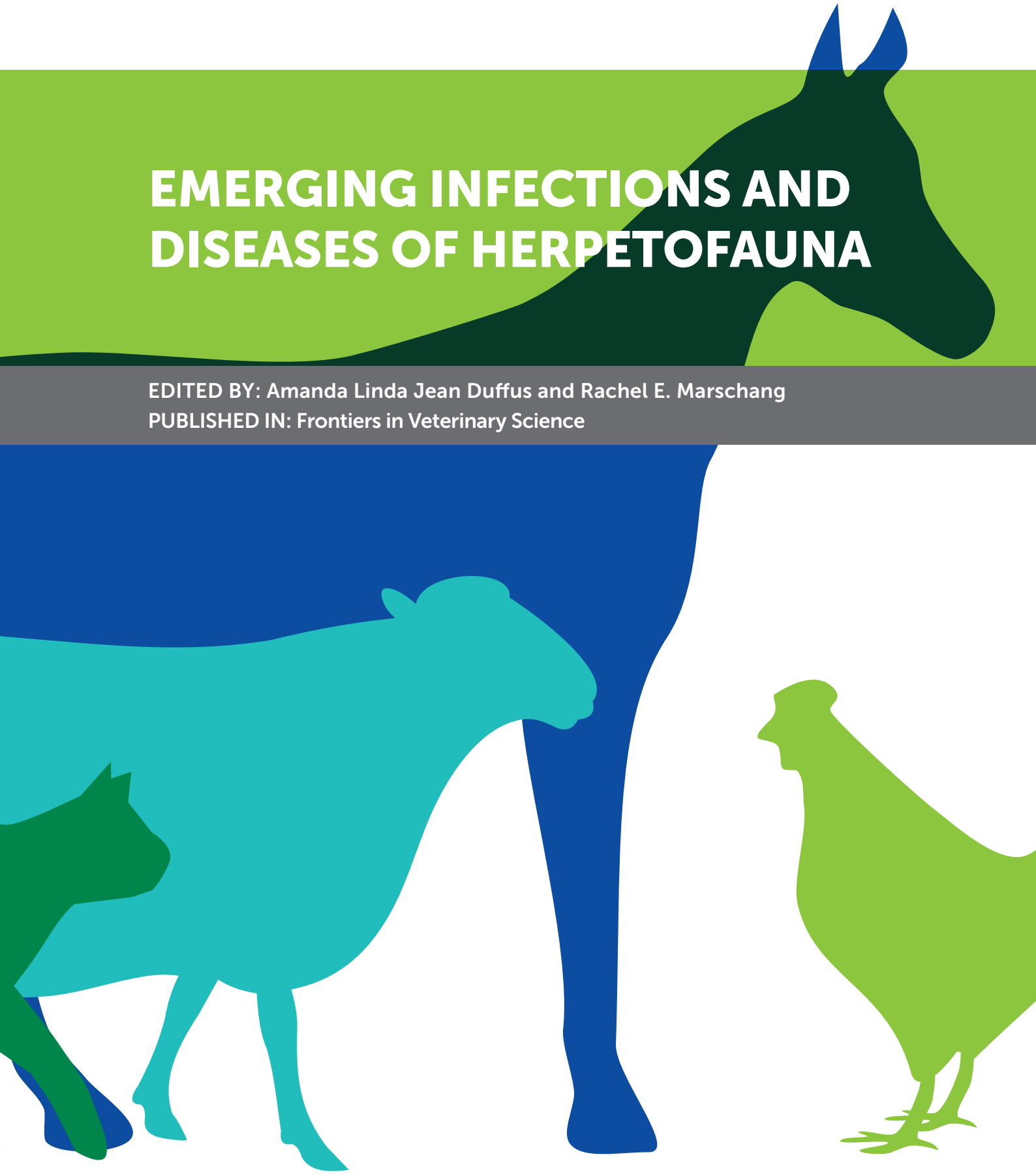
EMERGING INFECTIONS AND DISEASES OF HERPETOFAUNA

EDITED BY: Amanda Linda Jean Duffus and Rachel E. Marschang
PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-473-4
DOI 10.3389/978-2-83250-473-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

EMERGING INFECTIONS AND DISEASES OF HERPETOFAUNA

Topic Editors:
Amanda Linda Jean Duffus, Gordon State College, United States
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Citation: Duffus, A. L. J., Marschang, R. E., eds. (2022). Emerging Infections and Diseases of Herpetofauna. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-473-4

Table of Contents

frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 27 September 2022
DOI 10.3389/fvets.2022.909616

OPEN ACCESS

EDITED BY
Ferran Jori,
UMRASTRE—CIRAD, France

REVIEWED BY
Sean Michael Perry,
Louisiana State University,
United States
Francesco Carlo Origgi,
University of Bern, Switzerland

*CORRESPONDENCE
Amanda L. J. Duffus
aduffus@gordonstate.edu

SPECIALTY SECTION
This article was submitted to Zoological Medicine, a section of the journal Frontiers in Veterinary Science

RECEIVED 31 March 2022
ACCEPTED 26 August 2022
PUBLISHED 27 September 2022

CITATION
Allain SJR, Duffus ALJ and Marschang RE (2022) Editorial: Emerging infections and diseases of herpetofauna.
Front. Vet. Sci. 9:909616.
doi: 10.3389/fvets.2022.909616

Editorial: Emerging infections and diseases of herpetofauna

Steven J. R. Allain[1], Amanda L. J. Duffus[2]* and Rachel E. Marschang[3]

[1]Durrell Institute of Conservation and Ecology, University of Kent, Canterbury, United Kingdom, [2]Health of Herpetofauna Communities Research Group, Department of Natural Sciences, Gordon State College, Barnesville, GA, United States, [3]Laboklin GmbH & Co., KG, Bad Kissingen, Germany

KEYWORDS

***Batrachochytrium dendrobatidis*, *B. salamandrivorans*, herpesvirus, ophidiomycosis, ranavirus, reptile, amphibian**

Editorial on the Research Topic
Emerging infections and diseases of herpetofauna

We are in the midst of a period of unprecedented global biodiversity declines, which has been dubbed the sixth mass extinction (1–3). While many factors are contributing to these extreme declines, habitat loss and anthropogenic environmental change appear to be the largest drivers for many vertebrates [e.g., mammals, (3)]. There are an increasing number of infectious agents being identified in both amphibians and reptiles around the globe, and more data links infection and disease for many of these emerging pathogens. Human activities also influence infectious disease epidemiology, with interactions between captive animals, those in trade, and wild animals affecting infection dynamics. One important factor in increasing our understanding of these interactions is a better overview of the infection status of both wild and captive amphibian and reptile populations and communities.

The emergence of fungal infections and their resultant mycoses, has, for over the past two decades, been associated with enigmatic declines in several taxa. In amphibians, the emergence of chytridiomycosis, the disease caused by infection with *Batrachochytrium dendrobatidis* (*Bd*), was first described in 1998 (4) and has now contributed to the declines of over 500 species and the extinction of at least 90 [(5), but see Lambert et al. (6)]. A second amphibian chytrid, *Batrachochytrium salamandrivorans* (*Bsal*), was described by Martel et al. (7) when it caused unprecedented declines in European Fire Salamanders (*Salamandra salamandra*). It now threatens salamander populations around the globe, although it is currently limited to Europe and Asia.

A large portion of this Research Topic on emerging infections and diseases of herpetofauna is appropriately dedicated to studies and reviews of data on *Bd* and *Bsal*. Olson et al. detail the importance of global tracking databases for *Bd* and the transition of Bd-maps.net to AmphibianDisease.org, which tracks both *Bd* and *Bsal* infections (also see Koo et al.). Olson et al. also examine the occurrence of *Bd* in amphibians around the globe. Koo et al. discuss the utility, functionality, and importance of the AmphibianDisease.org database.

Urbina et al. examine the short- and long-term effects of *Bd* exposure on embryos. Sheets et al. examine the phenotypic responses of several strains of *Bd* to a temperature gradient. Alvarado-Rybak et al. investigate a *Bd*-associated mortality event in native Chilean frogs in a captive breeding program. Cowgill et al. study the effects of *Bd* at the community level in the Pacific Northwest of the USA. Belasen et al. perform a meta-analysis that supports previous findings that historical coexistence between host and endemic *Bd* lineages is associated with less disease and mortality, but that more recent coexistence with the *Bd* global pandemic lineage is not. These studies highlight the diversity of Research Topics that continue to be investigated.

Fungal pathogens are also a cause for concern in reptile medicine and conservation. In snakes, *Ophidiomyces ophidiicola* (*Oo*), the causative agent of ophidiomycosis (formerly known as snake fungal disease) has caused disease outbreaks in wild and captive animals (8). In North America, *Oo* has been associated with the decline of several snake species (9), and it has also been detected in wild European snakes (10). Understanding the distribution and impact of *Oo* globally is an important Research Topic that clearly requires additional study. In this issue, Davy et al. explore *Oo* infections in Ontario, Canada, and their results suggest that earlier assertions that *Oo* is endemic and widespread in the area are likely correct.

Viruses also pose a large threat to amphibians and reptiles around the globe. Herpesviruses have been reported in both amphibians and reptiles [e.g., Okoh et al.; (11)]. In amphibians, proliferative dermatitis has been associated with newly described herpesviruses in common frogs (*Rana temporaria*) and common toads (*Bufo bufo*) (11, 12), but relatively little is known about the biology of herpesviruses in amphibians. In reptiles, herpesviruses have been described in a wide range of species, most commonly in chelonians, and their clinical impact appears to depend on many different factors. In recent years, the number of genetically distinct herpesviruses described in reptiles has grown rapidly and includes descriptions in wild and captive animals (Okoh et al.). The overview in this issue of herpesviruses detected in captive chelonians in Europe in recent years (Leineweber et al.) indicates a complex dynamic, with the pet trade playing a role in the dissemination of viruses to new parts of the world.

Ranaviruses are globally distributed pathogens of amphibians and reptiles [see Duffus et al. (13)]. Causing infection and disease in many species, ranaviruses are a threat to populations of amphibians and reptiles around the globe. We are still determining the real geographic range and number of species affected (e.g., Box et al.). Ranaviruses are a group of pathogens that can cause population declines [e.g., *R. temporaria*; ((14) Bosch et al.)] and in models can persist within populations while the declines happen [e.g., *R. temporaria*; (15)] or completely decimate the tadpole population [e.g., *Lithobates sylvaticus*; (16)]. Additionally, we have little knowledge of how ranaviruses act in amphibian communities. Bienentreu et al. investigate how ranaviruses act in low-diversity amphibian communities in northern Canada. Importantly, they find that species richness impacts infection prevalence, suggesting that an increased number of species in a community has an amplification effect on infection rates.

Viruses in the order *Nidovirales*, family *Tobaniviridae*, and subfamily *Serpentovirinae*, were first reported as a cause of severe respiratory disease in pythons in 2014 [(17); see Marschang et al. (18) for an overview]. These viruses have since been shown to be a common cause of disease in many snake species with high prevalences reported in captive pythons in the United States and Europe. Similar viruses have been described in association with respiratory disease in other squamates including wild-caught shingleback lizards (*Tiliqua rugosa*) in Australia (19), and captive veiled chameleons (*Chamaeleo calyptratus*) in the United States (20). A related virus was also found during a large die-off of Bellinger River snapping turtles (*Myuchelys georgesi*) in Australia (21). Although the virus isolated from those animals was not proven to have caused the mortalities, it was hypothesized to have played an important role. The episode appeared to impact the viability of the wild population. The fast pace at which nidoviruses have been discovered in reptile hosts and the changes and advances made in recent years to the taxonomy of this group make the review of this topic published by Parrish et al. in this issue particularly timely.

In addition to the growing knowledge base on the diversity and impacts of these fungi and viruses, new potential pathogens are being described in growing numbers in herpetofauna [see e.g., (22)]. In this issue, Tillis et al. describe a novel species of *Actinomyces* associated with granulomatous disease in captive ball pythons (*Python regius*). Their study indicates a possible sexual transmission and impact on the breeding of this popular species. New infectious disease threats in herpetofauna are likely to continue to be discovered in the future. In addition to these discoveries, researchers continue to investigate the complex connections between specific pathogens, diverse hosts, life stages, environmental factors, disease development, and pathogen dissemination. The diversity of topics covered within this special issue is representative of the infectious disease threats that amphibian and reptile populations face around the globe both in the wild and in captivity. This provides an important synopsis of current knowledge, while also helping to expand that information base for future researchers.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

SA was supported by the Natural Environment Research Council through the EnvEast Doctoral Training Partnership (Grant No. NE/L002582/1).

Acknowledgments

We would like to thank all of the authors for their contributions.

Conflict of interest

Author RM is employed by Laboklin GmbH & Co. KG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Ceballos G, Ehrlich PR, Barnosky AD, García A, Pringle RM, Palmer TM. Accelerated modern human–induced species losses: entering the sixth mass extinction. *Sci Adv*. (2015) 1:e1400253. doi: 10.1126/sciadv.1400253

2. McCallum ML. Vertebrate biodiversity losses point to a sixth mass extinction. *Biodiver Conserv*. (2015) 24:2497–519. doi: 10.1007/s10531-015-0940-6

3. Ceballos G, Ehrlich PR, Dirzo R. Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proc Natl Acad Sci USA*. (2017) 114:E6089–96. doi: 10.1073/pnas.1704949114

4. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci USA*. (1998) 95:9031–6. doi: 10.1073/pnas.95.15.9031

5. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel AN, Beukema W, et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*. (2019) 363:1459–63. doi: 10.1126/science.aav0379

6. Lambert MR, Womack MC, Byrne AQ, Hernández-Gómez O, Noss CF, Rothstein AP, et al. Comment on "amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity". *Science*. (2020) 367:aay1838. doi: 10.1126/science.aay1838

7. Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, et al. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. In: *Proceedings of the National Academy of Sciences*. 201307356. doi: 10.1073/pnas.1307356110

8. Lorch JM, Knowles S, Lankton JS, Michell K, Edwards JL, Kapfer JM, et al. Snake fungal disease: an emerging threat to wild snakes. *Philos Trans R Soc B Biol Sci*. (2016) 371:20150457. doi: 10.1098/rstb.2015.0457

9. Haynes E, Chandler HC, Stegenga BS, Adamovicz L, Ospina E, Zerpa-Catanho D, et al. Ophidiomycosis surveillance of snakes in Georgia, USA reveals new host species and taxonomic associations with disease. *Sci Rep*. (2020) 10:10870. doi: 10.1038/s41598-020-67800-1

10. Franklinos LH, Lorch JM, Bohuski E, Fernandez JR, Wright ON, Fitzpatrick L, et al. Emerging fungal pathogen *Ophidiomyces ophiodiicola* in wild European snakes. *Sci Rep*. (2017) 7:3844. doi: 10.1038/s41598-017-03352-1

11. Origgi FC, Schmidt BR, Lohmann P, Otten P, Akdesir E, Gaschen V, et al. Ranid herpesvirus 3 and proliferative dermatitis in free-ranging wild common frogs (Rana temporaria). *Vet Pathol*. (2017) 54:686–94. doi: 10.1177/0300985817705176

12. Origgi FC, Schmidt BR, Lohmann P, Otten P, Meier RK, Pisano SRR, et al. Bufonid herpesvirus 1 (BfHV1) associated dermatitis and mortality in free ranging common toads (Bufo bufo) in Switzerland. *Sci Rep*. (2018) 8:14737. doi: 10.1038/s41598-018-32841-0

13. Duffus AL, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, et al. Distribution and host range of ranaviruses. In: Gray MJ, Chinchar VG, editors. *Ranaviruses*. Cham: Springer (2015). p. 9–57. doi: 10.1007/978-3-319-13755-1_2

14. Teacher AG, Cunningham AA, Garner TW. Assessing the long-term impact of ranavirus infection in wild common frog populations. *Anim Conserv*. (2010) 13:514–22. doi: 10.1111/j.1469-1795.2010.00373.x

15. Duffus AL, Garner TW, Nichols RA, Standridge JP, Earl JE. Modelling ranavirus transmission in populations of common frogs (Rana temporaria) in the United Kingdom. *Viruses*. (2019) 11:556. doi: 10.3390/v11060556

16. Peace A, O'Regan SM, Spatz JA, Reilly PN, Hill RD, Carter ED, et al. A highly invasive chimeric ranavirus can decimate tadpole populations rapidly through multiple transmission pathways. *Ecol Modell*. (2019) 410:108777. doi: 10.1016/j.ecolmodel.2019.108777

17. Stenglein MD, Jacobson ER, Wozniak EJ, Wellehan JF, Kincaid A, Gordon M, et al. Ball python nidovirus: a candidate etiologic agent for severe respiratory disease in python regius. *MBio*. (2014) 5:e01484–14. doi: 10.1128/mBio.01484-14

18. Marschang RE, Meddings JI, Ariel E. Chapter 13 viruses of reptiles. In: Hurst CJ, editors. *Studies in Viral Ecology*. 2nd edition. Hoboken, NJ: Wiley-Blackwell (2021). doi: 10.1002/9781119608370.ch13

19. O'Dea MA, Jackson B, Jackson C, Xavier P, Warren K. Discovery and partial genomic characterization of a novel nidovirus associated with respiratory disease in wild shingleback lizards (*Tiliqua rugosa*). *PLoS ONE*. (2016) 11:e0165209. doi: 10.1371/journal.pone.0165209

20. Hoon-Hanks LL, Stöhr AC, Anderson AJ, Evans DE, Nevarez JG, Díaz RE, et al. Serpentovirus (nidovirus) and orthoreovirus coinfection in captive veiled chameleons (*Chamaeleo calyptratus*) with respiratory disease. *Viruses*. (2020) 12:1329. doi: 10.3390/v12111329

21. Zhang J, Finlaison DS, Frost MJ, Gestier S, Gu X, Hall J, et al. Identification of a novel nidovirus as a potential cause of large scale mortalities in the endangered bellinger river snapping turtle (*Myuchelys georgesi*). *PLoS ONE*. (2018) 13:e0205209. doi: 10.1371/journal.pone.0205209

22. Woodburn DB, Miller AN, Allender MC, Maddox CW, Terio KA. Emydomyces testavorans, a new genus and species of onygenalean fungus isolated from shell lesions of freshwater aquatic turtles. *J Clin Microbiol*. (2019) 57:e00628–18. doi: 10.1128/JCM.00628-18

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 08 January 2021
doi: 10.3389/fvets.2020.594600

Oral, Cloacal, and Hemipenal Actinomycosis in Captive Ball Pythons *(Python regius)*

Steven B. Tillis[1], Marley E. Iredale[1], April L. Childress[1], Erin A. Graham[1†], James F. X. Wellehan[1], Ramiro Isaza[2] and Robert J. Ossiboff[1]*

[1] Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL, United States, [2] Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, United States

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Reviewed by:
Frank Pasmans, Ghent University, Belgium
Jinshan Jin, National Center for Toxicological Research (FDA), United States

***Correspondence:**
Robert J. Ossiboff
rossiboff@ufl.edu

†Present address:
Erin A. Graham, Tifton Veterinary Diagnostic and Investigational Laboratory, College of Veterinary Medicine, University of Georgia, Tifton, Georgia

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 13 August 2020
Accepted: 03 December 2020
Published: 08 January 2021

Citation:
Tillis SB, Iredale ME, Childress AL, Graham EA, Wellehan JFX, Isaza R and Ossiboff RJ (2021) Oral, Cloacal, and Hemipenal Actinomycosis in Captive Ball Pythons (Python regius). Front. Vet. Sci. 7:594600. doi: 10.3389/fvets.2020.594600

Ball pythons (*Python regius*) are one of the most commonly kept and bred reptiles in captivity. In a large ball python breeding colony, a unique syndrome characterized by granulomatous inflammation of the cloaca and hemipenes (phalli) was observed in 140 of 481 (29.1%) breeding males, but only one of 1,446 breeding females. Lesions were absent in virgin males ($n = 201$) and virgin females ($n = 293$). On postmortem examination ($n = 13$, 12 males, 1 female), numerous well-defined mucosal and submucosal granulomas were present in the hemipenes (males) and cloaca (males and female). Extension into the coelomic cavity and liver was noted in a subset of these animals. An additional small subset of breeder animals (6/2027; 0.3%) presented with oral and mandibular swellings. Postmortem examination ($n = 4$, all female) showed oral lesions histologically indistinguishable from the cloacal/hemipenal lesions. Aerobic bacterial culture of a hepatic granuloma of one snake resulted in the isolation of filamentous, Gram-positive bacilli; amplification, and sequencing of the 16S rRNA gene and subsequent phylogenetic analysis of the isolate identified the bacterium as a novel species of *Actinomyces*. Screening of cloacal and oral granulomas using a specific, heminested 16S rRNA PCR assay confirmed the presence of the agent in all 17 snakes, as well as in cloacal swabs taken at the time of necropsy in 11/13 snakes. The *Actinomyces* sp. was also identified by PCR of cloacal swabs of unaffected snakes ($n = 94$) from the affected colony and two unrelated, grossly unaffected breeding colonies. In the affected colony, 65.5% of breeding animals ($n = 23$) but only 11.9% of virgin animals ($n = 42$) tested PCR positive, with breeding status being a significant predictor of bacterium presence ($P < 0.00001$). This study characterizes a granulomatous mucosal disease syndrome of breeding male ball pythons associated with a novel *Actinomyces*. In stark contrast to male snakes, the presence of the bacterium in both breeding and virgin females was very rarely associated with clinical disease. Though additional studies are necessary, these data suggest a role for the novel bacterium in the disease process, a predilection for clinical disease in male snakes, and the potential for sexual transmission of the disease.

Keywords: actinomycosis, cloacitis, granuloma, hemipenis, reproductive disease, reptile, sexually transmitted disease, snake

INTRODUCTION

The captive reptile trade is a large and diverse market within the United States. Over 4.7 million households hold 13.6 million pet reptiles, comprising a market with annual revenues of over a billion dollars (1). Among captive reptiles, ball pythons *(Python regius)* have become one of the most popular pet reptiles kept and bred in captivity due to their small size, mild disposition, hardy nature, and variety of colors (2). Large colonies of these pythons numbering in the hundreds to thousands are kept and bred to supply pet trade demands. Currently over 6,000 color combinations are listed, with hundreds of unique genetic mutations combined to create new and unique phenotypes (3). Shifts in market demands for various color phenotypes require a breeding colony to keep significant numbers of snakes with diverse genetic backgrounds to produce offspring with the highest market demand annually.

In captive snake breeding colonies, several important reproductive diseases can occur. Reproductive diseases common in breeding females are often sequela of dystocia and include ovostasis (egg binding), oviductal prolapse, and egg-yolk coelomitis. In breeding males, common reproductive pathology includes hemipenal prolapse and hemipenal trauma, potentially caused by strenuous reproductive activity and aggressive copulation. Cloacitis associated with reproduction and associated courtship and copulation can be a major disease in both sexes (4).

Currently, no sexually transmitted diseases of a bacterial or viral nature in non-avian reptiles are formally described in the literature. Although sexual transmission of nematodes has been documented in wild *Anolis* lizards (5), there is additional concern for the potential sexual transmission of various reptile pathogens, including protozoa, bacteria, and other potential pathogens (4). However, bacterial venereal diseases are well-documented in birds (6). Among poultry species, a variety of sexually transmitted bacteria have been described including *Mycoplasma meleagridis* in turkeys, *M. iowae* in chickens, and *M. cloacale* in domestic geese (6). Moreover, contamination of chicken and turkey semen with *Salmonella* and *Campylobacter* spp. can contribute to the spread of these agents in both artificial and traditional breeding methods (7–9).

Members of the phylum Actinobacteria are often filamentous and fastidious and are relatively poorly identified by standard biochemical methods. The phylum contains some highly significant animal pathogens, including the genera *Mycobacterium* and *Nocardia*. *Actinomyces* is a bacterial genus containing at least 45 species in the phylum Actinobacteria, order Actinomycetales, family Actinomycetaceae. Members of the *Actinomyces* are common inhabitants of oral and genital mucosa in animal species, including humans (7, 10, 11). Furthermore, members of Actinobacteria have been identified both in the cloaca of birds (12) and in the gut microbiomes of various agamids (13). While they are most commonly innocuous members of the mucosal microbiome, certain species are also causative agents of inflammatory processes (actinomycoses) of the oral cavity, reproductive tract, abdomen, and subcutis (7, 14). Still other Actinobacteria such as those in the genus *Devriesea* have been shown to be directly involved with the formation of dermal lesions in agamid lizards (15).

This study identifies and characterizes a novel *Actinomyces* species associated with a granulomatous disease syndrome of oral, cloacal, and hemipenal tissues in captive ball pythons by epidemiological, pathological, and molecular modalities.

MATERIALS AND METHODS

Snake Population and Clinical Investigation

The investigated population is a large commercial production colony of ball pythons (*Python regius*) consisting mostly of animals with captive-bred origins. The colony consists of ∼2,000 breeding animals and produces neonates to supply the pet trade. Facility health records and animal information are maintained in a Microsoft Access database. In this colony, a breeding group consists of 2 males assigned to breed 6 females each year. Starting in February, males are introduced to females until either the female ovulates or the season ends in August. Each male is separately introduced to one female at a time and is moved to the next female in the breeding group 3 times a week, spending at least 2 days with each female. In this way, each of the two males is exposed to each female in the breeding group at least once every 2 weeks during the breeding season.

Anecdotal recollection dates the first recognition of the disease syndrome of male ball pythons (initially noted by the producer as abnormal cloacal irritation) a decade or more prior to this study. At that time, disease status determination was limited to observations of sporadic cases of occult irritation on the external perimeter of the cloaca in a small portion of breeding males. In attempts to potentially remedy the irritation, the practice of manually everting breeding males' hemipenes was adopted in the facility to check for any adhered substrate. Through this practice, a spectrum of gross syndrome expression was noted to include irritation and small masses on the hemipenes, in addition to the irritation previously noted around the cloaca.

To determine a farm-level prevalence of the disease syndrome, a complete census of all 481 breeding males and 1,446 breeding females on the farm was performed in September 2017. The cloacae and manually extruded hemipenes (males only) of all breeding snakes were examined for presence of any masses. Data recorded for the census included gross disease presence (present/absent), sex (male/female), and age (years). The association between disease presence and sex was evaluated with a Chi-squared analysis, with significance defined as $p < 0.05$. All statistical calculations for this study were performed with R Studio.

In January 2018, in preparation for the breeding season, the producer removed 168 breeding males from the breeding colony, which included 87 animals identified in the 2017 census with the disease syndrome. The lone female identified with the disease syndrome in 2017 was also removed from the colony. One hundred and sixty six first-time breeding males were added to breeding groups for the 2018 season. For the 2018 season, the producer placed 388 animals with no previous signs of the disease syndrome in breeding groups with partnering males also without signs, and 38 males with no previous signs in breeding groups

with a partnering male that had signs of the disease syndrome in 2017.

To examine incidence of new cases as well as determine an updated prevalence, a second census was conducted in October 2018. This census included all 479 breeding males and 1,477 breeding females, as well as 201 virgin males and 293 virgin females. Data recorded for this census included 2018 gross disease presence (present/absent), 2017 gross disease presence (present/absent/not applicable), sex (male/female), age (years), breeding status (breeder/virgin), and, for breeding males, breeding partner gross disease presence (present/absent). The association between gross disease presence and sex, gross disease presence and breeding status, and gross disease presence and breeding partner gross disease presence was evaluated with a Chi-squared analysis, with significance defined as $p < 0.05$. Finally, in September 2019 a smaller subset of animals in the colony were identified with swelling in the oral or nasal cavity. A third and final census was performed on all 2,027 breeding animals in the facility to determine the prevalence of facial swelling.

For molecular diagnostic investigation on a possible etiology, cloacal swabs were taken from 10 breeding female ball pythons, 12 virgin females, and 30 virgin males as well as 12 males and one female with the disease syndrome. The 10 breeding females had known exposure with a male with the disease syndrome and were selected using stratified pseudorandom sampling wherein a facility employee picked an animal from each cluster of caging organized by genetic morphological phenotype. Virgin animals in the affected colony were chosen by generating a list of applicable animals and assigning each a sequential numerical digit with animals selected at random, using https://www.randomizer.org. At the time of swabbing, disease status was confirmed as present or absent via gross examination of the cloaca and hemipenes. Variables collected for analysis include cloacal swab PCR bacterial presence (present/absent), gross disease presence (present/absent), sex (male/female), breeding status (breeder/virgin), and source colony status (colony absent of disease syndrome/disease syndrome present in colony). The association between cloacal bacterial presence and sex and cloacal bacterial presence and breeding status was determined with a Chi-squared analysis with a significance of 0.05. Additionally, an unweighted Cohen's Kappa test was performed to determine the level of agreement between cloacal bacterial presence and lesion presence. For males, the hemipenes were manually extruded, and the swab was run along both hemipenes and anterior and posterior portions of the internal cloacal cavity. For females, swabs were run along the anterior and posterior portions of the internal cloacal cavity. Swabs were placed in sterile microcentrifuge tubes and kept on ice until either frozen (−80°C) or immediately extracted.

Oral swabs were also collected from the affected colony to assess possible oral pathogen prevalence. Oral swabs were taken from two subsets of animals. The first subset was from 48 animals (12 virgin males, 12 virgin females, 12 breeder males, and 12 breeder females) selected at random by assigning each a sequential numerical digit and randomly chosen using https://www.randomizer.org. The second subset of animals included 37 snakes (17 breeder females, 4 breeder males, 8 virgin females, and 8 virgin males) whose cloaca had been sampled previously as part of cloacal swabbing described above. Variables collected for analysis include oral swab PCR bacterial presence (present/absent), sex (male/female), breeding status (breeder/virgin), and cloacal swab PCR bacterial presence (present/absent) for the second subset of animals. The association between oral bacterial presence and cloacal bacterial presence was determined with a Fisher's exact test with a significance of 0.05. Oral swabs were collected by running a Rayon-tipped plastic shaft swab along the oral mucosa at the labial margin, the trachea, the choana, and the caudal oral cavity and cranial esophagus. Swabs were placed in sterile microcentrifuge tubes and kept on ice until frozen (−80°C) or were immediately extracted.

Also included in this report are 2 smaller colonies consisting of ∼40 ball pythons each, with all animals of captive bred origins. These colonies have no history of the disease syndrome and use a breeding strategy similar to that outlined above but differ in having only 1 male in a given breeding rotation. For the purposes of this study, animals in all 3 colonies were classified into 4 groups: breeder males and females, and virgin males and females. For comparison and screening purposes, cloacal swabs were also taken from the two smaller colonies of ball pythons with no history of the disease syndrome with a combined total of 10 breeding males, 12 breeding females, 10 virgin males, and 10 virgin females. For the smaller colonies with no history of cloacal disease, nearly all demographically comparable animals were sampled to supply similar sample sizes.

Postmortem Examinations

Complete postmortem examinations were performed on 17 adult breeding animals with grossly apparent lesions; 13 snakes (12 males; 1 female) had cloacal/hemipenal lesions and four snakes (4 females) had oral lesions. All snakes were chemically euthanized by the Zoological Medicine Service at the University of Florida Small Animal Hospital for quality of life and/or population health concerns using AVMA approved euthanasia protocols (16). Samples of all tissues were collected and preserved in 10% neutral buffered formalin and submitted for routine processing and sectioning to the Histology Laboratory at the College of Veterinary Medicine, University of Florida. Samples of liver, kidney, heart, lung, spleen, and cloacal and visceral granulomas (when present) were collected and archived frozen (−80°C). In all 17 animals, the cloacal and hemipenal mucosa (males) or cloacal mucosa only (females) were swabbed with a single rayon-tipped plastic shaft swab (Puritan, Guilford, ME); in snakes with oral lesions, the oral mucosa was also swabbed with a separate swab. All swabs were archived frozen (−80°C) until testing. After a minimum of 24 h of fixation, tissues were processed routinely, cut at 5-μm sections, and stained with hematoxylin and eosin. Histochemical stains (Warthin-Starry, Twort's Gram, Brown and Brenn Gram, Giemsa, Gomori methenamine silver, and Fite-Faraco acid fast) were applied to select sections with granulomatous inflammation.

Bacterial Culture and 16S rRNA PCR

To decrease the chances of culturing a contaminant cloacal bacterium, a single hepatic granuloma was excised using sterile

methods and submitted for aerobic bacterial culture to the Clinical Microbiology Laboratory at the College of Veterinary Medicine, University of Florida. Samples from the excised mass were plated for culture on chocolate agar, Columbia blood agar (BAP), MacConkey agar (MAC), and colisitin with nalidixic acid (CNA). All plates were incubated at 25°C.

DNA from an isolated bacterial colony was extracted per manufacturer's recommendation using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). An approximately 1500-bp fragment of the small subunit ribosomal RNA (16S rRNA) gene was amplified by polymerase chain reaction (PCR) as previously described (17). Briefly, 1 μl of 20 μM sense primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and antisense primer 1492R (5′-GYT ACC TTG TTA CGA CTT-3′) (17) were added to 2 μl of 10 × PCR buffer, 0.8 μl of 50 mM magnesium chloride, 0.4 μl of 10 mM dNTP mixture, 0.1 μl of platinum *Taq* DNA polymerase, 12 μl of molecular grade water, and 3 μl of extracted nucleic acids (Invitrogen, Carlsbad, California). Thermocycler conditions were as follows: 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 10 min, 30 cycles of 95°C for 20 s followed by 50°C for 20 s followed by 72°C for 3 min; and 1 cycle at 72°C for 10 min followed by a hold stage at 4°C. Amplified products were visualized on a 1% agarose gel stained with ethidium bromide. Amplicons of the appropriate size were extracted using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, California) per manufacturer's recommendation and were submitted for Sanger sequencing to a commercial facility (Genewiz, South Plainfield, New Jersey). Sequences were edited and aligned using Geneious Prime (Auckland, New Zealand). Additional specific sequencing primers were designed and used for commercial sequencing (Genewiz) to get double coverage along the entire length of the amplicon. The resultant sequence was compared to the 16S rRNA sequences of representative species of common bacterial families available through BLASTn.

