Detailed final report

"Assessing the potential of a novel method of fighting chytridiomycosis, the most devastating emerging infectious disease of wildlife" (K-124375)

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Within the framework of the project we performed 18 experiments and a field study between November 2017 and October 2022, published 11 papers and got 1 in press (cumulative journal impact factor: 52.8). Two more manuscripts are under review and 9 are in preparation. We presented results at 5 international and 5 national conferences in 21 talks/posters. We also wrote 4 popular scientific papers and a book chapter in order to disseminate results towards the wider public as well. For details see the publication list ("Közlemények") and the brief descriptions listed below.

1) We wrote a review on the current state-of-the-art of the research field based on our grant application. We presented our considerations at the 29th International Congress for Conservation Biology, 2019, Kuala Lumpur, Malaysia. The paper was published in *Frontiers in Ecology and Evolution* in 2019.

2) We performed a laboratory-based study to determine combinations of treatment temperature and treatment duration that lead to the clearance of *Batrachochytrium dendrobatidis* (from here on Bd) infection in amphibians (see SP1a in the proposal). We also assessed potential side effects of elevated temperature on development speed and body mass at metamorphosis. Our results indicated that keeping tadpoles at 28 °C or higher for 6 days, or at 30 °C for 4 days was sufficient to clear the infection. Development of tadpoles was fastest at 27 and 28 °C, body mass at metamorphosis decreased with increasing temperature, especially beyond 28 °C. This study suggests that treating amphibian larvae at 28 °C for at least 6 days is a safe and effective approach to their disinfection. We presented results at the 20th Conference of the Hungarian Ethological Society, 2018, Kolozsvár, Romania and at the 21st European Congress of Herpetology, 2022, Belgrade, Serbia. We have started writing the manuscript, which we will submit in early 2023 to *Conservation Biology*.

3) We assessed the efficacy of thermal treatment of Bd-infected *Bufo bufo* metamorphs. Our results indicated that 30 °C effectively reduced Bd prevalence, but 28 °C did not. Infection intensity was significantly reduced at both 28 and 30 °C already after 3 days, but this reduction was more pronounced after 6 days of thermal treatment. All individuals exposed to 30 °C for 6 days and thereafter maintained at room temperature cleared the Bd infection by the tenth day. We presented results at the 6th Student Conference on Conservation Science, 2020, Tihany, and at the 22nd Conference of the Hungarian Ethological Society (MET), 2020, online. We have started writing the manuscript, which we will submit in late 2022 to *Animal Conservation*.

4) We investigated the thermal preference of *B. bufo* metamorphs and its effect on Bd prevalence and infection intensity following experimental infection. Initial infection prevalence was high, and it was decreased in the treatment where metamorphs had the opportunity to thermoregulate. Infection intensities followed the same pattern. We are

currently analysing video-recordings and are planning to write and submit the paper in late 2023 to *Animal Conservation*.

5) We investigated to what extent *B. bufo* metamorphs could clear Bd infection without treatment, and whether infection affected toxin production and body mass. Prevalence remained 100 % throughout the entire experimental period, but infection intensities stagnated first and started to decrease after three weeks. Infected animals produced lower amounts of toxins and their body mass was also lower as compared to controls. We presented results at the 12th Congress of the Hungarian Ecological Society (MÖK), 2021, Vác. The arising paper was published in *Journal of Zoology* in 2022.

6) We assessed the thermal preferences of tadpoles, determined the effect of environmental temperature on Bd infection prevalence, and tested for the presence of behavioural fever (see SP1b in the proposal). *B. bufo* tadpoles chose to spend most time at temperatures between 25 and 27 °C, which lowered infection prevalence by 60 % compared to individuals kept at a constant 20 °C: by the end of the experiment, 31 % of initially infected tadpoles still carried the fungus in the cool and homogeneous temperature environment, whereas only 12,5 % of them were still infected if they were presented with a temperature gradient. *R. dalmatina* tadpoles were highly resistant to Bd infection and preferred temperatures that were lower by ca. 4 °C as compared to *B. bufo* tadpoles. *H. arborea* tadpoles were intermediate in their resistance to Bd and preferred the highest temperatures, they spent most time at around 29 °C. Consequently, prevalence and infection intensity was lowest in this species in the treatment where they could effectively thermoregulate. We did not find signs of behavioural fever. These results suggest that tadpoles may prefer high enough temperatures to keep Bd infection under control if they have the opportunity to reach their preferred temperatures. We presented results at the 2021 Ecological Society of America Annual Meeting (online), and at the 20th Conference of the Hungarian Ethological Society, 2018, Kolozsvár, Romania. We will write and submit the manuscript in mid-2023 to *Conservation Biology*.

