Chytridiomycosis risk among Central European amphibians based on surveillance data

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ABSTRACT: The Czech Republic hosts a surprisingly rich biodiversity of amphibians representing the majority of amphibian species present in all of Central and Eastern Europe. Surveillance data of *Batrachochytrium dendrobatidis* (*Bd*) collected during 2008 to 2012 were analysed for basic patterns of prevalence and infection intensity among species, age groups and localities. In addition, an investigation was made into possible data bias due to varying PCR inhibition. Infection prevalence in the genus *Pelophylax* was significantly higher than in other sampled taxa, while *Bombina* and *Bufo* were infected with intermediate prevalence. Individual mortalities putatively caused by chytridiomycosis were detected in *Bombina* and *Bufo*, but not in *Pelophylax*. Differences among localities were seen to modulate the pathogen's infection rate and influence overall individual infection intensities. PCR inhibition occurred significantly more often in samples from the genus *Pelophylax* than in other tested taxa (*Bufo bufo*, *B. viridis*, *Bombina bombina*, *Pelobates fuscus* and *Rana dalmatina*). Although we found no completely inhibited samples within the genus *Bombina*, the infection loads were lower in the sample set processed without bovine serum albumin, suggesting some level of PCR inhibition. The combination of high *Bd* prevalence with no apparent deleterious effect and the high dispersal abilities of water frogs predispose them to act as vectors for chytridiomycosis. It is possible that the role of *Pelophylax* frogs in the spread of *Bd* is overlooked due to a large proportion of unrecognized false negatives, but this issue needs further confirmation.

KEY WORDS: Amphibian chytrid fungus · Czech Republic · PCR inhibition · Real-time Taqman PCR assay · Permutation analysis · GLM

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INTRODUCTION

Batrachochytrium dendrobatidis (Longcore et al. 1999), hereafter referred to as *Bd*, is a novel fungal pathogen that infects a large number of amphibian species (Olson et al. 2013), often causing a wide-scale impact on populations via direct mortalities (Bosch et al. 2001, Lips et al. 2006, Schloegel et al. 2006, Vredenburg et al. 2010). Susceptibility to *Bd*

infection varies substantially, as does its incidence among different taxa (Searle et al. 2011, Baláž et al. 2014). These differences appear to be influenced by geography, life history and habitat (Becker et al. 2012, Murray & Skerratt 2012). The pathogen's original host and biogeography context remains an unresolved issue that has received a great deal of attention (Weldon et al. 2004, Fisher 2009, Schloegel et al. 2012). It nevertheless seems clear that the major part

of the pathogen's impact is caused by novel genetic lineages that have recently spread around the world (James et al. 2009, Farrer et al. 2011, Schloegel et al. 2012). *Bd* has a broad distribution across Europe and has been detected in multiple amphibian taxa (Garner et al. 2005, Ohst et al. 2011, Baláž et al. 2014). The direct impact of chytridiomycosis, however, is localized to a few areas where mass mortalities (Bosch et al. 2007, Bielby et al. 2009, Rosa et al. 2013) and population declines (Stagni et al. 2004, Bosch & Rincon 2008, Bosch et al. 2013) have been observed.

The Czech Republic is inhabited by a rich assemblage of amphibian species (13 anurans and 8 caudates; taxonomy after Speybroeck et al. 2010). The area is flanked by distribution range margins (e.g. *Bufo calamita*) and includes patches of isolated populations separated by strong insular effects (e.g. *Bombina variegata* in western Bohemia, *Lissotriton montandoni* in the east and *L. helveticus* in the west). Most of the species have a very wide distribution across Europe, although exceptions include *L. montandoni*, *Triturus carnifex* and *T. dobrogicus*. Many populations within the country are considered vulnerable to declines or are believed already to have experienced declines (Mikátová & Vlašín 2002, Zavadil & Moravec 2003). All amphibians in the Czech Republic are legally protected and are under surveillance and direct conservation efforts, both of which are provided by a governmental body, the Nature Conservation Agency of the Czech Republic (e.g. Jeřábková et al. 2013).

