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ORIGINAL RESEARCH

Metamorphic common toads keep chytrid infection under control, but at a cost

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Abstract

Batrachochytrium dendrobatidis (*Bd*) causes chytridiomycosis, an infectious disease of amphibians, which has contributed to population declines in hundreds of species worldwide. Common toads (*Bufo bufo*) exhibit low resistance and relatively high tolerance to *Bd* infection, which may partly be attributable to bufadienolide toxins secreted in their granular skin glands. Bufadienolides are known to provide an effective defense against several pathogens, parasites, and predators. The toxin production of bufonids is a plastic trait, inducible by several environmental factors. Here, we experimentally infected juvenile common toads with *Bd* and investigated if the toadlets could clear the infection over time, whether the infection induced bufadienolide production, and whether the infection caused decreased body mass. We found that prevalence remained 100% throughout the entire experimental period, but infection intensity did not increase and it was significantly lower on day 30 than on day 20. At the same time, compared to controls, infected toadlets produced lesser amounts of bufadienolides and their body mass was also lower. These results suggest that although young toadlets may not be able to clear *Bd* infection on their own, they may be able to keep infection intensities under control. Nonetheless, even if toadlets do not succumb to the disease, the costs of chronic infection may still compromise their fitness.

Introduction

Infectious diseases pose a serious threat to biodiversity. They can have devastating effects on wildlife, livestock, and if they become zoonotic, on humans too (Daszak et al., 2000). Chytridiomycosis is an infectious disease of amphibians caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al., 1999) and *B. salamandrivorans* (*Bsal*) (Martel et al., 2013). It has contributed to severe population declines in hundreds of amphibian species and caused the extinction of at least 70 species over the last few decades (Lips, 2016; O'Hanlon et al., 2018; Scheele et al., 2019; Skerratt et al., 2007). In the last 50 years, *Bd* has spread worldwide and infected a host spectrum of most species in all three orders of amphibians (Scheele et al., 2019), while the more recently discovered and therefore less-studied *Bsal* is as yet confined to relatively small areas in the mainland of northwestern Europe and East Asia and mainly infects caudates (i.e., newts and salamanders; Martel et al., 2014; Spitzen-van der Sluijs et al., 2016; Thomas et al., 2019).

Batrachochytrium dendrobatidis infects keratinized epidermal cells of amphibians (Berger, Hyatt, et al., 2005), including the mouthparts of tadpoles and the entire skin surface of post-metamorphic individuals (Marantelli et al., 2004). The development of the infection in the skin comes with histopathological changes, including hyperkeratosis, hyperplasia, ulceration, and erosion (Berger, Hyatt, et al., 2005), which can lead to the disruption of normal skin function, systemic metabolic dysregulation, electrolyte imbalance, and, finally, cardiac arrest (Grogan, Robert, et al., 2018; Ohmer et al., 2017; Voyles et al., 2009). In many cases, infected individuals show no signs of disease (Daszak et al., 2004; Rothermel et al., 2008; Solís et al.,

2010), but can still suffer long term sublethal effects regarding jumping performance, testes morphology, body mass, and host water balance, that may lead to a reduction in their fitness (Bovo et al., 2016; Chatfield et al., 2013; Cheatsazan et al., 2013).

Amphibians exhibit various defense mechanisms against parasites and pathogens. Their immune system is highly developed and can launch both innate and adaptive immune responses (Grogan et al., 2020; Grogan, Skerratt, et al., 2018). The first line of defense is the epidermis, as it forms a physical barrier against pathogens and parasites (Grogan, Skerratt, et al., 2018; Varga et al., 2019). Mutualistic bacterial communities inhabiting the mucous skin surface provide additional protection, as some of their metabolites are active against different types of pathogens, including *Bd* (Becker et al., 2015; Brucker et al., 2008; Harris et al., 2009; Holden et al., 2015; Kueneman et al., 2016; Woodhams et al., 2014). Many amphibians also secrete defensive chemicals such as antimicrobial peptides, proteins, steroids, alkaloids, or biogenic amines onto their skin surface, which can provide highly effective defense against various pathogens and parasites (Daly, 1995; Gomes et al., 2007; König et al., 2015; Macfoy et al., 2005; Tempone et al., 2006).

