

Research article

Amphibian larvae benefit from a warm environment under simultaneous threat from chytridiomycosis and ranaviriosis

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Rising temperatures can facilitate epizootic outbreaks, but disease outbreaks may be suppressed if temperatures increase beyond the optimum of the pathogens while still within the temperature range that allows for effective immune function in hosts. The two most devastating pathogens of wild amphibians, *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses (*Rv*), co-occur in large areas, yet little is known about the consequences of their co-infection and how these consequences depend on temperature. Here we tested how exposure to *Bd* and subsequent exposure to *Rv*, followed by treatment at elevated temperatures (28 and 30°C versus 22°C) affected *Bd* and *Rv* prevalence, infection intensities, and resulting mortalities in larval agile frogs and common toads. We found multiple pieces of evidence that the presence of one pathogen influenced the prevalence and/or infection intensity of the other pathogen in both species, depending on temperature and initial *Rv* concentration. Generally, the 30°C treatment lowered the prevalence and infection intensity of both pathogens and, in agile frogs, this was mirrored by higher survival. These results suggest that if temperatures naturally increase or are artificially elevated beyond what is ideal for both *Bd* and *Rv*, amphibians may be able to control infections and survive even the simultaneous presence of their most dangerous pathogenic enemies.

Keywords: chytrid fungus, disease mitigation, *Ranavirus*, thermal optima mismatch, thermal tolerance, thermal treatment

Introduction

During recent decades amphibians have experienced dramatic declines (Scheele et al. 2019) and have become one of the most threatened vertebrate taxa, pushing them into the forefront of conservation efforts (Grant et al. 2019). The causes behind population



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declines and extinctions are complex (Collins 2010), with multiple stressors acting in synergy, but emerging infectious diseases likely play a decisive role (Fisher and Garner 2020). The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) was linked directly to the local extinction of dozens of amphibian species and caused the worst pandemic of wildlife ever recorded in history (Scheele et al. 2019, Fisher and Garner 2020). At the same time, epidemics caused by Ranaviruses (family *Iridoviridae*, hereafter *Rv*) have also led to mass mortality events, resulting in local extinctions of amphibians (Teacher et al. 2010, Kik et al. 2011, Price et al. 2014). More specifically, the globally distributed *Frog Virus 3* (FV3), along with the *Common midwife toad virus* (CMTV), whose distribution is currently restricted to Europe in the wild (Price et al. 2014, Thumsová et al. 2022) is responsible for amphibian declines on the Iberian Peninsula and perhaps elsewhere in Europe (Thumsová et al. 2022).

Typically, several pathogens are present in natural populations (Hoverman et al. 2012), and these organisms are likely to interactively determine consequences for hosts. Accordingly, co-infections are increasingly recognised as important drivers of disease dynamics (Romansic et al. 2017, Stutz et al. 2018). Co-infections can be disadvantageous, insignificant, or even beneficial to hosts, and multiple levels of interactions can influence the outcomes (Herczeg et al. 2021). Direct and indirect interactions among infectious agents within hosts include interference competition for physical space (Dobson and Roberts 1994) and exploitation competition for resources (Pedersen and Fenton 2007, Mideo 2009), as well as indirect interactions mediated by cross-reaction immunity and the immunosuppression of the host (Read and Taylor 2001, Lello et al. 2004). The outcome of co-infections is also shaped by the species-specific infectivity and virulence of pathogens (Mihaljevic et al. 2018), arrival order (i.e. 'priority effect' (Hoverman et al. 2013, Wuerthner, Hua and Hoverman 2017), species and body condition of hosts (Paré 2003, Johnson et al. 2019), and abiotic factors (Herczeg et al. 2021).

The highly pathogenic and widespread *Bd* and *Rv* frequently co-infect amphibians (Hoverman et al. 2012, Warne et al. 2016, Watters et al. 2018, Bosch et al. 2020). Naturally co-infected amphibians (e.g. American bullfrog and northern leopard frog) can experience higher *Bd* infection loads during simultaneous infection with *Rv* than individuals exclusively infected with *Bd* (Watters et al. 2018), while *Rv* infection intensity tends to be negatively associated with the probability of infection with *Bd*, but this relationship depends on frog taxa (Warne et al. 2016). Nonetheless, experimental studies scrutinising the effects of co-infection with *Bd* and *Rv* on mortality, morbidity, pathogen prevalence, and infection intensity are extremely scarce. The only such study to our knowledge (Ramsay and Rohr 2021) found non-additive effects of co-infection on pathogen loads but not on host growth, survival, and antibody response in post-metamorphic Cuban tree frog *Osteopilus septentrionalis*.

