

Ecosphere

Article

Disease Ecology

Impacts of sequential and simultaneous coinfection by major amphibian pathogens on disease outcomes

Evelyn Faust^{a,*}, Tamílie Carvalho^{a,b,*}, Francisco DeJesus Andino^c, Jacques Robert^c, Timothy Y. James^a

^aDepartment of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, 48109, USA

^bInstitute for Global Change Biology, School for Environment and Sustainability, University of Michigan, Ann Arbor, MI, 48109, USA

^cDepartment of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, 14642, USA

To whom correspondence should be addressed: ¹tamiliec@gmail.com

Evelyn Faust and Tamílie Carvalho contributed equally to the work reported here.

Handling Editor: Andrea Swei

Keywords: Coinfections; Disease dynamics; Batrachochytrium dendrobatidis; Ranavirus; amphibian disease; within-host interactions

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/ecs2.70365](https://doi.org/10.1002/ecs2.70365)

This article is protected by copyright. All rights reserved.

Abstract

In natural environments, hosts frequently experience infections from multiple pathogenic species or strains, significantly influencing disease dynamics. Despite shared susceptible hosts and overlapping distributions, the impacts of coinfections by the two most threatening global amphibian pathogens, *Batrachochytrium dendrobatidis* (Bd) and Ranavirus (Rv), remain largely understudied. This study offers new insights into how simultaneous and sequential exposures to Bd and Rv influence disease outcomes in an amphibian host under controlled experimental conditions. Our findings reveal that the sequence and timing of pathogen exposure can lead to contrasting outcomes. Animals previously exposed to Rv displayed the highest mortality following Bd infection, whereas simultaneous exposure to both pathogens resulted in higher survival compared to single infections. These findings suggest that priority effects, driven by differences in the timing and order of pathogen exposure, can exacerbate disease severity in amphibian populations, particularly in communities with persistent, sublethal Rv infections. This study highlights the critical role of pathogen interactions in shaping disease dynamics and emphasizes the importance of integrating coinfections into wildlife disease management strategies to mitigate biodiversity crises in amphibians and beyond.

1. Introduction

Coinfection by multiple pathogens is common in nature and is known to influence disease dynamics by affecting pathogen virulence (*i.e.*, harm caused to the host) and transmission potential (May and Nowak 1997, Read and Taylor 2001, de Roode et al. 2005b, Alizon et al. 2013). Varied disease outcomes can arise from multiple infections depending on complex within-host interactions between pathogens, including competition for resources, immunological interactions, or pathogen genetic recombination (Alizon et al. 2013). Moreover, the order and timing of infections can shape these dynamics; earlier infections may deplete host resources or modulate the host immune system, thereby influencing the severity and progression of later infections (de Roode et al. 2005a, Hoverman et al. 2013, Devevey et al. 2015, Clay et al. 2020). Consequently, 'overall virulence' – the virulence resulting from the interaction between all coinfecting pathogens and the host – can exceed the virulence of the most virulent pathogen, fall below that of the least virulent pathogen, or exist at an intermediate level (Alizon et al. 2013). Additionally, within-host competition will also influence baseline pathogen virulence through the evolution of increased virulence, driven by stronger selection pressure for transmission (May and Nowak 1997, Read and Taylor 2001, de Roode et al. 2005b, Alizon et al. 2013). Given these complexities, effective wildlife disease management must account for coinfections, as focusing solely on a single pathogen may overlook critical within-host interactions and their broader implications for disease outcomes.

Amphibians represent the most threatened class of vertebrate animals (Luedtke et al. 2023), having recently experienced major declines. The chytrid fungus *Batrachochytrium dendrobatidis* (Bd), the agent of the disease chytridiomycosis, plays a pivotal role in the ongoing and largest disease-associated amphibian biodiversity loss in history (Scheele et al. 2019). Bd is

an extreme generalist pathogen (Carvalho et al. 2024a) that infects the keratinized skin cells of hundreds of amphibian species, potentially causing host death by disrupting epidermal function, depleting electrolytes, and ultimately leading to cardiac failure (Voyles et al. 2011). Meanwhile, another major amphibian pathogen, known as FV3 or Frog Virus 3, belonging to the genus Ranavirus (Rv), is also linked to mass mortality events and local amphibian extinctions worldwide (Price et al. 2014, Rosa et al. 2017, Brunner et al. 2025). As a nucleocytoplasmic large double-stranded DNA virus, Rv primarily infects epithelial cells (Robert et al. 2005), potentially resulting in host mortality through hemorrhaging (Forzán et al. 2017).

Despite the significance of both pathogens, which commonly overlap in range and coinfect amphibian hosts (Hoverman et al. 2012, Warne et al. 2016, Ruggeri et al. 2024), current knowledge of Bd and Rv epidemiology often comes from isolated studies focusing on single-pathogen infections (Fisher and Garner 2020, Brunner et al. 2025). The limited number of studies examining their interactions has yielded contradictory results, making it challenging to draw definitive conclusions. Field studies have reported both facilitative interactions — where one pathogen increases the load of the other or enhances the likelihood of infection by the second pathogen in previously infected hosts (Love et al. 2016, Hartmann et al. 2024) — and neutral associations, where no interaction occurs between the pathogens (Warne et al. 2016, Olori et al. 2018, Bosch et al. 2020, Thumsová et al. 2024). Experimental studies investigating Bd–Rv interactions are particularly scarce and have so far been limited to two using a single host species, the Cuban treefrog (*Osteopilus septentrionalis*), which has a predominantly terrestrial adult phase and relies on water mainly for breeding. One experimental study demonstrated that higher Bd doses during simultaneous exposures reduced Rv load (*i.e.*, negative interactions) (Ramsay and Rohr 2023), whereas another study showed that coinfection with either pathogen

increased the load of the other (*i.e.*, facilitative interactions) (Ramsay and Rohr 2021). This latter study also revealed that the time lag between pathogen exposures can influence disease progression, specifically when Bd is the secondary pathogen (*i.e.*, priority effect driven by the timing of exposure). Thus, there is limited and contrasting information about how within-host interactions during coinfection by Bd and Rv may impact the spread, progression, and severity of diseases. Consequently, coinfections could represent an overlooked mechanism underlying disease outbreaks.

By comparing single vs. multiple infection outcomes, including both simultaneous and sequential exposures, we investigated how Bd-Rv coinfection can affect host mortality risk, disease progression (*i.e.*, pathogen load at early and late stages of infection), and host body condition — a key determinant of fitness. We used *Hymenochirus boettgeri*, a fully aquatic amphibian species, as our experimental model. This life history trait is especially relevant given that both Bd and Rv can be transmitted through water, and transmission dynamics may differ substantially between aquatic and terrestrial hosts — the latter being the focus of prior experimental work. Including a fully aquatic species helps address this gap and provides insight into how coinfection might progress in other aquatic or semi-aquatic amphibians. Our findings reveal that simultaneous Bd-Rv coinfections can benefit the host by delaying disease progression and extending host lifespan, whereas prior Rv infection exacerbates Bd virulence, likely due to facilitative within-host priority effects driven by differences in infection order. This study expands the experimental basis for predicting outcomes in Bd-Rv coinfections, shedding light on the underlying mechanisms driving disease outbreaks and providing valuable insights to better address the multifaceted nature of wildlife diseases.

2. Methods

All procedures described here were conducted at the University of Michigan Animal Care Facilities following protocols approved by the University's Institutional Animal Care and Use Committee (IACUC protocol PRO00011269).

2.1. Pathogens and Amphibian Host

We selected *Batrachochytrium dendrobatidis* strain JEL423 for this experiment, a strain belonging to the highly virulent global panzootic lineage (Bd-GPL) and isolated from Panama during a Bd outbreak event. The Bd isolate is cryo-preserved at -80 °C in the Collection of Zoosporic Eufungi at the University of Michigan (CZEUM) and was revived for our experiments. Bd was grown on 1% tryptone for approximately 5 days at 20 °C, at which point cultures had abundant active zoospores. We collected zoospores by flooding the plates with 2 mL of sterile distilled water, allowing approximately 30 minutes to stimulate zoospore release. Sterile 1% tryptone plates were flooded using the same procedure to obtain sham inocula for control animals and for animals receiving pathogen-free water baths on day 10 (see experimental design section). To prepare Bd inoculation solutions with the desired concentration, we quantified zoospores in a 20 µL subsample of the collected suspension based on the cell diameter (2.5–5.0 µm) using a Coulter Counter (Multisizer 4e, Beckman Coulter, USA). Zoospore viability was confirmed by visual inspection of active motility under a light microscope. All animals exposed to Bd received a dose of 6×10^4 zoospores / mL in a 3 mL bath of autoclaved pre-conditioned RO (reverse osmosis) water. This dose was chosen based on previous study conducted in our laboratory using the same host species (see below) and Bd isolate JEL423 (Carvalho et al. 2023b), suggesting that this exposure would lead to substantial infection without overwhelming the animals or obscuring interactions with Rv during coinfection.

