Experimental methodologies can affect pathogenicity of *Batrachochytrium salamandrivorans* infections

Rajeev Kumar¹, Daniel A. Malagon¹, Edward Davis Carter¹, Debra L. Miller¹,², Markese L. Bohanon¹, Joseph Patrick W. Cusaac¹, Anna C. Peterson¹, Matthew J. Gray*¹

¹ Center for Wildlife Health, University of Tennessee Institute of Agriculture, Knoxville, Tennessee, United States of America, ² Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee, United States of America

* mgray11@utk.edu

Abstract

Controlled experiments are one approach to understanding the pathogenicity of etiologic agents to susceptible hosts. The recently discovered fungal pathogen, *Batrachochytrium salamandrivorans* (*Bsal*), has resulted in a surge of experimental investigations because of its potential to impact global salamander biodiversity. However, variation in experimental methodologies could thwart knowledge advancement by introducing confounding factors that make comparisons difficult among studies. Thus, our objective was to evaluate if variation in experimental methods changed inferences made on the pathogenicity of *Bsal*. We tested whether passage duration of *Bsal* culture, exposure method of the host to *Bsal* (water bath vs. skin inoculation), *Bsal* culturing method (liquid vs. plated), host husbandry conditions (aquatic vs. terrestrial), and skin swabbing frequency influenced disease-induced mortality in a susceptible host species, the eastern newt (*Notophthalmus viridescens*). We found that disease-induced mortality was faster for eastern newts when exposed to a low passage isolate, when newts were housed in terrestrial environments, and if exposure to zoospores occurred via water bath. We did not detect differences in disease-induced mortality between culturing methods or swabbing frequencies. Our results illustrate the need to standardize methods among *Bsal* experiments. We provide suggestions for future *Bsal* experiments in the context of hypothesis testing and discuss the ecological implications of our results.

Introduction

*Batrachochytrium salamandrivorans* (*Bsal*) is an emerging fungal pathogen of global conservation concern [1–3]. *Bsal* has been identified as the causal agent in recent near extirpations of wild fire salamanders (*Salamandra salamandra*) in Belgium and the Netherlands [4, 5], and has been detected in live amphibians in captivity and moving through international trade [6–8]. Controlled experiments where hosts are exposed to *Bsal* under standardized conditions suggest that the pathogen has a broad host range, including several salamander and anuran...
Funding: This work was partially supported by the National Science Foundation Division of Environmental Biology (EID Grant # 1814520) and USDA National Institute of Food and Agriculture (Hatch Project 1012932) awarded to MJG and DLM. The sponsors did not play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. URLs: https://www.nsf.gov/funding/pgm_summ.jsp?pims_id=5269; https://nifa.usda.gov.

Competing interests: NO authors have competing interests.
Materials and methods

Methods common among experiments

Below are the methods common for all experiments unless noted otherwise. Sample sizes, *Bsal* doses, host life-stage and swabbing frequencies are in Table 1. Similar to Martel et al. [9] and Longo et al. [14], we used post-metamorphic eastern newts (*Notophthalmus viridescens*) for all experiments. Newts were collected from one site in Knox County, Tennessee, USA (Scientific Collection Permit #1504), and were confirmed to be *Bd* negative prior to the start of each experiment. We performed each experiment at 15˚C in environmental growth chambers, with relative humidity maintained between 80–90%. All water-bath exposed newts were exposed to *Bsal* in 100-mL containers with 1 mL inoculum and 9 mL autoclaved dechlorinated water. The *Bsal* used in our experiments was originally isolated by An Martel and Frank Pasmans from a morbid wild fire salamander in the Netherlands (isolate: AMFP13/1), and had been passaged (i.e., split) in cell culture ca. 20 times (P20) at the start of our experiments. The 200-passage isolate (P200) was maintained in culture and split ca. biweekly, while P20 was cryopreserved and revived for each experiment. We grew *Bsal* on TGhL plates and harvested zoospores by flooding each plate with 7 mL autoclaved dechlorinated water and filtering the suspended zoospores through a 20-um filter to remove zoosporangia. The target exposure dose was prepared by diluting the *Bsal* zoospores in autoclaved dechlorinated water (Table 1). For the exposure route, housing and swabbing experiments, we exposed animals to a single dose of 5x10⁶ zoospores. For passage and culture experiments, we used a lower dose of 1x10⁶ and 5x10⁵ zoospores because we were unable to harvest greater quantities for these experiments. These exposure doses were sufficient to cause infection and induce *Bsal* chytridiomycosis [9]. The control newts used for each experiment were exposed to autoclaved dechlorinated water under identical conditions. After a 24-hr exposure period, we removed the animals from the exposure containers and placed them in housing containers. We housed newts terrestrial in 710-mL plastic containers with a moist paper towel and PVC cover object. The exception was the housing experiment, where half of the newts were housed in circular 2-L containers with 300 mL of dechlorinated water. To minimize accumulation of nitrogenous waste, we transferred newts into clean containers and replaced all the materials every three days. We fed terrestrial housed newts small crickets corresponding to 8% of their body mass when containers were changed. Aquatically housed newts were fed bloodworms. We checked newts twice daily for gross signs of *Bsal* chytridiomycosis (e.g., necrotic lesions, skin sloughing, lethargy), and humanely euthanized individuals that lost righting reflex or at the end of the experiment.