Phylogenetic Analysis

The sequence of the novel ball python bacterium was aligned against all 45 characterized species of *Actinomyces*, 5 characterized species of the closely related genus *Trueperella*, and *Mobiluncus mulieris* (Genbank AJ576080) as an outgroup using multiple alignment using fast Fourier transform (MAFFT) (18). Maximum likelihood (gamma distributed rate variation with estimate of proportion of invariable sites with 1,000 bootstrap resamplings) and Bayesian (Mr. Bayes 3.2.6 with gamma distributed rate variation, 4 chains of 2×10^6 generations with 25% burn-in) were performed on the CIPRES server (19–21). Phylogenetic trees were visualized using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

Specific Heminested PCR of Tissue and Swab Samples

DNA from postmortem samples of the cloaca, oral mucosa, and liver containing granulomas was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Approximately 100 mg of affected tissue was lysed at 56°C for 16 h with proteinase K, and extractions were conducted according to the manufacturer's tissue protocol. DNA extraction from all swabs was performed using Prepman Ultra solution (Applied Biosystems, Foster City, California, US) using modified product protocols. One hundred fifty microliter of Prepman Ultra solution was added to the microcentrifuge tube with the swab and vortexed for 60 s. The tubes were then placed on a 95°C hotplate for 10 min. Once cooled to room temperature, a 1:10 dilution was made using 10 μl of Prepman and sample solution combined with 90 μl of molecular grade water for a final extraction volume of 100 μl.

A specific heminested PCR was developed for detection of the novel *Actinomyces* sp. The determined 16S sequence was compared to sequences of other *Actinomyces* and closely related genera present in Genbank (https://www.ncbi.nlm.nih.gov/genbank/) to develop novel *Actinomyces*-specific primers in the assay. The first round of primers in the protocol are sense primer ActBP154F (5′-GGA TAT TCT GTC TCC TGC GCA-3′) and antisense primer ActBP836R (5′-CTA CGG CGC GGA AAC CAT-3′). The second round in the heminested protocol utilizes the sense primer from the first round (ActBP154F) and antisense primer ActBP614R (5′-TAC CCA CCG CAA GCT GAA AG-3′). PCR reactions were performed with 2X PCRBIO HS Taq Mix kit (PCR Biosystems, Wayne, Pennsylvania) with 2 μl 10 μM of each primer, 25 μl of 2× PCRBIO HS Taq Mix solution, 18 μl of molecular grade water, and 3 μl of DNA extract (round one) or PCR product (round two). Cycle conditions for both rounds are as follows: 1 cycle at 95°C for 2 min, 40 cycles of 95°C for 20 s, annealing at 57°C for 20 s, and elongation at 72°C for 3 min; 1 cycle at 72°C for 10 min and a hold at 4°C. PCR products were run on a 1% agarose gel. DNA was extracted from excised bands with Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, California) per manufacturer's recommendation. All extracted products were submitted for Sanger sequencing (Genewiz). Sequences were edited and aligned using Geneious Prime (Auckland, New Zealand).

RESULTS

Snake Population and Clinical Investigation

Although irritation on the exterior of cloacas in male ball pythons was noted in the affected facility a decade or more prior to this study, in 2017 an apparent increase in the number of animals with cloacal masses was noted at the facility. Through the practice of manually everting breeding males' hemipenes, a spectrum of expression of the syndrome was observed (**Figure 1**). In contrast to unaffected snakes (**Figure 1A**), low (**Figure 1B**) to moderate (**Figure 1C**) numbers of 1–3-mm-diameter masses were present within the mucosa of the hemipenes. In the most severely affected individuals, the masses increased in both size (up to 10 mm diameter) and number and were present throughout the mucosa of the hemipenes and surrounding cloacal tissue (**Figure 1D**).

In the September 2017 cloacal exam census, a gross disease point prevalence of 29.1% was observed in the 481 breeding males in the farm (**Table 1**), expressing a range of severity in disease expression. Of the 140 affected males, 44 had lesions visible on both the hemipenes and the cloaca, with the remaining 96 males having gross lesions visible on the extruded hemipenes alone. In

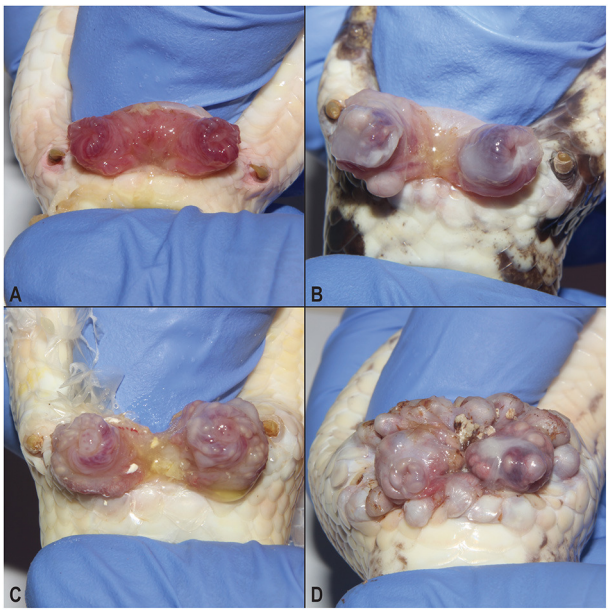


FIGURE 1 | Hemipenal lesions in breeding male ball pythons (*Python regius*). Normal everted ball python hemipenes **(A)** compared to a syndrome of hemipenal inflammation **(B–D)**. Inflammation and irritation ranged from mild **(B)**, to moderate **(C)**, to severe **(D)** which was associated with mass formation along the margins of the vent.

TABLE 1 | Epidemiology of gross lesion presence in affected colony from 2017 and 2018 censuses.

Variable	Category	Lesions present	Lesions absent	*P*
Year[a,b,c]	2017 breeder male	140 (29.1%)	341 (70.9%)	0.01067*
	2018 breeder male	105 (21.9%)	374 (78.1%)	
Sex[a,c]	Breeder male	105 (21.9%)	374 (78.1%)	<0.00001*
	Breeder female	1 (<0.1%)	1475 (>99.9%)	
Breeding	Virgin male	0 (0%)	201 (100%)	<0.00001*
status[a,c]	Breeding male	105 (21.9%)	374 (78.1%)	
Partner	2017 affected partner	12 (31.6%)	26 (68.4%)	0.00037*
status[a,c]	Unaffected partner	40 (10.3%)	348 (89.7%)	

[a] *Significance determined with Chi-square test, α =0.05.*
[b] *Data from 2017 census.*
[c] *Data from 2018 census.*
**Statistical significance.*

contrast, only 1 out of 1,446 breeding females presented with the disease syndrome visible on the cloaca. A Chi-square analysis of this data shows sex as being a statistically significant correlation ($P < 0.00001$) with clinical signs of the disease syndrome.

A second census completed by the producer after the completion of the breeding season in October 2018 found a 21.9% gross disease point prevalence in the 479 active breeding males. In contrast, 1 out of 1,477 breeding females had signs of the gross disease syndrome; no virgin males ($n = 201$) or virgin females ($n = 293$) had abnormal gross lesions. Of the 105 males affected with the disease syndrome in the 2018 census, 52 were new cases, consistent with an annual incidence rate of 12.8%; moreover, four

TABLE 2 | Prevalence and annual incidence of gross lesion presence of males in affected colony from 2018 census.

Age (Years)	*n*	Prevalence%	Annual incidence%
2	166	2.41	2.41
3	76	11.84	5.63
4	73	15.07	10.14
5	60	36.67	15.56
6	47	44.68	29.73
7+	57	66.67	50.00

of the new cases were of the 166 first-year breeding males. In comparison, none of the 24 virgin males of the same age cohort but not used for breeding in the 2018 season developed any gross signs ($p = 0.4421$). In contrast, only one female developed cloacal lesions in the same timeframe.

In comparison to 21.9% of breeding males ($n = 479$), none of the 201 virgin males showed any expression of disease syndrome, with breeding status correlating significantly ($P < 0.00001$) with the presence of the disease syndrome (**Table 1**). Of the animals that did not have lesions in 2017 but were placed in a breeding group with a symptomatic male for the 2018 season, 31.6% ($n = 38$) developed lesions in the following year, compared to 10.3% ($n = 388$) of males paired with a partner without apparent lesions; the presence of a breeding partner with clinical signs correlated significantly with the development of signs in the new cases ($P = <0.00001$; **Table 1**).

Table 2 shows disease syndrome prevalence and annual incidence compared to age in male ball pythons from the 2018 census. While only 2.41% of animals 2 years in age showed clinical signs in the 2018 census ($n = 166$), animals 3 years in age had a 11.84% prevalence ($n = 76$). Likewise, incidence increases from 2.41% for 2-year-old animals to 5.63% for animals 3 years in age. This positive trend continues for both incidence and prevalence as age increases, ramping up to 66.67% of animals 7 years in age or older expressing the disease syndrome with an annual incidence of 50% ($n = 57$).

Postmortem Examinations

Postmortem examinations of the affected snakes revealed multifocal to coalescing, pale tan, smooth, firm, pericloacal, hemipenal, and pericoelomic granulomas. The granulomas were present within the mucosa and submucosa of the hemipenes in males (**Figure 2A**), expanded the subcutis surrounding the vent, and variably tracked along the surface of the coelomic cavity deep into the muscle of the ventral body wall (**Figure 2B**). In animals with facial changes, granulomas were present in the submucosa and dermis of the rostrum and mandible that distorted the profile of the head and variably projected into the oral cavity (**Figure 2C**). Two of the 17 necropsied animals exhibited intracoelomic and/or intrahepatic granulomas of similar appearance.

Microscopic examination of the affected tissues identified well-demarcated, chronic granulomas within (i) the mucosa and submucosa of the hemipenes (**Figure 3A**); (ii) the mucosa,

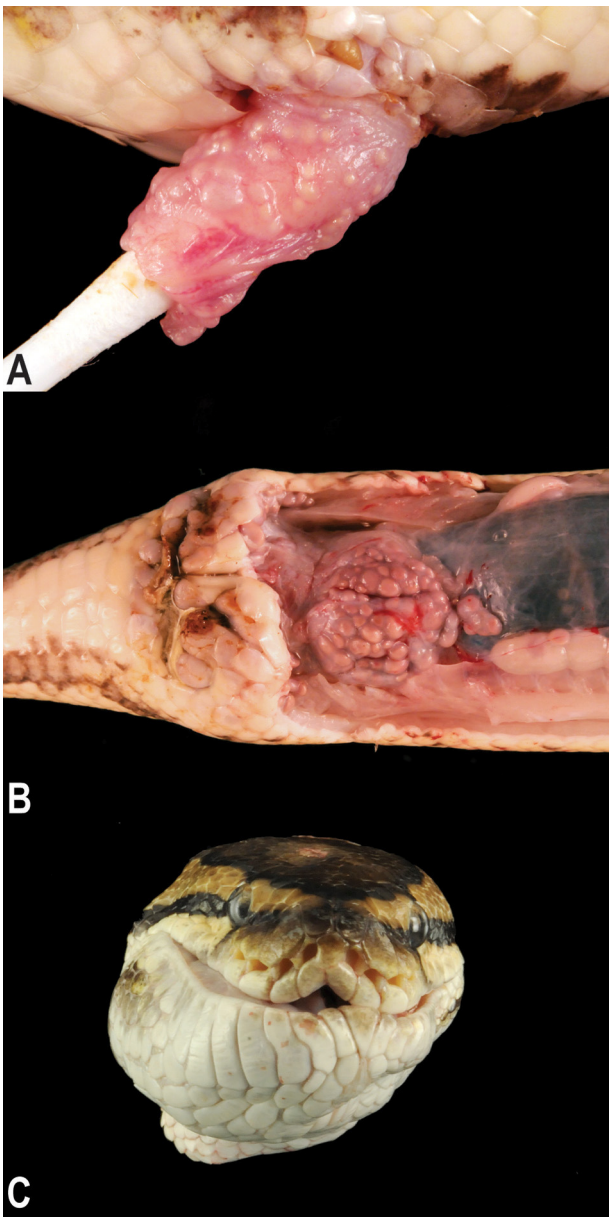


FIGURE 2 | Gross granulomatous inflammation of the hemipenes, cloaca, and oral cavity. Multifocal to coalescing chronic granulomas stud the mucosa of the hemipenis **(A)** are present within the subcutis surrounding the vent and track along the surface of the coelomic cavity deep into the muscle of the ventral body wall **(B)**. Large subcutaneous granulomas deep to the skin and mucosa of the mandible and rostrum distort the profile of the head **(C)**.

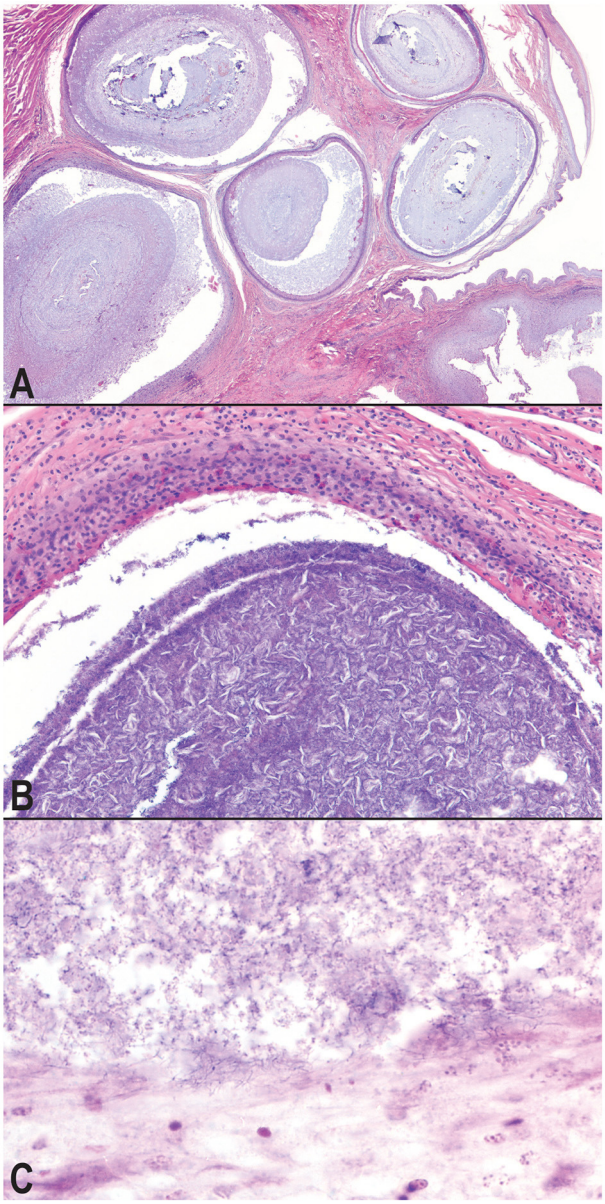


FIGURE 3 | Photomicrographs of granulomatous inflammation of the hemipenes and cloaca. Large, well-demarcated granulomas expand and compress the submucosa and mucosa of the hemipenes; hematoxylin and eosin, 20× magnification **(A)**. Granulomas surround cores of cellular debris, acicular (cholesterol) clefts, mineral, and mixed morphology bacteria; hematoxylin and eosin, 100× magnification **(B)**. A consistent subpopulation of filamentous, Gram-positive bacteria are present throughout the granulomas, and at the highest concentration of the margins of the granulomas with extension into the wall of the granuloma, Gram, 600× magnification **(C)**.

submucosa, dermis, and skeletal muscle surrounding the cloaca; (iii) within the serosa and mesentery of the intestines; (iv) within the oral mucosa, submucosa, dermis, and skeletal muscle of the mandible and rostrum; and (v) occasionally within the parenchyma of the liver and kidney. The granulomas were characterized by a central core of necrotic debris with basophilic, finely granular material (mineral) admixed with moderate to high numbers of clear, acicular spaces (cholesterol clefts; **Figure 3B**). Necrotic cores were surrounded by a collar of epithelioid macrophages that were often infiltrated by low to moderate numbers of lymphocytes and heterophils. Within granulomas, low to moderate numbers of mixed bacteria were often identified; most prominently present at the margins of the

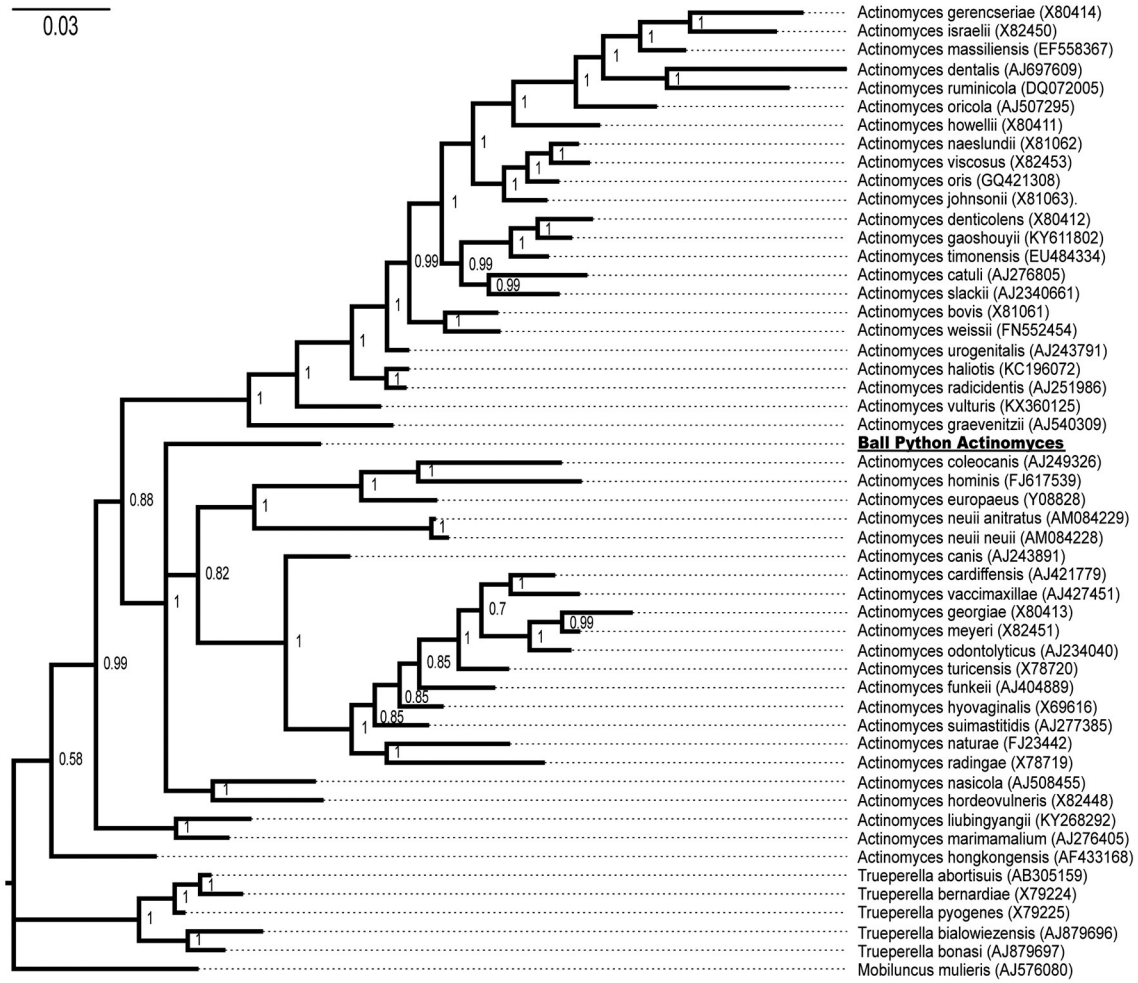


FIGURE 4 | Phylogenetic tree of the isolated ball python bacterium with members of the genera *Actinomyces*, *Trueperella*, and *Mobiluncus*. Genbank accession numbers are shown in parentheses at the end of the species name. Posterior probabilities are shown at branch points. The ball python *Actinomyces* species is underlined.

granulomas and extending through the granuloma capsule was a consistent subpopulation of fine, filamentous, Gram-positive bacterial rods, consistent with an actinomycete (**Figure 3C**). The cloacal granulomas of the lone grossly affected female snake, as well as the two snakes with the hepatic granulomas, and all of the snakes ($n = 4$) with oral granulomas were histologically identical to the granulomas present in the cloaca and hemipenes of the male snakes.

Bacterial Culture, 16S rRNA PCR, and Phylogenetic Analysis

Successful bacterial growth of the cultured hepatic granuloma was observed on BAP plates; colonies were pinpoint, white, pitting, and adhered to the media. Gram stain showed filamentous Gram-positive bacilli. PCR amplification and Sanger sequencing of the 16S small subunit ribosomal RNA gene resulted in a 1470-nt fragment after editing. A BLASTn search found that the sample most closely matched with members of *Actinomycetaceae* with the closest isolate being *Actinomyces hordeovulneris* (Genbank KF030211.1) with 91.7% nucleotide identity. The sequence was uploaded to GenBank (MT742158).

Both Bayesian (**Figure 4**) and maximum likelihood (data not shown) methods of phylogenetic inference placed the novel ball python bacterium within the genus *Actinomyces* with a Bayesian posterior probability of 100% (**Figure 4**) and a ML bootstrap value of 100% (data not shown), consistent with the isolated snake bacterium as a novel member of the genus *Actinomyces*. This *Actinomyces* will hereafter be referred to as Ball Python *Actinomyces* (BPA).

Specific BPA Heminested PCR of Tissue and Swab Samples

The BPA 16S rRNA heminested PCR resulted in an amplicon of 420 nucleotides after primer editing. All sequence data matched the homologous region of the sequence amplified from the isolated colony.

TABLE 3 | Epidemiology of BPA PCR status via swab in affected colony.

Variable	Category	Cloaca PCR positive	95% CI	*P*
Lesion presence[a]	Lesions	11/13 (84.6%)	54.6–98.1%	<0.00001*
	No lesions	9/52 (17.3%)	8.2–30.3%	
Sex[a]	Male	12/42 (28.6%)	15.7–44.6%	0.812
	Female	8/23 (34.8%)	16.4–57.3%	
Breeding status[a]	Virgin	5/42 (11.9%)	4.0–25.6%	<0.00001*
	Breeder	15/23 (65.5%)	42.7–83.6%	
Cloacal PCR vs. oral PCR[b]	Oral PCR Pos	2/3 (66.7%)	9.4–99.2%	0.2407
	Oral PCR Neg	10/34 (29.4%)	15.1–47.5%	

[a]*Significance determined with Chi-square test, $\alpha = 0.05$.*
[b]*Fisher's exact test, $\alpha = 0.05$.*
**Statistical significance.*

BPA was detected by PCR in cloacal/hemipenal granulomas of all 13 necropsied pythons with cloacal and hemipenal lesions. BPA was also detected in cloacal/hemipenal swabs taken from 10/12 (83.3%) snakes with cloacal/hemipenal lesions at necropsy. In the four snakes necropsied with oral/mandibular/facial granulomas, BPA was present in oral tissue in three (75%) of the snakes. BPA was further detected in oral swabs in three of the four (75%) snakes.

Despite a lack of the disease syndrome in the unaffected colonies, cloacal BPA prevalence between the unaffected and affected colonies appear quite similar. The PCR prevalence of BPA was 30.8% ($n = 65$) compared to 42.9% ($n = 42$) in the unaffected colonies. Breeder males within the unaffected colonies had a BPA prevalence of 90% ($n = 10$), compared to 83.3% ($n = 12$) of breeder males with the disease syndrome from the affected colony. Breeder females within the unaffected colonies had a BPA prevalence of 67.7% ($n = 12$), compared to 45.4% ($n = 11$) of breeder females exposed to males with the disease syndrome from the affected colony. Virgin animals in both unaffected and the affected colonies had a markedly lower prevalence of BPA, with 5% ($n = 20$) in unaffected colonies, and 11.9% in the affected colony ($n = 42$).

Swab BPA results from the affected colony are presented in **Table 3**. Within the affected colony, sex of the animal was not significantly correlated with ($p = 0.812$) the prevalence of BPA, being present in the cloaca of 28.6% of males ($n = 42$) and 34.8% of females ($n = 23$). In contrast, breeding status was significantly correlated ($p < 0.00001$) with the PCR prevalence of BPA, with 65.5% of breeding animals ($n = 23$) having the bacterium compared to 11.9% of virgin animals ($n = 42$). To determine the level of agreement between PCR results and lesion presence in the affected colony, an unweighted Cohen's Kappa test gave a value of 0.56 ($n = 65$, 95% CI [0.336, 0.784]). When expanded to include data from the 2 unaffected colonies, an unweighted Cohen's Kappa test gave a value of 0.138 [$n = 107$, 95% CI (0.138, 0.473)]. This signifies a weak agreement between the presence of BPA by PCR in the cloaca and presence of cloacal lesions.

In the final September 2019 oral examination census, 6 out of 2,027 breeding snakes (2.96%) had oral swelling, all of which were breeder females. PCR testing for BPA in oral swabs of 48 randomly selected animals (12 virgin males, 12 virgin females, 12 breeder males, and 12 breeder females) yielded only 2 snakes (4.2%) testing positive. Oral swabs taken from an additional 37 snakes (17 breeder females, 4 breeder males, 8 virgin females, and 8 virgin males) with prior testing for BPA in the cloaca yielded 5.4% being positive for BPA on both oral and cloacal swabs, 2.7% positive on just the oral swab, 27.0% positive on just the cloacal swab, and 64.9% negative on both swabs ($n = 37$). Fisher's exact test failed to find statistical significance between presence of BPA in the cloaca and presence of BPA in the oral cavity ($p = 0.2407$) (**Table 3**).

DISCUSSION

This study characterizes a granulomatous disease syndrome of the cloaca, external reproductive organs, and oral cavity of captive ball pythons (*Python regius*) associated with a novel *Actinomyces* species. The most interesting facet of the disease syndrome is the high proclivity in affecting the external genitals and cloaca of adult breeding male snakes. The histologic features of the lesion, the presence of intralesional, filamentous, Gram-positive bacteria, the aerobic bacterial culture of an actinomycete from affected tissues, and the detection of the cultured bacterium by PCR in affected tissues all support a role of the novel actinomycete in the observed disease syndrome.

Actinomyces are common inhabitants of the oral and genital mucosa in both human and animal species. Although often innocuous members of the resident microbiome, a disruption of the mucosal barrier can lead to clinical *Actinomyces* infections (7, 14). In animals, lumpy jaw is a well-characterized disease syndrome that can be caused by several species of *Actinomyces*; the disease is most commonly observed in ruminants, but it can also occur in other mammals including humans (10, 22–25). The lesions of lumpy jaw include facial inflammation and abscess formation with deterioration of underlying bone structures in the oral cavity and jaw. In humans, actinomycetes are known causative agents of pelvic inflammatory disease in females with intrauterine birth control devices (IUDs) (7, 26). Actinomyces infections are not limited to the oral cavity or reproductive tracts, however, and infections of the abdomen, neck, pleura, lungs, liver, kidney, appendix, caecum, skin, heart, meninges, and bone have all been reported (7). In all of these infections, a common characteristic is granulomatous inflammation often with a capsule of connective tissue and swelling (7, 10, 24). Both the observed cloacal/hemipenal and facial lesions of the snakes in this report exhibit features consistent with actinomycosis in other species.

Another facet in the pathogenesis of actinomycosis is the high propensity for polymicrobial infections (22, 27–30). 95.5% of cases of human cervicofacial actinomycoses ($n = 1997$) were polymicrobial mixed infections (22). This is consistent with the histologic appearance of mixed morphology bacteria in the granulomas present in necropsied animals presented here. Moreover, initial attempts to culture a cloacal granuloma from one of the initial presenting snakes resulted in mixed Gram-negative and Gram-positive growth (data not shown). The companion bacteria in polymicrobial actinomycosis are thought

to play a synergistic role in the development of actinomycosis (7, 14, 29), possibly by promoting infection through inhibiting host defense and optimizing ideal aerobic conditions (31). Actinomyces infections also commonly spread to adjacent tissue (27, 28, 32) with a subset of cases also presenting with hepatic infection (14, 32, 33). Both these attributes are consistent with what was observed in necropsied animals. While only a small subset ($n = 2$) of snakes had hepatic granulomas, regional extension of granulomas was a very common finding in the snakes (see **Figure 2B**).

While this is the first documentation of an *Actinomyces* species as a cause of granulomatous cloacal/hemipenal disease in snakes, this is not the first report of such lesions in a ball python. A figure in a review of snake reproduction by Stahl (4) showed similar lesions in the hemipenes of a male ball python. The biopsy was reported to reveal acid-fast positive bacterium; while this result was interpreted as mycobacteriosis, some *Actinomyces* spp. have been reported to exhibit acid-fast reactivity (34). Moreover, in addition to the large colony described in this paper, anecdotal observation of the disease syndrome has been reported in two other large (1000+ animal) colonies and two smaller (300+ animal) colonies (personal communication, Tillis). Lastly, a recent diagnostic biopsy submission to the Aquatic, Amphibian, and Reptile Pathology service at the University of Florida from a ball python was composed of cloacal granulomas histologically identical to those identified in this population and contained filamentous, Gram-positive bacilli (personal communication, Ossiboff). Together, these suggest that actinomycosis may not just be a sporadic event in this one breeding colony of ball pythons, and clinicians and hobbyists should be aware of this disease condition.

In the colony described here, the uptick in the disease syndrome that prompted this investigation was correlated with an increase in colony size of 22% from the year prior. Combined with the observations of a similar disease in other large collections, colony size may play a role in the prevalence of the disease syndrome. However, studies of disease transmission in livestock animal production have shown disease transmission rates to be independent of population size (35–38). Host density may have a larger role, and increase in population size without corresponding space increase may affect transmission rates (39). Farm-level surveys would be required to identify farm-level risk factors for the disease syndrome.

While both oral and cloacal/hemipenal lesions were observed in snakes in this colony, oral masses and PCR detection of BPA in oral swabs occurred at a markedly lower rate than that in the cloaca. This finding may suggest that the cloaca is either a more hospitable environment for BPA to grow, or frequent direct cloacal contact during breeding is a more efficient means of bacterial transmission within the colony. Although the environmental persistence of BPA and its role in transmission dynamics of the disease syndrome is unknown, presence of the disease syndrome was only seen in snakes with a history of breeding activity. Lack of the disease syndrome in virgin animals as well as the statistically significant increase in the development of the disease syndrome in unaffected males partnered with affected males could indicate venereal spread of the disease syndrome. An increased BPA prevalence was also associated with active breeding status animals. Due to the qualitative nature of the developed PCR test, BPA could only be confirmed as present or absent in the cloaca. Without a quantitative assay, relative BPA levels in the cloaca of PCR-positive animals with the disease syndrome cannot be compared to bacterial counts of BPA PCR positive animals without the disease syndrome.

The reproductive sequelae of the disease syndrome for affected snakes are unclear. Because this colony has 2 designated males per breeding group, fecundity can only be examined on the breeding group level. A decrease in fecundity brought on by the disease could be missed if the second male in the breeding group masked a deficit in fecundity in the partnering male or if fecundity is only affected in later-stage disease expression. Likewise, the culling of affected animals within the colony may mask late-stage disease progression that could ultimately result in higher untimely mortality. However, the clinical significance for the snakes in advanced stages of disease is unquestionable. Animals with severe disease exhibit other sequela to the disease, including mucosal and cutaneous ulceration, secondary infections, and possible obstipation.

Despite evidence for association with the disease syndrome, the prevalence of BPA did not differ greatly between the affected and unaffected colonies sampled in this study. This could suggest a multifactorial nature of the disease syndrome. One possible explanation for the formation of the disease syndrome presented here is that strenuous, repetitive, and long-term breeding of males at large production facilities can result in hemipenal mucosal damage. This mucosal damage paired with the presence of BPA could then lead to a higher chance of development of actinomycosis, as is well-documented in other species. This explanation would support males presenting with the disease syndrome at far higher rates than females, as well as why no virgin animals experienced the disease syndrome. Furthermore, the presence of a yet unknown co-infective agent, as is also common in actinomycoses in other species, could explain why BPA was associated with the disease syndrome in the affected colony but not in unaffected colonies. However, experimental infections and further studies of pathogenesis would be necessary to fulfill Koch's postulates and show a causative relationship between BPA and the disease syndrome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all prospective analysis/research was performed for diagnostic purposes for the health of the study collections at the

request of the collection owners. All diagnostic testing is covered under University of Florida IACUC protocol #201907944. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ST, MI, EG, and RO contributed to postmortem examination and interpretation and photographs. ST, AC, JW, and RO contributed to molecular characterization. ST and RI contributed to statistical analysis. All authors contributed to overall study design and writing of the manuscript and approve of the submitted version.

FUNDING

All expenses related to this study were either supported by the laboratories of JW and RO or as paid diagnostic submissions to the Aquatic, Amphibian, and Reptile Pathology Service at the College of Veterinary Medicine, University of Florida.

