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Amphibian mortality associated with chytridiomycosis in central Eastern Europe

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The amphibian disease chytridiomycosis, caused by two pathogenic fungal species (*Batrachochytrium dendrobatidis – Bd*; *B. salamandrivorans – Bsal*), is responsible for the infection of more than 1000 amphibian species worldwide (Monzon et al., 2020) and the decline of at least 500 species (Scheele et al., 2019). While the most affected continents seem to be the Americas and Australia (Olson et al., 2013; Scheele et al., 2019), massive die-offs have been reported in Europe, especially in western Europe (Bosch et al., 2001; Fisher et al., 2009; Martel et al., 2013). *Bd* is widespread with low prevalence in central Eastern Europe and the Balkans (Baláž et al., 2013; Vörös et al., 2018), as well as in northern Europe (Kärvemo et al., 2018; Meurling, 2019; Taugbøl et al., 2021) but no evidence of *Bd*-linked population declines has been recorded from these regions so far.

Hungary is one of the central Eastern European countries where intensive surveys have been undertaken to map the distribution of *Bd* (Vörös et al., 2018). During the period between 2009 and 2015, 1360 individuals of 16 taxa from 14 regions of the country were sampled. Nine regions were infected with *Bd* and the overall prevalence was 7.46%. Among the 16 taxa, populations of *Bombina variegata* (Linnaeus, 1758) and *Pelophylax ridibundus* (Pallas, 1771) showed the highest prevalence. Those results were consistent with the findings of Baláž et al. (2013), who concluded that members of the genus *Pelophylax* and the family Bombinatoridae (especially *B. variegata*) were good candidates for detecting *Bd*.

Bombina variegata occurs in the low elevation mountains of Hungary, with its island-like populations surrounded by those of *B. bombina* (Linnaeus, 1761) in the lowlands (Vörös et al., 2014). Because of hybridization between the two species, *Bombina* populations are popular research targets (e.g., Vörös et al., 2006; Plăiaşu et al., 2012; Gabor et al., 2017). Due to intensive research accompanied by long-term monitoring (Vörös et al., 2014), *B. variegata* is among the most surveyed species in Hungary, especially in certain regions of the country, such as the Mátra Mountains, the highest elevation part of the North Hungarian Mountain Range. Here we report, for the first time, a mortality event observed in a *B. variegata* population in the Mátra Mountains and bring evidence that lethal chytridiomycosis occurs in nature in this species even if it has not been reported before.

Material and Methods

In October 2019 and September 2020, during a regular habitat survey, eleven dying or dead juveniles and seven tadpoles of *Bombina variegata* with dekeratinized oral discs were found in and around four different temporary pools in the Mátra Mountains of Hungary (Table 1, Figs. 1, 2). The Mátra Mountains are the highest elevation area of the country (1014 m), with characteristic beech forests in the upper-elevation regions inhabited by *B. variegata*.

Both juveniles and tadpoles were collected and conserved in 70% ethanol, but three juveniles were chosen and stored in formaldehyde for histologic examination to confirm possible skin infections.

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Table 1. Data related to the occurrence of *Batrachochytrium dendrobatidis* (*Bd*) in populations of *Bombina variegata* found in four temporary pools in the Mátra Mountains southwest of Parád, Hungary. Infection load is expressed as the genomic equivalent of *Bd* zoospores, and locality information includes GPS coordinates using the WGS84 standard. We studied eighteen *B. variegata* individuals, and those marked with an asterisk (*) were fixed in formaldehyde for histological examination.