7) In a very similar experiment we assessed the thermal preference of *R. dalmatina* tadpoles infected with a Ranavirus. We are currently analysing molecular samples and video-recordings and are planning to write and submit the paper in late 2023 to *Animal Conservation*.

8) Many of our studies involve temperature manipulations, but there is very little information on what temperatures prevail in natural temporary ponds and are therefore available to anurans and their larvae. Therefore, we performed a study where we placed out temperature loggers between mid-March and mid-July in 2020 in twelve ponds in the Pilis-Visegrádi mountains. We observed extreme temporal and spatial variation. Temperatures above 28 °C were reached in most ponds on many days, and we measured water temperatures higher than 34 °C in three ponds. In the coolest pond, however, the maximum water temperature reached was only 23 °C. We will publish these results along with the study on temperature preferences of tadpoles (see previous point).

9) We assessed the influence of larval exposure to elevated temperature on sex ratio (see SP2a in the proposal) in two anuran amphibians: *Rana dalmatina* and *B. bufo*. Our results showed that in *R. dalmatina*, exposure to 28 °C at the end of larval development as well as exposure to 30 °C at any larval stage led to highly male-biased sex ratios, which was due to temperature-induced sex change. This masculinizing effect was not countered by a feminizing xenoestrogen present in environmentally relevant concentrations. Similar effects on phenotypic sex and mortality were not observed in *B. bufo*. We presented results at the 20th Conference of the Hungarian Ethological Society, 2018, Kolozsvár, Romania, the 21st Conference of the Hungarian Ethological Society, 2019, Mátrafüred, the 22nd Conference of the Hungarian Ethological Society, 2020, online, and at the 12th Congress of the Hungarian Ecological Society (MÖK), 2021, Vác. One paper on the interactive effects of the xenoestrogen and elevated temperature was published in *Environmental Pollution* in 2021. Another paper scrutinizing effects of temperature on early sexual development was published in *Science of the Total Environment* in 2022.

10) We performed an *in vitro* study to assess the upper thermal tolerance limit of Bd on a fine scale. Our results showed that 27 °C for at least three days may be sufficient to kill Bd that is present in the water. We presented results at the 20th Conference of the Hungarian Ethological Society, 2018, Kolozsvár, Romania and at the 6th Student Conference on Conservation Science, 2020, Tihany. The paper was published in *Mycologia* in 2022.

11) We tested potential methods for disinfecting anuran eggs by experimentally exposing embryos of *B. bufo* and *R. dalmatina* to three effective and widely used disinfectants of juvenile and adult amphibians and to four temperatures. Our results showed that voriconazole, chlorogen-sesquihydrate and temperature treatment cannot be recommended for the disinfection of amphibian eggs while chloramphenicol may be safely used. The paper was published in *Journal of Applied Toxicology* in 2021.

12) In collaboration with Spanish and Chilean colleagues, we measured CTmax values of Bd-infected and Bd-free tadpoles and terrestrial juveniles of *Alytes obstetricans* and examined whether brief exposure to their CTmax clears them from Bd infection. We showed that Bd can lower CTmax in tadpoles but not in toadlets, and that Bd infection is not cleared after brief exposure of tadpoles or toadlets to CTmax. The resulting paper was published in *Public Library of Science One* in 2019.