Co-infection with *Bd* and *Rv* occurs even though their thermal requirements are different. The optimum temperature

range of *Bd* falls between 17 and 25°C (Piotrowski et al. 2004), and its critical thermal maximum lies around 28°C (Johnson et al. 2003, Stevenson et al. 2013, Cohen et al. 2017, Voyles et al. 2017; for the *Bd* strain used here see Kásler et al. 2022). Therefore, *Bd* prevalence (Whitfield et al. 2013, Olori et al. 2018) and mortality attributable to *Bd* infection (Berger et al. 2004) are usually highest during moderately warm months and in habitats where the temperature does not become exceedingly high (Woodhams and Alford 2005, Becker et al. 2012), while high temperatures promote loss of infection and host survival (Berger et al. 2004). In contrast, the FV3 replicates successfully in vitro between 8 and 30°C, with a lower replication rate below 15°C and the highest rate at 30°C (Cunningham 2001). Indeed, deaths caused by *Rv* are more frequent during the summer months (Chinchar 2002), so temperature also appears to be a crucial determinant of ranaviruses dynamics (Price et al. 2019, Thumsová et al. 2022).

Temperature influences infection outcome not only through its effects on the growth of pathogens but also via its influence on amphibian hosts (Herczeg et al. 2021). The positive temperature dependence of the immune system of amphibians (Raffel et al. 2006, Raffel et al. 2013) may at least partly explain why individuals that occur in warmer areas can keep *Bd* infection at bay (Retallick et al. 2004, Kriger and Hero 2007, Kilpatrick et al. 2010). Accordingly, thermal treatments have proven helpful for clearing the *Bd* infection of amphibians in the laboratory (Ribas et al. 2009, Chatfield and Richards-Zawacki 2011, Geiger et al. 2011). However, a simple warmer-is-better rule does not apply universally to amphibian species facing chytridiomycosis, as the immune system of cold-adapted amphibians may be more effective against *Bd* at lower than at higher temperatures (Cohen et al. 2017, Sauer et al. 2018, Cohen et al. 2019). In the case of ranaviruses, elevating the temperature from 20 to 27°C increased *Rv* propagation, disease incidence, and mortality rate in the common frog *Rana temporaria* (Price et al. 2019).

Similarly, an increase in temperature from 10 to 25°C resulted in higher mortality and *Rv* copy numbers in tadpoles of four amphibian species exposed to FV3 (Brand et al. 2016). In contrast, *Rv* infection probability and mortality were lower at 22°C than at 14°C in two species of *Lithobates* frogs infected with three different FV3 strains (Echaubard et al. 2014). While these studies delivered clear evidence for the importance of temperature in the case of both chytridiomycosis and ranaviruses, contradictions in patterns may be due to interspecific differences in the temperature dependence of amphibian immune functions or to some other factor remaining to be explored. Importantly, manipulative studies testing how elevated temperature affects disease progression in amphibians co-infected with these two pathogens are lacking entirely.

Hence, to clarify the effects of high temperatures on disease progression in amphibians during single and sequential co-infection with *Bd* and *Rv*, we experimentally exposed tadpoles of agile frogs *Rana dalmatina* and common toads *Bufo bufo* to *Bd* and subsequently to *Rv*, treated them with

elevated temperatures for six days, and finally assessed infection patterns and survival. We thereby aimed to deliver information about the effects of high temperatures (i.e. 28 and 30°C) that occur during heat waves in temperate-climate ponds under natural conditions (Wells 2007, Indermaur et al. 2010, Geiger et al. 2017, Lambert et al. 2018 Lindauer et al. 2020) on the severity of consequences of *Bd* and *Rv* infection and, especially, on the outcomes of co-exposure. Finally, we intended to test the potential of localised heating, an in situ mitigation method relying on the thermal treatment of *Bd*-infected amphibians (Hettyey et al. 2019), by assessing whether elevated temperatures decrease *Bd* prevalence and intensity without resulting in elevated *Rv* infection loads and excessive mortality in the presence of *Rv*.

Material and methods

Experimental design and procedures

We applied a full-factorial design with three thermal treatments: 22, 28, and 30°C, combined with six infection treatments: uninfected control ('control'); exposed to *Bd* ('*Bd*'); exposed to *Rv* in a low concentration ('*Rv*-low'); exposed to *Rv* in a high concentration ('*Rv*-high'); sequentially co-exposed to *Bd* and *Rv* in a low concentration ('*Bd*+*Rv*-low'); and sequentially co-exposed to *Bd* and *Rv* in a high concentration ('*Bd*+*Rv*-high'). We replicated each treatment combination 20 times (two individuals from each of 10 families in each treatment combination) for a total of 360 animals per species. The experimental procedure started with a 19 days *Bd* treatment, followed by a 24 h *Rv* treatment and, finally, a six-day thermal treatment (for a schematic representation, Fig. 1).

