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Chytridiomycosis and climate change: exposure to *Batrachochytrium dendrobatidis* and mild winter conditions do not increase mortality in juvenile agile frogs during hibernation

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Abstract

Hibernation is often associated with high mortality, especially during early life stages, and losses can be exacerbated by unusual winter conditions or if animals enter hibernation carrying a disease. Here, we examined how overwintering amphibians may be affected by the combined effects of mild winters, which are projected to increase in frequency due to climate change, and of chytridiomycosis, a disease that has contributed to the decline of hundreds of species worldwide. We exposed juvenile agile frogs *Rana dalmatina* to *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of chytridiomycosis, 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) under laboratory conditions. Agile frogs proved to be highly resistant to *Bd* as 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. We observed lack of mortality in control and *Bd*-exposed groups also, in both types of winter. The two types of winter we simulated did not result in differing body mass loss either alone or in combination with experimental infection. In the *Bd*-exposed group, the two types of winter also did not cause differences in prevalence and infection intensity. However, among *Bd*-exposed frogs, individuals that were *Bd* negative when entering hibernation lost more body mass than their conspecifics that carried the fungus at the onset of overwintering. Based on our results, warming winter climate conditions, with or without *Bd* infection, do not decrease body mass and survival rate of hibernating agile frogs, and do not increase susceptibility of individuals to chytridiomycosis. It remains to be seen to what extent the relatively weak effects of milder winters can be generalized to other amphibians of the temperate climate zone.

Introduction

In cool climate zones, hibernation evolved as an adaptation to a seasonal decrease in ambient temperatures and food resources (Pinder, Storey, & Ultsch, 1992). Despite this highly effective evolutionary invention, the cold season remains a time period that exposes organisms to severe challenges. Overwintering amphibians, especially in juvenile life stages, often show high mortality rates (Resetarits, 1986; Sinsch, 1988), and unusual winter conditions or diseases can worsen this effect (Rumschlag & Boone, 2018; Wetsch *et al*., 2022). In addition, individuals surviving hibernation but exhibiting high pathogen loads upon emergence from hibernacula can act as reservoirs, especially so following short, mild winters, which are projected to increase in frequency as a result of climate change (Carvalho, Cardoso Pereira, & Rocha, 2021). Surprisingly, the question how changing overwintering conditions and diseases interact in shaping fitness of amphibians and how winter conditions affects infection prevalence and intensity upon emergence has largely remained unanswered.

Amphibians are among the most vulnerable vertebrate taxa, as 41% of the species are threatened with extinction (IUCN, 2022). Tropical species are most prone to decline (Stuart *et al*., 2004; Hof *et al*., 2011), but many species living in temperate regions are also threatened (Houlahan *et al*., 2000; Stuart *et al*., 2004). Beside anthropogenic

habitat change and habitat loss, infectious diseases and climate change are the factors that are most devastating for amphibians (Falaschi *et al*., 2019).

Chytridiomycosis, an infectious disease of amphibians has already led to the decline of over 500 species, including the presumed extinction of around 90 species (Skerratt *et al*., 2007; Lips, 2016; O'Hanlon *et al*., 2018; Scheele *et al*., 2019). It is caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*; Longcore, Pessier, & Nichols, 1999) and *B. salamandrivorans* (*Bsal*; Martel *et al*., 2013). *Batrachochytrium dendrobatidis* has spread worldwide in the last 50 years and infected a wide host spectrum in all three orders of amphibians (Scheele *et al*., 2019). In Hungary, *Bd* was first documented to be present in the wild in 2004 (Baláž *et al*., 2014), but severe population declines have not been documented yet (but see Harmos *et al*., 2021). Examination of archived samples of amphibians from museum collections dating back to between 1936 and 2005 suggested no previous occurrence of *Bd* in the country (Vörös *et al*., 2018). The fungus infects keratinized epidermal cells (Berger *et al*., 1998), which are confined to the mouthparts in tadpoles, but are spread out on the entire skin surface in post-metamorphic stages (Marantelli *et al*., 2004).