We estimated *Bsal* load at necropsy by swabbing the skin of newts following the standardized protocol used for *Bd* [20], and compared loads among treatments. Genomic DNA (gDNA) was extracted from each swab using Qiagen DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany). We estimated *Bsal* load using *Bsal* singleplex qPCR methods similar to those described in Blooi et al. [21]. All qPCR reactions were amplified using an Applied Biosystems QuantaStudio 6 Flex qPCR instrument (Thermo Fisher Scientific, USA). Each swab sample was run in duplicate and considered positive if both replicates amplified within 50 cycles. We also verified that newts were *Bd* negative at the start and end of each experiment using qPCR, because co-infection with *Bd* and *Bsal* can affect host survival [14]. For newts that died during the experiment, we confirmed *Bsal* chytridiomycosis by examining histological cross-sections of hematoxylin and eosin stained epidermal tissue [22]. Representative images of each experimental treatment are provided. We used *Bsal*-induced mortality confirmed by qPCR and histopathology as evidence of pathogenicity [22].
Table 1. Experiment, treatment, life-stage tested, exposure dose, exposure method, culture type, swabbing frequency, sample size (n), and descriptive statistics (mean and standard deviation, SD) for animals that died or survived the experiment. Also shown are Wilcox rank-sum and Kruskal-Wallis tests comparing Bsal loads of all animals that died during the experiment as well as test results comparing all animals used in each treatment.

| Experiment | Treatment | Life Stage | Exposure Dose | Exposure Culture Type | Swabbing Frequency | N | Dead Animal Bsal Copies/μL | Survived Animal Bsal Copies/μL | Necropsy Copies/μL – Treatment for Dead Animals | Necropsy Copies/μL – Treatment for All Animals |
|------------|-----------|------------|---------------|-----------------------|--------------------|---|--------------------------|-------------------------------|-----------------------------------------------|-----------------------------------------------|
| Passageway | Control   | Adult      | Autoclaved    | Water Bath            | Plate              | 6 | 0(5)                     | 0                            | 4                             | 0.08                           |
| Exposure   | Adult     | 1x10^6     | 10            | 46926.54              | 47720.7            |   |                          |                               |                                |                                |
|            | 200X Adult| 1x10^6     | 10            | 2078.95(3)            | 118048.33          |   |                          |                               |                                |                                |
| Culture Type | Control (Water Bath)** | Adult | Autoclaved | Water Bath | Plate | Every 6 Days | 5 | 0(2) | 0 | 2 | 0.1 | 14 | 0.6 |
|            | Water Bath+ | Adult | 5x10^6 | Water Bath | 6 | 57400.43(6) | 35511.13 |
|            | Pipette | Adult | 5x10^6 | Pipette | 6 | 28862.68(3) | 14059.314 |
|            | Liquid Eft | 1x10^5 | Liquid Broth | 8 | 3432.88(2) | 4854.83 |
|            | Plated Eft | 1x10^5 | Plate | 8 | 9410.3(5) | 12377.91 |
| Housing | Control (Aquatic) | Adult | Autoclaved | Water Bath | Plate | Every 6 Days | 6 | 0 | 0 | 10 | 0.91 | 15 | 0.7 |
|            | Control (Terrestrial)** | Adult | Autoclaved | Water Bath | Plate | Every 6 Days | 6 | 0 | 0 |
|            | Aquatic | Adult | 5x10^6 | 6 | 61648.91(3) | 33928.65 |
|            | Terrestrial* | Adult | 5x10^6 | 6 | 57400.43(6) | 35511.13 |
| Swabbing Frequency | Control (6 day swab)** | Adult | Autoclaved | Water Bath | Plate | Every 6 Days | 6 | 0(2) | 0 | 3.37 | 0.19 | 1.63 | 0.44 |
|            | Control (12 day swab) | Adult | Autoclaved | Water Bath | Plate | Every 12 Days| 6 | 0(2) | 0 |
|            | Control (Necropsy swab) | Adult | Autoclaved | Water Bath | Plate | Only Necropsy | 6 | 0(2) | 0 |
|            | 6 day swab+ | Adult | 5x10^6 | 6 | 57400.43(6) | 35511.13 |
|            | 12 day swab | Adult | 5x10^6 | 6 | 140061.79(5) | 24931.4 |
|            | Necropsy swab | Adult | 5x10^6 | Only Necropsy | 6 | 59445.86(6) | 37745.56 |

