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ORIGINAL PAPER

Plethodontid salamanders show variable disease dynamics in response to *Batrachochytrium salamandrivorans* chytridiomycosis

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Abstract Emerging infectious diseases are among the leading drivers of the sixth mass extinction. The recent invasion of a highly pathogenic chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), across Europe has led to salamander mass mortality. To date, it remains unclear whether *Bsal* will cause salamander mass mortalities in North America. Here, we tested the *Bsal* susceptibility of eight wild-caught salamander species (*Plethodon cinereus*, *P. glutinosus*, *P. montanus*, *P. cylindraceus*, *Desmognathus fuscus*, *D. wrighti, Eurycea wilderae*, and *Notophthalmus viridescens*) by inoculating individuals sequentially with a low (10,000 zoospores) and high (500,000 zoospores) *Bsal* dose. Overall, we found rapid and complete mortality of *N. viridescens* accompanied with high-*Bsal* infections (> 200,000 *Bsal* zoospores) and severe *Bsal* lesions distributed across the body and deep within the skin. In contrast, we found low mortality of plethodontid salamanders, where only 5 of 60 (8%) *Bsal*-exposed individuals died over the course of the experiment. In general, plethodontid salamanders experienced moderate *Bsal* infections (∼ 4000 *Bsal* zoospores) with small numbers of *Bsal*-

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type lesions limited to the head and lateral body. Following the first *Bsal* inoculation, we found that *Bd* co-infections negatively affected *Bsal* infections, suggesting cross reactivity of the immune system or competitive exclusion, but this pattern did not persist following the second inoculation. We also found that *Bsal* infection intensity decreased over time following the second higher *Bsal* inoculation, suggesting evidence of immune priming. Throughout the experiment, all species and treatments experienced stable or increasing body condition over time. Lastly, ancestral state reconstruction of *Bsal* susceptibility indicated that although the most recent common ancestor (MRCA) of the family Plethodontidae is resistant to *Bsal*, the MRCA of the genus *Plethodon* is tolerant of *Bsal*. This highlights the variation in *Bsal*-infection outcomes across Plethodontidae. Collectively, our results suggest that Plethodontidae salamanders differ in their *Bsal* susceptibility, with some species less impacted than others, which will likely have consequences for their conservation and management.

Keywords *Bsal* · Chytridiomycosis · Coinfection · Emerging infectious disease · Fungal disease · Salamander biodiversity hotspot · Susceptibility

Introduction

The recent introduction of several fungal pathogens to countries across the globe has devastated a number of taxa, including some of great economic and ecological importance (Fisher et al. 2012). For example, bat declines triggered by white-nose syndrome, which is caused by the fungal pathogen *Pseudogymnoascus destructans*, in North America are projected to cause agricultural losses greater than 3.7 billion USD per year (Boyles et al. 2011), and amphibian-related disease declines led by *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of chytridiomycosis, have disrupted resource-consumer dynamics and ecosystem processes (Whiles et al. 2006, 2013; Zipkin et al. 2020). In 2013, a second highly pathogenic fungus that causes chytridiomycosis, *Batrachochytrium salamandrivorans* (*Bsal*), was identified as the closest sister taxon to *Bd* (Martel et al. 2013). Both chytrid species, *Bd* and *Bsal*, are thought to have originated in Asia (Martel et al. 2014; O'Hanlon et al. 2018). To date, *Bsal* invasion in Europe, where it is a nonnative species, has caused salamander mass mortalities and population declines (Martel et al. 2013, 2014), but *Bsal* remains undetected in North America (Martel et al. 2013, 2014; Gray et al. 2015; Richgels et al. 2016; Spitzen-van der Sluijs et al. 2016; Klocke et al. 2017; Parrott et al. 2017; Waddle et al. 2020), where concerns are elevated because some of the world's salamander biodiversity hotspots occur in the United States.

From previous experiments and modeling exercises, it is expected that the emergence of *Bsal* in the United States may lead to widespread salamander declines (Yap et al. 2015; Richgels et al. 2016; Carter et al. 2020, 2021). Consequently, these losses and population declines may affect ecosystem structure and function (Hocking and Babbitt 2014) because amphibians are centrally nested in food webs, being important prey and consumers (Wyman 1998; Davic and Welsh 2004; Walton and Steckler 2005). In an effort to prevent *Bsal* invasion and/or mitigate the consequences of *Bsal* arrival into the United States, the *Bsal* Task Force 2020 are working to identify conservation actions and research priorities (< salamanderfungus.org >).

One research priority is to determine salamander species-specific susceptibility to *Bsal*. This information is useful for understanding the potential impacts of *Bsal* on salamander communities in the United States. However, with over 200 potential *Bsal*-susceptible salamander species in the United States, it is impractical to test them all in a single experiment (Lacey Act [1990]; 16 U.S.C. §§ 3371–3378 and 18 U.S.C. §§ 42–43). In this context, ancestral state reconstruction becomes an invaluable tool in predicting naïve pathogen susceptibility of closely-related species, especially if closely-related species are vulnerable to similar pathogens (Wiens et al. 2010).

Here, we experimentally exposed eight wild-caught temperate salamander species collected in the eastern United States to *Bsal* twice. First, we inoculated animals with a low *Bsal* dose (10,000 *Bsal* zoospores; Fig. 1), and we expected to observe signs of chytridiomycosis on species that are highly susceptible within 42 days, given the results of other *Bsal* experimental inoculations (Martel et al. 2014; Bletz et al. 2018; Carter et al. 2020; Malagon et al. 2020). Then, on day 42, we inoculated half of the previously exposed animals with a second higher *Bsal* dose (500,000 *Bsal*

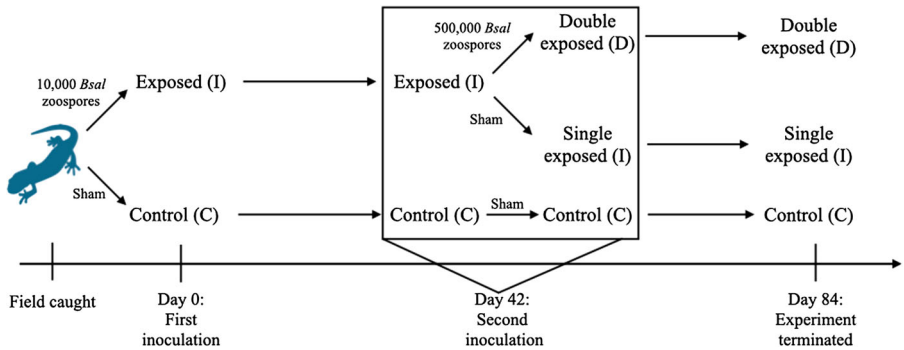

Fig. 1 Graphic depiction of the experimental design and schedule of *Bsal* inoculations. The first phase of the experiment ran from day 0 to 41, where a portion of salamanders were exposed to 10,000 *Bsal* zoospores (see Table 1 for sample sizes). The second phase of the experiment ran from day 42 to 84, where half of the individuals that were exposed to the first *Bsal* inoculation received a second higher *Bsal* inoculation (500,000 *Bsal* zoospores)

zoospores; Fig. 1) to determine whether *Bsal* chytridiomycosis develops in each species. It is unlikely that animals in nature are exposed to such high *Bsal* doses, but these exposure amounts often lead to infection loads in laboratory animals similar to what is detected on wild animals, highlighting the value of high *Bd*/*Bsal* exposure doses that mimic natural infection loads (Searle et al. 2011; Gervasi et al. 2013; Muletz-Wolz et al. 2019).

In this study, we quantified species survival, body condition, and *Bsal* growth rate. We used histology to determine the distribution of *Bsal* lesions across the body and within the skin. Our null hypothesis was that *Bsal* lesions would be distributed ubiquitously across the body and deep within the epidermis of susceptible and lethal species (see *Definitions* below). Susceptible species were expected to decrease body condition more quickly over time and have higher *Bsal* growth rates than tolerant or resistant species. We also hypothesized that tolerant and resistant species would have lesions isolated to particular parts of the body, similar to the distribution of *Bd* on *Pseudacris regilla* (Reeder et al. 2012). Lastly, we hypothesized that closely-related species would have more similar *Bsal* susceptibility than distantly-related species (Martel et al. 2014). Collectively, our results are useful in updating predictions and our understanding of *Bsal* risk to native U.S. amphibians.

Methods

Definitions

First, we define the difference between infection and disease. We define infection as the invasion of the body with organisms that have the potential to cause disease (Casadevall and Pirofski 2000). We define disease as the manifestation of the fight between the disease-producing or pathogenic organisms and the host with all its defense mechanisms (Casadevall and Pirofski 2000).

We follow the definitions of resistance, tolerance, susceptibility, and lethality per Martel et al. (2014). Resistance is defined as animals lacking infection and disease. Tolerance is defined as animals with infections but no disease. Susceptibility is defined as animals experiencing infection with clinical disease. Lastly, lethality is defined as infections that caused disease and mortality.

Field captures and animal husbandry

We collected 108 salamanders and obtained data from 97 salamanders of eight species: *Plethodon cinereus*, *P. glutinosus*, *P. montanus*, *P. cylindraceus*, *Desmognathus fuscus*, *D. wrighti, Eurycea wilderae*, and *Notophthalmus viridescens*, from Mount Rogers Recreational Area in Marion, VA between 04 and 06 June 2015 (see Table 1 for sample sizes). All species belong to the family Plethodontidae, except *N. viridescens* of the family Salamandridae. To determine if individuals carried field infections of *Bd* or

Table 1 Experimental and histological sample sizes of each species and treatment across the entirety of the experiment.

