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RESEARCH PAPER

Ophidiomyces ophidiicola in free-ranging and captive snakes in the Czech and Slovak Republics

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Abstract. *Ophidiomyces ophidiicola* (*Oo*) is a snake fungal pathogen that causes ophidiomycosis. The disease manifests as dermatomycosis and/or systemic mycosis, and can be fatal. It occurs in free-ranging snakes in Asia, Europe and the USA and has also been demonstrated in captive snakes. We tested for the presence of *Oo* in free-ranging snake populations in the Czech and Slovak Republics (n = 420) between 2019 and 2022, focusing mainly on grass snakes (*Natrix natrix*) and dice snakes (*Natrix tessellata*), as well as various captive exotic species (n = 207). After collecting skin swabs, we tested for *Oo* using the qPCR method. We confirmed fragmented occurrence of *Oo* in the Czech Republic (total prevalence 15%) and recorded *Oo* in the Slovak Republic for the first time (total prevalence 33.9%). The highest prevalence was observed in *N. tessellata* (20.2%), which appears to be the most susceptible species. The pathogen was not detected in captive snakes.

Key words: emerging disease, snake fungal disease, ophidiomycosis, *Natrix*, Europe

Introduction

Dermatomycoses, such as disseminated systemic mycoses of snakes, are emerging infectious diseases caused by obligate pathogenic fungi (Schmidt 2015) that are occasionally found in captive snakes (Sigler et al. 2013) and to varying degrees in wild snakes, and as such have relevance to snake conservation and veterinary care (Lorch et al. 2016, Schilliger et al. 2023). *Ophidiomyces ophidiicola* (Sigler et al. 2013; herein abbreviated as *Oo*), an ascomycetic fungus causing ophidiomycosis (Allain & Duffus 2019, Paré et al. 2021) that usually manifests as superficial dermatomycosis (Cheatwood et al. 2003, Lorch et al. 2015, Meier et al. 2018), is the most relevant and most often studied fungal pathogen of snakes (Sigler et al. 2013, Di Nicola et al. 2022). Ophidiomycosis was originally referred to as snake fungal disease (SFD) (Sleeman 2013, Lorch et al. 2015, Latney & Wellehan 2020) due to a set of typical clinical signs (Schilliger et al. 2023) that included crusting and ulcerative dermatitis of a typically yellow to brown colour (Allender et al. 2015a, Lorch et al. 2016, Franklinos et al. 2017, Long et al. 2019). In addition, the disease may progress to multifocal skin lesions, granulocytic inflammation and necrotic foci. The symptoms

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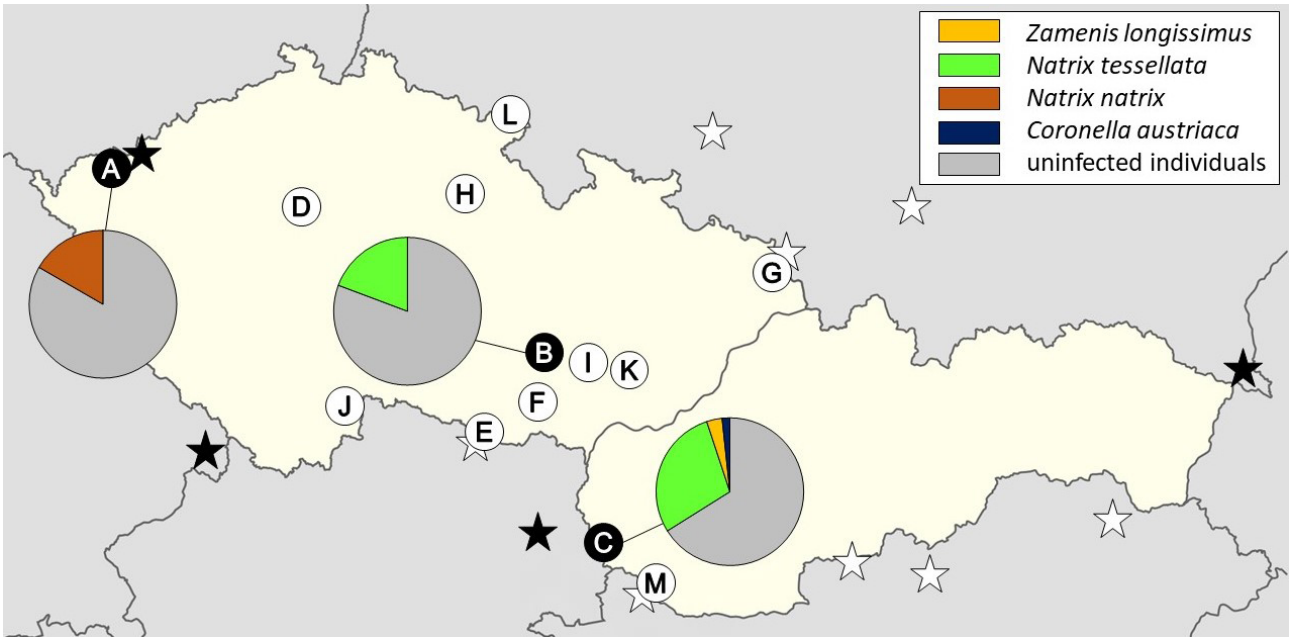

