additionally, after visual examination, snakes presenting skin lesions or deeper wounds were swabbed in duplicate specifically on the lesions themselves. The swabs were then stored separately. All the swabs were put in 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 delivered 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 lesions were present) from batches 1 and 3 were sent to Gaelle Blanvillain (protocol in annex 1). The other replicate of the swabbed lesions from batch 3, along with the swabs from batch 2 and 12 sheds from batch 4, were analysed by the author (NJ) at the Institute of Animal Pathology (ITPA), Vetsuisse faculty, University of Bern according to the protocol listed below.

Table 1: List of the different batches of samples with corresponding information

Batch of samples	Number of samples	Field responsible	Lacking information	Main laboratory investigator
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 (2 replicates of full body swabs + one replicate of swabbed lesions when present)
4	12 (sheds)	Nicolas Joudrier	Morphological data	Nicolas Joudrier

To collect material for the serological tests, blood collection was attempted on each individual, although not always successful. Blood collection was carried out using 1 mL syringes flushed with heparin before sampling. Between 0.1 and 1 mL (according to the size of the sampled snake) of blood was collected in the ventral tail vein. Blood samples were centrifugated at 13,000 rpm for 1 min as soon as possible after collection. The plasma and the red blood cells were separated and stored in different Eppendorf tubes and kept temporarily at -20°C before been stored at -80°C once delivered to the laboratory.

Sheds found in the field were also collected and kept temporarily at -20°C before storage at -80°C. They were then examined under a dissection microscope to determine the snake species when possible and the presence of lesions. If lesions were present, affected scales were collected using scalpel blade and placed into Eppendorf tubes.

To avoid contamination between sites and individuals and pathogen dispersion, we used different pairs of gloves and paper bags for every site, and every snake was manipulated wearing disposable plastic gloves. All capture and sampling were carried out with cantonal authorisations for the capture, as well as specific authorisation from the veterinary offices.

Detection of *Ophidiomyces ophiodiicola*:

Genetic tests were conducted to detect the occurence of *Oo* on the snakes. For that, DNA extraction was performed on the lesion swabs and scale samples from sheds using the DNeasy Qiagen extraction kit (Qiagen, Hobrechtikon, CH). The final DNA concentration was assessed by spectrophotometry with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Witec AG, Littau, CH). Conventional qualitative PCRs protocols were carried out to detect specific *Oo* genome sequences (Origgi et al., in press). Specifically, the protocols carried out included one targeting the 5.8-28s RNA internal transcribed spacer 2 (ITS), another the actin gene (ACT) and the third, the transcription elongation factor (TEF; see annex 2). A supplementary PCR protocol was carried out with broadly reacting primers against fungal sequences (panfungal; Borman et al., 2006). It was carried out in order to identify any

fungal agent, which might have been associated to detected lesions, for which *Oo* could not be detected (see annex 3). The positivity of the PCR products was assessed by resolving the specific amplicons in a 2% agarose gel.

When multiple DNA bands were detected in the agarose gel, the band of consistent size with the expected target was purified by gel band extraction using a gel extraction kit (PureLink Quick Gel Extraction Kit, Qiagen, Hobrechtikon, CH). In addition, when a sample tested negative together with a not visible primer dimer band, this was considered as a presumptive evidence of a reaction inhibition. Accordingly, these samples were re-tested increasing the final volume from 30 to 50 µl to dilute the putative inhibitors.

The positive samples were sent for Sanger sequencing to Microsynth AG (Belgach, CH) and the chromatograms were manually checked by the authors.

In order to determine the strains of the *Oo* from the positive samples, a phylogenetic analysis was performed on the obtained sequences. A maximum-likelihood phylogenetic tree was built using the software MEGA7 (Kumar et al., 2016) for the three genes ITS, ACT and TEF using 10'000 Bootstrap replicates.

Serology

One of the aims of this study was to assess a potential adaptive immune response (antibody production) against *Oo* in the infected snakes. Accordingly, an indirect Enzyme-linked immunosorbent assay (ELISA) was set up as described below.

Antigen

The antigen used in the ELISA test was a crude fungal extract obtained from colonies of *Oo* grown on Mycosel agar (Lucerna Biochem, CH) similarly to what described by Lorch et al. (2015). Briefly, the type strain MYCO-ARIZ AN0400001 was obtained by the ATCC repository and grown at 25°C for 10 to 30

days. The plates were then flooded with 10 ml of sterile PBS containing 0.05 Tween 20 and gently rotated to solubilize as much antigen as possible. The suspension was then collected and placed into a 50 ml falcon tube, after being filtered through two layers of a gauze. Following the suspension was centrifuged at 3,500g for 10 minutes. The supernatant was discarded and the pellet was resuspended in an equal amount of sterile PBS. The procedure was repeated three times until the pellet was resuspended in a final volume of 3 ml and the antigen (arthroconidia) quantified on a cell-counter chamber. The suspension was stored at -20°C after being diluted to $1x10^7$ conidia/ml. The antigen was tested at $1x10^6$/ml up to $1x10^0$/ml, using a suspension of a total of 50 µl per well.

Primary antibody

The primary antibody used in the indirect ELISA protocol was the serum of the snakes sampled for this investigation. The sera were tested at 1/12.5; 1/25 and 1/50 dilutions in blocking buffer (5% not fat dry milk in PBS)

Secondary antibody

A secondary Rabbit polyclonal antibody was developed to react against *Natrix tessellata*. IgY was obtained according to a similar protocol previously described (Origgi et al., 2001). Briefly, following purification by chromatography from whole snake serum of *Natrix tessellata*, IgY were inoculated subcutaneously in two rabbits (New Zeeland White) at time 0 and with two successive boosts at time 4 weeks and 8 weeks. Rabbits were bled at time 0, prior each boost and at the bleed out. Specificity of the polyclonal antibody was assessed by western blot and by Mass spectrometry (data not shown). The purified rabbit-anti- snake IgY were then labelled with Horseradish peroxidase (HRP) and resuspended in glycerol at 1 microgram/ml and stored at -20 until use. The cross-reactivity of the obtained polyclonal antibody was assessed against the following species of snakes by dot bot and ELISA (using the snake serum as antigen and the HRP-labeled polyclonal antibody as primary antibody): *N. tessellata*, *Natrix helvetica, Hierophis viridiflavus*, and *Vipera aspis* (tested by ELISA only).