The thermal optimum of *Bd* falls between 17 and 25°C (Piotrowski, Annis, & Longcore, 2004), but it can still grow at temperatures as low as 2°C (Voyles *et al*., 2017), and can recover after freezing (Voyles *et al*., 2017) which means it can persist on overwintering amphibians. Overwintering tadpoles and metamorphosed individuals can reportedly act as reservoirs of the pathogen (Briggs, Knapp, & Vredenburg, 2010; Narayan *et al*., 2014; Medina *et al*., 2015), facilitating the persistence of *Bd* in the population and spreading it in the next activity season to conspecifics or even to individuals of other amphibian species (Narayan *et al*., 2014; Medina *et al*., 2015; Gabor *et al*., 2017).

Besides infectious diseases, climate change also threatens amphibian populations (Reading, 2007; Blaustein *et al*., 2010). Because of their complex life cycle, ectothermic nature and highly permeable skin, changes in climatic conditions, especially in temperature and precipitation, can alter life-history traits of amphibians including breeding phenology (While & Uller, 2014; Combes *et al*., 2018), body condition (Reading, 2007), phenotypic sex (Eggert, 2004), and disease susceptibility (Pounds *et al*., 2006; Bosch *et al*., 2007; Rohr & Raffel, 2010; Cohen *et al*., 2017). Changes in winter conditions specifically, which were projected for temperate zones (i.e., milder temperatures and shorter cold periods; IPCC, 2022) may have beneficial effects on some amphibians (Üveges *et al*., 2016), but the interaction of a warming climate and infectious diseases on hibernating amphibians are practically unknown.

Here, we present an experimental test of the simultaneous effects of mild winter conditions and *Bd* infection on the survival and body mass change of hibernating juvenile agile frogs *Rana dalmatina* (Bonaparte, 1840), a species which is distributed across large areas of Europe, excluding the polar and boreal regions (Bonk *et al*., 2012). Agile frogs seem to be resistant to *Bd* infection (Baláž *et al*., 2014; Vörös *et al*., 2018; Ujszegi *et al*., 2021), but stressors, such as altered overwintering conditions may increase their susceptibility. We used juvenile frogs reared from the egg stage under seminatural conditions, performed experimental infections with *Bd* just before the start of hibernation, and exposed juveniles to winter conditions that are currently typical for the study area (1.5°C for 91 days; van Gelder *et al*., 1986; Sinsch, 1988; Holenweg & Reyer, 2000) or to conditions that are projected to become characteristic by around 2100 (4.5°C for 61 days; IPCC, 2022).

Materials and methods

Collection and rearing of animals

On 10th March 2020, we collected 120 eggs from each of 16 freshly laid egg clutches of the agile frog from two ponds in the Pilis-Visegrádi Mountains, Hungary (Garancsi-tó: 47.623457 N, 18.807393 E; Kerek-tó: 47.644794 N, 18.775291 E). We transported eggs to the Experimental Station of the Plant Protection Institute, Centre for Agricultural Research, Eötvös Loránd Research Network (Budapest, Hungary). We kept each clutch (hereafter family) separately in a plastic container (24 × 16 × 13 cm) filled with 1 L reconstituted soft water (RSW; USEPA, 2002) at a constant temperature of 19°C and a 12:12 h light:dark cycle. On 21st March, which was between 1 and 2 days after tadpoles reached the free-swimming state (development stage 25 according to Gosner, 1960), we placed 50 tadpoles separated by family into outdoor mesocosms. We set up mesocosms 2 weeks prior to the addition of tadpoles by filling plastic tubs (80 × 55 × 36 cm) with 130 L of aged tap water and adding 40 g dried beech *Fagus sylvatica* leaves to provide nutrients and refuges for tadpoles. To enhance algal growth and start up a self-sustaining ecosystem, we inoculated mesocosms with 1 L of pond water containing bacterio-, phyto-, and zooplankton. We covered mesocosms with mosquito screen lids to prevent colonization by predatory insects. When the first individuals reached developmental stage 42 (emergence of forelimbs, start of metamorphosis; 25 May), we removed leaves and monitored mesocosms daily for metamorphosing individuals, captured them using small dip nets and placed them into semitransparent, 45 L plastic boxes (56 × 39 × 28 cm; i.e., one box for each mesocosm) covered with a perforated lid and placed into a shady outdoor area. Boxes contained *c*. 0.5 L of aged tap water and were slightly tilted to provide both water covered as well as a dry area. When individuals completed metamorphosis (developmental stage 46), we chose 10 families exhibiting high survival rates and placed animals separated by family into 45 L plastic containers lined with wet paper towels as a substrate, and containing a large piece of egg carton to provide shelters. Twice a week we sprinkled boxes with RSW to maintain humidity and fed metamorphs with small crickets (*Acheta domesticus*, instar stage 1–2).