Bufonid toads produce steroid toxins called bufadienolides in granular glands of their skin from early larval development (Clarke, 1997; Hayes et al., 2009; Toledo & Jared, 1995; Ujszegi et al., 2017; Üveges et al., 2017). Bufadienolides do not only deter various predators (Hayes et al., 2009; Toledo & Jared, 1995) but may also show antimicrobial, antiprotozoal, and antifungal activity, providing some level of protection against pathogens and parasites, including *Bd* (Barnhart et al., 2017; Cunha Filho et al., 2005; Rodriguez et al., 2020, 2021; Tempone et al., 2008). Bufadienolide synthesis is a flexible trait, which is influenced by anthropogenic habitat change and pollution (Bókony et al., 2017, 2019), the presence of predators or competitors (Benard & Fordyce, 2003; Bókony et al., 2016, 2018; Hagman et al., 2009; Üveges et al., 2017, 2021), food availability (Üveges et al., 2017), and the age of individuals (Ujszegi et al., 2017, 2021; Üveges et al., 2017). However, little is known about the effects of pathogens on inducible bufadienolide synthesis. Ujszegi et al. (2020) demonstrated that toxin production of common toad (*Bufo bufo*) tadpoles is strongly related to the bacterial community structure of their aquatic habitat, which suggests that bufadienolide synthesis might be adjusted to the presence or absence of particular pathogens. There is also direct evidence that *Bd* infection can influence the amount of bufadienolides in the same species, and additionally, that the effect of infection can carry-over to post-metamorphic individuals: toadlets infected with *Bd* during their larval stage produce smaller quantities of defensive chemicals than their *Bd*-naïve conspecifics (Ujszegi et al., 2021). Nevertheless, it remains practically unknown whether common toad metamorphs infected with a *Bd* isolate from their distribution area are capable to clear the infection over time, whether the clearance of the pathogen results in other costs for the host, and if this isolate induces toxin production.

In this study, we investigated the dynamics of prevalence and infection intensity in juvenile common toads following experimental infection with *Bd*. We also explored the effects of infection on changes in toxin production and body mass of toadlets. Because *Bd* infection can lead to the disruption of normal skin function, we predicted, on the one hand, that it may negatively affect toxin production. On the other hand, if bufadienolides are effective against *Bd*, infected individuals may invest more into toxin synthesis, which can lead to the suppression of the pathogen and consequently to self-healing.

Materials and methods

Collection and rearing of toadlets

In April 2020, we collected ca. 100 eggs from each of five freshly laid egg-strings of *B. bufo* from a pond in Nagykovácsi, Hungary (Békás tó, 47.576292N, 18.868441E). We transported eggs to the experimental station of the Plant Protection Institute, Centre for Agricultural Research, Eötvös Loránd Research Network in Budapest (47.547778 N, 18.934722 E). We kept eggs separated by family in plastic boxes (24 × 16 × 13 cm) holding 1 L of reconstituted soft water (RSW; 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4 \times 2\ H_2O$, 61 mg/L $MgSO_4 \times 7\ H_2O$, 2 mg/L KCl added to reverse-osmosis filtered, UV-sterilized tap water; APHA-AWWA-WEF, 1992) at a constant temperature of 19°C and a 12:12 h light:-dark cycle. On 24 April, which was between 4 and 8 days after tadpoles reached the free-swimming stage (development stage 25 according to Gosner, 1960), we placed 55 tadpoles from each family into outdoor mesocosms (150 L plastic tubs, 57 × 39 × 28 cm), so that each tub only contained tadpoles from one family. Mesocosms provided semi-natural conditions for tadpoles: They were filled with 130 L aged tap water and contained 40 g dried beech (*Fagus sylvatica*) leaves and 1 L pond water, providing nutrients and refuge (e.g., Van Buskirk, 2012; Mikó et al., 2015; Üveges et al., 2021). When the first individuals reached developmental stage 42 (emergence of forelimbs, start of metamorphosis), we monitored mesocosms daily, captured metamorphs, and placed them into semitransparent, 45-L plastic boxes (one box for each mesocosm) equipped with a perforated lid. Boxes contained approximately 0.5 L of aged tap water and were slightly tilted to provide both water as well as dry areas. When individuals completed metamorphosis (developmental stage 46), we placed them individually into 2 L opaque plastic containers equipped with a perforated, translucent lid, lined with wet paper towels as a substrate, and containing a piece of egg carton as a shelter. We reared toadlets at 20°C (mean ± SD: 19.93 ± 1.48°C) and a 14:10 h light: dark cycle. Every other day we re-wetted paper towels and fed toadlets with crickets (*Acheta domestica*, instar stage 1–2) *ad libitum*.