"True" Positive and negative controls

Additionally to putative positive controls obtained from snakes showing consistent lesions and tested positive for the presence of intralesional *Oo* by PCR, along with sera from snakes with no detectable lesions and negative by PCR, we aimed to use as true negative and positive control sera, those obtained from *Oo* PCR negative snakes either inoculated with inactivated *Oo* (true positive) or sham inoculated with PBS (true negative). To obtain the true positive and negative sera six *N. tessellata* captured in the wild. They were tested by PCR as described above and checked for consistent SFD lesions. Inactivated *Oo* antigen was obtained by treating with one volume of 10% formalin 9 volumes of a suspension of *Oo* arthroconidia at 4°C overnight, followed by 5 washes to eliminate the formalin. The athroconidia were then prepared at a final concentration of 105/0.2 ml and stored at -20°C. A sample from the same batch was then seeded on Mycosel agar and monitored for a month to make sure that no growth was occurring.

The snakes were kept in two groups of three individuals in separated terrariums accordingly to the Swiss legislation. After some adaptation time (10 days), blood was taken from all the individuals as a time 0, then the positive group was injected with 0.2 mL of a suspension of a total of 10^5 arthrochonidia in PBS and the negative group with 0.2 mL of sterile PBS buffer. A second bleeding was carried out after 3 weeks post injection, prior the antigen boost. A third bleeding was planned at 8 weeks post injection. However, this could not be performed because of the unexpected death of all the snakes at week 5 (see annex 4). All the snakes were sampled at this time, but antibodies were obtained from the liver. More specifically 2 g of liver tissue were homogenized in a mortar with a pestle and quartz powder and 3 ml of cell-culture media Leibovitz's L15 (Gibco, Thermo scientific, CH). The suspension was then centrifugated twice at 1,200g and the supernatant was filtered using a 0.4 micron syringe filter and stored at -20°C until use.

ELISA protocol.

Ninety-six wells Elisa plates were covered with 50 µl of untreated suspension of O.o arthroconidia with concentration ranging at $1x10^6$ to $1x10^0$. The plates were then incubated at 4°C overnight to allow the biding of the antigen to the wells. After discarding the antigen, each well was blocked with 300 µl of a 5% suspension of not fat dry milk in PBS and incubated for one hour at room temperature or at 4°C overnight. The blocking buffer was then discarded and the wells were washed three times with 300 µl of PBS tween (0.05%). Fifty microliters per well of the primary sera (true positive and negative controls for the protocol tuning along with true positive sera confirmed by PCR; sample sera) was initially tested at different dilutions (1/12.5; 1/25; 1/50), and then added to the 96 well plate (in duplicate) and incubated at room temperature for one hour.

Following removal of the primary sera, each well was washed three times with 300 µl of PBS tween (0.05%). The secondary antibody was initially tested at different dilutions (1/250; 1/500; 1/1000) first without primary antibody to evaluate potential non-specific cross-reactivity. A total of 50 µl of the secondary antibody suspension diluted in PBS was added to each well and incubated at room temperature for one hour. The secondary serum was then discarded and the wells were washed three times with 300 microliters of PBS tween (0.05%). Development was carried out adding to each well a 100 µl suspension of 10 ml ABTS substrate buffer with 2.8 µL of hydrogen peroxide H_2O_2(Dr. GROGG, CH). The plates were read in an ELISA plate reader (VERSAmax microplate reader, Molecular Devices, Basel, CH) at a wavelength of 405 nm at time 0 and then every 5 minutes.

Dot Blot Protocol

As a second line of evidence of serological reaction, we complemented the ELISA protocol with a dot-blot test. Membranes of nitrocellulose (BioRad laboratories, Cressier, CH) were placed in a 24 well plate and covered with a mixed suspension of 2 µl of 105/ml crude arthoconidia and 2 µl of 106 autoclaved arthroconidia. The membranes were then blocked with 300 µl of the same blocking buffer used for the ELISA protocol for one hour at room temperature. Following three washes with 300 µl PBS-T, the optimized dilution of the primary serum worked out for the ELISA protocol was used for the dot blot, as well and similarly the secondary antibody. Membranes were developed by

chemiluminescence (Pierce™ ECL system, USA) and the readout was carried out using a chemiluminescence reader (c600, Azure Biosystems) with ten sequential reads, once every 10 seconds.

+ western blot

+ histology

Statistical analyses:

For every species part of the study, an habitat type (aquatic or terrestrial) was attributed according to the ecology of the species (see Table 2). To confirm the hypothesis that aquatic species are more likely to be affected by *Oo*, we used a generalised linear regression model (GLM) combined with an ANOVA (n=271). Additionally, to assess the impact of human disturbance on SFD incidence, we added two more variables in this model: "introduction", meaning if the species was introduced or is native to the site, and "human attendance". A level of human attendance (limited, medium or strong) was attributed to every site by Dr. Sylvain Ursenbacher according to the current knowledge and estimation of direct human impact (displacement, capture/release) on snake population on the site.

Another aim was to quantify the effect of seasonality on *Oo* incidence. For this, we defined the seasons as following: the spring season ranged from the 20th of March to the 20th of June, and the summer season from 21st of June to the 21st of September. The limited number of samples in Fall (n=4) and in Winter (n=4) did not allow us to include these seasons in the test. Moreover, we selected sites with a minimal number of samples for each season (n>5) and the retained sites were La Sauge and St-Blaise. The difference in the infection status of snakes in Spring and in Summer was then assessed using a Pearson's Chi-squared test.

Then, to determine if snakes with morphological indicators of low health are more likely to contract SFD, a Body Condition Index (BCI) was calculated for each one of the two main infected species (*Natrix Helvetica, Natrix tessellata*) for both sexes (n=174). This was done using the residuals of the linear regression of mass on snout-vent length (SVL), as in Gimmel et al. (2021). The effect of BCI on the

probability of infected snake was tested using a generalised linear regression model (GLM) combined with an ANOVA.

All statistical analyses were performed on R version 4.0.2.

RESULTS

Detection of *Ophidiomyces ophiodiicola*:

We found a total of 76 (21.9%) samples positive to *Oo* out of 271 individuals (68 live snakes out of 258 and 8 sheds out of 13) with PCR analyses, later confirmed by sequencing.

Oo has been found at different locations in all cantons part of the study. In the central Switzerland (cantons BE, OW, NI, SZ), the incidence of *Oo* was the highest with 45% (30 out of 66). In Romandy (western, cantons NE and VD; see Figure 1), there was the lowest *Oo* incidence with 21% of the samples being tested positive (29 out of 141). Finally, in the Swiss Italian region (South, canton Ticino), the incidence of *Oo* was 27% (17 out of 64). These differences in SFD incidence between the three regions were statistically significant (Chi-squared test, p-value < 0.005).

