

Mitigation of chytrid infection on tadpoles with the antimicrobial metabolites of

Xenorhabdus szentirmai

János Ujszegi^{1,2*}, Zsófia Boros^{1,3}, Krisztián Harnos⁴, Gábor Magos⁴, Ábris Tóth^{1,5}, Judit
Vörös⁶, Andrea Kásler^{1,2,7}

¹Department of Evolutionary Ecology, HUN-REN Centre for Agricultural Research, Plant
Protection Institute, H-1029, Budapest, Hungary

²Department of Systematic Zoology and Ecology, Eötvös Loránd University, H-1117,
Budapest, Hungary

³Department of Genetics, Eötvös Loránd University, H-1117, Budapest, Hungary

⁴Bükk National Park Directorate, H-3304, Eger, Hungary

⁵Department of Zoology, University of Veterinary Medicine Budapest, H-1078, Budapest,
Hungary

⁶Amphibian and Reptile Conservation Group, MME Birdlife Hungary, H-1121, Budapest,
Hungary

⁷Doctoral School of Biology, Institute of Biology, Eötvös Loránd University, H-1117
Budapest, Hungary

*Corresponding author

e-mail: ujszegi.janos@gmail.com

tel.: +36-1-3918607

fax: +36-1-3918653

ORCID ID: 0000-0002-6030-0772

Running head

Bacterial metabolites against chytrid infection

Abstract

Survival of amphibian assemblages is threatened by many factors. Among them, chytridiomycosis, the disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) has great importance, also threatening the populations of the yellow-bellied toad (*Bombina variegata*), which has a scattered, isolated distribution in Hungary. Treatment with secondary metabolites of the entomopathogenic bacterium, *Xenorhabdus szentirmai* is a promising method in the mitigation of chytridiomycosis, as recently demonstrated. To assess whether it may also be effective for lowering infection intensities in *B. variegata* tadpoles, we extracted cell-free culture media (CFCM) from liquid cultures of *X. szentirmai* and tested their antifungal efficacy at a dilution of 0.1 % (v/v), while also measuring possible short-term malign effects on tadpoles experimentally infected with Bd or sham infection. According to our results, CFCM treatment alone did not compromise tadpoles' survival probability, nor reduced the body mass and developmental speed of the individuals. At the same time, the treatment reduced the intensity and prevalence of Bd infection, but this effect was affected by the population of origin. The antimicrobial metabolites produced by *X. szentirmai* may therefore be suitable for the safe mitigation of chytridiomycosis in *B. variegata* tadpoles, but further studies are needed, aiming to monitor and increase the efficacy of this method.

Keywords

amphibian disinfection; EPN-EPB symbionts; frog-killing fungus; mitigation method, yellow-bellied toad

1. Introduction

Native amphibians are key taxa in many ecosystems serving as prey for predators (Burton & Likens 1975, Davic & Welsh 2004, Regester et al. 2006), consuming a wide array of invertebrates including pests and vector organisms (Khatiwada et al. 2016, Shuman-Goodier et al. 2019) and playing a weighty role in ecosystem services (Hocking & Babbitt 2014). Despite their significance, amphibians are going through a biodiversity crisis that started in the last century, becoming to be the most threatened vertebrate group in the present days (Monastersky 2014). The main causes of this decline are climate change, pollution, habitat loss, and emerging infectious diseases (Luedtke et al. 2023), which factors often exert a combined, synergistic negative effect on the populations of amphibian species (Koprivnikar 2010, Campbell Grant et al. 2016).

The natural defense mechanisms of amphibians are normally effective against a wide range of pathogens and parasites (Grogan et al., 2018), but pathogens introduced by human activities can have devastating effects on the naïve amphibian populations. Chytridiomycosis is the most serious infectious disease affecting amphibians (Scheele et al. 2019). The disease is caused by the chytrid fungi *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal), infecting the keratinous epidermal layers of the amphibian skin (Berger et al. 1998). These agents have already contributed to the decline or extinction of several hundred amphibian species and caused mass mortality events on all continents inhabited by amphibians (Van Rooij et al. 2015). Because Bsal has a much narrower distribution range (Lastra González et al. 2019), here we concentrate on the better-investigated and globally distributed Bd. The symptoms of heavy Bd infection are intensive sloughing or skin shedding, reddening on legs, and even ulcerations or skin lesions on ventral surfaces, ultimately loss of rightening reflex and body posture. The structural damage to the skin can impair skin breathing and osmoregulation, provoking shifts in electrolyte balance

and finally leading to cardiac arrest (Voyles et al. 2009). In general, anuran tadpoles are less susceptible to the disease than in later life stages (Van Rooij et al. 2015), because keratinous elements are exhibited only in their mouthparts (Marantelli et al. 2004), thus they can act as reservoirs at natural habitats (Kilpatrick et al. 2010, Walker et al. 2010). However, the presence of Bd sometimes contributes to the loss of keratinized mouthpart structures, therefore leading to reduced feeding abilities and even lower survival (Blaustein et al. 2005). Several countermeasures against the disease have been proposed so far (Johnson et al. 2003, Woodhams et al. 2003, Harris et al. 2006, Woodward et al. 2014), but a widely applicable mitigation method against chytridiomycosis has not been found yet (Woodhams et al. 2012, Garner et al. 2016).

The addition or supplementation of mutualistic skin bacteria (bioaugmentation) associated to amphibians' skin, that can prevent infections or disease propagation (Belden & Harris 2007, Krynak et al. 2016) is proposed to be a promising mitigation method against chytridiomycosis (Harris et al. 2009, Bletz et al. 2013, Rebollar et al. 2020), however, *in vivo* experiments usually reported moderate, or no mitigation effect against the disease (Woodhams et al. 2012, Küng et al. 2014, Rebollar et al. 2016). The target bacteria intended to settle on the new amphibian host can trigger an immune response (Rebollar et al. 2020), and may not establish or not produce the expected antifungal metabolites due to microbial competition or changing environmental factors experienced on the new host (Daskin et al. 2014, Robak & Richards-Zawacki 2018, Woodhams et al. 2018). Utilizing bacterial metabolites directly against Bd instead of trying to establish live cultures on amphibian hosts has also been tested (Bell et al. 2013, Madison et al. 2017, Ujszegi et al. 2023). This approach allows for avoiding most of the abovementioned problems, can be more safely controlled and the scope of the search for antifungal metabolites with broad-spectrum inhibition capabilities can be widened to cover microbial sources of non-amphibian origin too (Ujszegi et al. 2023).

Entomopathogenic nematode (EPN) species belonging to the *Steinernema* and *Heterorhabditis* genera are parasites of soil-dwelling insects (Akhurst 1982). Upon infection, EPN larvae release entomopathogenic bacteria (EPB) symbionts into the hemocoel of the insect host. These bacteria start to propagate and synthesize various secondary metabolites which suppress the insect's immune response and accelerate its death. Furthermore, EPBs produce antimicrobial metabolites that protect the cadaver against microbial food competitors (Forst et al. 1997, Ogier et al. 2020). Since EPBs are culturable apart from their EPN hosts, these antimicrobial agents can be further utilized against plant-, livestock-, and human pathogens (Furgani et al. 2008, Böszörményi et al. 2009, Vozik et al. 2015, Wenski et al. 2020, Fodor et al. 2022). Moreover, secondary metabolites in cell-free culture medium (CFCM) extracted from liquid cultures of *Xenorhabdus szentirmai* and *X. budapestensis* EPB species (Lengyel et al. 2005) have highly effective growth inhibition capabilities against *Bd in vitro*. In case of *X. szentirmai*, CFCM can also be safely applied for significant reduction of *Bd* infection intensity on juvenile *Bufo bufo* individuals *in vivo* (Ujszegi et al. 2023). These results highlight the possibility of utilizing EPB metabolites in the mitigation of chytridiomycosis.