*Indicates the experimental group was used for more than one comparison.
**Indicates the control group was used for more than one comparison.

https://doi.org/10.1371/journal.pone.0235370.1001
Experiment-specific methods
In order to minimize the total number of animals used for these experiments, we used newts exposed via water bath, housed terrestrially and swabbed every 6 days for comparisons of exposure route, housing conditions and swabbing frequency. These newts only differed from the other treatment group by the specific exposure method being compared (Table 1). For example, the water bath and pipette-exposed newts were exposed to the same passage isolate and zoospores harvested from plates, and newts were housed terrestrially after Bsal exposure and swabbed every six days.

Bsal passage history experiment. To test whether passage history of cultures affected Bsal pathogenicity, we randomly exposed newts to isolates from one of two culture treatments: 20 and 200 passages. We defined a passage as splitting cultures by inoculating 1 mL of TGhL broth containing suspended Bsals into 9 mL of new TGhL broth [23].

Bsal zoospore exposure route experiment. We tested if route of zoospore exposure influenced pathogenicity by randomly exposing newts to zoospores in water (as previously described) or by pipette inoculation. The pipette-inoculated newts were exposed by pipetting 1 mL of Bsals inoculum onto the dorsal aspect of the newt similar to Martel et al. [9].

Bsal culture type experiment. We tested for differences in Bsal pathogenicity between culturing methods by randomly exposing newts to either inoculum collected from TGhL plates (as previously described and done by Longo et al. [14]) or to zoospores harvested from TGhL broth media containing suspended Bsals similar to Martel et al. [9]. We filtered the broth media identical to TGhL plates to create the inoculum.

Housing experiment. To test whether housing conditions (terrestrial vs. aquatic) affected pathogenicity, we randomly assigned newts to either terrestrial containers (as described before) or to 2-L containers with 300 mL of aged dechlorinated water and a PVC cover object following the 24-hr exposure to Bsals.

Newt swabbing frequency experiment. Lastly, we tested whether swabbing frequency impacted Bsals pathogenicity by randomly assigning newts to one of three swabbing frequencies: swabbed only at necropsy, every six days or every 12 days. Swabbing technique was identical among treatments and followed Boyle et al. [20].

Statistical analyses
We compared median survival rates among treatments for each experiment using Kaplan-Meier analysis and the statistical software R (Version 3.6.1) [24]. We evaluated differences between two or more survival curves at $\alpha = 0.05$ using the "survdiff" function in the survival package [25, 26]. Hazard ratios were calculated using the “coxph” function in the survival package for a robust estimate of the magnitude of treatment differences [25]. We compared copies of Bsals DNA per uL extracted from swabs collected at necropsy using Wilcoxon rank sum tests or Kruskal-Wallis tests when comparing multiple groups, because data did not follow a normal distribution. If there were >2 treatments and the Kruskal-Wallis test was significant, we used Wilcoxon tests corrected with the Benjamin and Hochberg adjustment for post-hoc treatment comparisons. All Bsals copy comparisons were made using the stats package in R studio [24]. Data and R Code for all analyses are provided in the online supporting information.

Ethics statement
All husbandry and euthanasia procedures followed recommendations provided by the Association of Zoos and Aquariums and the American Veterinary Medical Association, and were approved by the University of Tennessee Institutional Animal Care and Use Committee.
(Protocol #2395). Newts that reached euthanasia endpoints were humanely euthanized via transdermal exposure to benzocaine hydrochloride.

