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NOTE

Batrachochytrium dendrobatidis is present in Poland and associated with reduced fitness in wild populations of *Pelophylax lessonae*

Krzysztof Kolenda[1], Anna Najbar[1], Maria Ogielska[1], Vojtech Baláž[2,3,*]

[1]Department of Evolutionary Biology and Conservation of Vertebrates, University of Wrocław, Sienkiewicza 21, 50-335 Wrocław, Poland

[2]Department of Ecology and Diseases of Game, Fish and Bees, and [3]Department of Biology and Wildlife Diseases Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, Brno 612 42, Czech Republic

ABSTRACT: The fungus *Batrachochytrium dendrobatidis* (*Bd*) is a pathogen associated with global declines of amphibians. We used qPCR to detect *Bd* in 255 samples from 10 Polish populations of 8 species. We found *Bd* infection in 3 species (*Bombina variegata*, *Pelophylax lessonae*, *P. esculentus*). The infection intensity in *P. lessonae* reached a maximum of 58 400 genomic equivalents of zoospores (GE), and the 2 most heavily infected individuals died. Previous observations of the populations that included infected individuals showed reduced body size, failure to reproduce, and mortalities of adults. These data highlight the importance of emerging diseases, and the need to recognize them as an important factor in conservation of the genus *Pelophylax* in Poland and Central and Eastern Europe.

KEY WORDS: Amphibian mortality · *Bombina* · Chytridiomycosis · Decline · Hybridogenetic complex · *Pelophylax* · Poland · Water frog

INTRODUCTION

Many amphibian species have experienced global decreases in the past 30 yr (Stuart et al. 2004). Emerging infectious diseases may play an important role in declines, population crashes, or even extinctions (Skerratt et al. 2007). The infectious agents of wild amphibians that cause concern are 2 chytrid fungi, viz. *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al. 1999) and *B. salamandrivorans* (Martel et al. 2013), and ranaviruses (Robert 2010). The occurrence and impact of *Bd* has been studied worldwide in the recent years (Olson et al. 2013), and this pathogen is widely distributed in Europe (Garner et al. 2005). However, most of the studies have concentrated on western and south-central European countries (as summarized by Baláž et al. 2014a), with little, if any, data available from Central and Eastern European countries.

Poland hosts 19 amphibian species (14 anurans and 5 caudates; Głowaciński & Rafiński 2003, Dufresnes et al. 2016). Thirteen of these have shown a global trend of population decline, but none is classified as globally threatened yet (IUCN 2015). A decrease in the number of local populations has been observed in southern and western Poland for more than half of the species, including those considered to be locally common (e.g. *Pelophylax lessonae*, *Rana temporaria*; Bonk & Pabijan 2010, Budzik et al. 2013, our unpubl. obs.). Polish amphibians are primarily threatened by

*Corresponding author: balazv@vfu.cz

habitat loss and degradation due to urbanization (Rybacki & Berger 2003, Budzik et al. 2013) and by water contamination (Rybacki & Berger 2003). In some localities (e.g. in the vicinity of Wrocław city), populations of *P. lessonae* and *R. temporaria* have been disappearing for several years, but the reasons for this are still poorly understood (authors' unpubl. data).

The presence and impact of pathogens in Polish populations of amphibians is largely unknown. To date, in the literature only Sura et al. (2012), referring to personal communication with T. Ohst, have reported that a German–Swiss research team detected *Bd* in 29 of 161 sampled water frogs from 5 localities, but georeferenced data are unavailable. Therefore, *Bd* in Poland should be treated as insufficiently investigated, although the fungus has commonly been observed in neighboring countries (Ohst et al. 2013, Baláž et al. 2014a). The aim of our study was a survey of the *Bd* presence in Poland, as it is a potential factor in fluctuations or decreases in local amphibian populations.

MATERIALS AND METHODS

Samples were collected in 2015 from 10 populations located in southwestern and northwestern Poland (Table 1, Fig. 1). In total, 255 individuals representing 8 amphibian species (*Bombina bombina*, *B. variegata*, *Bufo bufo*, *Pelophylax esculentus*, *P. lessonae*, *P. ridibundus*, *Rana temporaria*, and *Salamandra salamandra*) were analyzed. Animals were caught by hand using latex gloves. The sampling procedure followed the standard protocol recommended by Hyatt et al. (2007). Each individual was swabbed over the ventral part of the body (belly, legs, feet) using sterile cotton swabs (Dryswab®,

Table 1. *Batrachochytrium dendrobatidis* (*Bd*) detection in sampling sites representing the surveyed local populations of 8 amphibian species in Poland. GE: genomic equivalents of zoospores; NA: not applicable