In Hungary, at least one *Bd* strain belonging to the Global Pandemic Lineage (GPL) is present (O'Hanlon et al. 2018) with the highest prevalence in frogs belonging to the *Bombina* and *Pelophylax* genera (Vörös et al. 2018). No mass mortalities have been observed so far, but yearly amphibian population surveys are focusing only on the breeding season. Therefore, mass die-offs during metamorphosis, when amphibians are the most susceptible to chytridiomycosis may remain hidden since their body size is small, and can decompose quickly. It could happen in the area of the Bükk National Park (Northern Hungary), where a recent study documented several incidents of lethal chytridiomycosis in the case of metamorphosing and juvenile *Bombina variegata* individuals (Harmos et al. 2021). Thus, to

successfully combat Bd, the infection load should have been reduced already at the tadpole stage before individuals become more susceptible to the disease upon metamorphosis. In cooperation with the colleagues of the National Park, as a practical first step, we assessed whether *X. szentirmaii* CFCM may be effectively and safely applied for mitigation of infection intensity on *B. variegata* tadpoles experimentally exposed to Bd.

2. Materials & Methods

2.1. Culturing of bacteria

We prepared Luria broth agar (LBA) plates flooded with Luria broth (LB) (10 g casein peptone, 5 g yeast extract, 10 g sodium chloride, and 17 g agar dissolved in 1000 ml distilled water) as described previously (Ausubel et al. 1999). Indicator plates (LBTA) were supplemented with bromothymol blue and 2,3,5-Triphenyltetrazolium chloride and were used to distinguish antimicrobial peptide-producing (phase I) and non-producing (phase II) variants of *X. szentirmaii* (Leclerc & Boemare 1991). Fresh single-phase I colonies derived from frozen bacterial stocks were used for the experiment as previously described (Furgani et al. 2008, Böszörményi et al. 2009, Vozik et al. 2015). Microbiological media were obtained from Biolab Zrt. (Budapest, Hungary).

We cultured *X. szentirmaii* in liquid TGhLY medium (mTGhLY; 8 g tryptone, 2 g gelatine-hydrolysate, 4 g lactose, and 5 g yeast extract in 1000 ml distilled water) with a 7-day-old single colony grown on LBA. We adapted this method from an earlier study (Ujszegi et al. 2023) to provide optimal growth conditions in the same medium for both the Bd and the EPB with the addition of yeast extract. In all other respects, this medium is equivalent to the TGhL medium used for the culturing of Bd (see below). The EPB culture in this study was started with 5-10 ml of LB inoculated with a single colony of the respective bacterium picked from an LBTA indicator plate and incubated overnight at 28 °C in a water bath shaker (Lab-

Line Orbital Shaker Water Bath, Marshall Scientific, USA). Each late-log phase inoculum was then added to 200 ml mTGhLY into 400 ml tissue culture flasks to create scale-up cultures.

2.2. Preparation of cell-free culture medium (CFCM)

We incubated scale-up cultures of *Xenorhabdus szentirmai* for 7 days at 25 °C on an orbital shaker platform (Gallencamp, UK) With these preparation conditions, the production of antibiotic metabolites in *Xenorhabdus* cultures reaches a stationary phase in 5-6 days, containing the same amount of metabolites (Furgani et al. 2008, Böszörményi et al. 2009, Vozik et al. 2015). Then we centrifuged cultures at 6000 rpm for 20 minutes at 4 °C in 400 ml tubes using a JLA-10.500 type rotor (Avanti centrifuge J-26 XPI, Beckman Coulter, Indianapolis, USA). The supernatant was filtered through a sterile 0.22 µm nylon filter and centrifuged again at the same speed. We considered the resulting supernatant to be a cell-free culture medium (CFCM) of the antibiotic-producing *X. szentirmai*. To confirm that CFCM was indeed cell-free, we diluted at least two replicates with sterile 2× LB, incubated them along with the experimental samples, and checked for bacterial growth on LBA plates. We stored CFCM at 4 °C in glass bottles until further use.

2.3. Experimental procedures

In May 2023 we collected 170 *Bombina variegata* eggs from puddles and wheel tracks at four localities (four distinct populations) in the Mátra mountains, Hungary (Haluskási út: 47.8866° N, 19.9938° E; Marháti út: 47.8911° N, 20.0418° E; Somhegy: 47.8868° N, 20.0076° E; Hidasi erdészház: 47.8831° N, 19.9834° E). We transported eggs to the Experimental Station Juliannamajor of the Plant Protection Institute, Centre for Agricultural Research located on the outskirts of Budapest (47.5479° N, 18.9349° E). We placed eggs of

each of the four populations separately into plastic containers ($32 \times 22 \times 16$ cm) holding 0.7 l of reconstituted soft water (RSW; USEPA, 2002) at a constant temperature of 19.8 ± 0.4 °C and a light : dark cycle adjusted weekly to the conditions outside. Nine days after hatching, when all larvae reached development stage 25 (Gosner 1960), we started the experiment with 150 healthy-looking tadpoles (Haluskási út: N = 9 per treatment, 45 in total; Marháti út: N = 8 per treatment, 40 in total; Somhegy: N = 7 per treatment, 35 in total ; Hidasi erdészház: N = 6 per treatment, 30 in total). We reared tadpoles individually in opaque plastic boxes ($17 \times 12 \times 9$ cm) filled with 1 l RSW and fed them *ad libitum* with boiled and smashed spinach. Temperature in the laboratory was 18.2 ± 0.3 °C (mean $\pm$ SD) during the experiment. The light : dark cycle was adjusted weekly to outdoor conditions. We assigned tadpoles to five treatments (Table 1), using stratified randomization considering their population of origin: we exposed them to RSW (Treat 1), sterile TGhLY medium (Treat 2 and 3), or liquid culture of Bd (Treat 4 and 5; please see below for details) in the next 22 days. All hatchlings beyond those 150 that were arranged into the five treatments, were infected with Bd and kept the same way to increase the sample size of reference for initial Bd infection (see below). We arranged rearing boxes randomly on the laboratory shelves and changed water twice a week using different dip nets for each treatment to prevent cross-contamination. We monitored survival daily and noted any incidence of death.

Twenty-two days after the start of the experiment, we randomly selected five individuals from each treatment group, and humanely euthanized them together with all surplus individuals in a water bath containing 6.6 g l^{-1} tricaine-methanesulfonate (MS-222) buffered to neutral pH with the same amount of Na_2HPO_4 . We conserved dead individuals in 96 % EtOH and stored samples at 4 °C until further analysis as a reference for the initial Bd infection status of the animals before mitigation. We changed the boxes of the remaining individuals and started mitigation treatments by exposing them to either RSW, mTGhLY, or

CFCM at a dilution of 0.1 % (v/v) (Table1). We constantly treated animals for another 22 days re-administering treatments after each water change. On the last day of treatments, we gently blotted and weighed individuals to the nearest mg (OHAUS-PA213 analytical balance, Ohaus Europe Gmb, Nanikon, Switzerland), euthanized and preserved them as described above.