Although datasets on European and global mapping of the amphibian chytrid fungus have recently been analysed with interesting results as to geographic predictions (Olson et al. 2013) and infection risk among taxa (Baláž et al. 2014), more localized, well-analysed investigations for the area around the Czech Republic are lacking. The outcomes of *Bd* monitoring in this area from 2008 to 2012 have to date been limited to simple lists of positive and negative results (Civiš et al. 2012) and have been presented mostly at local conferences (e.g. Baláž et al. 2009).

In this study, we investigated differences in *Bd* prevalence among several amphibian taxa found in the Czech Republic using permutation analyses wherein background prevalence was compared to the prevalence observed

in the tested taxon. In this way, we were able to identify those taxa that carry infection most often and thus either have higher chances of acquiring the infection or bear it for longer time periods. Further analyses of the infection intensity patterns from these species enabled identifying the roles of age and locality in fungal growth. A description of the variance in PCR inhibition among different taxa is needed to enhance the robustness of the analyses.

MATERIALS AND METHODS

Sampling was conducted in 9 areas (Fig. 1) throughout the Czech Republic from 2010 to 2012, and the data thus obtained were enhanced with data collected previously (Civiš 2008, Civiš et al. 2012, Baláž et al. 2013, 2014). The overall dataset contained 1562 samples from 15 species, collected in habitats of various types (e.g. ephemeral puddles, man-made reservoirs, sky ponds on spoil banks, fish-breeding ponds, natural wetlands). Animals were caught by hand or with landing nets. Each individual was handled with a new pair of latex gloves. Nets and other equipment that were used were dried out completely or a new set was used between localities. The overall sampling procedure followed the method of Hyatt et al. (2007). All adult and juvenile amphibians were sampled by taking swabs from the pelvic patch and the ventral surface of the legs and feet using a fine-tipped swab (MW100; Medical Wire &
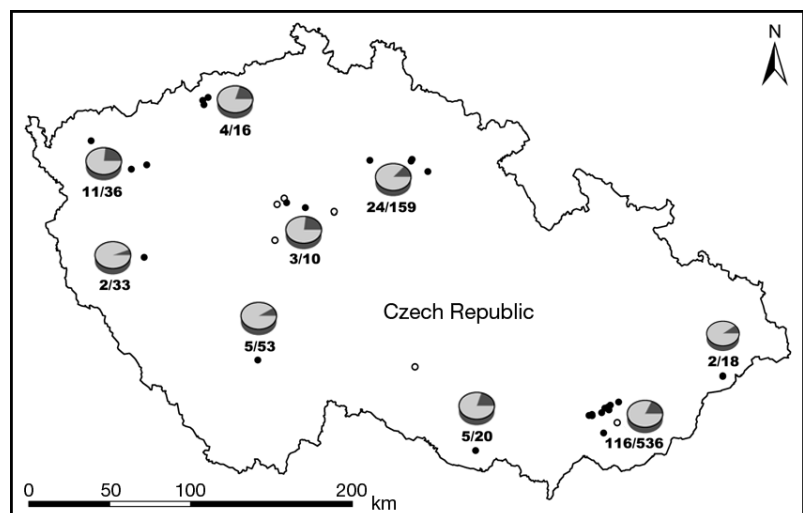

Fig. 1. Study area and individual sampling localities in the Czech Republic. Filled circles (●) represent positive localities; empty circles (○) show localities with no *Batrachochytrium dendrobatidis* detected based on all data collected during 2008–2012. Pie charts and numbers show the proportions of infected individuals (number/total) based on data produced by the upgraded protocol

Equipment). When sampling tadpoles, the mouth discs were swabbed. The swabs were left to dry before tube sealing and were later stored at 4°C until processing. Basic data on the sampled individuals were recorded, such as species, age group (adult; subadult = post-metamorphic animal not yet reaching maturity; tadpole), sex, locality and sample collection date.