Fig. 1. Map of sampling sites in the Czech and Slovak Republics. Symbols: black = positive sample, white = negative sample, stars = results of other studies. A = Pernink (n = 5), B = Brno Reservoir (n = 275), C = Bratislava – Devín, Gronárska Bay, Rusovce, Železná Studnička and Petržalka, (n = 59), D = Praha (n = 13), E = Podyjí, Šobes (n = 19), F = Pohořelice (n = 32), G = Havířov (n = 11), H = Plachta (n = 2), I = Člupy (n = 1), J = Majdalena (n = 1), K = Salaš (n = 1), L = Vižňov (n = 1), M = Bodíky (n = 2).

are often accompanied by frequent and defective moulting and, eventually, the emergence of risky behaviour in the form of behavioural fever (Lorch et al. 2015, Lind et al. 2022). The disease sometimes becomes chronic and affects the muscular and deeper body layers, with a tendency to form granulomas (Lorch et al. 2016). Organ lesions may also be common and systemic effects of the infection usually kill the affected host (Vissiennon et al. 1999, Allender et al. 2013, Dolinski et al. 2014, Robertson et al. 2016).

The disease first became noticed mainly due to a significant decline in the population of timber rattlesnakes (*Crotalus horridus*) in 2006 (Clark et al. 2011) and eastern massasaugas (*Sistrurus catenatus*) in 2008 (Allender et al. 2011) in the USA. Rajeev et al. (2009) described the causative agent as *Chrysosporium ophidiicola*, which was later reclassified as a separate genus *Ophidiomyces* (Sigler et al. 2013). In 2013, the term SFD was used for the first time (Sleeman 2013) and, since 2015, this has been used exclusively for *Oo* infections (Lorch et al. 2015). Retrospective analysis of museum specimens has demonstrated presence of *Oo* on a specimen of a scarlet snake (*Cemophora coccinea*) caught in Florida in 1945 (Lorch et al. 2021) and on a captive ball python (*Python regius*) in England in 1985 (Sigler et al. 2013).

Oo is now known from more than 62 snake species of nine families in 11 countries on four continents (Di Nicola et al. 2022), including more than 42 wild snake species (Blanvillain et al. 2022) on three continents. Ophidiomycosis is known to affect wild snake populations in North America (Chandler et al. 2019, Licitra et al. 2019, Allender et al. 2020, Haynes et al. 2020), and its presence has also been confirmed in Europe (Franklinos et al. 2017, Meier et al. 2018, Blanvillain et al. 2022) and Asia (Grioni et al. 2021, Sun et al. 2021). The disease has also been demonstrated in captive snakes in Europe (Vissiennon et al. 1999, Picquet et al. 2018), Asia (Takami et al. 2021), North America (Nichols et al. 1999, Bertelsen et al. 2005, Steeil et al. 2018) and Australia (Sigler et al. 2013, WHA Fact Sheet 2021). The most important cause of the worldwide spread of fungal diseases, and a likely cause of the spread of ophidiomycosis, is the animal trade (Allender et al. 2015b, Schillinger et al. 2023).

Knowledge on the distribution of *Oo* across Europe is still limited as only two surveillance studies have so far been performed (Franklinos et al. 2017, Blanvillain et al. 2022). Here, we extend the surveillance effort across the Czech and Slovak Republics, sampling both wild and captive snakes to assess both the presence of the disease and the potential risk of spill over to populations of wild snakes.