Genus	Species	Treatment	Experiment sample size	Histology sample size	*Bsal* + histology	*Bsal*– histology	*Bd* + histology	*Bd*– histology	*Bsal* + & *Bd* + histology	*Bsal*– & *Bd*– histology
Desmognathus	*fuscus*	C	4	2	0	2	0	2	0	2
Desmognathus	*fuscus*	I	10	2	0	2	0	2	0	2
Desmognathus	*fuscus*	D	5	3	0	3	0	3	0	3
Desmognathus	*wrighti*	C	4	2	0	2	0	2	0	2
Desmognathus	*wrighti*	I	11	3	0	3	0	3	0	3
Desmognathus	*wrighti*	D	6	4	0	4	0	4	0	4
Eurycea	*wilderae*	C	1	1	0	1	0	1	0	1
Eurycea	*wilderae*	I	2	2	1	1	1	1	1	1
Notophtalamus	*viridescens*	C	3	3	0	3	3	0	0	0
Notophtalamus	*viridescens*	I	10	8[†]	6	2	8	0	6	0
Plethodon	*cinereus*	C	4	1	0	1	0	1	0	1
Plethodon	*cinereus*	I	7	2	1	1	0	2	0	1
Plethodon	*cinereus*	D	4	1	1	0	0	1	0	0
Plethodon	*cylindraceus*	C	4	2	0	2	0	2	0	2
Plethodon	*cylindraceus*	I	10	1	0	1	0	1	0	1
Plethodon	*cylindraceus*	D	6	1	1	0	0	1	0	0
Plethodon	*glutinosus*	C	3	3	0	3	0	3	0	3
Plethodon	*glutinosus*	I	11	6	2	4	1	4	0	3
Plethodon	*glutinosus*	D	5	5	2	3	0	4	0	3
Plethodon	*montanus*	C	4	2	0	2	0	2	0	2
Plethodon	*montanus*	I	9	2	2	0	1	1	1	0
Plethodon	*montanus*	D	4	2	0	2	2	0	0	0

Treatment C = control, D = double exposed, I = single exposed. Note that the *Experiment sample size* column represents the total number of individuals ever in a treatment. On day 42, half of the exposed (I) individuals were exposed to a second higher dose of *Bsal*, comprising the double exposed (D) treatment (Fig. 1). To calculate total number of animals used per species, sum the control (C) and exposed (I) treatments only

[†]All 10 *Bsal*-exposed *N. viridescens* were examined histologically. However, two *Bsal*-exposed individuals had advanced autolysis (i.e., decomposition) which interfered with interpretation of the histology, so only eight are listed as being examined

Bsal, we swabbed all individuals within a day of capture and used qPCR to determine chytrid infection intensities (see *Molecular analyses* below for methods). For each individual, we recorded snout-to-vent and tail lengths using dial calipers to the nearest tenth of a mm, and we recorded body mass using an analytical scale to the nearest 0.01 g. During this process, individuals were placed in a fresh baseball card sleeve or Ziploc® bag to decrease the chances of cross-contamination. We did not treat any pre-existing chytrid infections with antifungal chemicals because we wanted to replicate the conditions that wild salamanders would experience if *Bsal* were to invade the eastern United States. In the laboratory, all plethodontid species were housed individually in plastic Ziploc® containers (25.4 cm × 25.4 cm × 10.2 cm) that included a plastic shelter and a moist, unbleached paper towel substrate, while fully aquatic adult newts, *N. viridescens*, were housed individually in the same plastic Ziploc® containers with 500 mL of sterile deionized water mixed with autoclaved, stream water collected in College Park, Maryland. We tilted each container such that animals were able to crawl onto dry surfaces or swim in their containers.

Animals were randomly assigned to one of six Percival® Incubators set to 12:12 h light:dark photoperiod, 15 °C, and 85% humidity. Temperatures of 15 °C maximize *Bsal* growth in vitro (Martel et al. 2013). We allowed salamanders to acclimate to these conditions for seven days before the start of the experiment when animals were exposed to *Bsal* (Fig. 1). We replaced all housing materials every seven days, changed water and fed amphibians crickets or fruit flies ad libitum every three days, and misted terraria daily.

Bsal cultures and inoculation

We used *Bsal* culture AMFP13/1, which was originally isolated from a dead *Salamandra salamandra* during the 2012 epizootic in the Netherlands (Martel et al. 2013). It was passaged approximately 10 times before it was used in the first *Bsal* inoculation (Fig. 1). The culture was passaged an additional two times before the second *Bsal* inoculation. We maintained the *Bsal* culture in 1% tryptone broth at 4°C and passaged it every three months. We grew *Bsal* on 1% tryptone agar plates, allowed them to grow for seven to 10 days at 15 °C, and we harvested and counted zoospores using a hemocytometer from those plates for salamander exposure following DiRenzo et al. (2014).

We assigned either one or four individuals from each species as control animals (C; Table 1; Fig. 1). The remaining individuals were used in the exposed treatment (I). On day 0 of the experiment, we inoculated the exposed treatment with 10,000 *Bsal* zoospores for 24 h, and we inoculated controls with a sham solution of 1% tryptone broth for 24 h (DiRenzo et al. 2014).

After inoculations, we monitored individuals daily for clinical symptoms of *Bsal* (i.e., lesions, lethargy, loss of appetite), and we swabbed all individuals once every three to four days using a sterile rayon swab (Dry Swab MW113, Medical Wire, Durham, NC, USA) following Hyatt et al. (2007). We swabbed the ventral side and each limb five times each. The same swab was used for *Bd* and *Bsal* testing using qPCR. We recorded body mass weekly using an analytical scale to the nearest 0.01 g. We euthanized individuals once they lost their righting reflex using a Benzocaine anesthetic followed by decapitation.

We expected to observe signs of *Bsal* chytridiomycosis in highly susceptible salamander species within 42 days, given the results of other *Bsal* experimental inoculations (Martel et al. 2014; Bletz et al. 2018; Carter et al. 2020; Malagon et al. 2020). Then, on day 42, we randomly selected half of the previously exposed animals to be inoculated with a second higher *Bsal* dose (500,000 *Bsal* zoospores; Fig. 1) to determine whether *Bsal* chytridiomycosis develops in each species. We refer to animals that were exposed during the first *Bsal* inoculation but not the second as single exposed (I); and we refer to animals that were exposed during the first and second *Bsal* inoculations as double exposed (D; Fig. 1). The second round of inoculations were conducted as previously described. On day 84, we terminated the experiment. All individuals were euthanized using a Benzocaine anesthetic followed by decapitation. We formalin-fixed specimens and sent them to the San Diego Zoo Institute for Conservation Research for histopathology by APP.

Molecular analyses

We used PrepMan Ultra® to extract DNA from swabs (Boyle et al. 2004). We ran separate qPCR reactions for *Bd* and *Bsal* in singlicate using Taqman qPCR

(Boyle et al. 2004; Hyatt et al. 2007; Blooi et al. 2013). The qPCR reactions were run separately for *Bd* and *Bsal* to avoid competition, in which a highly abundant template might have a higher likelihood of detection than a template in lower abundance (Thomas et al. 2018). We ran each plate with either *Bd* or *Bsal* standards of 0.1, 1, 10, 100, and 1000 zoospore genomic equivalents (ZGE) to determine presence and infection intensity. We used the same *Bsal* strain from the inoculations as standards. We categorized individuals as *Bsal* + or *Bd* + when infection intensities were greater than zero (Briggs et al. 2010). The qPCR assay consistently detects very small chytrid infections (0 − 1 zoospore genomic equivalents), representing very low levels of infection. To ensure that false positives were negligible, we included multiple negative controls (PCR water) in each qPCR plate and swabs collected from control group animals.

Histologic examination

We preserved all salamander carcasses in 10% neutral buffered formalin immediately after death or euthanasia. We processed all animal mortalities during the experiment to confirm cases of chytridiomycosis. The salamander carcasses were demineralized in hydrochloric acid (RDO Rapid Decalcifier, Apex Engineering Corp., Aurora, IL, USA) and transversely cut ("breadloafed") at 2–3 mm intervals with the entire body processed for histologic examination (a mean of 25 sections per animal and range of 17–40 sections). Tissues were automatically processed using a Tissue-Tek VIP vacuum infiltration processor (Sakura Finetek USA Inc., Torrance, CA, USA), embedded in paraffin and sectioned at 6 microns. Hematoxylin and eosin stained sections were examined by light microscopy by a veterinary pathologist blinded to *Bsal* infection status. All skin surfaces at each section were evaluated for the presence and distribution of chytrid fungal thalli, and the mean number of *Bd*-type and *Bsal*-type lesions per section were calculated. *Bd* and *Bsal*-type lesions were as defined by White et al. (2016). Briefly, characteristics of *Bd*-type lesions are epidermal hyperplasia and hyperkeratosis with chytrid thalli in the outer keratinized layers (*stratum corneum*), and those of *Bsal*-type lesions are invasion and necrosis of the epidermis with chytrid-type thalli throughout. So, in a mixed infection with *Bd* and *Bsal*, it would still be *Bsal* that causes the necrosis. An additional feature considered more typical of *Bsal* chytridiomycosis was the presence of colonial chytrid thalli with multiple internal septa.