On 8th July, we placed individuals separated by family (Table S1) into open-top outdoor enclosures located in a

forested area at the experimental station. Enclosures measured 3 × 3 m and were delimited by fences constructed of fine-meshed mosquito net. Fences were 60 cm high and were dug in 20 cm into the soil. Enclosures were not covered to allow small invertebrates to enter from above and serve as food for froglets, while a 15 cm wide overhanging, stretched mosquito net strip on the inner side of the fence prevented the frogs from climbing or jumping out. We equipped enclosures with a plastic automatic poultry waterer to provide water permanently. Every week we checked enclosures for incidental damage and refilled waterers if necessary. Every 2 weeks we supplemented naturally occurring food by adding small crickets (instar stage 2–3). Between 15 and 20 October, we captured surviving individuals by carefully searching enclosures thrice. We recaptured a total of 104 juveniles from the enclosures (details shown in Table S1). Three animals from enclosure 9 were excluded and one individual from enclosure 1 escaped when it was taken back to the laboratory, resulting in 100 individuals used in the experiment. We kept animals in the laboratory at 13°C (mean ± SD: 13.44 ± 0.73°C) until experimental exposure to *Bd*. We placed individuals into 2 L opaque plastic containers covered with a perforated, translucent lid, lined with wet paper towels as substrate and containing a piece of egg carton as shelter. We fed recaptured individuals every 2 days with small crickets (instar stage 2–3) until *Bd* exposure.

Maintenance of *Bd* culture and experimental exposure

We used the *Bd* isolate Hung_2014 of the hypervirulent Global Pandemic Lineage (GPL; O'Hanlon *et al*., 2018), granted to us by M.C. Fisher (Fungal Disease Epidemiology, Imperial College London, UK), which was isolated in 2014 by J. Vörös (Department of Zoology, Hungarian National History Museum, Budapest, Hungary) from a live specimen of *Bombina variegata* in the Bakony Mountains, Hungary. The culture was maintained at 4°C in 25 cm^2 cell culture flasks (Orange Scientific, Belgium) in liquid TGhL medium (8 g tryptone, 2 g gelatin hydrolysate, and 4 g lactose in 1 L distilled water) and passaged every 3 months.

Seven days before performing experimental infections, we inoculated 2 mL of the *Bd* culture into a 25 cm^2 cell culture flask containing 10 mL mTGhL and incubated at 20°C. After the 7 days incubation period, we estimated the zoospore concentration in the culture using a Bürker chamber and diluted the culture with sterile mTGhL to obtain a concentration of 750 000 zoospores/mL. We inoculated 1 mL of this culture into 50 plastic Petri dishes containing 9 mL RSW, resulting in a final concentration of 75.000 zoospores/mL. In parallel, we pipetted 10 mL of sterile mTGhL into another 50 Petri dishes for the control group. On 21st October, we measured the body mass of juvenile frogs (pre-hibernation body mass hereafter) to the nearest 0.01 g using a laboratory scale (Ohaus PA114) and placed 50–50 juvenile frogs individually into *Bd*-inoculated or control dishes. Five hours later, we placed animals back into their original containers. Animals were not fed thereafter to ensure that their digestive tract was empty upon entering hibernation. Five days later, we swabbed all individuals to assess the success of experimental infection and to check for accidental cross-contamination in control animals. We kept swabs in 96% EtOH at 4°C until molecular analysis.