Material and Methods

The main part of this work was performed at a single focal study site, the Brno Reservoir; however, samples were also collected samples from an additional 11

Table 1. Sites in the Czech Republic (CZ) and Slovak Republic (SK) and a general overview of free-ranging snake species, including their numbers, positivity and disease prevalence (95% CI). Sites visited repeatedly are marked *. Equivocal results (eq.) are included.

Site	GPS coordinates	Species	n	n positive	Prevalence (95% CI)
Brno Reservoir (CZ)*	(49.242° N, 16.507° E)	*Coronella austriaca*	6	1 eq.	0 (0-41.1)
		Natrix natrix	12	1 eq.	0 (0-24.3)
		Natrix tessellata	257	53; 23 eq.	20.6 (16.0-26.0)
Člupy (CZ)	(49.144° N, 16.976° E)	*Coronella austriaca*	1	0	0
Havířov (CZ)*	(49.802° N, 18.443° E)	*Natrix tessellata*	11	0	0 (0-26.5)
Majdalena (CZ)	(48.964° N, 14.863° E)	*Natrix natrix*	1	0	0
Pernink (CZ)	(50.391° N, 12.813° E)	*Natrix natrix*	1	1	0
		Vipera berus	4	0	0
Plachta (CZ)	(50.189° N, 15.857° E)	*Vipera berus*	2	0	0
Pohořelice (CZ)*	(48.963° N, 16.538° E)	*Natrix natrix*	32	0	0 (0-10.5)
Salaš (CZ)	(49.137° N, 17.361° E)	*Coronella austriaca*	1	0	0
Šobes (CZ)	(48.812° N, 15.977° E)	*Natrix tessellata*	16	0	0 (0-20.8)
		Zamenis longissimus	3	0	0
Vižňov (CZ)	(50.652° N, 16.260° E)	*Vipera berus*	1	0	0
Úholičky (CZ)*	(50.177° N, 14.353° E)	*Natrix natrix*	1	0	0
		Natrix tessellata	12	1 dead eq.	0 (0-24.3)
Bodíky (SK)	(47.900° N, 17.469° E)	*Natrix tessellata*	1	0	0
		Zamenis longissimus	1	0	0
Devín (SK)*	(48.172° N, 16.977° E)	*Coronella austriaca*	1	1	0
		Natrix tessellata	40	14	35 (21.2-51.3)
Gronárska Bay (SK)*	(48.160° N, 17.000° E)	*Natrix tessellata*	8	3	37.5 (11.1-71.1)
		Zamenis longissimus	3	2	0
Petržalka (SK)	(48.113° N, 17.137° E)	*Natrix tessellata*	1	0	0
Rusovce (SK)	(48.056° N, 17.153° E)	*Natrix natrix*	1	0	0
		Natrix tessellata	1	0	0
		Zamenis longissimus	1	0	0
Železná Studnička (SK)	(48.178°N, 17.074°E)	*Zamenis longissimus*	1	0	0
		Total	420	74	17.9 (14.4-21.9)

sites in the Czech Republic between 2019 and 2021, and six sites in the Slovak Republic in 2022 (Table 1). We also sampled snakes from seven breeders of exotic reptiles in the Czech Republic (Table 2, Fig. 1).

Snake collection and sampling

All manipulation with free-ranging snakes was undertaken wearing disposable gloves to prevent pathogen transmission and contamination of the samples. To facilitate manual handling, we sometimes used special snake tongs. At two repeatedly visited localities in the Czech Republic, we used artificial shelters to increase the probability of capture. All equipment was disinfected regularly with 70% ethanol to maintain sterility (Rzadkowska et al. 2016). Each snake was handled only for as long as necessary, after which it was released at the point of capture. We assessed the health status of each individual and collected swab samples for *Oo* detection using sterile swabs (MWE DrySwab, UK) moistened in deionised H_2O. We focused especially on the ventral scales, scales around the head and cloaca and skin lesions when present. The samples were placed in uniquely marked 2 ml screw top tubes (Sarstedt, Germany) with silica gel (P-Lab, Czech Republic) and stored at –20 °C until further use.

Sample analysis

We performed DNA isolation by repeated homogenisation (QIAGEN TissueLyserII, Germany)

Table 2. Total numbers of captive snake species tested, including their family classification.