Species	Site number	Site	Coordinates	Sample size	*Bd*+	Prevalence (95% CI)	GE			
							Min.–Max.	Mean	Median	SD
Rana temporaria	1	Domaszczyn	51° 11′ 32″ N, 17° 9′ 44″ E	11	0	0.00 (0.00–0.26)	0	0	0	NA
Bufo bufo	2	Wrocław	51° 7′ 24″ N, 17° 5′ 7″ E	35	0	0.00 (0.00–0.1)	0	0	0	NA
Rana temporaria	3	Ostrów Wielkopolski	51° 38′ 53″ N, 17° 44′ 54″ E	24	0	0.00 (0.00–0.14)	0	0	0	NA
Pelophylax ridibundus	3	Ostrów Wielkopolski	51° 38′ 53″ N, 17° 44′ 54″ E	1	0	NA	0	0	0	NA
Rana temporaria	4	Sulistrowice	50° 50′ 56″ N, 16° 44′ 51″ E	8	0	NA	0	0	0	NA
Pelophylax ridibundus	5	Ruda Milicka	51° 31′ 56″ N, 17° 20′ 13″ E	30	0	0.00 (0.00–0.11)	0	0	0	NA
Pelophylax esculentus	6	Wysoka Kamieńska	53° 49′ 54″ N, 14° 51′ 48″ E	31	0	0.00 (0.00–0.11)	0	0	0	NA
Pelophylax esculentus	7	Sanie	51° 24′ 22″ N, 16° 56′ 28″ E	22	0	0.00 (0.00–0.15)	0	0	0	NA
Pelophylax ridibundus	7	Sanie	51° 24′ 22″ N, 16° 56′ 28″ E	5	0	NA	0	0	0	NA
Pelophylax lessonae	7	Sanie	51° 24′ 22″ N, 16° 56′ 28″ E	3	0	NA	0	0	0	NA
Bombina bombina	7	Sanie	51° 24′ 22“ N, 16° 56′ 28″ E	2	0	NA	0	0	0	NA
Salamandra salamandra	8	Jarnołtówek	50° 16′ 37″ N, 17° 24′ 54″ E	21	0	0.00 (0.00–0.16)	0	0	0	NA
Bombina variegata	9	Konradów	50° 16′ 54″ N 17° 23′ 45″ E	30	15	0.5 (0.32–0.68)	0.83–13.35	4.41	3.49	3.96
Pelophylax lessonae	10	Raków	51° 10′ 28″ N, 17° 16′ 41″ E	29	29	1.0 (0.88–1.0)	0.4–58400	2041.73	45.6	10645.3
Pelophylax esculentus	10	Raków	51° 10′ 28″ N, 17° 16′ 41″ E	3	2	NA	7.77–292	149.89	149.89	NA
Total				255	46	0.18 (0.14–0.23)				

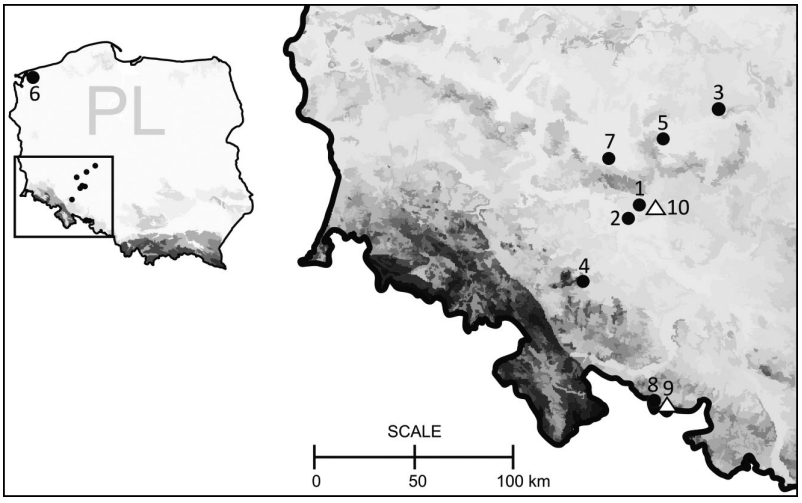

Fig. 1. Locations of sampling sites in Poland (upper left). See Table 1 for site details. Shading represents altitudinal structure of the area. Triangles mark sites where *Batrachochytrium dendrobatidis* (*Bd*)-positive samples were collected

MW100, Medical Wire & Equipment). The swabs were air-dried before tube closure or were preserved in 96% ethanol. Material was stored at −20°C until further analysis. We followed all applicable institutional and national guidelines for the care and use of animals.