## Results

Survival of eastern newts exposed to the P200 culture was significantly greater than newts exposed to the P20 culture ($X^2 = 11.4, P < 0.001$; Fig 1A). The odds of an individual dying when exposed to the P20 culture were 7.8X times greater than the P200 culture. Although Bsal loads were high in all animals that died ($\bar{x} = 36,577; SD = 45,791$ copies per uL), copies at necropsy did not differ significantly between treatments ($W = 28, P = 0.18$; S1 Fig).

Survival of newts exposed to zoospores via pipette inoculation was greater than newts inoculated via water bath ($X^2 = 11.6, P < 0.001$, Fig 1B). The odds of an individual dying when exposed to Bsal in a water bath were >100X times greater than pipette exposure on the dorsum. No significant differences in Bsal loads at necropsy were detected between these two treatments ($W = 14, P = 0.59$; S1 Fig).

We detected no differences in survival between liquid cultures and flooded plates ($X^2 = 1.9, P = 0.13$; Fig 1D). Bsal loads at necropsy were similar among treatments for animals exposed to zoospores harvested from liquid and plated cultures ($W = 16.5, P = 0.07$; S1 Fig).

Survival of Bsal-exposed newts was significantly greater for individuals housed aquatically compared to those housed terrestrially ($X^2 = 5.3, P = 0.02$, Fig 1C). The odds of an infected newt dying in terrestrial containers were 4X greater than newts housed aquatically. No

![Fig 1](https://doi.org/10.1371/journal.pone.0235370.g001)

(A-E). Kaplan–Meier survival curves showing survival of eastern newts (*Notophthalmus viridescens*) exposed to Bsal zoospores. Log-rank test ($X^2$) and $P$-values evaluating differences among survival curves for each experiment are shown for animals exposed to P20 or P200 isolates (A), animals exposed to Bsal via pipette or water bath inoculation (B), animals housed aquatically or terrestrially after exposure (C), animals exposed to Bsal zoospores harvested from TGhL agar plates or TGhL broth (D), and animals swabbed either every 6 days, every 12 days or only at necropsy (E).
differences were detected in Bsal loads at necropsy between housing treatments (W = 15, P = 0.69; S1 Fig).

We detected no differences in survival among swabbing frequencies (X^2 = 0.7, P = 0.7; Fig 1E). Bsal loads at necropsy were also similar among animals swabbed at different frequencies (X^2 = 1.54, P = 0.46; S1 Fig).

For all animals that died, we observed histological signs of Bsal chytridiomycosis (Fig 2). No control animals died during the study or were qPCR positive for Bsal DNA at the end of the experiment. Additionally, no animals tested positive for Bd infections at the start or end of the experiment.

Discussion

We found that Bsal-induced mortality was greater for eastern newts when exposed to the low passage isolate, when newts were exposed to zoospores via water bath, and when newts were housed in terrestrial environments. Newts exposed to the P20 isolate had greater odds of dying from Bsal chytridiomycosis compared to the P200 isolate, indicating differences in pathogenicity caused by passage history. Several studies on the genetically similar Bd chytrid fungus have reported loss of pathogen virulence associated with greater number of passages in culture [16, 17, 27]. Increased passage number can reduce zoospore production rate and total number of zoospores produced by zoosporangia [27]. Although this may have occurred in our study, we controlled for potential differences in production by enumerating zoospores and verifying viability using flow cytometry, and all individuals were exposed to a common dose. Reduced selection (i.e., attenuation) or differential expression of virulence genes in culture could have influenced pathogenicity [17], as suggested by studies comparing Bd that was recently isolated from wild hosts to Bd in cell culture. For example, Ellison et al. [16] found that Bd transcriptomes isolated from two infected amphibian hosts exhibited higher expression of genes associated with increased virulence when compared to a Bd culture grown in the lab. Rosenblum et al. [28] also reported that Bd cultured on frog skin displayed a greater number of genes coding for proteases that affect pathogenicity when compared to Bd cultured using tryptone media [29]. Hence, the differences that we observed in Bsal’s pathogenicity may have been driven by genomic changes (e.g., attenuation), phenotypic expression of virulence genes, or shifts in population composition of zoospores to less virulent types in the long-passage isolate.