Author Manuscript

Infectious Rv used in this experiment was produced in baby hamster (*Mesocricetus auratus*) kidney cell cultures (BHK-21) incubated at 30 °C, purified, and quantified via plaque assay. It was then stored at -80 °C in APBS (Phosphate Buffer Saline adjusted to amphibian osmolarity) in 100 µL aliquots to prevent loss from frequent thawing. To prepare virus inocula, stocks were thawed and diluted into 3 mL of autoclaved pre-conditioned RO water baths. Sham exposures included the same volume of APBS. All animals exposed to Rv received a dose of 5×10^5 plaque forming units (PFU) / mL, a dose chosen based on pilot results (Appendix S1: Figure S1) suggesting that this exposure would cause substantial infection without overwhelming Bd co-exposed animals or obscuring potential interactions.

We used post-metamorphic *H. boettgeri* (Anura; *Pipidae*) bred in our facility for experimental infections. This species has been established as a low-ecological impact model suitable for studying Bd (Carvalho et al. 2023b, 2025), but has never before been tested as a model for studying Rv. In a series of pilot experiments, we showed that *H. boettgeri* is susceptible to Rv (FV3) and can become infected via a minimally invasive water bath exposure (see Appendix S1 for more detailed methods and results from the pilot experiment; Appendix S1: Figures S1, S2 and S3). We selected adults with approximately the same size (SVL = 25.77 ± 1.45 mm), mass (1.10 ± 0.14 g), and age (7 months old), each treatment group comprising 5 males and 5 females for a total of 60 animals. None were previously removed from the uninfected healthy colony at our facility, and thus were originally naïve to both Bd and Rv. To confirm that all animals were uninfected prior to inoculation, a random 40% subset of each treatment and control group was tested on day 0 and returned negative results for both pathogens.

All animal care and housing procedures in this experiment were conducted following the methods outlined in a previous study (Carvalho et al. 2023b). To briefly summarize, animals

Author Manuscript

were individually housed in conditioned RO water in 1 L plastic tanks enriched with a 1 cm layer of gravel and a small PVC tube. Tank water was maintained at a controlled temperature of 20 °C, in a room with a 13:11 light-dark cycle. Daily, we checked a random 10% subset of tanks for water conductivity, temperature, and pH, ensuring they remained within an acceptable range. Additionally, we tested another random 10% subset for safe nitrate, nitrite, and ammonia levels at least biweekly. Three times per week, we performed water changes to remove excess nitrogenous wastes. Animals were also fed *ad libitum* three times weekly, alternating between gamma-irradiated bloodworms (used twice per week) and small-granule fish food (used once per week). Throughout the experiment, animals were carefully observed daily for symptoms of excess skin sloughing, hemorrhaging or lesions, erratic swimming, buoyancy issues, bloating, lack of righting response, and general lethargy. Individuals exhibiting a lack of righting response and/or moderate lethargy with other clinical symptoms were promptly euthanized using MS-222, as were surviving animals at experimental endpoint.

2.2. Experimental Design

The five experimental treatments comprised an Rv-only group, a Bd-only group, an Rv-first group ($Rv \rightarrow Bd$), a Bd-first group ($Bd \rightarrow Rv$), and a simultaneously inoculated group (BdRv; see inoculation schematic in Figure 1). Pathogen or sham exposures were performed on day 0 and day 10 by placing each animal into a 50 mL falcon tube for 3 hours with 3 mL of autoclaved pre-conditioned RO water and the inoculum corresponding to their treatment group for that day. Secondary inoculation at day 10 was intended to allow substantial establishment of the primary pathogen and the host immune response. Animals only receiving an inoculum on day 0 (*i.e.*, Bd, Rv, and BdRv) also received a sham exposure on day 10 to ensure consistent levels of bath-

induced stress across groups. After exposures, animals were returned to their individual tanks along with the contents of the water bath.

Skin swabs to detect Bd and Rv loads throughout disease progression were collected on day 0, day 10, day 20, and at time of death (variable) or at the experimental endpoint for surviving animals (day 34). Throughout this paper, ‘day 20’ of the experiment is occasionally referred to as day 10 post-exposure to one of the pathogens, reflecting the sequential inoculation timing between the groups. For each swab, we rinsed the animal with sterile water for 5 s and collected skin swabs (using new disposable gloves for each animal) following a standard protocol for detecting Bd (Hyatt et al. 2007). We demonstrated that the same skin swab protocol also enabled Rv load detection during the pilot experiment (Appendix S1: Figures S2 and S3). To track body condition changes over the course of disease, we also recorded mass and snout-vent-length (SVL) for all animals at the swab timepoints described above. However, for individuals found dead during the experiment (n = 9), mass and SVL were not recorded at time of death, as they could be altered by post-mortem water absorption.

2.3. *Bd and Rv Quantification from Swabs*

DNA was extracted from swabs using the Qiagen Blood and Tissue DNeasy extraction kit, following manufacturer guidelines with an added membrane-drying step and twice eluting 50 µL of UV-sterilized nuclease-free water for increased yield recovery. Pathogen intensity (load) was quantified using a standard qPCR assay on a QuantStudio 3 (Applied Biosystems) machine. For Bd, qPCR was performed following Boyle et al. (2004). For Rv quantification, we followed manufacturer recommendations for SYBR Green (BioRad SsoAdvanced Universal SYBR Green Supermix), using 2.5 µL of template DNA in 10 µL reactions, and primers targeting the Major

Capsid Protein gene region (FV3-090R-MCP (AN: Q67473 - 1442 bp) MH332773.1, primers: GTCCTTTAACACGGCATACT, GATAGGCAACACCAGCGAT).

Standard curves for Bd quantification were generated from serial dilutions (10^6 - 10^0 zoospore equivalents per 100 μ L) of a JEL423 culture DNA extraction. Standard curves for Rv were produced by inserting the FV3 Major Capsid Protein gene into a pGEM T-Easy Vector, transforming into DH10 β bacteria, and preparing serial dilutions (10^8 - 10^0 plasmids or MCP gene copies per 2.5 μ L). All standards and negative controls (UV-sterilized nuclease-free water) were run in triplicate to ensure accurate standard curve generation and potential contamination detection. Samples were run in duplicate to verify accurate quantification. The raw quantity of Bd from qPCR reflects the total zoospore equivalents extracted from a given swab into 100 μ L, while raw quantities of Rv were multiplied by 40 (dilution factor of standards volume:DNA extraction elution volume) in order to reflect the total MCP gene copies extracted from a given swab (proxy for viral particles). Mean pathogen loads of replicates were recorded for all positive samples.

Samples were considered Bd-positive with raw quantities ≥ 1 zoospore equivalent for both replicates, or 2 out of 3 if initial replicate inconsistency warranted repetition of that run. Samples were considered Rv-positive with adjusted quantities ≥ 80 MCP gene copies for both replicates, or 2 out of 3 if initial inconsistency warranted repetition. These thresholds were based on maximum levels of baseline detection observed in swabs from uninfected individuals tested before the experiment, likely arising from spurious amplification products. To maintain the integrity of the experimental treatments, some individuals were omitted from analysis because a positive Rv load was never detected by skin swab (1 animal from Rv group, 2 from Bd $\rightarrow$ Rv, 2 from Rv $\rightarrow$ Bd, and 2 from BdRv). For these animals, kidney/liver samples were also analyzed,

confirming the absence of Rv. Further details on viral detection from organs can be found in Appendix S1.

2.4. Statistical Analyses

To assess differences in host mortality among treatments, we built Kaplan-Meier survival curves for each treatment group using the function *survfit* from the *survival* package in R (Therneau 2024) and compared results using log-rank tests implemented by the *survdif* function also from the *survival* package. Post-hoc comparisons of survival curves were computed using the *pairwise_survdif* function from the *survminer* package (Kassambara et al. 2021), adjusting *p*-values for false discovery rate using the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

To evaluate differences in infection load among treatments at specific time points, we log-transformed qPCR-derived quantities and performed an analysis of variance (ANOVA) using the *aov* function from the *stats* package with treatments as fixed effects. We then performed Tukey's post hoc tests using the *TukeyHSD* function also from the *stats* package. Post hoc significance was validated using the *glht* function in the *multcomp* package (Hothorn et al. 2008). We used the same methods to compare body condition between treatments at each experimental time point. As a well-accepted representation of body condition (Brodeur et al. 2020), a Scaled Mass Index (SMI) was calculated from mass and SVL measurements (Peig and Green 2009). The scaling component (R) of the SMI equation, or the regression value of mass on SVL for the whole population, was calculated via Reduced Major Axis regression using online software (Bohonak and van der Linde 2004) and log-transformed inputs. This value was calculated separately for males (2.668) and females (2.397) and included measurements from all time

points. To assess the relative contributions of lifespan and treatment group on SMI differences at time of death, we ran and compared a series of linear regression models using the *lm* function in the *stats* package, implementing lifespan and/or treatment group, or the interaction of the two, as fixed effects. Paired *t* tests (*t.test* function) were used to determine differences in both pathogen load and SMI between time points. All analyses were conducted in R version 4.2.2 (R Core Team 2023).