Overwintering of juveniles

After swabbing, we placed animals individually into 50 mL centrifuge tubes filled with 20 mL of a 1:1 mixture of sterilized and dried soil and sand, moistened with 5 mL aged tap water to allow burying and prevent desiccation (for further details, see Üveges *et al*., 2016). We covered the openings of tubes with mosquito nets to allow air exchange and easy watering but at the same time prevented animals from escaping. We assigned juveniles from both the *Bd*-exposed and the control groups randomly to one of the two hibernation scenarios while keeping body masses balanced among treatments (GLM; $F = 0.01$, d.f. = 1, $P = 0.93$). On 30th October, we placed tubes into two laboratory refrigerators with forced air convection (Pol-Eko-Aparatura, Wodzisław Śląski, Poland) set to 10°C. Three days later, we lowered the temperature to 7.5°C and maintained it for 2 weeks to simulate transient autumn temperatures (Fig. 1). We initiated hibernation on 16th November by lowering the temperature to 4.5 and 1.5°C in the short and mild (SM) and in the long and cold (LC) winter scenario, respectively. We kept track of actual temperatures by recording them every 5 min using the refrigerators' inbuilt thermometers. Mean temperature ± SE during the SM and the LC treatments were 4.493 ± 0.004 and 1.479 ± 0.003°C, respectively. We sprinkled aged tap water into tubes twice a week to prevent desiccation. Sixty-one (SM treatment) and 91 days (LC treatment) after start of hibernation we raised the temperature to 7.5°C in the refrigerators. Animals were kept at this temperature for 10 days, then we measured their body mass again, and humanely killed them with the 'cooling then freezing' method (Shine *et al*., 2015). Subsequently, we took toe clips and preserved these in 96% EtOH at 4°C until molecular analysis.

Quantifying infection intensity

We lost one swab sample collected from a *Bd*-infected individual before hibernation. For the assessment of *Bd* infection prevalence and intensity, we homogenized swabs and toe clips of preserved animals. We extracted DNA with PrepMan Ultra Sample Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the instructions of Boyle *et al*. (2004), and stored extracts at −20°C until further analysis. Following a standard amplification methodology targeting the ITS-1/5.8 S 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 tenfold with PCR water. We ran samples in duplicate, and in case of contradictory results, we repeated reactions in another duplicate. If this again returned an equivocal result, we considered the sample to be *Bd* positive (Kriger,