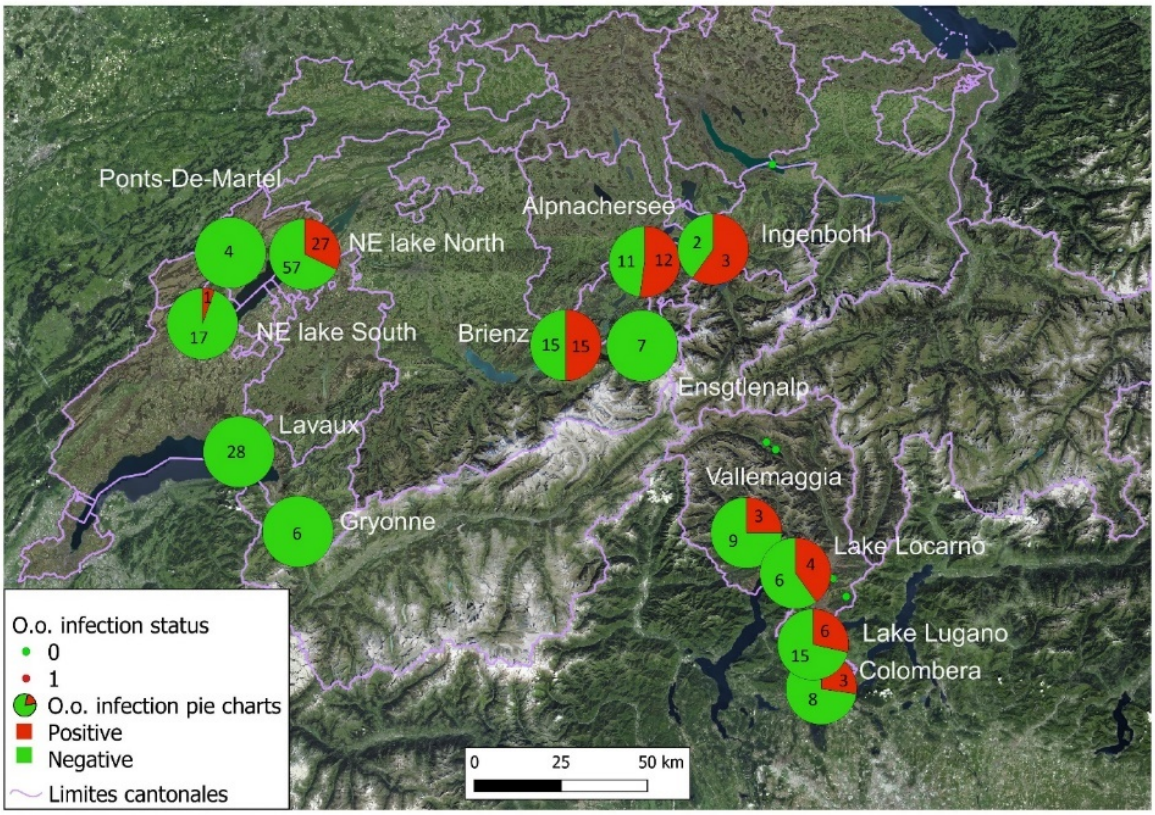


Figure 1: Mapping of the frequency (or occurrence) of Ophidiomyces ophiodiicola *infection in Switzerland. 0 stands for negative to Oo by PCR and 1 for positive by PCR. Made on Qgis 3.22.*

Lineages

Phylogenetic analyses of the strains confirmed the presence of isolates clustering either with the American or European clades (names according to Franklinos et al., 2017) in Switzerland consistently with what a recently published retrospective study found (Origgi et al. EID, in press). The strain of *Oo* was successfully assessed for 46 samples regrouped in 3 regions: Romandy (n=14), central Switzerland (n=21), and Swiss Italian (n=11). In Romandy, all 14 samples sequenced show the European strain, whereas the 11 samples sequenced in Ticino show the American strain. Additionnally, the Central Switzerland region include 10 samples with the American strain (sites “Alpnachersee” and “Brienz”; see Figure 2), 3 samples with the “European” strain (site “Ingenbohl”) and 8 other samples with an intermediate strain of *Oo* Indeed, for 8 out of 11 samples of the site “Brienz”, the *Oo* sequences show the SNIPs of the “American” strain for the genes ACT and TEF, while they show the European strain’s SNIP for the gene ITS. However, we will consider these intermediate strains as American strains

because of the more conserved nature of the ACT and TEF genes compared to ITS which is a non-coding sequence, until deeper study of this lineage.

Table 2: number of samples of the different lineages for the three studied regions

Region/number of samples	European lineage	American lineage	Intermediate lineage
Romandy	14	0	0
Central Switzerland	3	10	8
Ticino	0	11	0