ACKNOWLEDGMENTS

The authors would like to thank the collection owners, Jessy Castellanos-Gell, and the following laboratories at the University of Florida's College of Veterinary Medicine: Clinical Microbiology, Clinical Pathology, and Histology.

REFERENCES

1. Collis AH, Fenili RN. *The Modern U.S. Reptile Industry*. Washington, DC: Georgetown Economic Services, LLC (2011).
2. Barker DG, Barker TM. *Ball pythons: The History, Natural History*. VPI Library: Care and Breeding (2006).
3. Riis A, Bendtsen T. *World of Ball Pythons* [WWW *Document*] (2019).
4. Stahl SJ. Veterinary management of snake reproduction. *Vet Clin North Am*. (2002) 5:615–36. doi: 10.1016/S1094-9194(02)00017-8
5. Langford GJ, Willobee BA, Isidoro LF. Transmission, host specificity, and seasonal occurrence of cyrtosomum penneri (nematoda: Atractidae) in lizards from Florida. *J Parasitol*. (2013) 99:241–6. doi: 10.1645/12-30.1
6. Sheldon BC. Sexually transmitted disease in birds: occurrence and evolutionary significance. *Philos Trans*. (1993) 339:491–7. doi: 10.1098/rstb.1993.0044
7. Wallace RJ, Musher DM. Actinomycosis: an update. *Int J Dermatol*. (1977) 16:185–7. doi: 10.1111/j.1365-4362.1977.tb01849.x
8. Cox NA, Richardson LJ, Buhr RJ, Mcdaniel CD, Cosby DE, Wilson JL, et al. Apparent attachment of campylobacter and salmonella to broiler breeder rooster Spermatozoa. *Poult Sci*. (2000) 85:619–24. doi: 10.1093/ps/85.4.619
9. Donoghue AM, Blore PJ, Cole K, Loskutoff NM. Detection of Campylobacter or Salmonella in turkey semen and the ability of poultry semen extende. *Poult Sci*. (2004) 83:1728–33. doi: 10.1093/ps/83.10.1728
10. Bochev V, Angelova I, Tsankov N. Cervicofacial actinomycosis- report of two cases. *Nagoya J Med Sci*. (2003) 12:105–8.
11. Valour F, Chidiac C, Ferry T, Lyon Bone and Joint Infection Study Group. A 22-year-old woman with right lumpy jaw syndrome and fistula. *BMJ Case Rep*. (2015) 2015:bcr2014206557. doi: 10.1136/bcr-2014-206557
12. Kreisinger J, Cízková D, Kropácková L, Albrecht T. Cloacal microbiome structure in a long-distance migratory bird assessed using deep 16sRNA pyrosequencing. *PLoS ONE*. (2015) 10:137401. doi: 10.1371/journal.pone.0137401
13. Holmes IA, Monagan IV, Rabosky DL, Davis Rabosky AR. Metabolically similar cohorts of bacteria exhibit strong cooccurrence patterns with diet items and eukaryotic microbes in lizard guts. *Ecol Evol*. (2019) 9:12471–81. doi: 10.1002/ece3.5691
14. McBride WJH. Mandell, douglas and bennett's principles and practice of infectious diseases. *Sex Health*. (2010) 7:218. doi: 10.1071/SHv7n2_BR3
15. Hellebuyck T, Martel A, Chiers K, Haesebrouck F, Pasmans F. Devriesea agamarum causes dermatitis in bearded dragons (Pogona vitticeps). *Vet Microbiol*. (2009) 134:267–71. doi: 10.1016/j.vetmic.2008.08.021
16. Leary S, Underwood W, Anthony R, Cartner S, Grandin T, Greenacre C, et al. *AVMA Guidelines for the Euthanasia of Animals*. Schaumburg, IL: JAVMA (2020).
17. Schuurman T, De Boer RF, Kooistra-Smid AMD, Van Zwet AA. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. *J Clin Microbiol*. (2004) 42:734–40. doi: 10.1128/JCM.42.2.734-740.2004
18. Katoh K, Toh H. Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinform*. (2008) 9:212. doi: 10.1186/1471-2105-9-212
19. Stamatakis A, Hoover P, Rougemont J. A rapid bootstrap algorithm for the RAxML web servers. *Syst Biol*. (2008) 57:758–71. doi: 10.1080/10635150802429642
20. Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, et al. Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Syst Biol*. (2012) 61:539–42. doi: 10.1093/sysbio/sys029
21. Miller MA, Schwartz T, Pickett BE, He S, Klem EB, Scheuermann RH, et al. A RESTful API for access to phylogenetic tools via the CIPRES science gateway. *Evol Bioinforma*. (2015) 11:43–8. doi: 10.4137/EBO.S21501
22. Bartlett JG. Human cervicofacial actinomycoses: microbiological data for 1997 cases. *Infect Dis Clin Pract*. (2004) 12:146. doi: 10.1086/376621
23. Soto E, Arauz M, Gallagher CA, Illanes O. Nocardia cyriacigeorgica as the causative agent of mandibular osteomyelitis (lumpy jaw) in a cat. *J Vet Diagnostic Investig*. (2014) 26:580–4. doi: 10.1177/1040638714533117
24. Masand A, Kumar N, Patial V. Actinomycosis (lumpy jaw) in cow: a case report. *Comp Clin Path*. (2015) 24:541–3. doi: 10.1007/s00580-014-1939-1
25. Sotohira Y, Suzuki K, Sano T, Arai C, Asakawa M, Hayashi H. Stress assessment using hair cortisol of kangaroos affected by the lumpy jaw disease. *J Vet Med Sci*. (2017) 79:852–4. doi: 10.1292/jvms.16-0633
26. Kim YJ, Youm J, Kim JH, Jee BC. Actinomyces-like organisms in cervical smears: the association with intrauterine device and pelvic inflammatory diseases. *Obstet Gynecol Sci*. (2014). 57:393–6. doi: 10.5468/ogs.2014.57.5.393
27. Warren NG. Actinomycosis, nocardiosis, and actinomycetoma. *Dermatol Clin*. (1996) 14:85–95. doi: 10.1016/S0733-8635(05)70328-4
28. Sullivan DC, Chapman SW. Bacteria that masquerade as fungi: actinomycosis/Nocardia. *Proc Am Thorac Soc*. (2010) 7:216–21. doi: 10.1513/pats.200907-077AL
29. Breton P, Lustig S, Laurent F, Ader F, Boussel L, Senechal A, et al. Actinomycosis: etiology, clinical features, diagnosis, treatment, and management. *Infect Drug Resist*. (2014) 7:183–97. doi: 10.2147/IDR.S39601
30. Topić MB, Desnica B, Vicković N, Skuhala T, Bayer K, Bukovski S. The polymicrobial *Actinomyces naeslundii* and *Pseudomonas aeruginosa* sepsis in a patient with ulcerative colitis 2 months after colonoscopy. *Wien Klin Wochenschr*. (2014) 126:130–2. doi: 10.1007/s00508-013-0471-7
31. Wang Y-H, Tsai H-C, Lee SS-J, Mai M-H, Wann S-R, Chen Y-S, et al. Clinical manifestations of Kikuchi's disease in southern Taiwan. *J Microbiol Immunol Infect*. (2005) 38:35–40.
32. Wong JJ, Kinney TB, Miller FJ, Rivera-Sanfeliz G. Hepatic actinomycotic abscesses: diagnosis and management. *Am J Roentgenol*. (2006) 186:174–6. doi: 10.2214/AJR.04.1691
33. Ávila F, Santos V, Massinha P, Pereira JR, Quintanilha R, Figueiredo A, et al. Hepatic actinomycosis. *GE Port J Gastroenterol*. (2015) 22:19–23. doi: 10.1016/j.jpge.2014.08.002

34. Lowe RN, Azimi PH, McQuitty J. Acid-fast actinomyces in a child with pulmonary actinomycosis. *J Clin Microbiol*. (1980) 12:124–6. doi: 10.1128/JCM.12.1.124-126.1980
35. De Jong MCM, Heesterbeek JAP. How does transmission of infection depend on population size? *Publicat. Newton Institute*. (1994) 5:84–94.
36. Bojesen AM, Nielsen SS, Bisgaard M. Prevalence and transmission of haemolytic Gallibacterium species in chicken production systems with different biosecurity levels. *Avian Pathol*. (2003) 32:503–10. doi: 10.1080/0307945031000154107
37. Hethcote HW. The mathematics of infectious diseases. *SIAM Rev*. (2005) 42:599–653. doi: 10.1137/S0036144500371907
38. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C. Within-herd contact structure and transmission of *Mycobacterium avium* subspecies paratuberculosis in a persistently infected dairy cattle herd. *Prev Vet Med*. (2011) 100:116–25. doi: 10.1016/j.prevetmed.2011.02.004
39. Voutilainen L, Kallio ER, Niemimaa J, Vapalahti O, Henttonen H. Temporal dynamics of *Puumala hantavirus* infection in cyclic populations of bank voles. *Sci Rep*. (2016) 6:23853. doi: 10.1038/srep23853

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 05 May 2021
doi: 10.3389/fvets.2021.642894

Herpesviruses in Reptiles

God'spower Richard Okoh, Paul F. Horwood, David Whitmore and Ellen Ariel*

Division of Tropical Health and Medicine, College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia

Since the 1970s, several species of herpesviruses have been identified and associated with significant diseases in reptiles. Earlier discoveries placed these viruses into different taxonomic groups on the basis of morphological and biological characteristics, while advancements in molecular methods have led to more recent descriptions of novel reptilian herpesviruses, as well as providing insight into the phylogenetic relationship of these viruses. Herpesvirus infections in reptiles are often characterised by non-pathognomonic signs including stomatitis, encephalitis, conjunctivitis, hepatitis and proliferative lesions. With the exception of fibropapillomatosis in marine turtles, the absence of specific clinical signs has fostered misdiagnosis and underreporting of the actual disease burden in reptilian populations and hampered potential investigations that could lead to the effective control of these diseases. In addition, complex life histories, sampling bias and poor monitoring systems have limited the assessment of the impact of herpesvirus infections in wild populations and captive collections. Here we review the current published knowledge of the taxonomy, pathogenesis, pathology and epidemiology of reptilian herpesviruses.

Keywords: herpesviruses, reptiles, fibropapillomatosis, taxonomy, pathogenesis, pathology, epidemiology

OPEN ACCESS

Edited by:
Amanda Linda Jean Duffus, Gordon State College, United States

Reviewed by:
Francisco Rivera-Benítez, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico
Annie Page-Karjian, Florida Atlantic University, United States

****Correspondence:***
God'spower Richard Okoh
godspower.okoh@my.jcu.edu.au

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *17 December 2020*
Accepted: *06 April 2021*
Published: *05 May 2021*

Citation:
Okoh GR, Horwood PF, Whitmore D and Ariel E (2021) Herpesviruses in Reptiles. Front. Vet. Sci. 8:642894. doi: 10.3389/fvets.2021.642894

INTRODUCTION

Reptiles are a group of vertebrates (class Reptilia) that are adapted to a broad range of terrestrial and aquatic environments (1, 2). The group comprises over 11,000 extant species placed in four orders, namely: Testudines (turtles, tortoises, and terrapins); Squamata (lizards, snakes, and worm lizards); Crocodilia (crocodiles, alligators, gharials, and caimans); and Rhynchocephalia (tuatara) (3). Reptiles constitute an integral part of the natural ecosystem and play the roles of both pollinators and predators, as well as environmental health indicators (4). In addition to their ecological services, reptiles have become desirable for food, medicinal products, pet trade, leather goods and research applications (5–7). However, their existence and well-being have constantly been threatened by several factors, such as hunting, environmental pollution, loss of habitat, destructive non-native species, climate change, and infectious diseases (4, 8–10). Disease surveillance and research in wild populations of reptiles are associated with numerous challenges including difficulties in accessing samples or field data, misleading epidemiological data and missing population data, as well as political and cultural restrictions (11). These challenges well explain the use of captive wildlife as models in many studies to acquire epidemiological information, since diseases are comparable in both wild and captive animals (12–15). Nonetheless, more robust and ideal epidemiological data are obtained when free ranging animals are surveyed. Recently, researchers have taken a renewed interest in reptilian viruses, partly due to the role played by reptiles as reservoir hosts for zoonotic viruses, as well as improvements in viral diagnostic methods that, in turn, have increased understanding of viruses in reptiles (16–20).

Herpesviruses (HVs) are members of the family *Herpesviridae,* a large taxon of DNA viruses that have been described in most vertebrate animals, including reptiles (16, 21). Herpesviruses are enveloped viruses with an icosahedral nucleocapsid and a linear double-stranded genome of varying length from ∼124 to 259 kbp (22). Generally, HVs replicate within host cell nuclei and are able to remain latent in their natural hosts (17, 23). So far, reptilian HVs that have been identified and characterised all belong to the subfamily *Alphaherpesvirinae* (17, 24–26).

The occurrence of HV infections among reptiles has been widely documented and associated with stomatitis, tumors, encephalitis, conjunctivitis, hepatitis and mortalities (27, 28). Unfortunately, current treatment options of reptilian HVs are limited and the search for potent vaccines remains a herculean task; therefore, the adoption of preventative strategies is still the most efficient way of controlling these diseases. This review aims to assist in biosecurity planning as well as create a knowledge platform for decision makers and researchers by providing an overview of the taxonomy, pathogenesis, pathology and epidemiology of reptilian HVs.

METHODS

Databases such as Medline (Ovid), PubMed, and Scopus were searched using specific keywords and phrases including *Herpesviridae* infections, herpesvirus infection, fibropapillomatosis, grey-patch disease, loggerhead genital-respiratory herpesvirus, herpesvirus disease, reptiles, turtles, tortoise, snakes, lizards, alligators, and crocodiles (**Supplementary File 1**). To ensure that relevant publications were not missed, each subheading was searched independently on PubMed. Also, an additional literature search was conducted by assessing references of articles selected from previous databases. A summary of the search results is shown in **Figure 1**. Furthermore, we read the abstracts and full texts of the selected articles, extracted and analysed information on the diagnostic methods used and the reptilian HVs investigated from 1972 to September 8, 2020 (**Figures 2**, **3**; **Supplementary File 2**). Non-English, non-original research, guidelines, and review articles were excluded from the analysis.

BIBLIOMETRICS

We conducted bibliometric analyses of published articles on the topic of reptilian HVs using Vosviewer software (29) and the Web of Science Core Collection database. A total of 245 publications were downloaded from Web of Science Core Collection database using the following search terms; herpesvirus, turtle, lizard, snake, tortoise and crocodile. The strategy involved a combined use of the keywords, tags and Boolean operators to create query sets as follows: ALL= (herpesvirus) AND ALL= (turtle* OR lizard* OR snake* OR tortoise* OR crocodile*) with no limitations. USA had the highest number of research outputs with 149 (60.8%) articles. This was followed by Germany (28; 11.4%) and Australia (26; 10.6%) (**Figure 4**; **Table 1**). Using Vosviewer, we visualised the major keywords commonly used in the field of reptilian HVs and the link strengths between collaborating countries (**Figure 4**; **Supplementary File 3**). Of note, a low number of records on reptilian HVs were observed for some countries (Indonesia, Mexico and India) that have rich reptile diversity (**Table 1**) (3, 30, 31). Some of these countries also had little or no collaborations with the high research output countries (**Figure 4**), thus suggesting an under-reporting of reptilian HVs in these countries. Conversely, Germany has less reptile diversity with a higher number of records (**Table 1**). This observation could be attributed to the presence of established diagnostic resources or increased monitoring and reporting systems for reptilian diseases in the country.

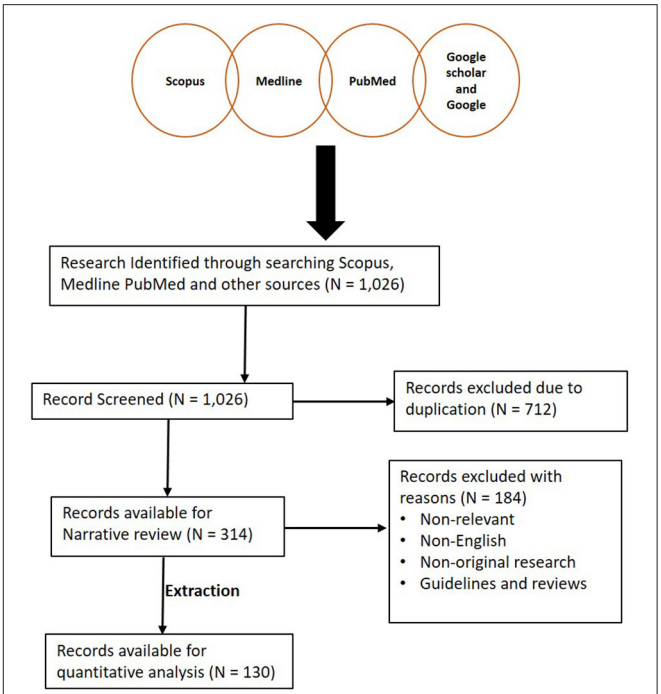


FIGURE 1 | Summary of literature search conducted. A total of 1,026 articles were initially screened and 712 articles were later excluded due to duplication. A total of 314 articles were then reviewed for this study. One hundred and thirty studies were then extracted for quantitative analysis.

TAXONOMY OF REPTILIAN HERPESVIRUSES

Reptilian HVs belong to the family *Herpesviridae,* a member of the order *Herpesvirales* (32). According to the 2019 *International Committee on Taxonomy of Viruses* (ICTV) classification, the subfamily *Alphaherpesvirinae* comprises five genera namely, *IItovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus,* and *Varicellovirus*. Only the genus *Scutavirus* contains species that cause HV diseases in reptiles and includes *Chelonid alphaherpesvirus 5* (ChHV-5) and *Testudinid alphaherpesvirus*

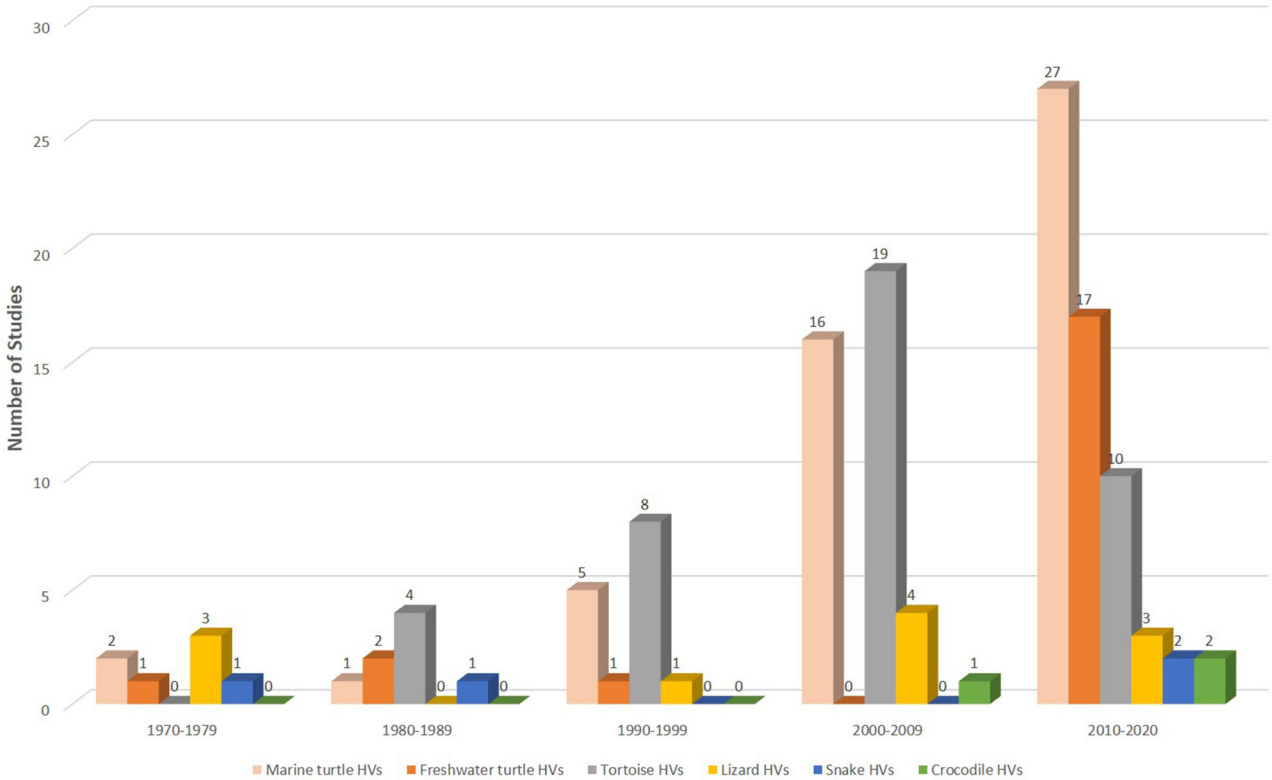


FIGURE 2 | Studies on HVs in reptiles from 1972 to September 8, 2020. Overall, more than a quarter ($n = 51$; 39%) of the 130 extracted studies were conducted in marine turtles since the 1970s. Of the 60 publications between 2010 and 2020, 45% ($n = 27$) were ChHV-5 related studies.

3 (TeHV-3). The species *Chelonid alphaherpesvirus 6* (ChHV-6) belongs to the subfamily *Alphaherpesvirinae* with unknown generic placement. The *Iguanid herpesvirus 2* (IgHV-2) that causes cytopathic infection in iguanids is of unknown generic and subfamilial placement (32–35).

Since the end of the twentieth century, advances in molecular and phylogenetic analyses have made it possible for novel reptilian HVs to be identified with proposed taxonomic placements (36). Novel HVs have been detected in freshwater turtles, including Emydoidea herpesvirus 1 (EBHV-1), Pelomedusid herpesvirus 1, Glyptemys herpesvirus 1 and 2 (GlyHV-1 and−2), Emydid herpesvirus 1 and 2 (EmyHV-1 and−2), and Terrapene herpesvirus 1 and 2 (TerHV-1 and−2) (28, 37–41). Loggerhead genital-respiratory herpesvirus (LGRV) and loggerhead orocutaneous herpesvirus (LOCV) were detected in loggerhead turtles (*Caretta caretta*) and the genus *Chelonivirus* was proposed for these viruses and other related chelonian HVs (42). Also, tortoise HV species (TeHV-1,−2,−3,−4) have been identified and placed in the proposed genus Chelonivirus (43–46), although TeHV-3 has been formally assigned to the genus *Scutavirus* (32). Other unassigned reptilian HVs in the family *Herpesviridae* include the Iguanid herpesvirus 1 (IgHV-1), Gerrhosaurid herpesvirus 1-3, Varanid herpesvirus (VHV-1,−2,−3) and Helodermatid herpesvirus 1 (HeHV-1) in lizard species (25, 47–51), Opheodrys herpesvirus 1 in snakes (52), Crocodyline herpesvirus 1-3 (CrHV-1,−2,−3) in crocodiles (53), and, Chelonid herpesvirus 1-4 (ChHV-1,−2,−3,−4) in green turtles (*Chelonia mydas*-ChHV-1) (54), freshwater turtles (*Clemmys marmorata*-ChHV-2; *Chrysemis picta*-ChHV-3) (55, 56), and Argentine tortoise (*Geochelone chilensis*-ChHV-4) (57). Notably, some of these unassigned HVs were identified decades ago based on their morphological and biological characteristics using techniques (virus isolation, electron microscopy and histopathology) that were available at that time, thus making it challenging to place them taxonomically. We conducted a phylogenetic analysis to illustrate the relationship between the unassigned reptilian HVs and currently assigned HVs using amino acid sequences of HV-DNA-dependent DNA polymerase (37 complete and 17 partial sequences) from the NCBI website (https://www.ncbi.nlm.nih.gov/). As previously described (37, 58), the analysis showed that the unassigned reptilian HVs form a monophyletic group with members of the subfamily *Alphaherpesvirinae*. Freshwater HVs showed a close phylogenetic relationship with the tortoise HVs while the lizard HVs indicated high variations (**Figure 5**). Overall, given the variations shown by the unassigned reptilian HVs, it remains a matter of scientific deliberation whether these viruses should be assigned into one genus such as TeHV-3 and ChHV-5 or into different genera, although we envisage the latter would be the case.

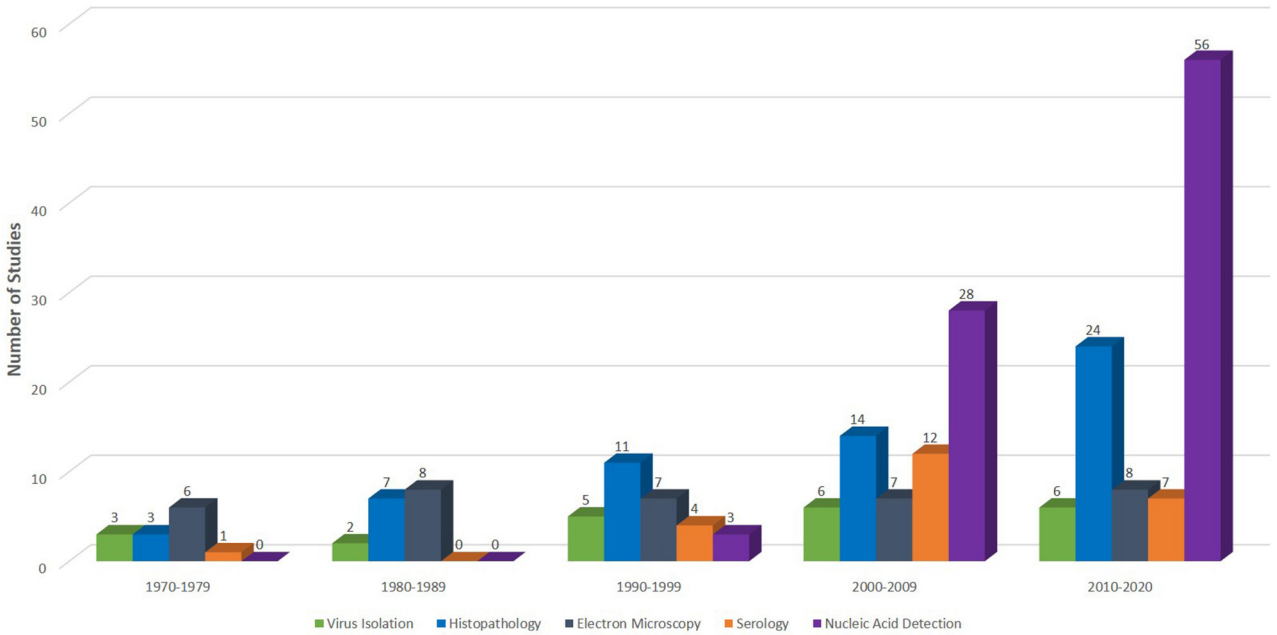


FIGURE 3 | Diagnostic methods used in different studies from inception 1970 to September 8, 2020. Of the 130 studies extracted for this quantitative analysis, 22 (17%), 59 (45%), 36 (28%), 24 (18%), and 87 (67%) used virus isolation, histopathology, electron microscopy, serology, and nucleic acid detection assays, respectively, for various investigations of reptilian HVs.

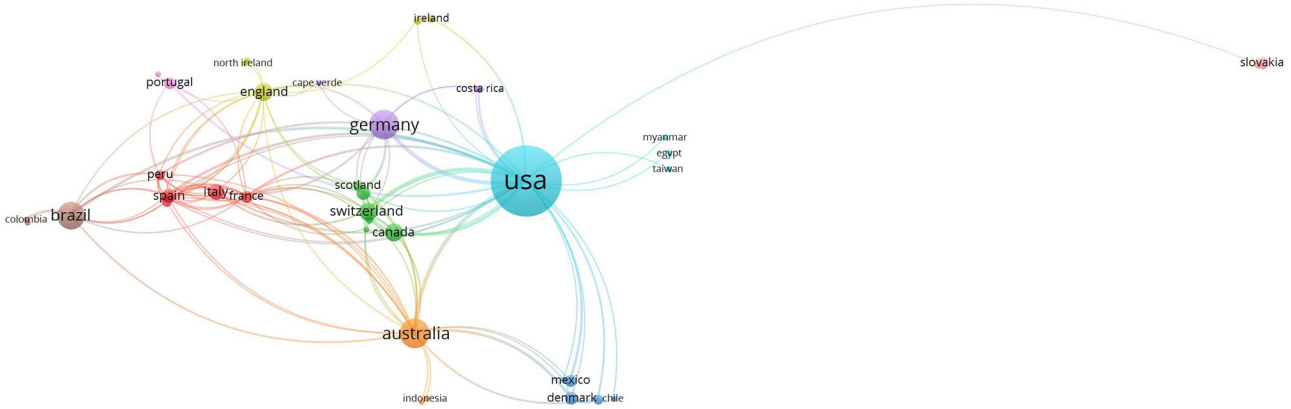


FIGURE 4 | Links between collaborating authors from different countries with research output from inception to 2020. The circular coloured nodes represent countries and the node size indicates number of publications from the country. The lines between nodes indicate authorship collaborations between countries and the widths of these lines indicate the link strength.

VIRION AND GENOME ORGANIZATION

All members of the family *Herpesviridae* share a common virion architecture, comprising a monopartite, linear, double stranded DNA core enclosed within an icosahedral capsid with a $T = 16$ symmetry (60–62). The capsid is tightly wrapped by a proteinaceous tegument, which, in turn, is surrounded by an envelope containing polyamines, lipids, and essential antigenic glycoproteins (62, 63). Unlike reptilian HVs, the atomic structures of human alphaherpesviruses have mostly been described. For instance, a cryo-electron microscopy (Cryo-EM) resolved the atomic structure of human simplexviruses (HSV-1 and−2), which comprise capsid organisation (hexons, pentons and triplexes), capsid proteins (VP5, VP19C, VP23, and VP26) and tegument proteins (pUL17, pUL25, and pUL36) (64–66). Although the atomic structures of reptilian HVs have not specifically been resolved, the resolved structures of other alphaherpesviruses provide insights, since their genomes have many similarities (36, 67). Consequently, the insights could guide future

TABLE 1 | Bibliographic data on reptilian herpesviruses based on the number of articles from different countries.

Countries/Regions	Records	% of 245	No. of reptiles species (3,31)	*Rank
USA	149	60.8	1,147	2
Germany	28	11.4	29	31
Australia	26	10.6	1,159	1
Brazil	23	9.4	878	5
Switzerland	11	4.5	27	32
Italy	10	4.1	65	24
Canada	9	3.7	57	25
United Kingdom	15	6.1	6	43
Belgium	6	2.4	11	41
Spain	6	2.4	78	23
Denmark	5	2.0	12	40
France	4	1.6	181	15
Mexico	4	1.6	1,021	3
Portugal	4	1.6	44	27
Slovakia	4	1.6	16	37
Ecuador	3	1.2	541	10
Japan	3	1.2	111	22
China	3	1.2	605	8
Peru	3	1.2	586	9
Austria	2	0.8	18	36
Costa Rica	2	0.8	467	12
Czech Republic	2	0.8	15	38
India	2	0.8	889	4
Ireland	2	0.8	21	35
Netherlands	2	0.8	23	34
Barbados	1	0.4	13	39
Cape Verde	1	0.4	51	26
Chile	1	0.4	179	16
Colombia	1	0.4	654	7
Croatia	1	0.4	42	28
Egypt	1	0.4	133	20
French Guiana	1	0.4	177	17
Indonesia	1	0.4	798	6
Israel	1	0.4	148	19
Myanmar	1	0.4	374	13
Nicaragua	1	0.4	218	14
Norway	1	0.4	10	42
Romania	1	0.4	31	30
Seychelles	1	0.4	37	29
South Africa	1	0.4	529	11
South Korea	1	0.4	26	33
Taiwan	1	0.4	123	21
Turkey	1	0.4	150	18
Turks Caicos	1	0.4	12	40

**Ranking was conducted based on the number of reptile species by countries identified from our bibliometric search and not based on the global ranking by Butler (31).*

research in the atomic structure resolutions of reptilian HVs, which in turn could serve as a baseline for reptilian HV vaccinology.

Although the complete nucleotide sequences for most reptilian HVs are yet to be obtained, genomic features can be inferred from other fully sequenced alphaherpesviruses owing to sequence homology (36, 67). All alphaherpesvirus genome structures contain unique long (U_L) and short (U_S) sequences and each are flanked by both terminal (TR_L, TR_S) and internal (IR_L, IR_S,) inverted repeat regions, giving the general configuration TR_L-U_L-IR_L-IR_S-U_S-TR_S (68). The complete genome of two TeHV-3 strains (1976 and 4295) was recently sequenced. The 1976 strain was shown to have a novel inverted repeat (TR_T, IR_T) and unique (U_T) regions (69). The genome is approximately 160 kbp, encodes at least 107 open reading frames (ORFs) and consists of U_L (107,928 bp) and U_S (20,375 bp) regions. The U_L is bound to its right by the U_S adjoined to inverted repeats (IR_S and TR_S; 8,536 bp) and to its left by a third unique region (U_T; 12,595 bp), which is also bordered by inverted repeats (TR_T and IR_T; 1,194 bp) to give the overall layout TR_T-U_T-IR_T-U_L-IR_S-U_S-TR_S (69). However, this differs from the type D configuration earlier attributed to this species (70). In another study, the complete nucleotide sequence of a Bacterial Artificial Chromosome (BAC) containing the entire genome of ChHV-5 (cloned in pTARBAC2.1) was obtained and it showed a different configuration (U_L-IR_S-U_S-TR_S) from that of TeHV-3, even though they both belong to the genus *Scutavirus* (71). Moreover, the genome characterisation of strain 4295 identified regions containing genes that could be involved in viral pathogenesis or virulence (69). This is an important finding as these regions could serve as therapeutic or diagnostic targets in future research. Similarly, evidence of recombination among strains of ChHV-5 documented by Morrison et al. could lead to increased virulence and transmission of ChHV-5 variants (72) and these events may remain undetected in sea turtle populations. Therefore, it is pertinent to strengthen current diagnostic approaches to allow for more comprehensive geographical surveys and characterisation of HVs. Also, as new and affordable diagnostic techniques are being developed and improved upon, we expect more novel structures of reptilian HVs to be reported.