Maintenance of *Bd* culture and experimental exposure

We used the H-2014 *Bd*GPL isolate obtained from a living specimen of *Bombina variegata* co-occurring with *B. bufo* in the Pannonian Basin (Bakony Mountains), Hungary in 2014 by J. Vörös (Department of Zoology, Hungarian National History

Museum, Budapest, Hungary), courtesy of M.C. Fisher (Fungal Disease Epidemiology, Imperial College London, UK). The culture was maintained at 4°C in cell culture flasks (25 cm^2, closed cap; Orange Scientific, Belgium) using mTGhL liquid medium (8 g/L tryptone, 2 g/L gelatin hydrolysate, 4 g/L lactose in distilled water) and passaged every 3 months.

Seven days before experimental infection of toadlets, we inoculated 2 mL *Bd* culture into a 25-cm^2 cell culture flask containing 10 mL mTGhL and incubated it at 20°C. After the 7-day incubation period, we estimated the zoospore concentration in the culture by using a standard hemocytometer. We inoculated 1 mL of the unfiltered culture into 60 plastic Petri dishes containing 9 mL RSW, resulting in a concentration of minimum 50 000 zoospores/mL in each dish. In parallel, we inoculated 1 mL of sterile mTGhL into another 60 dishes containing 9 mL RSW for the control group.

Ten days after completion of metamorphosis, we measured the body mass of toadlets using a laboratory scale to the nearest 0.1 mg and bathed 60 toadlets individually in *Bd*-inoculated dishes and another 60 toadlets in control dishes for 5 hours. This time interval has been previously proven to be sufficient for experimental infection of common toad metamorphs with *Bd* (Garner et al., 2009). After exposure, we placed toadlets back into their containers. Five days later, we swabbed all individuals (Brem et al., 2007) to assess experimental infection success and to test if cross-contamination of control individuals occurred. We swabbed the rear feet, the ventral surface of thighs, and the ventral abdominal surface of each individual by sweeping five times along the entire length of each of the five areas. Ten, 20, and 30 days after each infection challenge, we randomly selected and measured 20 control and 20 *Bd*-infected individuals, and ultimately, euthanized them using the "cooling then freezing" method (Shine et al., 2015). Finally, we preserved individuals in 96% EtOH and kept samples at 4°C until further processing.

Quantifying infection intensity

For the assessment of infection prevalence and intensity, we homogenized toe clips of preserved toadlets, extracted DNA using the PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the instructions of Boyle et al. (2004), and stored extracts at −20°C until further analyses. We used toe clips because toes of metamorphosed individuals are likely to carry the fungus in high loads if infected and can therefore be used for the assessment of infection (Berger, Speare, et al., 2005; Garner et al., 2009; Hyatt et al., 2007). To enhance the precision of estimates, we used the tips of all toes of both hind feet of toadlets. Following a standard amplification methodology targeting the ITS-1/5.8S rDNA region (Boyle et al., 2004), we ran real-time quantitative polymerase chain reactions (qPCR) on a BioRad CFX96 Touch Real-Time PCR System. To avoid PCR inhibition by ingredients of PrepMan, we diluted samples 10-fold with PCR water. We ran samples in duplicate, and in case of contradictory results, we repeated reactions in another duplicate. If it again returned an equivocal result, we considered that sample to be *Bd* positive. Genomic equivalent (GE) values of *Bd*, estimating infection intensities, were obtained from standard curves based on five dilutions of a standard (DNA extracted from zoospores; 1000, 100, 10, 1, and 0.1 zoospore genomic equivalents; courtesy of J. Bosch; Museo Nacional de Ciencias Naturales, Madrid, Spain).

Analysis of bufadienolides

For the analysis of bufadienolides, we homogenized whole bodies of the toadlets with a homogenizer (IKA ULTRA-TURRAX T25) equipped with a dispersing tool (IKA S12N-7S). Afterward, we dried samples *in vacuo* at 45°C using a rotary evaporator (Büchi Rotavapor R-134, Flawil, Switzerland), weighed their dry mass, and re-dissolved samples in 1 mL absolute HPLC-grade methanol, aided by brief exposure to ultrasound in a bath sonicator (Tesla UC005AJ1). Finally, we filtered samples through FilterBio nylon syringe filters (pore size = 0.22 μm) and stored them at −20°C until further analyses.