Laboratory sample processing took place at the University of Veterinary and Pharmaceutical Sciences Brno following the guidelines of Boyle et al. (2004) and Hyatt et al. (2007) with some modifications as mentioned below. The infection status was detected using quantitative polymerase chain reaction (qPCR) with *Bd*-specific primers and a TaqMan probe (Boyle et al. 2004). Bovine serum albumin (BSA) was added to reduce PCR inhibition (Garland et al. 2010). The reactions were run in triplicate on a Light Cycler 480 II system with reaction volumes of 10 µl and using original reagents designed for the Light Cycler system. A sample was considered positive according to criteria described by Hyatt et al. (2007) 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. Samples considered positive were compared to a standard GE curve ranging between 0.1 and 100. The genomic standards used during this study were all obtained from the Institute of Zoology, Zoological Society of London.

Part of the dataset originated from previously published results which had been obtained by the original method of Boyle et al. (2004) without the addition of BSA or the detection of inhibition. We therefore needed to account for the difference between these 2

datasets. We rechecked part of the samples from 2010 using 2 reaction settings — one containing the internal positive control (IPC; Hyatt et al. 2007) to detect the presence of PCR inhibition, and the second containing BSA (Garland et al. 2010) to detect potential positives that had not been detected originally. As we were limited in resources and in the availability of samples from the earlier study, we used only sample subsets. To assess whether taxonomy played a role in the incidence of PCR inhibition, we used samples from 6 taxa (*Bufo bufo* n = 30, *B. viridis* n = 43, *Bombina bombina* n = 43, *Pelophylax* spp. n = 39, *Pelobates fuscus* n = 9, *Rana dalmatina* n = 8).

The data from individual species were grouped by genera in the case of *Pelophylax*, *Rana* and *Bufo* in order to obtain fewer groups with larger numbers of data points. Grouping *Pelophylax* was also done due to difficulties in identifying juvenile specimens of this genus. Using permutation analysis, as described in detail by Bielby et al. (2006) and Baláž et al. (2014), we tested which of the taxa showed a higher than average probability of carrying infection. For this analysis, the average prevalence of the dataset as a whole was used as the background prevalence. When the observed number of infected individuals for the checked taxon lay in the 2.5% tail at either end of the null distribution, it was categorized as either being infected less or more often than the background prevalence. To demonstrate possible bias due to the presence of false negatives, we performed the permutation tests both on the complete dataset (2008–2012, Table 1) and on the dataset that contained only data obtained by the improved detection method (2010–2012, Table 2). Confidence intervals and frequency analysis by chi-squared tests

Table 1. Results of permutation tests and summary statistics of infection intensity using the complete dataset and chi-squared test on nonrandomness of *Bd* incidence among taxa. *Bd*+: number of samples positive for *Batrachochytrium dendrobatidis*. p-values of permutation analyses for over-infection and under-infection (–: taxon with no significant trend; ↑: taxon infected significantly more often; ↓: taxon infected less often than the background prevalence). GE: genomic equivalents of zoospores; NA: not applicable

Taxon	Sample size	*Bd*+	Prevalence (95% CI)	p ↑	p ↓	Result	GE Mean	GE Median	GE SD
Bombina bombina	513	72	0.14 (0.11–0.17)	0.2704	0.7844	–	10.86	1.41	26.94
Bombina variegata	425	85	0.20 (0.17–0.24)	0.0000	1.0000	↑	335.76	16.40	1099.68
Pelophylax spp.	173	36	0.21 (0.15–0.28)	0.0025	0.9987	↑	348.52	17.31	1322.29
Bufo spp.	260	12	0.05 (0.03–0.08)	1.0000	0.0000	↓	1226.49	21.09	3945.94
Rana spp.	29	0	0.00 (0.00–0.12)	1.0000	0.0168	↓	0	0	NA
Pelobates fuscus	31	0	0.00 (0.00–0.11)	1.0000	0.0130	↓	0	0	NA
Pleurodelinae	109	1	0.01 (0.00–0.05)	1.0000	0.0000	↓	3.5	3.5	NA
Salamandra salamandra	22	0	0.00 (0.00–0.15)	1.0000	0.0470	↓	0	0	NA
Total	1562	206	0.13 (0.12–0.15)						