We collected 100 eggs from each of ten freshly laid clutches (hereafter, sibling groups) from two natural populations of

both species and transported them to the laboratory, where we reared embryos until hatching. Five days after hatching (development stage 25 according to Gosner (Gosner 1960)), we divided sibling groups into groups of 10 larvae and placed tadpoles into plastic rearing containers holding 10 L of reconstituted soft water i.e. RSW (USEPA 2002); see Supporting information for details on animal collection and husbandry). We randomly assigned rearing containers to *Bd* treatments, with one container representing each treatment by sibling group combination. We performed the first infection with *Bd* on day 1 and, after that, renewed *Bd* concentrations following each water change until performing infections with *Rv*, which resulted in a total of five occasions of *Bd* addition. On each occasion, we exposed tadpoles to approximately 2000 zoospores \times ml⁻¹ of *Bd* directly in their rearing containers, while the control tadpoles received sterile broth (see Supporting information for a detailed description of experimental infection with *Bd*). We re-exposed tadpoles to *Bd* after each water change to mimic natural conditions, where zoospores are constantly present in the aquatic environment, and to maximize the likelihood of infection.

On day 19, we haphazardly selected eight tadpoles from each rearing container and randomly assigned them to *Rv* \times temperature combinations. Then we exposed tadpoles to FV3 by applying one of two concentrations, i.e. *Rv*-low (6.12×10^3 plaque-forming unit (pfu) \times ml⁻¹) and *Rv*-high (6.25×10^5 pfu \times ml⁻¹) during 24 h exposures where tadpoles were challenged individually in plastic cups containing RSW and the corresponding concentration of *Rv*, while control tadpoles received sham extract (see Supporting information for more details on experimental infection with *Rv*). Based on previous studies on different but phylogenetically related host species (wood frog *Lithobates sylvaticus*) we expected high mortality of agile frog tadpoles during *Rv* infection (Warne et al. 2011, Earl and Gray 2014); in contrast, such an effect was not experienced in agile frogs and common toads after repeated

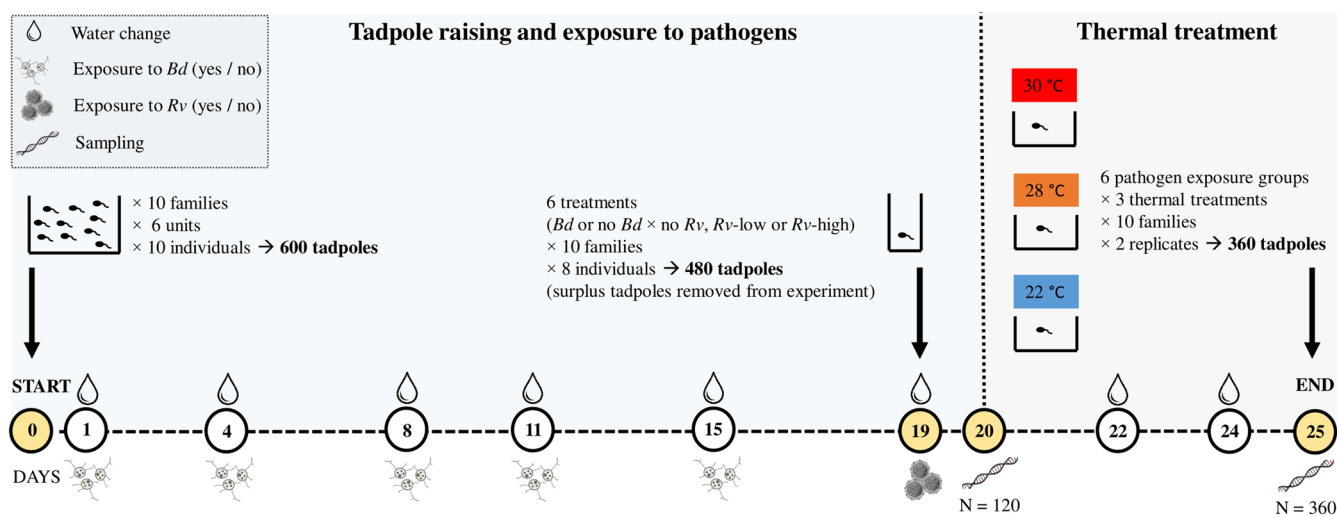


Figure 1. Schematic representation of the experimental procedure. *Bd* = *Batrachochytrium dendrobatidis*; *Rv* = *Ranavirus*.

Bd exposure in early age (Ujszegi et al. 2021). Therefore, we applied different exposure durations (continued exposure to *Bd* and one brief exposure to *Rv*). Furthermore, we exposed tadpoles to *Rv* at two concentrations (low and high) because there is a gap in the literature regarding susceptibility to *Rv* and the necessary exposure concentrations and duration in our host species. Subsequently, to assess pre-thermal treatment (hereafter pre-treatment) infection prevalence and intensity for both pathogens, we haphazardly selected 20 tadpoles from each of the six infection treatment groups (12 tadpoles from each sibling group, 120 individuals in total) and preserved them in 96% ethanol.