We analyzed bufadienolides using high-performance liquid chromatography coupled with a diode-array detector and electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS). We identified the chromatographic peaks as bufadienolides based on their characteristic UV spectra (Hayes et al., 2009) and by comparing their retention times and mass spectra to those of the following commercially acquired standards: arenobufagin, bufalin, bufogenin, bufotalin, cinobufagin, cinobufotalin, digitoxigenin, gamabufotalin, marinobufotoxin (courtesy of Dr. R. Capon, University of Queensland, Brisbane, Australia), and telocinobufagin. We performed HPLC–MS measurements on a Shimadzu LC–MS 2020 instrument (Shimadzu, Kyoto, Japan) that consisted of a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a diode array detector, and a single-quadrupole mass analyzer with electrospray ionization. The separation and detection method was similar to that published in Ujszegi et al. (2021), but narrower column (100 mm × 2.1 mm Kinetex C18 2.6 μm), 0.3 mL/min flow rate, 5 μL injections, and the following gradient program was used: 0–1 min: 15–20% B; 1–14 min: 20–35% B; 14–16 min: 35–60% B; 16.1–20 min: 100% B; 20.1–25 min: 15% B, where eluent A was 5% aqueous acetonitrile with 0.05% formic acid and eluent B was acetonitrile with 0.05% formic acid. Data were acquired and processed using the LabSolutions 5.42v software (Shimadzu).

We calculated the number of bufadienolide compounds (NBC) for each individual by assuming a compound to be present if the signal-to-noise ratio (S/N) of its peak was at least three. We estimated the quantity of each compound from the area values of chromatogram peaks based on the calibration curve of the bufotalin standard and summed these values to obtain an estimate of total bufadienolide quantity (TBQ) for each individual.

Statistical analysis

No toadlets died during the experiment, resulting in a total sample size of 120 [two treatments (controls vs.

infected) × three sampling dates × 20 replicates]. We averaged *Bd* GE values obtained from qPCR runs within samples and subsequently rank-transformed the obtained values across samples, because GE values that fall outside the standard interpolation curve are not reliably estimated. We found a strong correlation between the body mass and the TBQ of individuals (Pearson's $R = 0.88$, $t = 19.91$, $df = 118$, $P < 0.001$); thus, we calculated body mass-corrected TBQ values (mcTBQ) by dividing TBQ value with the body mass measured at the end of the experiment for each individual. We calculated the relative change in body mass by subtracting the body mass at infection from the body mass measured at the end of the experiment and then dividing this difference with the body mass at infection for each individual.

All statistical analyses were conducted in "R" (version 4.0.3; R Core Team, 2020). We analyzed differences in infection intensity among infected individuals using linear mixed-effects models (LMM) ("lme" function of the "nlme" package; Ezzet & Pinheiro, 2007) on the rank-transformed GE values containing time until sampling as a fixed factor and family as a random factor. To assess intergroup differences, we used pairwise comparisons with linear contrasts for our dependent variable calculated from our LMM model using the "emmeans" function of the "emmeans" package (Lenth et al., 2021). We also analyzed the effects of *Bd* infection on body mass, NBC, TBQ, and mcTBQ using linear mixed effects models implemented with the "lme" function in "nlme", with body mass, NBC, TBQ, or mcTBQ entered in the models as dependent variables, infection (control or infected), time until sampling (10, 20, or 30 days) and their interaction entered as fixed effects, and family as a random factor. We allowed for intergroup differences in variance between the two infection groups within each sampling occasion by including the "weights" argument with the "varIdent" function, because graphical model diagnostics indicated heterogeneous variances. Subsequently, using the model estimates, we tested the overall effects of infection and time until sampling by calculating linear contrasts while applying the false discovery rate (FDR) correction to adjust *P* values for multiple comparisons (Pike, 2011). We analyzed the effects of infection intensity on TBQ and mcTBQ using linear mixed effects models implemented with the "lme" function in "nlme", with TBQ or mcTBQ entered in the models as dependent variables, rank-transformed GE values, time until sampling (10, 20, or 30 days) and their interaction as fixed effects, and family as a random factor.

Results

Results of the qPCRs of the swabs taken on day 5 and of the tissue samples taken on days 10, 20, and 30 showed that there was no cross-contamination. On day 5, all except four toadlets tested positive for *Bd* in the infected group, whereas at any later sampling *Bd* prevalence was 100%, although none of the individuals showed typical signs of chytridiomycosis (e.g., intense skin shedding, loss of appetite, inactivity, and abnormal posture; Berger et al., 1998, 2005). Pairwise comparisons of ranked *Bd* GE values showed that infection intensity did not differ after 10 and 20, or 10 and 30 days, but it was significantly lower on day 30 than on day 20 (Fig. 1, Table 1). All control individuals tested negative for *Bd*.

We did not detect a difference in body mass between control and infected individuals 10 days after infection, but on day 20 and 30, the body mass of *Bd*-infected individuals was significantly lower than that of controls (Fig. 2A, Table 2). Relative change in body mass was positive in all groups and at all sampling occasions, with only two individuals losing weight, while this change was significantly lower in infected than in control individuals on day 10 (Fig. 2B, Table 2).