TRANSMISSION AND PATHOGENESIS

Several modes of reptilian HV transmission have been postulated including vertical, horizontal and mechanical transmissions (73–75). Marenzoni et al. reported the first evidence of vertical transmission of TeHV-3 in a captive breeding facility (14). In this study, one hatchling born in isolation from the egg laid by an infected tortoise (*Testudo hermanni hermanni*) presented with conjunctivitis and tested positive by specific polymerase chain reaction (PCR) targeting the partial sequence of the UL39 gene of TeHV-3 (14). In other studies, Jones and colleagues provided molecular evidence for the horizontal transmission of ChHV-5 in green turtles by demonstrating the molecular link between viral variants and foraging grounds (76, 77). Furthermore, experimental studies have revealed the possible transmission of reptilian HVs by direct contact with infectious

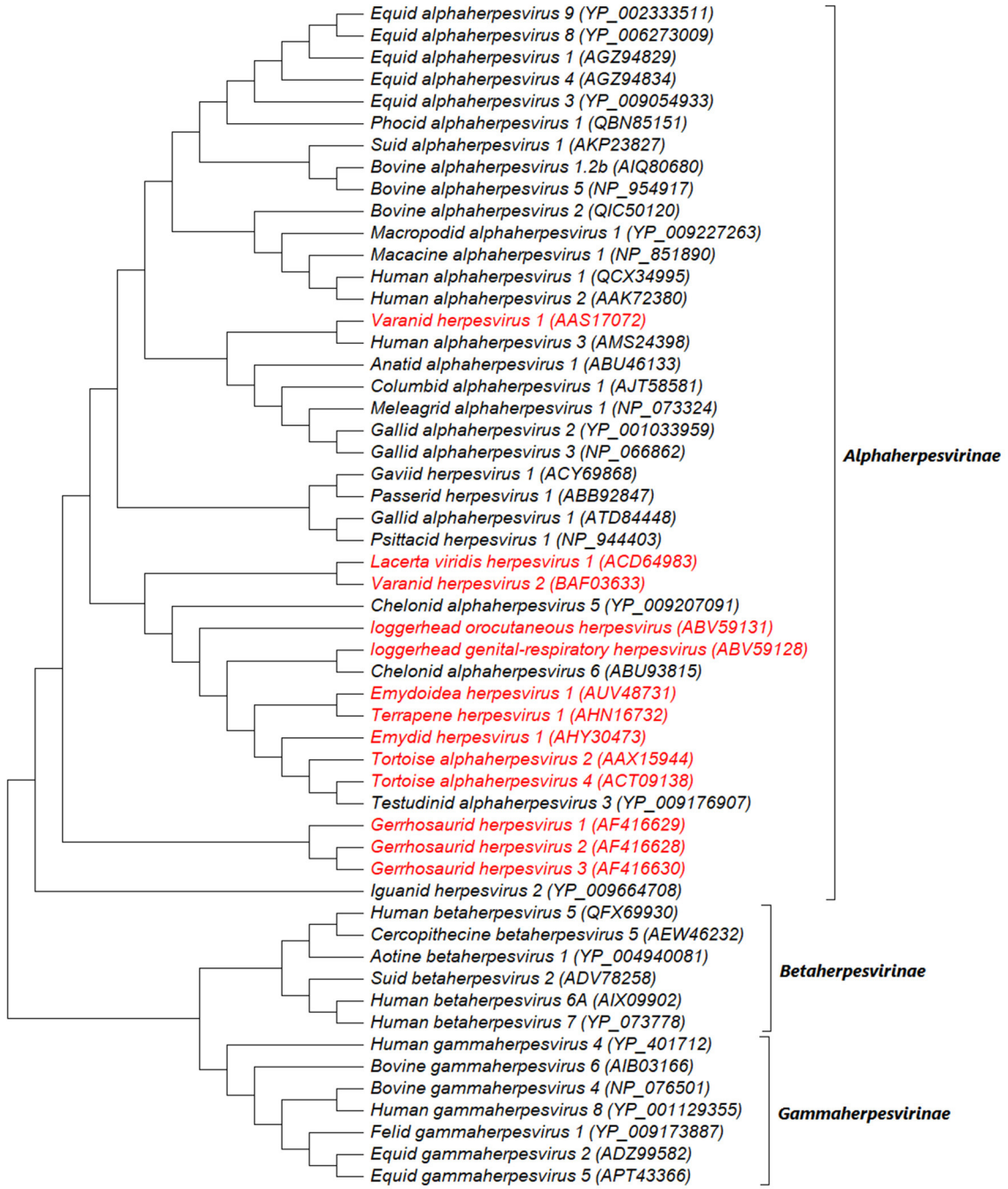


FIGURE 5 | Midpoint rooted maximum likelihood phylogenetic tree of predicted amino acid sequences of HV-DNA-dependent DNA polymerase. The unassigned reptilian HVs are shown in red and cluster within the subfamily *Alphaherpesvirinae*. This analysis involved 54 amino acid sequences (37 complete and 17 partial sequences) downloaded from NCBI website (https://www.ncbi.nlm.nih.gov/) and aligned by ClustalW. There were a total of 1,443 positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (−60139.09) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (59).

secretions (78–80) or indirectly via vectors and water (81–84). By linking viral shedding patterns or frequency to disease occurrence, we could trace the most probable transmission mode of reptilian HVs. For instance, in captive enclosures, HVs could be easily transmitted via contact with secretions or contaminated materials, even at low shedding rates, and

the removal of infected animals and infectious materials could stop the spread of the virus. In the wild, different transmission agents such as vectors, fomites and superspreaders could interplay to compensate for the low contact rate and infrequent shedding of some HVs (85). Because of the managerial implications, it is therefore important to further investigate the roles of these factors in the transmission of HVs in wild reptiles.

Four pathogenic mechanisms are highly conserved among all HVs and include: (1) intranuclear replication and capsid assembly; (2) expression of DNA metabolic and synthetic enzymes; (3) cell destruction following the release of viral progenies; and (4) the maintenance of latency in natural host cells (63, 86, 87). Generally, HV infections begin with viral entry, which is followed either by localisation or systemic spread (87). An experimental transmission study described the systemic dissemination of tortoise HVs (isolates HV 1976 and HV 4295/7R/95) (78). Following experimental infection via intranasal and intramuscular routes, the HVs elicited clinical signs and were detected by PCR in tissue samples from the respiratory, digestive and urogenital tracts, central nervous system (CNS), heart and spleen (78). Fibropapillomatosis (FP) and grey patch disease (GPD) are both associated with clinical signs that could be attributed to local destruction of infected cells due to replication and progeny release (54, 80, 88–90). Evidentially, two studies demonstrated the local replication of ChHV-5 by detecting certain biomarkers (eosinophilic intranuclear inclusions, F-VP26, DNA, and mRNA transcripts) within fibropapillomatous lesions (85, 91).

Unfortunately, the specific mechanisms involved in host cell invasion, immune evasion, localisation and spread of reptilian HVs have not been fully elucidated. However, recent molecular studies have provided insights into some virulence factors associated with reptilian HVs (69–71). Briefly, glycoproteins B (gB), gC, gD, gH, gK gL, gM, and gN have been hypothesised to function in host cell attachment and entry (69–71). gB and gC are capable of binding to heparan sulphate proteoglycans that are present on the surface of many cells, thus aiding viral adsorption and penetration into different cell types (69, 92). The interactions of gB, gD, gH, gK, gL, gM, and gN have also been postulated to mediate membrane fusion and viral entry into the cell (69, 70, 92). Glycoproteins B, E, H, and L are involved in viral cell to cell spread, which could occur through intercellular bridges or intra-axonal transport, thus circumventing humoral immune responses (69, 70). Similarly, the gC can bind to the third complement component (C3b) to block the alternative pathway complement activation (69, 93). The gE and gI in HSV-1 inhibit the normal function of antibodies by building up a complex that acts as an Fc-receptor (94). However, the immunosuppression mechanism of the gE homologue in reptilian HVs is not yet clear. Finally, the F-M04 and F-sial proteins were recently identified in ChHV-5 and thought to play a role in FP pathogenesis; however, the specific mechanisms involved are not yet understood (71). Future research should consolidate characterisation of reptilian HVs in order to increase the understanding of host-pathogen interactions and improve clinical interventions.

CLINICAL AND PATHOLOGICAL SIGNS

Herpesvirus infections have been described in reptiles with a range of clinical manifestations (16). To provide an overview, the clinical signs and the gross and histological lesions associated with reptilian HVs are summarised in **Table 2**. Some of the more detailed descriptions are from sea turtles, tortoises and crocodiles. Grey patch disease and FP, characterised by coalescing greyish papular skin lesions (spreading patches) and branching papillary tumours (**Figure 6**), respectively, have been reported in sea turtles (42, 54, 108, 109). Lung-eye-trachea disease (LETD) with a clinical course of 2–3 weeks has been seen in green sea turtles (98). Lung-eye-trachea diseased turtles often present with pneumonia, stridor and caseation of the eyes, oropharynx and trachea (98). In freshwater turtles, HV infections are associated with hepatic necrosis, and proliferative and/or ulcerative lesions of the skin and shell (**Figure 7**) (37, 38, 110, 111). Infections in tortoises result in ulcerative to diphtheroid-necrotizing stomatitis, conjunctivitis, glossitis, rhinitis, dyspnoea, liver disease and neurological disease and could be accompanied by anorexia, regurgitation, neck oedema, lethargy and death (**Figure 8**) (112–115). Papillomas, stomatitis, and hepatitis are commonly described in lizards infected with HV (25, 47–49, 116, 117). Recently, five green snakes (*Opheodrys vernalis*) housed together presented with oropharyngeal squamous cell carcinoma and molecular analysis confirmed the presence of a novel Opheodrys herpesvirus-1 (*Alphaherpesvirinae*) (52). In another study, a lymphoid follicular cloacal inflammation in juvenile alligators was initially associated with tortoise HV. However, the HV (Genbank accession AY913769.1) was later determined to be a likely laboratory contamination and the actual causative agent is still unknown (118). Similarly, Hyndman et al. identified three novel HVs associated with conjunctivitis and/or pharyngitis (CP), systemic lymphoid proliferation with non-suppurative encephalitis (SLPE), and lymphonodular skin lesions (LNS) in farmed saltwater crocodiles (*Crocodylus porosus*) and captive freshwater crocodiles (*Crocodylus johnstoni*) (53). Obviously, HVs can induce significant diseases in both captive and wild reptiles; therefore, there is a need to develop rapid diagnostic tests that will aid disease surveillance and reporting in order to maintain safe biosecurity measures and reduce spread.

EPIDEMIOLOGY

Epidemiological studies of HVs in wild reptiles could be challenging due to a lack of sensitive diagnostics for the detection of unknown or novel species, especially in resource-limited regions. HV infections are commonly characterised by non-specific clinical signs in most reptiles, thus making diagnoses on the basis of clinical signs alone difficult. An exception to this is FP, in which the presence of cutaneous tumours gives an indication of the disease; hence, more FP-associated HV data have been reported in recent decades (**Figure 2**; **Supplementary File 2**). Even so, the complete disease impact on wild populations could be currently underestimated due to the underreporting of outbreaks, sampling bias and poor monitoring systems. For instance, there is a paucity of information for FP epidemiology

TABLE 2 | Clinical presentations, gross, and histological lesions of reptilian HV species.

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
ChHV-1	• Green sea turtles (*Chelonia mydas*)	• Benign papular lesions on the neck and flippers • Spreading grayish patches to large areas of the epidermal surface • Death may occur	• Benign Papules • Spreading gray patches	• Intranuclear inclusions found in epidermal keratinocytes	(54, 95, 96)
ChHV-2	• Pacific pond turtles *(Clemmys marmorata*)	• Lethargy • Anorexia • Muscular weakness • Coma • Subcutaneous haemorrhages • Death	• Hepatomegaly • Pallor of kidney • Subcutaneous Petechial and ecchymotic haemorrhages	• Hepatic necrosis • Intranuclear inclusion bodies • Lymphocytic aggregation in liver, kidney, and spleen • Moderately hyperplastic spleen	(55)
ChHV-3	• Painted turtles (*Chrysemys picta*)	• Abscessation • Death	• Pulmonary edema • Friable and greenish-brown liver • Distended gall bladder • Congested spleen • Shell rot lesions on plastron	• Foci of necrosis on the liver and infundibular septa • Hepatocytes containing Eosinophilic intranuclear inclusions • Granulocytic and mononuclear infiltrations	(56)
ChHV-4	• Argentine tortoise (*Geochelone chilensis*)	• Acute death • Nasal discharge • Ocular discharge • Regurgitation • Anorexia • Lethargy • Necrotizing stomatitis	• Necrotizing lesions • Serous atrophy of fat • Pale liver	• Diffuse area of necrosis in mucosal epithelium • Accumulation of necrotic cellular debris and fibrin • Infiltration of Inflammatory cells • Eosinophilic intranuclear inclusions within degenerating epithelial cells and other tissues • Vacuolar degeneration of hepatocytes	(57)
ChHV-5	• Green sea turtle (*Chelonia mydas*) • Loggerhead sea turtle (*Caretta caretta*) • Hawksbill turtle (*Eretmochelys imbricata*) • Leatherback turtle (*Dermochelys coriacea*) • Olive ridley sea turtle (*Lepidochelys olivacea*) • Kemp's ridley sea turtle (*Lepidochelys kempii*) • Flatback turtle (*Natator depressus*)	• Tumours on the inguine, tail, flippers, axillae, chin, neck, eyelids, corneas, carapace and plastron	• Single to multiple raised cutaneous masses that are verrucous, smooth, sessile or pedunculated • Ulcerated and necrotic large masses • Pigmented cutaneous tumours • Spherical, smooth, firm, white, or gelatinous and translucent nodules in the lungs, kidneys, liver, heart, and gastrointestinal tract	• Papillary epidermal and dermal hyperplasia • Orthokeratotic hyperkeratosis • Hypertrophied epithelial cells overlying vascularized fibrous stroma • Epithelial necrosis and multifocal areas of ballooning degeneration • Lymphocytes and plasma cells infiltrations • Melanophores within the masses • Eosinophilic intranuclear inclusions	(97)
ChHV-6	• Green sea turtles (*Chelonia mydas*)	• Gasping • Buoyancy abnormalities • Inability to dive • Lethargy • Caseous exudate covering the eyes, glottis and trachea • Death	• Emphysematous areas in the lungs • Caseous exudate in the eyes, glottis and trachea • Multifocal raised white nodules in the liver	• Necrotic lesions in the glottis, tracheal and lungs • Periglottal accumulations of necrotic cellular debris and fibrin • Infiltrations of heterophils, lymphocytes, and plasma cells in periglottal submucosa • Periglottal and tracheal epithelial proliferative and/or squamous metaplastic changes • Syncytial giant cells in tracheal mucosa and major airways of lungs • Thickened interstitium	(98)

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
				• Hypertrophic and hyperplastic cells with enlarged vacuolated nuclei lining the airways • Amphophilic intranuclear inclusions	
TeHV-1	• Horsfield tortoises (*Testudo horsfieldii*) • Pancake tortoises (*Malacochersus tornieri*)	• Cervical extension • Laboured breathing • Respiratory murmur • Oral and nasal discharge • Reddish-white fibrinous coating of the tongue • Death	• Yellowish-white pseudomembrane in the mouth, pharynx and glottis • Hepatomegaly and ecchymotic liver • Pseudomembrane formation in the stomach	• Diffuse areas of degeneration and necrosis in tongue and pharynx and larynx • Necrotic cellular debris and fibrin accumulation • Inflammatory cells infiltrations • Eosinophilic or amphophilic inclusion bodies	(99–101)
TeHV-2	• Desert tortoises (*Gopherus agassizii*)	• Anorexia • Lethargy • Necrotizing stomatitis	• Oral plaques	• Eosinophilic and amphophilic intranuclear inclusions in superficial epithelial cells • Thick coagulum over the epithelial surfaces of the mouth, pharynx, and trachea • Infiltration of heterophils, lymphocytes, plasma cells, and macrophages • Granulation of oropharyngeal tissue following epithelial loss	(44)
TeHV-3	• Greek Tortoises (*Testudo graeca*) • Hermann's Tortoises (*Testudo hermanni*)	• Nasal and oral discharges • Rhinitis • Dyspnoea • Conjunctivitis associated with blepharospasm • Diphtheroid-necrotizing stomatitis • Glossitis • Pharyngitis • CNS involvement (Circling, head tilt, lethargy, circling, paralysis and incoordination) • Deaths	• Stomatitis with yellowish oral plaques • Rhinitis with foamy nasal discharge • Conjunctivitis	• Oesophageal hyperplasia • Hyperplasia and hyperkeratosis in the oral mucosa • Sloughing of the epithelial cells and multifocal erosion • Glottal epithelial ulceration, hyperplasia and necrosis • Heterophilic pustules. • Amphophilic intranuclear inclusion bodies • Heterophilic bronchitis and pneumonia • Nuclear degeneration changes of the hepatocytes • Ballooning degeneration renal and digestive organs	(14, 78, 102, 103)
TeHV-4*	• Bowsprit tortoise (*Chersina angulata*) • Leopard tortoise (*Stigmochelys pardalis*)	• Asymptomatic in some cases. • Respiratory distress • Increased salivation	• No data	• No data	(43, 104)
LGRV	• Loggerhead sea turtles (*Caretta caretta*)	• Moribund state • Lethargy and quadriparesis • Emaciation • Abnormal gait • Death	• Colon impaction • Fibrinonecrotic colitis • Linear ulcers around the base of the base of the phallus • Multifocal ulcers along the mucocutaneous junction of the eyelids • Circumferential ulcer around the entire mucocutaneous junction of the cloaca • Ulcerative gastritis	• Epithelial hyperplasia. • Ballooning degeneration and syncytial cell formation within basal layers of the epithelium • Intranuclear eosinophilic inclusion bodies • Heterophilic inflammation	

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
LOCV	• Loggerhead sea turtles (*Caretta caretta*)	• Moribund state • Lethargy, bradycardia, hypoventilation, and aspiration pneumonia. • Death	• Deep multifocal ulcers around the rostral aspect of the tongue • Multifocal pale cutaneous plaques with erythematous borders on the ventral neck region • Tenacious exudates covering some plaques • Liver pallor	• Eosinophilic intranuclear inclusion bodies • Necrosis of the epithelium and extend into the underlying lingual collagen • Heterophilic inflammation • Epidermal hyperplasia • Hyperkeratosis • Intraepithelial pustules • Multifocal serocellular crust • Sloughed epithelial cells in the airways	(42)
EBHV-1*	• Blanding's turtles (*Emydoidea blandingii*)	Asymptomatic	• No data	• No data	(39)
GlyHV-1*	• Bog turtles (*Glyptemys muhlenbergii*)	Asymptomatic	• No data	• No data	(37)
GlyHV-2*	• Wood turtles (*Glyptemys insculpta*)	Asymptomatic	• No data	• No data	(37)
EmyHV-1	• Eastern river cooter (*Pseudemys concinna*) • Northern map turtle (*Graptemys geographica*) • Painted turtles (*Chrysemys picta*)	• Weakness • Frothy nasal discharge • Acute death	• Dark red, wet, and heavy lungs • Thickened, wet, and gelatinous cranial aspect of the lungs • Trace amount of watery fluid in the trachea • Diffusely tan, and slightly rounded lobular edges of the liver	• Hepatic lipidosis • Intranuclear inclusion bodies • Necrotic lesions in the lungs, liver and spleen • Granulocytic and lymphocytic interstitial infiltrations • Acute congestion with multifocal haemorrhage	(28, 105)
EmyHV-2*	• Bog turtle (*Glyptemys muhlenbergii*) • Spotted turtles (*Clemmys guttata*)	Asymptomatic	• No data	• No data	(37)
TerHV-1*	• Eastern box turtles (*Terrapene carolina carolina*)	• Lethargy • Dehydration • Dyspnoea • Moribund state with fibronecrotic stomatitis and cloacitis • Conjunctivitis • Blepharoedema • Death	• No data	• Necrosis, ulceration and syncytia formation of the pharyngeal mucosal epithelium • Eosinophilic to amphophilic intranuclear inclusions	(40)
TerHV-2	• Eastern box turtles (*Terrapene carolina carolina*)	• Papillomatous skin lesions • Anorexia	• Cutaneous papillomas	• Papillary hyperplasia of the epithelium • Infiltrations of lymphocytes, plasma cells, and heterophils • Epithelium covered by keratin and cell debris	(38)
Pelomedusid HV-1*	• West African mud turtles (*Pelusios castaneus*)	• Asymptomatic	• No data	• No data	(41)
IgHV-1	• Green iguana (*Iguana iguana*)	• Acute death	• Thin body • Generalized muscle wasting • Loss of fat store	• Hepatocellular necrosis • Hepatic syncytia • Eosinophilic intranuclear inclusions • Stomach and intestinal ulceration and necrosis • Acute renal tubular necrosis • Splenic lymphoid atrophy or hypoplasia	(33, 50, 51)

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
IgHV-2	• San Esteban Chuckwalla (*Sauromalus varius*)	• Acute death	• Haemorrhage in the lung • Congestion of airway • Pale liver	• Diffuse hepatic necrosis, eosinophilic intranuclear inclusions • Multifocal necrosis of the spleen • Interstitial infiltrations of muscles by mononuclear leucocyte • Fibrosis of muscle and gingiva	(34)
Gerrhosaurid HV-1	• Sudan plated lizard (*Gerrhosaurus major*)	• Glossal stomatitis • Severe dyspnoea	• Raised and tanned periglottal tongue • Little body fat	• Glottal trachea of granulocytic and lymphocytic inflammation with erosion of overlying epithelium	(47)
Gerrhosaurid HV-2*	• Black-lined plated lizard (*Gerrhosaurus nigrolineatus*)	• Labial stomatitis	• No data	• No data	(47)
Gerrhosaurid HV-3*	• Sudan plated lizard (*Gerrhosaurus major*)	• Chronic labial proliferative and ulcerative growth	• No data	• No data	(47)
VHV-1	• Green tree monitor lizards (*Varanus prasinus*)	• Proliferative and Ulcerative stomatitis/gingivitis • Squamous cell carcinomas	• Small white chalky plaques in the coelomic membrane, thoracic musculature, liver, kidneys, heart, and joints fascial plane • Gingival proliferation • Mucosal hyperplasia • Fibrinous exudate on the serosa of the gall bladder • Oral villous-like proliferation with patches of focal erythema	• Mucosal epithelial proliferation • Severe pulmonary, myocardial, hepatic, and renal vascular thrombosis • Sloughed tubular endothelial cells • Gingival necrosis • Hepatic lipidosis • Hepatic and renal amyloidosis	(48)
VHV-3	• Monitor Lizards (*Varanus spp.*)	• Acute death	• Yellow-tan or white viscous material and white, thick material in the intestine and distal colon, respectively • Multiple soft, white particles (2–3 mm) in intestinal tract • Diffuse pale-brown liver with multiple flat, tan pinpoint foci on the capsular surface	• Acute, multifocal, coagulative necrosis in the lamina propria of the small intestine • Acute, multifocal hepatocellular coagulative necrosis • Eosinophilic intranuclear inclusions in the small intestine and liver	(49)
HeHV-1	• Gila monster (*Heloderma suspectum*)	• Intraoral mass • Loss of weight	• Gingival nodule • Muscle atrophy	• Anastomosing epithelial cords • Proliferative gingival tissues • Eosinophilic and birefringent material within mass	(25)
Elapid HV-1	• Siamese cobra (*Naja naja kaouthia*)	• Thick tenacious venom (low grade venom)	• Enlarged venom gland • Thick venom exudates	• Venom glands are lined by degenerated epithelial cells • Mononuclear cell infiltration of gland subepithelium • Debris, degenerated cells and venom in the lumina of glands • Intranuclear inclusions	(106)
Opheodrys HV-1	• Smooth green snakes (*Opheodrys vernalis*)	• Oropharyngeal squamous cell carcinoma	• Pale tan, multinodular masses on oropharyngeal mucosa • Brown friable accumulations on tumour surface	• Distorted oropharyngeal mucosa and submucosa by epithelial neoplasm • Islands of neoplastic epithelial cells containing keratin cores • Anisocytosis and anisokaryosis of neoplastic epithelial cells	(52)

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
				• Squamous differentiation, keratin pearls, prominent intercellular bridges • Heterophilic inflammation and surface compact keratin layers	
CrHV-1	• Saltwater crocodiles (*Crocodylus porosus*)	• Conjunctivitis-pharyngitis (CP).	• Reddening and swelling of the conjunctivae of the eyelids and nictitating membrane • Cornea opacity and rupture • Fibrinocaseous conjunctival, lingual and oropharyngeal exudates	• Epithelial Hyperplasia, erosion, or ulceration of the conjunctiva, pharynx and larynx with cellular infiltrations • Lymphocyte, heterophil, and macrophage infiltrations of cornea, iris, and conjunctival, pharyngeal and laryngeal epithelium	(53, 107)
CrHV-2	• Saltwater crocodiles (*Crocodylus porosus*)	• Conjunctivitis-pharyngitis (CP) • Concurrent skin ulcers. • Systemic lymphoid proliferation and encephalitis (SLPE) • Lymphnodular skin (LNS)	**CP** • Gross lesions of CP syndrome as described above **SLPE** • Poor body condition • Splenomegaly. • Pulmonary edema **LNS** • Pale, soft, raised, well-delineated foci on lateral abdominal scales with occasional ulcerated surface covered in caseous exudate • Pale pink soft glistening tissue between the epidermis and deep dermal collagen • Enlarged tonsils with multinodular appearance • Discrete soft white foci in the subepithelial tissue of the conjunctiva • Multinodular swelling of the cloacal mucosa • Discrete white soft foci in the parenchyma of the myocardium, liver, or kidney	**CP** • Histological lesions of CP syndrome as described above **SLPE** • Lymphohistiocytic and macrophage infiltration of pulmonary septae, hepatic periportal regions, pancreatic interstitium, gastrointestinal submucosa, pericardium, epicardium, iris, wall of large blood vessels and brain • Hyperplastic lymphocytic conjunctivitis **LNS** • Expansion and displacement of collagen of the superficial and mid-dermis by intense multinodular mononuclear cell infiltrate • Epithelial hyperplasia of the tonsils will lymphocytes and macrophage infiltrations • Dense lymphohistiocytic aggregates of myocardium, liver, or kidney	(53, 107)
CrHV-3	Freshwater crocodiles (*Crocodylus johnstoni*)	• Systemic lymphoid proliferation	• Gross lesions of SLPE described above	• Histological lesions of SLPE described above	(53, 107)

ChHV, Chelonid herpesvirus; TeHV, Testudinid herpesvirus; LGRV, loggerhead genital-respiratory herpesvirus; LOCV, loggerhead orocutaneous herpesvirus; EBHV, Emydoidea herpesvirus; GlyHV, Glyptemys herpesvirus; EmyHV, Emydid herpesvirus; TerHV, Terrapene herpesvirus; IgHV, Iguanid herpesvirus; VHV, Varanid herpesvirus; HeHV, Helodermatid herpesvirus; CrHV, Crocodyline herpesvirus.

*****To the best of our knowledge, the gross or histological lesions of some novel viruses have either not been detected, or were reported while this manuscript was being written.*

at the pelagic phase of life in sea turtles as most studies are biassed towards sampling nearshore juveniles and adult females at foraging grounds or nesting beaches. Nevertheless, wildlife workers and researchers who, despite numerous challenges, have provided considerable epidemiological data targeted at conservation efforts towards endangered species should be commended. An overview of some of the epidemiological information including the prevalence and demography of both wild and captive reptilian HVs is discussed in this section.

Herpesviruses are linked to different diseases of marine turtles, including FP, LETD and GPD (54, 82). FP is a debilitating disease characterised by the development of tumours (119, 120). Depending on the location of the tumours, FP can have detrimental effects (109, 121). On the basis of prevalence and distribution, Tagliolatto et al. reported a prevalence rate of 43% for FP in green turtles captured in a foraging area in south-eastern Brazil (121). Adnyana et al. recorded 22% overall prevalence in green turtles in Indonesia and also observed that the prevalence rate of FP was higher among turtles from waters adjacent to densely populated regions compared to those collected from waters remote from urbanised regions of Indonesia (122). These findings indicate that the epidemiology of FP in marine turtles vary between geographical regions and may be linked to anthropogenic activity. This theory is supported by the findings in another study, which attributed the variation of FP prevalence to environmental cofactors that vary among local habitats (123). A study associated the geographical distribution of FP with the genomic variation of HVs in marine turtles, and observed four forms of the virus corresponding to Atlantic Ocean, west Pacific, mid-Pacific, and east Pacific (124). A similar study conducted in Australian waters identified different genotypes along the east coast of Queensland. Such differences in strains may also effectuate different levels of pathogenicity between strains (76, 77) and account for variation in reported

FIGURE 6 | Fibropapillomatosis in green turtle (*Chelonia mydas*). Photo by Dr. Karina Jones.

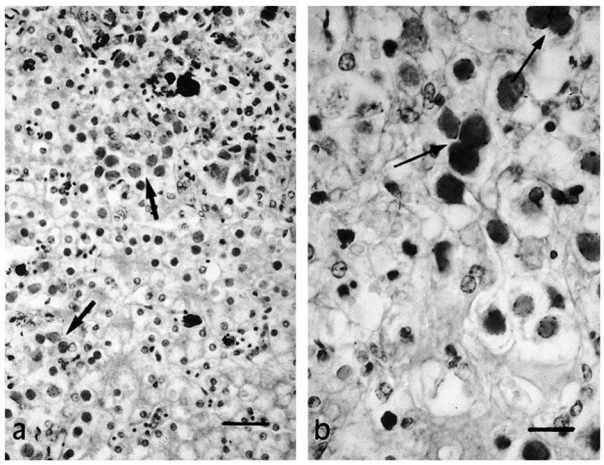


FIGURE 8 | Necrotic foci **(a)** and syncytial formation **(b)** in HV infected hepatocytes of a tortoise (*Testudo horsfieldii*). "Adapted from Hepatitis Associated with Herpes Viral Infection in the Tortoise (*Testudo horsfieldii*)" by Hervás et al. (112). Copyright 2021 by John Wiley and Sons. Reprinted with permission.

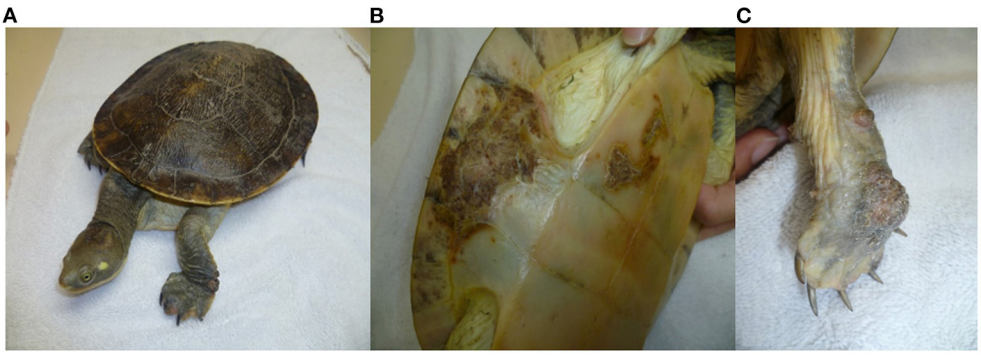


FIGURE 7 | Herpesvirus infection in freshwater turtle (*Emydura macquarii krefftii*) presented with proliferative and ulcerative lesions of the skin **(A)**, proliferative and crusted lesions on the bridge of the shell **(B)**, and proliferative lesion on the palmar aspect of the right forefoot **(C)**. "Adapted from Herpesvirus in a captive Australian Krefft's river turtle (*Emydura macquarii krefftii*)" by Cowan et al. (110). Copyright 2021 by John Wiley and Sons. Reprinted with permission.

prevalence in different regions. Also, given that the immune system of reptiles is dependent on temperature (125, 126), the variation in the prevalence rates of reptilian HVs across regions could be associated with differences in regional climate types. Comprehensive reviews of the epidemiology of FP in marine turtles have been documented elsewhere (73, 97, 127–129). The host immune status influences the clinical course of a disease, as immuno-deficient populations are more likely to succumb to disease outbreaks. Serosurveys have been conducted to determine the immune status of populations and to provide evidence of past and ongoing HV infections (130, 131). Seroepidemiological studies in three localities in Florida revealed high anti-ChHV seroprevalences (up to 100%) in both FP and non-FP sea turtles (81, 132). Contrastingly, seropositivity to ChHV-5 was dependent on the tumour status in turtles from Hawaii (133). This variation was mainly attributed to differences in the pathogenicity of ChHV-5 subtypes from the two regions (133). In another report, an epizootic of LETD in confined juvenile green sea turtles resulted in 8 to 38% mortality, thus posing significant conservation and management concerns (98, 134). The LETD impact on free ranging sea turtles has not been investigated; however, seroprevalence rates of 13% and 22% were reported in two studies, respectively (134, 135).