3. Results

Survival probability was significantly different between treatment groups (log-rank test: $X^2 = 68.9$, $df = 5$, $p < 0.002$; Figure 2A; Appendix S1: Table S1). Animals exposed to Bd-only or Bd prior to Rv succumbed to infection the fastest, with nearly identical survival probability throughout the infection course (Figure 2A; Appendix S1: Table S1). Notably, there was also no statistical difference in survival between the control group with 0 deaths and the Rv-only group with 2 deaths ($p > 0.05$), suggesting that mortality was primarily associated with Bd infection. This reduced mortality in the Rv-only group, compared to the pilot study, is likely due to the slightly lower viral dose used in the main experiment. Further supporting Bd as the main driver of mortality, Rv loads in coinfecting animals at time of death (Appendix S1: Figure S4) were typically lower than Rv loads at time of death from our pilot experiment (Appendix S1: Figure S3), during which singly infected animals did experience Rv-associated mortality. Furthermore, all individuals surviving until experimental endpoint (day 34) had completely cleared Rv infection (load < 80 MCP gene copies), except one animal with a low viral load (184.32 MCP gene copies). Because these results indicate that Bd was the primary pathogen influencing host

mortality, here we present analyses of survival probability, lifespan, and Bd infection load in terms of days following exposure to Bd.

As exposure to Bd in the Rv→Bd group occurred 10 days after the beginning of the experiment, we excluded these 10 days in our reanalysis of the survival curve. After this adjustment, survival probability following exposure to Bd was still significantly different between treatment groups (Log-rank test: $X^2 = 64.5$, $df = 4$, $p < 0.004$; Figure 2B; Appendix S1: Table S2). Animals previously infected with Rv (hereafter considered a pre-existing, low-level infection) succumbed the fastest to Bd once exposed, faster than the Bd-alone group by approximately 4.1 ± 4.04 days (mean difference in lifespan $\pm$ standard deviation). On the other hand, animals simultaneously exposed to Bd and Rv survived longer than the Bd-only group by approximately 5.1 ± 4.51 days (mean difference in lifespan $\pm$ standard deviation). Animals in the control group showed no symptoms of either disease and all tested negative for both pathogens at the end of the experiment, suggesting that mortality was directly associated with infection from experimental inoculation.

Bd establishment (*i.e.*, load on day 10 after Bd exposure) was hindered in simultaneously coinfecting animals (BdRv group) compared to other Bd-exposed treatment groups ($F = 6.65$, $df = 3, 30$, $p = 0.001$; Figure 3; Appendix S1: Table S3). Meanwhile, sequential inoculation groups (*i.e.*, Bd→Rv and Rv→Bd) were not statistically different from the Bd-only group in terms of Bd load establishment ($p > 0.05$; Appendix S1: Table S3). There were also no significant differences in Rv loads between Rv-exposed treatment groups at any given swab time point following exposure to Rv, nor any within-group differences in Rv loads between early (day 10) and late (time of death) time points (Appendix S1: Figure S4, Table S4).

While Bd load was lower for simultaneously coinfecting BdRv animals at the early stage of disease, Bd-exposed individuals had similar Bd loads at time of death regardless of treatment group ($F = 1.40$, $df = 3, 29$, $p > 0.05$; Figure 4). However, as host lifespan varied between Bd-exposed groups ($F = 14.44$, $df = 3, 30$, $p < 0.001$; Figure 4; Appendix S1: Table S5), the growth rate of Bd load also varied between groups. In other words, the Bd loads that were reached in only 10 days post-exposure (on average) in the Rv→Bd group, were reached in a mean of 19.5 days in the simultaneously inoculated group, and 14.2 and 14.4 days in the Bd-only and Bd→Rv groups, respectively (Appendix S1: Table S6). Bd therefore grew the fastest in individuals previously infected with Rv (Rv→Bd), slowest in simultaneously inoculated animals (BdRv), and nearly the same between the Bd-only and Bd→Rv groups, suggesting that the transmission potential of Bd is increased in animals previously infected with Rv. However, Bd infecting the Bd→Rv group only grew for approximately 4 days in the presence of Rv prior to death. This possibly indicates that the harm caused by this dosage of Bd during those first 10 days overshadowed the consequences of coinfection, making it difficult to draw conclusions about the effects of late exposure coinfecting Rv.

At day 10, there was no significant variation in body condition (assessed as SMI in grams) among treatments or the control group ($p > 0.05$; Figure 5). At time of death (TOD), body condition of the Rv→Bd group appeared lower than the control and Rv-only groups (Tukey's HSD adjusted p -values of 0.058 and 0.065, respectively, after removing one high Rv→Bd outlier; Figure 5). If not for their infection status of the Rv→Bd group, a longer total-experiment lifespan (*i.e.* longer than that of Bd-alone) would otherwise predict more host growth over time, demonstrated by the upward body condition trend from day 10 to endpoint in the control and Rv-only groups (Figure 5). While these are interesting preliminary trends, we believe the short

timeframe of this experiment may obscure body condition comparisons, none of which were statistically significant ($p > 0.05$). The residual variation in TOD body condition that was not explained by host lifespan was also not well explained by treatment group ($F = 0.57$, $df = 5, 38$, $p > 0.05$). There were no significant differences in body condition measured at day 0 ($p > 0.05$).

4. Discussion

Bd and Rv individually threaten amphibian communities worldwide (Scheele et al. 2019, Brunner et al. 2025), and theory predicts that mixed infections have the potential to substantially alter epidemiological dynamics (May and Nowak 1997, Alizon et al. 2013). However, despite being common in wild amphibians, it remains unclear how Bd-Rv coinfections impact infectious processes within and between hosts. Here, we provide new insights into how coinfections can lead to differential outcomes in disease progression and host mortality, depending on the timing and order of exposures. Most notably, we found that a pre-existing Rv infection significantly worsened disease outcomes following exposure to Bd, suggesting a facilitative priority effect due to the order of infection. In contrast, simultaneous co-exposure to both pathogens protected hosts compared to single Bd infections. These findings highlight the potential for disproportionate Bd-associated damage to communities with otherwise low-level, persistent Rv infections. We emphasize the importance of incorporating coinfection dynamics into disease models and management strategies, alongside enhanced monitoring of both pathogens. This approach will enhance disease risk assessments and support more targeted conservation actions, such as optimizing intervention timing and identifying priority areas to mitigate the impact of these pathogens in the field.

4.1. Pre-existing Rv Infection Exacerbates Bd Infection

Our results suggest that a pre-existing Rv infection can exacerbate host mortality and Bd growth rates over the course of coinfection. Notably, even a sublethal Rv dose incurred this decrease in resistance to Bd – a highly relevant result given the demonstrated persistence of subclinical Rv infections in nature (Duffus et al. 2015, Brunner et al. 2025), and the underestimation of subclinical viral prevalence more generally (Lachish et al. 2012). Possibly contributing to its persistence, Rv is also known to exhibit quiescence (a non-replicating latent phase) in the macrophages of otherwise healthy *Xenopus laevis* adults for several months (Robert et al. 2007, 2014). Thus, communities harboring persistent Rv – perhaps maintained by viral quiescence, or by tolerant reservoir hosts like the American Bullfrog (*Aquarana catesbeiana*) (Brunner et al. 2019) – may be at higher mortality risk from Bd, underscoring the importance of managing Bd in these regions.

Additionally, we should consider how the seasonal overlap of Bd and Rv in the field might facilitate certain outcomes over others. Rv-associated die-offs are often reported during the summer months (Brunner et al. 2025), and the severity of Rv infections can correlate with higher temperatures (Price et al. 2017) than those associated with Bd (Neely et al. 2020, Carvalho et al. 2024b). Thus, we speculate that coinfection risks may peak during the transition from warmer to colder temperatures, when Rv infections remain high and Bd begins to rise, increasing the likelihood of the "Rv-first" scenario worsening Bd outcomes. This period may represent a critical window for implementing interventions to control Rv or Bd, thereby preventing the onset of coinfection. However, temperature-induced changes to amphibian immune function may create a disconnect between pathogen replication optima and host survival (Rojas et al. 2005, Neely et al. 2020), complicating predictions along with host susceptibility

factors like species phylogeny, breeding ecology, and life history (Hoverman et al. 2011, Blaustein et al. 2018, Carvalho et al. 2024b), ultimately calling for more empirical work.

Several underlying mechanisms may explain why pre-existing Rv infection predisposes hosts to worse disease outcomes. First, battling Rv infection for 10 days may overwork the immune system and deplete host energy stores, weakening the subsequent fight against Bd. The somewhat lower body condition at time of death in the Rv→Bd group suggests that hosts sacrifice growth or resource acquisition for immune processes, reflecting a well-established energy tradeoff (Lochmiller and Deerenberg 2000). Moreover, the inflammation characteristic of Rv infections may escalate disease and lower host survival (Grayfer et al. 2015) by compounding immunopathology (tissue damage) when combined with the inflammatory cascade triggered by Bd (Grogan et al. 2018). Ramsay & Rohr (2021) found that Bd load from a secondary infection increased with time since the start of a previous Rv infection, suggesting that this burden on hosts may amplify with longer time lags between exposures. Alternatively, Rv may actively suppress the immune system and weaken the host's ability to fight Bd as a secondary pathogen. In fact, several Rv-encoded proteins or protein homologs are known to dampen immune function by modulating inflammatory molecules (Chinchar et al. 2009, Besch et al. 2009). While Bd is also known to suppress the host immune system, we did not resolve its potential effects on secondary Rv infection because animals only lived an average of four days following Rv exposure, not different from the Bd-only group in terms of survival.