We detected 47 different bufadienolide compounds in *B. bufo* metamorph extracts (mean ± SE: 38 ± 0.42; Table S1). We found that the number of bufadienolide compounds did not differ significantly between infected versus control individuals at any sampling occasion (Fig. 3A, Table 2). Total bufadienolide quantity also did not differ significantly between control and infected individuals on day 10, but it was lower in

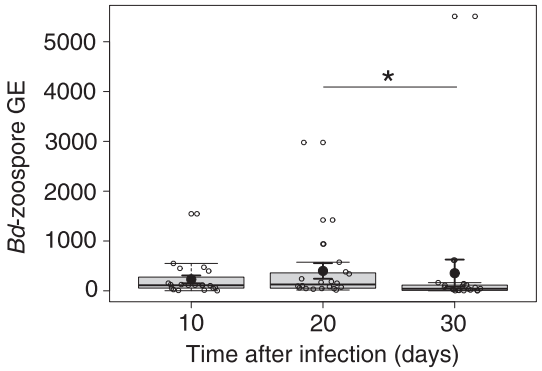

Figure 1 Intensity of *Batrachochytrium dendrobatidis* infection in common toads as zoospore genomic equivalents (GE). In each boxplot, the thick horizontal line and the box represent the median and the interquartile range, respectively; whiskers extend to the upper and lower quartile ± 1.5 × interquartile range, and open circles represent extreme data points. Error bars represent means ±SE. A significant difference between groups is indicated by an asterisk (* = $P < 0.05$).

Table 1 *Batrachochytrium dendrobatidis* infection intensities as the mean ± SE of genomic equivalent (GE) values and of rank transformed GE (rGE) values

Sampling occasion (days after start)	Mean GE ± SE	Mean rGE ± SE
10	229 ± 187	32.8 ± 3.75
20	399 ± 187	36.1 ± 3.75
30	354 ± 187	22.6 ± 3.75

Contrast	Estimate ± SE	*t*	*P*
10–20	−3.33 ± 5.30	−0.63	0.81
10–30	10.15 ± 5.30	1.91	0.15
20–30	13.47 ± 5.30	2.54	**0.04**

Comparisons between groups were calculated using linear contrasts based on the rank transformed GE values. A significant difference between groups is highlighted in bold. $N = 20$ in all experimental groups.

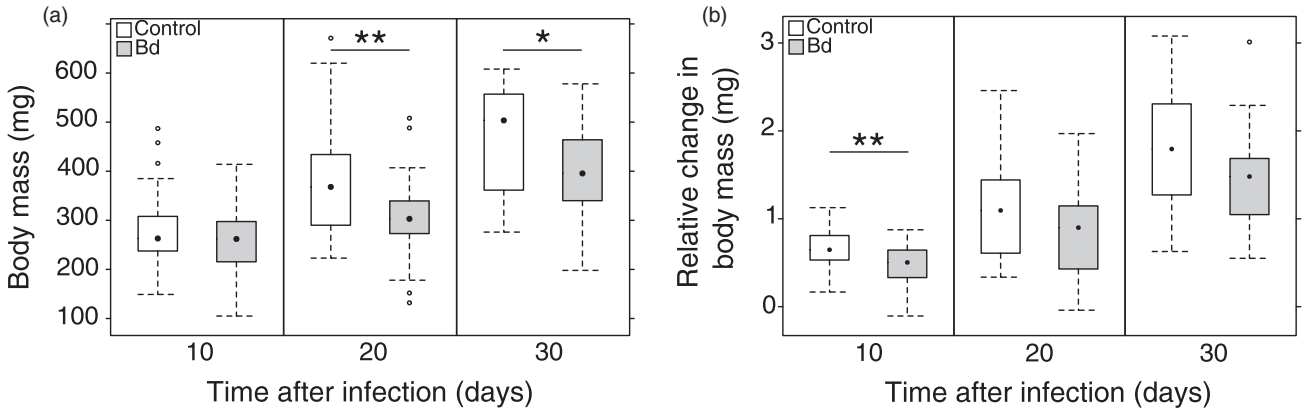

Figure 2 Body mass (a) and relative change in body mass (b) of toads infected with *Batrachochytrium dendrobatidis* during the experiment. Dots represent medians, boxes represent interquartiles, whiskers extend to the upper and lower quartile ± 1.5 × interquartile range, and open circles represent extreme data points. Significant differences between groups are indicated by asterisks (* = $P < 0.05$, ** = $P < 0.01$).