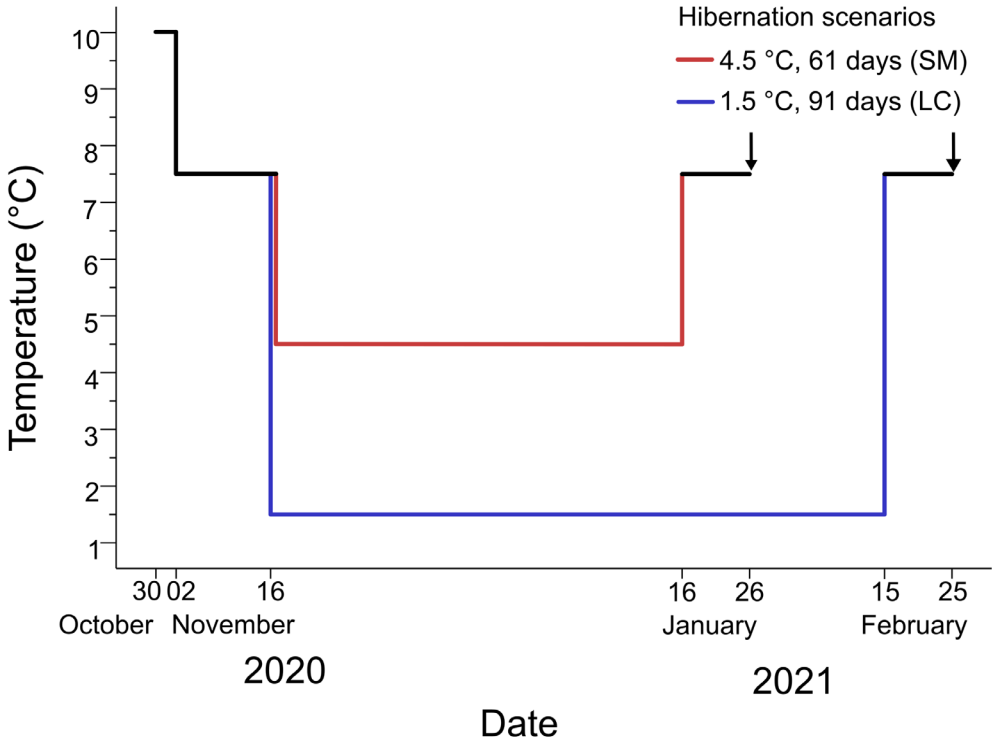

Figure 1 Schematic representation of the overwintering procedures of juvenile agile frogs. Arrows show the dates of weighing after hibernation.

Hero, & Ashton, 2006). Genomic equivalent (GE) values of *Bd*, estimating infection intensities, were obtained from standard curves based on four dilutions of a standard (100, 10, 1, and 0.1 zoospore genomic equivalents; courtesy of J. Bosch; Museo Nacional de Ciencias Naturales, Madrid, Spain). To obtain infection intensity estimates, we averaged *Bd* GE values obtained from qPCR runs within samples of toe clips. We used data obtained by swabbing only for the assessment of prevalence because the *in vivo* applicable method of swabbing does not allow a reliable estimation of infection intensities (Clare *et al.*, 2016).

Statistical analyses

All statistical analyses were conducted in 'R' (version 4.1.0, R Core Team, 2020). We analyzed the effects of *Bd* exposure on the change of body mass using general linear models, with change in body mass during hibernation entered as the dependent variable, infection treatment, winter scenario and their interaction as categorical fixed factors, and pre-hibernation body mass as a continuous covariate. As number of individuals in families showed great variance and in some families it was very low, we could not insert family as a random factor in the model, just as a fixed factor. We also built a similar model where we entered pre-hibernation infection status (*Bd* positive or *Bd* negative, irrespective of infection treatment) instead of infection treatment. To assess intergroup differences, we used pairwise comparison with linear contrasts using the 'emmeans' function of the 'emmeans' package (Lenth *et al.*, 2021) and calculated 84% confidence intervals which, if not overlapping, indicate significant differences (Payton, Greenstone, & Schenker, 2003; Julious, 2004). In all analyses, we applied a backward stepwise model simplification procedure to avoid potential problems due to the inclusion of nonsignificant terms (Grafen & Hails, 2002; Engqvist, 2005). We note, however, that the full models did not deliver qualitatively different results regarding the main variables.

Results

Mortality during hibernation was very low as only one animal, an uninfected control in the LC treatment, died, so we did not analyze survival. In the infected groups, the molecular analysis of swabs indicated *Bd* infection in 18 individuals: nine individuals in both the LC and the SM winter scenario out of 25 and 24 samples, respectively (Fig. 2a). Thus, pre-hibernation prevalence of *Bd* infection was 36 and 37.5% in the *Bd*-exposed groups. After hibernation, only four individuals tested positive for *Bd*: two in the LC and two in the SM winter scenario, corresponding to 8% of the *Bd*-exposed individuals in both winter scenarios (Fig. 2b). Infection intensities were very low in all four positive cases (5.2 and 31.82 GE in the LC scenario; 0.03 and 7.53 GE in the SM scenario). Interestingly, only one individual out of these four tested positive before hibernation. None of the swabs and none of the tissue samples of control individuals tested positive, indicating a lack of cross-contamination.