Perhaps in conjunction with indirect immune mechanisms, diseased state physiology may also contribute to worsened outcomes. For example, Bd infection is correlated with lower plasma osmolarity in several species (Voyles et al. 2007, Marcum et al. 2010), resulting from electrolytic dysregulation that ultimately leads to cardiac failure. Aligning with Bd pathogenesis (Miller et

al. 2008), Rv infection causes hemorrhaging in the kidneys' glomeruli and renal tubules, possibly preventing electrolyte reabsorption back into the blood (Bollinger et al. 1999, Docherty et al. 2003). When hosts are initially infected with Rv, physiological changes like osmotic stress may magnify the pathophysiology from a secondary Bd infection, potentially explaining the heightened host mortality in the Rv→Bd group. Further investigation of these proposed mechanisms can help uncover context and interventions for harmful coinfection dynamics.

4.2. Simultaneous Pathogen Co-exposure Protects Hosts

When animals were exposed simultaneously to both pathogens, we observed the opposite disease outcome pattern compared to the Rv→Bd group: a slower establishment of Bd, which led to increased host lifespan. Meanwhile, Rv load went unchanged. This relative protection from simultaneous co-exposure may have resulted from within-host competition between pathogens or elevated immune reactivity to the combined dose (Watters et al. 2018), which may help mitigate disease severity.

Evaluating the feasibility of competition as a mechanism involves considering the host niches that each pathogen occupies: while the kidney is a major replication site for Rv, amphibians can also develop a detectable skin infection (Robert et al. 2011), the target site for Bd. Moreover, recent findings suggest that Rv can bind to, aggregate, and infect Bd, potentially reducing Bd's ability to infect hosts' skin during co-exposure (De Jesús Andino et al. 2024). However, the authors found that FV3 infection following Bd infection increased Bd loads and host mortality. The effect of Rv hyperparasitism on Bd infectivity may therefore depend on exposure timing: perhaps simultaneous co-exposure reverses the Bd virulence enhancement demonstrated in certain sequentially inoculated groups. It is possible that a combination of

interference or local resource competition between co-exposed pathogens in the skin may diminish Bd load, at least during earlier stages in disease (*i.e.*, day 10). Although we assessed Bd zoospore viability prior to inoculum preparation, we did not evaluate viability after combining Bd and Rv in the co-exposure treatment. Thus, we cannot rule out the possibility that direct pathogen–pathogen interactions in the inoculum may have influenced initial Bd infectivity.

Alternatively, or alongside pathogen reduction from competition, cross-reactive immunity (immunity combating both infections) may indirectly enhance host protection. For example, it is possible that simultaneous exposure amplifies production of cross-reactive antimicrobial peptides (AMPs) in amphibian skin (such as magainin II (Rollins-Smith 2009)), quelling initial infections more effectively. In the sequential-inoculation group, AMP production initially stimulated by Rv may have subsided by the time Bd was introduced, failing to provide the same level of protection. Cross-reactive immunity promoted by simultaneous exposure may alternatively involve type I interferons (IFNs), which could provide context-dependent protection (McNab et al. 2015) when induced by both Rv and Bd in the skin (Wendel et al. 2017, Grogan et al. 2020). Perhaps the IFN response to Rv had subsided by the time Bd was introduced as a secondary pathogen in the sequential-inoculation group, reducing cross-reactive synergy. Once again, further exploration of these proposed mechanisms can help explain timing-dependent outcomes and their effect on natural communities.

4.3. Conclusion

Our findings reveal a potential threat that warrants immediate attention and action. In terms of disease severity, amphibian communities with a history of persistent Rv may be more vulnerable to Bd than communities without Rv, highlighting the significant danger of Bd in these

areas and the urgent need for more Rv monitoring. These insights call for improved monitoring programs that account for both pathogens and their interactions across multiple time scales.

While additional surveillance and modeling efforts are necessary to predict outcomes at population and community scales, individual-level effects of coinfection have the potential to influence downstream transmission, epidemic severity, and pathogen virulence evolution. We acknowledge that coinfection outcomes may vary depending on host species, particularly given differences in host susceptibility and immune responses. However, as the first experimental study to investigate coinfection by Bd and Rv in a fully aquatic amphibian host, our findings offer valuable insight into how coinfection might unfold in other susceptible species with similar natural histories, such as threatened species in the genus *Telmatobius*. Due to previously limited surveillance and/or the plausibly high lethality of coinfection preventing its detection, coinfection may have been overlooked as a factor in past Bd outbreaks and amphibian declines.

Our study contributes to a growing body of research in amphibians demonstrating that coinfections can influence multiple aspects of host–pathogen dynamics, including host survival, pathogen transmission, and shifts in associated microbial communities (Medina et al. 2021, Herczeg et al. 2021, Ramsay and Rohr 2023, Carvalho et al. 2023a). Thus, incorporating coinfection dynamics into conservation planning is crucial for mitigating future disease outbreaks and preserving vulnerable amphibian populations. In combination with more field monitoring, these experimental coinfection outcome predictions aid the implementation of more efficient management strategies. While our research focuses on amphibians, the insights gained here have broader implications for understanding pathogen dynamics and managing disease outbreaks in other wildlife and even human populations.

Acknowledgements. We thank members of the U. Michigan Unit for Laboratory Animal Medicine for their advice and support (Dr. Jill Keller, Kate Clifton, Allison Kulka, and Josh Kirk), Michelle Orozco-Quime for laboratory support, and the frogs that were involved in the study. The authors acknowledge generous support from the Gordon and Betty Moore Foundation Award number 9337 (10.37807/GBMF9337) and the Canadian Institute for Advanced Research program Fungal Kingdom: Threats and Opportunities. The *Xenopus laevis* Research Resource for Immunobiology is supported by the National Institute of Health (R24-AI-05983).

Author Contribution

EF, TC and TJ conceptualized the study with contributions from FDA and JR. EF, TC and FDA conducted the laboratory methods. EF drafted the initial manuscript and performed most of the analysis. EF and TC analyzed the data and continued drafting the manuscript. All authors carried out the study and critically revised the manuscript.

Declaration of Interest.

The authors declare no competing interest.

DATA AVAILABILITY STATEMENT

Data (Faust et al., 2025) are available from Dryad: <https://doi.org/10.5061/dryad.70rxwdc71>.

References

- Alizon, S., J. C. de Roode, and Y. Michalakis. 2013. Multiple infections and the evolution of virulence. *Ecology Letters* 16:556–567.
- Benjamini, Y., and Y. Hochberg. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)* 57:289–300.
- Besch, R., H. Poeck, T. Hohenauer, D. Senft, G. Häcker, C. Berking, V. Hornung, S. Endres, T. Ruzicka, S. Rothenfusser, and G. Hartmann. 2009. Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells. *The Journal of Clinical Investigation* 119:2399–2411.
- Blaustein, A. R., J. Urbina, P. W. Snyder, E. Reynolds, T. Dang, J. T. Hoverman, B. Han, D. H. Olson, C. Searle, and N. M. Hambalek. 2018. Effects of Emerging Infectious Diseases on Amphibians: A Review of Experimental Studies. *Diversity* 10:81.
- Bohonak, A. J., and K. van der Linde. 2004. RMA: Software for Reduced Major Axis regression.
- Bollinger, T. K., J. Mao, D. Schock, R. M. Brigham, and V. G. Chinchar. 1999. PATHOLOGY, ISOLATION, AND PRELIMINARY MOLECULAR CHARACTERIZATION OF A NOVEL IRIDOVIRUS FROM TIGER SALAMANDERS IN SASKATCHEWAN. *Journal of Wildlife Diseases* 35:413–429.
- Bosch, J., C. Monsalve-Carcaño, S. J. Price, and J. Bielby. 2020. Single infection with *Batrachochytrium dendrobatidis* or Ranavirus does not increase probability of co-infection in a montane community of amphibians. *Scientific Reports* 10:21115.
- Boyle, D. G., D. B. Boyle, V. Olsen, J. a. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60:141–148.
- Brodeur, J. C., M. J. Damonte, J. Vera Candioti, M. B. Poliserpi, M. F. D'Andrea, and M. F. Bahl. 2020. Frog body condition: Basic assumptions, comparison of methods and

characterization of natural variability with field data from *Leptodactylus latrans*.

Ecological Indicators 112:106098.

Brunner, J. L., A. D. Olson, J. G. Rice, S. E. Meiners, M. J. L. Sage, J. A. Cundiff, C. S.

Goldberg, and A. P. Pessier. 2019. Ranavirus infection dynamics and shedding in American bullfrogs: consequences for spread and detection in trade. *Diseases of Aquatic Organisms* 135:135–150.

Brunner, J. L., A. Storfer, E. H. Le Sage, T. W. J. Garner, M. J. Gray, and J. T. Hoverman. 2025.

Ranavirus Ecology: From Individual Infections to Population Epidemiology to Community Impacts. Pages 231–270 in M. J. Gray and V. G. Chinchir, editors. *Ranaviruses: Emerging Pathogens of Ectothermic Vertebrates*. Springer Nature Switzerland, Cham.