Table 2 Responses of common toads to *Batrachochytrium dendrobatidis* infection

Response	Sampling occasion (days after start)	Contrast	Mean ± SE	Estimate ± SE	*t*	*P*
Body mass (mg)	10	Control	286 ± 25.51	30 ± 17.25	1.71	0.09
		Bd	256 ± 22.18			
	20	Control	385 ± 28.33	77 ± 25.89	2.99	**0.004**
		Bd	308 ± 26.71			
	30	Control	460 ± 28.24	63 ± 25.62	2.47	**0.02**
		Bd	397 ± 26.53			
Relative change in body mass (mg)	10	Control	0.65 ± 0.07	0.18 ± 0.06	3.02	**0.003**
		Bd	0.47 ± 0.07			
	20	Control	1.10 ± 0.12	0.20 ± 0.17	1.20	0.24
		Bd	0.90 ± 0.14			
	30	Control	1.81 ± 0.15	0.32 ± 0.19	1.71	0.09
		Bd	1.49 ± 0.14			
NBC	10	Control	39.0 ± 1.27	1.10 ± 2.038	0.54	0.59
		Bd	38.0 ± 1.61			
	20	Control	37.4 ± 0.67	0.15 ± 1.172	0.13	0.90
		Bd	37.2 ± 0.98			
	30	Control	38.4 ± 0.61	0.20 ± 0.836	0.23	0.82
		Bd	38.2 ± 0.65			
TBQ (ng)	10	Control	31578 ± 4790	4033 ± 3986	1.01	0.31
		Bd	27545 ± 3662			
	20	Control	98924 ± 12282	34070 ± 13258	2.57	**0.01**
		Bd	64854 ± 6737			
	30	Control	132471 ± 12201	41094 ± 15022	2.74	**0.007**
		Bd	91377 ± 9863			
mcTBQ (ng/mg)	10	Control	105 ± 7.96	−1.3 ± 7.94	−0.16	0.87
		Bd	106 ± 5.54			
	20	Control	241 ± 18.03	39.5 ± 22.03	1.79	0.08
		Bd	201 ± 13.84			
	30	Control	278 ± 16.66	57.8 ± 22.17	2.61	**0.01**
		Bd	220 ± 15.66			

Pre-planned comparisons of body mass, relative change in body mass, number of bufadienolide compounds (NBC), total bufadienolide quantity (TBQ), and mass-corrected total bufadienolide quantity (mcTBQ) of toadlets at each sampling occasion between control and infected individuals. Significant differences between groups are highlighted in bold. $N = 20$ in all experimental groups.