Pond No.	Animal ID	Infection Load	Latitude	Longitude	Age	Date	Sample type
1	BVHK1*	-	47.8866°N	19.9868°E	juvenile	28 September 2019	toe clip in formalin
1	BVHK2*	-	47.8866°N	19.9868°E	juvenile	28 September 2019	toe clip in formalin
1	BVHK3	37.6	47.8866°N	19.9868°E	juvenile	28 September 2019	toe clip in ethanol
1	BVHK4	34.8	47.8866°N	19.9868°E	juvenile	28 September 2019	toe clip in ethanol
2	HK01*	-	47.8830°N	19.9930°E	juvenile	13 September 2020	toe clip in formalin
1	HK02	7.5	47.8866°N	19.9868°E	juvenile	4 October 2019	toe clip in ethanol
1	HK03	249.9	47.8866°N	19.9868°E	juvenile	4 October 2019	toe clip in ethanol
1	HK04	13.2	47.8866°N	19.9868°E	juvenile	4 October 2019	toe clip in ethanol
1	HK05	506.0	47.8866°N	19.9868°E	juvenile	4 October 2019	toe clip in ethanol
1	HK06	91.5	47.8866°N	19.9868°E	juvenile	4 October 2019	toe clip in ethanol
1	HK07	2.3	47.8866°N	19.9868°E	juvenile	4 October 2019	toe clip in ethanol
3	HK08	128.1	47.8831°N	19.9939°E	tadpole	19 September 2020	mouth part in ethanol
4	HK09	1249.8	47.8818°N	19.9880°E	tadpole	19 September 2020	mouth part in ethanol
4	HK10	5236.5	47.8818°N	19.9880°E	tadpole	19 September 2020	mouth part in ethanol
4	HK11	1523.8	47.8818°N	19.9880°E	tadpole	19 September 2020	mouth part in ethanol
4	HK12	4316.1	47.8818°N	19.9880°E	tadpole	19 September 2020	mouth part in ethanol
4	HK13	1160.9	47.8818°N	19.9880°E	tadpole	19 September 2020	mouth part in ethanol
4	HK14	314.9	47.8818°N	19.9880°E	tadpole	19 September 2020	mouth part in ethanol

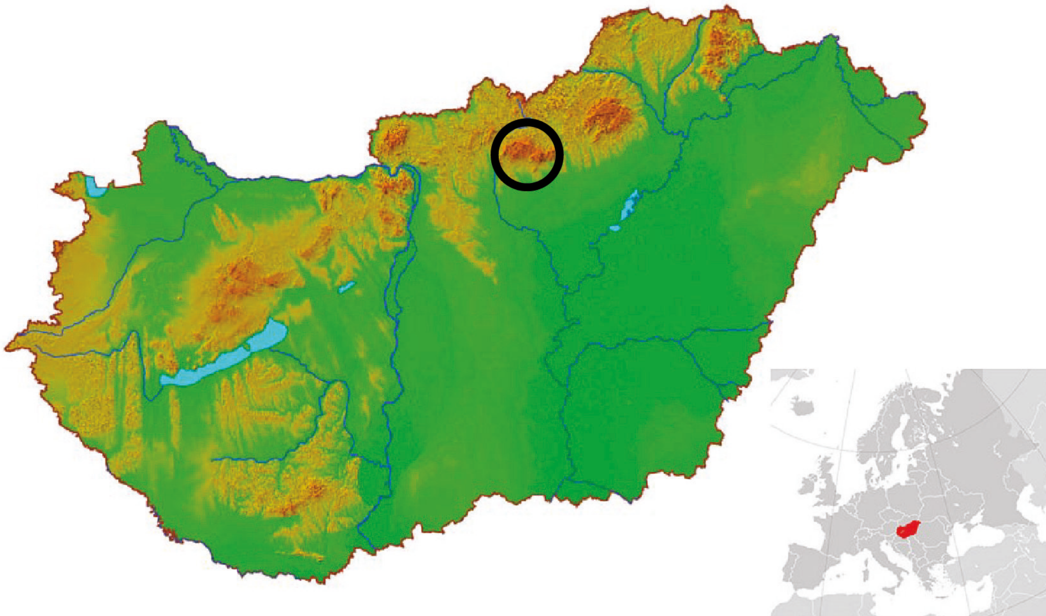

Figure 1. Map of Hungary, with a red circle indicating the location of the four temporary pools in the Mátra Mountains near Parád, Hungary, where *Bombina variegata* with chytridiomycosis were discovered.