Similarly, HV infections are causing increasingly significant concerns in non-marine chelonians (27, 39). Herpesviruses have been implicated as the cause of severe clinical signs and acute death in terrestrial and freshwater turtles (**Table 2**) (28, 40, 105, 110, 111, 136). Although, HVs have been associated with latent infections in their natural hosts, infections in young, immunosuppressed or non-adapted hosts could result in the development of significant diseases (137). Therefore, monitoring the disease impact on both wild and captive endangered species has become pertinent. In an epidemiological study conducted in Tennessee and Illinois, USA, 128 of 409 free-ranging eastern box turtles (*Terrapene Carolina Carolina*) tested positive for TeHV-1 using TaqMan quantitative PCR, and the detection rate varied widely between seasons (138). Another study reported 48.3% prevalence of HV infections in endangered populations of bog (*Glyptemys muhlenbergii*), wood (*G. insculpta*), and spotted (*Clemmys guttata*) turtles in the northeastern United States (37). Furthermore, tortoise HVs have been associated with high mortality and morbidity (104, 113, 139–141). Different HV species were identified to cause the death of a large number of pancake (*Malacochersus tornieri*), Horsfield (*Testudo horsfieldii*), Hermann's (*Testudo hermanni*), and Egyptian tortoises (*Testudo kleinmanni*) during spontaneous outbreaks in Japan, Italy and Germany, respectively (99, 102, 142). Species dependent susceptibility to HV was reported in a tortoise colony in which *T. graeca* and *T. horsfieldii* appeared to be unaffected by the HV species that caused the death of other tortoises in the same colony (113). A possible explanation could be that the causative HV species is well-adapted in these tortoises and they could be transmitting the virus to naïve or non-adapted tortoises. Of the four tortoise HV species (TeHV1-4), TeHV-3 appears to be the most pathogenic and frequently described, causing lethal disease in different tortoise species (14, 142–145). In a recent assessment of the incidence of chelonian HVs in Europe, more than half (54%) of all the detected chelonian viruses were TeHV-3 (146). Again, seroprevalence rates of 27% and 31% were reported for TeHV-1 and TeHV-3, respectively, in different populations of desert tortoises in California (147, 148). Despite the significance of HV infections, we observed that the disease is still grossly under-studied in some countries (**Table 1**). Thus, insufficient data and underreporting have made it difficult to assess the geographical patterns of the HV epidemiology in non-marine chelonians and other reptiles.

Herpesviruses have also been described in various species of squamates and crocodilians (25, 47, 48, 52, 106, 116–118, 149). A recent outbreak of a lethal HV infection in a private facility housing 127 snakes resulted in the death of all 71 horned vipers at the premises after a brief illness (150). An earlier study also implicated HV in the death of some boa constrictors within the first year of life (151). Herpesvirus-induced deaths have been reported in different species of lizards with case fatalities nearing 100% (33, 49, 152). As stated earlier, HV infections in crocodiles are associated with CP, SLPE and LNS syndromes (53). Another study strongly linked HV infection to SLPE and CP syndromes in farmed Australian saltwater crocodiles, with the highest prevalence rates of 94 and 54%, respectively (107). Crocodiles are intensively farmed for commercial purposes in Australia; therefore, the occurrence of HVs in crocodiles has both epidemiological and economic implications (53, 153).

Finally, we extracted a total of 130 articles, of which 39% (51 articles) and 32% (41 articles) were studies that investigated HVs in marine turtles and tortoises, respectively. A total of 21 (16%) studies investigated HVs in freshwater turtles. HVs were least studied in lizards (8%; 11 articles), snakes (3%; 4 articles) and crocodiles (2%; 3 articles) (**Figure 2**). The scant studies of HVs in some reptilian species since the 1970s could be attributed to the unavailability of reagents or sensitive diagnostic assays required to investigate reptilian diseases in remote areas or the lack of interest to investigate HVs in reptiles because of their relatively low socio-economic importance. Therefore, future efforts should be directed towards enhancing collaborations among government agencies, researchers and wildlife workers with a view to creating awareness, increasing access to reagents and sensitive assays, and ultimately conserving endangered reptiles.

DIAGNOSIS

A timeline of reptilian HV diagnosis showed that traditional assays including histopathology, virus isolation (VI) and electron microscopy (EM) have been the mainstays in the diagnosis of reptilian HVs (**Supplementary File 4**). Many studies have reported the use of these techniques since the 1970s for the investigation of reptilian HVs. A breakdown of the number of studies that have used these methods to detect reptilian HVs is shown in **Figure 3**. Molecular diagnosis of reptilian HVs started two decades ago and has been used increasingly since then (**Figure 3**; **Supplementary File 4**). The advent of molecular diagnostic techniques has provided insight into the genetic characteristics and the phylogenetic relationship of most reptilian

HVs. This section highlights some important characteristics of the various techniques used in the diagnosis of reptilian HVs.

Diagnosis of reptilian HVs is tentatively made on the basis of patient history, clinical signs, and gross and histological lesions (16). However, this is not always the case, as host-adapted HVs can cause subclinical, mild or latent infections in their natural hosts, and the demonstration of intranuclear inclusions is not pathognomonic of reptilian HV infections (154). Intranuclear inclusions are frequently associated with other reptilian viruses including adenoviruses and papillomaviruses (155–157). Earlier researchers used EM to confirm the presence of reptilian HV infections by demonstrating the ultrastructure of the viral particles in fixed, cut and stained sections of tissue samples (51, 54, 98, 106, 116, 136, 140, 158). More recently, EM has been used to confirm a necrotic hepatitis associated with HV infection in a tortoise with no clinical signs or lesions in the respiratory tract, oral cavity or other organs (112). The need for high technical capacities and the high cost of electron microscopes limit the use of EM for epidemiological and diagnostic purposes especially in resource-limited areas. Despite these limitations, EM remains a powerful detection tool in most high-class virology laboratories.

Reptilian HVs have been isolated in cell culture and identified on the basis of their cytopathic effects (50, 98, 107, 159). For instance, tortoise HVs were isolated from pharyngeal swabs, trachea, kidney, oesophagus, tongue, stomach, and intestine, and caused cytolysis and rounding of cells in terrapene heart cells (TH-1) (160). In another study, detachment and foci of enlarged, rounded, refractile cells were produced following inoculation of tissue and swab supernatants in turtle heart cells (142). ChHV-5, which historically has been resistant to replication in conventional cultures, produced *de novo* ballooning degeneration and eosinophilic intranuclear inclusion in plugs and organotypic skin cultures (89). This observation implies that ChHV-5 remains latent in conventional cultures and requires replication of the turtle skin to grow *in vitro* (89). Aside from the fact that CPE are not obtained for non-cytopathic viruses, cell culture is susceptible to both chemical and biological contaminations, which in turn affect its sensitivity and specificity. Also, diagnostic turnaround could be delayed for slow-growing viruses. Therefore, it should not be solely relied upon for the epidemiological investigations of HVs.

Following primary infections in reptiles, a strong non-specific (innate) immune response that includes lysozymes, leukocytes, natural antibodies (NAbs), antimicrobial peptides, and the complement pathway, is quickly stimulated (126, 161). No specific information is currently documented about adaptive cell mediated immunity to HV infections. Unlike mammals, in reptiles a less robust and slower humoral response (IgA, IgD, IgM, and IgY) is stimulated after the innate immune system is activated (126, 154). In tortoises, neutralising antibodies to HV infection were detectable in serum at least 4 weeks post-exposure (162). These serum neutralising antibodies did not appear to confer immunity to reinfection or recrudescence (78). Later seroconversion was observed (four months to one year) in green turtles (*Chelonia mydas*) that were experimentally infected with ChHV (81, 132). Generally, the detection of anti-herpesvirus antibodies in a single sample could indicate previous or latent infection, while rising antibody titre in paired samples collected at least 6 weeks apart indicates active infection (154, 163). Humoral antibodies are detected by serological assays such as serum neutralisation (SN) tests, ELISA, and immunoperoxidase (IP) assays (132, 164–166). The SN test is considered the reference test for anti-herpesvirus antibody detection but has limitations such as a delayed turnaround, inherent assay arduity and the requirement for standard isolates (162). ELISAs with high sensitivity and specificity have been developed and deployed in various seroepidemiological studies (44, 81, 132, 147, 162, 166). However, a high degree of cross-reactivity that potentially affects assay specificity has been demonstrated among different tortoise HV isolates used as antigens in the ELISA (147, 162). Cross-reactivity could also occur in other reptilian HVs that share similar antigenic epitopes, giving false positive results and, thus, leading to unnecessary post-exposure interventions. Overall, serological diagnostic techniques are not useful for the early diagnosis of reptilian HVs because of the delay in antibody response and the need for paired serum sample collection weeks apart with accurate timing. However, it can play an important role in retrospective studies and in the diagnosis of latent or asymptomatic patients.

Recent epidemiological studies have largely relied on molecular methods to identify potential genetic and environmental risk factors associated with reptilian HVs (24, 80, 138, 167–169). Species-specific PCR-based assays targeting specific gene segments of reptilian HVs have been developed and validated (78, 170). Lindeman et al. developed two quantitative PCR assays and recorded a detection limit as low as 1 viral copy per reaction using primers that targeted the EBHV-1 specific segment of DNA polymerase gene (U_L30) (39). In another study, two TaqMan PCR assays developed to target the U_L30 gene of TerHV-1 detected 10 viral copies per reaction (171). Conventional and heminested PCR assays using tortoise HV-specific primers have been developed with assay sensitivity of 10^3 and 10^1 DNA copies, respectively (172). Alternatively, consensus PCR techniques developed by VanDevanter et al. have been employed for the molecular screening and novel detection of reptilian HV species (39, 43, 100, 173–177). Although the molecular assays for the diagnosis of reptilian HVs have demonstrated excellent performance, their use still presents a major challenge in remote areas due to high cost, complexity of instrumentation, aseptic technique requirement and the need for electricity to operate PCR machines.

In order to accurately estimate the magnitude and scope of a disease outbreak or occurrence, case definition (that is, standard criteria for categorising diseases) would need to be established. One of the ways to achieve this is to make available rapid, sensitive and affordable assays for confirming the presence of diseases. Rapid diagnostic immunoassays that use lateral flow or chromatographic strategies should be developed for the rapid diagnosis of reptilian HV infections in the field or point of care (POC) settings. This approach could overcome some of the above-mentioned diagnostic challenges, especially in low resource areas. However, the use of lateral flow immunoassays for viral detection in other species have been marred by low and varying sensitivities (178–181). Sensitive molecular-based

rapid assays are relatively expensive and yet to be employed for the diagnosis of reptilian HVs (182–185). We would propose an ultrasensitive format that combines PCR and immunoassay but then it can be argued that such a laboratory-based system is less rapid and has limited use in low-class laboratories (186, 187). Rapid detection techniques such as Microfluidic chip immunoassay and Smartphone-based rapid telemonitoring system (SBRTS) are fast becoming powerful tools in the diagnosis of viral infections (188–196). Of particular interest, is the SBRTS that combines biosensor and smartphone functionalities to produce a rapid, sensitive and cheap detection system (197). SBRTS has an average turnaround of 30 min, overcomes inherent problems associated with sample handling and preparation, and can remotely monitor and report data on disease occurrence, thus making it suitable for use in resource-limited countries (193, 197). This assay if employed could tick all the boxes for the epidemiological investigation and reporting of reptilian HVs.

Herpesvirus diagnostic and epidemiological data should be interpreted with prudence because of the possible influence of coinfection variables that could cause the reactivation of seemingly latent HV infections. For instance, some studies have reported the detection of co-pathogens in reptiles showing clinical signs, some of which are typical of HV infections (27, 38, 146, 168, 173, 198, 199). These observations imply that the detection of HVs may not be the actual cause of the current disease, but because the immune system is compromised by other pathogens, the HVs recrudesce and become easier to detect. Both latency (decreases apparent prevalence and significance) and coinfections (increase apparent prevalence and may also falsely assign the clinical signs to the HV) will have an influence on the disease picture. Therefore, we recommend that biosecurity and conservation measures should include a multiplex pathogen detection model whenever possible in order to fully assess the health of reptilian populations.

TREATMENT, PREVENTION, AND CONTROL

Surgical excision, carbon dioxide (CO_2) laser surgery and cryosurgery are some of the commonly used therapeutic strategies for the management of HV-associated tumours (25, 110, 111, 120, 200–202). High rates of recurrence and the risk of secondary bacterial infections have greatly reduced the efficacy of surgical excision (200, 203). CO_2 laser surgery, which combines laser excision and ablation of tumours, has shown improved intraoperative and postoperative outcomes and is therefore the treatment of choice (120, 201, 204). Non-surgical approaches including electrochemotherapy (ECT) and photodynamic therapy (PDT) with no known recurrence have recently been employed as alternatives in the treatment of FP (205, 206).

Several authors have recommended the use of acyclovir complemented by fluid and antibiotic therapies for the effective treatment of tortoise HV infection (143, 207–209). Marschang et al. showed that acyclovir and ganciclovir effectively inhibited HV replication *in vitro* at a single dose or repeated daily dose of 25 or 50 μg/mL (142). Similarly, the *in vitro* activities of acyclovir and ganciclovir were recently tested and shown to be effective against TeHV-3; however, the safety of these drugs is yet to be demonstrated in tortoises (210). Based on the toxicity (on liver and kidney cells) and other biochemical data, this same study showed that eprociclovir is not suitable for use as anti-TeHV-3 in Hermann's tortoises and further *in vivo* assessment of other potential antiviral drugs was recommended (210).

Recently, an autogenous vaccine therapy was proposed and used for the treatment of HV-associated papillomatosis in Williams' mud turtle (*Pelusios williamsi*) (111). The autogenous vaccine, which was aseptically prepared from excised fresh tissue induced substantial areas of necrosis of the papillomatous lesions, thus indicating the efficacy of the vaccine (111). Autogenous vaccines potentially contain relevant neoantigens that comparatively improve their efficacy (211). However, their use could be limited by lack of sufficient tumours (in patients) needed to produce adequate vaccine doses. Also, no standard protocol exists for autogenous vaccine production and delivery, and patients' tumours may progress beyond the intervention stage before the vaccine becomes ready for delivery. Allogeneic vaccines on the other hand, can overcome some of the aforementioned challenges; however, they may lack the advantageous self-neoantigens (211). In the past, an inactivated vaccine was evaluated against tortoise HV without success as no significant rise in antibody was detected in vaccinated tortoises after 369 days post vaccination (160). DNA or mRNA based vaccines have the capacity to induce both humoral and cellular immune responses and have shown promising outcomes against some animal and human diseases (212–215). Although vaccine research and development could be costly, laborious and time-consuming, the nucleic acid vaccines hold the potential to significantly reduce HV-associated losses in captive collections and wild reptiles of conservation concerns.

Prevention is of utmost importance in the management of reptilian HV infections, since death may still occur following therapeutic interventions and recovered animals remain latent carriers (143, 163). Unfortunately, there are no established preventive or control measures for HV infections in wild populations of reptiles (200), which consequently presents a major conservation challenge. Environmental factors including degraded water quality caused by pollutants, increased water temperature, natural biotoxins, and high dietary arginine concentrations due to microalgae bloom have arguably been linked as cofactors in the development of FP in sea turtles (73, 119, 216–221). Therefore, adopting conservation actions needed to regulate water and species management, as well as regulating human activities leading to climate change, would be sensible.

In captive reptiles, quarantine procedures and adequate testing of new acquisitions are strongly recommended (153, 163, 167). All previously infected or HV seropositive animals should be treated as latent carriers and potential shedders to naïve populations, as factors including stress, bad husbandry, illness or immunosuppression could reactivate the virus (14, 163, 167, 200). Generally, strict hygiene practises and adequate biosecurity should be followed in all facilities housing reptiles (162, 172, 222–224).

AUTHOR CONTRIBUTIONS

GO and EA conceived and designed this review. GO wrote the manuscript, analysed the data, and prepared figures and tables. EA, PH, and DW contributed to the concept and reviewed drafts of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This review was supported by the College of Public Health, Medical and Veterinary Sciences Higher Degree Research Enhancement Scheme, James Cook University, Australia.

ACKNOWLEDGMENTS

The authors would like to especially thank Professor Elizabeth Tynan for providing editorial support to this review. Authors' special thanks also goes to Dr. Fredric Frye for providing us with the full text of some articles used in this review. Finally, we would like to thank James Cook University librarians for their technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.642894/full#supplementary-material

Supplementary File 1 | Literature review search terms and strategies.

Supplementary File 2 | Data extraction for literature review.

Supplementary File 3 | Co-occurrence map of all keywords from 1975 to 2021. We found a total of 93 keywords with a minimum of 5 occurrences. Node size represents the number of occurrences of each keyword. The lines denote the total link strength between keywords, and it is proportional with the width. The line colours denote five different keyword clusters. The clusters gave an indication of the main areas research interest of reptilian HVs.

Supplementary File 4 | A timeline of reptilian HV diagnosis. A review of articles conducted from 1972 to 2020 showed that VI, EM, histopathology, and serologic assays have been used for the detection of reptilian HVs since 1972. Nucleic acid detection assay (PCR) was first reported in 1998 for detection of ChHV-5 in fibropapillomas. Events were chosen based on information from the extracted articles (**Supplementary File 2**).

REFERENCES

1. Rasmussen AR, Murphy JC, Ompi M, Gibbons JW, Uetz P. Marine reptiles. *PLoS ONE.* (2011) 6:e27373. doi: 10.1371/journal.pone.0027373
2. Cogger H. *Reptiles and Amphibians of Australia*. Collingwood: Csiro Publishing (2014).
3. The Reptile Database [Internet]. editor [cited Febrruary 09, 2021]. Available from: http://www.reptile-database.org.
4. Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, et al. The Global Decline of Reptiles, Déjà Vu Amphibians: reptile species are declining on a global scale. Six significant threats to reptile populations are habitat loss and degradation, introduced invasive species, environmental pollution, disease, unsustainable use, and global climate change. *BioScience.* (2000) 50:653–66. doi: 10.1641/0006-3568(2000) 050[0653:TGDORD]2.0.CO;2
5. Zug GR, Dowling HG. *Reptile*. Encyclopædia Britannica, Inc. (2020). Available online at: https://www.britannica.com/animal/reptile
6. González JA, Amich F, Postigo-Mota S, Vallejo JR. The use of wild vertebrates in contemporary Spanish ethnoveterinary medicine. *J Ethnopharmacol.* (2016) 191:135–51. doi: 10.1016/j.jep.2016.06.025
7. Robinson JE, Griffiths RA, John FAS, Roberts DL. Dynamics of the global trade in live reptiles: shifting trends in production and consequences for sustainability. *Biol Conserv.* (2015) 184:42–50. doi: 10.1016/j.biocon.2014.12.019
8. Todd BD, Willson JD, Gibbons JW. The global status of reptiles and causes of their decline. *Ecotoxicol Amphibians Reptiles.* (2010) 47:67. doi: 10.1201/EBK1420064162-c3
9. Van Der Ploeg JAN, Van Weerd M, Persoon GA. A cultural history of crocodiles in the Philippines: towards a new peace pact? *Environ History.* (2011) 17:229–64. doi: 10.3197/096734011X12997574043008
10. Reynolds J, Peres C. Overexploitation. In: Groom MJ, Meffe GK, Carroll CR, editors. *Principles of Conservation Biology*. Oxford, MA: Sinauer (2006). p. 253–77.
11. Ryser-Degiorgis M-P. Wildlife health investigations: needs, challenges and recommendations. *BMC Vet Res.* (2013) 9:223. doi: 10.1186/1746-6148-9-223
12. Munson L, Cook RA. Monitoring, investigation, and surveillance of diseases in captive wildlife. *J Zoo Wildl Med.* (1993) 24:281–90.
13. Haines H, Kleese WC. Effect of water temperature on a herpesvirus infection of sea turtles. *Infect Immunity.* (1977) 15:756–9. doi: 10.1128/IAI.15.3.756-759.1977
14. Marenzoni ML, Santoni L, Felici A, Maresca C, Stefanetti V, Sforna M, et al. Clinical, virological and epidemiological characterization of an outbreak of Testudinid Herpesvirus 3 in a chelonian captive breeding facility: lessons learned and first evidence of TeHV3 vertical transmission. *PLoS ONE.* (2018) 13:e0197169. doi: 10.1371/journal.pone.0197169
15. Hausmann JC, Wack AN, Allender MC, Cranfield MR, Murphy KJ, Barrett K, et al. Experimental challenge study of Fv3-like ranavirus infection in previously Fv3-like ranavirus infected eastern box turtles (Terrapene Carolina Carolina) to assess infection and survival. *J Zoo Wildl Med.* (2015) 46:732–46. doi: 10.1638/2015-0022.1
16. Ariel E. Viruses in reptiles. *Vet Res.* (2011) 42:100. doi: 10.1186/1297-9716-42-100
17. Marschang RE. Viruses infecting reptiles. *Viruses.* (2011) 3:2087–126. doi: 10.3390/v3112087
18. Dahlin CR, Hughes DF, Meshaka WE, Jr., Coleman C, Henning JD. Wild snakes harbor West Nile virus. *One Health.* (2016) 2:136–8. doi: 10.1016/j.onehlt.2016.09.003
19. Machain-Williams C, Padilla-Paz SE, Weber M, Cetina-Trejo R, Juarez-Ordaz JA, Loroño-Pino MA, et al. Antibodies to West Nile virus in wild and farmed crocodiles in southeastern Mexico. *J Wildl Dis.* (2013) 49:690–3. doi: 10.7589/2012-11-290
20. Habarugira G, Suen WW, Hobson-Peters J, Hall RA, Bielefeldt-Ohmann H. West Nile Virus: an update on pathobiology, epidemiology, diagnostics, control and "One Health" Implications. *Pathogens.* (2020) 9:589. doi: 10.3390/pathogens9070589
21. Mettenleiter TC, Keil GM, Fuchs W. *Molecular Biology of Animal Herpesviruses. Animal Viruses: Molecular Biology*. Norfolk: Caister Academic Press (2008) p. P531.
22. Payne S. Chapter 34 - family herpesviridae. In: Payne S, editor. *Viruses*. Cambridge: Academic Press (2017) p. 269–78.
23. Jacobson ER. *Infectious Diseases and Pathology of Reptiles: Color Atlas and Text*. CRC Press (2007).
24. Aplasca AC, Titus V, Ossiboff RJ, Murphy L, Seimon TA, Ingerman K, et al. Health assessment of free-ranging chelonians in an urban

section of the Bronx river, New York, USA. *J Wildl Dis.* (2019) 55:352–62. doi: 10.7589/2017-12-304
25. Goe AM, Heard DJ, Abbott JR, de Mello Souza CH, Taylor KR, Sthay JN, et al. Surgical management of an odontogenic tumor in a banded Gila monster (*Heloderma suspectum* cinctum) with a novel herpesvirus. *Vet Q.* (2016) 36:109–14. doi: 10.1080/01652176.2016.1153169
26. Sharma V, Mobeen F, Prakash T. Comparative genomics of herpesviridae family to look for potential signatures of human infecting strains. *Int J Genomics.* (2016) 2016:9543274. doi: 10.1155/2016/9543274
27. Adamovicz L, Allender MC, Archer G, Rzadkowska M, Boers K, Phillips C, et al. Investigation of multiple mortality events in eastern box turtles (*Terrapene carolina* carolina). *PLoS ONE.* (2018) 13:e0195617. doi: 10.1371/journal.pone.0195617
28. Jungwirth N, Bodewes R, Osterhaus AD, Baumgartner W, Wohlsein P. First report of a new alphaherpesvirus in a freshwater turtle (*Pseudemys concinna* concinna) kept in Germany. *Vet Microbiol.* (2014) 170:403–7. doi: 10.1016/j.vetmic.2014.02.029
29. van Eck NJ, Waltman L. Software survey: VOSviewer, a computer program for bibliometric mapping. *Scientometrics.* (2010) 84:523–8. doi: 10.1007/s11192-009-0146-3
30. Fischetti M. Where the reptiles are. *Sci Am.* (2018) 318:80. doi: 10.1038/scientificamerican0418-80
31. Butler RA. *Total Number of Reptile Species by Country.* (2019). Available online at: https://rainforests.mongabay.com/03reptiles.htm.
32. International Committee on Taxonomy of Viruses. *Virus Taxonomy.* (2019). Available online at: https://talk.ictvonline.org/taxonomy/
33. Wilkinson M, Cline M, Jerome WG. Cytopathic herpesvirus infection in a green iguana (Iguana iguana). *J Zoo Wildl Med.* (2005) 36:724–6. doi: 10.1638/03-067.1
34. Wellehan JF, Jarchow JL, Reggiardo C, Jacobson ER. A novel herpesvirus associated with hepatic necrosis in a San Esteban Chuckwalla, *Sauromalus varius*. *J Herpetol Med Surg.* (2003) 13:15–9. doi: 10.5818/1529-9651.13.3.15
35. Gatherer D, Benkō M, Brandt C, Bryant N, Dastjerdi A, Depledge D, et al. *18 New Species in the Family Herpesviridae*, Order Herpesvirales (2018). Available online at: https://talk.ictvonline.org/ictv/proposals/2018.009D.A.v1.Herpesviridae_18sp.zip
36. McGeoch DJ, Gatherer D. Integrating reptilian herpesviruses into the family herpesviridae. *J Virol.* (2005) 79:725–31. doi: 10.1128/JVI.79.2.725-731.2005
37. Ossiboff RJ, Raphael BL, Ammazzalorso AD, Seimon TA, Newton AL, Chang TY, et al. Three novel herpesviruses of endangered Clemmys and Glyptemys turtles. *PLoS ONE.* (2015) 10:e0122901. doi: 10.1371/journal.pone.0122901
38. Yonkers SB, Schneider R, Reavill DR, Archer LL, Childress AL, Wellehan JF, Jr. Coinfection with a novel fibropapilloma-associated herpesvirus and a novel *Spirorchis* sp. in an eastern box turtle (*Terrapene carolina*) in Florida. *J Vet Diagn Investig.* (2015) 27:408–13. doi: 10.1177/1040638715589612
39. Lindemann DM, Allender MC, Thompson D, Adamovicz L, Dzhaman E. Development and validation of a quantitative PCR assay for detection of Emydoidea herpesvirus 1 in free-ranging Blanding's turtles (*Emydoidea blandingii*). *J Virol Methods.* (2018) 254:40–5. doi: 10.1016/j.jviromet.2018.01.006
40. Sim RR, Norton TM, Bronson E, Allender MC, Stedman N, Childress AL, et al. Identification of a novel herpesvirus in captive Eastern box turtles (Terrapene carolina carolina). *Veterinary Microbiology.* (2015) 175:218–23. doi: 10.1016/j.vetmic.2014.11.029
41. Marschang RE, Heckers KO, Heynol V, Weider K, Behncke H. [Herpesvirus detection in clinically healthy West African mud turtles (*Pelusios castaneus*)]. *Tierarztliche Praxis Ausgabe K, Kleintiere/Heimtiere.* (2015) 43:166–9. doi: 10.15654/TPK-140575
42. Stacy BA, Wellehan JF, Foley AM, Coberley SS, Herbst LH, Manire CA, et al. Two herpesviruses associated with disease in wild Atlantic loggerhead sea turtles (*Caretta caretta*). *Vet Microbiol.* (2008) 126:63–73. doi: 10.1016/j.vetmic.2007.07.002
43. Bicknese EJ, Childress AL, Wellehan JF, Jr. A novel herpesvirus of the proposed genus Chelonivirus from an asymptomatic bowsprit tortoise (*Chersina angulata*). *J Zoo Wildl Med.* (2010) 41:353–8. doi: 10.1638/2009-0214R.1
44. Johnson AJ, Pessier AP, Wellehan JF, Brown R, Jacobson ER. Identification of a novel herpesvirus from a California desert tortoise (*Gopherus agassizii*). *Vet Microbiol.* (2005) 111:107–16. doi: 10.1016/j.vetmic.2005.09.008
45. Marschang RE, Frost JW, Gravendyck M, Kaleta EF. Comparison of 16 chelonid herpesviruses by virus neutralization tests and restriction endonuclease digestion of viral DNA. *J Vet Med Ser B.* (2001) 48:393–9. doi: 10.1046/j.1439-0450.2001.00450.x
46. Marschang RE, Gleiser CB, Papp T, Pfitzner AJ, Bohm R, Roth BN. Comparison of 11 herpesvirus isolates from tortoises using partial sequences from three conserved genes. *Vet Microbiol.* (2006) 117:258–66. doi: 10.1016/j.vetmic.2006.06.009
47. Wellehan JF, Nichols DK, Li LL, Kapur V. Three novel herpesviruses associated with stomatitis in Sudan plated lizards (Gerrhosaurus major) and a black-lined plated lizard (*Gerrhosaurus nigrolineatus*). *J Zoo Wildl Med.* (2004) 35:50–4. doi: 10.1638/03-011
48. Wellehan JF, Johnson AJ, Latimer KS, Whiteside DP, Crawshaw GJ, Detrisac CJ, et al. Varanid herpesvirus 1: a novel herpesvirus associated with proliferative stomatitis in green tree monitors (*Varanus prasinus*). *Vet Microbiol.* (2005) 105:83–92. doi: 10.1016/j.vetmic.2004.10.012
49. Hughes-Hanks JM, Schommer SK, Mitchell WJ, Shaw DP. Hepatitis and enteritis caused by a novel herpesvirus in two monitor lizards (*Varanus* spp.). *J Vet Diagn Investig.* (2010) 22:295–9. doi: 10.1177/104063871002200224
50. Clark HF, Karzon DT. Iguana virus, a herpes-like virus isolated from cultured cells of a lizard, Iguana iguana. *Infect Immunity.* (1972) 5:559–69. doi: 10.1128/IAI.5.4.559-569.1972
51. Zeigel RF, Clark HF. Electron microscopy observations on a new herpes-type virus isolated from *Iguana iguana* and propagated in reptilian cells *in vitro*. *Infect Immunity.* (1972) 5:570–82. doi: 10.1128/IAI.5.4.570-582.1972
52. Lovstad JN, Ossiboff RJ, Kinsel MJ, Gamble KC. Novel herpesvirus associated with oropharyngeal squamous cell carcinoma in smooth green snakes (*Opheodrys vernalis*). *Vet Pathol.* (2019) 56:630–5. doi: 10.1177/0300985819837722
53. Hyndman TH, Shilton CM, Wellehan JF, Jr., Davis S, Isberg SR, et al. Molecular identification of three novel herpesviruses found in Australian farmed saltwater crocodiles (*Crocodylus porosus*) and Australian captive freshwater crocodiles (*Crocodylus johnstoni*). *Vet Microbiol.* (2015) 181:183–9. doi: 10.1016/j.vetmic.2015.09.013
54. Rebell G, Rywlin A, Haines H. A herpesvirus-type agent associated with skin lesions of green sea turtles in aquaculture. *Am J Vet Res.* (1975) 36:1221–4.
55. Frye FL, Oshiro LS, Dutra FR, Carney JD. Herpesvirus-like infection in two Pacific pond turtles. *J Am Vet Med Assoc.* (1977) 171:882–4.
56. Cox WR, Rapley WA, Barker IK. Herpesvirus-like infection in a painted turtle (*Chrysemys picta*). *J Wildl Dis.* (1980) 16:445–9. doi: 10.7589/0090-3558-16.3.445
57. Jacobson ER, Clubb S, Gaskin JM, Gardiner C. Herpesvirus-like infection in Argentine tortoises. *J Am Vet Med Assoc.* (1985) 187:1227–9.
58. Davison AJ. Herpesviruses: general features. In: Mahy BWJ, Van Regenmortel MHV, editors. *Encyclopedia of Virology*. Oxford: Academic Press (2008). p. 430–6.
59. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol.* (2018) 35:1547–9. doi: 10.1093/molbev/msy096
60. Bowman BR, Baker ML, Rixon FJ, Chiu W, Quiocho FA. Structure of the herpesvirus major capsid protein. *EMBO J.* (2003) 22:757–65. doi: 10.1093/emboj/cdg086
61. Caspar DL, Klug A. Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp Quant Biol.* (1962) 27:1–24. doi: 10.1101/SQB.1962.027.001.005
62. Richard J. *Herpesviruses: Chapter 68*. Whitley: University of Texas Medical Branch at Galveston (1996).
63. Roizmann B, Desrosiers R, Fleckenstein B, Lopez C, Minson A, Studdert M. The family Herpesviridae: an update. *Arch Virol.* (1992) 123:425–49. doi: 10.1007/BF01317276
64. Yuan S, Wang J, Zhu D, Wang N, Gao Q, Chen W, et al. Cryo-EM structure of a herpesvirus capsid at 3.1 Å. *Science.* (2018) 360:eaao7283. doi: 10.1126/science.aao7283