Family	Taxon	n	n positive	Prevalence
Boidae	*Acrantophis dumerili*	2	0	0
	Boa constrictor (*constrictor, longicauda*)	8	0	0
	Boa imperator imperator	2	0	0
	Candoia aspera	1	0	0
	Candoia paulsoni (*paulsoni, tasmai*)	7	0	0
	Epicrates maurus	11	0	0
	Eryx colubrinus loveridgei	15	0	0
Colubridae	*Boiga cyanea*	2	0	0
	Dasypeltis fasciata	1	0	0
	Elaphe climacophora	4	0	0
	Elaphe dione	4	0	0
	Elaphe schrenckii	4	0	0
	Euprepiophis mandarinus	15	0	0
	Gonyosoma boulengeri	6	0	0
	Gonyosoma oxycephalum	5	0	0
	Gonyosoma prasinum	4	0	0
	Hemorrhois ravergieri	4	0	0
	Lampropeltis knoblochi	2	0	0
	Lampropeltis nelsoni	3	0	0
	Lampropeltis nigra	11	0	0
	Lampropeltis triangulum (campbeli, hondurensis)	16	0	0
	Lampropeltis mexicana	2	0	0
	Lampropeltis pyromelana	5	0	0
	Oreocryptophis porphyraceus (coxi, laticinctus, pulchra)	26	0	0
	Orthriophis moellendorffi	4	0	0
	Orthriophis taeniurus	1	0	0
	Pantherophis obsoletus	3	0	0
	Pantherophis guttatus	3	0	0
	Philodryas baroni	6	0	0
	Pituophis catenifer sayi	2	0	0
	Telescopus semiannulatus	6	0	0
	Zamenis situla	2	0	0
Elapidae	*Aspidelaps lubricus*	3	0	0
Lamprophiidae	*Boaedon capensis*	3	0	0
	Boaedon fuliginosus	4	0	0
Pseudoxyrhophiidae	*Madagascarophis meridionalis*	3	0	0
Pythonidae	*Aspidites melanocephalus*	1	0	0
	Aspidites ramsayi	1	0	0
	Liasis mackloti	1	0	0
	Morelia viridis	1	0	0
	Python anchietae	1	0	0
Viperidae	*Sistrurus miliarius barbouri*	2	0	0
	Total	207	0	0