On day 20, we started thermal treatments as described in Ujszegi et al. (2022) and monitored tadpoles daily to record any mortality events. We discarded dead individuals from further analysis because it is unknown how pathogen loads and their detectability change shortly after death of the host, and because of quick body decomposition at high temperatures preventing a precise necropsy. We placed the 2 L rearing boxes in 80 × 60 × 12 cm trays filled with tap water to a depth of 8 cm (water level was lower than in rearing boxes by ca 2 cm to avoid floating of the latter) and subsequently turning on submersible aquarium heaters (Tetra HT 200 in 28°C treatments and Tetra HT 300 in 30°C treatments) and water pumps (Tetra WP 300) placed opposite to each other on the longitudinal axis of trays. Thereby, water temperature increased gradually to the desired level in ca two hours, allowing tadpoles to adjust to increasing temperatures. After heating up, the temperature did not change over time and varied only a little among/within trays (Supporting information), as documented by automated temperature loggers (Onset HOBO Pendant Temperature/Light 8K) placed into one-third of the trays (i.e. 12 out of 36). Actual water temperatures in the tadpole rearing boxes were overall 21.4 ± 0.72, 28.16 ± 0.24, and 30.13 ± 0.35°C (mean ± SD) in the three temperature treatments, respectively. During the six days of thermal treatment, we changed water twice with RSW pre-heated to the temperature of the respective thermal treatment group. We fed tadpoles with a lowered amount of spinach (one-third of the amount provided during the rearing period) to avoid water fouling and anoxia at high temperatures. Six days after the start of thermal treatments, we terminated the experiment by preserving all surviving tadpoles in 96% ethanol.

We extracted *Bd* DNA from dissected mouthparts and *Rv* DNA from liver tissue. We assessed infection prevalence and intensity using qPCR following standard amplification methodologies for *Bd* (Boyle et al. 2004) and for *Rv* (Stilwell et al. 2018). When the qPCR result was equivocal, we repeated reactions in duplicate. If we obtained an equivocal result again, we considered the sample positive (Kriger et al. 2006) (Supporting information).

Statistical analyses

We analysed data on the two species separately. We calculated the prevalence data with 95% confidence intervals using

QPWeb ver. 1.0.15 (Reiczigel et al. 2019) (Supporting information). For all other analyses, we used the R computing environment, ver. 4.0.4 (www.r-project.org).

Before thermal treatment, *Bd* prevalence was low in both species, so we did not perform statistical analyses on pre-treatment *Bd* prevalence and infection intensity. In case of pre-treatment *Rv* prevalence, we tested the effects of the applied *Rv* concentration and of previous exposure to *Bd* using Fisher's exact tests. When analysing pre-treatment *Rv* infection intensity, we used linear mixed-effects models (LMM; *lme* function of the 'nlme' package) to assess the effects of *Rv* concentration and *Bd* co-infection, as well as their interaction.

To analyse prevalence and pathogen load after thermal treatment, we included only those treatment groups that had been exposed to the given pathogen. We used generalised linear mixed-effects models (GLMM) to test the effects of treatments on pathogen prevalence. The model for *Rv* prevalence in agile frogs contained thermal treatment (22, 28 or 30°C), *Rv* concentration (low or high) and co-exposure to *Bd* (yes or no) as fixed factors and all two-way interactions (there was not enough variance in the data to allow model fit for testing the three-way interaction). The model for *Bd* prevalence in common toads contained thermal treatment and a three-category factor that combined the information on the presence and concentration of *Rv* (no *Rv*, low *Rv*, high *Rv*) and the interaction of the two fixed factors. In both models, we entered sibling group as a random factor. We assumed a binomial error distribution and used a logit link function. We fitted the models applying maximum likelihood estimation using the *glmmTMB* function of the package 'glmmTMB' (Brooks et al. 2017) and checked model-fit diagnostics using the 'DHARMA' package (Hartig 2020). We did not run such analyses for *Bd* prevalence in agile frogs because zero prevalence in the majority of treatment groups would have led to very high estimation uncertainty (separation) and inability to test interactions.

Within *Rv*-positive agile frogs we analysed the effect of treatments on infection intensity after thermal treatment using an LMM, allowing the variances to differ among treatment groups (*varIdent* function) because graphical model diagnostics indicated heterogeneous variances. The model contained the natural log-transformed *Rv* infection intensity as a dependent variable, thermal treatment, *Rv* concentration, *Bd* co-exposure, and their two- and three-way interactions as fixed factors, and sibling group as a random factor. For *Rv* infection load in common toads, we used the same modelling approach, but we tested only the main effects because there was not enough variation in the data for testing interactions (i.e. prevalence was zero in 6 out of 12 treatment combinations, causing separation in binomial models). Low prevalence prohibited the analyses of infection intensity for *Bd* in both species.