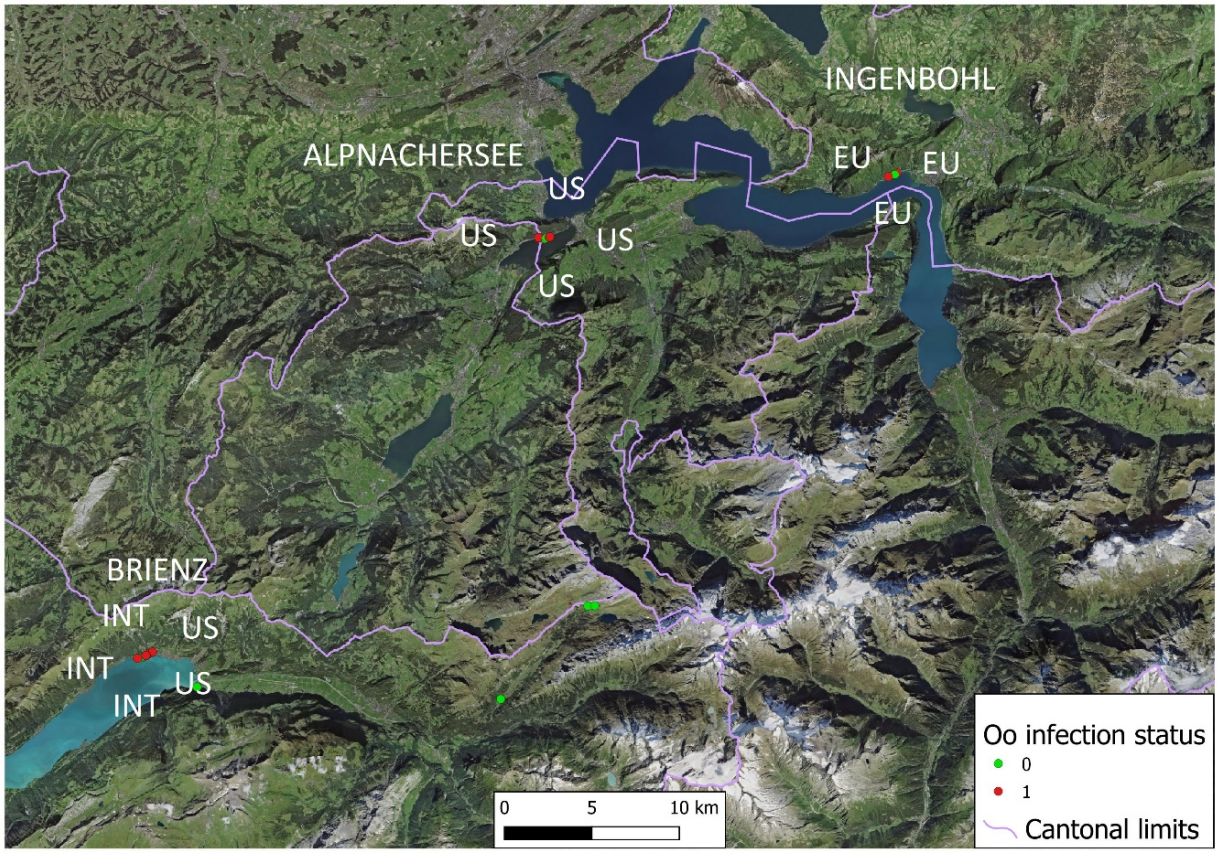


Figure 2: Oo Strains in the Central Switzerland. US stands for the American strain, EU for the European one and INT for the intermediate American strain. Made on Qgis 3.22.

The ACT and TEF phylogenetic trees showed a clear dividing line between the two strains (see annexe 5), whereas the ITS phylogenetic tree additionally shows the intermediate American strain (See Figure 3).

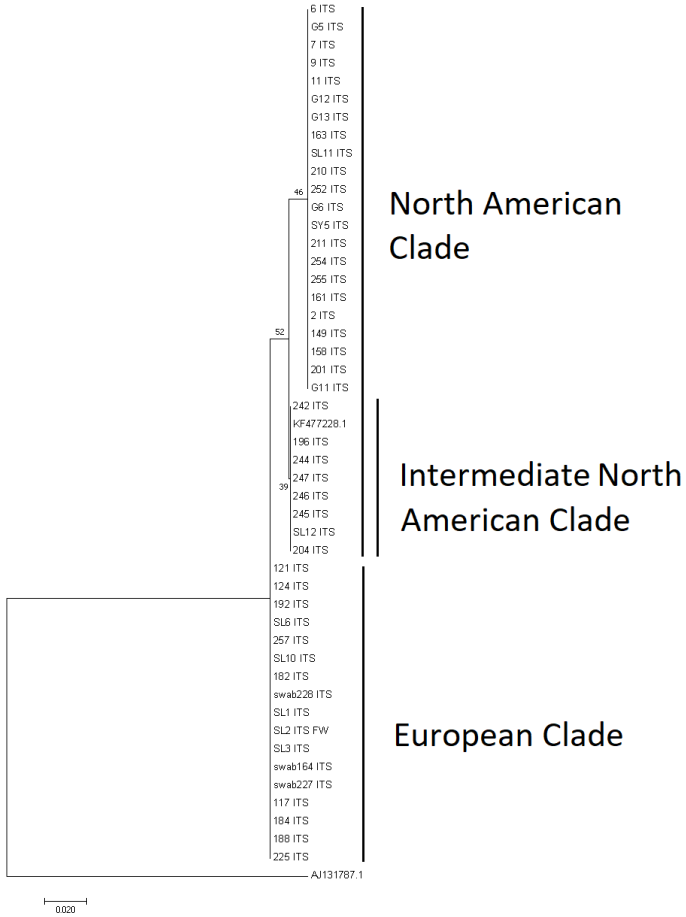


Figure 3: Maximum likelihood *Phylogenetic tree of the ITS gene with the associated* Bootstrap *value at the nods. AJ311787.1 is the outgroup sequence (*Pseudoamauroascus australiensis *5.8S rRNA gene and internal transcribed spacers 1 and 2, strain FMR 5482).*

Associated factors

The habitat type, or if the snake is mainly aquatic or terrestrial (see Table 3), had a significant effect on the incidence of SFD (Chisq = 23.52, Pr(>Chisq) = 1.233e-06). Aquatic snakes were more likely infected by *Oo* (overall mean of infection = 0.35) than terrestrial snakes (mean = 0.072; see Figure 4), as hypothesised. The level of human attendance was also significantly explaining the variation of SFD frequency (Chisq = 10.80, Pr(>Chisq) < 0.005), with the strong level of attendance being associated with a higher infection probability (see Figure 4), which confirms our hypothesis.

On the contrary, if the species was introduced or was native to the site had no significant effect on infection frequency (Chisq = 1.75, Pr(>Chisq) > 0.05).

Regarding the effect of season, we found no significant difference between the Spring and the Summer seasons (Pearson's Chi-squared test, X-squared = 0.61, p-value = 0.44).

Table 3: number of positives and negatives for every species included in the study and the associated habitat type. (aq) stands for aquatic habitat type and (ter) for terrestrial one.

Oo infection status	Coronella austriaca (ter)	Hierophis viridiflavus (ter)	Natrix Helvetica (aq)	Natrix maura (aq)	Natrix tessellate (aq)	Natrix sp. (aq)	Vipera aspis (ter)	Vipera berus (ter)	Zamenis longissimus (ter)
0	3	40	78	4	49	0	9	7	5
1	0	5	35	3	32	1	0	0	0

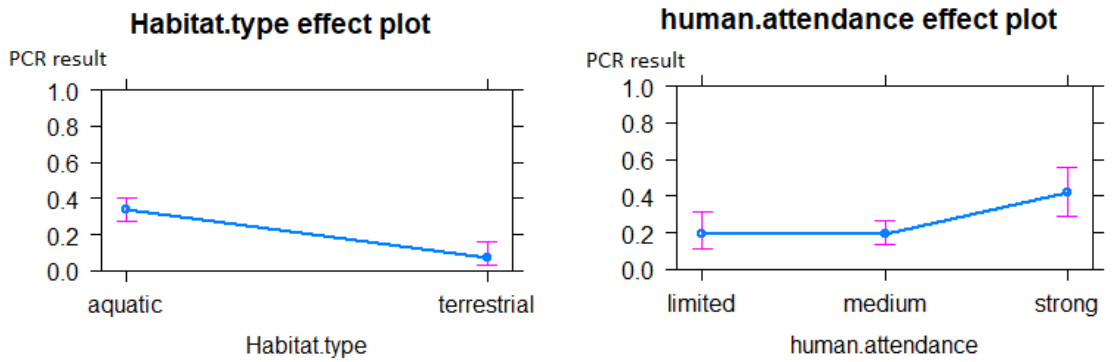


Figure 4: variability of the detection of Oo with PCR for different habitat types and different level of human attendance. Made using the package "effects" on R v4.0.2

The BCI, calculated for *Natrix tessellata* and *Natrix Helvetica* (n=174) with the residuals of the linear regression of mass on snout-vent length (SVL), had no significant correlation with SFD incidence (Chisq = 0.93, Pr(>Chisq) = 0.33).