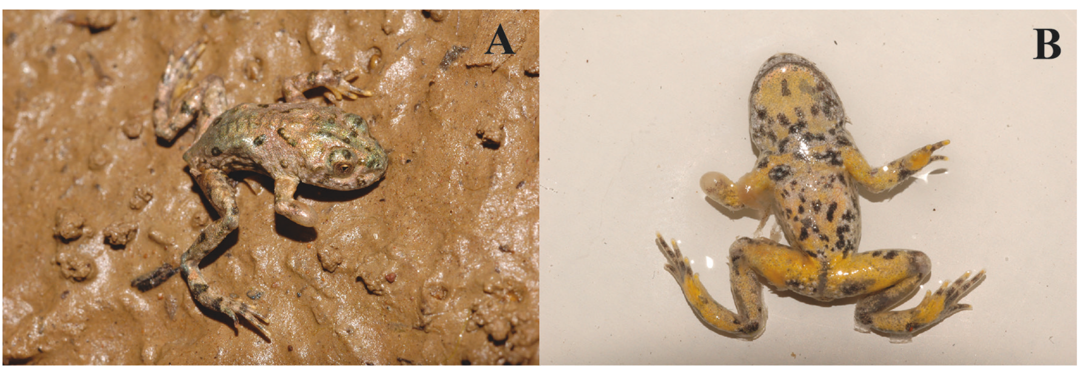

Figure 2. A juvenile *Bombina variegata* (BVHK2) from the Mátra Mountains near Parád, Hungary, presenting with an infection of chytridiomycosis. (A) The frog is still alive and shows the clinical signs of the disease, including lethargy, abnormal body position, especially of the hind legs, and loss of appetite. (B) The recently deceased frog showing excessive shedding. Photos by Krisztián Harmos.

Photographs of oral discs of six tadpoles were taken to document dekeratinization. Toe clips of juveniles and oral discs of tadpoles were then cut using sterilized scissors and were used for extraction of DNA, applying PrepMan Ultra reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Extractions were diluted to 1:10 before qPCR amplification following Boyle et al. (2004). Negative controls and standards with known concentrations were used in each plate.

The infection load of each sample was assessed directly by the machine software according to the reference function obtained with standards of known concentration of zoospores. Samples were considered to be positive if the infection load was ≥ 0.1 genomic equivalents (hereafter GE) of zoospores and the amplification curves presented a robust sigmoidal shape. Tissue samples were fixed in 4% neutral buffered formalin before been processed for histologic examination, and four µm sections were stained with hematoxylin and eosin and examined by light microscopy.

Results

Remains of keratinized oral disc structure could be observed in three tadpoles (HK8–10) while total lack of keratinization in both upper and lower jaw sheaths as well as in tooth rows was found in three specimens (HK11–13; Fig. 3). All fifteen specimens tested positive for *Bd* (Table 1). The mean, median, and standard deviation of GE values were 991.5, 249.9, and 1625, respectively. Despite the tissue alteration due to the alcoholic fixative, histological observation confirmed that *Bd* had invaded the epidermis in all the three examined animals, from superficial to transmural lesions, covering the *stratum corneum* and *stratum granulosum* (Fig. 4). Characteristic epidermal hyperplasia related to *Bd* infections was observed mainly in individual BVHK2 (Fig. 4B). Epithelial cells contained a high concentration of intracytoplasmic fungal forms in all analysed individuals. Different stages of chytrid development were detected: homogenous immature stage (Fig. 4B, black arrow), zoosporangia containing zoospores (Fig. 4A), zoosporangia with discharged papillae (Fig. 4B, white arrow), empty zoosporangia formed after zoospores have discharged (Fig. 4C), and zoospores divided by an internal septum (Fig. 4C, white arrows).

Discussion

Amphibian declines were first empirically demonstrated in the 1990s and since then multiple factors have been identified that contribute to the loss of amphibian diversity (e.g., Blaustein et al., 2011). Among these are climate change, agricultural chemical use, emerging infectious diseases, habitat destruction, and invasive species. Chytridiomycosis is one of the major causes of population declines worldwide, but Europe has been described as a continent not heavily impacted by the disease (Scheele et al., 2019). In Hungary, as in many other countries of Europe, it is suspected that *Bd* may have silently contributed to the slow disappearance of amphibian populations (Vörös et al., 2014, 2018), but so far, we have lacked empirical evidence of disease-related mortalities, as nobody has been investigating its sublethal nor indirect effects. The data we present herein are consistent with mortality due to chytridiomycosis in several individuals of *B. variegata* in Hungary.