Newts exposed to Bsal via 24-hr water bath had >100X greater odds of dying due to the pathogen than individuals exposed by directly pipetting the pathogen on the animal’s dorsum, which may be related to a greater exposed skin surface area in water for pathogen encystment. If so, increased encystment could have led to faster and more severe disease development. It is possible that viability of zoospores pipetted onto the dorsum of an animal also declined more rapidly than zoospores in a water bath, because Bsal is predominantly an aquatic pathogen and viability of Bsal zoospores decreases rapidly on dry substrate [4]. Thus, infection efficiency of Bsal zoospores in water may have contributed to the differences in newts resisting infection. Ecologically, these results suggest that transmission of Bsal may be greater in water than through direct transfer of zoospores occurring from host-to-host contact.

Exposure to Bsal zoospores collected by flooding TGhL agar plates resulted in greater final mortality (62.5%) than exposure to zoospores grown in and collected from TGhL broth (25%).
Although these differences in mortality were not statistically significant, they represent a 2.5-fold difference in experimental outcomes. Harvesting Bsal from TGhL agar plates might more closely resemble the natural life cycle of Bsal (i.e., zoospore encysts in the epidermis of the host, forms a zoosporangium, and it releases zoospores [5]). Growing Bsal in TGhL broth may represent an alteration from the typical life cycle and select for zoospores and zoosporangia that grow well when immersed in a nutrient solution rather than when adhered to a substrate, including skin. TGhL broth cultures also might result in mixed-aged cultures with fewer infectious motile zoospores compared to more synchronized, even-aged zoospore release on agar plates.

Eastern newts that were housed terrestrially had 4X greater odds of experiencing Bsal-induced mortality than those housed aquatically. Although adult eastern newts can be found in terrestrial environments [30, 31], this age class is found most often in aquatic systems [32]. Our study animals were collected from a permanent wetland (i.e., pond), thus the terrestrial environment may have resulted in greater host stress. Increased stress can compromise immune function and thus potentially facilitate greater zoospore infection and disease progression [33]. We also observed that aquatically housed newts were able to shed their skin more easily, which may decrease infection loads and thereby reduce the severity of chytridiomycosis because zoospores are shed into the environment rather than being confined to the animal’s skin. Skin shedding has been hypothesized as a resistance mechanism for chytrid infections [34], because shed skin can contain infectious zoospores hence possibly reduce reinfection of the host. Ecologically, the greater pathogenicity of Bsal in the terrestrial environment suggests that if infected newts leave the aquatic environment (e.g., during pond drying), their likelihood of dying from Bsal chytridiomycosis will increase. Species that mostly or entirely use the terrestrial environment (e.g., Salamandra salamandra) also may be at greater risk.

Lastly, swabbing frequency had no apparent effect on survival or Bsal loads. Although swabbing can remove zoospores [18], it likely does not remove all zoosporangia, which can extend deeper into the stratum corneum and stratum granulosum [35, 36]. In histological cross-sections, we observed removal of epidermal layers, presumably from swabbing, and the presence of zoosporangia thereafter. Although we did not measure indicators of stress response, it is likely that newts which were never swabbed experienced less stress than swabbed individuals; however, perhaps acute presence of immunosuppressive stress hormones, associated with handling, were offset by some zoospore removal during swabbing [33, 37].

Collectively, our results might provide some additional insight into the differences observed between Martel et al. [9] and Longo et al. [14]. In particular, the isolate used by Martel et al. [9] was lower passage and they housed newts terrestrially; whereas, newts had a choice between aquatic and terrestrial environments in Longo et al. [14]. These methodological differences between the two studies might explain why Martel et al. [9] observed greater mortality than Longo et al. [14] even though the same species was challenged. Interestingly, Longo et al. [14] exposed newts to zoospores in a water bath, yet observed less mortality than Martel et al. [9] who pipetted the pathogen on the dorsum of newts. Hence, exposure method might have less of an effect on Bsal pathogenicity than isolate passage duration and housing conditions.