65. Brown JC, Newcomb WW. Herpesvirus capsid assembly: insights from structural analysis. *Curr Opin Virol.* (2011) 1:142–9. doi: 10.1016/j.coviro.2011.06.003
66. Dai X, Zhou ZH. Structure of the herpes simplex virus 1 capsid with associated tegument protein complexes. *Science.* (2018) 360:eaao7298. doi: 10.1126/science.aao7298
67. Mocarski ES Jr. Comparative analysis of herpesvirus-common proteins. *In*: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, et al., editors. *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*. Cambridge: Cambridge University Press (2007).
68. Hay J, Ruyechan WT. Alphaherpesvirus DNA replication. *Human herpesviruses: Biology, Therapy, and Immunoprophylaxis.* Cambridge: Cambridge University Press (2007).
69. Gandar F, Wilkie GS, Gatherer D, Kerr K, Marlier D, Diez M, et al. The genome of a tortoise herpesvirus (Testudinid Herpesvirus 3) has a novel structure and contains a large region that is not required for replication *in vitro* or virulence *in vivo*. *J Virol.* (2015) 89:11438–56. doi: 10.1128/JVI.01794-15
70. Origgi FC, Tecilla M, Pilo P, Aloisio F, Otten P, Aguilar-Bultet L, et al. A genomic approach to unravel host-pathogen interaction in chelonians: the example of testudinid herpesvirus 3. *PLoS ONE.* (2015) 10:e0134897. doi: 10.1371/journal.pone.0134897
71. Ackermann M, Koriabine M, Hartmann-Fritsch F, de Jong PJ, Lewis TD, Schetle N, et al. The genome of Chelonid herpesvirus 5 harbors atypical genes. *PLoS ONE.* (2012) 7:e46623. doi: 10.1371/journal.pone.0046623
72. Morrison CL, Iwanowicz L, Work TM, Fahsbender E, Breitbart M, Adams C, et al. Genomic evolution, recombination, and inter-strain diversity of chelonid alphaherpesvirus 5 from Florida and Hawaii green sea turtles with fibropapillomatosis. *PeerJ.* (2018) 2018:e4386. doi: 10.7717/peerj.4386
73. Jones K, Ariel E, Burgess G, Read M. A review of fibropapillomatosis in Green turtles (*Chelonia mydas*). *Vet J.* (2016) 212:48–57. doi: 10.1016/j.tvjl.2015.10.041
74. O'Rourke DP, Lertpiriyapong K. Chapter 19 - biology and diseases of reptiles. In: Fox JG, Anderson LC, Otto GM, Pritchett-Corning KR, Whary MT, editors. *Laboratory Animal Medicine,* 3rd ed. Boston, MA: Academic Press (2015) p. 967–1013.
75. Muller M, Sachsse W, Zangger N. [Herpesvirus epidemic in Greek (*Testudo hermanni*) and Moorish land tortoises (*Testudo graeca*) in Switzerland]. *Schweizer Arch Tierheilkunde.* (1990) 132:199–203.
76. Jones K, Burgess G, Budd AM, Huerlimann R, Mashkour N, Ariel E. Molecular evidence for horizontal transmission of chelonid alphaherpesvirus 5 at green turtle (*Chelonia mydas*) foraging grounds in Queensland, Australia. *PLoS ONE.* (2020) 15:e0227268. doi: 10.1371/journal.pone.0227268
77. Ariel E, Nainu F, Jones K, Juntunen K, Bell I, Gaston J, et al. Phylogenetic variation of chelonid alphaherpesvirus 5 (ChHV5) in Populations of Green Turtles *Chelonia mydas* along the Queensland Coast, Australia. *J Aquat Anim Health.* (2017) 29:150–7. doi: 10.1080/08997659.2017.1330783
78. Origgi FC, Romero CH, Bloom DC, Klein PA, Gaskin JM, Tucker SJ, et al. Experimental transmission of a herpesvirus in Greek tortoises (*Testudo graeca*). *Vet Pathol.* (2004) 41:50–61. doi: 10.1354/vp.41-1-50
79. Chaves A, Aguirre AA, Blanco-Peña K, Moreira-Soto A, Monge O, Torres AM, et al. Examining the role of transmission of chelonid alphaherpesvirus 5. *EcoHealth.* (2017) 14:530–41. doi: 10.1007/s10393-017-1248-7
80. Monezi TA, Mehnert DU, de Moura EM, Muller NM, Garrafa P, Matushima ER, et al. Chelonid herpesvirus 5 in secretions and tumor tissues from green turtles (Chelonia mydas) from Southeastern Brazil: a ten-year study. *Vet Microbiol.* (2016) 186:150–6. doi: 10.1016/j.vetmic.2016.02.020
81. Herbst LH, Greiner EC, Ehrhart LM, Bagley DA, Klein PA. Serological association between spirorchidiasis, herpesvirus infection, and fibropapillomatosis in green turtles from Florida. *J Wildl Dis.* (1998) 34:496–507. doi: 10.7589/0090-3558-34.3.496
82. Curry SS, Brown DR, Gaskin JM, Jacobson ER, Ehrhart LM, Blahak S, et al. Persistent infectivity of a disease-associated herpesvirus in green turtles after exposure to seawater. *J Wildl Dis.* (2000) 36:792–7. doi: 10.7589/0090-3558-36.4.792
83. Greenblatt RJ, Work TM, Balazs GH, Sutton CA, Casey RN, Casey JW. The Ozobranchus leech is a candidate mechanical vector for the fibropapilloma-associated turtle herpesvirus found latently infecting skin tumors on Hawaiian green turtles (*Chelonia mydas*). *Virology.* (2004) 321:101–10. doi: 10.1016/j.virol.2003.12.026
84. Lu Y, Yu Q, Zamzow JP, Wang Y, Losey GS, Balazs GH, et al. Detection of green turtle herpesviral sequence in Saddleback Wrasse Thalassoma Duperrey: a possible mode of transmission of green turtle fibropapilloma. *J Aquat Anim Health.* (2000) 12:58–63. doi: 10.1577/1548-8667(2000)012<0058:DOGTHS>2.0.CO;2
85. Work TM, Dagenais J, Balazs GH, Schettle N, Ackermann M. Dynamics of virus shedding and *in situ* confirmation of chelonid herpesvirus 5 in Hawaiian green turtles with fibropapillomatosis. *Vet Pathol.* (2015) 52:1195–201. doi: 10.1177/0300985814560236
86. Whitley R. *Chapter 68: Herpesviruses*. *Medical Microbiology*. Galveston, TX: University of Texas Medical Branch at Galveston (1996).
87. Davison AJ. *Herpesviruses: General Features*?. *Reference Module in Biomedical Sciences*. Elsevier (2014).
88. Page-Karjian A, Gottdenker NL, Whitfield J, Herbst L, Norton TM, Ritchie B. Potential noncutaneous sites of chelonid herpesvirus 5 persistence and shedding in green sea turtles *Chelonia mydas*. *J Aquat Animal Health.* (2017) 29:136–42. doi: 10.1080/08997659.2017.1321590
89. Work TM, Dagenais J, Weatherby TM, Balazs GH, Ackermann M. *In vitro* replication of chelonid herpesvirus 5 in organotypic skin cultures from Hawaiian green turtles (*Chelonia mydas*). *J Virol.* (2017) 91:01. doi: 10.1128/JVI.00404-17
90. Herbst LH, Jacobson ER, Klein PA, Balazs GH, Moretti R, Brown T, et al. Comparative pathology and pathogenesis of spontaneous and experimentally induced fibropapillomas of green turtles (*Chelonia mydas*). *Vet Pathol.* (1999) 36:551–64. doi: 10.1354/vp.36-6-551
91. Kang KI, Torres-Velez FJ, Zhang J, Moore PA, Moore DP, Rivera S, et al. Localization of fibropapilloma-associated turtle herpesvirus in green turtles (Chelonia mydas) by *in-situ* hybridization. *J Comp Pathol.* (2008) 139:218–25. doi: 10.1016/j.jcpa.2008.07.003
92. Engels M, Ackermann M. Pathogenesis of ruminant herpesvirus infections. *Vet Microbiol.* (1996) 53:3–15. doi: 10.1016/S0378-1135(96)01230-8
93. Huemer HP, Larcher C, van Drunen Littel-van den Hurk S, Babiuk LA. Species selective interaction of Alphaherpesvirinae with the "unspecific" immune system of the host. *Arch Virol.* (1993) 130:353–64. doi: 10.1007/BF01309666
94. Jacobs L. Glycoprotein E of pseudorabies virus and homologous proteins in other alphaherpesvirinae. *Arch Virol.* (1994) 137:209–28. doi: 10.1007/BF01309470
95. Haines H. A herpesvirus disease of green sea turtles in aquaculture. *Mar Fish Rev.* (1978) 40:330–37.
96. Haines HG, Rywlin A, Rebell G. A herpesvirus disease of farmed green turtles (*Chelonia mydas*). *J World Aquac Soc.* (1974) 5:183–95. doi: 10.1111/j.1749-7345.1974.tb00189.x
97. Herbst LH. Fibropapillomatosis of marine turtles. *Ann Rev Fish Dis.* (1994) 4:389–425. doi: 10.1016/0959-8030(94)90037-X
98. Jacobson ER, Gaskin JM, Roelke M, Greiner EC, Allen J. Conjunctivitis, tracheitis, and pneumonia associated with herpesvirus infection in green sea turtles. *J Am Vet Med Assoc.* (1986) 189:1020–3.
99. Une Y, Uemura K, Nakano Y, Kamiie J, Ishibashi T, Nomura Y. Herpesvirus infection in tortoises (*Malacochersus tornieri* and *Testudo horsfieldii*). *Vet Pathol.* (1999) 36:624–7. doi: 10.1354/vp.36-6-624
100. Une Y, Murakami M, Uemura K, Fujitani H, Ishibashi T, Nomura Y. Polymerase chain reaction (PCR) for the detection of herpesvirus in tortoises. *J Vet Med Sci.* (2000) 62:905–7. doi: 10.1292/jvms.62.905
101. Stöhr AC, Marschang RE. Detection of a tortoise herpesvirus type 1 in a Hermann's Tortoise (*Testudo hermanni* boettgeri) in Germany. *J Herpetol Med Surg.* (2010) 20:61–3. doi: 10.5818/1529-9651-20.2.61
102. Marschang RE, Papp T, Ferretti L, Hochscheid S, Bentivegna F. Detection and partial characterization of herpesviruses from Egyptian tortoises (*Testudo kleinmanni*) imported into Italy from Libya. *J Zoo Wildl Med.* (2009) 40:211–3. doi: 10.1638/2008-0045.1
103. Heldstab A, Bestetti G. Herpesviridae causing glossitis and meningoencephalitis in land tortoises (*Testudo hermanni*). *Herpetopathologia.* (1989) 1:5–9.

104. Kolesnik E, Mittenzwei F, Marschang RE. Detection of testudinid herpesvirus type 4 in a leopard tortoise (*Stigmochelys pardalis*). *Tierarztliche Praxis Ausgabe K Kleintiere/Heimtiere.* (2016) 44:283–6. doi: 10.15654/TPK-150843
105. Ossiboff RJ, Newton AL, Seimon TA, Moore RP, McAloose D. Emydid herpesvirus 1 infection in northern map turtles (*Graptemys geographica*) and painted turtles (*Chrysemys picta*). *J Vet Diagn Investig.* (2015) 27:392–5. doi: 10.1177/1040638715584793
106. Simpson CF, Jacobson ER, Gaskin JM. Herpesvirus-like infection of the venom gland of Siamese cobras. *J Am Vet Med Assoc.* (1979) 175:941–3.
107. Shilton CM, Jerrett IV, Davis S, Walsh S, Benedict S, Isberg SR, et al. Diagnostic investigation of new disease syndromes in farmed Australian saltwater crocodiles (Crocodylus porosus) reveals associations with herpesviral infection. *J Vet Diagn Investig.* (2016) 28:279–90. doi: 10.1177/1040638716642268
108. Aguirre AA, Spraker TR, Chaves A, Du Toit L, Eure W, Balazs GH. Pathology of fibropapillomatosis in olive ridley turtles lepidochelys olivacea nesting in costa rica. *J Aquat Anim Health.* (1999) 11:283–9. doi: 10.1577/1548-8667(1999) 011<0283:POFIOR>2.0.CO;2
109. Cardenas DM, Cucalon RV, Medina-Magues LG, Jones K, Aleman RA, Alfaro-Nunez A, et al. Fibropapillomatosis in a Green Sea Turtle (*Chelonia mydas*) from the Southeastern Pacific. *J Wildl Dis.* (2019) 55:169–73. doi: 10.7589/2017-12-295
110. Cowan ML, Raidal SR, Peters A. Herpesvirus in a captive Australian Krefft's river turtle (*Emydura macquarii* krefftii). *Aust Vet J.* (2015) 93:46–9. doi: 10.1111/avj.12290
111. Siroky P, Frye FL, Dvorakova N, Hostovsky M, Prokop H, Kulich P. Herpesvirus associated dermal papillomatosis in Williams' mud turtle *Pelusios williamsi* with effects of autogenous vaccine therapy. *J Vet Med Sci.* (2018) 80:1248–54. doi: 10.1292/jvms.18-0126
112. Hervas J, Sanchez-Cordon PJ, de Chacon Lara F, Carrasco L, Gomez-Villamandos JC. Hepatitis associated with herpes viral infection in the tortoise (*Testudo horsfieldii*). *J Vet Med Ser B.* (2002) 49:111–4. doi: 10.1046/j.1439-0450.2002.00522.x
113. Hunt CJ. Herpesvirus outbreak in a group of mediterranean tortoises (*Testudo* spp). *Vet Clin N Am Exot Anim Pract.* (2006) 9:569–74. doi: 10.1016/j.cvex.2006.05.018
114. Braune S, Geiss V, Thiel W. A new herpesvirus-caused disease in tortoises. *Tierarztliche Praxis.* (1989) 17:416–9.
115. McArthur S, Blahak S, Köelle P, Jacobson ER, Marschang RE, Origgi F, et al. Chelonian herpesvirus. *J Herpetol Med Surg.* (2002) 12:14–31. doi: 10.5818/1529-9651.12.2.14
116. Raynaud A, Adrian M. [Cutaneous lesions with papillomatous structure associated with viruses in the green lizard (*Lacerta viridis* Laur.)]. *Comptes Rendus Hebdomadaires Seances l'Academie Sciences - D: Sciences Naturelles.* (1976) 283:845–7.
117. Literak I, Robesova B, Majlathova V, Majlath I, Kulich P, Fabian P, et al. Herpesvirus-associated papillomatosis in a green lizard. *J Wildl Dis.* (2010) 46:257–61. doi: 10.7589/0090-3558-46.1.257
118. Govett PD, Harms CA, Johnson AJ, Latimer KS, Wellehan JF, Fatzinger MH, et al. Lymphoid follicular cloacal inflammation associated with a novel herpesvirus in juvenile alligators (*Alligator mississippiensis*). *J Vet Diagn Investig.* (2005) 17:474–9. doi: 10.1177/104063870501700513
119. dos Santos RG, Martins AS, Torezani E, Baptistotte C, da Nóbrega FJ, Horta PA, et al. Relationship between fibropapillomatosis and environmental quality: a case study with *Chelonia mydas* of Brazil. *Dis Aquat Organ.* (2010) 89:87–95. doi: 10.3354/dao02178
120. Page-Karjian A, Norton TM, Krimer P, Groner M, Nelson SE, Jr., et al. Factors influencing survivorship of rehabilitating green sea turtles (Chelonia mydas) with fibropapillomatosis. *J Zoo Wildl Med.* (2014) 45:507–19. doi: 10.1638/2013-0132R1.1
121. Tagliolatto AB, Guimarães SM, Lobo-Hajdu G, Monteiro-Neto C. Characterization of fibropapillomatosis in green turtles *Chelonia mydas* (Cheloniidae) captured in a foraging area in southeastern Brazil. *Dis Aquat Organ.* (2016) 121:233–40. doi: 10.3354/dao03020
122. Adnyana W, Ladds PW, Blair D. Observations of fibropapillomatosis in green turtles (*Chelonia mydas*) in Indonesia. *Aust Vet J.* (1997) 75:736–42. doi: 10.1111/j.1751-0813.1997.tb12258.x
123. Ene A, Su M, Lemaire S, Rose C, Schaff S, Moretti R, et al. Distribution of chelonid fibropapillomatosis-associated herpesvirus variants in Florida: molecular genetic evidence for infection of turtles following recruitment to neritic developmental habitats. *J Wildl Dis.* (2005) 41:489–97. doi: 10.7589/0090-3558-41.3.489
124. Greenblatt RJ, Quackenbush SL, Casey RN, Rovnak J, Balazs GH, Work TM, et al. Genomic variation of the fibropapilloma-associated marine turtle herpesvirus across seven geographic areas and three host species. *J Virol.* (2005) 79:1125–32. doi: 10.1128/JVI.79.2.1125-1132.2005
125. Zimmerman LM, Vogel LA, Bowden RM. Understanding the vertebrate immune system: insights from the reptilian perspective. *J Exp Biol.* (2010) 213:661. doi: 10.1242/jeb.038315
126. Rios FM, Zimmerman LM. *Immunology of Reptiles*. eLS, Chichester: John Wiley & Sons, Ltd. (2015). p. 1–7.
127. Herbst LH, Klein PA. Green Turtle fibropapillomatosis: challenges to assessing the role of environmental cofactors. *Environ Health Perspect.* (1995) 103(Suppl 4):27–30. doi: 10.1289/ehp.95103s427
128. Herbst L, Ene A, Su M, Desalle R, Lenz J. Tumor outbreaks in marine turtles are not due to recent herpesvirus mutations. *Curr Biol.* (2004) 14:R697–9. doi: 10.1016/j.cub.2004.08.040
129. Jones AG. Sea turtles: old viruses and new tricks. *Curr Biol.* (2004) 14:R842–3. doi: 10.1016/j.cub.2004.09.038
130. Winter AK, Martinez ME, Cutts FT, Moss WJ, Ferrari MJ, McKee A, et al. Benefits and challenges in using seroprevalence data to inform models for measles and rubella elimination. *J Infect Dis.* (2018) 218:355–64. doi: 10.1093/infdis/jiy137
131. Willimann A. *The prevalence of antibodies against envelope proteins of Chelonid herpesvirus 5 is inconsistent with the current understanding of the pathogenesis and epidemiology of fibropapillomatosis in marine turtles* (Doctoral dissertation), University of Zurich, Zurich (2018).
132. Herbst LH, Lemaire S, Ene AR, Heslin DJ, Ehrhart LM, Bagley DA, et al. Use of baculovirus-expressed glycoprotein H in an enzyme-linked immunosorbent assay developed to assess exposure to chelonid fibropapillomatosis-associated herpesvirus and its relationship to the prevalence of fibropapillomatosis in sea turtles. *Clin Vaccine Immunol.* (2008) 15:843–51. doi: 10.1128/CVI.00438-07
133. Work TM, Dagenais J, Willimann A, Balazs G, Mansfield K, Ackermann M. Differences in antibody responses against chelonid alphaherpesvirus 5 (CHHV5) suggest differences in virus biology in CHHV5-seropositive green turtles from Hawaii and CHHV5-seropositive green turtles from Florida. *J Virol.* (2020) 94:e00404-17. doi: 10.1128/JVI.01658-19
134. Coberley SS, Herbst LH, Brown DR, Ehrhart LM, Bagley DA, Schaf SA, et al. Detection of antibodies to a disease-associated herpesvirus of the green turtle, *Chelonia mydas*. *J Clin Microbiol.* (2001) 39:3572–7. doi: 10.1128/JCM.39.10.3572-3577.2001
135. Coberley SS, Herbst LH, Ehrhart LM, Bagley DA, Hirama S, Jacobson ER, et al. Survey of Florida green turtles for exposure to a disease-associated herpesvirus. *Dis Aquat Org.* (2001) 47:159–67. doi: 10.3354/dao047159
136. Jacobson ER, Gaskin JM, Wahlquist H. Herpesvirus-like infection in map turtles. *J Am Vet Med Assoc.* (1982) 181:1322–4.
137. Divers SJ, Stahl SJ. *Mader's Reptile and Amphibian Medicine and Surgery-E-Book*. Amsterdam: Elsevier Health Sciences (2018).
138. Kane LP, Allender MC, Archer G, Dzhaman E, Pauley J, Moore AR, et al. Prevalence of terrapene herpesvirus 1 in free-ranging eastern box turtles (*Terrapene carolina* Carolina) in Tennessee and Illinois, USA. *J Wildl Dis.* (2017) 53:285–95. doi: 10.7589/2016-06-138
139. Pettan-Brewer KC, Drew ML, Ramsay E, Mohr FC, Lowenstine LJ. Herpesvirus particles associated with oral and respiratory lesions in a California desert tortoise (*Gopherus agassizii*). *J Wildl Dis.* (1996) 32:521–6. doi: 10.7589/0090-3558-32.3.521
140. Lange H, Herbst W, Wiechert JM, Schliesser T. [Electron microscopic detection of herpesviruses in a mass death of Greek tortoises (Testudo hermanni) and four-toed turtles (*Agrionemys horsfieldii*)]. *Tierarztliche Praxis.* (1989) 17:319–21.
141. Muro J, Ramis A, Pastor J, Velarde R, Tarres J, Lavin S. Chronic rhinitis associated with herpesviral infection in captive spur-thighed tortoises from Spain. *J Wildl Dis.* (1998) 34:487–95. doi: 10.7589/0090-3558-34.3.487

142. Marschang RE, Gravendyck M, Kaleta EF. Herpesviruses in tortoises: investigations into virus isolation and the treatment of viral stomatitis in Testudo hermanni and *T. graeca*. *Zentralblatt Fuer Veterinaermed Reihe B.* (1997) 44:385–94. doi: 10.1111/j.1439-0450.1997.tb00989.x
143. Origgi FC, Rigoni D, editors. A tortoise herpesvirus outbreak in Italy: history, diagnosis, therapy and follow up. In: *Proceedings of the 10th Conference of the Association of Reptilian and Amphibian Veterinarians.* Minneapolis, MI. (2003).
144. Origgi FC. Testudinid Herpesviruses: a review. *J Herpetol Med Surg.* (2012) 22:42–54. doi: 10.5818/1529-9651-22.1-2.42
145. Marschang RE. Emerging reptile viruses. Fowler's Zoo and Wild Animal Medicine. *Curr Ther.* (2019) 9:267. doi: 10.1016/B978-0-323-55228-8.00039-4
146. Kolesnik E, Obiegala A, Marschang RE. Detection of Mycoplasma spp., herpesviruses, topiviruses, and ferlaviruses in samples from chelonians in Europe. *J Vet Diagn Investig.* (2017) 29:820–32. doi: 10.1177/1040638717722387
147. Jacobson ER, Berry KH, Wellehan JF, Jr., Origgi F, Childress AL, et al. Serologic and molecular evidence for Testudinid herpesvirus 2 infection in wild Agassiz's desert tortoises, *Gopherus agassizii*. *J Wildl Dis.* (2012) 48:747–57. doi: 10.7589/0090-3558-48.3.747
148. Johnson AJ, Morafka DJ, Jacobson ER. Seroprevalence of *Mycoplasma agassizii* and tortoise herpesvirus in captive desert tortoises (*Gopherus agassizii*) from the Greater Barstow Area, Mojave Desert, California. *J Arid Environ.* (2006) 67:192–201. doi: 10.1016/j.jaridenv.2006.09.025
149. McCowan C, Shepherdley C, Slocombe RF. Herpesvirus-like particles in the skin of a saltwater crocodile (*Crocodylus porosus*). *Aust Vet J.* (2004) 82:375–7. doi: 10.1111/j.1751-0813.2004.tb11109.x
150. Catoi C, Gal AF, Taulescu MA, Palmieri C, Catoi AF. Lethal herpesvirosis in 16 captive horned vipers (*Vipera ammodytes* ammodytes): pathological and ultrastructural findings. *J Comp Pathol.* (2014) 150:341–4. doi: 10.1016/j.jcpa.2013.10.002
151. Hauser B, Mettler F, Rubel A. Herpesvirus-like infection in two young Boas. *J Comp Pathol.* (1983) 93:515–9. doi: 10.1016/0021-9975(83)90057-9
152. Watson GL. Herpesvirus in red-headed (common) agamas (*Agama agama*). *J Vet Diagn Investig.* (1993) 5:444–5. doi: 10.1177/104063879300500326
153. Lott MJ, Moore RL, Milic NL, Power M, Shilton CM, Isberg SR. Dermatological conditions of farmed Crocodilians: a review of pathogenic agents and their proposed impact on skin quality. *Vet Microbiol.* (2018) 225:89–100. doi: 10.1016/j.vetmic.2018.09.022
154. Divers SJ, Mader DR. *Reptile Medicine and Surgery-E-Book*. Amsterdam: Elsevier Health Sciences (2005).
155. Farkas SL, Gál J. Adenovirus and mycoplasma infection in an ornate box turtle (*Terrapene ornata* ornata) in Hungary. *Vet Microbiol.* (2009) 138:169–73. doi: 10.1016/j.vetmic.2009.03.016
156. Manire CA, Stacy BA, Kinsel MJ, Daniel HT, Anderson ET, Wellehan JF, Jr. Proliferative dermatitis in a loggerhead turtle, *Caretta caretta*, and a green turtle, *Chelonia mydas*, associated with novel papillomaviruses. *Vet Microbiol.* (2008) 130:227–37. doi: 10.1016/j.vetmic.2008.01.013
157. Hyndman T, Shilton CM. Molecular detection of two adenoviruses associated with disease in Australian lizards. *Aust Vet J.* (2011) 89:232–5. doi: 10.1111/j.1751-0813.2011.00712.x
158. Harper PA, Hammond DC, Heuschele WP. A herpesvirus-like agent associated with a pharyngeal abscess in a desert tortoise. *J Wildl Dis.* (1982) 18:491–4. doi: 10.7589/0090-3558-18.4.491
159. Drury SE, Gough RE, Kay AV, McArthur SD. Detection and isolation of a herpesvirus from a spur-thighed tortoise (*Testudo graeca*) in the UK. *Vet Rec.* (1999) 145:586–8. doi: 10.1136/vr.145.20.586
160. Marschang RE, Milde K, Bellavista M. Virus isolation and vaccination of Mediterranean tortoises against a chelonid herpesvirus in a chronically infected population in Italy. *Deutsche Tierarztliche Wochenschrift.* (2001) 108:376–9.
161. Zimmerman LM, Bowden RM, Vogel LA. Red-eared slider turtles lack response to immunization with keyhole limpet hemocyanin but have high levels of natural antibodies. *ISRN Zool.* (2013) 2013:858941. doi: 10.1155/2013/858941
162. Origgi FC, Klein PA, Mathes K, Blahak S, Marschang RE, Tucker SJ, et al. Enzyme-linked immunosorbent assay for detecting herpesvirus exposure in Mediterranean tortoises (spur-thighed tortoise [*Testudo graeca*] and Hermann's tortoise [*Testudo hermanni*]). *J Clin Microbiol.* (2001) 39:3156–63. doi: 10.1128/JCM.39.9.3156-3163.2001
163. Pasmans F, Blahak S, Martel A, Pantchev N. Introducing reptiles into a captive collection: the role of the veterinarian. *Vet J.* (2008) 175:53–68. doi: 10.1016/j.tvjl.2006.12.009
164. Origgi FC, Klein PA, Tucker SJ, Jacobson ER. Application of immunoperoxidase-based techniques to detect herpesvirus infection in tortoises. *J Vet Diagn Investig.* (2003) 15:133–40. doi: 10.1177/104063870301500207
165. Marschang RE, Schneider RM. Antibodies to viruses in wild-caught spur-thighed tortoises (*Testudo graeca*) in Turkey. *Vet Rec.* (2007) 161:102–3. doi: 10.1136/vr.161.3.102
166. Nie YC, Lu CP. Antibody against Testudo herpesvirus is not common in Chinese soft-shelled turtles. *Zentralblatt Fuer Veterinaermed Reihe B.* (1999) 46:731–4. doi: 10.1046/j.1439-0450.1999.00292.x
167. Martel A, Blahak S, Vissenaekens H, Pasmans F. Reintroduction of clinically healthy tortoises: the herpesvirus Trojan horse. *J Wildl Dis.* (2009) 45:218–20. doi: 10.7589/0090-3558-45.1.218
168. Sim RR, Allender MC, Crawford LK, Wack AN, Murphy KJ, Mankowski JL, et al. Ranavirus epizootic in captive eastern box turtles (*Terrapene carolina* carolina) with concurrent herpesvirus and mycoplasma infection: management and monitoring. *J Zoo Wildl Med.* (2016) 47:256–70. doi: 10.1638/2015-0048.1
169. Lawrance MF, Mansfield KL, Sutton E, Savage AE. Molecular evolution of fibropapilloma-associated herpesviruses infecting juvenile green and loggerhead sea turtles. *Virology.* (2018) 521:190–7. doi: 10.1016/j.virol.2018.06.012
170. Braun J, Schrenzel M, Witte C, Gokool L, Burchell J, Rideout BA. Molecular methods to detect *Mycoplasma* spp. and Testudinid herpesvirus 2 in desert tortoises (*Gopherus agassizii*) and implications for disease management. *J Wildl Dis.* (2014) 50:757–66. doi: 10.7589/2013-09-231
171. Kane LP, Bunick D, Abd-Eldaim M, Dzhaman E, Allender MC. Development and validation of quantitative PCR for detection of Terrapene herpesvirus 1 utilizing free-ranging eastern box turtles (*Terrapene carolina* carolina). *J Virol Methods.* (2016) 232:57–61. doi: 10.1016/j.jviromet.2016.02.002
172. Murakami M, Matsuba C, Une Y, Nomura Y, Fujitani H. Development of species-specific PCR techniques for the detection of tortoise herpesvirus. *J Vet Diagn Investig.* (2001) 13:513–6. doi: 10.1177/104063870101300610
173. Salinas M, Francino O, Sanchez A, Altet L. Mycoplasma and herpesvirus PCR detection in tortoises with rhinitis-stomatitis complex in Spain. *J Wildl Dis.* (2011) 47:195–200. doi: 10.7589/0090-3558-47.1.195
174. Lu Y, Wang Y, Yu Q, Aguirre AA, Balazs GH, Nerurkar VR, et al. Detection of herpesviral sequences in tissues of green turtles with fibropapilloma by polymerase chain reaction. *Arch Virol.* (2000) 145:1885–93. doi: 10.1007/s007050070063
175. Lackovich JK, Brown DR, Homer BL, Garber RL, Mader DR, Moretti RH, et al. Association of herpesvirus with fibropapillomatosis of the green turtle *Chelonia mydas* and the loggerhead turtle Caretta caretta in Florida. *Dis Aquat Organ.* (1999) 37:89–97. doi: 10.3354/dao037089
176. Quackenbush SL, Work TM, Balazs GH, Casey RN, Rovnak J, Chaves A, et al. Three closely related herpesviruses are associated with fibropapillomatosis in marine turtles. *Virology.* (1998) 246:392–9. doi: 10.1006/viro.1998.9207
177. VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, et al. Detection and analysis of diverse herpesviral species by consensus primer PCR. *J Clin Microbiol.* (1996) 34:1666–71. doi: 10.1128/JCM.34.7.1666-1671.1996
178. Fedorko DP, Nelson NA, McAuliffe JM, Subbarao K. Performance of rapid tests for detection of avian influenza A virus types H5N1 and H9N2. *J Clin Microbiol.* (2006) 44:1596–7. doi: 10.1128/JCM.44.4.1596-1597.2006
179. Padgett KA, Cahoon-Young B, Carney R, Woods L, Read D, Husted S, et al. Field and laboratory evaluation of diagnostic assays for detecting West Nile virus in oropharyngeal swabs from California wild birds. *Vector Borne Zoonot Dis.* (2006) 6:183–91. doi: 10.1089/vbz.2006.6.183
180. Yu J, Lin Y, Cao Y, Li X, Liao D, Ye Y, et al. Development and application of a colloidal gold test strip for the rapid detection of the infectious laryngotracheitis virus. *Poult Sci.* (2020) 99:2407–15. doi: 10.1016/j.psj.2019.11.066