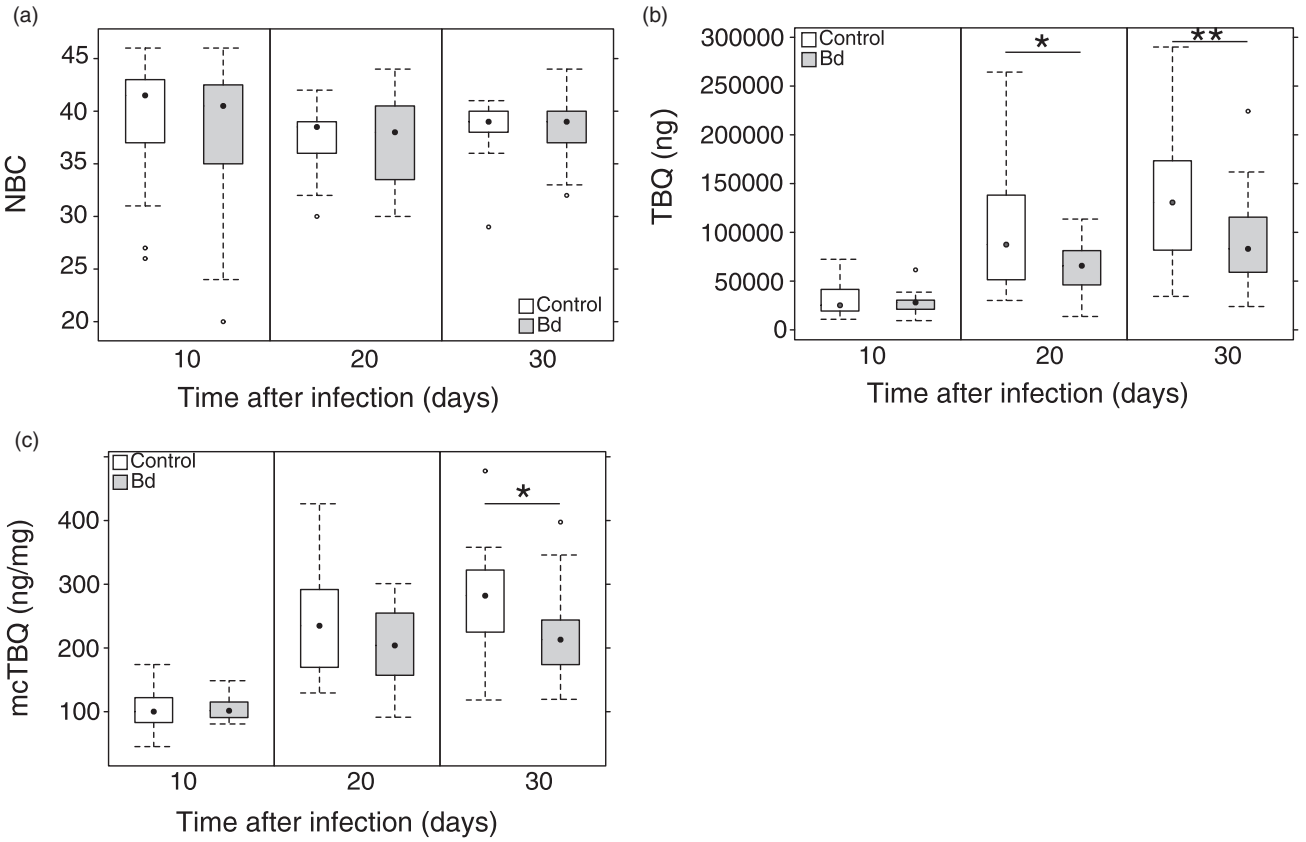

Figure 3 Number of bufadienolide compounds (a), total bufadienolide quantity (b), and mass-corrected total bufadienolide quantity (c) of toads infected with *Batrachochytrium dendrobatidis* during the experiment. Dots represent medians, boxes represent interquartiles, whiskers extend to the upper and lower quartile ± 1.5 × interquartile range, and open circles represent extreme data points. Significant differences between groups are indicated by asterisks (* = $P < 0.05$, ** = $P < 0.01$).

infected individuals than in controls on days 20 and 30 (Fig. 3B, Table 2). In the case of mass-corrected total bufadienolide quantity, this difference was significant on day 30, but not earlier (Fig. 3C, Table 2). Neither TBQ nor mcTBQ was affected by infection intensity represented as rank-transformed GE values at any sampling occasion (Table S2).

Discussion

Our study showed that common toads are tolerant but not resistant (Woodhams et al., 2011) to infection with a *Bd* isolate co-occurring with them in the Pannonian Basin. Toadlets exposed to *Bd* 10 days after metamorphosis and kept at 20°C for extended periods (i.e., 10, 20, or 30 days) all carried the fungus. However, *Bd* infection intensities did not show an exponential increase, as expected in case of uncontrolled pathogen growth, but stagnated between days 10 and 20 at relatively low levels that are harmless for amphibians (Briggs et al., 2010; Vredenburg et al., 2010), and even decreased between days 20 and 30. At the same time, infection with *Bd* had a negative effect on body mass and on total bufadienolide quantity. Thus, while common toad metamorphs appear to be able to keep *Bd*-infection under control, costs arising from infection manifest in important life-history traits.

Common toad metamorphs could not clear *Bd* infection, as prevalence was 100% in all infected groups, even 30 days after exposure to the pathogen. Our findings correspond to Bielby et al. (2015) who found 80% *Bd* prevalence 24 days after infection challenge, and to Garner et al. (2009) in which 100% prevalence was documented 40 days after exposure, both in *Bufo* metamorphs. Altogether, these results confirmed the notion that common toads are not resistant to chytridiomycosis. Interestingly, the previous experiments observed high levels of mortality ranging between 50 and 100% in *Bd*-infected toadlets. In our study, all individuals survived and none of them displayed the typical signs of chytridiomycosis (Berger et al., 1998; Berger, Speare, et al., 2005). The discrepancy in observed mortalities may be attributable to differences in the virulence of the *Bd* isolates used, but common toad populations may also vary in their susceptibility to infection. Our results suggest that common toad metamorphs from the tested populations are adapted to the *Bd* lineage that is present in their area. Even though the *Bd* prevalence was exceptionally high, the toadlets could keep infection intensities at a low

level, and therefore, they were capable to avoid more serious disease outcomes.