Serology

Assessment of optimal primary antibody dilution

Using an initial dilution of the antigen set arbitrarily at 10^5 arthroconidia/ml, we covered each of the microtiter wells with 50 microliters of the suspension. Of the primary sera dilutions assessed ranging from 1/12.5 to 1/50, the dilution of 1/12.5 was the one providing the stronger reactivity and accordingly, was chosen as ideal primary sera dilution (see Figure X).

Assessment of optimal secondary antibody dilution

Of the different dilutions tested of the secondary antibody, ranging from 1/250 to 1/1000, the dilution providing the highest reactivity with the lowest background was that corresponding to 1/500, which was the dilution, which was eventually chosen for the testing of all the field samples (Francesco).

Assessment of optimal antigen amount

Once the optimal dilutions of primary and secondary antibodies were obtained, they were tested against different dilutions of antigen ranging from $3x10^6$ to 10^1. The antigen concentration which provided the overall highest and most consistent OD reading across the presumptive positive sera from the available snakes (*Natrix* sp., including those experimentally infected and *H. viridiflavus*) was that of 10^6/ml (see figure Y).

Positive control sera

We considered presumptive positive sera, those obtained from snakes that tested positive for *Oo* by PCR and which showed consistent lesions with intralesional fungi. Animals fulfilling these requirements were a single *Natrix helvetica* and a single *H. viridiflavus*. Additionally, we performed an inoculation study in naïve *N. tessellata*, putatively negative for *Oo* (PCR negative) in order to obtain "true" positive and negative sera samples. Unfortunately, all the snakes in the study (n=6) died suddenly at 5 weeks post injection of unrelated cause to SFD. Accordingly, it was unclear if seroconversion would actually have occurred, given that snakes are known to fully seroconvert in approximately 8 weeks (Lock et al., 2003). Assessment of the parameters described above was carried out using all the putative positive samples available (2 naturally infected snakes and a pool of the three *N. tessellata* inoculated with inactivated *Oo*) and selecting as putative negative sera those from different PCR negative *Natrix sp*.

and *H. viridiflavus* along with a pool from the three *N. tessellata* inoculated with PBS (negative controls). Finally, normalization of the ELISA results across microtiters plates (to control for interpolate variability) was carried out using the OD reading of the each of the positive sera used, which were arbitrarily considered equal to 1. OD reading was recorded at 2 different time points, besides at time 0.

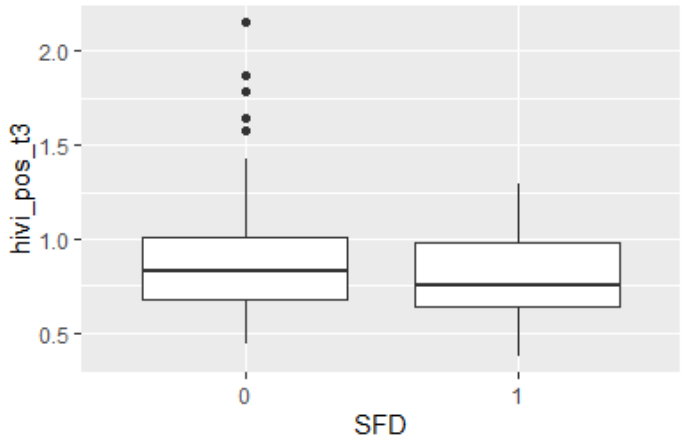
ELISA results of the field samples

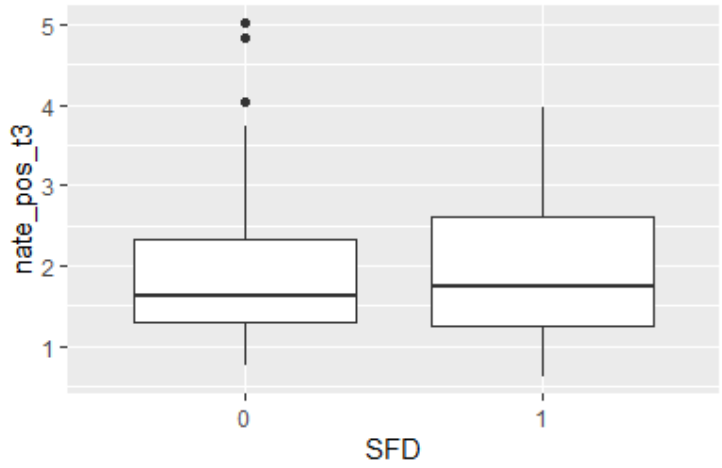
A total of 226 serum samples including 38 *Hierophis viridiflavus*, 98 *Natrix Helvetica*, 2 *Natrix maura,* 75 *Natrix tessellata*, 5 *Vipera aspis*, 4 *Vipera berus*, and 4 *Zamenis longissimus* were tested by ELISA.

In absence of any other serological test that could be used as gold standard comparison for the ELISA results, we matched the normalized OD readings with the PCR results, fully aware of the caveats associated with it. Overall, the PCR positive snakes and those testing negative, showed relatively comparable OD readings (see Figure 5), suggesting either a lack of reactivity against *Oo* by the infected snakes or/and the occurrence of non-specific serologic reactivity of the snakes against this fungus. This lack of significant difference between positive and negative PCR samples as assessed by ELISA was confirmed by using a Student's t-test for independent samples on the normalisation of the three controls, each at the three different timings. None of the test showed a significant correlation (see Table 4) but the one normalized with the *Hierophis viridiflavus* serum at time 3 (p-value = 0.03) for which the mean OD reading values were higher for samples tested negative for *Oo* (mean of negative tested samples: 0.884, mean of positive tested samples: 0.804).