Statistical analyses

For all analyses, we removed data from three individuals that were exposed to *Bsal* but were *Bsal* negative for the entirety of the experiment because it is unclear if these animals were truly never infected, if they became infected and cleared their infection quickly, or if their *Bsal* infections were undetectable (Miller et al. 2012; DiRenzo et al. 2018). In addition, because these animals were exposed to *Bsal*, and therefore not treated equally to other control animals, we did not include them in either the survivorship or body condition analyses. For summary statistics, we averaged *Bsal* infection intensity at time of euthanasia or death by species. We calculated the number of individuals that were *Bd* + at least once during the experiment, and we calculated the number of individuals that were simultaneously infected with *Bd* and *Bsal* (i.e., a single swab was *Bd* + and *Bsal* +).

Survival analysis

Given the low number of deaths experienced by most species during the entirety of the experiment, we did not perform any statistical analysis on species survivorship. Therefore, we report the average days to death of *N. viridescens*, the only species that experienced substantial mortality. For all other species, we present data following the first and second inoculations separately, and we used the function *survfit*() in package *survival* (Therneau 2015) to draw Kaplan-Meier survival curves. We also report the few plethodontid individuals that experienced mortality.

First inoculation analyses

To analyze *Bsal* growth rate by species, we used a linear mixed effects model using function *lme*() in package *nlme* (Pinheiro et al. 2019). We $\log_{10}$ transformed the *Bsal* infection intensity detected on each individual at each swabbing event using $\log_{10}$(*Bsal* zoospores + 1), and we used it as the response variable in the following analysis. We included species, experimental day, $\log_{10}$ (*Bd* zoospores + 1)

at the previous swabbing event, and the interaction species * experimental day as fixed effects. We also included salamander ID as a random intercept, corresponding to initial *Bsal* zoospore infection. We standardized experimental day by subtracting the mean and dividing by the standard deviation. We interpreted the slope estimate of the linear mixed effects model as the *Bsal* growth rate (DiRenzo et al. 2014), which we defined as the daily increase in *Bsal* zoospore load. To determine if there were differences in *Bsal* growth rate among species, we tested for the significance of the interaction (species * experimental day) term using an analysis of variance. If $p < 0.05$, then, we performed a post-hoc analysis to determine pairwise differences in *Bsal* growth rate. To perform the post-hoc analysis, first, we estimated the marginal mean effect of *Bsal* growth rate by species using the function *emtrends* () in the package *emmeans* (Lenth 2019). Then, to generate a compact letter display of all pairwise comparisons of least-squares means of *Bsal* growth rate by species, we used the function *cld* () in package *multcomp* (Hothorn et al. 2008). The function *cld* () adjusts *p* values for multiple comparisons using the tukey method.

We calculated body condition using the residuals taken from the ln (snout-to-vent length) versus ln (body mass) relationship separately for each species (Green 2001; Schulte-Hostedde et al. 2005). To determine changes in salamander body condition over time, we used a linear mixed effects model with body condition as the response variable, and species, experimental day, treatment, species * day interaction, species * treatment interaction, and treatment * day interaction as explanatory variables. The two-way interactions were included to allow the slope estimate to vary by either species or treatment. We included salamander ID as a random intercept, and we standardized experimental day as described above. Similar to above, we also tested for the significance of interactions using an analysis of variance, and if $p < 0.05$, then, we performed post-hoc analyses. We used the same packages and methods as stated in the previous paragraph for pairwise comparisons.

Second inoculation analyses

As above, we used a linear mixed effects model to compare *Bsal* growth rates between single and double exposed treatments across species. We used $\log_{10}$(*Bsal* zoospores + 1) as the response variable, and we included species, experimental day, treatment, $\log_{10}$ (*Bd* zoospores + 1) at the previous swabbing event, species * day interaction, species * treatment interaction, treatment * day interaction, and the three-way interaction species * treatment * day as explanatory variables. The two-way and three-way interactions were included to allow the slope estimate to vary by species and treatment. We included salamander ID as a random intercept, and we standardized experimental day as described above. We removed *E. wilderae* and *N. viridescens* from analyses because there was only one exposed individual remaining per species.

As before, we calculated body condition using the residuals taken from the snout-to-vent length versus mass relationship separately for each species. To determine changes in salamander body condition over time by treatment and species, we used a linear mixed effects model with body condition as the response variable, and we included species, experimental day, treatment, species * day interaction, species * treatment interaction, and treatment * day interaction as explanatory variables. Again, the two-way interactions were included to allow the slope estimate to vary by either species or treatment. We included salamander ID as a random intercept, and we standardized experimental day as described above. We used the same packages and methods as stated in above to determine significance of interaction terms and to make pairwise comparisons among species.

Ancestral state reconstruction of *Bsal* susceptibility

To assess naïve *Bsal* susceptibility across the salamander phylogeny and to determine whether certain taxonomic groups evolved resistance or tolerance to *Bsal* infection, we combined our results with those from Martel et al. (2014) and reconstructed the response to *Bsal* infection on the salamander phylogeny (Table 2). Two species overlapped between our study and Martel et al. (2014), *N. viridescens* and *P. glutinosus*, for which we used our experimental results. Our study and Martel et al. (2014) both found *N. viridescens* as "lethal", but we differed in our conclusions for *P. glutinosus*. Martel et al. (2014) determine *P. glutinosus* was resistant because 0 of 5 individuals become infected, sick, or died following *Bsal* exposure. We found that *P. glutinosus* did

Table 2 Summary of average ± standard error chytrid loads at death, alongside a summary of *Batrachochytrium salamandrivorans* (*Bsal*) histology and *Bsal* growth rates following the first inoculation. Average *Batrachochytrium dendrobatidis* (*Bd*) and *Bsal* loads at death were calculated using data when either individuals died or when the experiment was terminated on day 84.

Genus	Species	Treatment	Average *Bsal* load at death	SE *Bsal* load at death	*Bd* load at death	SE *Bd* load at death	*Bsal* histology	*Bsal* growth rate	*Bsal* susceptibility
Desmognathus	*fuscus*	I	0.00	0.00	0.00	0.00	None	(−)	Resistant
Desmognathus	*fuscus*	D	3.62	2.41	26.72	17.81			
Desmognathus	*wrighti*	I	0.00	0.00	0.00	0.00	None	(−)	Resistant
Desmognathus	*wrighti*	D	711.72	525.64	0.00	0.00			
Eurycea	*wilderae*	I	0.00	0.00	0.00	0.00	Lethal	(+)	Lethal
Notophtalamus	*viridescens*	I	211,008.95	93,399.37	856,068.12	382,480.46	Lethal	(+)	Lethal
Plethodon	*cinereus*	I	106.59	63.45	0.00	0.00	Minimal	(0)	Tolerant
Plethodon	*cinereus*	D	3653.07	1886.01	0.00	0.00			
Plethodon	*cylindraceus*	I	23.44	11.17	0.00	0.00	Minimal	(0)	Tolerant
Plethodon	*cylindraceus*	D	1074.26	738.72	0.00	0.00			
Plethodon	*glutinosus*	I	7.77	5.24	0.00	0.00	Minimal	(0)	Tolerant
Plethodon	*glutinosus*	D	56.42	36.77	0.00	0.00			
Plethodon	*montanus*	I	2845.09	1544.01	0.00	0.00	Minimal	(+)	Tolerant
Plethodon	*montanus*	D	542.82	383.83	0.00	0.00			

Bsal susceptibility is coded using data from *Bsal* histology (signs of disease: none, lethal, minimal) and *Bsal* growth rate (positive [+], negative [−], or stable [0]; Supporting Information S1). See *Definitions* in the Methods for more information on *Bsal* susceptibility categorization. Treatment D = double exposed, I = single exposure

become infected and carried a moderate *Bsal* load over time, but *P. glutinosus* experienced minimal skin lesions (see *Results* below). Therefore, we assigned *P. glutinosus* as tolerant (Table 2).

We downloaded and pruned the Amphibia tree from Timetree to only include species for which we had *Bsal* susceptibility data (Kumar et al. 2017). The final tree included 40 amphibian species. We used the *Bsal* susceptibility data obtained following the first inoculation (day 0–41) because animals across the two studies were inoculated with the same number of zoospores (10,000 *Bsal* zoospores). We did not analyze the results following the second inoculation in a phylogenetic context.

We compared the fit of three discrete trait models using the *AICcmodavg* 2.0–4 package (Mazerolle 2017). We assessed the fit for evolutionary models that hypothesize: (1) all transition rates were equal (ER); (2) forward and reverse transitions were equal (SYM); and (3) all transition rates were different (ARD) using corrected Akaike information criterion (AICc). The final ancestral state reconstruction analysis was done using the *ace* () function in the *ape* package (Paradis and Schliep 2018) under the ER model. The *ace* () function uses a Markov model employing a maximum likelihood approach. In this analysis, the marginal ancestral states are returned, which are given as the proportion of the total likelihood calculated for each state at each node. We used Pagel's lambda with the *fitDiscrete* () function in the package *geiger* (Harmon et al. 2008) to determine if there was a phylogenetic signal in naïve *Bsal* susceptibility (i.e., closely-related species experienced more similar *Bsal* susceptibility than distantly-related species).

Results

General patterns of *Bd* and *Bsal* infection

On the day animals were brought into the laboratory, all individuals tested *Bsal*-negative, and only eight *N. viridescens* tested *Bd*-positive. During the experiment, we found that approximately 66% of individuals tested *Bd*-positive (64 of 97) at least once (Fig. S1), indicating that most *Bd* infections emerged and became detectable in the laboratory. However, the *Bd* infections were often low-intensity infections and only detected once or twice on any given individual (Fig. S1).