In our study, all examined tadpoles exhibited dekeratinization of oral structures, as well as a high infection load of *Bd*. Dekeratinization of tadpole oral regions as a consequence of a severe *Bd* infection, has been revealed in numerous studies (e.g., Berger et al., 1998; Nichols et al., 2001; Marantelli et al., 2004). However, caution should be taken when using dekeratinization as a direct link to chytridiomycosis because other stressors can also produce this result. Dekeratinization varies among species and is not present in the early stages of the disease (Blaustein et al., 2005; Navarro-Lozano et al., 2018). Therefore, dekeratinization by itself should not be used as a general proxy to detect *Bd* in tadpoles but, if detected, more accurate diagnostic techniques, such as PCR analysis or histology, can be employed. All tadpoles analysed

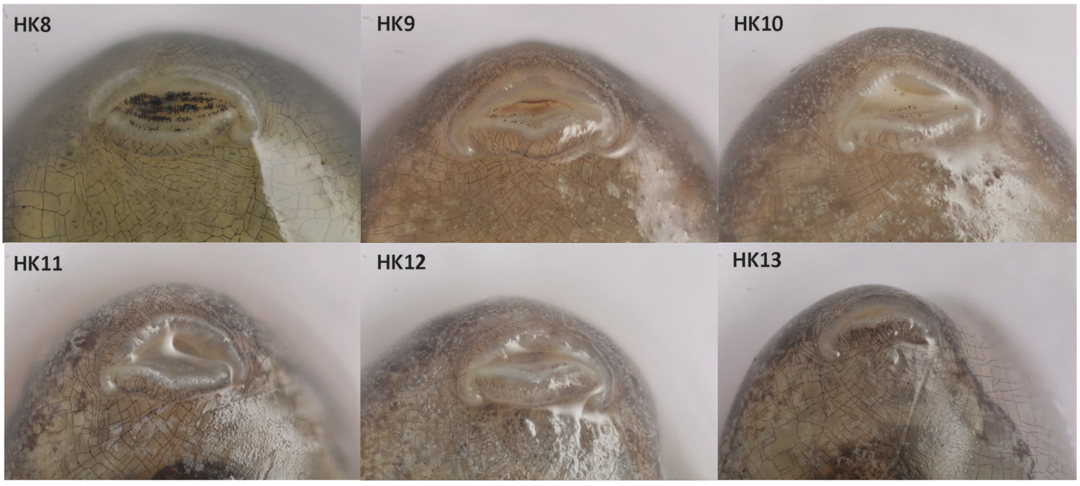

Figure 3. Tadpole oral discs featuring dekeratinization in six *Bombina variegata* individuals from the Mátra Mountains near Parád, Hungary. For individual identification, see Table 1. Photos by Tamás Németh.

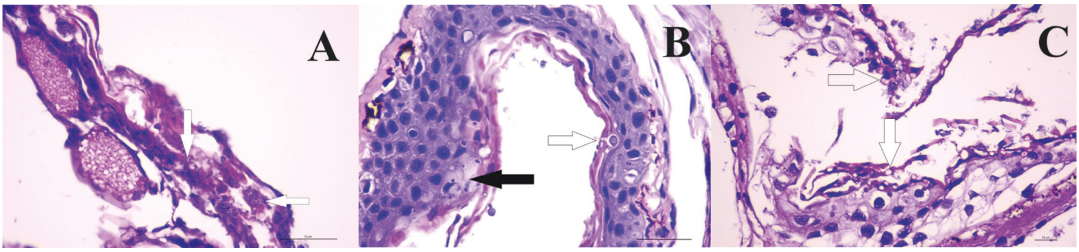

Figure 4. Histological sections of skin from three *Bombina variegata* juveniles from the Mátra Mountains near Parád, Hungary, stained with hematoxylin and eosin. (A) Aggregates of zoosporangia containing numerous zoospores (arrows) in the *stratum corneum* and *stratum granulosum* are shown in Specimen BVHK1. (B) The focal area of epidermal hyperplasia with numerous immature spherical zoospores (black arrow) and a single zoosporangium with a discharge papilla (white arrow) is shown in Specimen BVHK2. (C) Heavily infected, sloughed *stratum corneum* with mostly empty zoosporangia, formed after zoospores have been discharged, some with an internal septum (arrow), is shown in skin from Specimen BHK01 (C). Scale bars represent 50 µm in (A) and (B), 20 µm in (C). For details of examined individuals, see Table 1.

here contained a significant number of zoospores (up to 5200), which at that level usually produce mortality in the closely related genus *Alytes* (J. Bosch, pers. obs.).