Carvalho, T., A. M. Belasen, L. F. Toledo, and T. Y. James. 2024a. Coevolution of a generalist pathogen with many hosts: the case of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Current Opinion in Microbiology* 78:102435.

Carvalho, T., D. Medina, and T. Y. James. 2025. Successful Transmission and Isolation of a Fungal Pathogen From Wild Frogs to a Captive Amphibian Model Species: Fine Scale Pathogen Genetic Diversity and Infection-Induced Changes in Skin Bacteria. *Environmental Microbiology* 27:e70136.

Carvalho, T., D. Medina, L. P. Ribeiro, D. Rodriguez, T. S. Jenkinson, C. G. Becker, L. F.

Toledo, and J. L. Hite. 2023a. Coinfection with chytrid genotypes drives divergent infection dynamics reflecting regional distribution patterns. *Communications Biology* 6:941.

Carvalho, T., D. Medina, R. Rebouças, C. G. Becker, and L. F. Toledo. 2024b. Thermal

mismatch explains fungal disease dynamics in Brazilian frogs. *Perspectives in Ecology and Conservation* 22:72–78.

- Carvalho, T., C. Si, R. A. Clemons, E. Faust, and T. Y. James. 2023b. Amphibian Hymenochirus boettgeri as an experimental model for infection studies with the chytrid fungus Batrachochytrium dendrobatidis. *Virulence* 14:2270252.
- Chinchar, V. G., A. Hyatt, T. Miyazaki, and T. Williams. 2009. Family Iridoviridae: Poor Viral Relations No Longer. Pages 123–170 in J. L. Van Etten, editor. *Lesser Known Large dsDNA Viruses*. Springer, Berlin, Heidelberg.
- Clay, P. A., M. A. Duffy, and V. H. W. Rudolf. 2020. Within-host priority effects and epidemic timing determine outbreak severity in co-infected populations. *Proceedings of the Royal Society B: Biological Sciences* 287:20200046.
- De Jesús Andino, F., A. Davydenko, R. J. Webb, and J. Robert. 2024. The Binding, Infection, and Promoted Growth of Batrachochytrium dendrobatidis by the Ranavirus FV3. *Viruses* 16:154.
- Devevey, G., T. Dang, C. J. Graves, S. Murray, and D. Brisson. 2015. First arrived takes all: inhibitory priority effects dominate competition between co-infecting Borrelia burgdorferi strains. *BMC Microbiology* 15:61.
- Docherty, D. E., C. U. Meteyer, J. Wang, J. Mao, S. T. Case, and V. G. Chinchar. 2003. DIAGNOSTIC AND MOLECULAR EVALUATION OF THREE IRIDOVIRUS-ASSOCIATED SALAMANDER MORTALITY EVENTS. *Journal of Wildlife Diseases* 39:556–566.
- Duffus, A. L. J., T. B. Waltzek, A. C. Stöhr, M. C. Allender, M. Gotesman, R. J. Whittington, P. Hick, M. K. Hines, and R. E. Marschang. 2015. Distribution and Host Range of Ranaviruses. Pages 9–57 in M. J. Gray and V. G. Chinchar, editors. *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Springer International Publishing, Cham.
- Faust, E. 2025. <https://BioRender.com/waqee1x>

- Faust, E., T. Carvalho, F DeJesus Andino, J. Robert, and T. Y. James. 2025. Data from: Impacts of sequential and simultaneous coinfection by major amphibian pathogens on disease outcomes. Dataset. Dryad. <https://doi.org/10.5061/dryad.70rxwdc71>
- Fisher, M. C., and T. W. J. Garner. 2020. Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology* 18:332–343.
- Forzán, M. J., K. M. Jones, E. Ariel, R. J. Whittington, J. Wood, R. J. F. Markham, and P.-Y. Daoust. 2017. Pathogenesis of Frog Virus 3 (Ranavirus, Iridoviridae) Infection in Wood Frogs (*Rana sylvatica*). *Veterinary Pathology* 54:531–548.
- Grayfer, L., E.-S. Edholm, F. De Jesús Andino, V. G. Chinchar, and J. Robert. 2015. Ranavirus Host Immunity and Immune Evasion. Pages 141–170 *in* M. J. Gray and V. G. Chinchar, editors. *Ranaviruses*. Springer International Publishing, Cham.
- Grogan, L. F., J. E. Humphries, J. Robert, C. M. Lanctôt, C. J. Nock, D. A. Newell, and H. I. McCallum. 2020. Immunological Aspects of Chytridiomycosis. *Journal of Fungi* 6.
- Grogan, L. F., J. Robert, L. Berger, L. F. Skerratt, B. C. Scheele, J. G. Castley, D. A. Newell, and H. I. McCallum. 2018. Review of the Amphibian Immune Response to Chytridiomycosis, and Future Directions. *Frontiers in Immunology* 9.
- Hartmann, A. M., K. Sash, E. P. Hill, N. M. Claunch, M. L. Maddox, S. McGrath-Blaser, C. C. McKinstry, R. J. Ossiboff, and A. V. Longo. 2024. Partitioning the influence of host specificity in amphibian populations threatened by multiple emerging infectious diseases. *Biological Conservation* 296:110685.
- Herczeg, D., J. Ujszegi, A. Kásler, D. Holly, and A. Hettyey. 2021. Host–multiparasite interactions in amphibians: a review. *Parasites & Vectors* 14:296.
- Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous Inference in General Parametric Models. *Biometrical Journal* 50:346–363.

- Hoverman, J. T., M. J. Gray, N. A. Haislip, and D. L. Miller. 2011. Phylogeny, Life History, and Ecology Contribute to Differences in Amphibian Susceptibility to Ranaviruses. *EcoHealth* 8:301–319.
- Hoverman, J. T., B. J. Hoyer, and P. T. J. Johnson. 2013. Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. *Oecologia* 173:1471–1480.
- Hoverman, J. T., J. R. Mihaljevic, K. L. D. Richgels, J. L. Kerby, and P. T. J. Johnson. 2012. Widespread Co-occurrence of Virulent Pathogens Within California Amphibian Communities. *EcoHealth* 9:288–292.
- Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, R. Campbell, G. Marantelli, F. Gleason, and A. Colling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 73:175–192.
- Kassambara, A., M. Kosinski, P. Biecek, and S. Fabian. 2021, March 9. *survminer: Drawing Survival Curves using “ggplot2.”*
- Lachish, S., A. M. Gopalaswamy, S. C. L. Knowles, and B. C. Sheldon. 2012. Site-occupancy modelling as a novel framework for assessing test sensitivity and estimating wildlife disease prevalence from imperfect diagnostic tests. *Methods in Ecology and Evolution* 3:339–348.
- Lochmiller, R. L., and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* 88:87–98.
- Love, C. N., M. E. Winzeler, R. Beasley, D. E. Scott, S. O. Nunziata, and S. L. Lance. 2016. Patterns of amphibian infection prevalence across wetlands on the Savannah River Site, South Carolina, USA. *Diseases of Aquatic Organisms* 121:1–14.
- Luedtke, J. A., J. Chanson, K. Neam, L. Hobin, A. O. Maciel, A. Catenazzi, A. Borzée, A. Hamidy, A. Aowphol, A. Jean, Á. Sosa-Bartuano, A. Fong G., A. de Silva, A. Fouquet, A.

- Angulo, A. A. Kidov, A. Muñoz Saravia, A. C. Diesmos, A. Tominaga, B. Shrestha, B. Gratwicke, B. Tjaturadi, C. C. Martínez Rivera, C. R. Vásquez Almazán, C. Señaris, S. R. Chandramouli, C. Strüßmann, C. F. Cortez Fernández, C. Azat, C. J. Hoskin, C. Hilton-Taylor, D. L. Whyte, D. J. Gower, D. H. Olson, D. F. Cisneros-Heredia, D. J. Santana, E. Nagombi, E. Najafi-Majd, E. S. H. Quah, F. Bolaños, F. Xie, F. Brusquetti, F. S. Álvarez, F. Andreone, F. Glaw, F. E. Castañeda, F. Kraus, G. Parra-Olea, G. Chaves, G. F. Medina-Rangel, G. González-Durán, H. M. Ortega-Andrade, I. F. Machado, I. Das, I. R. Dias, J. N. Urbina-Cardona, J. Crnobrnja-Isailović, J.-H. Yang, J. Jianping, J. T. Wangyal, J. J. L. Rowley, J. Measey, K. Vasudevan, K. O. Chan, K. V. Gururaja, K. Ovaska, L. C. Warr, L. Canseco-Márquez, L. F. Toledo, L. M. Díaz, M. M. H. Khan, M. Meegaskumbura, M. E. Acevedo, M. F. Napoli, M. A. Ponce, M. Vaira, M. Lampo, M. H. Yáñez-Muñoz, M. D. Scherz, M.-O. Rödel, M. Matsui, M. Fildor, M. D. Kusriini, M. F. Ahmed, M. Rais, N. G. Kouamé, N. García, N. L. Gonwouo, P. A. Burrowes, P. Y. Imbun, P. Wagner, P. J. R. Kok, R. L. Joglar, R. J. Auguste, R. A. Brandão, R. Ibáñez, R. von May, S. B. Hedges, S. D. Biju, S. R. Ganesh, S. Wren, S. Das, S. V. Flechas, S. L. Ashpole, S. J. Robleto-Hernández, S. P. Loader, S. J. Incháustegui, S. Garg, S. Phimmachak, S. J. Richards, T. Slimani, T. Osborne-Naikatini, T. P. F. Abreu-Jardim, T. H. Condez, T. R. De Carvalho, T. P. Cutajar, T. W. Pierson, T. Q. Nguyen, U. Kaya, Z. Yuan, B. Long, P. Langhammer, and S. N. Stuart. 2023. Ongoing declines for the world's amphibians in the face of emerging threats. *Nature* 622:308–314.
- Marcum, R. D., S. St-Hilaire, P. J. Murphy, and K. J. Rodnick. 2010. Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus (Bufo) boreas boreas*. *Diseases of Aquatic Organisms* 91:17–21.
- May, R. M., and M. A. Nowak. 1997. Coinfection and the evolution of parasite virulence. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 261:209–215.