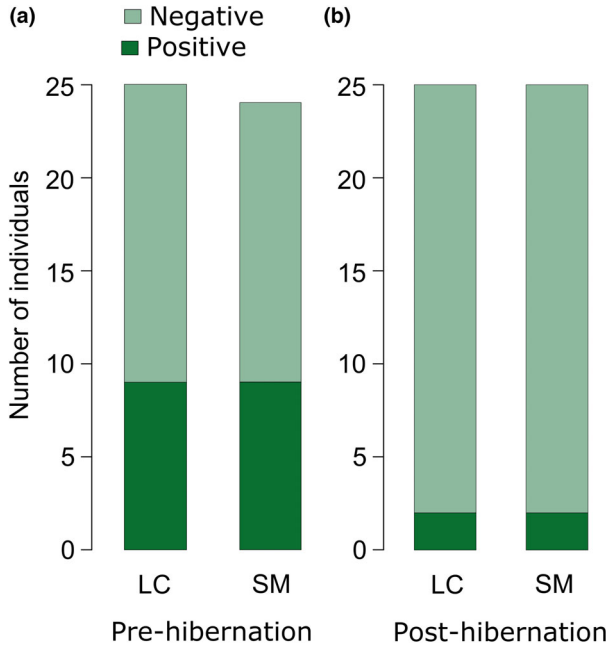

Figure 2 Proportion of exposed versus infected individuals (exposed group, *N* total = 50) before hibernation based on swab samples **(a)** and after hibernation based on tissue samples **(b)**. LC = 'long, cold' winter; SM = 'short, mild' winter.

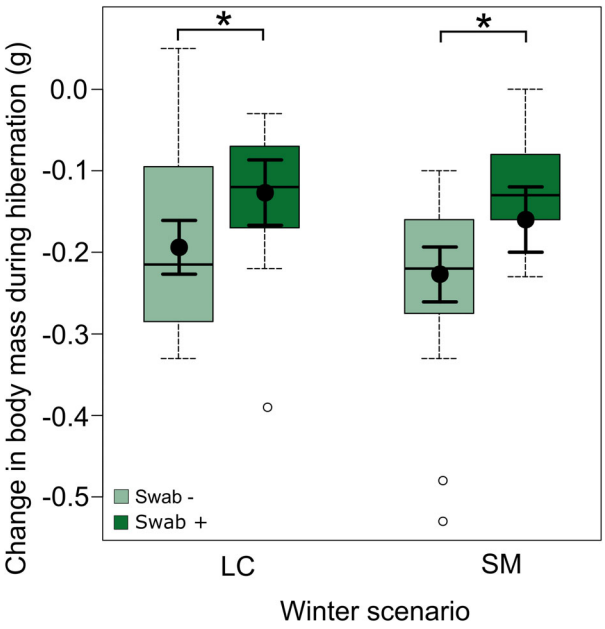

Figure 3 Change in body mass during hibernation of individuals exposed to *Batrachochytrium dendrobatidis* (*N* = 50). Infection status was based on pre-hibernation swab samples. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, and open circles represent outliers [deviating from the boundary of the interquartile range (IQR) by more than 1.5× IQR]. Error bars depict means and 84% confidence intervals. Significant differences ($P < 0.05$) between groups are indicated by asterisks. LC = 'long, cold' winter; SM = 'short, mild' winter.