To process the samples, we followed Boyle et al. (2004) and Hyatt et al. (2007). The presence of *Bd* DNA was detected using quantitative PCR (qPCR) (Boyle et al. 2004) with a reaction mix containing bovine serum albumin (BSA) to reduce PCR inhibition (Garland et al. 2010). A sample was considered to be positive for the presence of *Bd* when measured genomic equivalents of zoospores (GE) representing infection intensity were over 0.1 and the sample produced a clear sigmoidal growth curve of fluorescence in both wells. For quantification, we used genomic standards equivalent to 0.1, 1, 10, and 100 zoospores per 5 µl, which were made from stock *Bd* DNA of the GPL lineage, strain IA042 (Ibon Acherito, Pyrenees, collected in 2004), obtained from the Institute of Zoology, Zoological Society of London.

RESULTS

Bd was detected in 46 of 255 samples (18%). Three species inhabiting 2 sites were infected: *Bombina variegata* from Konradów village in the Opawskie Mountains (rural area), and *Pelophylax lessonae* and *P. esculentus* from Raków village near Wrocław city (a complex of fishponds surrounded by forest and fields; Table 1, Fig. 1). Prevalence reached 50% and 100% in *B. variegata* and *P. lessonae*, respectively. One of 3 individuals of *P. esculentus* was infected (Table 1). Infection intensity in *B. variegata* was low (Table 1), and none of the animals sampled showed any symptoms of disease. In the case of *P. lessonae*, the GE values reached from 0.4 to 58 400 ± 10 645 (SD) (Table 1). Two individuals, including the one with the highest GE, showed symptoms of disease: weakness, emaciation, skin dryness, and swelling with gas. Both were taken to the laboratory but died a few days after sampling. The *P. esculentus* with an infection intensity of 292 GE had 2 open wounds on the head and inflammatory changes in the eyes, neither of which are symptoms that are commonly associated with chytridiomycosis.

DISCUSSION

Our results presented here fit with the present knowledge of *Bd* presence in the area of Central and Eastern Europe, with 1 locality of *Pelophylax* species that deserve closer investigation as a result of their high level of infection and its impacts. The taxa that tested positive for *Bd* in Poland, *Bombina variegata* and 2 species of the genus *Pelophylax,* have previously been identified as good targets for *Bd* surveillance (Baláž et al. 2014a), and the proportion of infected sites also corresponds with previous results from other countries in the region (Ohst et al. 2013, Baláž et al. 2014b). The infection prevalence and intensity in *B. variegata* in Poland closely reflects the status observed for the species across its whole distribution range (Vörös et al. 2013, Baláž et al. 2014a, Vojar et al. 2017).

The case of *P. lessonae* from Raków is exceptional in our *Bd*-screening results, particularly when taken in conjunction with additional data on the local population. Several studies of the *P. esculentus* complex from the Raków population have been conducted since 2006 (Skierska 2011). Between 2006 and 2008, ~900 individuals were sampled to investigate their population life history. The results revealed that the body size of adults was in general slightly smaller in this population (females: 36.4–67.4 mm, males: 36.5–76.1 mm; Skierska 2011) when compared to other Polish populations of the genus (females: 48–78 mm, Juszczyk 1987; and 45–83 mm, Berger 2008; for

males: 45–75 mm, Juszczyk 1987; and 43–75 mm, Berger 2008). In addition, 50 individuals were found dead over the period from 2006 to 2008 (K. Skierska pers. obs.), but the cause was not determined and carcasses were not collected. Later studies conducted in 2015, using artificial crosses of *P. lessonae* from Raków, revealed spermatozoids produced by 4 of 7 collected males which were in very bad condition or dead. Additionally, 1 of 3 females included in the crossing procedure laid deformed eggs. While both males and females from this site were artificially crossed, fertilization either did not occur at all, or less than 50% of eggs survived (authors' unpubl. data). A *Bd* infection rate of 100% with infection intensity levels indicating acute fatal chytridiomycosis (Vredenburg et al. 2010) provide evidence that at the Raków site, *P. lessonae* may be highly susceptible to the effects of chytridiomycosis and is above the average level of risk for European amphibians. However, it is not clear whether the infection by *Bd* is the trigger of the observed condition of the *Pelophylax* population in Raków. The simplistic causality relationship between pathogen presence and disease in our study material is contradicted by experimental exposures where the genus *Pelophylax* proved to be tolerant to *Bd* infection in laboratory conditions (Woodhams et al. 2012). Some of the observed symptoms are not known to be associated with chytridiomycosis but may often be found in ranavirus-infected amphibians (Miller et al. 2015).

In conclusion, *Bd* is present in Polish amphibians and, in at least 1 locality, is linked to reduced fitness and signs of disease. As we did not test for other pathogens (e.g. ranaviruses) or stressors in this study, we do not claim that *Bd* is the only factor explaining the observed mortality, population decrease, or health problems. However, the levels of infection are consistent with a link between *Bd* and reduced health in this site and species. Future research will aim to collect data on other possible stressors and provide a health assessment of the infected populations. The water frog complex has been an important model for hybridogenetic speciation and is likely to become an important research area of interest for wildlife conservation medicine.

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