2.4. Maintaining *Bd* cultures and experimental exposure

We used the global pandemic lineage (GPL) of *Bd*. The isolate (Hung_2014) originated from a *B. variegata* collected alive in 2014 by J. Vörös in the Bakony Mountains, Hungary, and isolated by M.C. Fisher and colleagues (Imperial College London, London, UK). We maintained parallel cultures in TGhL medium (mTGhL; 8 g tryptone, 2 g gelatine-hydrolysate, and 4 g lactose in 1000 ml distilled water) in 25 cm² cell culture flasks at 4 °C and passaged them every three months into sterile mTGhL.

One week before performing experimental infections, we inoculated 100 ml mTGhLY with 2 ml of *Bd* stock culture in a 175 cm² cell culture flask and incubated it for seven days at 21 °C. We assessed the concentration of intact zoospores using a Bürker chamber at ×400 magnification before every inoculation. During inoculation of tadpoles' rearing boxes, the mean initial concentrations were $\sim 7.5 \times 10^5$ zoospores (zsp) ml⁻¹ in the flasks. After each water change, we inoculated 1 ml of these cultures into the tadpoles' rearing boxes holding 1 l RSW, resulting in ~ 750 zsp ml⁻¹ concentration in the rearing boxes. We inoculated controls with the same quantity of sterile mTGhLY or RSW according to the treatments. Contaminated water and equipment were disinfected overnight with VirkonS before disposal (Johnson et al. 2003).

2.5. Assessment of infection intensity

We cut out, then homogenized the whole mouthparts of the preserved tadpoles, extracted DNA from samples using PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to previous recommendations (Boyle et al. 2004), and stored extracted DNA at -20 °C until further analyses. We assessed infection intensity using real-time quantitative polymerase chain reaction (qPCR) following a standard amplification methodology targeting the ITS-1/5.8S rDNA region (Boyle et al. 2004) on a BioRad CFX96 Touch Real-Time PCR System (BioRad Laboratories, Hercules, USA). To avoid PCR inhibition by ingredients of PrepMan, we diluted samples ten-fold with double-distilled water. We ran samples in duplicate, and in case of equivocal results, we repeated reactions in duplicate. If this again returned an equivocal result, we considered the sample to be Bd positive (Kriger et al. 2006). Genomic equivalent (GE) values were estimated from standard curves based on five dilutions of a standard (1000, 100, 10, 1, and 0.1 zoospore GE; provided by J. Bosch; CSIC-University of Oviedo, Spain).

2.6. Statistical analyses

From the analyses, we excluded all the reference individuals preserved for inspecting initial Bd infection status, and a further individual from the infection analyses which started metamorphosis before the end of the experiment. We assessed treatment effects on survival, the stage of larval development, body mass, Bd prevalence, and infection intensity. For each dependent variable, we ran a model (see model specifications below) with treatment and population of origin (population hereafter) as categorical fixed factors and their interaction. For the analysis of survival, we used Cox's proportional hazards model (R package 'coxme') with treatment as the explanatory variable. The structure of the data did not allow for including population as another fixed factor. We entered the number of days until death as the dependent variable and individuals that survived until the termination of the experiment were

treated as censored observations. To analyse variation in the stage of larval development and body mass, we used general linear models (LM; ‘lm’ function of the ‘nlme’ package). In the analysis of body mass, we further included developmental stage as a covariate. To analyse Bd prevalence, we used generalized linear models (‘glm’ function of the ‘nlme’ package) with binomial distribution and logit link function. For the investigation of infection intensity, we averaged GE values obtained from qPCR runs for each sample and analysed resulting estimates corrected for body mass using generalized linear mixed models (GLMM) with negative binomial distribution and a log link function using the ‘glmmTMB’ package (Brooks et al. 2017). All tests were two-tailed, and we checked model fits in the case of all dependent variables by visual inspection of diagnostic plots. We applied a backward stepwise model simplification procedure (Grafen & Hails 2002) to avoid potential problems due to the inclusion of non-significant terms (Engqvist 2005). We used the significance value of $\alpha = 0.05$. In case of significant effect by any grouping explanatory variable, we applied post-hoc tests by calculating pre-planned linear contrasts (Ruxton & Beauchamp 2008), correcting the significance threshold for multiple testing using the false discovery rate (FDR) method (Pike 2011). All analyses were conducted in ‘R’ (version 4.0.5).

3. Results

Survival was not affected by any of the treatments (Cox model: $z = -1.24$, $p = 0.21$; Fig 1). The stage of larval development was significantly affected by treatment (LM: $F_{4,94} = 17.19$, $p < 0.001$; Fig 2A) and by population ($F_{3,94} = 4.27$, $p < 0.001$), but not their interaction ($F_{12,82} = 0.58$, $p = 0.86$). Pairwise comparisons revealed that developmental speed was significantly lower in the group Treat 1 compared with all other groups, but Bd exposure and / or CFCM treatment did not affect it (Table 2). Body mass of the tadpoles at the end of the experiment was not affected by population (LM: $F_{3,92} = 1.41$, $p = 0.25$) or its interaction with

treatment (LM: $F_{12,80} = 1.01$, $p = 0.45$), but it was significantly affected by treatment alone ($F_{4,95} = 35.15$, $p < 0.001$; Fig 2B) and developmental stage (LM: $F_{1,95} = 42.87$, $p < 0.001$). Pairwise comparisons revealed that Treat 1 significantly reduced body mass compared to all other treatment groups. However, Bd exposure or treatment with CFCM did not affect body mass (Table 3, Fig 2B).

Since all tested individuals from uninfected treatment groups remained Bd negative, we can exclude the possibility of cross-contamination. Bd prevalence was 71 % in experimentally infected individuals chosen as reference for initial Bd infection right before the start of the CFCM treatment, with an average infection intensity of 14.7 (0.0-111.8) GE (median and interquartile range) for all individuals. CFCM treatment resulted in significantly lower Bd prevalence (GLM: $z = -2.83$, $p = 0.009$; 54 % vs. 89 % in the non-treated group) and infection intensity ($z = -3.65$, $p < 0.001$; 0.14 [0.0-1.18] GE/gram body weight [GE/gbw; median and interquartile range]; Fig 3) compared to the Bd exposed group without CFCM treatment (0.59 [0.13-1.87] GE gbw⁻¹ [median and interquartile range]; Fig 3). However, the population also affected this antifungal effect since its interaction with treatment was also significant in the case of infection intensity ($z = 3.58$, $p < 0.001$) and marginally non-significant in the case of Bd prevalence ($z = 1.83$, $p = 0.067$). Omitting this interaction, the effect of population alone on Bd prevalence was significant ($z = 2.29$, $p = 0.022$).

4. Discussion

Treatment with CFCM of the *X. szentirmai* EPB had no short term negative effect on the measured life history traits of *Bombina variegata* tadpoles, similarly to former results on juvenile *Bufo bufo* individuals exposed to much more concentrated CFCM solutions (Ujszegi et al. 2023). Moreover, microbial medium at the applied dilution seemed to exert a beneficial effect in the terms of developmental speed and body mass of the tadpoles, compared with

conspicuous reared in pure RSW throughout the experiment. This is likely due to the absorption of extra nutritional compounds from the media through their gut, which accelerated tadpoles' growth and development. However, whether CFCM treatment has other negative effects on the long run for the treated amphibians, for example through the alteration of skin microbiome, is still need to be investigated in future studies.