Our results highlight the importance of standardizing methods in Bsal experiments if results are going to be compared among studies, or at a minimum acknowledging how methodological differences could lead to biases in interpreting disease outcomes. Given our results, we provide suggestions for future Bsal exposure experiments. We recommend that low-passage (<20 passages) inoculum be used for all experiments to facilitate study comparisons, unless the objective is to understand Bsal evolution or gene expression in culture. We also recommend flooding TGhL agar plates to collect zoospores, and that the exposure route be chosen to mimic the most likely transmission pathway in nature. For example, transmission of
Bsal in fire salamanders likely occurs most often during terrestrial breeding events via contact [4, 38]. Hence, pipetting inoculum on the animal might represent the most realistic route of exposure as it more closely mimics a direct contact scenario. Exposure to Bsal in a water bath likely represents a common transmission pathway for aquatic species such as adult eastern newts. Similarly, we recommend that the housing conditions represent the most likely environment of the host, and for hosts that use both environments, the option to enter and leave water should be provided. Lastly, we recommend that the standardized swabbing protocol for Bd is followed [18]; however, swabbing frequency should depend on the study objectives. For studies where tracking infection dynamics is essential, swabbing once per week should capture changes in prevalence and loads given that the Bsal infected animals typically survive for several weeks [9, 10], allowing for load comparisons over time. However, swabbing can affect histological interpretation of disease progression by removing skin layers (DLM, person. observ.). Given that swabbing frequency did not impact Bsal-induced mortality in our study, swabbing a subset of individuals for infection data and using a different set of non-swabbed animals for histological examination and disease determination might be an appropriate methodological design.

One caveat of all methods used throughout this series of challenge experiments is that they do not necessarily reflect the conditions amphibians experience as they encounter pathogens in a natural environment. However, in order to understand the complexities of natural disease systems, it is often useful to evaluate possible factors individually and in combination with a controlled, common-garden experimental design then scale-up influential factors to mesocosm or natural experiments. Reducing methodological differences among controlled studies increases the likelihood that outcomes observed reflect true biological processes.

Supporting information

S1 Fig. (TIF)
S2 Fig. (JPG)
S3 Fig. (JPG)
S4 Fig. (JPG)

Acknowledgments

We would like to acknowledge Dr. Jeffrey Kovac and the University of Tennessee College Scholars Program for providing support to DAM. We thank Dr. Bobby Simpson and Alex Anderson of the University of Tennessee East Tennessee Research and Education Center for laboratory and logistical support. We also thank Brian Gleaves, Ciara Sheets, Ana Towe, and Bailee Augustino for assistance with animal care and data collection. Appreciation is extended to Allison Byrne and two anonymous reviewers for providing helpful comments on earlier versions of our manuscript.

Author Contributions

Conceptualization: Rajeev Kumar, Daniel A. Malagon, Edward Davis Carter, Debra L. Miller, Matthew J. Gray.
Data curation: Edward Davis Carter.

Formal analysis: Edward Davis Carter.

Funding acquisition: Matthew J. Gray.

Investigation: Rajeev Kumar, Daniel A. Malagon, Edward Davis Carter, Markese L. Bohanon, Matthew J. Gray.

Methodology: Rajeev Kumar, Daniel A. Malagon, Edward Davis Carter, Debra L. Miller, Markese L. Bohanon, Joseph Patrick W. Cusaac, Matthew J. Gray.

Project administration: Debra L. Miller, Matthew J. Gray.

Resources: Debra L. Miller, Matthew J. Gray.

Supervision: Debra L. Miller, Joseph Patrick W. Cusaac, Anna C. Peterson, Matthew J. Gray.

Validation: Debra L. Miller, Matthew J. Gray.

Visualization: Edward Davis Carter.

Writing – original draft: Rajeev Kumar, Daniel A. Malagon, Edward Davis Carter, Debra L. Miller, Markese L. Bohanon, Joseph Patrick W. Cusaac, Anna C. Peterson, Matthew J. Gray.

Writing – review & editing: Rajeev Kumar, Daniel A. Malagon, Edward Davis Carter, Debra L. Miller, Anna C. Peterson, Matthew J. Gray.