Chi-squared statistic = 69.81, df = 7, minimum expected count = 2.9, p < 0.001

Table 2. Results of permutation tests and summary statistics of infection intensity using only the data of reactions containing bovine serum albumin to limit PCR inhibition and chi-squared test on non-randomness of *Bd* incidence among the taxa. *Bd*+: number of samples positive for *Batrachochytrium dendrobatidis*. p-values of permutation analyses for over-infection and under-infection (–: taxon with no significant trend; ↑: taxon infected significantly more often; ↓: taxon infected less often than the background prevalence). GE: genomic equivalents of zoospores; NA: not applicable

Taxon	Sample size	*Bd*+	Prevalence (95% CI)	p ↑	p ↓	Result	GE Mean	GE Median	GE SD
Bombina bombina	289	44	0.15 (0.11–0.20)	0.9731	0.0413	–	13.38	2.41	31.85
Bombina variegata	399	80	0.2 (0.16–0.24)	0.1861	0.8585	–	335.62	17.78	1131.02
Pelophylax spp.	109	34	0.31 (0.23–0.41)	0.0006	0.9996	↑	367.87	17.31	1359.22
Bufo spp.	39	11	0.28 (0.16–0.45)	0.0868	0.9588	–	1337.93	36.75	4118.68
Pelobates fuscus	27	0	0 (0.00–0.12)	1	0.0017	↓	0	0	NA
Pleurodelinae	39	1	0.03 (0.00–0.14)	0.9996	0.0025	↓	3.5	3.5	NA
Salamandra salamandra	12	0	0 (0.00–0.24)	NA	NA		0	0	NA
Total	914	170	19 (0.16–0.21)						

Chi-squared statistic = 32.06, df = 6, minimum expected count = 2.2, p < 0.001

were computed using the Quantitative Parasitology application (Rózsa et al. 2000). A chi-squared test was used to check for non-randomness among results from the attempt to detect PCR inhibition in the limited sample set (Table 3).

To check for yearly changes detected in our dataset, we plotted the data from both species of the genus *Bombina*, as that taxon has been shown to be a reliable target for surveillance (Baláž et al. 2014), and eventual trends in temporal changes can be expected to be most apparent in that genus. Using the data from 3 selected taxa (*B. bombina*, *B. variegata* and *Pelophylax* spp.) obtained only with the use of BSA, we tested whether infection intensity differed among age groups and localities and whether there was an interaction between these 2 factors. We used generalized linear models (GLMs) with infection intensity (GE values) as a response variable with negative bi-

nomial distribution (Crawley 2007) and age group and locality as explanatory variables. To be able to use the GE values, which are by rule fractional values (each being a mean of 3 qPCR reactions), with a negative binomial distribution operating only with whole numbers, we first transformed the values. This was done by rounding each GE value to 2 decimal places and then multiplying them by 100. Simple rounding into integers could conceal differences within small infection loads and cause some of the data points to become 0 values. The permutation analyses and GLMs were performed in R statistical freeware, version 2.15.0 (R Development Core Team 2009).