The observed high infection rate (100%) in juveniles is concordant with other studies conducted in different parts of Europe and in the laboratory. High prevalence and/or intensity of infection have been reported for juveniles of *B. variegata* across its distributional range. Stagni et al. (2004) found mortality of *B. variegata pachypus* juveniles (raised from tadpoles in captivity) caused by chytridiomycosis at the northeastern limit of the species' range in Italy. Gál et al. (2012) and Baláž et al. (2013) showed that *B. variegata* juveniles were heavily infected in the Bakony Mountains of Hungary (with reported prevalences of 37% and 50%, respectively). During research conducted on the landscape context of *Bd* distribution in Romania (Scheele et al., 2015), juveniles exhibited higher prevalence (14.8%) than adults (2.7%). Finally, Spitzen-van der Sluijs et al. (2017) proved that during seven-year survey in the Netherlands, younger life stages (larvae and juveniles) showed higher prevalence (18.7% and 40%, respectively) than older life stages (sub-adults 5.5%, adults 6.6%).

In light of these results and other reported episodes of mass mortalities of *B. variagata pachypus* in Italy (among juveniles collected as tadpoles, raised in laboratory conditions) (Stagni et al., 2004), we urge that specific surveys to detect mass mortalities related to *Bd* in susceptible species of central Eastern Europe are conducted. Even when a considerable number of surveys are conducted every year in amphibian populations of central Eastern Europe to evaluate their population trends, no specific surveys are carried out to evaluate the potential effect of chytridiomycosis. In fact, most surveys, if not all, are focusing on the breeding season to count adult specimens, even though in most cases mass mortalities caused by *Bd* occur at the end of the metamorphosis. Then, tiny carcasses of susceptible species can easily disappear unnoticed, as they decompose rapidly. We suggest that specific surveys to evaluate the real effects of *Bd* should be developed and harmonized across Europe to be able to compare its incidence among countries/continents and to provide effective conservation measures.

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References

Baláž, V., Vörös, J., Civiš, P., Vojar, J., Hettyey, A., Sós, A., et al. (2013): Assessing risk and guidance on monitoring of *Batrachochytrium dendrobatidis* in Europe through identification of taxonomic selectivity of infection. Conservation Biology **28**: 213–223.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., et al. (1998): Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America **95**: 9031–9036.

Blaustein, A.R., Romansic, J.M., Scheessele, E.A., Han, B.A., Pessier, A.P., Longcore, J.E. (2005): Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. Conservation Biology **19**(5): 1460–1468.

Blaustein, A.R., Han, B., Relyea, R.A., Johnson, P. (2011): The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences **1223**: 108–119.

Bosch, J., Martínez-Solano, I., García-París, M. (2001): Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation **97**: 331–337.

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D. (2004): Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms **60**: 141–148.

Fisher, M.C., Garner, T., Walker, J. (2009): Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time and host. Annual Review of Microbiology **63**: 291–310.

Gabor, C., Forsburg, Z., Vörös, J., Serrano-Laguna, C., Bosch, J. (2017): Differences in chytridiomycosis infection costs between two amphibian species from Central Europe. Amphibia-Reptilia **38**: 250–256.

Gál, J.T., Szabó, K., Vörös, J. (2012): Kitridiomikózis vizsgálata egy magas-bakonyi vizes élőhely kétéltű közösségén [Investigation of chytridiomycosis in an amphibian community in a high Bakony wetland]. Állattani Közlemények [Zoological Notices] **97**: 47–59.