Change in body mass during hibernation was not significantly affected by exposure to *Bd* ($F = 0.18$, d.f. = 1, $P = 0.67$), by winter scenario ($F = 0.96$, d.f. = 1, $P = 0.33$) or by their interaction (F = 0.28, d.f. = 1, $P = 0.59$; Fig. S1), but it was significantly affected by pre-hibernation body mass ($F = 46.41$, d.f. = 1, $P < 0.001$) and family of origin ($F = 2.69$, d.f. = 8, $P = 0.01$). Pre-hibernation body mass showed negative correlation with the change of body mass during hibernation ($b = -0.15$, SE = 0.02). On the other hand, within the *Bd*-exposed groups infection status before entering hibernation had a significant effect on the change in body mass during hibernation ($F = 8.44$, d.f. = 1, $P = 0.006$): individuals with negative swabs lost more mass than individuals with positive swabs (Fig. 3). Also, winter scenario ($F = 0.48$, d.f. = 1, $P = 0.49$) and family of origin ($F = 0.91$, d.f. = 8, $P = 0.52$) did not influence body mass loss during hibernation, and the interaction between infection status before hibernation and winter scenario was nonsignificant as well ($F = 3.21$, d.f. = 1, $P = 0.08$).

Discussion

Juvenile agile frogs showed an intermediate level of resistance to the fungus, as out of the 50 animals experimentally exposed to *Bd* 18 returned *Bd*-positive swabs 5 days later. It is consistent with results obtained in field studies reporting low prevalence of *Bd* infection among adult agile frogs in natural populations (Baláž *et al.*, 2014; Vörös *et al.*, 2018), and is coherent with results of recent experimental studies also reporting low prevalence following experimental infection (Ujszegi *et al.*, 2021; Herczeg *et al.*, 2022). Clearly, the assessment of infection status using the noninvasive method of swabbing has its limits (Clare *et al.*, 2016), which is also supported by our result that three individuals that appeared to be *Bd*-negative before hibernation were positive after hibernation. Nonetheless, even if our estimate on *Bd* prevalence based on swabs is only a rough one, it indicates that many, but not all individuals were able to suppress the infection below the limit of detectability or to avoid becoming infected in the first place.

Out of the 50 individuals exposed to *Bd*, and in contrast to the 18 individuals testing positive just before start of the simulated winter, only four juvenile frogs were positive at the end of hibernation, all exhibiting very low infection intensities. This result clearly suggests that *Bd* infection does not increase in intensity during overwintering but rather becomes suppressed by the immune system of agile frog juveniles, perhaps combined with the effect of unfavorable temperatures falling way below the optimum of the fungus. Although the number of individuals testing positive for *Bd* after hibernation was very low, the practically identical and very low prevalence and infection intensity values observed in animals subjected to the two winter scenarios put forward the conclusion that neither a long and cold winter, nor a short and mild winter largely boosted *Bd* proliferation. On the other hand, our results are in contrast with another

previous study, which found that 'poor' (warmer) overwintering temperature regime increased the probability of *Bd* infection in the common toad (Garner, Rowcliffe, & Fisher, 2011). Finally, the observation that three individuals that tested *Bd* negative before hibernation were positive after hibernation raises the possibility that, although agile frog juveniles appear to benefit from hibernation when facing *Bd* infection, even individuals that enter hibernation with very low infection intensities can carry the fungus upon emergence from hibernacula. Nonetheless, it has to be noted that in our study the juvenile frogs hibernated individually, while aggregated hibernation is known to occur in Ranids (Wells, 2007). This is important because the persistence and transmission of *Bd* during overwintering may be enhanced if several individuals use the same hibernaculum. Future studies testing for an effect of aggregated versus solitary hibernation will clearly enhance our understanding of the fate of infections during overwintering.