13) We investigated the effect of elevated temperatures on the survival and infection load of *R. dalmatina* and *B. bufo* larvae experimentally (co-)infected with Bd and a ranavirus (see SP2b in the proposal). We found that *R. dalmatina* tadpoles were highly resistant to Bd infection, but neither resistant nor tolerant to ranavirus infection, while *B. bufo* larvae exhibited intermediate resistance and high tolerance to Bd infection, and were not resistant, but highly tolerant to the ranavirus. Furthermore, the presence of one pathogen influenced the prevalence and/or infection intensity of the other pathogen in both species, depending on temperature and initial ranavirus concentration. Generally, the 30 °C treatment lowered the prevalence and infection intensity of both pathogens, and, in *R. dalmatina*, this was mirrored by higher survival. We wrote a manuscript which

is currently under review at *Philosophical Transactions of the Royal Society B - Biological Sciences* following an invitation to contribute to a special issue.

14) With the contribution of a Fulbright visiting fellow (Caitlin R. Gabor, Texas State University, USA) we performed an experiment in 2019 to assess how Bd infection affected stress-hormone (CORT) levels of tadpoles and metamorphs of *R. dalmatina*, *B. bufo*, and *Hyla arborea*. Infected *B. bufo* released higher amounts of CORT than their non-infected conspecifics, while there was no such pattern detectable in *R. dalmatina* or in *H. arborea*. Because of a large overlap in CORT levels between Bd-infected and non-infected *B. bufo*, measuring stress-hormone levels to assess infection prevalence in a non-invasive way will not be an operable approach. We presented results at the 12th Congress of the Hungarian Ecological Society (MÖK), 2021, Vác, and at the 22nd Conference of the Hungarian Ethological Society (MET), 2020, online. We have started writing the manuscript, which we will submit in early 2023 to *Integrative and Comparative Biology*.

15) We reviewed the literature reporting on co-infections in amphibians. We summarized what was known about within-host interactions among parasites, which environmental and intrinsic factors may influence the outcomes, and what effects co-infections can have on hosts. The paper was published in *Parasites and Vectors* in 2021.

16) In a study performed in 2016 we investigated effects of larval exposure to Bd on chemical defences in *R. dalmatina* and *B. bufo*. Exposure to Bd during the larval stage did not result in enhanced synthesis of the antimicrobial peptide Brevinin-1Da in *R. dalmatina* tadpoles or in increased production of bufadienolides in *B. bufo* tadpoles. However, exposure to Bd during the larval stage had a carry-over effect: both *R. dalmatina* and *B. bufo* froglets contained smaller quantities of defensive chemicals than their Bd-naïve conspecifics. We presented results at the 29th International Congress for Conservation Biology, 2019, Kuala Lumpur, Malaysia. The paper was published in *BMC Ecology and Evolution* in 2021.

17) In a correlative study involving 16 natural populations performed in 2015 we revealed strong relationships between the number of bufadienolide compounds as well as the total quantity of bufadienolides and bacterial community structure of the aquatic habitat. The paper was published in *Journal of Chemical Ecology* in 2020.

18) We investigated fitness-related traits in larvae and juveniles that underwent spontaneous female-to-male sex reversal in two of our laboratory-based experiments. We found only small differences in early life growth, development, and larval behaviour between sex-reversed and sex-concordant individuals, suggesting that fitness-effects of sex reversal manifesting during the juvenile life-stage may not be dramatic. The paper was published in *Frontiers in Ecology and Evolution* in 2021.

19) We studied the Bd-growth inhibitory potential of biosynthetic metabolites of an entomopathogenic bacterium *in vitro* and *in vivo*. We extracted cell-free culture media (CFCM) from liquid cultures of X. szentirmaii and tested their disinfecting efficacy at dilutions of 10 and 2 %, while also measuring possible malign effects on juvenile *B. bufo* experimentally infected with Bd. We detected no negative effects on survival and body

mass of uninfected toads. However, among infected individuals, survival was higher and infection intensity was lower following the 10 % CFCM treatment, compared to toads treated with no, or 2 % CFCM solution. We presented results at the 6th European Congress of Conservation Biology, 2022, Prague, Czech Republic and at the 13th Hungarian Conservation Biology Conference, 2022, Pécs. We wrote a manuscript which is currently under review at *Conservation Biology*.