RESULTS

Infection by *Bd* was detected in 9 of 15 tested species and in all 9 areas, but not at all localities (Fig. 1). Infection intensity ranged from the lower limit of detection up to 13 750 ± 21 (SD) GE, found in *Bufo viridis.* This individual died shortly after sampling (for more information on the context of this case, see Baláž et al. 2013). High infection loads were most commonly found in subadults of the genus *Pelophylax* and in *Bombina variegata*. The only case of field-observed mortality occurred in *B. variegata* with GE 8294 ± 3261 at the end of September 2012. This was at a site with a very high abundance of both *B. variegata* and *Pelophylax* spp. juveniles of both taxa showing high *Bd* prevalence and in general high infection intensities (Chřiby, Halenkovice Valley; Tables S1 & S2 in the Supplement; www.int-res.com/articles/suppl/d112p001_supp.pdf). This constituted the case of the highest infection intensity found in

Table 3. Inhibition incidence among selected amphibian taxa and chi-squared test on non-randomness of its occurrence among the taxa

Species	N	Complete PCR inhibition	Proportion of inhibited samples (95% CI)
Bufo viridis	43	5	0.12 (0.05–0.12)
Bufo bufo	30	7	0.23 (0.11–0.42)
Bombina bombina	43	0	0.00 (0.00–0.09)
Pelophylax spp.	39	29	0.74 (0.58–0.86)
Pelobates fuscus	9	0	0.00 (0.00–0.32)
Rana dalmatina	8	0	0.00 (0.00–0.36)
Total	172	41	0.24 (0.18–0.31)

Chi-square statistic = 77.144, df = 5, minimum expected count = 1.9, p < 0.001

this species within our complete dataset. No mortalities were found in the field among *Pelophylax* spp. despite GE values reaching up to 7655 ± 2213.

According to the complete dataset, *Bd* was encountered most often in *B. bombina*, *B. variegata* and in the genus *Pelophylax* (Table 1). When data from detections with reduced PCR inhibition were analysed separately, however, the results showed striking differences: prevalence in *Pelophylax* spp. was 1.5 times higher than in the complete dataset, and prevalence in *Bufo* spp. increased 6-fold (likely influenced by the limited sample size of *Bufo* in the second dataset). However, prevalence in neither *Bombina* species showed any major change (Table 2). The difference can be explained in part by the variable incidence of PCR inhibition among taxa. Although the overall rate of inhibition (24 %) was similar to previously published results (Hyatt et al. 2007), there was a significant difference within the 6 species we included in the analysis (chi-squared statistic = 77.144, p < 0.001). The highest proportion of completely inhibited samples was found in the genus *Pelophylax* (74 % of samples being inhibited), and both *B. bufo* and *B. viridis* contained inhibited samples as well. In the sample sets of *Rana dalmatina*, *Pelobates fuscus* and *B. bombina*, meanwhile, all IPC reactions performed similarly to the standards (Table 3). The dataset of yearly changes in prevalence based on *Bombina* spp. results showed no significant trends (Fig. 2). The effect of BSA treatment on GE values detected in positive samples was significant according to the Mann-Whitney test.
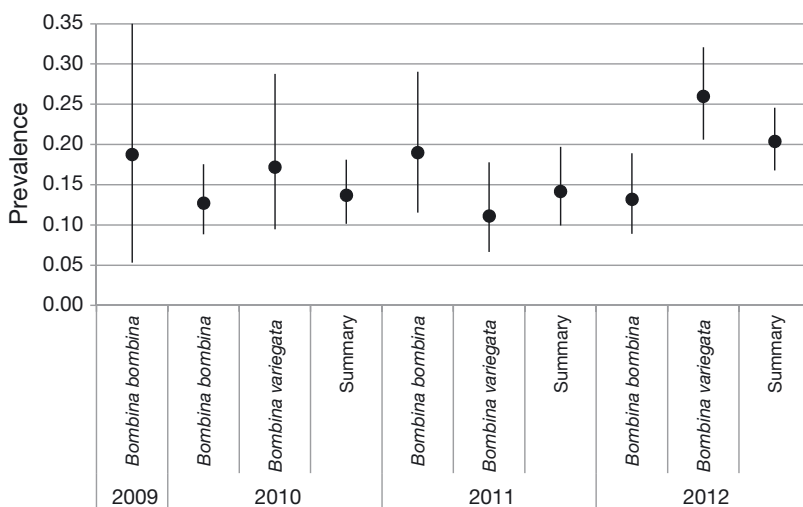