Of the animals exposed to *Bsal*, 95% of individuals were *Bsal*-positive at least once (70 of 73). At the first swabbing event, on days 3 and 4, the average *Bsal* load across all *Bsal*-exposed individuals was 3.08 ± 1.45 zoospores (mean ± standard error), and the average *Bd* load across all individuals was 3,488.82 ± 1,894.70 zoospores. At the last swabbing event, day 81, the average *Bsal* load across *Bsal*-exposed individuals was 720.52 ± 301.35 zoospores, and average *Bd* loads were 179.96 ± 178.61 zoospores.

Variation in *Bsal* lesion distribution across resistant, tolerant, and lethal species

Histologic lesions of *Bsal* chytridiomycosis were observed in *N. viridescens*, all *Plethodon* species, and one of two *Bsal*-exposed *E. wilderae* (Table 1). There was no histologic evidence of *Bsal* chytridiomycosis in the exposed *Desmognathus* spp.

The lesions were numerous in 6 of 8 *Bsal*-exposed *N. viridescens* examined histologically and in 1 of 3 *E. wilderae* with a range of 29–136 individual skin lesions per affected animal (1.4–2.8 lesions per section examined). In contrast, lesions were observed in only 10 of 20 exposed *Plethodon* spp. examined histologically with a range of one to two individual skin lesions per affected animal (0.04–0.09 lesions per section examined).

Bsal-lesions in *N. viridescens* and *E. wilderae* were distributed in multiple locations over the head, dorsal, lateral and ventral body and feet. *Bsal*-lesions in *N. viridescens* and the affected *E. wilderae* very frequently extended one-half to the full-thickness of the epidermis (Fig. 2a). Note that *Bsal*-type lesions were only detected in one of the two *E. wilderae* individuals that were exposed to *Bsal*. The other *E. wilderae* individual inoculated with *Bsal* had no lesions detected.

Lesions in the *Plethodon* spp. were present most often on the head (dorsum, nasolabial region, eyelid and lip) and lateral body at the dorsal insertion of the hindlimb and less frequently on the ventral body and feet. Note that swabs used to test for chytrid infections were collected from the ventral body and feet. *Bsal*-type lesions in *Plethodon* spp. were superficial

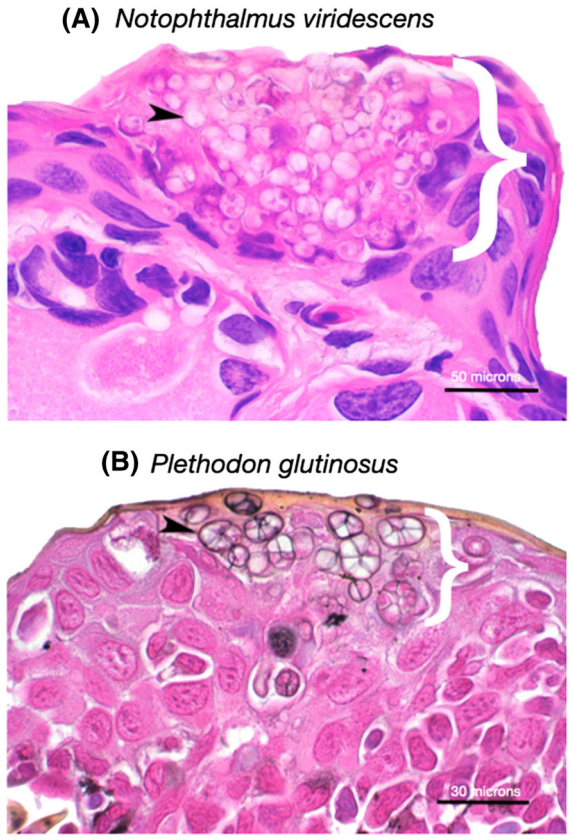

Fig. 2 Skin histology from an infected **A** *N. viridescens* and **B** *P. glutinosus*. Both individuals were experimentally exposed to *Bsal*. The *N. viridescens* individual has full thickness epidermal necrosis with myriad chytrid fungal thalli (black arrowhead) typical of *Bsal* chytridiomycosis. The *P. glutinosus* individual has necrosis limited to the superficial epidermis with numerous large chytrid fungal thalli that have multiple internal septa typical of *Bsal* chytridiomycosis. Brackets indicate depth of *Bsal* infection. Scale bar (A) = 50 microns, scale bar (B) = 30 microns

(Fig. 2b) and only in a single instance extended beyond the upper one-third of the epidermis.

Lesions typical of *Bd*-type chytridiomycosis were observed in *N. viridescens*, *P. montanus*, *P. glutinosus* and *E. wilderae* (same individual with the *Bsal*-lesions). Only in *N. viridescens* and *E. wilderae* did these lesions extend over large areas of the skin. *Bd*-type lesions in the *Plethodon* spp. were limited to small multifocal areas 3–4 cells wide.

High mortality of *N. viridescens* and high host survival of most plethodontids

Following the first inoculation, *N. viridescens* was the only species to substantially experience mortality (Fig. S2). *Notophthalmus viridescens* died within 19.90 ± 2.78 days following exposure to the low dose of *Bsal* (day range = 8−46 days; Fig. S2, S3). *Eurycea wilderae* was the only other species that experienced mortality following the first inoculation, where one of the two *Bsal*-exposed individuals died (Fig. S1). Following the second inoculation with a higher *Bsal* dose, one individual of each of the following treatment species combinations experienced mortality: 1 single-exposed *P. glutinosus*, 1 single-exposed *P. montanus*, 1 double-exposed *D. fuscus*, 1 double-exposed *D. wrighti* (Fig. S3).

Bsal growth rates varied by species following first low dose inoculation

We found that *Bsal* growth rate differed by species following the first inoculation (Supporting Information S1). *Bsal* growth rates were highest on *N. viridescens*, followed by *E. wilderae* and *P. montanus* (Fig. 3, Table 1; Supporting Information S1). Each of these three species had slope coefficients that were positive, and their 95% CIs did not overlap zero. *Bsal* growth rates did not change over time for *P. cylindraceus*, *P. glutinosus*, and *P. cinereus*, where the 95% CI of slope coefficients overlapped zero; and *Bsal* growth rates decreased for both *Desmognathus* species, where all 95% CI of slope coefficients were negative and did not overlap zero (Supporting Information S1).

We did not detect differences in body condition over time among treatments (Supporting Information S2; Fig. S4), but we did detect differences in body condition over time among species. All species showed trends of either stable or increasing body condition over time (Supporting Information S2; Fig. S4), with *P. montanus* and *N. viridescens* showing the highest, most positive change in body condition estimates over time and *D. fuscus* and *D. wrighti* showing stable body condition over time.

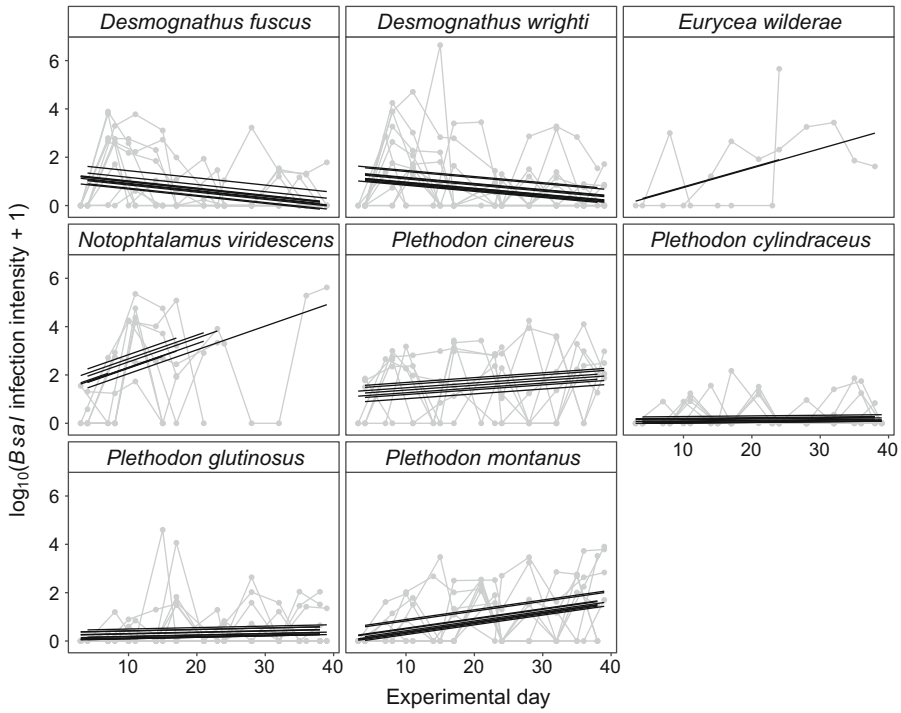

Fig. 3 First phase (days 0–41): *Bsal* infection intensity over time for eight wild-caught salamander species from the eastern United States. Each gray point represents a swab sample collected from a salamander individual, and gray lines connect the multiple swabs collected from each individual. The black lines represent fitted estimates for each individual of each species generated from the linear mixed effects model with individual as a random effect (Supporting Information S1)

Bsal infections decreased following second high dose inoculation

Following the second inoculation, we no longer detected differences in *Bsal* growth rate among species, but we did detect a significant difference in *Bsal* growth rate between the single and double exposed treatments (Fig. 4). We found that animals exposed twice to *Bsal* had a negative *Bsal* growth rate, whereas animals exposed once to *Bsal* had stable infections (Supporting Information S3).

Similar to the results following the first inoculation, we did not detect differences in body condition over time among treatments (Supporting Information S4; Fig. S5), but we did detect differences in body condition over time among species. All species showed trends of increasing body condition over time (Supporting Information S4; Fig. S5), with *P. montanus* and *P. glutinosus* showing the highest, most positive change in body condition over time.