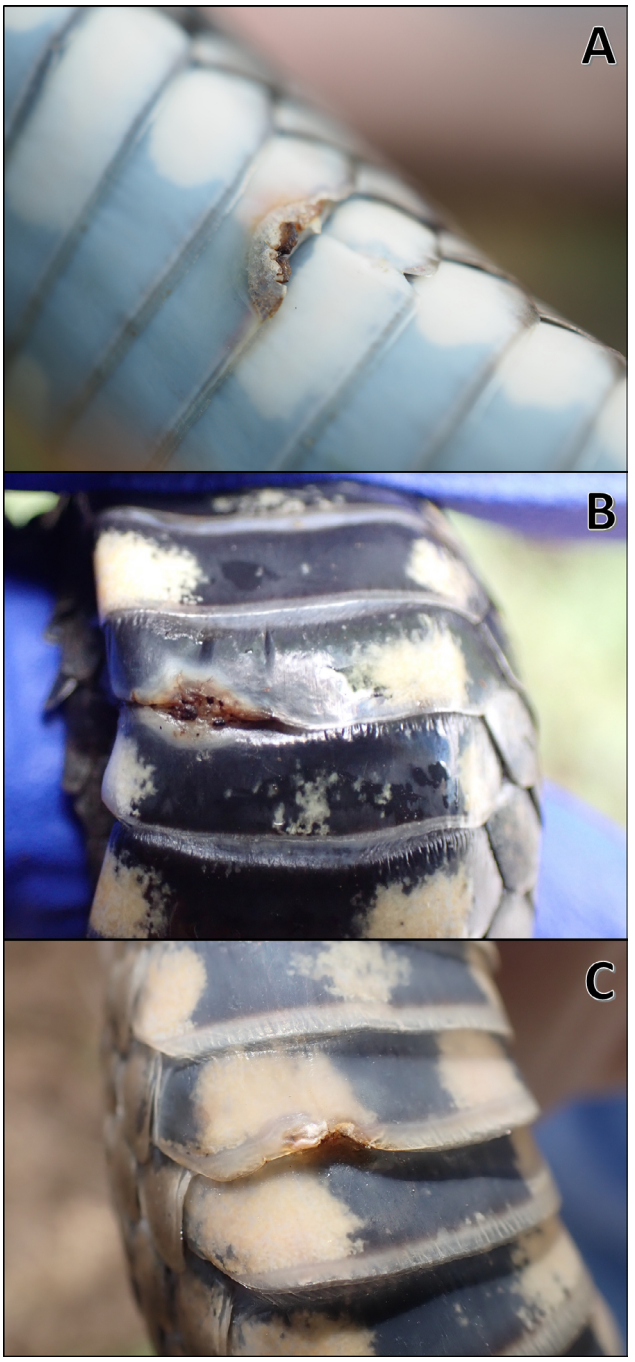

Fig. 2. A) Lesion of the ventral scale of *Natrix natrix* from Pernink (Czech Republic). B) Example of a typical skin lesion on *Natrix tessellata* at Brno Reservoir (Czech Republic). C) Ventral scale lesion on *Natrix tessellata* at Devín (Slovak Republic).

of swabs using zirconium beads in PrepMan™ (ThermoFisher, USA), followed by incubation in a thermoblock (Labnet AccuBlock, USA), according to Bohuski et al. (2015). Presence of the pathogen's DNA in the sample was assessed using a quantitative polymerase chain reaction (qPCR) using a specific probe targeting the relevant ITS region, the reaction mixture containing deionised H_2O, Roche Probes Master (Roche Diagnostics, Switzerland), 0.9 µM Oo-rt-ITS-F primer, 0.9 µM Oo-rt-ITS-R primer, 0.15 µM Oo-rt-ITS-P TaqMan FAM probe, 0.3 µM bovine serum albumin and 5 µl of 10 × diluted DNA at a total volume of 25 µl. A custom made linear double-stranded DNA fragment of the target sequence gBlock (Integrated DNA technologies, California, USA) was used as a positive control. A LightCycler 480 system (Roche Diagnostics, Switzerland), and later a qTower system (Analytik Jena, Germany), were used for the qPCR analysis, the qPCR program and fluorescence measurements being set according to the protocol of Bohuski et al. (2015). All samples were tested in duplicate. A sample was considered positive when the Cp/Ct value was < 40, while samples amplified later were considered equivocal and samples with no amplification were considered negative. If only a single well was amplified, the sample was retested. We calculated prevalence with a 95% confidence interval (CI) using the Quantitative Parasitology program (v.3.0), using Sterne's exact method for sample sets over five.

Results

Overall, we analysed 420 swab samples from 420 wild snakes representing five species belonging to two families (*Coronella austriaca* n = 9; *Natrix natrix* n = 48; *Natrix tessellata* n = 347; *Vipera berus* n = 7; *Zamenis longissimus* n = 9). We confirmed qPCR positivity at two sites in the Czech Republic and two in the Slovak Republic (Table 1). *Oo* was most commonly identified in *N. tessellata*, with an overall prevalence of 20.2% (95% CI 16.3-24.8%); even in this apparently infection-sensitive species, however, we did not find *Oo* at all tested sites. In the Slovak Republic, the pathogen was detected at two sites close to the River Danube; however, neither the pathogen nor clinical signs of ophidiomycosis were detected at the other four sites investigated. Total *Oo* prevalence in the Czech Republic was 15.0% and 33.9% in the Slovak Republic.

In total, we tested 207 captive snakes from 42 species of seven families (Table 2). In no case were clinical signs consistent with ophidiomycosis observed and qPCR testing failed to detect the pathogen in any captive snake.

Discussion

Recent findings suggest the likelihood that *Oo* is already globally distributed (Burbrink et al. 2017). Some studies, especially in the USA (Chandler et al. 2019, Fuchs et al. 2020, Harding et al. 2022), describe a high overall prevalence of *Oo* (sometimes over 50%)

and a subsequent 50% decrease in the abundance some isolated rattlesnake populations (Clark et al. 2011). However, similar declines associated with this pathogen have not been described elsewhere (Davy et al. 2021). In comparison, prevalence in wild European snakes is significantly lower at around 8.7% (n = 1,254) (Blanvillain et al. 2022). Our results for overall prevalence of *Oo* in the Czech Republic are somewhat similar at 15.0% (n = 361). We attribute the higher overall prevalence of *Oo* observed in snakes from the Slovak Republic (33.9%, n = 59) to our focus on one main site within a limited area, and the relatively small number of samples analysed from that country. We also realise that many samples were collected somewhat haphazardly and in small numbers per site; nevertheless, we consider them useful for further studies (e.g. meta-analyses).