Table 4: correlation between Oo infection status of the snakes (tested by PCR) and the OD reading values of the ELISA test

	Time 0		Time 1		Time 2	
Control sample for normalisation	t	p-value	t	p-value	t	p-value
Hierophis viridiflavus	0.67	0.50	1.82	0.07	2.18	0.03
Natrix helvetica	-1.75	0.08	0.10	0.92	0.46	0.65
Natrix tessellata	-1.12	0.27	-1.25	0.22	-0.72	0.47





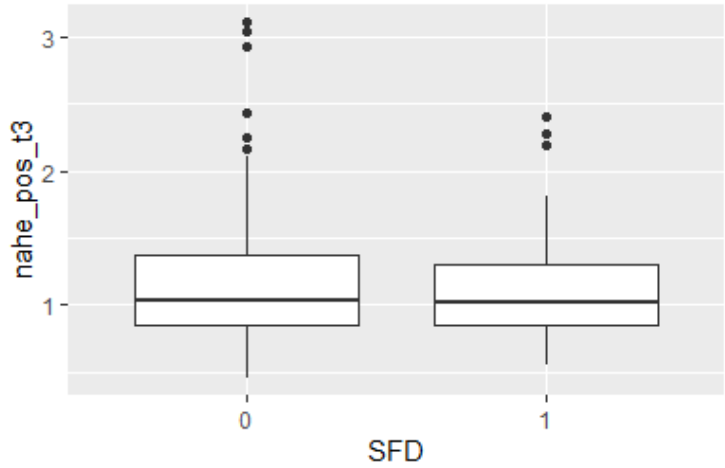


Figure 5: Standardized (respectively to Hierophis viridiflavus*,* Natrix tessellata *and* Natrix helvetica*) OD readings of every sample at time 3 with the associated PCR result. 0 stands for negative to Oo and 1 for positive to Oo. Made using boxplot function of ggplot2 package in R.*

Dot blots

The same sera, which were tested by ELISA, were also tested by DOT blot. Batches of 5 sera at a time were used for each membrane. None of the tested batch showed any obvious reactivity.

Combined serological results

The combined serological results including the novel ELISA test and the dot bot are highly suggestive of a lack of specific reactivity versus *Oo* by the infected snakes.

DISCUSSION

This is, to the best of our knowledge, the most extensive study to date carried out on *Oo* in Europe, and the first study to complement the molecular examination with a first investigation into the adaptive immune response to *Oo.* We confirmed the presence of *Oo* and the associated disease (SFD) in wild Swiss populations of snakes as suspected by the unique case from Meier et al. (2018) and the museum samples screening recently carried out by Origgi and colleagues (EID, in press).

During this study, we sampled a large sample size of snakes (n=271) of different species and from different regions of Switzerland. The overall incidence of SFD was significantly high (21.9%). According to our study sites, the most affected region was the central Switzerland (German speaking part), with a SFD incidence reaching 45% among the 66 snakes tested. Interestingly, along with evidence of the presence of the two currently known lineages of *Oo* (American and European) in Switzerland, consistently with Origgi et al. (in press), we found a putative new lineage, which clusters in an intermediate clade between the American and the European one (Figure 2). This presumptive intermediate lineage was detected in the same area where also the American lineage was observed, but also at a relative close distance from a site where only the American and the European clades were

present. It is not clear if this intermediate strain does actually represent an actual third independent clade, unrelated to the two known up to date and which evolve independently, or if it actually represents an intermediate form of the fungus obtained by the recombination between the American and the European clade. Further studies are needed to clarify this aspect. However, other strains with the same SNPs of various geographical origin are present in Genbank, suggesting that this intermediate lineage might represent an actual independent clade, coevolved together with the other two.

We assessed the correlation between the SFD infection status of the snakes (tested by PCR) and morphological and ecological features. First, we found a strong significant difference between aquatic and terrestrial species. Indeed, the aquatic species (*Natrix maura, Natrix Helvetica, Natrix tessellata*) were more likely to be infected by *Oo* than terrestrial species. The only terrestrial species that was tested positive was *Hierophis viridiflavus.* This result is consistent with previous studies (McKenzie et al., 2019) describing a higher occurrence of SFD in aquatic animals or following abnormally wet years (Clark et al., 2011) and with the ecology of most fungal pathogenic organisms, which growth is favoured in humid conditions (Schumacher, 2003).

Human disturbance seemed to have a complex impact on SFD incidence. If the species was introduced or is native to a site did not correlate with the probability of observing an infected snake, which is contrary to our hypothesis. Indeed, we expected that species that were released (most of the time illegally) would be more likely to be vector of the infectious agents because of their unknown original site and housing conditions. On the contrary, the human attendance, defined as the level of capture/release/displacement, had a significant impact on SFD incidence. Snakes in sites with strong human attendance were more likely to be infected with *Oo*. This result is to be considered carefully because of the uncertain classification of the sites, due to the potential past or on-going illegal activities that would not be to the knowledge of the conservation and protection services.

We did not find any effect of seasonality on the infection probability, although this is not consistent with previous studies (McCoy et al., 2017; McKenzie et al., 2019). However, our data set did not permit us to test for Fall and Winter seasons, a limitation which did not allow us to be able to fully compare

our data to those of McKenzie et al. (2019), where the authors highlighted a significant difference between Summer and Fall but not Summer and Spring.

The body condition, calculated with the residuals of the regression of SVL against mass, was also not significant in explaining the variation in *Oo* infection's rate. We hypothesized that the individuals with the lowest body conditions would be more affected by SFD. This would have led to two opposed interpretations: 1) snakes with low body condition are more sensitive to infectious and thus more likely to contract SFD. 2) SFD would have a negative impact on the health of the snakes and thus would be the cause of lower body condition, via reducing the feeding abilities for example. On the contrary to our finding, McCoy et al. (2017) found that snakes contracting SFD were in lower body condition compared to the overall population. Further study is required to estimate the impact of SFD on the health and the ecology of snakes in Switzerland.

Out of the 271 snakes sampled, 112 (41%) were presenting lesions consistent with SFD, the others presented either no macroscopic detectable lesions or other lesions not consistent with SFD. Of 112 snakes with lesions, 71 (63%) tested positive to *Oo* by PCR. This means that around 40% of snakes with lesions consistent with SFD are likely to be infected with another infectious agent, including fungi (see example in Annex 7 Image 1). The SFD negative lesions, once tested with a panfungal PCR and sequencing allowed us to find 8 other fungal organisms on the lesions (see Annex 3). The actual clinical significance of these agents would need to be assessed by histopathology to draw a conclusive assessment on their role as relevant pathogens. Interestingly, in a previous study, the investigation of a snake presenting several macroscopic lesions, found in the French speaking part of Switzerland revealed the presence of up to 18 species of fungi in the lesions, including several opportunistic pathogens (Dubey et al., 2022).

Interestingly, 5 out of 159 snakes without lesion tested positive for *Oo*, possibly suggesting that: 1) the snakes were in early stage of infection. 2) direct contact between infected and not infected snakes can allow a superficial load of *Oo* to be transferred on the uninfected individual and to be sufficient to give

a positivity by PCR. 3) the efficiency of clearing the superficial infection by shedding is not complete and leaves residual *Oo*.