Prevalence of chytridiomycosis can reach high values in *B. variegata* at several natural habitats across its whole distribution (Sztatecsny & Glaser 2011, Baláz et al. 2014, Kolenda et al. 2017, Vojar et al. 2017, Vörös et al. 2018), likely contributing to local population declines (Canestrelli et al. 2013). Additionally, mortality events that have been documented (Baláz et al. 2014, Harnos et al. 2021) further emphasize that intervention may be necessary soon to prevent populations from catastrophic losses. We demonstrated, that *X. szentirmaii* CFCM solution applied at a concentration as low as 0.1 % v/v is still able to inhibit Bd growth *in vivo* in *B. variegata* tadpoles. This extreme effectiveness is in line with former findings, where dilution close to 0.1 % CFCM of the same microbe still showed *in vitro* growth inhibition of Bd (Ujszegi et al. 2023). However, growth inhibition capabilities varied between the host's populations of origin. Specimens of *B. variegata* secrete potent AMPs in their skin (Mignogna et al. 1993), and intraspecific differences in AMP synthesis can reflect the differences of populations in the sensitivity to chytridiomycosis in other amphibians (Tennessen et al. 2009, Bradley et al. 2015). Therefore different *B. variegata* populations may differ in their immunity and skin-secreted AMP repertoire, which affect their tolerance of Bd infection, and probably differently interact with antimicrobial metabolites in the CFCM. Also, distinct populations may harbor different skin microbiome compositions (Woodhams et al. 2014) variously affecting, and interacting with the beneficial effect of the CFCM treatment. Improvement of the method's efficacy to reliably achieve a more uniform effect could help to remove this population effect very likely.

One possible option for enhancing effectivity would be the application of a more concentrated CFCM solution, but this can be problematic upon constantly treating tadpoles, exposing them to CFCM through their ambient water. Since CFCM is a culture medium, its ingredients can promote excessive bacterial bloom in the water already at the dilution of 0.5 % v/v, which compromises dissolved oxygen level, threatening the tadpoles' health (Ujszegi et al. unpublished). Furthermore, CFCM concentrated more than 15% can be harmful to individuals directly (Ujszegi et al. 2023) thus taken together, this direction to enhance the effectiveness of CFCM treatment on amphibians is not recommended. An alternative option could be the treatment of tadpoles in separate enclosures out of their rearing water for a limited time on several consecutive occasions to reduce pathogen load or clear infection. In this case, since tadpoles are exposed to CFCM in fresh water on every occasion and only for a limited time, more concentrated CFCM could be used to enhance effectivity, such as in the case of juvenile *B. bufo* individuals (Ujszegi et al. 2023). This direction deserves more detailed research and could be a very useful method in the laboratory, or in captive breeding facilities where disinfection is crucial for preventing disease outbreaks (Kueneman et al. 2022), even in the case of *B. variegata* (Stagni et al. 2004). However, for such CFCM treatments, all necessary instruments are better available indoors, and the number of individuals is more manageable than in the field, therefore *in situ* application of this direction is very limited.

Since a low level of infection usually causes no clinical signs or mortality (Vredenburg et al. 2010, Cheng et al. 2011) and amphibian populations can coexist with Bd (Rowley & Alford 2013), a complete clearance of infection may not be essential. It is possible, that the efficiency achieved by the treatment with 0.1 % *X. szentirmai* CFCM can even be sufficient for the mitigation of chytridiomycosis at *B. variegata* habitats, because co-existence with enzootic Bd may lead to immunization (Ramsey et al. 2010, McMahon et al. 2014) and allow

for adaptation to the disease via the spread of resistance alleles (Bataille et al. 2015, Savage & Zamudio 2016, Voyles et al. 2018) or alternative reproductive strategies (Spitzen-Van Der Sluijs et al. 2017) in the populations. Whether the observed effect is sufficient for halting mortality events by Bd in natural populations, and whether the addition of CFCM would be harmful to other aquatic organisms besides amphibians (such as pond vegetation and macroinvertebrates) at the habitats should be long-term monitored. Given that CFCM has got wide array of antimicrobial activity, and the media contains many extra nutrients, investigating whether the treatment causes long-term shifts in the aquatic microbial composition and therefore in the flow of energy and nutrients would be also important before routinely applying this method at natural habitats.

Since a universally applicable mitigation method against chytridiomycosis for most of the host species with the same level of efficacy won't be found very likely (Garner et al. 2016), researchers and nature conservation specialists must focus their effort on species that are endangered, or largely affected by chytridiomycosis. Scientists should find locally adaptable and successful *in situ* mitigation methods designed for the focal species of conservation (e.g. Thumsová et al., 2024), considering their special needs and characteristics. Going back to *B. variegata*, these frogs mostly live in small water bodies and wheel track puddles. In such environments, the desired CFCM amount (0.1 % v/v) could be easily achieved needing no more than some litres for the treatment of the whole water body. CFCM can still be produced quickly and cheaply in such quantities. Furthermore, due to its extreme thermostability, CFCM does not require special handling during transportation and would last for a long time after application. Based on these characteristics and our results, improving this method to be applied for *in situ* mitigation purposes to preserve *B. variegata* populations would be worthwhile in the future.

Acknowledgments

We are thankful to A. Hettyey, Z. Mikó, N. Ujhegyi, D. Herczeg, and M. Szederkényi for their assistance and help during the experiment, A. Hettyey for providing the institutional background for the experiment, V. Bókony for help in statistics and M. Z. Németh, and all colleagues from the Department of the Plant Pathology for allowing us to use their lab facilities. T. Vellai provided us with the laboratory capacity, and A. Fodor helped with professional guidance at the Department of Genetics, Eötvös Loránd University. Zoospore genomic equivalent standards were kindly offered by J. Bosch, and Bd isolate was sent by M. C. Fisher. The research was supported by the Lendület Programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012) and the New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development, and Innovation Fund (ÚNKP-23-4 for U.J., ÚNKP-22-3 and ÚNKP-23-4 for A. K., ÚNKP-23-6 for Á. T.). Open access funding was provided by Eötvös Loránd University. The Ethical Commission of the Plant Protection Institute approved experimental procedures and research was carried out according to the permits issued by the Government Agency of Pest County (PEI/001/1797-3/2015, PE/EA/00270-6/2023) and the Government Agency of Heves County (HE/TVO/00022-10/2023). The authors have no conflict of interest to declare.

Literature cited

Akhurst RJ (1982) Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J Gen Microbiol 128:3061-3065.

Ausubel FM, Brent R, Kingston RE, David D, Moore DD, Seidman JG, Smith JA, Struhl K (1999) Short Protocols in Molecular Biology: A Compendium of Methods from Current

Protocols in Molecular Biology. Ausubel FM, Brent R, Kingston RE, David D, Moore DD, Seidman JG, Smith JA, Struhl K (eds) John Wiley & Sons, New York.

Baláz V, Vörös J, Civiš P, Vojar J, Hettyey A, Sós E, Dankovics R, Jehle R, Christiansen DG, Clare F, Fisher MC, Garner TWJ, Bielby J (2014) Assessing Risk and Guidance on Monitoring of *Batrachochytrium dendrobatidis* in Europe through Identification of Taxonomic Selectivity of Infection. *Conserv Biol* 28:213–223.

Bataille A, Cashins SD, Grogan L, Skerratt LF, Hunter D, McFadden M, Scheele B, Brannelly LA, Macris A, Harlow PS, Bell S, Berger L, Waldman B (2015) Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. *Proc R Soc B Biol Sci* 282:20143127.

Belden LK, Harris RN (2007) Infectious diseases in wildlife: The community ecology context. *Front Ecol Environ* 5:533–539.

Bell SC, Alford RA, Garland S, Padilla G, Thomas AD (2013) Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis Aquat Organ* 103:77–85.

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin LC, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci U S A* 95:9031–9036.

Blaustein AR, Romansic JM, Scheessele EA, Han BA, Pessier AP, Longcore JE (2005) Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conserv Biol* 19:1460–1468.

Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, Minbiole KPC, Harris RN (2013) Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. *Ecol Lett* 16:807–820.

Böszörményi E, Érsek T, Fodor A, Fodor AM, Földes LS, Hevesi M, Hogan JS, Katona Z, Klein MG, Kormány A, Pekár S, Szentirmai A, Sztaricskai F, Taylor RAJ (2009) Isolation and activity of *Xenorhabdus* antimicrobial compounds against the plant pathogens *Erwinia amylovora* and *Phytophthora nicotianae*. J Appl Microbiol 107:746–759.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ 60:141–148.

Bradley PW, Gervasi SS, Hua J, Cothran RD, Relyea RA, Olson DH, Blaustein AR (2015) Differences in sensitivity to the fungal pathogen *Batrachochytrium dendrobatidis* among amphibian populations. Conserv Biol 29:1347–1356.

Brooks ME, Kristensen K, Benthem KJ van, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Maechler M, Bolker BM (2017) GlmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R J 9:378–400.

Burton TM, Likens GE (1975) Energy flow and nutrient cycling in salamander populations in the Hubbard Brook Experimental Forest, New Hampshire. Ecology 56:1068–1080.

Campbell Grant EH, Miller DAW, Schmidt BR, Adams MJ, Amburgey SM, Chambert T, Cruickshank SS, Fisher RN, Green DM, Hossack BR, Johnson PTJ, Joseph MB, Rittenhouse TAG, Ryan ME, Waddle HJ, Walls SC, Bailey LL, Fellers GM, Gorman TA, Ray AM, Pilliod DS, Price SJ, Saenz D, Sadinski W, Muths E (2016) Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. Sci Rep 6:25625.

Canestrelli D, Zampiglia M, Nascetti G (2013) Widespread occurrence of *Batrachochytrium dendrobatidis* in contemporary and historical samples of the endangered *Bombina pachypus* along the Italian Peninsula. PLoS One 8:e63349.

Cheng TL, Rovito SM, Wake DB, Vredenburg VT (2011) Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc Natl Acad Sci U S A* 108:9502–9507.

Daskin JH, Bell SC, Schwarzkopf L, Alford RA (2014) Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians – implications for disease management and patterns of decline. *PLoS One* 9:e100378.

Davic RD, Welsh HH (2004) On the ecological roles of salamanders. *Annu Rev Ecol Evol Syst* 35:405–434.

Engqvist L (2005) The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim Behav* 70:967–971.

Fodor A, Gualtieri M, Zeller M, Tarasco E, Klein MG, Fodor AM, Haynes L, Lengyel K, Forst SA, Furgani GM, Karaffa L, Vellai T (2022) Type strains of entomopathogenic Nematode-symbiotic bacterium species , *Xenorhabdus szentirmaii* (EMC) and *X. budapestensis* (EMA), are exceptional sources of thermotolerant-antimicrobial peptides (by both), and Iodinine (by EMC). *Pathogens* 11:342.

Forst S, Neelson KH, Boemare N, Stackebrandt E (1997) *Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs. *Annu Rev Microbiol* 51:47–72.

Furgani G, Böszörményi E, Fodor A, Máthé-Fodor A, Forst S, Hogan JS, Katona Z, Klein MG, Stackebrandt E, Szentirmai A, Sztaricskai F, Wolf SL (2008) *Xenorhabdus* antibiotics: A comparative analysis and potential utility for controlling mastitis caused by bacteria. *J Appl Microbiol* 104:745–758.

Garner TW, Schmidt BR, Martel A, Pasmans F, Muths E, Cunningham AA, Weldon C, Fisher MC, Bosch J (2016) Mitigating amphibian chytridiomycoses in nature. *Philos Trans R Soc B Biol Sci* 371:20160207.

Gosner KL (1960) A simplified table for staging anuran embryos larvae with notes on

476 identification. *Herpetologica* 16:183–190.

477 Grafen A, Hails R (2002) *Modern statistics for the life sciences*. Oxford University Press,
478 Oxford ; New York.

479 Harmos K, Bosch J, Thumsová B, Martínez-Silvestre A, Velarde R, Vörös J (2021)
480 Amphibian mortality associated with chytridiomycosis in central Eastern Europe.
481 *Herpetol Notes* 14:1213–1218.

482 Harris RN, James TY, Lauer A, Simon MA, Patel A (2006) Amphibian pathogen
483 *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian
484 species. *Ecohealth* 3:53–56.

485 Harris RN, Lauer A, Simon MA, Banning JL, Alford RA (2009) Addition of antifungal skin
486 bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis Aquat Organ*
487 83:11–16.

488 Hocking DJ, Babbitt KJ (2014) Amphibian contributions to ecosystem services. *Herpetol*
489 *Conserv Biol* 9:1–17.

490 Johnson ML, Berger L, Philips L, Speare R (2003) Fungicidal effects of chemical
491 disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium*
492 *dendrobatidis*. *Dis Aquat Organ* 57:255–260.

493 Khatiwada JR, Ghimire S, Paudel Khatiwada S, Paudel B, Bischof R, Jiang J, Haugaasen T
494 (2016) Frogs as potential biological control agents in the rice fields of Chitwan, Nepal.
495 *Agric Ecosyst Environ* 230:307–314.

496 Kilpatrick AM, Briggs CJ, Daszak P (2010) The ecology and impact of chytridiomycosis: an
497 emerging disease of amphibians. *Trends Ecol Evol* 25:109–118.

498 Kolenda K, Najbar A, Ogielska M, Baláz V (2017) *Batrachochytrium dendrobatidis* is
499 present in Poland and associated with reduced fitness in wild populations of *Pelophylax*
500 *lessonae*. *Dis Aquat Organ* 124:241–245.

501 Koprivnikar J (2010) Interactions of environmental stressors impact survival and
502 development of parasitized larval amphibians. *Ecol Appl* 20:2263–2272.

503 Kriger KM, Hero J-M, Ashton KJ (2006) Cost efficiency in the detection of chytridiomycosis
504 using PCR assay. *Dis Aquat Organ* 71:149–154.

505 Krynak KL, Burke DJ, Benard MF (2016) Landscape and water characteristics correlate with
506 immune defense traits across Blanchard’s cricket frog (*Acris blanchardi*) populations.
507 *Biol Conserv* 193:153–167.

508 Kueneman J, Bletz M, Becker M, Gratwicke B, Garcés OA, Hertz A, Holden WM, Ibáñez R,
509 Loudon A, McKenzie V, Parfrey L, Sheafor B, Rollins-smith LA, Richards-zawacki C,
510 Voyles J, Woodhams DC (2022) Effects of captivity and rewilding on amphibian skin
511 microbiomes. *Biol Conserv* 271:109576.

512 Küng D, Bigler L, Davis LR, Gratwicke B, Griffith E, Woodhams DC (2014) Stability of
513 microbiota facilitated by host immune regulation: Informing probiotic strategies to
514 manage amphibian disease. *PLoS One* 9:e87101.

515 Lastra González D, Baláž V, Solský M, Thumsová B, Kolenda K, Najbar A, Najbar B,
516 Kautman M, Chajma P, Balogová M, Vojar J (2019) Recent findings of potentially
517 lethal salamander fungus *Batrachochytrium salamandrivorans*. *Emerg Infect Dis*
518 25:1416–1418.

519 Leclerc MC, Boemare NE (1991) Plasmids and phase variation in *Xenorhabdus* spp. *Appl*
520 *Environ Microbiol* 57:2597–2601.