References

1. Yap TA, Koo MS, Ambrose RF, Wake DB, Vredenburg VT. Averting a North American biodiversity crisis: A newly described pathogen poses a major threat to salamanders via trade. Science (80-). 2015; 349: 481–482. https://doi.org/10.1126/science.aab1052

2. Richgels KLD, Russell RE, Adams MJ, White CL, Grant EHC. Spatial variation in risk and consequence of Batrachochytrium salamandrivorans introduction in the USA. R Soc Open Sci. 2016; 3. https://doi.org/10.1098/rsos.150616

3. Gray MJ, Lewis JP, Nanjappa P, Klocke B, Pasmans F, Martel A, et al. Batrachochytrium salamandrivorans: The North American Response and a Call for Action. PLoS Pathogens. 2015. https://doi.org/10.1371/journal.ppat.1005251

4. Stegen G, Pasmans F, Schmidt BR, Rouffaer LO, Van Praet S, Schaub M, et al. Drivers of salamander extirpation mediated by Batrachochytrium salamandrivorans. Nature. 2017; 544: 353–356. https://doi.org/10.1038/nature22059

5. Martel A, Spitzen-Van Der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, et al. Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A. 2013; 110: 15325–15329. https://doi.org/10.1073/pnas.1307356110

6. Sabino-Pinto J, Bietz M, Hendrix R, Perl RGB, Martel A, Pasmans F, et al. First detection of the emerging fungal pathogen Batrachochytrium salamandrivorans in Germany. Amphib Reptil. 2015; 36: 411–416. https://doi.org/10.1163/15685381-00003008

7. Nguyen TT, Nguyen T Van, Ziegler T, Pasmans F, Martel A. Trade in wild anurans vectors the urodelan pathogen Batrachochytrium salamandrivorans into Europe. Amphib Reptil. 2017; 38: 554–556. https://doi.org/10.1163/15685381-0003125

8. Fitzpatrick LD, Pasmans F, Martel A, Cunningham AA. Epidemiological tracing of Batrachochytrium salamandrivorans identifies widespread infection and associated mortalities in private amphibian collections. Sci Rep. 2018; 8. https://doi.org/10.1038/s41598-018-31800-z

9. Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, et al. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science (80-). 2014; 346: 630–631. https://doi.org/10.1126/science.1258268
10. Carter ED, Miller DL, Peterson AC, Sutton WB, Cusac JPW, Spatz JA, et al. Conservation risk of Batrachochytrium salamandrivorans to endemic lungless salamanders. Conserv Lett. 2019; https://doi.org/10.1111/conl.12675

11. Johnson PTJ, Ostfeld RS, Keesing F. Frontiers in research on biodiversity and disease. Ecol Lett. 2015; 18: 1119–1133. https://doi.org/10.1111/ele.12479

12. Gray MJ, Chinchar VG. Ranaviruses: lethal pathogens of ectothermic vertebrates. Choice Rev Online. 2015; 53: 53-1272–53–1272. https://doi.org/10.5860/choice.192841

13. Sauer EL, Cohen JM, Lajeunesse MJ, McMahon TA, Civitello DJ, Knutie SA, et al. A meta-analysis reveals temperature, dose, life stage, and taxonomy influence host susceptibility to a fungal parasite. Ecology. 2020; 101. https://doi.org/10.1002/ency.2979

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

15. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science (80-). 2019; 363: 1459–1463. https://doi.org/10.1126/science.aaq0379

16. Ellison AR, DiRenzo GV., McDonald CA, Lips KR, Zamudio KR. First in vivo Batrachochytrium dendrobatidis transcriptomes reveal mechanisms of host exploitation, host-specific gene expression, and expressed genotype shifts. G3 Genes, Genomes, Genet. 2017; 7: 269–278. https://doi.org/10.1534/g3.116.035873

17. Refsnyder JM, Poorten TJ, Langhammer PF, Burrowes PA, Rosenblum EB. Genomic correlates of virulence attenuation in the deadly amphibian chytrid fungus, Batrachochytrium dendrobatidis. G3 Genomes, Genes, Genet. 2015; 5: 2291–2298. https://doi.org/10.1534/g3.115.021808

18. Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, et al. Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms. 2007. pp. 175–192. https://doi.org/10.3354/dao073175

19. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. Science. American Association for the Advancement of Science; 2012. pp. 936–941. https://doi.org/10.1126/science.1214935

20. Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ. 2004; 60: 141–148. https://doi.org/10.3354/dao060141

21. Blooi M, Pasmans F, Longcore JE, Spitzen-Van Der Sluijs A, Vercammen F, Martel A. Duplex real-Time PCR for rapid simultaneous detection of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans in amphibian samples. J Clin Microbiol. 2013; 51: 4173–4177. https://doi.org/10.1128/JCM.02313-13