- McNab, F., K. Mayer-Barber, A. Sher, A. Wack, and A. O'Garra. 2015. Type I interferons in infectious disease. *Nature Reviews Immunology* 15:87–103.
- Medina, D., S. E. Greenspan, T. Carvalho, C. G. Becker, and L. F. Toledo. 2021. Co-infecting pathogen lineages have additive effects on host bacterial communities. *FEMS Microbiology Ecology* 97:fiab030.
- Miller, D. L., S. Rajeev, M. Brookins, J. Cook, L. Whittington, and C. A. Baldwin. 2008. Concurrent Infection with Ranavirus, *Batrachochytrium dendrobatidis*, and *Aeromonas* in a Captive Anuran Colony. *Journal of Zoo and Wildlife Medicine* 39:445–449.
- Neely, W. J., S. E. Greenspan, L. P. Ribeiro, T. Carvalho, R. A. Martins, D. Rodriguez, J. R. Rohr, C. F. B. Haddad, L. F. Toledo, and C. G. Becker. 2020. Synergistic effects of warming and disease linked to high mortality in cool-adapted terrestrial frogs. *Biological Conservation* 245:108521.
- Olori, J. C., R. Netzband, N. McKean, J. Lowery, K. Parsons, and S. T. Windstam. 2018. Multi-year dynamics of ranavirus, chytridiomycosis, and co-infections in a temperate host assemblage of amphibians. *Diseases of Aquatic Organisms* 130:187–197.
- Peig, J., and A. J. Green. 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos* 118:1883–1891.
- Price, S. J., E. Ariel, A. Maclaine, G. M. Rosa, M. J. Gray, J. L. Brunner, and T. W. J. Garner. 2017. From fish to frogs and beyond: Impact and host range of emergent ranaviruses. *Virology* 511:272–279.
- Price, S. J., T. W. J. Garner, R. A. Nichols, F. Balloux, C. Ayres, A. Mora-Cabello de Alba, and J. Bosch. 2014. Collapse of Amphibian Communities Due to an Introduced Ranavirus. *Current Biology* 24:2586–2591.
- R Core Team. 2023. R: A Language and Environment for Statistical Computing.

- Ramsay, C., and J. R. Rohr. 2021. The application of community ecology theory to co-infections in wildlife hosts. *Ecology* 102:e03253.
- Ramsay, C., and J. R. Rohr. 2023. Identity and density of parasite exposures alter the outcome of coinfections: Implications for management. *Journal of Applied Ecology* 60:205–214.
- Read, A. F., and L. H. Taylor. 2001. The Ecology of Genetically Diverse Infections. *Science* 292:1099–1102.
- Robert, J., L. Abramowitz, J. Gantress, and H. D. Morales. 2007. XENOPUS LAEVIS: A POSSIBLE VECTOR OF RANAVIRUS INFECTION? *Journal of Wildlife Diseases* 43:645–652.
- Robert, J., E. George, F. De Jesús Andino, and G. Chen. 2011. Waterborne infectivity of the Ranavirus frog virus 3 in *Xenopus laevis*. *Virology* 417:410–417.
- Robert, J., L. Grayfer, E.-S. Edholm, B. Ward, and F. De Jesús Andino. 2014. Inflammation-Induced Reactivation of the Ranavirus Frog Virus 3 in Asymptomatic *Xenopus laevis*. *PLoS ONE* 9:e112904.
- Robert, J., H. Morales, W. Buck, N. Cohen, S. Marr, and J. Gantress. 2005. Adaptive immunity and histopathology in frog virus 3-infected *Xenopus*. *Virology* 332:667–675.
- Rojas, S., K. Richards, J. K. Jancovich, and E. W. Davidson. 2005. Influence of temperature on Ranavirus infection in larval salamanders *Ambystoma tigrinum*. *Diseases of Aquatic Organisms* 63:95–100.
- Rollins-Smith, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1788:1593–1599.
- de Roode, J. C., M. E. H. Helinski, M. A. Anwar, and A. F. Read. 2005a. Dynamics of Multiple Infection and Within-Host Competition in Genetically Diverse Malaria Infections. *The American Naturalist* 166:531–542.

- de Roode, J. C., R. Pansini, S. J. Cheesman, M. E. H. Helinski, S. Huijben, A. R. Wargo, A. S. Bell, B. H. K. Chan, D. Walliker, and A. F. Read. 2005b. Virulence and competitive ability in genetically diverse malaria infections. *Proceedings of the National Academy of Sciences* 102:7624–7628.
- Rosa, G. M., J. Sabino-Pinto, T. G. Laurentino, A. Martel, F. Pasmans, R. Rebelo, R. A. Griffiths, A. C. Stöhr, R. E. Marschang, S. J. Price, T. W. J. Garner, and J. Bosch. 2017. Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. *Scientific Reports* 7:43260.
- Ruggeri, J., M. R. Pontes, L. P. Ribeiro, K. L. Gendreau, R. L. M. Sousa, and L. F. Toledo. 2024. Predominant prevalence of Ranavirus in southern Brazil, a region with widespread occurrence of the amphibian chytrid. *Animal Conservation* 27:338–349.
- Scheele, B. C., F. Pasmans, L. F. Skerratt, L. Berger, A. Martel, W. Beukema, A. A. Acevedo, P. A. Burrowes, T. Carvalho, A. Catenazzi, I. De la Riva, M. C. Fisher, S. V. Flechas, C. N. Foster, P. Frías-Álvarez, T. W. J. Garner, B. Gratwicke, J. M. Guayasamin, M. Hirschfeld, J. E. Kolby, T. A. Kosch, E. La Marca, D. B. Lindenmayer, K. R. Lips, A. V. Longo, R. Maneyro, C. A. McDonald, J. Mendelson, P. Palacios-Rodriguez, G. Parra-Olea, C. L. Richards-Zawacki, M.-O. Rödel, S. M. Rovito, C. Soto-Azat, L. F. Toledo, J. Voyles, C. Weldon, S. M. Whitfield, M. Wilkinson, K. R. Zamudio, and S. Canessa. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* 363:1459–1463.
- Therneau, T. M. 2024. A Package for Survival Analysis in R.
- Thumsová, B., G. Alarcos, C. Ayres, G. M. Rosa, and J. Bosch. 2024. Relationship between two pathogens in an amphibian community that experienced mass mortalities. *Conservation Biology* 38:e14196.

- Voyles, J., L. Berger, S. Young, R. Speare, R. Webb, J. Warner, D. Rudd, R. Campbell, and L. F. Skerratt. 2007. Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* 77:113–118.
- Voyles, J., E. B. Rosenblum, and L. Berger. 2011. Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity. *Microbes and Infection* 13:25–32.
- Warne, R. W., B. LaBumbard, S. LaGrange, V. T. Vredenburg, and A. Catenazzi. 2016. Co-Infection by Chytrid Fungus and Ranaviruses in Wild and Harvested Frogs in the Tropical Andes. *PLOS ONE* 11:e0145864.
- Watters, J. L., D. R. Davis, T. Yuri, and C. D. Siler. 2018. Concurrent Infection of *Batrachochytrium dendrobatidis* and Ranavirus among Native Amphibians from Northeastern Oklahoma, USA. *Journal of Aquatic Animal Health* 30:291–301.
- Wendel, E. S., A. Yaparla, D. V. Koubourli, and L. Grayfer. 2017. Amphibian (*Xenopus laevis*) tadpoles and adult frogs mount distinct interferon responses to the Frog Virus 3 ranavirus. *Virology* 503:12–20.

Figure 1. Schematic of inoculation process. Treatment group abbreviations are shown at the top. Bd refers to single exposure to *Batrachochytrium dendrobatidis* (Bd), Rv to single exposure to *Ranavirus* (Rv), Bd → Rv indicates exposure to Bd followed by Rv, Rv → Bd indicates exposure to Rv followed by Bd, and BdRv represents simultaneous exposure to both pathogens. “+ buffers” refers to the addition of APBS and sterile tryptone plate flooding solution for sham inoculation. Illustrative elements were created with BioRender.com (Faust, 2025).