521 Lengyel K, Lang E, Fodor A, Szállás E, Schumann P, Stackebrandt E (2005) Description of
522 four novel species of *Xenorhabdus*, family *Enterobacteriaceae*: *Xenorhabdus*
523 *budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and
524 *Xenorhabdus szentirmaii* sp. nov. *Syst Appl Microbiol* 28:115–122.

525 Luedtke JA, Chanson J, Neam K, Hobin L, Maciel AO, Catenazzi A, Borzée A, Hamidy A,

Aowphol A, Jean A, Sosa-Bartuano Á, Fong G A, de Silva A, Fouquet A, Angulo A, Kidov AA, Muñoz Saravia A, Diesmos AC, Tominaga A, Shrestha B, Gratwicke B, Tjaturadi B, Martínez Rivera CC, Vásquez Almazán CR, Señaris C, Chandramouli SR, Strüssmann C, Cortez Fernández CF, Azat C, Hoskin CJ, Hilton-Taylor C, Whyte DL, Gower DJ, Olson DH, Cisneros-Heredia DF, Santana DJ, Nagombi E, Najafi-Majd E, Quah ESH, Bolaños F, Xie F, Brusquetti F, Álvarez FS, Andreone F, Glaw F, Castañeda FE, Kraus F, Parra-Olea G, Chaves G, Medina-Rangel GF, González-Durán G, Ortega-Andrade HM, Machado IF, Das I, Dias IR, Urbina-Cardona JN, Crnobrnja-Isailović J, Yang J-H, Jianping J, Wangyal JT, Rowley JLL, Measey J, Vasudevan K, Chan KO, Gururaja KV, Ovaska K, Warr LC, Canseco-Márquez L, Toledo LF, Díaz LM, Khan MMH, Meegaskumbura M, Acevedo ME, Napoli MF, Ponce MA, Vaira M, Lampo M, Yáñez-Muñoz MH, Scherz MD, Rödel M-O, Matsui M, Fildor M, Kusrini MD, Ahmed MF, Rais M, Kouamé NG, García N, Gonwouo NL, Burrowes PA, Imbun PY, Wagner P, Kok PJR, Joglar RL, Auguste RJ, Brandão RA, Ibáñez R, von May R, Hedges SB, Biju SD, Ganesh SR, Wren S, Das S, Flechas S V, Ashpole SL, Robleto-Hernández SJ, Loader SP, Incháustegui SJ, Garg S, Phimmachak S, Richards SJ, Slimani T, Osborne-Naikatini T, Abreu-Jardim TPF, Condez TH, De Carvalho TR, Cutajar TP, Pierson TW, Nguyen TQ, Kaya U, Yuan Z, Long B, Langhammer P, Stuart SN (2023) Ongoing declines for the world's amphibians in the face of emerging threats. *Nature* 622:308–314.

Madison JD, Berg EA, Abarca JG, Whitfield SM, Gorbatenko O, Pinto A, Kerby JL (2017) Characterization of *Batrachochytrium dendrobatidis* inhibiting bacteria from amphibian populations in Costa Rica. *Front Microbiol* 8:1–13.

Marantelli G, Berger L, Speare R, Keegan L (2004) Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific*

551 Conserv Biol 10:173–179.

552 McMahon TA, Sears BF, Venesky MD, Bessler SM, Brown JM, Deutsch K, Halstead NT,
553 Lentz G, Tenouri N, Young S, Civitello DJ, Ortega N, Fites JS, Reinert LK, Rollins-
554 Smith LA, Raffel TR, Rohr JR (2014) Amphibians acquire resistance to live and dead
555 fungus overcoming fungal immunosuppression. Nature 511:224–227.

556 Mignogna G, Simmaco M, Kreil G, Barra D (1993) Antibacterial and haemolytic peptides
557 containing D-alloisoleucine from the skin of *Bombina variegata*. EMBO J 12:4829–
558 4832.

559 Monastersky R (2014) Biodiversity: Life a status report. Nature 516:158–161.

560 O’Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, Kosch TA, Murray
561 KA, Brankovics B, Fumagalli M, Martin MD, Wales N, Alvarado-rybak M, Bates KA,
562 Berger L, Böll S, Brookes L, Clare F, Courtois EA, Cunningham AA, Doherty-bone
563 TM, Ghosh P, Gower DJ, Hintz WE, Höglund J, Jenkinson TS, Lin C, Laurila A, Loyau
564 A, Martel A, Meurling S, Miaud C, Minting P, Pasmans F, Schmeller DS, Schmidt BR,
565 Shelton JMG, Skerratt LF, Smith F, Soto-azat C, Spagnoletti M, Tessa G, Toledo LF,
566 Valenzuela-sánchez A, Verster R, Vörös J, Webb RJ, Wierzbicki C, Wombwell E,
567 Zamudio KR, Aanensen DM, James TY, Gilbert MTP, Weldon C, Bosch J, Balloux F,
568 Garner TWJ, Fisher MC (2018) Recent Asian origin of chytrid fungi causing global
569 amphibian declines. Science (80-) 360:621–627.

570 Ogier JC, Pagès S, Frayssinet M, Gaudriault S (2020) Entomopathogenic nematode-
571 associated microbiota: From monoxenic paradigm to pathobiome. Microbiome 8:25.

572 Pike N (2011) Using false discovery rates for multiple comparisons in ecology and evolution.
573 Methods Ecol Evol 2:278–282.

574 Ramsey JP, Reinert LK, Harper LK, Woodhams DC, Rollins-Smith LA (2010) Immune
575 defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian

declines, in the South African clawed frog, *Xenopus laevis*. Infect Immun 78:3981–
3992.

Rebollar EA, Martínez-Ugalde E, Orta AH (2020) The amphibian skin microbiome and its
protective role against chytridiomycosis. Herpetologica 76:167–177.

Rebollar EA, Simonetti SJ, Shoemaker WR, Harris RN (2016) Direct and indirect horizontal
transmission of the antifungal probiotic bacterium *Janthinobacterium lividum* on Green
frog (*Lithobates clamitans*) tadpoles. Appl Environ Microbiol 82:2457–2466.

Regester KJ, Lips KR, Whiles MR (2006) Energy flow and subsidies associated with the
complex life cycle of Ambystomatid salamanders in ponds and adjacent forest in
Southern Illinois. Oecologia 147:303–314.

Robak MJ, Richards-Zawacki CL (2018) Temperature-dependent effects of cutaneous
bacteria on a frog’s tolerance of fungal infection. Front Microbiol 9:Article 410.

Van Rooij P, Martel A, Haesebrouck F, Pasmans F (2015) Amphibian chytridiomycosis: A
review with focus on fungus-host interactions. Vet Res 46:1–22.

Rowley JJ, Alford RA (2013) Hot bodies protect amphibians against chytrid infection in
nature. Sci Rep 3:1515.

Ruxton GD, Beauchamp G (2008) Time for some a priori thinking about post hoc testing.
Behav Ecol 19:690–693.

Savage AE, Zamudio KR (2016) Adaptive tolerance to a pathogenic fungus drives major
histocompatibility complex evolution in natural amphibian populations. Proc R Soc B
Biol Sci 283:20153115.

Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, Acevedo AA,
Burrowes PA, Carvalho T, Catenazzi A, De la Riva I, Fisher MC, Flechas S V, Foster
CN, Frías-Álvarez P, Garner TWJ, Gratwicke B, Guayasamin JM, Hirschfeld M, Kolby
JE, Kosch TA, La Marca E, Lindenmayer DB, Lips KR, Longo A V, Maneyro R,

McDonald CA, Mendelson III J, Palacios-Rodriguez P, Parra-Olea G, Richards-zawacki CL, Rödel M-O, Rovito SM, Soto-Azat C, Toledo LF, Voyles J, Weldon C, Whitfield SM, Wilkinson M, Zamudio KR, Canessa (2019) Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* (80-) 363:1459–1463.