We observed almost zero mortality during hibernation in both winter scenarios. In a study on common toad juveniles, Üveges *et al*. (2016) found that individuals experienced nearly 40% mortality when hibernated in the laboratory at 1.5°C for 91 days. In a field study, a population of *ex situ* bred, reintroduced *R. muscosa* showed over 70% mortality during the hibernation period (Hammond *et al*., 2021). Amphibian species differ widely in their ability to cope with low temperatures, with many species of the family *Ranidae* being freeze tolerant, while species of the family *Bufonidae* being typically freeze intolerant (Voituron *et al*., 2009).

Nearly all individuals lost body mass during hibernation, but this mass-loss did not differ between winter scenarios, indicating that mild winters do not have a negative effect on the body mass of overwintering juvenile agile frogs. Üveges *et al*. (2016) found that shorter winter and milder hibernation temperature had synergistic beneficial effects on body mass change during hibernation of common toad juveniles. In contrast, Holenweg & Reyer (2000) documented in a field study that individuals of two members of the genus *Pelophylax* (*P. lessonae* and *P. esculentus*) lost more weight during warm than in cold winters. These contradictory results suggest that amphibians show species-specific responses to changing winter conditions, which may at least partly be due to differences in their strategies to cope with the cold season and in their ability to tolerate freezing (Storey & Storey, 1986). On the other hand, inherent differences between the closely controlled and highly artificial conditions experienced by amphibians in the laboratory versus the variable natural environment in field-based experiments may also have contributed to these inconsistencies.

When comparing mass loss of individuals in the *Bd*-exposed group that either tested *Bd* positive or *Bd* negative before hibernation, we found that *Bd* negative individuals lost more mass during hibernation irrespective of the winter scenario. It is possible that maintaining such a high immune function that it can fend off or quickly clear *Bd* infection carries higher costs to individuals than the maintenance of a less effective immune function combined with any direct costs of the infection, and this resulted in greater body mass loss in individuals that were able to fully clear *Bd* infection. Because there is a trade-off between investment in immune function and in other life-history traits and functions (Martin, Weil, & Nelson, 2008), it is possible that fending off or overcoming the infection led to the enhanced decrease in body mass (for a similar result, see Cheatsazan *et al*., 2013). Amphibians can increase their feeding activity when infected with *Bd*, resulting in body mass changes which are equal between infected and control individuals (Hess *et al*., 2015). During hibernation, food is not available, so such a compensation of increased physiological demands is impossible, probably leading to the manifestation of costs in terms of enhanced body mass loss.

Our results suggest that winters that become milder and shorter in temperate regions due to climate change probably will not cause declines in agile frog populations via enhanced body mass loss or by leading to a proliferation of *Bd* in infected animals. Individuals showed strong resistance against *Bd* and the fungus hardly persisted on them even under short, mild winter conditions, when the temperature can promote *Bd* growth. We predict that overwintering agile frogs will not become important reservoirs of *Bd*, even not under changing winter conditions. However, we only tested the effects of two winter durations and two temperatures and not in a fully crossed design, so that further studies are needed to scrutinize the effects of variation in overwintering conditions. Another avenue that would urgently need to be followed is to investigate the extent to which our result suggesting weak effects of changing overwintering conditions can be generalized to other amphibians of the temperate zone: *Bd* infection and changing overwintering conditions may have more severe consequences in amphibians that are more susceptible to chytridiomycosis or more sensitive to the hibernation environment.

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Author contributions

A.K. and A.H. conceived and designed the experiments. A.K., J.U., and D.Ho. performed the experiments. K.A, J.U., D.He, and D.Ho. performed the molecular analysis. K.A. analyzed the data. K.A. and A.H. wrote the paper; other authors provided editorial advice.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

TABLE S1. Number of animals introduced into outdoor enclosures and the number of recaptured individuals. Each enclosure contained members of a different family (i.e., sibling group).

Figure S1. Change in body mass during hibernation by treatments (all individuals, $N = 100$: 25/treatment group). Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, and open circles represent outliers [deviating from the boundary of the interquartile range (IQR) by more than 1.5× IQR]. Error bars depict means and 84% confidence intervals.