Regarding the serological study, we wanted to provide a critical tool to assess the presence of an adaptive immunological response to SFD in snakes, and a complementary tool to PCRs in order to study the incidence of *Oo* in captive and wild snakes. This was to our knowledge the first attempt to set up an ELISA protocol for free ranging colubrids. We successfully produced an anti-IgY immunoglobulin by developing a polyclonal serum from rabbits. The results of the different ELISA tests we conducted showed high variability across samples but also within samples, complicating the repeatability of the experiment and the interpretation of the results. We did not find any correlation between the infection status of the snakes and the associated OD reading values. The injection study, which was carried out to clarify if an immune response against *Oo* was possible and to obtain "true" positive and negative control sera, could not be completed because a sudden and unpredicted death of the snakes, complicating the statement about the presence of a humoral response against the fungus. Our preliminary evidences suggest that the adaptive immune response is not elicited by the *Oo* infection in free ranging snakes. The predominant distribution of the SFD associated lesions to the superficial skin, might actually prevent the contact of the fungus with the antigen presenting cell, that would mediate the intervention of the adaptive immune response. Accordingly, the immune response to *Oo* would be predominantly innate. Furthermore, some of the animal that tested positive by PCR might have been recently infected and without circulating antibody against the fungus. At the same time, animals testing negative by PCR, might have circulating antibodies against the fungus from a previous clinical disease. Finally, we cannot rule out that snake might have circulating humoral factors broadly reacting against common fungal antigen, which would make the discrimination of a specific reaction to the fungus extremely difficult. Additionally, experiments are on the way to better clarify these aspects.

Conclusion

This study is the broadest investigation of the presence, distribution and ecology of *Oo* and of the associated disease in Europe. Our results show that *Oo* is widely distributed in Switzerland with a high overall incidence. We found the presence of the American and the European lineages as well as an intermediate lineage/putative third independent clade in the Swiss territory. We found that aquatic species were more likely to be infected with *Oo* and that strong human attendance could be instrumental to the introduction of *Oo* in wild snake's populations. We have developed the first ELISA test for the detection of anti *Oo* immunoglobulins in colubrid snakes. Our results are consistent with a possible lack of an adaptive humoral response following *Oo* infection. These preliminary data will be a critical baseline for all the future molecular and serological studies on *Oo* in Switzerland and in Europe.

Conservation implications

We recommend to every person involved in study or field, requiring wild snakes' manipulation to wear disposable gloves, and to strictly follow the rule of one pair of catching gloves per site when possible, more particularly in humid habitats. We recommend disinfecting the hands and all the material in contact with the snakes with ethanol 70% for at least 5 min (Rzadkowska et al., 2016). When translocating wild animals, we suggest establishing a quarantine protocol and testing by PCR the presence of *Oo* on the concerned animals.

Further studies are needed to determine the effects of SFD on the health, ecology and behaviour of wild snakes to improve their conservation.

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Authorisations

Veterinary authorisation number VD3718; ID (National number): 33612

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Annexes

Annex 1: PCR Protocol used by Gaelle Blanvillain: not received yet

Annex 2: PCR Protocol and primers used by Nicolas Joudrier

PCR primers:

1. ITS:
   FW: 5'-TGTCCGAGCGTCATTGCAACC
   RV: 5'-AACAGATTCCCATACACTCAGACACC
2. ACT:
   FW: 5'-TTAGATTTCCAGCAAGAGATCCAGACTG
   RV: 5'-CCAAGACGCTGGGTTGGAAAAG
3. TEF
   FW: 5'-CCAGCCCAACTATCAAACTTTGGC
   RV: 5'-TGATACCACGCTCACGCTCGG
4. PANFUNGAL
   FW: 5'-GAGTCGAGTTGTTTGGGAATGC
   RV: 5'-GGTCCGTGTTTCAAGACGG

PCR settings:

Primer	ITS	ACT/TEF	PANFUNGAL
Nb of cycles	35	40	35
Pol activation time (in minute:seconde) /temperature (in °C)	3:00 at at 95	3:00 at 94	3:00 at 95
Denaturation time/temperature	0:30 at 95	0:30 at 94	0:30 at 95
Annealing time/temperature	0:30 at 52	0:30 at 52	0:30 at 57
Extension time/temperature	0:30 at 72	0:30 at 72	0:30 at 72
Final extension	10:00 at 72	10:00 at 72	10:00 at 72

Protocol:

A total of 100 ng DNA per sample was added to each reaction tube containing a total volume of 30 µl comprising 3uL of 10X reaction buffer BD + 3 uL of 25mM $MgCl_2$ + 0.5 uL Forward primer + 0.5 uL reverse primer (both at 100 micromolar) + 0.4 uL dNTP mix 10 mM + 0.2 uL DNApolymerase 5 U/ul) and DD water up to 30 microliters.

Annex 3: other fungal organisms found on the swabbed lesions, found by using Panfugal PCR.

Fungal organism	GenBank accession number
Cladosporium sp.	MH877884.1 MT636935.1 MN396196.1 MT636938.1
Cadophora bubakii	MH871230.1
Pleosporales sp	MH877884.1
Phacidium pseudophacidioides	NG_069195.1
Keratinophyton indicum	MH872218.1
Phaeothecoidiella missouriensis	NG_069924.1
Verrucocladosporium dirinae	MH874471.1
Dothiora coronicola	NG_081525.1

Annex 4: necropsy findings of the 6 deceased Natrix 34essellate *of the injection study*

			Histological examination			
Natrix tessellata	Weight (g)	Length (cm)	skin	heart	liver	lung
1	174.5	100	Dermatitis, heterophilic, multifocal, mild to moderate with intralesional arthropods (mites)	-Hemorrhages, multifocal to coalescent, moderate, subacute -Pericarditis, heterophilic, multifocal, mild to moderate, chronic	atrophy, moderate, diffuse, subacute to chronic	Endoparasitism (nematodes), moderate, multifocal, chronic
2	200	96	Similar to snake 1	Similar to snake 1	Similar to snake 1	Similar to snake 1
3	188	97	Similar to snake 1	-Hemorrhages, mild, multifocal, subacute -Pericarditis, heterophilic, multifocal, minimal, subacute to chronic	Similar to snake 1	Similar to snake 1
4	270	96	Dermatitis, heterophilic, multifocal, mild with		Similar to snake 1	Endoparasitism (nematodes), mild, multifocal, chronic

			intralesional arthropods (mites)			
5	197	100	Similar to snake 1, with no visible mites in the sections	Hemorrhages, multifocal, mild, subacute		
6	149.5	94	Similar to snake 1 with no visible mites in the sections	-Hemorrhages, mild, multifocal, subacute -Pericarditis, heterophilic, minimal, multifocal, chronic	Similar to snake 1	Lung: Endoparasitism (nematodes), mild, multifocal, chronic