Shuman-Goodier ME, Diaz MI, Almazan ML, Singleton GR, Hadi BA, Propper CR (2019) Ecosystem hero and villain: Native frog consumes rice pests, while the invasive cane toad feasts on beneficial arthropods. *Agric Ecosyst Environ* 279:100–108.

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. *Proc R Soc B Biol Sci* 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? *Ital J Zool* 71:151–154.

Sztatecsny M, Glaser F (2011) From the eastern lowlands to the western mountains: First records of the chytrid fungus *Batrachochytrium dendrobatidis* in wild amphibian populations from Austria. *Herpetol J* 21:87–90.

Tennessen JA, Woodhams DC, Chaurand P, Reinert LK, Billheimer D, Shyr Y, Caprioli RM, Blouin MS, Rollins-Smith LA (2009) Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens. *Dev Comp Immunol* 33:1247–1257.

Thumsová B, González-Miras E, Rubio Á, Granados I, Bates KA, Bosch J (2024) Chemical disinfection as a simple and reliable method to control the amphibian chytrid fungus at breeding points of endangered amphibians. *Sci Rep* 14:5151.

Ujszegi J, Boros Z, Fodor A, Vajna B, Hettyey A (2023) Metabolites of *Xenorhabdus*

bacteria are potent candidates for mitigating amphibian chytridiomycosis. *AMB Express* 13:88.

USEPA (2002) Section 7: Dilution water. In: *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 5th ed. Office of Water, U.S. Environmental Protection Agency, Washington, D.C., EPA-821-R-02-012, p 33

Vojar J, Havlíková B, Solský M, Jablonski D, Iković V, Baláž V (2017) Distribution, prevalence, and amphibian hosts of *Batrachochytrium dendrobatidis* in the Balkans. *Salamandra* 53:44–49.

Vörös J, Herczeg D, Fülöp A, Gál J, Dán Á, Harnos K, Bosch J (2018) *Batrachochytrium dendrobatidis* in Hungary: an overview of recent and historical occurrence. *Acta Herpetol* 13:125–140.

Voyles J, Woodhams DC, Saenz V, Byrne AQ, Perez R, Rios-Sotelo G, Ryan MJ, Bletz MC, Sobell FA, McLetchie S, Reinert L, Rosenblum EB, Rollins-Smith LA, Ibáñez R, Ray JM, Griffith EJ, Ross H, Richards-Zawacki CL (2018) Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. *Science* (80-) 359:1517–1519.

Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook D, Webb R, Alford RA, Skerratt LF, Speare R (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* (80-) 326:582–585.

Vozik D, Bélafi-Bakó K, Hevesi M, Böszörményi E, Fodor A (2015) Effectiveness of a peptide-rich fraction from *Xenorhabdus budapestensis* culture against fire blight disease on apple blossoms. *Not Bot Horti Agrobot Cluj-Napoca* 43:547–553.

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc Natl Acad Sci* 107:9689–9694.

- Walker SF, Bosch J, Gomez V, Garner TW, Cunningham AA, Schmeller DS, Ninyerola M, Henk DA, Ginestet C, Arthur C, Fisher MC (2010) Factors driving pathogenicity versus prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* and chytridiomycosis in Iberia. *Ecol Lett* 13:372–382.
- Wenski SL, Cimen H, Berghaus N, Fuchs SW, Hazir S, Bode HB (2020) Fabclavine diversity in *Xenorhabdus* bacteria. *Beilstein J Org Chem* 16:956–965.
- Woodhams DC, Alford RA, Marantelli G (2003) Emerging disease of amphibians cured by elevated body temperature. *Dis Aquat Organ* 55:65–67.
- Woodhams DC, Brandt H, Baumgartner S, Kielgast J, Küpfer E, Tobler U, Davis LR, Schmidt BR, Bel C, Hodel S, Knight R, McKenzie V (2014) Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One* 9:e96375.
- Woodhams DC, Geiger CC, Reinert LK, Rollins-Smith LA, Lam B, Harris RN, Briggs CJ, Vredenburg VT, Voyles J (2012) Treatment of amphibians infected with chytrid fungus: Learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. *Dis Aquat Organ* 98:11–25.
- Woodhams DC, LaBumbard BC, Barnhart KL, Becker MH, Bletz MC, Escobar LA, Flechas S V, Forman ME, Iannetta AA, Joyce MD, Rabemananjara F, Gratwicke B, Vences M, Minbiole KP (2018) Prodigiosin, violacein, and volatile organic compounds produced by widespread cutaneous bacteria of amphibians can inhibit two *Batrachochytrium* fungal pathogens. *Microb Ecol* 75:1049–1062.
- Woodward A, Berger L, Skerratt LF (2014) In vitro sensitivity of the amphibian pathogen *Batrachochytrium dendrobatidis* to antifungal therapeutics. *Res Vet Sci* 97:365–367.

Table 1 Treatment combinations

	medium	Bd exposure	CFCM treatment
Treat 1	no	no	no
Treat 2	yes	no	no
Treat 3	yes	no	yes
Treat 4	yes	yes	no
Treat 5	yes	yes	yes

679 Table 2 Pairwise comparisons for the effects of treatments on developmental stage

Comparison	Hazard ratio $\pm$ SE	P
Treat 1 / Treat 2	- 5.285 $\pm$ 0.68	<0.001
Treat 1 / Treat 3	- 4.481 $\pm$ 0.67	<0.001
Treat 1 / Treat 4	- 4.244 $\pm$ 0.67	<0.001
Treat 1 / Treat 5	- 4.148 $\pm$ 0.67	<0.001
Treat 2 / Treat 3	0.804 $\pm$ 0.66	0.325
Treat 2 / Treat 4	1.041 $\pm$ 0.66	0.194
Treat 2 / Treat 5	1.137 $\pm$ 0.67	0.181
Treat 3 / Treat 4	0.237 $\pm$ 0.65	0.797
Treat 3 / Treat 5	0.333 $\pm$ 0.65	0.763
Treat 4 / Treat 5	0.096 $\pm$ 0.65	0.884

680

681

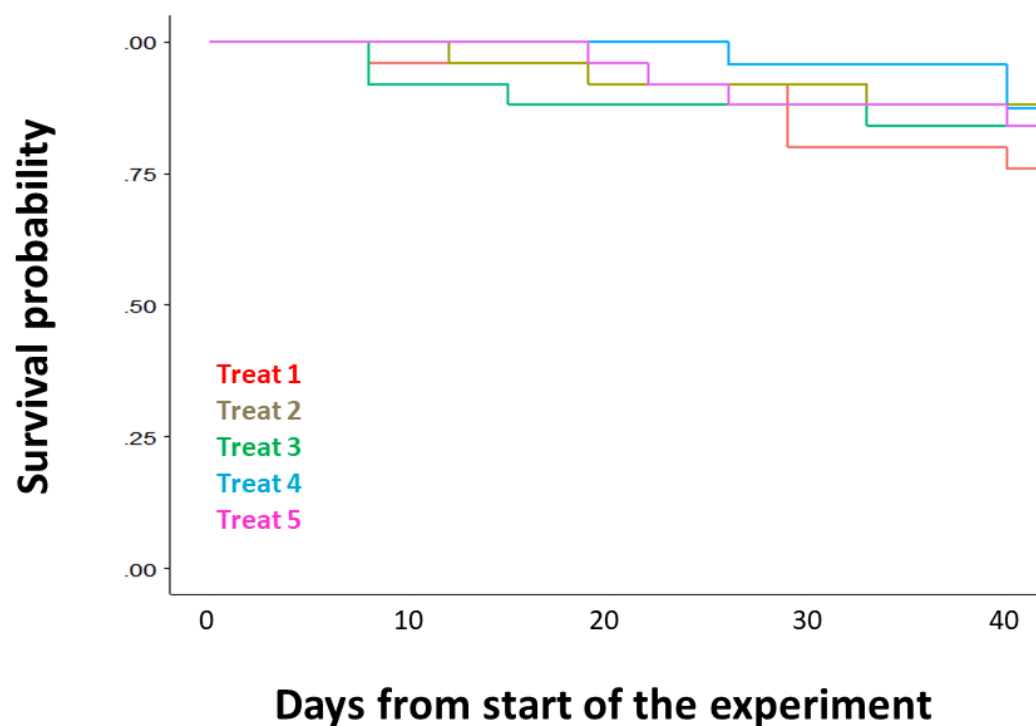
682 Table 3 Pairwise comparisons for the effects of treatments on body mass