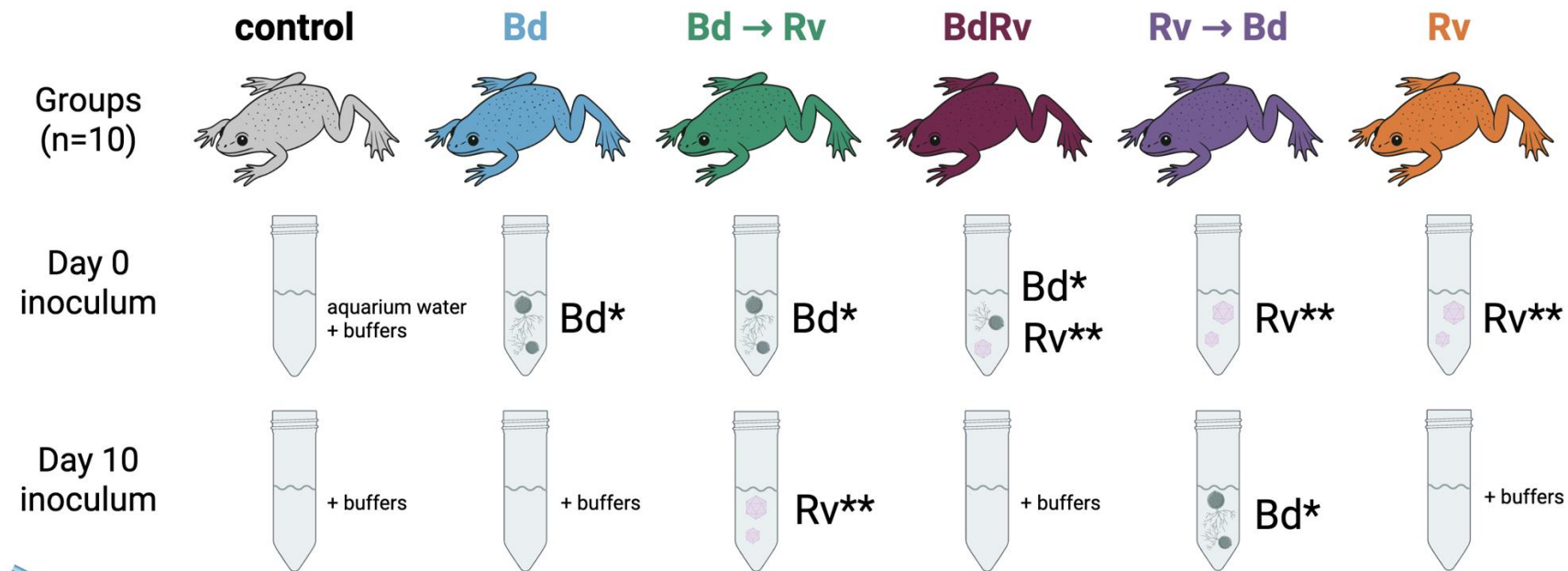
Figure 2. Survival probability curves for each treatment group, with time shown (A) in days since first exposure (*i.e.*, experiment day 0) and (B) in days following exposure to *Batrachochytrium dendrobatidis* (Bd). Different letters denote significant differences between groups based on post hoc Tukey’s honestly significant differences (HSD) test (see adjusted *p* values in Appendix S1: Tables S1 and S2).

Figure 3. Log-transformed *Batrachochytrium dendrobatidis* (Bd) loads (zoospore equivalents) from individual skin swabs performed 10 days after Bd exposure, shown for each treatment group. Each point represents an individual frog; larger points indicate group means and vertical lines show standard errors. Different letters denote significant differences between groups based on post hoc Tukey’s honestly significant differences (HSD) test (see adjusted *p* values in Appendix S1: Table S3).

Figure 4. Comparison across treatment groups of mean *Batrachochytrium dendrobatidis* (Bd) load (zoospore equivalents) from individual skin swabs performed at time of death, displayed on the x-axis as days after exposure to Bd. On both axes, error bars indicate the mean value (Bd

load or lifespan) $\pm$ standard deviation. Different letters denote significant lifespan differences between groups based on post hoc Tukey's honestly significant differences (HSD) test (see adjusted p values in Appendix S1: Table S5). Mean Bd load at time of death was similar among the treatment groups.

Figure 5. Scaled mass indices (SMI) for individual frogs, shown across treatment groups and timepoints. Each point represents an individual; horizontal bars and vertical lines indicate group means $\pm$ SE. "Day 10" indicates SMI measured 10 days after exposure to the first pathogen, and "endpoint/TOD" corresponds to measurements taken at the experimental endpoint (day 34) or at the animal's time of death.