To analyse the survival of agile frog tadpoles during thermal treatment, we ran a mixed-effects Cox's proportional hazards model (COXME; *coxme* function of the 'coxme' package), entering sibling group as a random effect (Therneau

and Grambsch 2000). We entered survival as an ordinal categorical dependent variable ranging 1–6 (each category representing the day of death during heat treatment, one being the first 24 h); individuals that survived to the end of thermal treatment were treated as censored observations. We included thermal treatment, *Rv* concentration, *Bd* co-exposure, and their two- and three-way interactions as predictors. Because mortality of common toads was negligible (1.1%; Supporting information), we did not analyse their survival.

We applied a backward stepwise model selection procedure to reduce noise in parameter estimates due to the inclusion of non-significant terms (Grafen and Hails 2002, Engqvist 2005). We obtained statistics for excluded terms by re-entering them to the final model. For these steps, we used type-3 analysis-of-deviance tables (*Anova* function of the ‘car’ package). To perform pairwise comparisons, we calculated linear contrasts from the final models using the *emmeans* function of the ‘emmeans’ package while applying the false discovery rate (FDR) correction method to adjust p values for multiple comparisons (Pike 2011, Lenth et al. 2021).

Results

Agile frogs

Pre-treatment *Bd* prevalence was extremely low: two out of 60 *Bd*-exposed individuals carried the fungus. Pre-treatment *Bd* infection intensities were also very low (Supporting information). In contrast, the pre-treatment prevalence of *Rv* in *Rv*-exposed tadpoles was between 73.7 and 100% (Supporting information) and *Rv* infection intensities were low to moderate (Supporting information).

By the end of the six days of thermal treatments, *Bd* prevalence in *Bd*-exposed agile frog tadpoles remained low, with only three tadpoles testing positive, all in the *Bd* + *Rv*-low treatment (Supporting information). Infection intensity of *Bd* was also very low after thermal treatments (< 12.2 genomic equivalents (GE); Supporting information). Cross-contamination was not detected in either infection group except for one individual in the control group after 30°C thermal treatment, but this tadpole also exhibited very low *Rv* infection intensity (28 pfu × ml⁻¹; Supporting information).

The prevalence of *Rv* in *Rv*-exposed tadpoles after thermal treatments varied between 21.4 and 100% (Fig. 2A, Supporting information) and was higher in the *Rv*-high treatment (GLMM; $\chi^2_1 = 14.49$, $p < 0.001$) and in tadpoles not co-exposed to *Bd* ($\chi^2_1 = 13.34$, $p < 0.001$). There was a tendency for thermal treatment to affect *Rv* prevalence ($\chi^2_2 = 4.62$, $p = 0.099$), where the lowest infection probability was at 28 and the highest at 22°C (Supporting information). The two-way interactions were non-significant (all $p > 0.37$).

Ranavirus infection intensities were high and were positively affected by *Rv* concentration (LMM; $\chi^2_1 = 15.34$, $p < 0.001$), whereas the main effects of thermal treatment ($\chi^2_2 = 4.34$, $p = 0.11$) and previous exposure to *Bd* ($\chi^2_1 = 2.24$, $p = 0.14$) were non-significant. The two-way interactions were all non-significant as well (all $p > 0.17$), but the three-way interaction between thermal treatment, previous exposure to *Bd*, and *Rv* concentration was significant ($\chi^2_2 = 7.85$, $p = 0.02$; Fig. 2A). To scrutinise the pattern behind this interaction we separately analysed the treatment groups exposed to the low and the high *Rv* concentration. In the treatment groups exposed to the low *Rv* concentration, the interaction between previous exposure to *Bd* and thermal