181. Chen H, Zhang X, Jin Z, Huang L, Dan H, Xiao W, et al. Differential diagnosis of PRV-infected versus vaccinated pigs using a novel EuNPs-virus antigen probe-based blocking fluorescent lateral flow immunoassay. *Biosens Bioelectr.* (2020) 155:112101. doi: 10.1016/j.bios.2020.112101
182. Lemieux B, Li Y, Kong H, Tang Y-W. Near instrument-free, simple molecular device for rapid detection of herpes simplex viruses. *Expert Rev Mol Diagn.* (2012) 12:437–43. doi: 10.1586/erm.12.34
183. Kohda C, Chiba N, Shimokoba K, Mizuno K, Negoro T, Nakano Y, et al. A simple smart amplification assay for the rapid detection of human cytomegalovirus in the urine of neonates. *J Virol Methods.* (2014) 208:160–5. doi: 10.1016/j.jviromet.2014.07.034
184. Shojaei TR, Tabatabaei M, Shawky S, Salleh MAM, Bald D. A review on emerging diagnostic assay for viral detection: the case of avian influenza virus. *Mol Biol Rep.* (2015) 42:187–99. doi: 10.1007/s11033-014-3758-5
185. Voermans JJC, Seven-Deniz S, Fraaij PLA, van der Eijk AA, Koopmans MPG, Pas SD. Performance evaluation of a rapid molecular diagnostic, MultiCode based, sample-to-answer assay for the simultaneous detection of Influenza A, B and respiratory syncytial viruses. *J Clin Virol.* (2016) 85:65–70. doi: 10.1016/j.jcv.2016.10.019
186. Loose FN, Breitbach A, Bertalan I, Rüster D, Truyen U, Speck S. Diagnostic validation of a rapid and field-applicable PCR-lateral flow test system for point-of-care detection of cyprinid herpesvirus 3 (CyHV-3). *PLoS One.* (2020) 15:e0241420. doi: 10.1371/journal.pone.0241420
187. Wang H, Sun M, Xu D, Podok P, Xie J, Jiang Y, et al. Rapid visual detection of cyprinid herpesvirus 2 by recombinase polymerase amplification combined with a lateral flow dipstick. *J Fish Dis.* (2018) 28:28. doi: 10.1111/jfd.12808
188. Sun F, Ganguli A, Nguyen J, Brisbin R, Shanmugam K, Hirschberg DL, et al. Smartphone-based multiplex 30-minute nucleic acid test of live virus from nasal swab extract. *Lab Chip.* (2020) 20:1621–7. doi: 10.1039/D0LC00304B
189. Zhu H, Fohlerová Z, Pekárek J, Basova E, NeuŽil P. Recent advances in lab-on-a-chip technologies for viral diagnosis. *Biosens Bioelectr.* (2020) 153:112041. doi: 10.1016/j.bios.2020.112041
190. Phillips EA, Moehling TJ, Ejendal KFK, Hoilett OS, Byers KM, Basing LA, et al. Microfluidic rapid and autonomous analytical device (microRAAD) to detect HIV from whole blood samples. *Lab Chip.* (2019) 19:3375–86. doi: 10.1039/C9LC00506D
191. Rodriguez-Moncayo R, Cedillo-Alcantar DF, Guevara-Pantoja PE, Chavez-Pineda OG, Hernandez-Ortiz JA, Amador-Hernandez JU, et al. A high-throughput multiplexed microfluidic device for COVID-19 serology assays. *Lab Chip.* (2021) 21:93–104. doi: 10.1039/D0LC01068E
192. Yu X, Xia Y, Tang Y, Zhang WL, Yeh YT, Lu H, et al. A nanostructured microfluidic immunoassay platform for highly sensitive infectious pathogen detection. *Small.* (2017) 13:1700425. doi: 10.1002/smll.201700425
193. Natesan M, Wu SW, Chen CI, Jensen SMR, Karlovac N, Dyas BK, et al. A smartphone-based rapid telemonitoring system for Ebola and Marburg disease surveillance. *ACS Sens.* (2019) 4:61–8. doi: 10.1021/acssensors.8b00842
194. Priye A, Bird SW, Light YK, Ball CS, Negrete OA, Meagher RJ. A smartphone-based diagnostic platform for rapid detection of Zika, Chikungunya, and Dengue viruses. *Sci Rep.* (2017) 7:44778. doi: 10.1038/srep44778
195. Yeo SJ, Cuc BT, Sung HW, Park H. Evaluation of a smartphone-based rapid fluorescent diagnostic system for H9N2 virus in specific-pathogen-free chickens. *Arch Virol.* (2016) 161:2249–56. doi: 10.1007/s00705-016-2922-8
196. Yeo SJ, Kang H, Dao TD, Cuc BT, Nguyen ATV, Tien TTT, et al. Development of a smartphone-based rapid dual fluorescent diagnostic system for the simultaneous detection of influenza A and H5 subtype in avian influenza A-infected patients. *Theranostics.* (2018) 8:6132–48. doi: 10.7150/thno.28027
197. Zhao W, Tian S, Huang L, Liu K, Dong L, Guo J. A smartphone-based biomedical sensory system. *Analyst.* (2020) 145:2873–91. doi: 10.1039/C9AN02294E
198. Soares JF, Chalker VJ, Erles K, Holtby S, Waters M, McArthur S. Prevalence of *Mycoplasma agassizii* and Chelonian herpesvirus in captive tortoises (*Testudo* sp.) in the United Kingdom. *J Zoo Wildl Med.* (2004) 35:25–33. doi: 10.1638/02-092
199. Archer GA, Phillips CA, Adamovicz L, Band M, Byrd J, Allender MC. Detection of copathogens in free-ranging Eastern Box Turtles (*Terrapene carolina* carolina) in Illinois and Tennessee. *J Zoo Wildl Med.* (2017) 48:1127–34. doi: 10.1638/2017-0148R.1
200. CABI. *Fibropapillomatosis of Sea Turtles [original text by Chris A. Whitehouse]*. Wallingford: CAB International (2019). Available online at: www.cabi.org/isc
201. Raiti P. Carbon Dioxide (CO_2) Laser treatment of cutaneous papillomas in a common snapping turtle, *Chelydra serpentina*. *J Zoo Wildl Med.* (2008) 39:252–65. doi: 10.1638/2007-0055R.1
202. Schroeder BA, Witherington BE. *Proceedings of the Thirteenth Annual Symposium on Sea Turtle Biology and Conservation*. NOAA Technical Memorandum NMFS-SEFSC-341. Jekyll Island, GA (1994). p. 281.
203. Page-Karjian A, Perrault JR, Zirkelbach B, Pescatore J, Riley R, Stadler M, et al. Tumor re-growth, case outcome, and tumor scoring systems in rehabilitated green turtles with fibropapillomatosis. *Dis Aquat Org.* (2019) 137:101–8. doi: 10.3354/dao03426
204. Glazkova A. Treating sea turtle fibropapillomatosis with CO_2 laser surgery. *Veterinary Practice News*. (2015). p. 34–5.
205. Brunner CH, Dutra G, Silva CB, Silveira LM, Martins Mde F. Electrochemotherapy for the treatment of fibropapillomas in *Chelonia mydas*. *J Zoo Wildl Med.* (2014) 45:213–8. doi: 10.1638/2010-0125.1
206. Sellera FP, Sabino CP, Fernandes LT, Pogliani FC, Teixeira CR, Dutra GH, et al. Green turtle (*Chelonia mydas*) cutaneous fibropapillomatosis treatment by photodynamic therapy. *Mar Turt Newsl.* (2014) 142:1–31.
207. McArthur SDJ. An acyclovir trial in *Testudo* sp. In: *Proceedings of the BVZS, Spring Meeting, Emerging Diseases* London (2000).
208. Origgi F. *Herpesvirus in Tortoises. Reptile Medicine and Surgery*, 2nd ed. St Louis, MO: Saunders (2006). p. 814–21.
209. Wright K, editor. How I treat herpesvirus in tortoises, small animal and exotics. In: *Proceedings of the North American Veterinary Conference*. Orlando, FL: The North American Veterinary Conference (2008).
210. Gandar F, Marlier D, Vanderplasschen A. *In vitro* and *in vivo* assessment of eprociclovir as antiviral treatment against testudinid herpesvirus 3 in Hermann's tortoise (*Testudo hermanni*). *Res Vet Sci.* (2019) 124:20–3. doi: 10.1016/j.rvsc.2019.02.001
211. Sondak VK, Sabel MS, Mulé JJ. Allogeneic and autologous melanoma vaccines: where have we been and where are we going? *Clin Cancer Res.* (2006) 12:2337s. doi: 10.1158/1078-0432.CCR-05-2555
212. Leal L, Guardo AC, Morón-López S, Salgado M, Mothe B, Heirman C, et al. Phase I clinical trial of an intranodally administered mRNA-based therapeutic vaccine against HIV-1 infection. *AIDS.* (2018) 32:2533–45. doi: 10.1097/QAD.0000000000002026
213. Deb R, Dey S, Madhan Mohan C, Gaikwad S, Kamble N, Khulape SA, et al. Development and evaluation of a *Salmonella typhimurium* flagellin based chimeric DNA vaccine against infectious bursal disease of poultry. *Res Vet Sci.* (2015) 102:7–14. doi: 10.1016/j.rvsc.2015.07.004
214. Brisse M, Vrba SM, Kirk N, Liang Y, Ly H. Emerging concepts and technologies in vaccine development. *Front Immunol.* (2020) 11:583077. doi: 10.3389/fimmu.2020.583077
215. Jackson LA, Anderson EJ, Rouphael NG, Roberts PC, Makhene M, Coler RN, et al. An mRNA vaccine against SARS-CoV-2 - preliminary report. *N Engl J Med.* (2020) 383:1920–31. doi: 10.1056/NEJMoa2022483
216. Foley AM, Schroeder BA, Redlow AE, Fick-Child KJ, Teas WG. Fibropapillomatosis in stranded green turtles (*Chelonia mydas*) from the eastern United States (1980-98): trends and associations with environmental factors. *J Wildl Dis.* (2005) 41:29–41. doi: 10.7589/0090-3558-41.1.29
217. Aguirre AA, Balazs GH, Zimmerman B, Galey FD. Organic contaminants and trace metals in the tissues of green turtles (*Chelonia mydas*) afflicted with fibropapillomas in the Hawaiian Islands. *Mar Pollut Bull.* (1994) 28:109–14. doi: 10.1016/0025-326X(94)90547-9
218. Van Houtan KS, Hargrove SK, Balazs GH. Land use, macroalgae, and a tumor-forming disease in marine turtles. *PLoS ONE.* (2010) 5:29. doi: 10.1371/journal.pone.0012900

219. Landsberg JH, Balazs GH, Steidinger KA, Baden DG, Work TM, Russell DJ. The potential role of natural tumor promoters in marine turtle fibropapillomatosis. *J Aquat Anim Health.* (1999) 11:199–210. doi: 10.1577/1548-8667(1999) 011<0199:TPRONT>2.0.CO;2
220. Arthur K, Limpus C, Balazs G, Capper A, Udy J, Shaw G, et al. The exposure of green turtles (*Chelonia mydas*) to tumour promoting compounds produced by the cyanobacterium *Lyngbya majuscula* and their potential role in the aetiology of fibropapillomatosis. *Harmful Algae.* (2008) 7:114–25. doi: 10.1016/j.hal.2007.06.001
221. Van Houtan KS, Smith CM, Dailer ML, Kawachi M. Eutrophication and the dietary promotion of sea turtle tumors. *PeerJ.* (2014) 2:e602. doi: 10.7717/peerj.602
222. Stahl SJ, editor. Reptile production medicine. *Semin Avian Exot Pet Med.* (2001) 10:140–50. doi: 10.1053/saep.2001.24256
223. International Union for Conservation of Nature (IUCN). *Red List of Threatened Species.* (2020). Available online at: https://www.iucnredlist.org
224. Teifke JP, Lohr CV, Marschang RE, Osterrieder N, Posthaus H. Detection of chelonid herpesvirus DNA by nonradioactive *in situ* hybridization in tissues from tortoises suffering from stomatitis-rhinitis complex in Europe and North America. *Vet Pathol.* (2000) 37:377–85. doi: 10.1354/vp.37-5-377

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 31 May 2021
doi: 10.3389/fvets.2021.665805

Revisiting Ophidiomycosis (Snake Fungal Disease) After a Decade of Targeted Research

Christina M. Davy[1,2], Leonard Shirose[3,4], Doug Campbell[3,4], Rachel Dillon[2], Christina McKenzie[3,4], Nicole Nemeth[3,4,5], Tony Braithwaite[6], Hugh Cai[7], Tarra Degazio[8], Tammy Dobbie[8], Sean Egan[9], Heather Fotherby[10], Jacqueline D. Litzgus[11], Pilar Manorome[12], Steve Marks[13], James E. Paterson[2], Lynne Sigler[14], Durda Slavic[7], Emily Slavik[12], John Urquhart[15] and Claire Jardine[3,4]*

[1] Wildlife Research and Monitoring Section, Ontario Ministry of Natural Resources, Peterborough, ON, Canada, [2] Environmental and Life Sciences Program, Trent University, Peterborough, ON, Canada, [3] Department of Pathobiology, University of Guelph, Guelph, ON, Canada, [4] Canadian Wildlife Health Cooperative – Ontario/Nunavut, Guelph, ON, Canada, [5] Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, GA, United States, [6] Kingsville Animal Hospital, Kingsville, ON, Canada, [7] Animal Health Laboratory, University of Guelph, Guelph, ON, Canada, [8] Point Pelee National Park, Leamington, ON, Canada, [9] Egan Fife Animal Hospital, Chatham, ON, Canada, [10] Natural Resource Solutions Inc., Waterloo, ON, Canada, [11] Department of Biology, Laurentian University, Sudbury, ON, Canada, [12] Ontario Parks, Ontario Ministry of Natural Resources, Peterborough, ON, Canada, [13] Essex County Field Naturalists' Club, c/o Ojibway Nature Centre, Windsor, ON, Canada, [14] Agriculture, Life and Environmental Sciences, University of Alberta, Edmonton, AB, Canada, [15] Blazing Star Environmental, Oshawa, ON, Canada

OPEN ACCESS

Edited by:
Amanda Linda Jean Duffus, Gordon State College, United States

Reviewed by:
Dean Konjević, University of Zagreb, Croatia
Houston Chandler, The Orianne Society, United States

***Correspondence:**
Christina M. Davy
christina.davy@ontario.ca

Specialty section:
This article was submitted to Zoological Medicine, a section of the journal Frontiers in Veterinary Science

Received: *09 February 2021*
Accepted: *09 April 2021*
Published: *31 May 2021*

Citation:
Davy CM, Shirose L, Campbell D, Dillon R, McKenzie C, Nemeth N, Braithwaite T, Cai H, Degazio T, Dobbie T, Egan S, Fotherby H, Litzgus JD, Manorome P, Marks S, Paterson JE, Sigler L, Slavic D, Slavik E, Urquhart J and Jardine C (2021) Revisiting Ophidiomycosis (Snake Fungal Disease) After a Decade of Targeted Research. Front. Vet. Sci. 8:665805. doi: 10.3389/fvets.2021.665805

Emerging infectious diseases (EIDs) are typically characterized by novelty (recent detection) and by increasing incidence, distribution, and/or pathogenicity. Ophidiomycosis, also called snake fungal disease, is caused by the fungus *Ophidiomyces ophidiicola* (formerly "*ophiodiicola*"). Ophidiomycosis has been characterized as an EID and as a potential threat to populations of Nearctic snakes, sparking over a decade of targeted research. However, the severity of this threat is unclear. We reviewed the available literature to quantify incidence and effects of ophidiomycosis in Nearctic snakes, and to evaluate whether the evidence supports the ongoing characterization of ophidiomycosis as an EID. Data from Canada remain scarce, so we supplemented the literature review with surveys for *O. ophidiicola* in the Canadian Great Lakes region. Peer-reviewed reports of clinical signs consistent with ophidiomycosis in free-ranging, Nearctic snakes date back to at least 1998, and retrospective molecular testing of samples extend the earliest confirmed record to 1986. Diagnostic criteria varied among publications ($n = 33$), confounding quantitative comparisons. Ophidiomycosis was diagnosed or suspected in 36/121 captive snakes and was fatal in over half of cases (66.7%). This result may implicate captivity-related stress as a risk factor for mortality from ophidiomycosis, but could also reflect reporting bias (i.e., infections are more likely to be detected in captive snakes, and severe cases are more likely to be reported). In contrast, ophidiomycosis was diagnosed or suspected in 441/2,384 free-ranging snakes, with mortality observed in 43 (9.8 %). Ophidiomycosis was only speculatively linked to population declines, and we found no evidence that the prevalence of the pathogen or disease increased over the past decade of targeted

research. Supplemental surveys and molecular (qPCR) testing in Ontario, Canada detected *O. ophidiicola* on 76 of 657 free-ranging snakes sampled across ∼136,000 km^2. The pathogen was detected at most sites despite limited and haphazard sampling. No large-scale mortality was observed. Current evidence supports previous suggestions that the pathogen is a widespread, previously unrecognized endemic, rather than a novel pathogen. Ophidiomycosis may not pose an imminent threat to Nearctic snakes, but further research should investigate potential sublethal effects of ophidiomycosis such as altered reproductive success that could impact population growth, and explore whether shifting environmental conditions may alter host susceptibility.

Keywords: emerging infectious disease, fungal pathogen, ophidiomycosis, *Ophidiomyces ophidiicola*, reptile, snake, qPCR

INTRODUCTION

Emerging infectious diseases are defined as diseases that have newly appeared in a population or are increasing in incidence or geographic range (1). Those affecting animals and plants (or fauna and flora) can present a major challenge to biodiversity conservation (2, 3). Fungal pathogens, in particular, are increasing in frequency (4). Impacts of fungal epidemics on biodiversity include devastating declines in American chestnut (*Castanea dentata*) populations infected with *Cryphonectria parasitica,* extinctions in some amphibian populations infected with *Batrachochytrium dendrobatidis* and *B. salamandrivorans,* and declines in some bat species infected with *Pseudogymnoascus destructans* (5). Ophidiomycosis (also called snake fungal disease) is a disease of snakes caused by *Ophidiomyces ophidiicola* [formerly "*ophiodiicola*"; Kirk (6)] (7–10). Ophidiomycosis has frequently been compared to epidemics such as bat white-nose syndrome and amphibian chytridiomycosis, and is often referred to as an emerging infectious disease that could threaten the conservation of Nearctic (North American) snakes (7, 11–16).

Ophidiomycosis occurs in wild and captive snakes in North America, Great Britain, Europe and Australia (8, 9, 17, 18) and a distinct strain of *O. ophidiicola* has been described from snakes in Europe (14). Ophidiomycosis in free-ranging snakes was first described in eastern massasauga rattlesnakes (*Sistrurus catenatus*) in North America (7), and has now been identified in snakes throughout the eastern USA, from Florida to Massachusetts to Wisconsin (8, 19–21). The fungus appears to be an opportunistic pathogen with a wide tolerance of environmental conditions (11). Experimental infection of cottonmouths (*Agkistrodon piscivorus*) and corn snakes (*Pantherophis guttatus)* with North American strains of *O. ophidiicola* caused dermal lesions consistent with those observed in wild snakes (22–24).

Clinical signs of ophidiomycosis range from a mild dermatitis with hyperkeratosis, scabs and crusts (**Figure 1**) to premature shedding of the skin, subcutaneous nodules, and corneal opacities (11). The range of clinical signs and their potential overlap with other conditions makes diagnosis difficult. Impacts to infected snakes vary with the severity of clinical signs, which can differ among individuals even in controlled, experimental infections (22, 23, 25). Facial lesions may interfere with feeding ability (22) and vision, and infection of the nasolabial pits of Viperidae could affect their heat-sensing ability, although this effect has not been directly tested. Severe cases of ophidiomycosis can end in mortality. The current standard for diagnosis of ophidiomycosis is detection of *O. ophidiicola* through real-time polymerase chain reaction [qPCR; (26)] combined with histopathological detection of fungal hyphae in lesions, particularly if diagnostic arthroconidia are present (8, 18, 27).

Research into ophidiomycosis has been mobilized by concern that *O. ophidiicola* causes or has the potential to cause widespread morbidity and mortality in free-ranging snakes (7, 11, 14–16, 23, 28). Ophidiomycosis clearly causes or contributes to mortality of snakes under some circumstances (22–25). However, it is unclear whether the available evidence indicates an impact of ophidiomycosis on the viability of snake populations (i.e., population-level effects). We reviewed the literature on ophidiomycosis in free-ranging, Nearctic snakes from a conservation ecology perspective to ask: (1) whether the available evidence supports the qualification of ophidiomycosis as an emerging infectious disease of immediate conservation concern; and (2) whether the evidence supports consideration of this disease as a current threat to populations of wild snakes. Reports of ophidiomycosis in snakes in Canada remain scarce [but see McKenzie et al. (25); Paré and Sigler (8)]. Therefore, we supplemented our literature review with a field survey of ophidiomycosis prevalence in free-ranging snakes from the Great Lakes region of Ontario, Canada, which allowed us to explore the northern distribution of the pathogen and the disease.

MATERIALS AND METHODS

The first confirmed case of ophidiomycosis from Canada was an adult, female eastern foxsnake (*Pantherophis vulpinus*) found in Point Pelee National Park, Essex County, Ontario, in late March 2015 (8). Physical examination of the snake revealed a large number of dry, crusted scabs along the dorsal midline and ventral surface of the head. The cloaca was nearly occluded by dried scabs. Skin samples and swabs were collected when the snake was taken into veterinary care. The snake underwent ecdysis while in care and the shed skin provided a further diagnostic sample. All

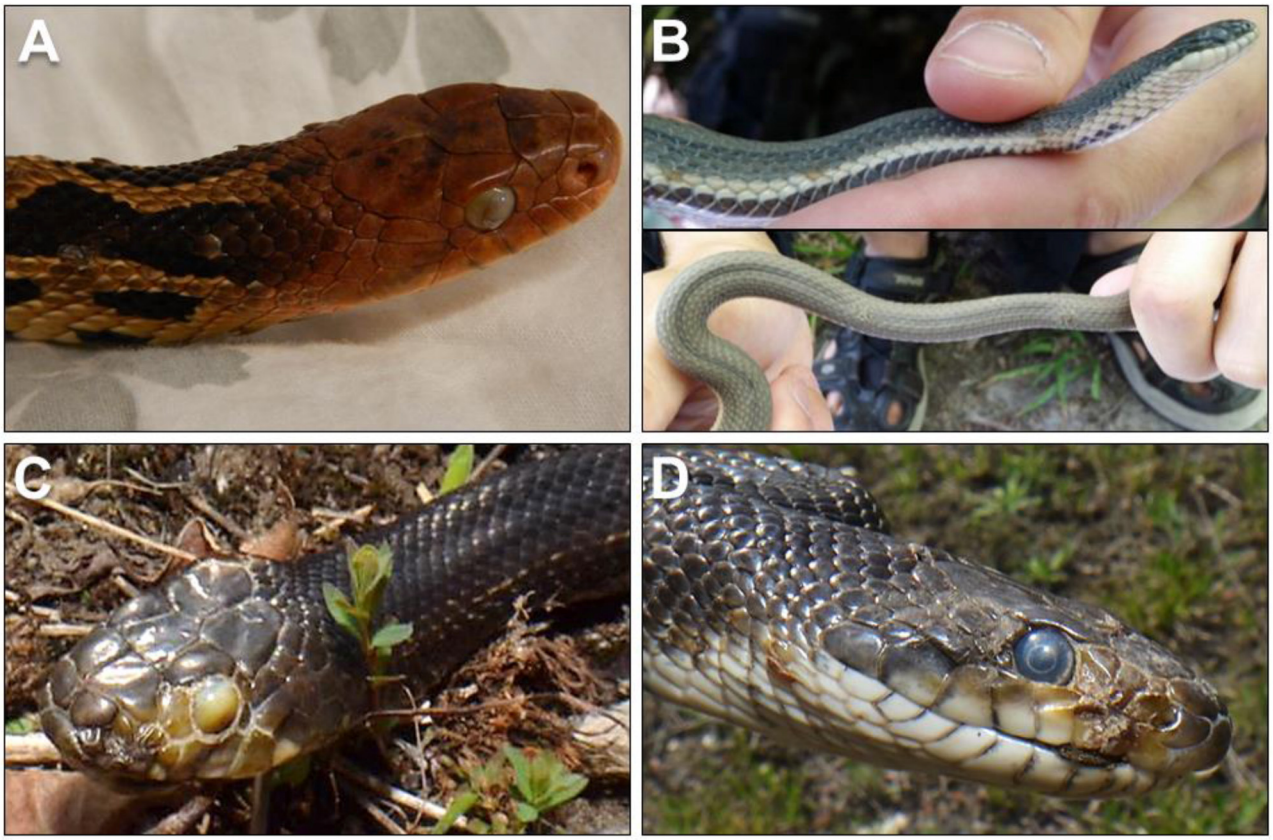


FIGURE 1 | (A) Ophidiomycosis manifesting in gross lesions on the skin and eye of an eastern foxsnake (*Pantherophis vulpinus*) with a plaque of fungal hyphae growing over the eye (photo: Sheeva Nakhaie). **(B)** Ophidiomycosis on a queensnake (*Regina septemvittata;* photos: Heather Fotherby); **(C,D)** Gray ratsnake (*Pantherophis spiloides;* photos: Kenny Ruelland) from southeastern Ontario, Canada with suspect ophidiomycosis.

samples were cultured for fungus at the diagnostic bacteriology laboratory of the Animal Health Laboratory (AHL) of the University of Guelph. A fungus identified as a *Chrysosporium* sp. was cultured from the skin. The isolate was confirmed to be *O. ophidiicola* by morphology and sequencing at the University of Alberta (L. Sigler) and is stored as UAMH 11863 in the UAMH Center for Global Microfungal Biodiversity (https://www.uamh.ca/index.html). Samples from this isolate were used to validate the qPCR assay conducted at the AHL.

Researchers in Canada also began to examine individuals in their study populations for signs of *O. ophidiicola* infection following the description of ophidiomycosis in free-ranging snakes in the United States (7) but different approaches were taken. Some projects in southern Ontario collected swab samples from the body surfaces of all captured snakes, regardless of whether the individuals showed signs of disease. Other projects directly swabbed only those individuals that exhibited gross lesions consistent with ophidiomycosis. Swabs were stored in lysis buffer (typically at room temperature) prior to analysis. Some projects were also able to access veterinary facilities where biopsies of gross lesions could be taken under sterile conditions, with subsequent release of the potentially affected snakes. Swabs and frozen biopsies were submitted to the Canadian Wildlife Health Cooperative (CWHC) in Guelph, Ontario for testing. From 2012 to 2016 the CWHC also received swab and tissue samples from snake carcasses submitted for diagnostic evaluation, following incidental mortalities observed in the field (predation, road mortality, or mortality with no obvious cause of death). The presence of *O. ophidiicola* on swabs or tissue samples was tested using the qPCR assay described by Allender et al. (26). Histopathology was used to investigate the presence of fungal hyphae and associated microscopic lesions in tissue samples, allowing diagnosis of ophidiomycosis following the criteria currently applied by the CWHC (**Table 1**). The CWHC criteria are similar to the diagnostic criteria proposed by Baker et al. (27), but do not require confirmation of arthroconidia to meet the diagnostic threshold.

To assess the incidence of ophidiomycosis in free-ranging, Nearctic snakes and explore whether occurrence increased over time, we searched for peer-reviewed scientific literature in Google Scholar. We used the search string ["ophidiomycosis" OR "snake fungal disease" OR "*Ophidiomyces ophiodiicola*"], and included all papers identified by this search as of 10 July 2020. We used the original spelling of the species epithet (i.e., *O. ophiodiicola*)

TABLE 1 | Criteria applied to diagnose ophidiomycosis in snakes ($n = 657$) from the Great Lakes region of Ontario, Canada.

Presence of gross lesions	Histological examination of lesions detects fungal hyphae consistent with *O. ophidiicola*	*O. ophidiicola* detected through qPCR/culture	Diagnosis
Yes or No	No	No	Negative
Yes or No	–	No	Not detected
–	–	Yes	Detected
Yes	–	Yes	Suspect Ophidiomycosis
Yes	Yes	–	Suspect Ophidiomycosis
–	Yes	Yes	Ophidiomycosis
Yes	Yes	Yes	Ophidiomycosis

in the search because the change to '*ophidiicola*' occurred very recently (6).

We reviewed all documents and retained those that provided records of ophidiomycosis in Nearctic snakes. We also accessed and reviewed publications that the initial set of studies mentioned as potential, previously unrecognized cases of ophidiomycosis. We summarized detections of the pathogen and the disease in the literature following the criteria in **Table 1**, which are currently used by the CWHC in Canadian surveillance of ophidiomycosis.

To assess the incidence of mortality of Nearctic snakes from ophidiomycosis, we also summarized records of mortality in the studies we reviewed. Several studies described situations in which snakes showing clinical signs of ophidiomycosis were taken into captivity for treatment. It was not possible to estimate what the outcome would have been if these snakes had been left in the wild. Other studies described mortality in free-ranging snakes that showed clinical signs of ophidiomycosis, but did not confirm the presence of the pathogen with molecular testing, culture, or histopathology. In these cases, it was not possible to be certain that the mortality was caused by ophidiomycosis. To enable an estimate of mortality incidence among studies, we assumed that all reported mortalities of snakes diagnosed with ophidiomycosis or suspected ophidiomycosis were caused by the disease. This assumption may result in an overestimate of the severity of ophidiomycosis. However, it allowed us to account for variation and related uncertainty in diagnostic criteria used among studies, and to ensure we did not inadvertently underestimate mortality associated with ophidiomycosis.

Finally, we tested whether the reported prevalence of pathogen detection (through PCR, qPCR, or cultures) or the reported prevalence of gross lesions had changed over the past decade of targeted research. We used generalized linear mixed effects models with binomial error structures, and with sampling year as a fixed effect, to test shifts in prevalence reported in the published literature (i.e., not including the additional testing we conducted on Canadian snakes, as described above). Some studies described samples collected in multiple years without reporting yearly prevalence, so we used the mean sampling year of the study for multi-year studies (e.g., samples from 2016 to 2017 = 2017.5). We scaled sampling year prior to model fitting (mean = 0, SD = 1). We included random intercepts and random slopes for species, to account for likely interspecific variation in host-pathogen interactions or environmental variation linked to species' habitat preferences.

RESULTS

Detection of *O. ophidiicola* and Ophidiomycosis in Canada

We collected swab or tissue samples opportunistically from 657 snakes (13 species) across southern Ontario (**Figure 2**). Gross lesions were observed on 116 individuals (18%, **Table 2**). We detected *O. ophidiicola* by qPCR on 76 snakes (11.6%), including *Nerodia sipedon, Pantherophis spiloides, P. vulpinus, Regina septemvittata, Sistrurus catenatus, Storeria dekayi,* and *Thamnophis sirtalis*. *Ophidiomyces ophidiicola* was not detected on *Heterodon platirhinos*, *Lampropeltis triangulum*, *Storeria occipitomaculata,* or *Thamnophis butleri,* but sample sizes for these species were low (**Table 2**). A swab from one *T. butleri* with highly suggestive lesions tested negative; a tissue sample was not available from this individual.

We confirmed ophidiomycosis through a combination of histopathology and positive qPCR results in 14 snakes (11 *P. vulpinus, two P. spiloides* and one *R. septemvittata*; **Table 2**). Suspected ophidiomycosis was observed in 38 additional individuals. Ophidiomycosis was diagnosed as the likely cause of death in two snakes (one *P. vulpinus* and one *P. spiloides*), and two further snakes with suspected ophidiomycosis also died (one *P. vulpinus* and one *T. butleri*). A final *P. vulpinus* with ophidiomycosis died in the course of a surgery to replace an implanted radio-transmitter. Necropsy revealed that this individual also had an unusually enlarged heart and ophidiomycosis was likely not the proximate cause of death. Nevertheless, we counted this case as a mortality associated with ophidiomycosis. The other snakes with confirmed or suspected ophidiomycosis included one road-killed individual, and 44 snakes that were alive and behaving normally at the time of sampling (**Table 2**).

Evaluation of Pathogen and Disease Prevalence in Wild Snakes, and Mortality in the Wild

We identified 33 peer-reviewed studies published from 2003 to July 2020 that included original observations of free-ranging, Nearctic snakes exhibiting confirmed or probable ophidiomycosis or "snake fungal disease," or that detected *O. ophidiicola* in free-ranging, Nearctic snakes (42 species; **Supplementary Table 1**). The earliest observation of clinical signs consistent with ophidiomycosis that we found in the peer-reviewed literature occurred in 1997-98 (19b). However, retrospective molecular testing detected *O. ophidiicola* in a sample collected from a corn snake (*Pantherophis guttatus*) in 1986 (18).

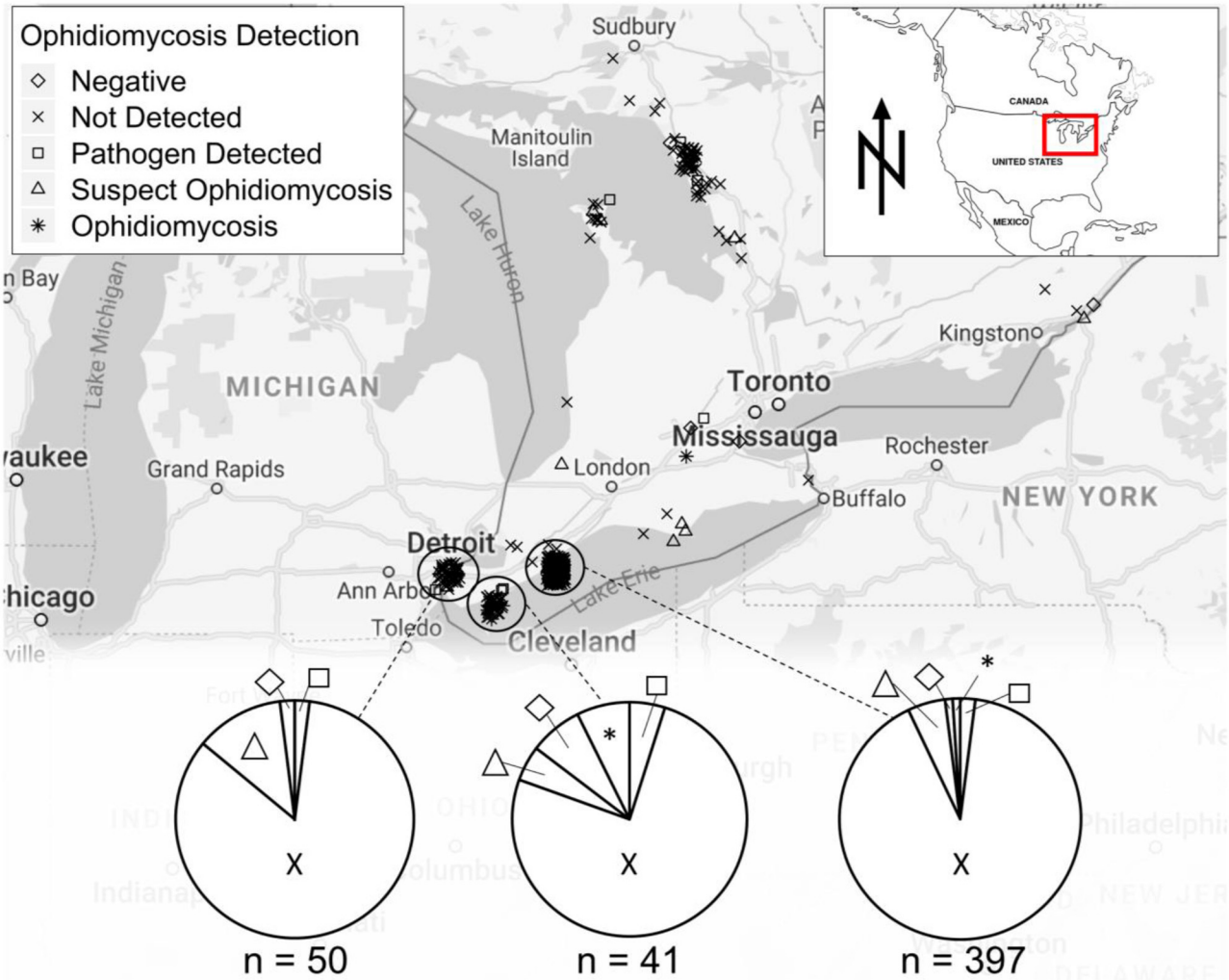


FIGURE 2 | Sampling locations for snakes tested for ophidiomycosis in the Great Lakes region of Ontario, Canada, showing diagnoses based on the criteria in **Table 1**. Locations are shown for 542 snakes for which specific coordinates were available. A further 115 samples were tested and included in **Table 2** but are not shown on the map as they were were submitted to the Canadian Wildlife Health Center without precise location data.