Bd infection intensity negatively impacted *Bsal* infection intensity

Of all the *Bsal*-exposed animals during the experiment, 44% tested positive for *Bd* and *Bsal* simultaneously at least once (31 of 70 individuals; Fig. S1). The only species that consistently tested *Bd* positive was *N. viridescens* (Fig. S1). During the first phase of the experiment (day 0–41), we detected a negative relationship between the $\log_{10}$(*Bd* infection intensity + 1) at the previous time step ($t - 1$) and $\log_{10}$(*Bsal* infection intensity + 1) at the current time step (t; Fig. 5), suggesting that there may be immune cross-reactivity or competitive exclusion occurring between the chytrids (Supporting Information S1); however, we did not detect this pattern during the second phase of the experiment (Supporting Information S3).

Susceptibility to *Bsal* is variable in Plethodontidae

The ER model fit (i.e., all transition rates were equal; AICc = 84.00) was better than either the SYM (i.e., forward and reverse transitions were equal; AICc = 88.60) or ARD (i.e., all transition rates were different; AICc = 104.77) model fits. The ancestral state reconstruction supports the hypothesis that *Bsal* was likely lethal for the most recent common ancestor (MRCA) of Salamandridae (Fig. 6). In addition, the MRCA of the genus *Plethodon* was mostly likely tolerant,

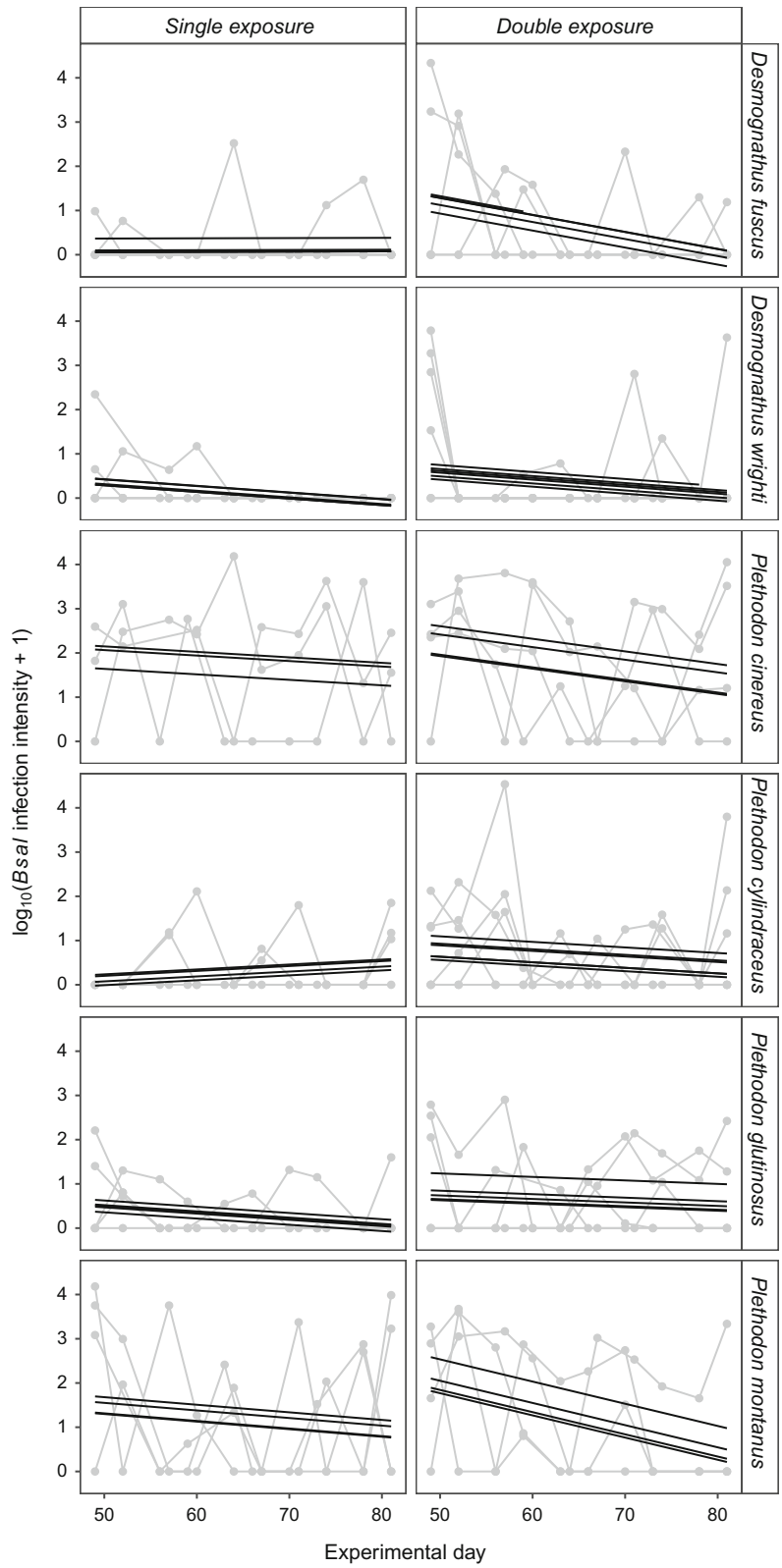

Fig. 4 Second phase (days 42–84): *Bsal* infection intensity over time for six salamander species across two treatments (single and double exposure). Animals in the single exposure treatment were inoculated with a single low *Bsal* dose (left), whereas animals in the double exposure treatment were inoculated with a low and high *Bsal* dose (right; Fig. 1). Each gray point represents a swab sample collected from an individual salamander, and gray lines connect the multiple swabs collected from an individual over time. The black lines represent fitted estimates to each individual and species generated from the linear mixed effects model. By day 46, all exposed *N. viridescens* had died, and only two *E. wilderae* remained (one control, one single exposed); therefore, those species were not included in this analysis. *Bsal* growth rates significantly differed between treatments but not differ among species. The double exposure treatment had a negative slope coefficient that did not overlap zero, whereas the single exposure treatment had a slope coefficient that overlapped zero (Supporting Information S3)

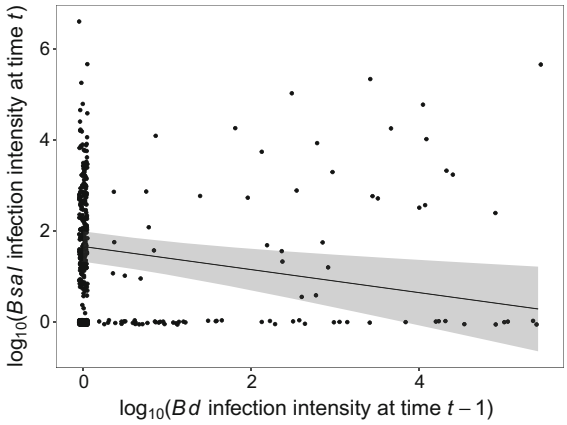

Fig. 5 First phase (days 0–41): The negative relationship between *Bsal* infection intensity and *Bd* infection intensity on the $\log_{10}$ -$\log_{10}$ scale. Note that we added one to all values (i.e., + 1), but we omitted this information from the axes labels to reduce confusion. When *Bd* infection intensity at time $t - 1$ was higher, we detected lower *Bsal* infection intensities at time *t*. Points are jittered to show their density at different locations. Note that jittering the points makes some values appear slightly negative. The solid black line represents the average model prediction, and the gray shading is the 95% confidence interval. Statistics can be found in Supporting Information S1

whereas the MRCA of Plethodontidae was mostly likely resistant.

The ER model provided a better fit of trait evolution (AICc = 81.40) than a model estimated under a phylogeny with a lambda of zero (i.e., a complete polytomy at the ancestral node; AICc = 105.51), indicating that there is phylogenetic signal to *Bsal* susceptibility (ΔAICc = 24.11), such that closely-related species experience more similar *Bsal* susceptibility than distantly-related species.

Discussion

To date, *Bsal* has not been detected in the United States (Waddle et al. 2020), but if and when it invades, some plethodontid species may harbor non-lethal infections and serve as *Bsal* reservoirs, whereas other more susceptible salamanders, such *N. viridescens* and other plethodontids, may experience rapid mortality. As expected, we observed signs of *Bsal* chytridiomycosis on highly susceptible salamander species, such as *N. viridescens*, within 42 days post-*Bsal* inoculation. We found complete mortality of *N. viridescens* accompanied with high *Bsal* infections (> 200,000 *Bsal* zoospores), which contrasted the low mortality of plethodontid salamanders, where 5 of 60 (8%) *Bsal*-exposed individuals died. These individuals were characterized by moderate *Bsal* infections (∼ 4,000 *Bsal* zoospores). Similarly, as expected, we found that *Bsal* lesions were distributed ubiquitously across the body and deep within the epidermis of susceptible and lethal species (see *Definitions* in Methods). We also expected and observed that tolerant and resistant species would have lesions isolated to particular regions of the body, similar to the patchy distribution of *Bd* on *Pseudacris regilla* (Reeder et al. 2012). Particularly, the *Bsal*-type lesions on most of the *Plethodon* spp. were considerably fewer in number, less severe, and more superficial across the skin than the *Bsal*-type lesions detected on *N. viridescens* and the one infected *E. wilderae*. In contrast, we did not find that body condition declined over time for any species, but we did observe that more closely-related salamander species had more similar *Bsal* susceptibility than distantly-related species (Martel et al. 2014). Our results indicate that the family Plethodontidae showed variable disease dynamics in response to *Bsal* infection.