Comparison	Hazard ratio $\pm$ SE	P
Treat 1 / Treat 2	- 222.35 $\pm$ 49.8	<0.001
Treat 1 / Treat 3	- 224.35 $\pm$ 46.8	<0.001
Treat 1 / Treat 4	- 169.24 $\pm$ 46.3	0.001
Treat 1 / Treat 5	- 167.00 $\pm$ 46.3	0.001
Treat 2 / Treat 3	- 2.25 $\pm$ 38.5	0.954
Treat 2 / Treat 4	53.11 $\pm$ 38.6	0.216
Treat 2 / Treat 5	55.35 $\pm$ 38.6	0.216
Treat 3 / Treat 4	55.36 $\pm$ 37.8	0.216
Treat 3 / Treat 5	57.60 $\pm$ 37.8	0.216
Treat 4 / Treat 5	2.24 $\pm$ 37.8	0.954

683

684

685 Ujszegi et al. Fig 1



686

687 Figure 1: Survival of *Bombina variegata* tadpoles over the experiment in the five treatments.

688 For explanation of treatments please see Table 1.

689

Ujszegi et al. Fig 2

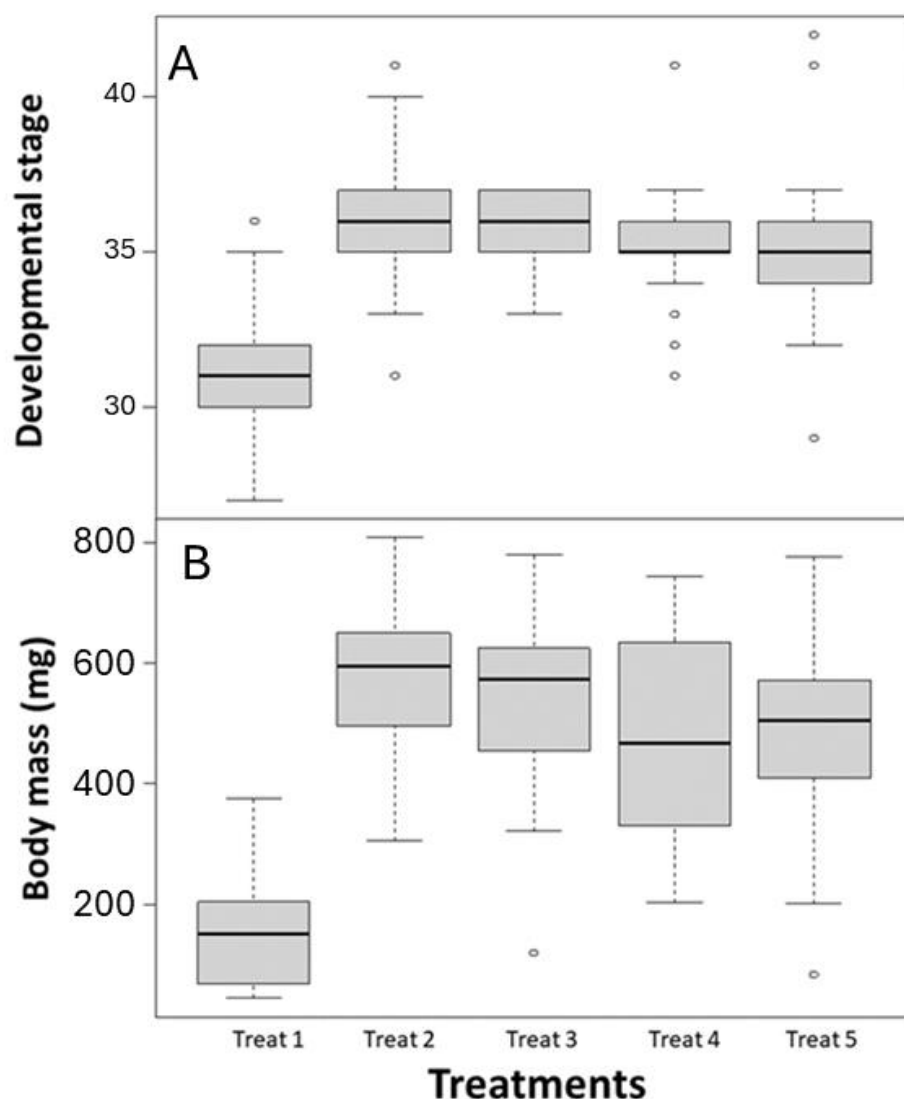


Figure 2: Responses of *Bombina variegata* tadpoles to the five treatments in terms of the (A) developmental stage (Gosner) and (B) body mass at the termination of the experiment. In boxplots, horizontal lines and boxes represent medians and interquartile ranges (IQR), respectively, while whiskers extend to $IQR \pm 1.5 \times IQR$ and dots indicate more extreme data points. For explanation of treatments please see Table 1.

Ujszegi et al. Fig 3

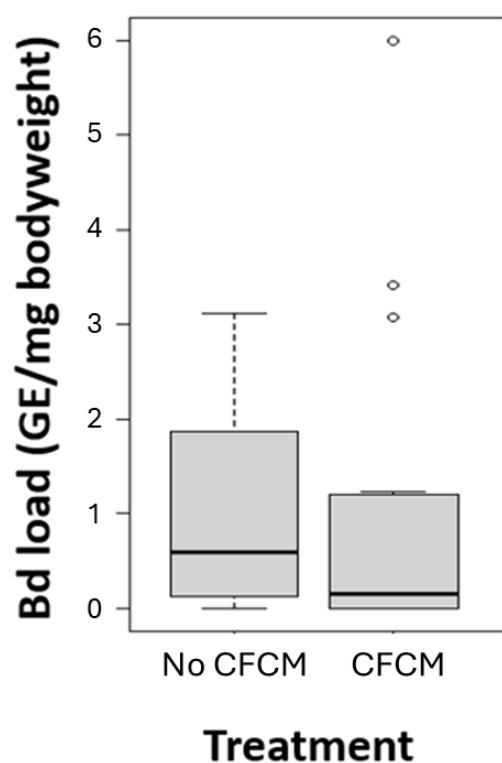


Figure 3: Tadpoles' infection intensities from the two Bd exposed groups (Treat 4 and Treat5) in the absence and the presence of 0.1 % (v/v) cell-free culture medium (CFCM) treatment. Horizontal lines and boxes represent medians and interquartile ranges (IQR), respectively, while whiskers extend to $IQR \pm 1.5 \times IQR$ and dots indicate more extreme data points.