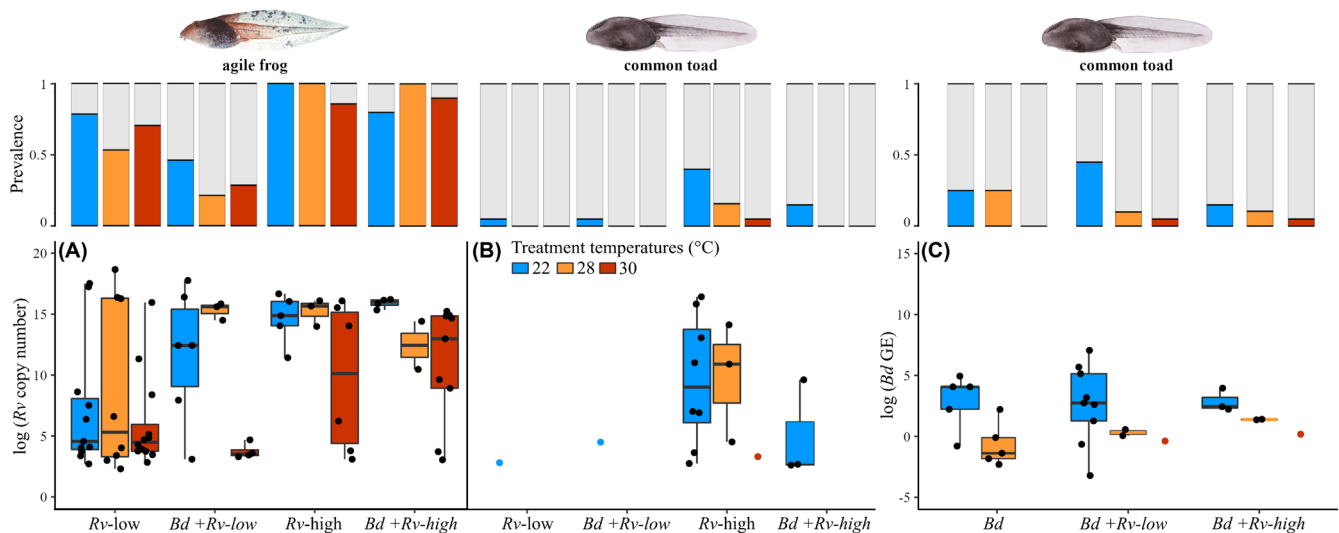


Figure 2. (A) *Ranavirus* (*Rv*) infection intensities in *Rv*-positive agile frog tadpoles with corresponding prevalences and (B and C) *Rv* and *Batrachochytrium dendrobatidis* (*Bd*) infection intensities in *Rv*- and *Bd*-positive common toads with corresponding prevalences of pathogens after the six days of thermal treatment, following exposure to infection treatments. The *Rv* and *Bd* infection intensity data (black dots) were natural log-transformed. Treatment groups with only one intensity data point represented as coloured dots. Horizontal lines represent medians, boxes represent interquartile ranges, and whiskers represent minimum–maximum ranges.

treatment was significant ($\chi^2_2 = 13.12$, $p = 0.001$), where *Rv* infection intensity tended to be higher in the previously *Bd*-exposed treatment groups, but this effect was abolished by the 30°C thermal treatment (Fig. 2A, Supporting information). In treatment groups exposed to the high *Rv* concentration, the two-way interaction between previous exposure to *Bd* and thermal treatment did not reach significance ($\chi^2_2 = 3.48$, $p = 0.18$), and previous exposure to *Bd* did not have an effect ($\chi^2_1 = 1.49$, $p = 0.22$), but thermal treatment did ($\chi^2_2 = 14.29$, $p < 0.001$). Tadpoles in the 30°C treatment exhibited lower *Rv* copy numbers than those maintained at 22°C (Fig. 2A, Supporting information).

Survival of agile frog tadpoles was significantly influenced by thermal treatments (COXME; $\chi^2_2 = 11.48$, $p = 0.003$): it increased to 61.5% at 30°C from ca 43.5% at 22–28°C (Fig. 3, Supporting information). The effect of *Rv* concentration was also significant ($\chi^2_1 = 52.83$, $p < 0.001$): survival probability was 2.7 times higher in the *Rv*-low treatment than in the *Rv*-high treatment (Fig. 3, Supporting information). Previous co-exposure to *Bd* did not affect survival ($\chi^2_1 = 1.24$, $p = 0.27$) and none of the interactions were significant (all $p > 0.27$).

Common toads

The pre-treatment prevalence of *Bd* varied between 5 and 42%, and *Bd* infection intensities remained low until the start of thermal treatments (Supporting information). At the same time, the pre-treatment prevalence of *Rv* varied between 20 and 100% and pre-treatment *Rv* infection intensities were low to moderate (Supporting information).

After thermal treatments, the prevalence of *Bd* in *Bd*-exposed common toad tadpoles varied between 0 and 45% (Supporting information). The thermal treatment (GLMM;

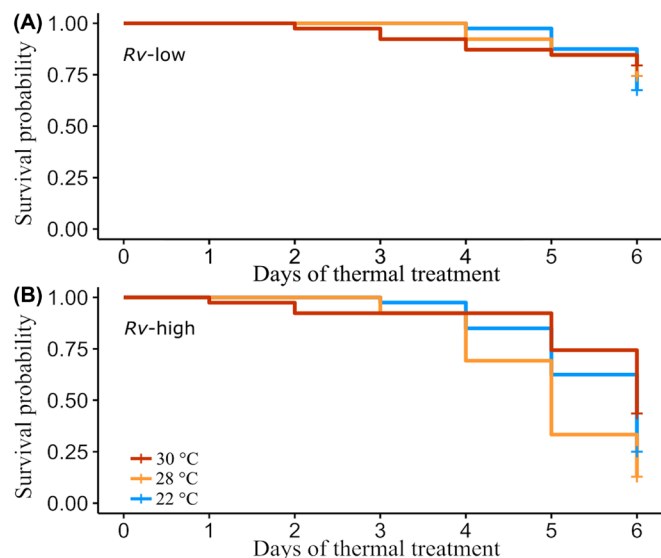


Figure 3. Survival probability of agile frog tadpoles (A) exposed to low *Rv* concentrations or (B) exposed to high *Rv* concentrations during the six days of thermal treatment visualised by Kaplan–Meier curves.