Following the first *Bsal* inoculation, we found that *Bd* infections had a negative impact on *Bsal* infections, which could be the result of either cross reactivity of the immune system or competitive exclusion, but we did not observe this trend following the second higher *Bsal* inoculation. We note that *N. viridescens* had *Bd* infections longer than any of the other species, and their *Bd* loads were considerably higher than any other species (Fig. S1), making it possible that this species was driving the trend. We no longer detected the negative relationship between *Bd* and *Bsal* infection intensity following the second higher *Bsal* inoculation, when all of the *N. viridescens* individuals had died. In contrast to these results, one study found that simultaneous co-infections of *Bd* and *Bsal* led to 78% mortality of *N. viridescens* over 18-weeks, which was driven by the persistence of *Bsal* because newts cleared *Bd* infection within a month (Longo et al. 2019). A number of different mechanisms could act singly or in concert to produce these differences in results. For example, Longo et al. (2019) treated wild-caught salamanders with an anti-fungal to clear chytrid infections upon entry to the laboratory, whereas we did not use anti-fungals. The use (or absence) of anti-fungals could have affected the

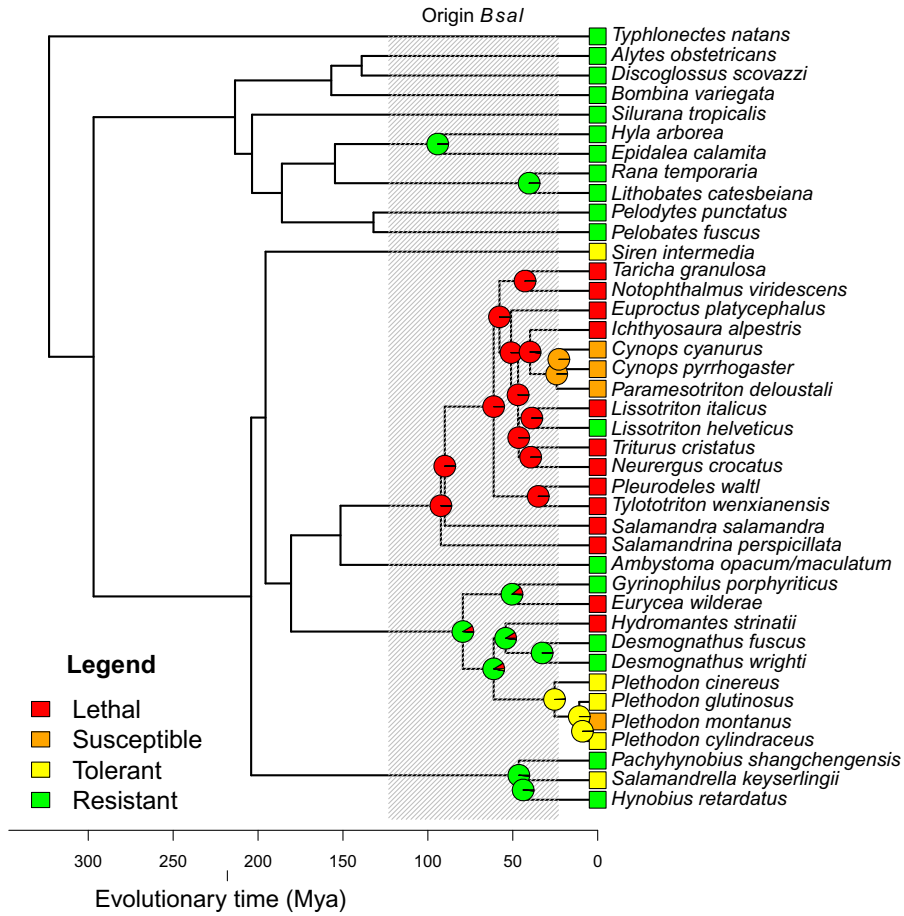

Fig. 6 Ancestral state reconstruction of *Bsal* susceptibility in 40 amphibian species. The origin of *Bsal* is highlighted by the grey box (Martel et al. 2014). Because most salamander species did not historically come into contact with *Bsal*, we do not interpret the ancestral state reconstruction as the evolution of host response to disease. Instead, we use ancestral state reconstruction to inform potential disease susceptibility of closely-related species. Colored boxes on tip ends indicate the current state, and the pie charts on nodes correspond to the scaled likelihoods calculated in the ancestral state reconstruction from the model of best fit (ER model)

salamander microbiome or stimulated their immune system. Alternatively, differences in the *Bd* genotype could produce variations in co-infection outcomes between the studies, where particular strains may be more virulent than others (Rosenblum et al. 2013). In our study, animals came in from the field infected with a local *Bd* strain, whereas Longo et al. (2019) used *Bd* isolate ALKL1, which was isolated from *N. viridescens* in Virginia, USA. Collectively, these two studies demonstrate how pathogen co-infections can generate different disease outcomes.

We found that exposure to a second higher *Bsal* dose led to decreases in *Bsal* infection intensity over time, whereas animals only exposed once to a lower *Bsal* dose sustained infections over time. This is surprising because we expected that the second higher dose would induce *Bsal* chytridiomycosis, but instead, the first lower dose may have primed the immune system for the second higher dose. Unfortunately, our experimental design was not set up to evaluate the effects of immune priming because we do not know how naïve animals respond to the higher dose. For this type of comparison, we should have also inoculated half of the control treatment on day 42 with the second higher *Bsal* dose. Without this information, it is difficult to gain any meaningful insight about priming from this experimental design. However, our experiment serves as a template for future experiments to

examine the possibility of immune priming in treating *Bsal* infections.

Contrary to our expectations, we did not find significant decreases in body condition over time across species or treatments. One potential explanation is that the relatively short duration of the experiment did not capture long-term effects of disease on body condition. Unfortunately, we cannot compare our results to other studies because none of the other *Bsal* susceptibility trials report changes in body condition following *Bsal* exposure (Martel et al. 2014; Longo et al. 2019; Carter et al. 2020; Kumar et al. 2020; Malagon et al. 2020). The closest comparison that can be made is with Carter et al. (2020), who reported that, across all species (*Eurycea wilderae*, *E. lucifuga*, and *Pseudotriton ruber*) and populations, salamanders at the highest *Bsal* dose consumed 11–69% less food than control animals, which could manifest as a decline in body condition over time. However, most of these salamanders experienced disease-induced mortality within 42–79 days, making the changes in body condition over the long term a moot point. In our study, we did not observe body condition declines or high mortality rates across all species. It is likely that we fed salamanders adequately, and that they were not resource limited, given the stable or increasing body condition over time.

Alternatively, it could be that tolerant hosts experience good body condition, even at high pathogen loads (Sánchez et al. 2018). All *Plethodon* species tested in this experiment were either susceptible or tolerant to *Bsal* infection. Although the mechanisms that lead to tolerance are not well understood, environmental factors, such as food availability, may mediate tolerance (Medzhitov et al. 2012). This explanation, however, does not explain the stable or increasing body condition of the species that experienced rapid disease-induced mortality, such as *N. viridescens*. Therefore, it would be interesting for future *Bsal* studies to also compare changes in body condition over time alongside pathogen load to better determine the effects of *Bsal* on host fitness.

If *Bsal* were to enter the United States, our results suggest that *Bsal*-tolerant plethodontid species could serve as *Bsal* vectors and/or reservoirs, while other plethodontid species, such as *Eurycea* (Carter et al. 2020), could experience region-specific declines. Along these same lines, Carter et al. (2020) found that *Bsal* susceptibility of *E. wilderae* varied by population. Similarly, we document that *Bsal* susceptibility of *E. wilderae* varied within a population, with one of two individuals succumbing to *Bsal* chytridiomycosis and the other showing no observable signs of disease. Therefore, with such a high variability in *Bsal* infection responses across the Plethodontidae family tested thus far (18%; 5 of 28 genera; Martel et al. 2014; Carter et al. 2020; and this study), there is a strong need for more *Bsal* susceptibility trials of other Plethodontidae species across populations.

If *Bsal* emerges in North America, our results also suggest that *N. viridescens* will likely be an acute pathogen supershedder (i.e., sheds more infectious propagules than other species within a short timeframe), similar to the role of *Atelopus zeteki* in the *Bd* system (DiRenzo et al. 2014). In *N. viridescens*, we documented an exponential increase in *Bsal* infection intensities to orders of magnitude higher than any other salamander species tested thus far (Martel et al. 2014; Carter et al. 2020). The complex life history of *N. viridescens*, where individuals disperse among subpopulations distributed across a landscape of ponds and upland habitats (Gill 1978) could make them a key vector of *Bsal* dispersal from aquatic to terrestrial habitats. These characteristics make *N. viridescens* likely to disproportionately contribute to *Bsal* transmission and pathogen spillover to sympatric amphibian species (Streicker et al. 2013), including plethodontids. It is unclear, however, if a species such as *N. viridescens* that succumbs to disease-induced mortality quickly and sheds many infectious propagules contributes disproportionately more to pathogen transmission than other longer-lived species that shed a small number of infectious propagules over longer periods of time.

In the eastern United States, information on salamander *Bsal* susceptibility is critically needed to understand the effects of *Bsal* emergence in these communities. Martel et al. (2014) tested 35 amphibian species for *Bsal* susceptibility, but only six of these species are found in the eastern United States, one of the world's salamander biodiversity hotspots. Using this information, the U.S. Fish and Wildlife Service (USFWS) published an interim rule listing 201 salamander species as injurious wildlife under the Lacey Act (1990; 16 U.S.C. §§ 3371–3378 and 18 U.S.C. §§ 42–43), which prohibits the importation and interstate transport of all listed salamander species to

prevent the unintended introduction of *Bsal* into the United States. More information on *Bsal* susceptibility of other species would be useful in updating this list.