We found that *Bd*-infected toadlets grew more slowly during the experiment than their control groupmates and, as a result, weighed less 20 and 30 days after exposure to the pathogen. This result is in contrast with previous studies, which did not find an effect of *Bd*-exposure on the growth of *B. bufo* metamorphs (Bielby et al., 2015; Ujszegi et al., 2021). Bielby et al. (2015) infected individuals after metamorphosis and reared them for 24 days, but they used lower infection burdens: Even in their high dose treatment, toadlets were exposed only to a concentration of 530 zoospores/mL, while in our study, we used an approximately 50 000 zoospores/mL concentration of *Bd* in the infected group, during the same length of exposure. Ujszegi et al. (2021) may not have detected an effect of previous *Bd*-exposure because of different timing: They infected tadpoles and observed growth later on, 14 days after metamorphosis, whereas in the present study, we infected individuals 10 days after metamorphosis and measured body mass 10, 20, and 30 days later. Also, Ujszegi et al. (2021) applied a lower sample size ($N = 12$ in all treatment groups vs. $N = 20$ here), resulting in lower statistical power. Furthermore, both Bielby et al. (2015) and Ujszegi et al. (2021) used the *Bd* isolate IA-042, which was isolated from a dead *Alytes obstetricans* found in Spain during a mass mortality event in 2004, whereas we challenged the toadlets with the isolate H-2014 which was isolated from a live specimen of *Bombina variegata* in Hungary in 2014. So that the geographic origin, the host species, and their history since isolation (duration of culturing, number of passages, etc.) all differ between the two isolates. These discrepancies may have contributed to the differences between the effects of infection in these previous studies. Nonetheless, our results align to those of Garner et al. (2009) documenting decreased body mass at metamorphosis in experimentally infected *B. bufo* tadpoles compared to control individuals. Lower body mass at metamorphosis has been linked to decreased survival probability and reproductive success (Altwegg & Reyer, 2003; Cabrera-Guzmán et al., 2013). Consequently, a period of restrained growth of toads immediately after metamorphosis is also very likely to negatively affect the fitness of individuals. However, it is important to note that there is a two-way interaction between body mass and infection probability: Decreased fitness of individuals presupposed by lower body mass contributes to higher probability of subsequent infection and mortality (Carey et al., 2006; Garner et al., 2011).

Infection with *Bd* did not result in altered toxin compound numbers, as NBC did not differ between infected and control individuals at either sampling occasion. This is in line with the results of Ujszegi et al. (2021), the only similar study testing the effect of experimental infection on toxin production in an amphibian species. More generally, this result corresponds to the findings of previous studies that the number of bufadienolide compounds appears to be a genetically fixed or at least less plastic trait and that it is less affected by environmental factors (Bókony et al., 2017, 2018; Hettyey et al., 2019). Nonetheless, TBQ and mcTBQ were both lower in infected individuals than in controls by 30 days post-infection. Ujszegi et al. (2021) did not detect a similar pattern 14 days after infection, which is in agreement with our results. Based on the previous study and our findings, approximately 15–20 days are necessary after exposure for the negative effect to manifest. Total bufadienolide quantity produced by individuals is known to be a plastic trait that can be induced by several factors, including conspecifics, predator presence, or exposure to environmental contaminants (Bókony et al., 2017, 2018; Hettyey et al., 2019). However, while other stress factors caused upregulated toxin synthesis, infection with *Bd* resulted in lowered bufadienolide quantities. Although we do not explicitly know the ultimate cause and physiological mechanisms behind the observed decrease in TBQ, this might be the consequence or cost of *Bd* infection due to the disruption of normal skin function or trade-offs with other fitness traits which are important for battling the fungus. Nonetheless, a weakened chemical defense is likely to lower survival probabilities and thereby the fitness of affected individuals, by rendering them more vulnerable to pathogens, parasites, competition, and predators (Barnhart et al., 2017; Bókony et al., 2018; Cunha Filho et al., 2005; Hayes et al., 2009; Toledo & Jared, 1995; Üveges et al., 2021).

In conclusion, these results suggest that metamorphic common toads of our study population can keep infection intensities within tolerable limits when facing a co-occurring isolate of *Bd*. Nonetheless, negative fitness effects of *Bd*-infection can manifest through reduced body mass and lowered toxin synthesis. These impacts of the pathogen may be less conspicuous than the mass mortalities sometimes observed in less tolerant amphibian populations and species, but may persist and accumulate through time and thereby have strong long-lasting negative effects on these populations.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Percentages of common toad metamorphs containing each bufadienolide compound detected in our study. Seven compounds were unambiguously identified based on standards. We detected another 40, unidentified bufadienolides (unkx) based on their characteristic UV spectra. Analytical properties of the detected compounds are provided. M/z = mass/charge.

Table S2. The effect of infection intensity on total bufadienolide quantity (TBQ) and mass-corrected total bufadienolide quantity (mcTBQ) at different sampling occasions. Infection intensity was represented by rank transformed GE values. Results are based on linear mixed effects models. $N = 20$ in all experimental groups.