$\chi^2_2 = 10.943$, $p = 0.004$) significantly influenced *Bd* prevalence, but the presence and concentration of *Rv* did not ($\chi^2_2 = 2.379$, $p = 0.304$; Fig. 2C). The prevalence of *Bd* was higher in tadpoles treated at 22°C compared to those kept at 30°C (Supporting information). The interaction between thermal treatment and the presence and concentration of *Rv* was non-significant ($\chi^2_4 = 3.681$, $p = 0.45$).

The intensity of infection with *Bd* was highest after 22°C treatments in all *Bd*-exposed groups, with a dramatic drop in GE values in groups receiving 28 and 30°C treatments (Fig. 2C, Supporting information). The effect of co-exposure to *Rv* could not be assessed across all thermal treatments due to the very low prevalence observed at higher temperatures, but within the 22°C thermal treatment it was non-significant ($\chi^2_2 = 0.065$, $p = 0.968$).

The prevalence of *Rv* in *Rv*-exposed tadpoles varied between 0 and 40% (Fig. 2B, Supporting information) and was significantly influenced by thermal treatment ($\chi^2_2 = 11.92$, $p = 0.002$), *Bd* co-exposure ($\chi^2_1 = 5.43$, $p = 0.02$), and *Rv* concentration ($\chi^2_1 = 8.84$, $p = 0.003$). The prevalence of *Rv* was higher when tadpoles were treated at 22°C compared to 28 or 30°C, while it did not differ between animals treated at 28 and 30°C (Fig. 2B, Supporting information). Also, *Rv* prevalence was higher in the absence of *Bd* co-exposure and after receiving a high *Rv* concentration (Supporting information). Copy number of *Rv* was highest after the 22°C treatment, especially when tadpoles had been exposed to the high *Rv* concentration in the absence of *Bd* (Fig. 2B).

Discussion

Previous reports showed that interactions between *Bd* and *Rv* during co-infection can be negative and positive and can also result in neutral co-existence (Herczeg et al. 2021). Under natural circumstances, the secondary invader may encounter already triggered immune functions of the host, resulting in lowered replication. On the other hand, if the pathogen arriving second faces immune responses that are weakened by infection by the preceding pathogen, replication of the secondary agent may be facilitated (Ramsay and Rohr 2021). In the present study, previous exposure to *Bd* decreased *Rv* prevalence in both species. In contrast, *Bd* prevalence was not consistently affected by a single exposure to *Rv* that followed a prolonged exposure to *Bd*. However, some of the interactive effects of the two pathogens were modulated by the concentration of *Rv* inoculation and the subsequent thermal treatment. Generally, high *Rv* concentrations resulted in enhanced *Rv* prevalence in both hosts and higher viral loads in agile frogs. The dose of inoculum is a crucial factor that increases virulence and decreases average survival time towards high doses in amphibians (Brunner et al. 2005), which corresponds to our findings. The other general trend in our results was that the lowest infection prevalence and intensities were observed at 30°C, suggesting that the replication of the pathogens was lowest at 30°C. The outcome of these three effects (i.e. interactive effects of pathogens, *Rv*

concentration, and temperature) was complex. In agile frogs, *Bd* co-exposure increased *Rv* intensity in the low-*Rv* treatment at lower temperatures, but this effect was reversed at 30°C. This pattern was not observed in agile frog tadpoles exposed to high *Rv* concentration. In common toads, both high temperature treatments resulted in zero *Rv* prevalence in all groups excepting the combination of high *Rv* concentration and absence of *Bd* exposure. These findings highlight that co-infections can alter disease dynamics and they may do so in a temperature-dependent way.