We caution readers to be wary when interpreting the results of laboratory studies and making inference about ecological dynamics in the wild because it is important to acknowledge the environmental mismatch between these settings and their impacts on results. The environmental mismatch between wild and laboratory conditions could skew estimates of salamander disease susceptibility. We also acknowledge that the *Bsal* dose used in our inoculations is high, and that animals in the field are likely not exposed to such high doses; but, animals inoculated with high doses in the laboratory experience similar infection dynamics as animals in the wild (Martel et al. 2013; Stegen et al. 2017). In this study, we caught all salamanders in the wild, brought them back into the laboratory, and kept them at a constant, fairly cold, temperature (15 °C), at which *Bsal* growth is maximized and within the thermal breadth of *Plethodon* salamanders (Clay & Gifford 2018). At Mount Rogers Recreational Area (the collection locality), the range of minimum to maximum air temperatures contain or are below 15 °C for 10 of 12 months of the year (< https://www.timeanddate.com/weather/@4782784/climate >). Unfortunately, lab experiments are one of the only ways to gauge host susceptibility before the invasion of a pathogen in a new area (Martel et al. 2014; Stegen et al. 2017; Carter et al. 2020).

We also caution readers to be wary when comparing host susceptibilities across laboratory studies. The observed differences in *Bsal* susceptibility across or within species could be a result of confounding factors, such as collection region, pathogen dose, inoculation procedure, and temperature. For example, *Bsal* susceptibility is known to vary regionally within at least one species (*E. wilderae*; Carter et al. 2020, also used in this study), and it could be that the observed regional differences within species may actually be a product of undescribed species complexes (Jacobs 1987; Camp et al. 2000; Pierson and Miele 2019). In another instance, a recent study examined how laboratory procedures influence disease-induced mortality (Kumar et al. 2020). Specifically, they found that *N. viridescens* experienced disease-induced mortality more quickly when exposed to a low passage isolate, when housed in terrestrial environments, and if exposed to zoospores via a water bath. They did not detect differences in disease-induced mortality with respect to culturing method or swabbing frequency. As the number of experimental *Bsal* susceptibility trials increases, these are all factors to keep in mind when comparing results across studies.

Lastly, we want to highlight that two characteristics of our observed infection intensity data may be explained by the same phenomena. The first characteristic relates to how *Bd* infections were absent and then emerged in the laboratory; and the second characteristic relates to individual variability of *Bd* and *Bsal* infection intensities over time (i.e., infection intensities bounce up and down over time). These two characteristics may be explained by the imperfect sampling process (Miller et al. 2012; Lachish et al. 2012; DiRenzo et al. 2018). From a number of studies, it has been documented that if an individual has a low infection intensity, then the likelihood of missing an infection is high (Miller et al. 2012; Lachish et al. 2012; DiRenzo et al. 2018). It has also been well documented that animals that are brought into the laboratory from the field can test *Bd*-negative upon arrival, and later, animals test *Bd*-positive (Mendoza-Almeralla et al. 2016; Byrne et al. 2018; Longo et al. 2019). One reason for this pattern is that laboratory conditions may exacerbate previously undetectable *Bd* infections (Mendoza-Almeralla et al. 2016). Variability in observed *Bd* and *Bsal* infection intensities over time can also be the result of the sampling or diagnostic process (DiRenzo et al. 2019). Although many scientists try to standardize data collection, the reality is that small differences during sample collection or diagnostic testing can result in measurement error of infection intensities. For example, differences in how and where animals are swabbed or pipetting variability among scientists can lead to observed non-standard progressions of disease. Although some studies collapse disease progression over time using means and standard error to show smoother disease progressions (Gervasi and Hunt 2014; Warne et al. 2016; Byrne et al. 2018), high variability of individual-level infection intensity is common (Stice and Briggs 2010; DiRenzo et al. 2014; Mendoza-Almeralla et al. 2016). One way to account for measurement error of infection intensities and imperfect pathogen detection are state-space models (DiRenzo et al. 2019), but these models are computationally expensive and data hungry, making them impractical in

some circumstances. In practice, these models are also much more valuable in field settings, where individual infection history is unknown, rather than laboratory settings, where treatments are applied.

Here, we document variable disease dynamics by plethodontid salamanders in response to *Bsal* infection and hyper-sensitivity to *Bsal* by *N. viridescens*. Our results support previous studies that demonstrated high susceptibility of *N. viridescens* to *Bsal* (Martel et al. 2014; Longo et al. 2019; Kumar et al. 2020; Malagon et al. 2020), with animals developing clinical signs of *Bsal* chytridiomycosis and dying quickly. Most Plethodontidae individuals carried moderate *Bsal* infections over time which may have long term sublethal effects and may serve as reservoirs that can infect or re-infect others. Plethodontidae, containing $\sim$ 68% of described salamander species, is among the most diverse salamander families with respect to morphology, ecology, and behavior (AmphibiaWeb), and plethodontid salamanders are one of the most abundant vertebrate predators in eastern North American forests and headwaters (Davic & Welsh 2004; Walton & Steckler 2005). Our results indicate that salamander susceptibility to *Bsal* is variable across plethodontid species, and if *Bsal* were to enter North America, these infection responses have consequences for dynamic, adaptive management actions.

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Author contributions GVD helped design and perform the experiment, complete the statistical analyses, and wrote the first draft of the manuscript. JAG helped with the ancestral state reconstruction and edited the manuscript. KRL helped design, perform the experiment, and edited the manuscript. AVL analyzed swab samples in the laboratory and edited the manuscript. CRM-W helped design and perform the experiment and edited the manuscript. APP interpreted histopathology and edited the manuscript. All authors gave final approval for publication.

Data availability All data and code for analyses can be reproduced and accessed at the github repository: https://github.com/Grace89/Bsal

Declarations

Conflict of interest We have no competing interests.

References

Bletz MC, Kelly M, Sabino-pinto J et al (2018) Disruption of skin microbiota contributes to salamander disease. Proc R Soc B 285:20180758

Blooi M, Pasmans F, Longcore J et al (2013) Duplex real-time PCR for rapid simultaneous detection of Batrachochytrium dendrobatidis and B. salamandrivorans in amphibian samples. J Clin Microbiol 51:4173–4177. https://doi.org/10.1128/JCM.02313-13

Boyle DG, Boyle DB, Olsen V et al (2004) Rapid quantitative detection of chytridiomycosis. Dis Aquat Organ 60:141–148

Boyles JG, Cryan PM, McCracken GF, Kunz TK (2011) Economic importance of bats in agriculture. Science. https://doi.org/10.1126/science.1201366

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proc Natl Acad Sci U S A 107:9695–9700. https://doi.org/10.1073/pnas.0912886107

Byrne AQ, Poorten TJ, Voyles J et al (2018) Opening the file drawer: Unexpected insights from a chytrid infection experiment. PLoS ONE 13:e0196851. https://doi.org/10.1371/journal.pone.0196851

Camp CD, Marshall JL, Landau KR et al (2000) Sympatric occurrence of two species of the two-lined salamander (Eurycea bislineata) complex. Copeia 2000:572–578. https://doi.org/10.1643/0045-8511(2000)000[0572:SOOTSO]2.0.CO;2

Carter ED, Cusaac JPW, Bohanon M et al (2020) Conservation risk of Batrachochytrium salamandrivorans to endemic lungless salamanders. Conserv Lett 13:e12675. https://doi.org/10.1111/conl.12675

Carter ED, Bletz MC, Le Sage M et al (2021) Winter is coming – Temperature affects immune defenses and susceptibility to Batrachochytrium salamandrivorans. PLoS Pathog 17:e1009234. https://doi.org/10.1371/journal.ppat.1009234

Casadevall A, Pirofski LA (2000) Host-pathogen interactions: Basic concepts of microbial commensalism, colonization, infection, and disease. Infect Immun 68:6511–6518. https://doi.org/10.1128/IAI.68.12.6511-6518.2000

Clay TA, Gifford ME (2018) Thermal constraints of energy assimilation on geographical ranges among lungless salamanders of North America. J Biogeogr 45:1664–1674. https://doi.org/10.1111/jbi.13347

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

DiRenzo GV, Langhammer PF, Zamudio KR, Lips KR (2014) Fungal infection intensity and zoospore output of Atelopus zeteki, a potential acute chytrid supershedder. PLoS ONE 9:e93356. https://doi.org/10.1371/journal.pone.0093356

DiRenzo GV, Campbell Grant EH, Longo AV et al (2018) Imperfect pathogen detection from non-invasive skin swabs biases disease inference. Methods Ecol Evol 9:380–389. https://doi.org/10.1111/2041-210X.12868

DiRenzo GV, Che-Castaldo C, Saunders SP et al (2019) Disease-structured N-mixture models: A practical guide to model disease dynamics using count data. Ecol Evol 9:899–909. https://doi.org/10.1002/ece3.4849

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484(7393):186–194. https://doi.org/10.1038/nature10947

Gervasi S, Hunt E (2014) Temporal patterns in immunity, infection load and disease susceptibility: understanding the drivers of host responses in the amphibian-chytrid fungus system. Funct. https://doi.org/10.1111/1365-2435.12194

Gervasi S, Gondhalekar C, Olson DH, Blaustein AR (2013) Host identity matters in the amphibian-Batrachochytrium dendrobatidis System: fine-scale patterns of variation in responses to a multi-host pathogen. PLoS ONE 8:e54490. https://doi.org/10.1371/journal.pone.0054490