20) We examined how overwintering amphibians may be affected by the combined effects of changing overwintering conditions and Bd infection. We exposed juvenile *R. dalmatina* to Bd and subsequently subjected them to either a long, cold winter (1.5 °C for 91 days) or a short, mild winter (4.5 °C for 61 days). Only 37 % of Bd-exposed individuals became infected as determined before hibernation, and prevalence further decreased to 8 % by the end of hibernation, with individuals showing very low infection intensity values. There was virtually no mortality either in the control or in the Bd-exposed groups in both types of winter. Further, overwintering conditions did not affect Bd prevalence or infection intensity. We presented results at the 13th Hungarian Conservation Biology Conference, 2022, Pécs. We wrote a manuscript which is in press at *Animal Conservation* and will be published in 2023.

21) We assessed the effects of pesticide exposure on the susceptibility of *R. dalmatina* to Bd and a ranavirus. We reared tadpoles in outdoor microcosms and exposed them to environmentally relevant concentrations of one of six pesticides (cypermethrin, fenoxycarb, terbuthylazine, pendimethalin, tebuconazole, copper hydroxide). Following metamorphosis, we experimentally infected juveniles with Bd or a ranavirus, and ten days later noted survival, measured body mass and preserved animals. We also subjected tadpoles to various concentrations of the same pesticides in a classic laboratory-based ecotoxicological experiment. In the laboratory, cypermethrin (insecticide, neurotoxin) and Cu(OH)2 (fungicide, enzyme system inhibitor) were highly toxic to agile frog larvae, but only at concentrations 10× higher than those found in aquatic habitats, whereas the other tested pesticides seemed to be harmless. At ecologically relevant concentrations, none of the pesticides had an effect in outdoor microcosms and previous exposure to pesticides did not result in enhanced susceptibility to the pathogens either. We presented results at the 21st European Congress of Herpetology, 2022, Belgrade, Serbia. We will write and submit the manuscript in late 2023 to *Animal Conservation*.

22) We investigated whether juvenile *R. dalmatina* and *B. bufo* can recognize and avoid ranavirus- or Bd-infected conspecifics, respectively. We raised tadpoles in outdoor mesocosms. Ten days after metamorphosis, we experimentally infected juveniles with Bd or a ranavirus. Another ten days later we performed choice tests in classic two-way choice arenas and video-recorded animal movements. In parallel, we also assessed potential changes in the odour of juveniles following experimental infection by repeatedly collecting dynamic headspace solid-phase microextraction followed by GC-MS. We are in the process of analysing video-recordings, molecular samples and odour composition and are planning to write a paper and submit it in late 2023 to *Proceedings of the Royal Society B – Biological Sciences*.

23) We tested the effectiveness of a prototype of submersible heated refugia designed to help anuran tadpoles in keeping Bd infection under control (see SP3a in the proposal). We raised *B. bufo* tadpoles under laboratory conditions, infected them experimentally with Bd, and finally provided them with submersible refugia. Refugia were either heated to 30 °C for six days or were left unheated, and tadpoles could enter and leave refugia at their will. We observed how much tadpoles used refugia and assessed the effect of the availability of heated refugia on Bd infection. Infected tadpoles did not use heated refugia more than their non-infected conspecifics. The availability of heated refugia did not lower the prevalence of Bd, but it lowered infection intensity. We presented results at the 24th Conference of the Hungarian Ethological Society (MET), 2022, Budapest. We will write and submit the manuscript in mid-2023 to *Animal Conservation*.

The following conference presentations are not mentioned in the list of publications („Közlemények") to avoid inflation of the list, as we later published the same results in the form of scientific articles or presented them at more important conferences:

Two oral presentations and two posters at the 20th Conference of the Hungarian Ethological Society (MET), 2018, Kolozsvár – Cluj Napoca, Romania.

Two oral presentations at the 29th International Congress for Conservation Biology, 2019, Kuala Lumpur, Malaysia.

One oral presentation at the 21st Conference of the Hungarian Ethological Society (MET), 2019, Mátrafüred, Hungary.

Two oral presentations at the 22nd Conference of the Hungarian Ethological Society (MET), 2020, online, Hungary.

One oral presentation at the 6th Student Conference on Conservation Science, 2020, Tihany, Hungary.

Two oral presentations and one poster at the 12th Congress of the Hungarian Ecological Society (MÖK), Vác, Hungary.