Kärvemo, S., Meurling, S., Berger, D., Höglund, J., Laurila, A. (2018): Effects of host species and environmental factors on the prevalence of *Batrachochytrium dendrobatidis* in northern Europe. PLoS ONE **13:** *e0199852*.

Marantelli, G., Berger, L., Speare, R., Keegan, L. (2004): Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. Pacific Conservation Biology **10**: 173–179.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., et al. (2013): *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences of the United States of America **110**: 15325–15329.

Meurling, S. (2019): The response in native wildlife to an invading pathogen: Swedish amphibians and *Batrachochytrium dendrobatidis*. Unpublished PhD thesis, Uppsala University, Uppsala, Sweden.

Monzon, F.C, Rödel, M.-O., Jeschke, J.M. (2020): Tracking *Batrachochytrium dendrobatidis* infection across the globe. Eco-Health **17**: 270–279.

Navarro-Lozano, A., Sánchez-Domene, D., Rossa-Feres, D.C., Bosch, J., Sawaya, R.J. (2018): Are oral deformities in tadpoles accurate indicators of anuran chytridiomycosis? PLoS ONE **13**(1): e0190955.

Nichols, D.K., Lamirande, E.W., Pessier, A.P., Longcore, J.E. (2001): Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. Journal of Wildlife Diseases **37**(1): 1–11.

Olson, D.H., Aanensen, D.M., Ronnenber, K.L., Powell, C.I., Walker, S.F., Bielby, J., et al. (2013): Mapping the global emergence of *Batrachochytrium dendrobatidis,* the amphibian chytrid fungus. PLoS ONE **8**: e56802.

Plăiaşu, R., Vörös, J., Băncilă, R. (2012): Fluctuating asymmetry as a tool in identifying population stress in Hungarian populations of *Bombina bombina*, *B. variegata* and their hybrids. Acta Zoologica Academiae Scientiarum Hungaricae **58**(4): 361–368.

Scheele, B.C., Hunter, D.A., Skerratt, L.F., Brannelly, L.A., Driscoll, D.A. (2015): Low impact of chytridiomycosis on frog recruitment enables persistence in refuges despite high adult mortality. Biological Conservation **182**: 36–43.

Scheele, B., Pasmans, F., Skerratt, L., Berger, L., Martel, A., Beukema, W. (2019): Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science **363**(6434): 1459–1463.

Spitzen-van der Sluijs, A., Canessa, S., Martel, A., Pasmans, F. (2017): Fragile coexistence of a global chytrid pathogen with amphibian populations is mediated by environment and demography. Proceedings of the Royal Society B: Biological Sciences **284**: 20171444.

Stagni, G., Dall’Olio, R., Fusini, U., Mazzotti, S., Scoccianti, C., Serra, A. (2004): Declining populations of Apennine yellow-bellied toad *Bombina pachypus* in the northern Apennines (Italy): Is *Batrachochytrium dendrobatidis* the main cause? Italian Journal of Zoology **71**: 151–154.

Taugbøl, A., Bærum, K.M., Dervo, B.K., Fossøy, F. (2021): The first detection of the fungal pathogen *Batrachochytrium dendrobatidis* in Norway with no evidence of population declines for great crested and smooth newts based on modeling on traditional trapping data. Environmental DNA **3**: (pagination pending).

Vörös, J., Alcobendas, M., Martínez-Solano, I., García-París, M. (2006): Evolution of *Bombina bombina* and *Bombina variegata* (Anura: Discoglossidae) in the Carpathian Basin: a history of repeated mt-DNA introgression across species. Molecular Phylogenetics and Evolution **38**(3): 705–715.

Vörös, J., Kiss, I., Puky, M. (2014): Amphibian declines and conservation in Hungary. In: Amphibian Biology: Status of Decline of Amphibians: Eastern Hemisphere. Amphibians, p. 99–130. Heatwole, H., Wilkinson, J.W., Eds., Exeter, United Kingdom, Pelagic Publishing.

Vörös, J., Herczeg, D., Fülöp, A., Gál, J.T., Dán, Á., Harmos, K., Bosch, J. (2018): *Batrachochytrium dendrobatidis* in Hungary: a review of recent and historical occurence. Acta Herpetologica **13**(2): 125–140.

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