Day 0, 10, 20, time of death: skin swabs for detecting fungal and viral loads

*Bd dose: 6×10^4 zoospores/mL in 3mL
 **Rv dose: 5×10^4 PFU/mL in 3mL

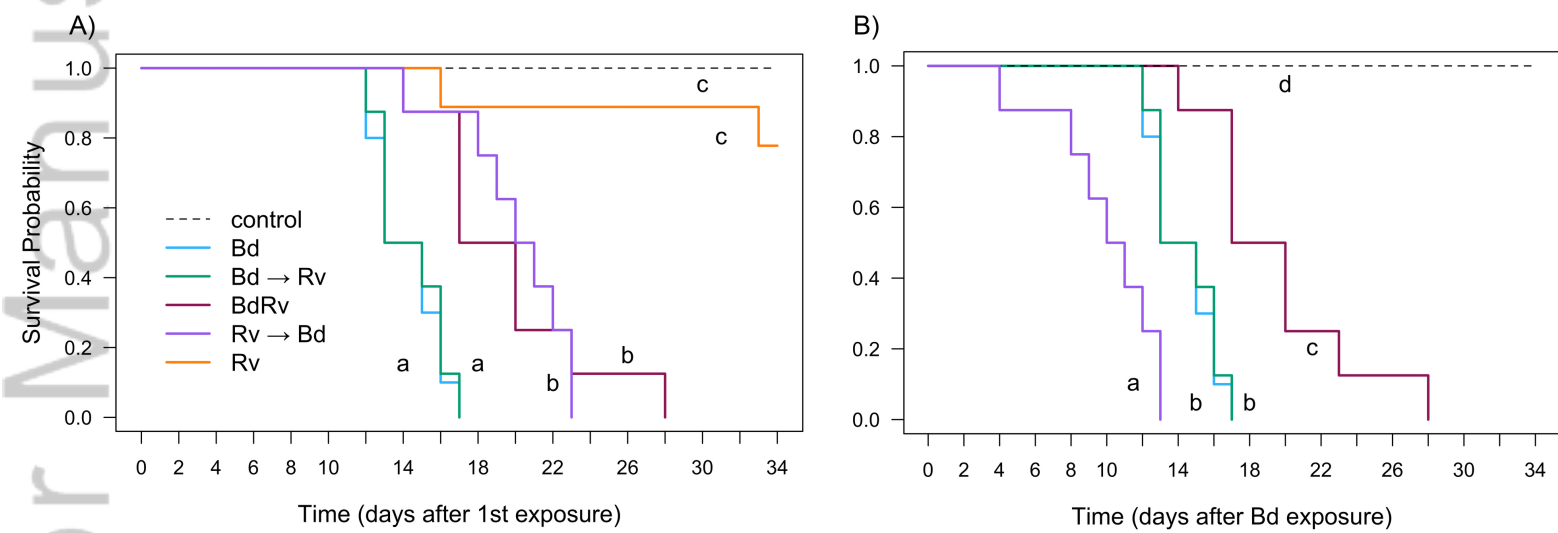


Figure_2.jpg

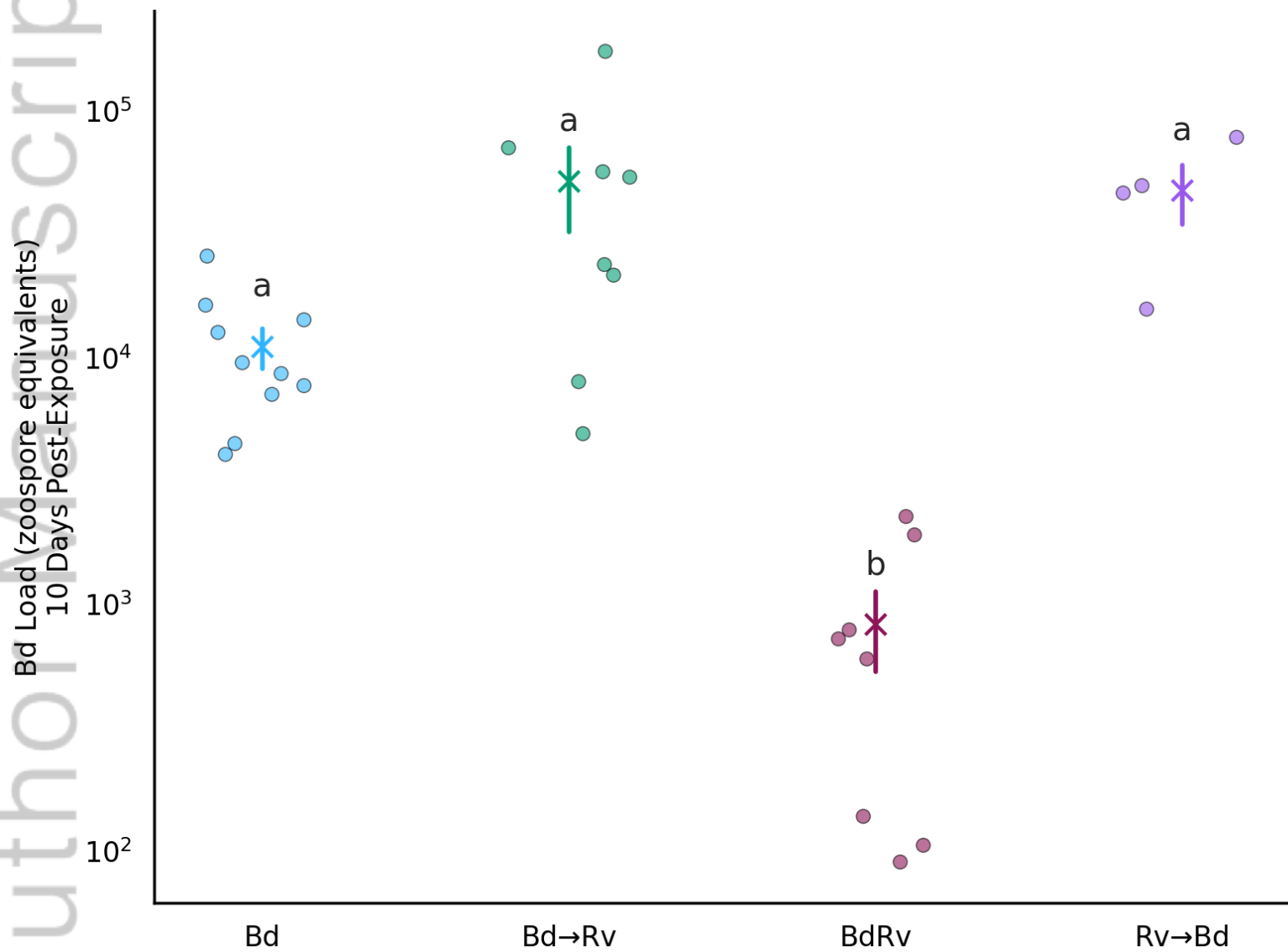
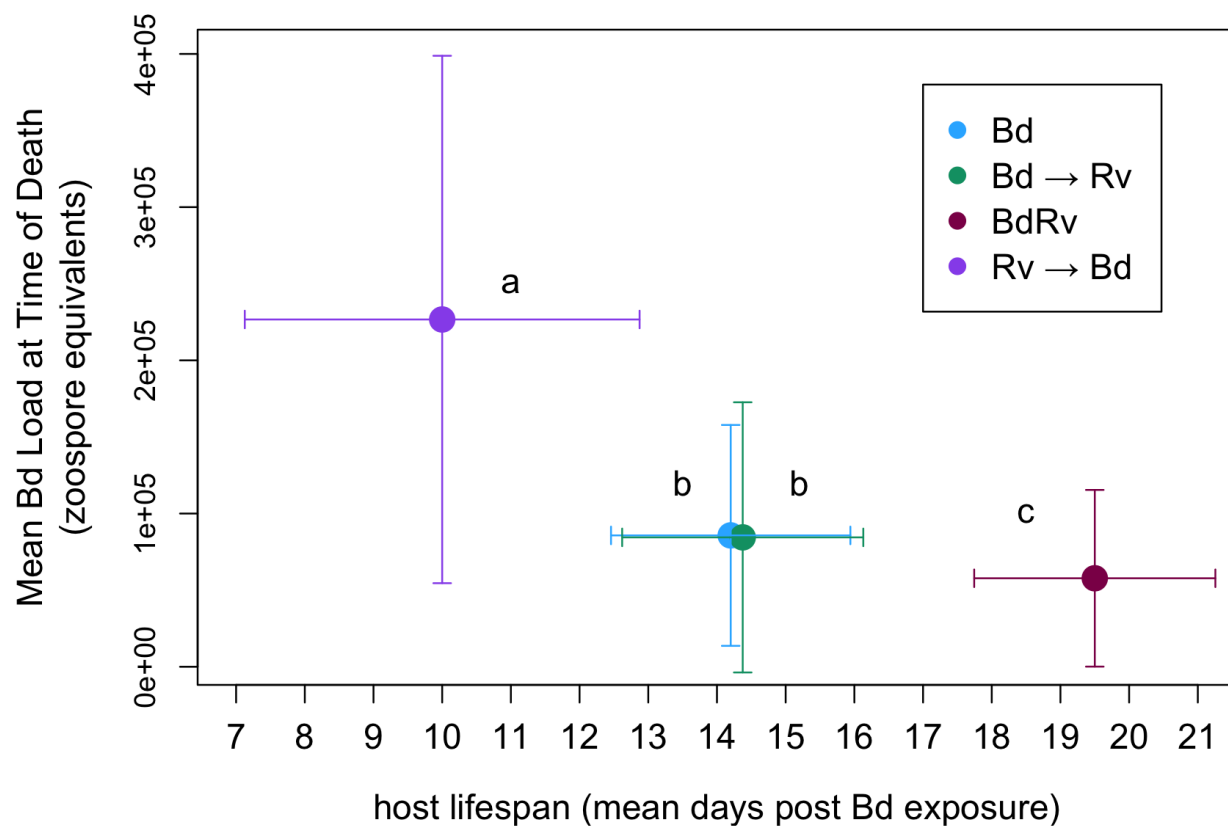


Figure_3.png



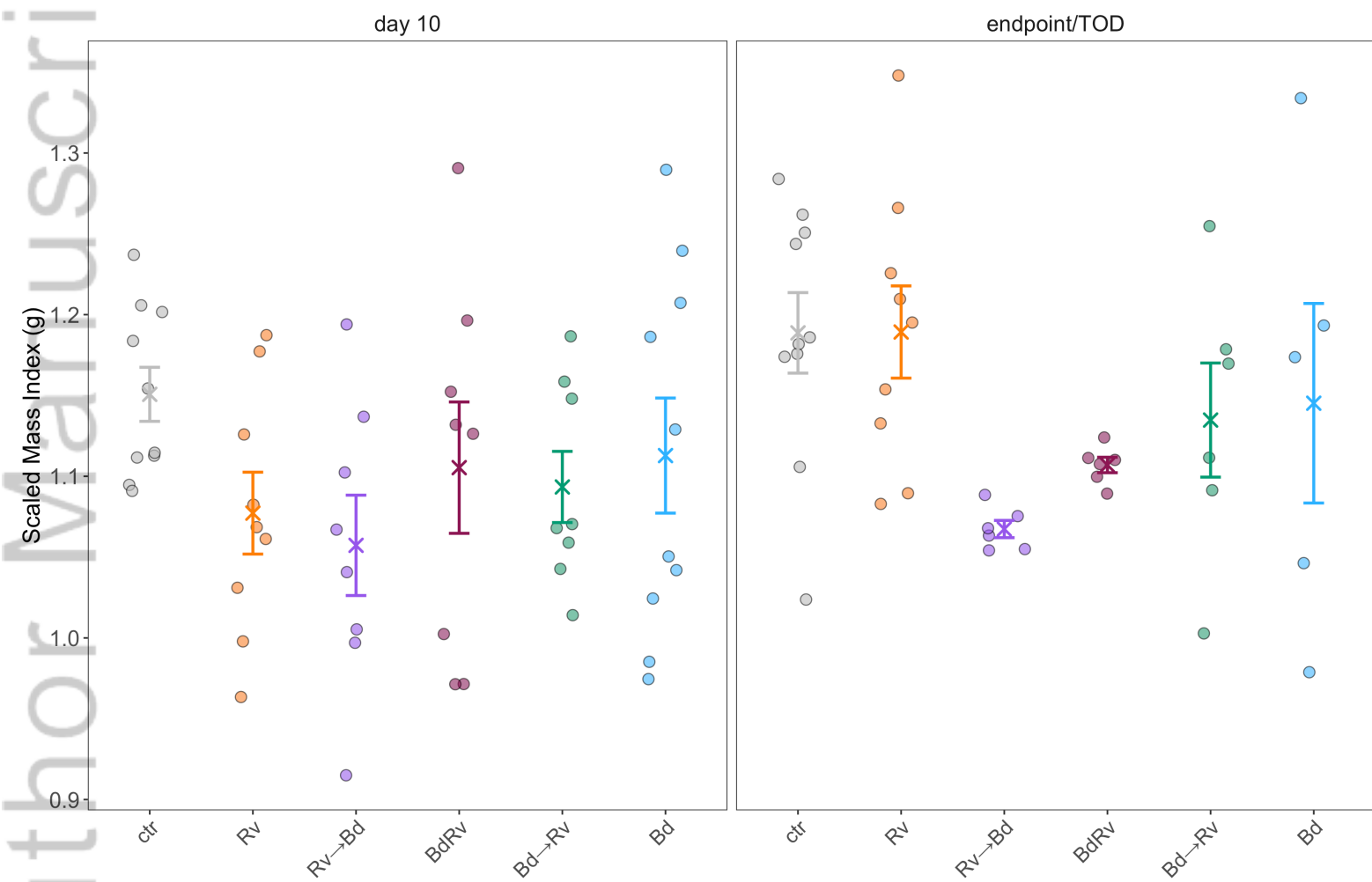


Figure_5.png