Our results indicate that the thermal tolerance of the two pathogens might not be as different as previously thought. In the case of single *Bd* infections, a series of in vivo studies performed on larval and adult frogs demonstrated that elevated temperatures (approximately 26–30°C) could reduce *Bd* growth, enhance survival, or clear the pathogen burden depending on the host species, the applied temperature, and the duration of thermal treatment (Ribas et al. 2009, Chatfield and Richards-Zawacki 2011, Geiger et al. 2011). In contrast, the thermophilic nature of *Rv* documented by in vitro studies (Cunningham 2001) and supported by the observation that mortality is highest in the summer months (Chinchar 2002) put forward the hypothesis that *Rv* would become more virulent at higher temperatures, irrespective of co-infection with *Bd*. In contrast to this prediction, in our study, both host species exhibited lowered infection prevalence and intensity of *Rv* at 30°C. This had a crucial effect on fitness because, among the agile frog tadpoles exposed to *Rv*, those treated at 30°C had the lowest mortality. These findings have several implications for important conservation issues. First, temperature variability associated with anthropogenic climate change is one of the most current problems that can dramatically impact wildlife. More specifically, the interactions between climate warming and disease outbreaks have caused declines or even extinctions in several ectothermic hosts, including amphibians (Lafferty et al. 2004, Bruno et al. 2007, Rohr and Raffel 2010). A recent study by Thumsová et al. (2022) found strong evidence that climate warming may trigger outbreaks of CMTV. Our study found direct experimental evidence that if the temperature becomes higher than what is ideal for the pathogens (i.e. close to 30°C), it can reduce disease risk in amphibian larvae under simultaneous threat to two widespread pathogens. Finally, the artificial elevation of environmental temperature beyond the pathogens' optimum could serve as a basis for in situ conservation actions against chytridiomycosis and ranavirus (Hettyey et al. 2019).

Our study also revealed interspecific differences in pathogen resistance and tolerance. We found that agile frogs were highly resistant to the chytrid fungus but susceptible to ranaviral infection. At the same time, common toads were moderately resistant to both pathogens. These differences in prevalence were mirrored by patterns in mortality. Agile frogs exposed to *Rv* suffered considerable and concentration-dependent mortality in accordance with other amphibian-ranavirus systems (Hua et al. 2017): tadpoles that received high concentrations were less likely to survive than tadpoles

challenged with low concentrations (Brunner et al. 2005). In contrast, common toads experienced negligible mortality regardless of pathogen exposure. A previous experiment investigating the chemical defences against *Bd* reported a similarly low susceptibility to the fungus in the early life stages of agile frogs compared to common toads (Ujszegi et al. 2021). The susceptibility of amphibian species to chytridiomycosis has been related to the presence/absence of cytolytic skin-secreted antimicrobial peptide (AMP) profiles (Woodhams et al. 2007, Tennessen et al. 2009). Accordingly, the resistance of agile frogs to *Bd* might be related to their AMP production (e.g. Brevinin-1 Da; Conlon et al. 2004). However, as agile frogs in our study carried *Rv* at high prevalence, this line of defence appeared ineffective against *Rv* infection. AMPs can directly inactivate plaque formation of FV3 in vitro, but they do not inhibit viral replication in infected cells (Chinchar et al. 2001). In contrast, bufonid toads lack skin-secreted AMPs (Conlon 2011) but, from early larval development, they produce bufadienolide compounds (Üveges et al. 2017) with antimicrobial activity, which can protect against *Bd* (Cunha Filho et al. 2005, Barnhart et al. 2017) and might protect against *Rv*. Furthermore, while AMPs are present exclusively on skin surfaces and may be effective against skin invaders such as the chytrid fungus, bufadienolides are present not only in the skin but also in internal organs (Halliday et al. 2009) and thus might more successfully mitigate pathogens that target internal organs such as *Rv*. The effects of bufadienolides on *Rv* replication or co-infection with other pathogens are not yet explored but may hold promising potential for battling diseases.

In summary, our results suggest that high temperatures may be beneficial to amphibians exposed to both *Bd* and *Rv*. Also, while previous exposure to *Bd* affected *Rv* prevalence and – in some treatment combinations – *Rv* infection intensity as well, superinfection with *Rv* did not influence *Bd* infection intensity. Finally, temperature and co-infection appeared to also interact in their effects on pathogen load and disease progression. Nonetheless, in our study, both species exhibited relatively low prevalence and infection intensities except for *Rv* in agile frogs, so we urge further experimental studies on more susceptible species to scrutinise the effects of co-infection and external factors modulating its outcomes in amphibians. Finally, because the pathogenicity, virulence, and thermal ecology may vary between different lineages of the pathogens, future studies should also consider including other *Rv* and *Bd* strains in experiments exploring their temperature dependence, as well as in experiments scrutinizing the effects of temperature on infection probability and disease progression upon co-infection.

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Author contributions

Dávid Herczeg: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Investigation (equal); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead). **Dóra Holly:** Investigation (equal); Writing – review and editing (supporting). **Andrea Kásler:** Investigation (equal); Writing – review and editing (supporting). **Veronika Bókonyi:** Formal analysis (equal); Writing – review and editing (equal). **Tibor Papp:** Investigation (equal); Writing – review and editing (equal). **Hunor Takács-Vágó:** Investigation (equal). **János Ujszegi:** Investigation (equal); Writing – review and editing (equal). **Attila Hettyey:** Conceptualization (equal); Funding acquisition (lead); Writing – original draft (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Figshare Digital Repository: <https://doi.org/10.6084/m9.figshare.21702218.v1> (Herczeg et al. 2023).

Supporting information

The Supporting information associated with this article is available with the online version.

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