Fig. 2. Temporal changes in *Batrachochytrium dendrobatidis* prevalence within *Bombina* spp. No differences were detectable between years based on a chi-squared test of year summary results (chi-squared statistic = 0.41, df = 3, p = 0.94)

Table 4. Effects of locality (loc), age stage (age) and their interaction on *Batrachochytrium dendrobatidis* infection load (measured as genomic equivalents of zoospores) in 3 taxa with sufficient data. A separate generalized linear model was used for each taxon. In the case of *Pelophylax* spp., it was not possible to compute the interaction between the locality and age stage, because not all age stages occurred at all localities

Variable	*Bombina bombina*		*Bombina variegata*		*Pelophylax* spp.	
	df	p	df	p	df	p
Loc	5	$<10^{-3}$	7	0.001	2	$<10^{-5}$
Age	1	$<10^{-4}$	2	$<10^{-6}$	1	0.43
Loc:Age	2	0.22	3	$<10^{-4}$	–	–

The GLM results for all 3 tested taxa showed that both locality and age influenced the detected GE value (Table 4). The interaction of locality and age was significant for *B. variegata* but not significant for *B. bombina*. The effect of locality on infection load was very strong for all 3 taxa, suggesting that site factors affected fungal proliferation and resulted in high infection intensities.

DISCUSSION

Altogether 19 of the 21 species present in the Czech Republic have been reported to carry infection by the chytrid fungus either in the country directly or in its surroundings (Ohst et al. 2011, Sztatecsny & Glaser 2011, Baláž et al. 2014). This finding is in accordance with the trend that widely spread species have higher chances of acquiring infection (Murray & Skerratt 2012) and enables analysis of infection risk among different hosts. While all common amphibians in the Czech Republic may become infected, only 2 genera frequently carry the pathogen: *Bombina* and *Pelophylax*. Permutation analyses of both datasets used showed significantly higher prevalence within the genus *Pelophylax*. The position of *Bombina* species changed between datasets, but in general, they can be considered good target species for *Bd* surveillance as they carried constant intermediate infection rates. The remaining taxa did not contain sufficiently large sam-

ple sizes to be considered definitive, yet it is clear that the genus *Bufo* should receive further pathogen surveillance.

The detected taxonomic pattern of infection is partially in accordance with our previous results (Baláž et al. 2014) as well as other published literature (Ohst et al. 2011, Sztatecsny & Glaser 2011). Differences between taxa in proneness to infection are found even in localities inhabited by several species, such as at the Temelín locality (details in Table S1). This supports the role of biological differences in the ability of the studied amphibians to avoid or to clear infection.

There is a great risk of biased results if data collected by different methods are incorporated into one analysis. The differences in sensitivity and specificity among available detection methods (histology, standard PCR, qPCR) are generally known (Hyatt et al. 2007) and should be taken into account. This applies even to molecular DNA detection methods with only minor modifications. More importantly, the observation that taxa differ non-randomly in the amount of PCR inhibition warns against the use of such data. The source of PCR inhibitors remains unknown. The environment is generally regarded as the main source of substances hampering PCR reactions (Hyatt et al. 2007), but in our study, samples of *B. bombina* and *Pelophylax* spp. differed despite having been collected at the same locality in practically identical circumstances. Our sampling was far from sufficient to enable re-calculating previous results obtained using the original protocol (e.g. Civiš et al. 2012), and it is also clear that factors other than taxonomy could influence inhibition. We perceive this as an important problem of present and future meta-analyses based on data shared among different research teams (Fisher et al. 2009, Olson et al. 2013, Baláž et al. 2014). The databases should specify the exact detection methods used (or whether inhibition was accounted for) if researchers are to avoid assessing false patterns of pathogen presence.