Gill DE (1978) The metapopulation ecology of the red-spotted newt, Notophthalmus viridescens (Rafinesque). Ecol Monogr 48:145–166

Gray MJ, Lewis JP, Nanjappa P et al (2015) Batrachochytrium salamandrivorans: The North American Response and a Call for Action. PLoS Pathog 11:1–9. https://doi.org/10.1371/journal.ppat.1005251

Green A (2001) Mass / length residuals: Measures of body condition or generators of spurious results? Ecology 82:1473–1483

Harmon LJ, Weir JT, Brock CD et al (2008) GEIGER: investigating evolutionary radiations. Bioinformatics 24:129–131

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

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biometrical J 50:346–363

Hyatt AD, Boyle DG, Olsen V et al (2007) Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. Dis Aquat Organ 73:175–192. https://doi.org/10.3354/dao073175

Jacobs JF (1987) A preliminary investigation of geographic genetic variation and systematics of the two-lined salamander, Eurycea bislineata (Green). Herpetologicae 43:423–446

Klocke B, Becker M, Lewis J et al (2017) Batrachochytrium salamandrivorans not detected in US survey of pet salamanders. Sci. Rep. 7:13132

Kumar S, Stecher G, Suleski M, Hedges SB (2017) TimeTree: A resource for timelines, timetrees, and divergence times. Mol Biol Evol 34:1812–1819. https://doi.org/10.1093/molbev/msx116

Kumar R, Malagon DA, Carter ED et al (2020) Experimental methodologies can affect pathogenicity of Batrachochytrium salamandrivorans infections. PLoS ONE 15:e0235370. https://doi.org/10.1101/2020.06.16.154328

Lachish S, Gopalaswamy AM, Knowles SCL, Sheldon BC (2012) Site-occupancy modelling as a novel framework for assessing test sensitivity and estimating wildlife disease prevalence from imperfect diagnostic tests. Methods Ecol Evol 3:339–348. https://doi.org/10.1111/j.2041-210X.2011.00156.x

Lenth R (2019) emmeans: Estimated marginal means, aka least-squares means. In: R Packag. version 1.3.5.1

Longo AV, Fleischer RC, Lips KR (2019) Double trouble: co-infections of chytrid fungi will severely impact widely distributed newts. Biol Invasions 21:2233–2245. https://doi.org/10.1007/s10530-019-01973-3

Malagon DA, Melara LA, Prosper OF et al (2020) Host density and habitat structure influence host contact rates and Batrachochytrium salamandrivorans transmission. Sci Rep 10:5584. https://doi.org/10.1038/s41598-020-62351-x

Martel A, Spitzen-van der Sluijs A, Blooi M et al (2013) Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci 110:15325–15329. https://doi.org/10.1073/pnas.1307356110

Martel A, Blooi M, Adriaensen C et al (2014) Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science. https://doi.org/10.1126/science.1258268

Mazerolle MJ (2017) AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). R package version 2.1–1

Medzhitov R, Schneider DS, Soares MP (2012) Disease tolerance as a defense strategy. Science. https://doi.org/10.1126/science.1214935

Mendoza-Almeralla C, López-Velázquez A, Longo AV, Parra-Olea G (2016) Temperature treatments boost subclinical infections of Batrachochytrium dendrobatidis in a Mexican salamander (Pseudoeurycea leprosa). Rev Mex Biodivers 87:171–179. https://doi.org/10.1016/j.rmb.2016.01.020

Miller DAW, Talley BL, Lips KR, Campbell Grant EH (2012) Estimating patterns and drivers of infection prevalence and intensity when detection is imperfect and sampling error occurs. Methods Ecol Evol 3:850–859. https://doi.org/10.1111/j.2041-210X.2012.00216.x

Muletz-Wolz CR, Fleischer RC, Lips KR (2019) Fungal disease and temperature alter skin microbiome structure in an experimental salamander system. Mol Ecol 28:2917–2931. https://doi.org/10.1111/mec.15122

O'Hanlon SJ, Rieux A, Farrer RA et al (2018) Recent Asian origin of chytrid fungi causing global amphibian declines. Science 80(360):621–627

Paradis E, Schliep K (2018) ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35:526–528

Parrott JC, Shepack A, Burkart D et al (2017) Survey of pathogenic chytrid fungi (Batrachochytrium dendrobatidis and B. salamandrivorans) in salamanders from three mountain ranges in Europe and the Americas. EcoHealth 14:296–302. https://doi.org/10.1007/s10393-016-1188-7

Pierson TW, Miele A (2019) Reproduction and life history of two-lined salamanders (Eurycea CF. aquatica) from the upper Tennessee river valley, USA. Herpetol Conserv Biol 14:111–118

Pinheiro J, Bates D, DebRoy S et al (2019) nlme: Linear and Nonlinear Mixed Effects Models. R Packag. version 3:1–140

Reeder NMM, Pessier AP, Vredenburg VT (2012) A reservoir species for the emerging amphibian pathogen Batrachochytrium dendrobatidis thrives in a landscape decimated by disease. PLoS ONE 7:e33567. https://doi.org/10.1371/journal.pone.0033567

Richgels KLD, Russell RE, Adams MJ et al (2016) Spatial variation in risk and consequence of Batrachochytrium salamandrivorans introduction in the USA. R Soc Open Sci 3:150616. https://doi.org/10.1098/rsos.150616

Rosenblum EB, James TY, Zamudio KR et al (2013) Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. Proc Natl Acad Sci U S A 110:9385–9390. https://doi.org/10.1073/pnas.1300130110

Sánchez CA, Becker DJ, Teitelbaum CS et al (2018) On the relationship between body condition and parasite infection in wildlife: a review and meta-analysis. Ecol Lett 21:1869–1884. https://doi.org/10.1111/ele.13160

Schulte-Hostedde AI, Zinner B, Millar JS, Hickling GJ (2005) Restitution of mass-size residuals: validating body condition indices. Ecology 86:155–163. https://doi.org/10.1890/04-0232

Searle CL, Gervasi SS, Hua J et al (2011) Differential host susceptibility to Batrachochytrium dendrobatidis, an emerging amphibian pathogen. Conserv Biol 25:965–974. https://doi.org/10.1111/j.1523-1739.2011.01708.x

Spitzen-van der Sluijs A, Martel A, Asselberghs J et al (2016) Expanding distribution of lethal amphibian fungus Batrachochytrium salamandrivorans in Europe. Emerg Infect Dis 22:1286–1288. https://doi.org/10.3201/eid2207.160109

Stegen G, Pasmans F, Schmidt BR et al (2017) Drivers of salamander extirpation mediated by Batrachochytrium salamandrivorans. Nature 544:353–356. https://doi.org/10.1038/nature22059

Stice MJ, Briggs CJ (2010) Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus Batrachochytrium dendrobatidis. J Wildl Dis 46:70–77

Streicker DG, Fenton A, Pedersen AB (2013) Differential sources of host species heterogeneity influence the transmission and control of multihost parasites. Ecol Lett 16:975–984. https://doi.org/10.1111/ele.12122

Therneau T (2015) _A Package for Survival Analysis in S_. version 2.38

Thomas V, Blooi M, Van Rooij P et al (2018) Recommendations on diagnostic tools for Batrachochytrium salamandrivorans. Transbound Emerg Dis 65:e478–e488. https://doi.org/10.1111/tbed.12787

Waddle JH, Grear DA, Mosher BA et al (2020) Batrachochytrium salamandrivorans (Bsal) not detected in an intensive survey of wild North American amphibians. Sci Rep 10:13012. https://doi.org/10.1038/s41598-020-69486-x

Walton BM, Steckler S (2005) Contrasting effects ofsalamanders on forest- floor macro- and mesofauna in laboratory microcosms. Pedobiologia (Jena) 49:51–60

Warne RW, LaBumbard B, LaGrange S et al (2016) Co-infection by chytrid fungus and ranaviruses in wild and harvested frogs in the tropical andes. PLoS ONE 11:1–15. https://doi.org/10.1371/journal.pone.0145864

Whiles MR, Lips KR, Pringle CM et al (2006) The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. Front Ecol Environ 4:27–34

Whiles MR, Hall RO, Dodds WK et al (2013) Disease-driven amphibian declines alter ecosystem processes in a tropical stream. Ecosystems 16:146–157. https://doi.org/10.1007/s10021-012-9602-7

White CL, Forzan MJ, Pessier AP, Allender MC, Ballard JR, Catenazzi A, Fenton H, Martel A, Pasmans F, Miller DL, Ossiboff RJ, Richgels KLDKJ (2016) A case definition for Batrachochytrium salamandrivorans chytridiomycosis. Herpetol Rev 47:207–209

Wiens JJ, Ackerly DD, Allen AP et al (2010) Niche conservatism as an emerging principle in ecology and conservation biology. Ecol Lett 13:1310–1324. https://doi.org/10.1111/j.1461-0248.2010.01515.x

Wyman RL (1998) Experimental assessment of salamanders as predators of detrital food webs: effects on invertebrates, decomposition and the carbon cycle. Biodivers Conserv 7:641–650

Yap TA, Koo MS, Ambrose RF et al (2015) Averting a North American biodiversity crisis. Science 80(349):481–482. https://doi.org/10.1126/science.aab1052

Zipkin EF, DiRenzo GV, Ray JM et al (2020) Tropical snake diversity collapses after widespread amphibian loss. Science 80(367):814–816. https://doi.org/10.1126/science.aay5733