Natrix tessellata	Macroscopical examination
1	- moderate to severe acariasis -surrounding the heart are multifocal mild to moderate haemorrhages -kidneys and liver show moderately pale mahogany -site of inoculation unremarkable -discoloration of the scales extending from 10 cm to 20 cm to the head. -Pin-point discoloration diffused along the entire body
2	-light tan discoloration of the scales extending 20 cm to 24 cm distally to the head and softening of the ventral lateral scales left from midline -pin-point discoloration diffused along the entire body - severe acariasis -multifocal mild haemorrhages surrounding the heart -liver and kidney are pale mahogany -injection site unremarkable
3	-Moderate to severe acariasis -light tan discoloration of the scales extending 19 cm to 24 cm distally to the head in the ventral lateral region right from midline -pin-point discoloration diffused along the entire body -multifocal mild haemorrhages surrounding the heart -liver and kidneys are pale mahogany -injection site subcutaneously unremarkable
4	-moderate acariasis -pin-point discoloration diffused along the entire body -multiple chronic 0.5 cm wide light tan discoloration consistent with foci of chronic dermatosis -multifocal mild haemorrhages surrounding the heart -5 egg follicles -subcutaneous injection site unremarkable
5	-moderate to severe acariasis -pin-point discoloration diffused along the entire body -no sign of haemorrhages around the heart -kidneys and liver lightly discoloured -injection site unremarkable -multifocal red discolorations (haemorrhages) present on the inner aspect of the dorsal lateral scales
6	-Moderate to severe acariasis -pin-point discoloration diffused along the entire body -no visible external anomaly -subcutaneous injection site unremarkable -moderate multifocal haemorrhages in the heart -light mahogany discoloration of the kidneys and liver.

Snake number 2 also showed granuloma focal, severe and chronic in the Kidney.

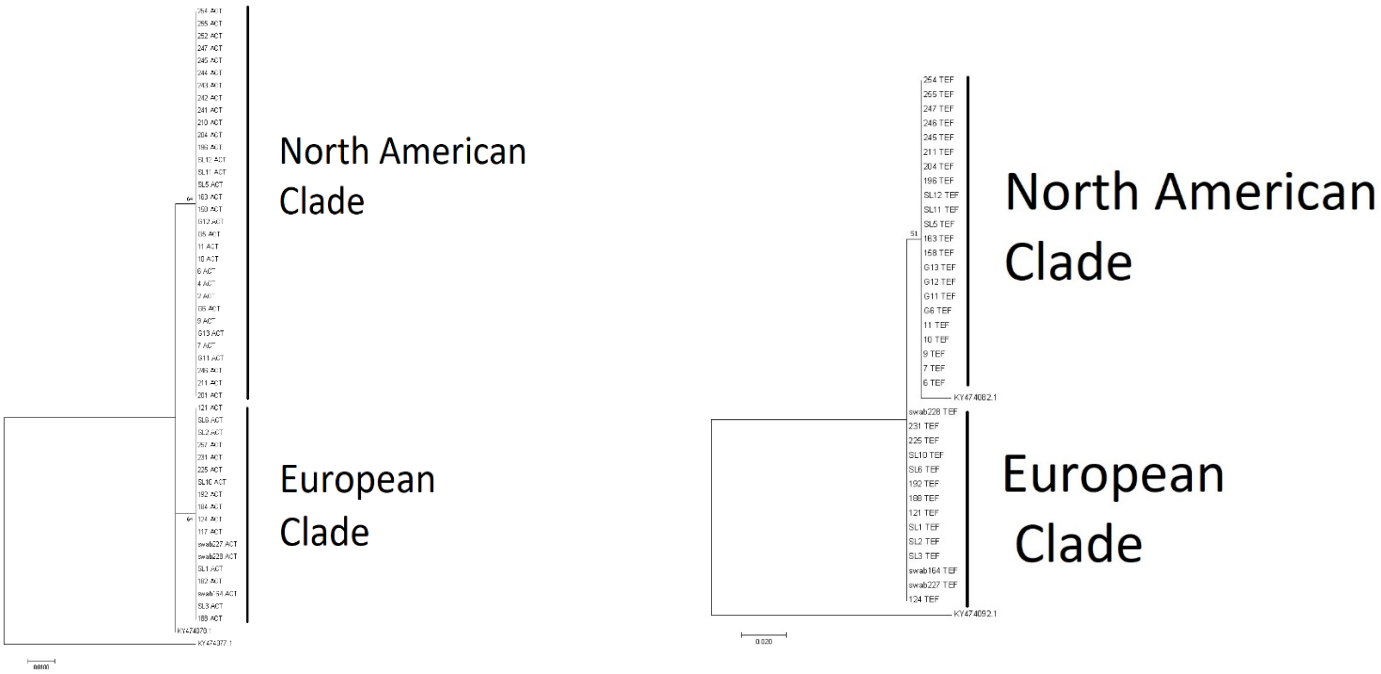
The organs which are not mentioned showed no significant findings.

Additional comment:

The most consistent changes across all the snakes were a dermatitis (6/6), most likely secondary to the mite's infestation observed grossly and confirmed also histologically, in several individuals (4/6). The extent and severity of these lesions and the amount of mites observed grossly, are consistent with a significant clinical finding in all the snakes. The second most consistent change was the presence of hemorrhages (5/6) and a mild to moderate pericarditis in the heart (4/6). Although this lesion could be

clinically relevant, the extent of the hemorrhages is unlikely to cause a cardiac tamponade and no evidence of systemic infection (sepsis) was observed (negative bacteriological examination on all the snakes). Of interest was the presence of embryonated nematode eggs in the lung of several snakes. The liver showed signs of atrophy, consistent with an energetic deficit. We could not identify significant changes neither macroscopically, nor histologically in the region of the injection site in none of the snakes.

Annex 5: Maximum likelihOod Phylogenetic tree of the ACT (left) and TEF (right) genes with the associated BOotstrap value at the nods. The outgroup fungal organism is Pseudoamauroascus australiensis.



Annex 6: Elisa results:

Table for the choice of antigen dilution and for the choice and primary serum dilution.

Annex 7: pictures of lesions observed during the survey

Image 1: Pictures of Natrix tessellata presenting lesions similar to SFD but that were tested negative by PCR (site Lavaux, VD). Pictures by Nicolas Joudrier.

Image2: pictures of the individual (Natrix tessellata) presenting the most severe lesions of the study and tested positive to SFD. Pictures by Nicolas Joudrier.