The observation of greater variability in infection intensity among localities shows that the external environment plays a role in infection outcomes. The strong interaction between age and locality in *B. variegata* suggests that different habitat conditions modulate infection between age groups, with some conditions posing a risk to adults and others to juveniles. Furthermore, given the variable chances of different taxa carrying the pathogen, species composition and abundance are environmental factors that should be recognized. Amphibians from sites with more species present tend to have higher chytrid prevalence and infection loads (Table S2).

The difference in infection prevalence between *B. bombina* and *B. variegata* is intriguing. The scarcity of cases of high infection loads detected in *B. bombina* may imply that this species possesses an ability to limit fungal growth. On the other hand, *B. variegata* and frogs of the genus *Pelophylax* repeatedly carried infection loads on the order of thousands of GE. Whether there is a difference between the parental species (*P. lessonae* and *P. ridibundus*) and the hybridogenetic *P. esculentus* could not be answered using our data. Previously published literature does provide some support for that idea, however. *Pelophylax* spp. have been reported as carriers of the infection with no observed morbidities or mortalities (Di Rosa et al. 2007, Baláž et al. 2014), and Woodhams et al. (2012) showed no differences in prevalence between naturally infected *P. lessonae* and *P. esculentus*. The experimental work on the latter 2 species revealed a susceptibility to infection but high resistance to the disease itself, and mortality associated with chytridiomycosis was exceptional even in animals artificially infected with high pathogen doses (Woodhams et al. 2012). Ranids in general are known to have strikingly diverse responses to *Bd* infection. For example, mass mortalities have been reported among *Rana muscosa* in California, USA (Briggs et al. 2010), while practically no infections of this genus have been reported in Europe (Baláž et al. 2014). The genus *Pelophylax* is a good candidate for further *Bd* research despite there being no apparent effect of the fungal disease on that population.

B. variegata, on the other hand, has faced declines in local populations (Stagni et al. 2004, Canestrelli et al. 2013) linked to *Bd* presence. Whether the anecdotal report of a population decrease by as much as 90% among *B. variegata* in western parts of the Czech Republic during the 1990s (Mikátová & Vlašín 2002) was linked to the pathogen is a question requiring further historical analysis. There is limited evidence of *B. variegata* juveniles experiencing chytrid-linked mortality during hibernation (R. Rozínek unpubl. data). Four years of surveillance in the Czech Republic, however, support the idea that the level of prevalence in both *Bombina* species is in a stable, probably endemic, stage of pathogen dynamics.

Given the observed patterns of infection prevalence, infection intensity and detected mortality, we can assume that the 2 taxa *Pelophylax* spp. and *B. variegata* are most important for the spread of *Bd*, as well as its persistence and impact in the area. In years with exceptionally good conditions, *Pelophylax* froglets are produced in the thousands and disperse to all available bodies of water — including even such

biologically suboptimal sites as flowing water, ephemeral puddles and intensive fish-production ponds (V. Baláž pers. obs.). Although no study has investigated the dispersal distances achieved by juveniles, adult *Pelophylax* frogs are known to move several kilometres (Tunner 1991, Holenweg Peter 2001). If juvenile frogs carry infection, then several factors triggering chytridiomycosis outbreaks can occur (Briggs et al. 2010) and allow the pathogen to reach high infection intensities and re-infection rates. These include over-crowding of puddles and immigration of new amphibians susceptible to the infection. If disease-susceptible species are present in such circumstances, we can expect them to experience mortality events caused by the disease, as observed from our data in the cases of *B. variegata* and *B. viridis*. Well-meaning projects involving the construction of water bodies that support large *Pelophylax* populations in habitats where these frogs would otherwise be rare or absent may facilitate disease transmission and outbreaks, which could eventually lead to declines in more susceptible species. Furthermore, *Pelophylax* are common in fish-production ponds and live fish transports, which constitute an important mode of pathogen transmission (Peeler et al. 2011). If conservation measures and disease mitigation actions are planned using data biased by non-random PCR inhibition, even species that have a great impact on the spread and persistence of the pathogen could be listed as unimportant and thus omitted.

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