Clinical symptoms were only observed in the form of small focal skin lesions (Fig. 2), with no observations of severely affected snakes or individuals with ophidiomycosis apparently impairing their general health. We also failed to observe dying snakes or carcasses with ophidiomycosis. We recognise, however, that finding such snakes can be difficult as they tend to be less active than healthy animals (Tetzlaff et al. 2017, Harding et al. 2022). On the other hand, we did find snakes with no obvious signs of ophidiomycosis but with healed scars, especially on the ventral scales, as previously described by Blanvillain et al. (2022).

Snakes with symptoms were observed more often in the period before and after brumation, suggesting spread of the pathogen among individuals as they aggregate at hibernacula (Guthrie et al. 2016, Lorch et al. 2016, Chandler et al. 2019). However, brumation itself is unlikely to have a significant effect on the development of lesions or the disease (McKenzie et al. 2020). Interestingly, Walker et al. (2019) has previously isolated the causative agent of *Oo* from soil. Our observation of many infected individuals in large snake populations even in the middle of summer suggests that population density is an important factor in *Oo* epidemiology.

Most samples collected for this study came from snakes of the genus *Natrix* due to their habitat preference, availability and ease of handling. At the same time, species of this genus show the highest prevalence of the disease in Europe (Blanvillain et al. 2022), indicating a possible connection between higher prevalence and water-bound snakes (McKenzie et al. 2019). This is also consistent with studies of some US rattlesnakes associated with marshes and wetlands (Blanvillain et al. 2022) and may indicate a pathogenic habitat preference (Lorch et al. 2016, McKenzie et al. 2019, Fuchs et al. 2020).

Our results correspond with the recently published pattern of local *Oo* presence and/or absence at sites geographically neighbouring our own (Blanvillain et al. 2022). The origin and distribution of *Oo* in Europe, as well as its effect on its European host species, is currently not well understood (Allain & Duffus 2019). However, recent studies suggest that the Eurasian phylogenetic clades of *Oo* are likely to be older than the American clades, and that the pathogen may have been introduced to North America with captive snakes. Captive snakes in Australia, Europe and North America all share *Oo* strains with wild snakes in the eastern US, demonstrating circulation of these strains on snakes in captivity (Ladner et al. 2022). The distinctiveness of some *Oo* clades isolated in southeast Asia (Sun et al. 2021) suggests an origin in that region (Ladner et al. 2022). On the other hand, the similarity of other Asian clades with European strains in free-ranging and captive snakes may indicate the direction of pathogen spread (Blanvillain et al. 2022). The breeders of the snakes tested here often handle animals as part of international trade, including imported snakes from Asia and the US, and some are also field herpetologists. Despite this, we were able to rule out presence of *Oo* in the snakes of these breeders.

To conclude, our results demonstrate fragmented occurrence of *Oo* in free-ranging snakes in the Czech and Slovak Republics. Consequently, we assume that *Oo* is probably not a continuously distributed pathogen but may instead form natural foci of disease. Under such circumstances, it is important to prevent contamination of uninvaded sites by at least maintaining basic biosecurity measures, i.e. cleaning and disinfection of equipment, limiting the amount of manipulation and preventing translocation of snakes between populations. Rzadkowska et al. (2016) has previously demonstrated that 3% sodium hypochlorite or ≥ 70% ethanol are effective against *Oo* spores. The lack of clinical signs or qPCR positivity for *Oo* in a collection of more than 200 captive snakes from seven different breeders suggests an absence of the pathogen in captive exotic snakes in the Czech Republic at the time of this study. As knowledge of the diversity, prevalence and impacts of emerging wildlife diseases is continuously being improved and expanded, however, the results of a single study should not be considered definitive. This is

particularly true regarding the situation of *Oo* in Europe, which still lacks an evaluation of pathological impacts on individual health and its importance for endangered species conservation.

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Author Contributions

M. Přibyl, V. Baláž and J. Pikula initiated and designed the study and drafted the manuscript; M. Přibyl, R. Kabelka, P.M. Hanzlík performed the laboratory analysis; R. Kabelka and M. Přibyl produced the figures; P. Mikulíček, N. Folk assisted in expansion of the study to Slovakia; all co-authors were involved in sample collection and later in the manuscript preparation.

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