22. Thomas V, Blooi M, Van Rooij P, Van Praet S, Verbrugghe E, Grasselli E, et al. Recommendations on diagnostic tools for Batrachochytrium salamandrivorans. Transbound Emerg Dis. 2018; 65: e478–e488. https://doi.org/10.1111/tbed.12787

23. Voyles J, Johnson LR, Briggs CJ, Cashins SD, Alford RA, Berger L, et al. Experimental evolution alters the rate and temporal pattern of population growth in Batrachochytrium dendrobatidis, a lethal fungal pathogen of amphibians. Ecol Evol. 2014; 4: 3633–3641. https://doi.org/10.1002/ece3.1199

24. R Core Team. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria URL http://www.R-project.org/; 2014; R Foundation for Statistical Computing.

25. Therneau TM, Grambsch PM. Modeling Survival Data: Extending the Cox Model | Terry M. Therneau| Springer. Statistics for Biology and Health. 2000.

26. Jager KJ, Van Dijk PC, Zoccali C, Dekker FW. The analysis of survival data: The Kaplan-Meier method. Kidney Int. 2008; 74: 560–565. https://doi.org/10.1038/ki.2008.217

27. Langhammer PF, Lips KR, Burrowes PA, Tunstall T, Palmer CM, Collins JP. A Fungal Pathogen of Amphibians, Batrachochytrium dendrobatidis, Attenuates in Pathogenicity with In Vitro Passages. PLoS One. 2013; 8. https://doi.org/10.1371/journal.pone.0077630

28. Rosenblum EB, Poorten TJ, Joneson S, Settles M. Substrate-Specific Gene Expression in Batrachochytrium dendrobatidis, the Chytrid Pathogen of Amphibians. PLoS One. 2012; 7. https://doi.org/10.1371/journal.pone.0049924

29. Farrer RA, Martel A, Verbrugghe E, Abouelil A, Ducatelle R, Longcore JE, et al. Genomic innovations linked to infection strategies across emerging pathogenic chytrid fungi. Nat Commun. 2017; 8. https://doi.org/10.1038/ncomms14742
30. Walters PJ, Greenwald L. Physiological Adaptations of Aquatic Newts (Notophthalmus viridescens) to a Terrestrial Environment. Physiol Zool. 1977; 50: 88–98. https://doi.org/10.1086/physzool.50.2.30152549

31. Roe AW, Grayson KL. Terrestrial Movements and Habitat Use of Juvenile and Emigrating Adult Eastern Red-Spotted Newts, Notophthalmus Viridescens. J Herpetol. 2008; 42: 22–30. https://doi.org/10.1670/07-040.1

32. Gill DE. The Metapopulation Ecology of the Red-Spotted Newt, Notophthalmus viridescens (Rafinesque). Ecol Monogr. 1978; 48: 145–166. https://doi.org/10.2307/2937297

33. Rollins-Smith LA. Amphibian immunity–stress, disease, and climate change. Dev Comp Immunol. 2017; 66: 111–119. https://doi.org/10.1016/j.dci.2016.07.002

34. Ohmer MEB, Cramp RL, Russo CJM, White CR, Franklin CE. Skin sloughing in susceptible and resistant amphibians regulates infection with a fungal pathogen. Sci Rep. 2017; 7. https://doi.org/10.1038/s41598-017-03605-z

35. Van Rooij P, Martel A, Haesebrouck F, Pasmans F. Amphibian chytridiomycosis: A review with focus on fungus-host interactions. Veterinary Research. 2015. https://doi.org/10.1186/s13567-015-0266-0

36. Ossiboff RJ, Towe AE, Brown MA, Longo A V., Lips KR, Miller DL, et al. Differentiating Batrachochytrium dendrobatidis and B. salamandrivorans in Amphibian Chytridiomycosis Using RNAScope® in situ Hybridization. Front Vet Sci. 2019; 6: 304. https://doi.org/10.3389/fvets.2019.00304

37. Graham SP, Kelehear C, Brown GP, Shine R. Corticosterone-immune interactions during captive stress in invading Australian cane toads (Rhinella marina). Horm Behav. 2012; 62: 146–153. https://doi.org/10.1016/j.yhbeh.2012.06.001

38. Schmidt BR, Bozzuto C, Lötters S, Steinfartz S. Dynamics of host populations affected by the emerging fungal pathogen Batrachochytrium salamandrivorans. R Soc Open Sci. 2017; 4. https://doi.org/10.1098/rsos.160801