Inferring rates of mortality associated with ophidiomycosis from the available data, we found that reported case fatality was typically high in studies that described one or several severely affected individuals. These studies also tended to describe outcomes in snakes that were taken into captivity. Summing mortalities reported in 33 peer-reviewed studies and the data from Canada reported above, ophidiomycosis was confirmed or suspected of causing the deaths of 72 individual snakes between 1999 and 2020 (**Supplementary Table 1**). Ophidiomycosis was diagnosed or suspected in 36/121 captive snakes and was fatal in 66.7% of cases involving snakes kept in (or brought into) captivity (**Supplementary Table 1**). In contrast, ophidiomycosis was diagnosed or suspected in 441/2,384 free-ranging snakes, with mortality directly observed in 43 of these individuals (9.8 %) (**Supplementary Table 1**).

Studies did not consistently report the same variables or apply the same diagnostic criteria (**Supplementary Table 1**), which limited direct comparison of prevalence, severity of clinical signs, and outcomes among populations. Nevertheless, we were able to test whether the reported prevalence of pathogen detection (through PCR, qPCR, or cultures) had changed from 2008 to 2018. Prevalence of *O. ophidiicola* reported in peer-reviewed studies declined over this time ($\chi^2 = 13.86$, df $= 1$, $P < 0.0001$). The reported prevalence of the pathogen (variance of random intercepts $= 1.09$) and the slope of how reported prevalence of the pathogen changed through time (variance of random slopes $= 0.68$) varied among the 42 species. We also tested whether the reported prevalence of gross lesions had declined from 2007 to 2018 (observations of gross lesions consistent with ophidiomycosis predated molecular detection of the pathogen). Overall, model-predicted reported prevalence of gross lesions declined between 2007 and 2018 ($\chi^2 = 18.20$, df $= 1$, $P < 0.0001$). Reported prevalence of gross lesions (variance of random intercepts $= 0.54$) and the slope of how reported

TABLE 2 | Clinical signs of ophidiomycosis (presence of gross lesions and of hyphae in lesions by histopathology) and detection of *Ophidiomyces ophidiicola* through qPCR, from 657 snakes in southern Ontario, Canada, for which samples were submitted to the Canadian Wildlife Health Cooperative between 2012 and 2018.

		Gross lesions present			*O. ophidiicola* detected (qPCR)?			Presence of fungal hyphae in lesions				Diagnosis			
	Total indiyiduals examined/sampled	No	Yes	Proportion of snakes with gross lesions	No	Yes	Proportion of swabs positive by qPCR	Total biopsies examined	No	Yes	Detected	Negative	Not detected	Suspect ophidiomycosis	Ophidiomycosis
Diodophis punctatus	1	1		0	1		0						1		
Heterodon platirhinos	2	2		0	2		0	1	1			1	1		
Lampropeltis triangulum	3	3		0	3		0	2	2			2	1		
Nerodia sipedon	24	17	7	0.29	19	5	0.21				4		19	1	
Pantherophis spiloides	9	7	2	0.22	3	6	0.67	3	1	2	4	1	2		2
Pantherophis vulpinus	216	159	57	0.26	169	47	0.22	21	10	11	10	7	161	27	11
Regina septemvittata	16	1	15	0.94	7	9	0.56	1		1	1		7	7	1
Sistrurus catenatus	83	83		0	80	3	0.02	2	2		3	1	79		
Storeria dekayi	75	69	6	0.08	74	1	0.01				1		74		
Storeria occipitomaculata	1	1		0	1		0						1		
Thamnophis butleri	15	13	2	0.13	15		0	2	1	1			14	1	
Thamnophis sauritus	26	23	3	0.12	24	2	0.08				1		24	1	
Thamnophis sirtalis	186	163	23	0.12	183	3	0.02				2		183	1	
Total	657	541	116	0.18	582	76	0.12	32	16	15	26	12	567	38	14

Some snakes were marked during mark-recapture surveys; others were sampled opportunistically and then released. It is unlikely but not impossible that some individuals were sampled more than once.

prevalence of gross lesions changed through time (variance of random slopes = 1.69) also varied among species.

Surveillance for *O. ophidiicola* increased substantially after 2011 (**Figure 3**). Our model accounted for variation in sample sizes and the number of projects collecting samples in each year, but we also reran the model including only samples collected after 2011, to ensure that our model results were not biased by increased sampling effort. This post-2011 model produced similar results to the original model that included all years.

DISCUSSION

The data summarized here suggest that ophidiomycosis is now most accurately understood as a relatively common but previously unrecognized disease of free-ranging Nearctic snakes, rather than as an EID of immediate conservation concern. Supporting evidence includes (1) that researchers were not looking for *O. ophidiicola* or ophidiomycosis prior to 2011; (2) that retrospective analyses confirm the fungus was present in samples collected from free-ranging snakes prior to 2011 (e.g., 9, 18); (3) that the available data do not suggest high fatality rates among free-ranging snakes with ophidiomycosis (**Supplementary Table 1**); (4) that the available data do not suggest an increase in the prevalence of ophidiomycosis over time, or a change in the Nearctic distribution of the fungus or disease, despite occasional observations of individual mortality potentially caused by the disease (7, 29, 30); and (5) that our field data demonstrate that low, relatively haphazard sampling effort (i.e., low number of samples collected) is sufficient to detect *O. ophidiicola* across a wide range of species and locations in southern Canada, at the northern limit of its reported Nearctic range. However, surveys with a robust, spatially explicit design would be required to truly establish the Nearctic range limits of *O. ophidiicola* and ophidiomycosis, and may still reveal an expanding distribution or shifting prevalence.

Diagnostic criteria for ophidiomycosis varied widely among the studies we reviewed (**Supplementary Table 1**). Some studies diagnosed "snake fungal disease" based solely on the observation of gross lesions, while others required qPCR confirmation of pathogen presence, and histopathological confirmation of fungal hyphae and/or arthroconidia in lesions. The variation in diagnostic criteria and sampling designs precludes direct comparisons of ophidiomycosis prevalence among Nearctic snakes, and highlights the importance of applying consistent criteria among studies. We also acknowledge that swabbing the skin or lesions for *O. ophidiicola* has an imperfect detection rate by qPCR (31, 32) and is generally not useful for culture. For example, swabs taken from experimentally infected cottonmouths or corn snakes did not always test positive for *O. ophidiicola* (22), and swabs of wild snakes sometimes fail to detect the fungus when it is present (32). Ideally, studies relying on swabbing to assess pathogen prevalence should estimate and report their detection accuracy. Once more standardized data are available, modeling spatio-temporal variation in disease and

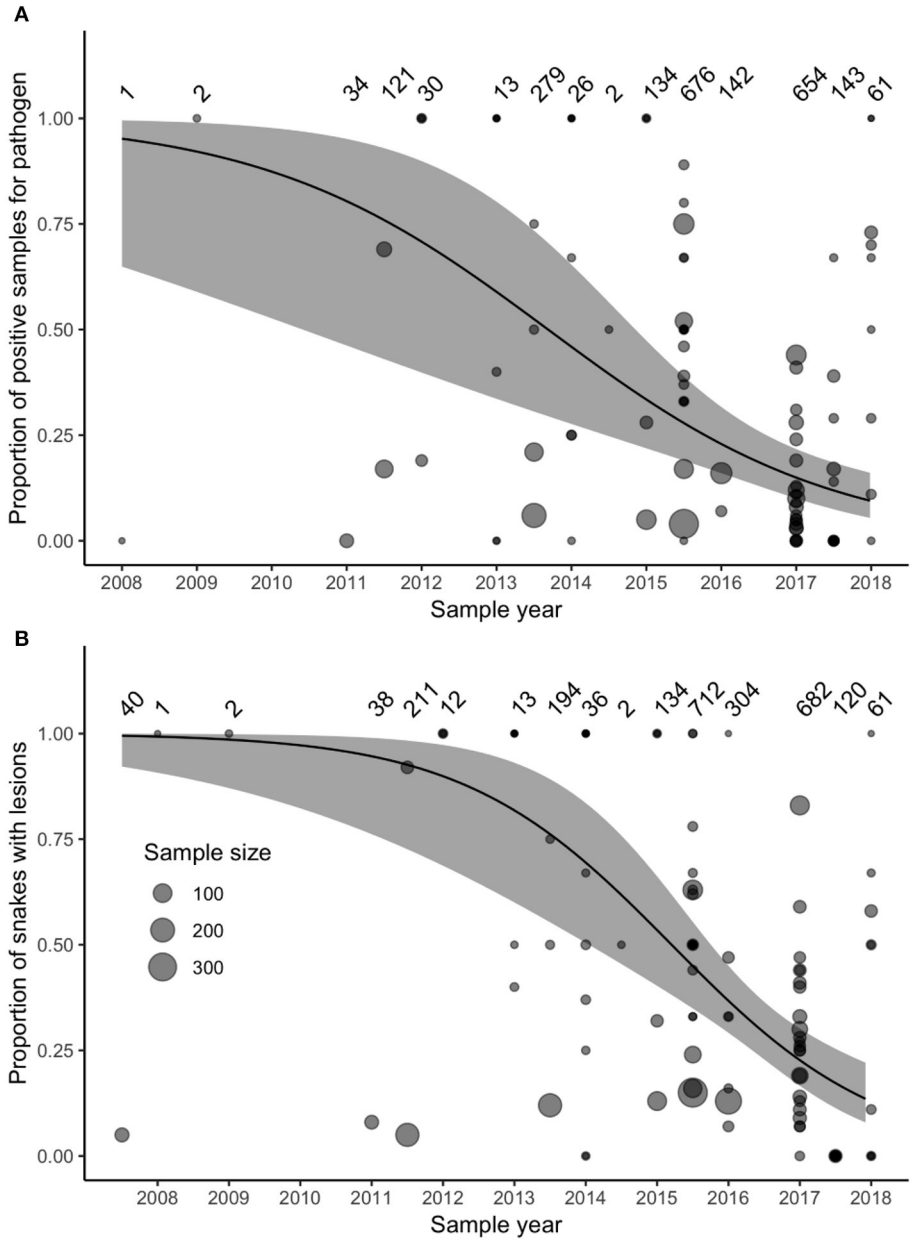


FIGURE 3 | (A) The prevalence of *Ophidiomyces ophidiicola* (detected through PCR, qPCR, or culture) in samples collected between 2008 and 2018 and reported in the peer-reviewed literature did not increase over time. **(B)** The prevalence of reported observations of gross lesions suggestive of ophidiomycosis did not increase between 2007 and 2018. Data represent samples collected from 42 species (**Supplementary Table 1**). Size of points varies with sample size reported in each study, sample sizes for each time-point are noted the top of each panel, and the solid lines and gray-shaded areas indicate the fixed-effect model predictions and 95% confidence intervals.

pathogen prevalence may reveal drivers of ophidiomycosis in free-ranging snakes, and identify vulnerable species or regions on which ophidiomycosis may have a greater impact.

When we tallied all mortalities or possible mortalities associated with ophidiomycosis reported in the literature, and the five possible mortalities reported here from our survey of snakes in southern Ontario, we found only 72 reported mortalities associated with ophidiomycosis in Nearctic snakes from 1999 to 2020 (**Supplementary Table 1**). This number includes snakes whose proximate causes of death were not certain. We acknowledge that most mortality in free-ranging snakes is likely to go undetected due to the cryptic behavior of many snake species, and low probability of detection for sick or dead animals in the wild. We also acknowledge that

robust, longitudinal studies are required to assess potential impacts of ophidiomycosis on fecundity, and on behavior that might increase mortality risk (for example, increased basking while snakes recover could increase risk of depredation). These potential, indirect effects on survivorship deserve further research. Nevertheless, based on available reports of mortality associated with ophidiomycosis in free-ranging snakes, this disease does not appear to be devastating snake populations at this time.

Many studies of ophidiomycosis to date (including ours) focus on individuals that were selected for examination because gross lesions or other clinical signs were observed. Twenty-four of the tallied mortalities were snakes that were taken into captivity for observation, and died in captivity. Outcomes in captivity may not reflect survivorship in free-ranging, infected snakes, and the apparent association between captivity and ophidiomycosis-associated mortality suggests that stress associated with captivity may exacerbate the effects of the disease (33). Furthermore, ophidiomycosis appears to have a high case fatality rate in cases with severe clinical signs, but this conclusion relies heavily on case studies with very small sample sizes [e.g., see Table 1 in Lorch et al. (15)] that do not allow estimates of per capita mortality rates from ophidiomycosis in affected, free-ranging populations. The most robust published survey on the prevalence of potential ophidiomycosis in a wild population (19) reports fungal dermatitis and stomatitis in 9.8% of captured *Sistrurus miliarius barbouri* ($>$ 10,000 captures of $>$ 600 individuals). The pathogen involved was not confirmed by culture or qPCR, but a histopathology image showing arthroconidia erumpent from a skin lesion clearly identifies *O. ophidiicola* (18, 19). No mortalities were associated with the disease, but other effects (e.g., on fecundity or behavior) were not explored. More recently, declines in a population of *C. horridus* in New Hampshire were tentatively associated with observations of a fungal disease in some individuals (29). This study is frequently cited as evidence for population-level effects of ophidiomycosis, and we counted these cases as ophidiomycosis-associated mortalities in our summary, in keeping with our conservative approach. However, we note that Clark et al. (29) did not identify the pathogen, and attributed the observed mortality to a combination of inbreeding, disease, and extreme climate events, not unequivocally to disease. We found no other evidence of likely population declines associated with ophidiomycosis.

Multiple factors may determine the impacts of ophidiomycosis on free-ranging snakes (15). We acknowledge that radio-telemetry studies in ophidiomycosis-exposed populations should be carried out cautiously to ensure that attachment or implantation of transmitters does not increase susceptibility to ophidiomycosis (7, 30). Nevertheless, the impact of ophidiomycosis on individual fitness in free-ranging snakes can only be evaluated by comparing the survivorship, behavior and reproductive output of infected and uninfected individuals in their natural habitats (34, 35). Research on population-level effects of ophidiomycosis will benefit from future studies clearly reporting the following information, where possible: (1) the total number of free-ranging snakes examined, and the survey method; (2) the number of snakes observed with gross lesions; (3) the number of snakes with and without gross lesions that were swabbed and/or biopsied, (4) the number of these samples that tested positive for *O. ophidiicola* using qPCR*;* (5) the number of samples for which histopathology and qPCR confirmed a diagnosis of ophidiomycosis, and (6) the specific diagnostic criteria used.

Our results confirm the presence of *O. ophidiicola* and ophidiomycosis in several snake species across a wide geographic area in Ontario and extend the known distribution of the pathogen substantially northward (**Figure 2**). Some snake species in which *O. ophidiicola* was detected are listed as species at risk in Canada, including *P. spiloides, P. vulpinus,* and *S. catenatus*. If ophidiomycosis poses a current or future threat to population persistence, then the presence of the disease is a conservation concern for Canadian snakes. Fortunately, the available evidence does not suggest that it is causing substantial mortality of free-ranging snakes in Canada at this time. However, ongoing surveillance of ophidiomycosis in free-ranging snakes is advised, as rapidly shifting environmental conditions may alter host susceptibility to wildlife diseases.

Evidence from long-term studies indicates that global declines of many reptile species are severe and broad in terms of both geographic scope and range of taxa affected (15). In a study of 17 snake populations in the UK, France, Italy, Nigeria, and Australia between 1995 and 2009, two-thirds of the monitored populations collapsed and have shown no signs of recovery over the nearly a decade following the populations' declines (36). In Canada, COSEWIC lists 18 of the 33 distinct subspecies of snake as being at risk (http://www.cosewic.gc.ca/). The cause of any specific decline can be a complex interaction of cumulative and sometimes synergistic factors. Globally, reptile populations declines are driven by a combination of threats, including habitat loss and degradation, introduced invasive species, environmental pollution, global climate change, unsustainable use and persecution, and disease and parasitism (37–39). In this framework, the emergence of a widespread and virulent infectious disease could exacerbate existing population declines. However, the data summarized here do not support the prioritization of ophidiomycosis as a conservation crisis. We did not find evidence for population-level impacts of ophidiomycosis on Nearctic snakes. Indeed, individuals in some populations of *S. catenatus* and eastern indigo snakes (*Drymarchon couperi*) appear to tolerate ophidiomycosis (32, 40), and free-ranging *P. vulpinus* with relatively severe clinical signs of ophidiomycosis that are left in the wild can often resolve their clinical signs with no direct effect on fitness (34). Nevertheless, the interactions between ophidiomycosis and other pressures (i.e., syndemics) merit further research, as diseases can interact with other threats such as climate change and landscape modification to exacerbate population declines (41, 42).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ontario Ministry of Natural Resources and Forestry, Wildlife Animal Care Committee.

AUTHOR CONTRIBUTIONS

CD and LSh conceived the study. CD, RD, TB, TDe, TDo, SE, HF, JL, PM, SM, JP, ES, and JU collected samples from wild snakes for diagnostic testing. LSi identified the pathogen on its first documented appearance in Canada, and provided mycology expertise to the study. LSh, DC, CM, NN, and CJ assessed and diagnosed specimens. HC and DS conducted qPCR assays to detect the pathogen. RD, JP, CM, and CD conducted the literature review. RD and JP conducted statistical analyses and generated maps of pathogen distribution. CD, LSh, and LSi led the writing of the manuscript. All authors edited and approved the manuscript prior to submission.

FUNDING

This study was supported by the Government of Ontario and the Canadian Wildlife Health Cooperative.

ACKNOWLEDGMENTS

We thank Monique Aarts, Hower Blair, Leah Brown, Patricia Charlebois-Page, Coral Frenette-Ling, Shuya Huang, Sean Hudson, Hannah McCurdy-Adams, Sheeva Nakhaie, Kyle Ritchie, and Amelia Whitear for assistance in the field. Thanks also to Amanda Duffus and Rachel Marschang for inviting us to submit this paper, and to the two reviewers for suggestions that helped to improve our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.665805/full#supplementary-material

REFERENCES

1. Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis.* (1995) 1:7–15. doi: 10.3201/eid0101.950102
2. Berger L, Roberts AA, Voyles J, Longcore JE, Murray KA, Skerratt LF. History and recent progress on chytridiomycosis in amphibians. *Fungal Ecol.* (2015) 19:89–99. doi: 10.1016/j.funeco.2015.09.007
3. Hoberg EP, Brooks DR. Evolution in action: climate change, biodiversity dynamics and emerging infectious disease. *Philos Trans R Soc B-Biol Sci.* (2015) 370:7. doi: 10.1098/rstb.2013.0553
4. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, et al. Emerging fungal threats to animal, plant and ecosystem health. *Nature.* (2012) 484:186–94. doi: 10.1038/nature10947
5. Voyles J, Kilpatrick AM, Collins JP, Fisher MC, Frick WF, McCallum H, et al. Moving beyond too little, too late: managing emerging infectious diseases in wild populations requires international policy and partnerships. *Ecohealth.* (2015) 12:404–7. doi: 10.1007/s10393-014-0980-5
6. Kirk P. *Ophidiomyces ophidiicola*. *Index Fungorum*. (2020). Available online at: http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=637774 (accessed March 30, 2021).
7. Allender MC, Dreslik M, Wylie S, Phillips C, Wylie D, Maddox C, et al. *Chrysosporium sp*. infection in Eastern Massasauga Rattlesnakes. *Emerg Infect Dis.* (2011) 17:2383–4. doi: 10.3201/eid1712.110240
8. Paré JA, Sigler L. An overview of reptile fungal pathogens in the genera *Nannizziopsis, Paranannizziopsis*, and *Ophidiomyces*. *J Herpetol Med Surg.* (2016) 26:46–53. doi: 10.5818/1529-9651-26.1-2.46
9. Rajeev S, Sutton DA, Wickes BL, Miller DL, Giri D, Meter M, et al. Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola*, from a mycotic granuloma of a Black Rat Snake *(Elaphe obsoleta obsoleta)*. *J Clin Microbiol.* (2009) 47:1264–8. doi: 10.1128/JCM.01751-08
10. Sigler L. *Ophidiomyces* Sigler, Hambleton & Pare, gen.nov. gen. nov. IF550166 and IF550167. *Index Fungorum* 19 (2013).
11. Allender MCC, Raudabaugh DBB, Gleason FHH, Miller ANN, Fisher M. The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. *Fungal Ecol.* (2015) 17:187–96. doi: 10.1016/j.funeco.2015.05.003
12. Cornelison CT, Cherney B, Gabriel KT. Contact-Independent antagonism of *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease by *Rhodococcus rhodochrous* DAP 96253 and select volatile organic compounds. *J Vet Sci Technol.* (2016) 7. doi: 10.4172/2157-7579.1000397. [Epub ahead of print].
13. Eskew EA, Todd BD. Parallels in amphibian and bat declines from pathogenic fungi. *Emerg Infect Dis.* (2013) 19:379–85. doi: 10.3201/eid1903.120707
14. Franklinos LHV, Lorch JM, Bohuski E, Rod J, Wright ON, Fitzpatrick L, et al. Emerging fungal pathogen *Ophidiomyces ophiodiicola* in wild European snakes. *Sci Rep.* (2017) 7:1–7. doi: 10.1038/s41598-017-03352-1
15. Lorch JM, Knowles S, Lankton JS, Michell K, Edwards JL, Kapfer JM, et al. Snake fungal disease: an emerging threat to wild snakes. *Philos Trans R Soc Lond B Biol Sci.* (2016) 371. doi: 10.1098/rstb.2015.0457. [Epub ahead of print].
16. Sutherland WJ, Aveling R, Brooks TM, Clout M, Dicks LV, Fellman L, et al. A horizon scan of global conservation issues for 2014. *Trends Ecol Evol.* (2014) 29:15–22. doi: 10.1016/j.tree.2013.11.004
17. Allain SJR, Duffus ALJ. Emerging infectious disease threats to European herpetofauna. *Herpetol J.* (2019) 29:189–206. doi: 10.33256/hj29.4.189-206
18. Sigler L, Hambleton S, Paré A. Molecular characterization of reptile pathogens currently known as members of the *Chrysosporium* anamorph of *Nannizziopsis vriesii* complex and relationship with some human-associated isolates. *J Clin Microbiol.* (2013) 51:3338–57. doi: 10.1128/JCM.01465-13
19. Cheatwood JL, Jacobson ER, May PG, Farrell TM, Homer BL, Samuelson D, et al. An outbreak of fungal dermatitis and stomatitis in a free-ranging population of pigmy rattlesnakes (Sistrurus miliarius barbouri) in Florida. *J Wildl Dis.* (2003) 39:329–37. doi: 10.7589/0090-3558-39.2.329
20. McBride MP, Wojick KB, Georoff TA, Kimbro J, Garner MM, Wang X, et al. Ophidiomyces ophiodiicola dermatitis in eight free-ranging Timber Rattlesnakes *(Crotalus horridus)* From Massachusetts. *J Zoo Wildl Med.* (2015) 46:86–94. doi: 10.1638/2012-0248R2.1
21. Stengle A. Habitat selection, connectivity, and population genetics of a Timber Rattlesnake (*Crotauls horridus*) metapopulation in southwestern Massachussets. PhD thesis, University of Massachusets (2018).
22. Allender MC, Baker S, Wylie D, Loper D, Dreslik MJ, Phillips CA, et al. Development of snake fungal disease after experimental challenge with *Ophidiomyces ophiodiicola* in cottonmouths (*Agkistrodon piscivorous*). *PLoS ONE.* (2015) 10:e0140193. doi: 10.1371/journal.pone.0140193
23. Lorch J, Lankton J, Werner K, Falendysz EA, McCurley K, Blehert DS. Experimental infection of snakes with *Ophidiomyces ophiodiicola* causes pathological changes that typify snake fungal disease. *MBio.* (2015) 6:1–9. doi: 10.1128/mBio.01534-15

24. McKenzie CM, Oesterle PT, Stevens B, Shirose L, Mastromonaco G, Lillie BN, et al. Ophidiomycosis in red cornsnakes (*Pantherophis guttatus*): potential roles of brumation and temperature on pathogenesis and transmission. *Vet Pathol*. (2020) 57:825–37. doi: 10.1177/0300985820953423
25. McKenzie C, Oesterle PT, Stevens B, Shirose L, Lillie BN, Davy CM, et al. Pathology associated with Ophidiomycosis in snakes in Ontario, Canada. *Can Vet J*. (2020) 61:957–62.
26. Allender MCC, Bunick D, Dzhaman E, Burrus L, Maddox C. Development and use of a real-time polymerase chain reaction assay for the detection of *Ophidiomyces ophiodiicola* in snakes. *J Vet Diagnostic Investig.* (2015) 27:217–20. doi: 10.1177/1040638715573983
27. Baker SJ, Haynes E, Gramhofer M, Stanford K, Bailey S, Christman M, et al. Case definition and diagnostic testing for snake fungal disease. *Herpetol Rev*. (2019) 50:279–85.
28. Burbrink FT, Lorch JM, Lips KR. Host susceptibility to snake fungal disease is highly dispersed across phylogenetic and functional trait space. *Sci Adv*. (2017) 3: e1701387. doi: 10.1126/sciadv.1701387
29. Clark RW, Marchand MN, Clifford BJ, Stechert R, Stephens S. Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: interactions between climate change, disease, and loss of genetic diversity. *Biol Conserv*. (2011) 144:886–91. doi: 10.1016/j.biocon.2010.12.001
30. Tetzlaff S, Allender M, Ravesi M, Smith J. First report of snake fungal disease from Michigan, USA involving Massasaugas, *Sistrurus catenatus* (Rafinesque 1818). *Herpetol Notes.* (2015) 8:31–3.
31. DiRenzo GV, Campbell Grant EH, Longo AV, Che-Castaldo C, Zamudio KR, Lips KR. Imperfect pathogen detection from non-invasive skin swabs biases disease inference. *Methods Ecol Evol.* (2018) 9:380–9. doi: 10.1111/2041-210X.12868
32. Hileman ET, Allender MC, Bradke DR, Faust LJ, Moore JA, Ravesi MJ, et al. Estimation of *Ophidiomyces* prevalence to evaluate snake fungal disease risk. *J. Wildl. Manage.* (2018) 82:173–81. doi: 10.1002/jwmg.21345
33. Allender MC, Phillips CA, Baker SJ, Wylie DB, Narotsky A, Dreslik MJ. Hematology in an Eastern massasauga (*Sistrurus catenatus*) population and the emergence of *Ophidiomyces* in Illinois, USA. *J Wildl Dis.* (2016) 52:258–69. doi: 10.7589/2015-02-049
34. Dillon R. *Disease Ecology of Ophidiomycosis in Free-Ranging Snakes*. Masters thesis, Trent University, Peteroborugh, Ontario (2020).
35. Tetzlaff SJ, Ravesi MJ, Allender MC, Carter ET, DeGregorio BA, Josimovich JM. Snake fungal disease affects behavior of free-ranging Massasauga Rattlesnakes (*Sistrurus catenatus*). *Herpetol Conserv Biol.* (2017) 12:624–34.
36. Reading CJ, Luiselli LM, Akani GC. Are snake populations in widespread decline? *Biol Lett.* (2010) 6:777–780. doi: 10.1098/rsbl.2010.0373
37. Böhm M, Collen B, Baillie JEM, Bowles P, Chanson J, Cox N, et al. The conservation status of the world's reptiles. *Biol Conserv.* (2013) 157:372–85. doi: 10.1016/j.biocon.2012.07.015
38. Falaschi M, Manenti R, Thuiller W, Ficetola GF. Continental-scale determinants of population trends in European amphibians and reptiles. *Glob Chang Biol.* (2019) 25:3504–15. doi: 10.1111/gcb.14739
39. Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, et al. The global decline of reptiles, déjà vu amphibians. *Bioscience.* (2000) 50:653–66. doi: 10.1641/0006-3568(2000)050[0653:TGDORD]2.0.CO;2
40. Chandler HC, Allender MC, Stegenga BS, Haynes E, Ospina E, Stevenson DJ. Ophidiomycosis prevalence in Georgia's Eastern Indigo Snake (*Drymarchon couperi*) populations. *PLoS ONE.* (2019) 14:e0218351. doi: 10.1371/journal.pone.0218351
41. Guégan JF, Ayouba A, Cappelle J, De Thoisy. B. Forests and emerging infectious diseases: unleashing the beast within. *Environ Res Lett.* (2020) 15. doi: 10.1088/1748-9326/ab8dd7. [Epub ahead of print].
42. Heffernan C. The climate change-infectious disease nexus: is it time for climate change syndemics? *Anim Health Res Rev.* (2013) 14:151–4. doi: 10.1017/S1466252313000133

Conflict of Interest: HF was employed by the company Natural Resource Solutions Incorporated.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 21 June 2021
doi: 10.3389/fvets.2021.645491

Long-Term Monitoring of Amphibian Populations of a National Park in Northern Spain Reveals Negative Persisting Effects of *Ranavirus*, but Not *Batrachochytrium dendrobatidis*

Jaime Bosch[1,2*], *Amparo Mora-Cabello de Alba*[3], *Susana Marquínez*[3], *Stephen J. Price*[4], *Barbora Thumsová*[1,2] *and Jon Bielby*[5]

[1] Research Unit of Biodiversity (Consejo Superior de Investigaciones Científicas, Universidad de Oviedo, Principado de Asturias), Oviedo University, Mieres, Spain, [2] Museo Nacional de Ciencias Naturales-Consejo Superior de Investigaciones Científicas , Madrid, Spain, [3] Parque Nacional Picos de Europa, Cangas de Onís, Spain, [4] Genetic Institute, University College London, London, United Kingdom, [5] School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool, United Kingdom

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Reviewed by:
Benoit De Thoisy, Institut Pasteur de la Guyane, French Guiana
Dean Konjević, University of Zagreb, Croatia
Sylvain Larrat, Fauna Vet Service, France

***Correspondence:**
Jaime Bosch
bosch@mncn.csic.es

Specialty section:
This article was submitted to Zoological Medicine, a section of the journal Frontiers in Veterinary Science

Received: *23 December 2020*
Accepted: *21 May 2021*
Published: *21 June 2021*

Citation:
Bosch J, Mora-Cabello de Alba A, Marquínez S, Price SJ, Thumsová B and Bielby J (2021) Long-Term Monitoring of Amphibian Populations of a National Park in Northern Spain Reveals Negative Persisting Effects of Ranavirus, but Not Batrachochytrium dendrobatidis. Front. Vet. Sci. 8:645491. doi: 10.3389/fvets.2021.645491

Amphibians are the most highly threatened vertebrates, and emerging pathogens are a serious threat to their conservation. Amphibian chytrid fungi and the viruses of the *Ranavirus* genus are causing disease outbreaks worldwide, including in protected areas such as National Parks. However, we lack information about their effect over amphibian populations in the long-term, and sometimes these mortality episodes are considered as transient events without serious consequences over longer time-spans. Here, we relate the occurrence of both pathogens with the population trends of 24 amphibian populations at 15 sites across a national Park in northern Spain over a 14-year period. Just one out 24 populations presents a positive population trend being free of both pathogens, while seven populations exposed to one or two pathogens experienced strong declines during the study period. The rest of the study populations (16) remain stable, and these tend to be of species that are not susceptible to the pathogen present or are free of pathogens. Our study is consistent with infectious diseases playing an important role in dictating amphibian population trends and emphasizes the need to adopt measures to control these pathogens in nature. We highlight that sites housing species carrying *Ranavirus* seems to have experienced more severe population-level effects compared to those with the amphibian chytrid fungus, and that ranaviruses could be just as, or more important, other more high-profile amphibian emerging pathogens.

Keywords: ranaviruses, chytrid fungus, amphibian declines, emerging diseases, population trends

INTRODUCTION

Over a quarter of known amphibian species face an elevated risk of extinction (1, 2). Further, many widespread species listed at Least Concern have experienced declines during the last few decades [e.g., (3, 4)]. A growing body of evidence suggests that infectious pathogens have been a major factor in the decline of numerous species and populations (5, 6).

Multiple pathogen types seem to have been involved in these declines, most notably fungi and viruses. Fungi have been highlighted as a major causal agent in vertebrate declines (6–8), with amphibians being severely affected. Viruses are another group of pathogens that have had a disproportionate effect on wildlife health (5), with some species having a high risk of spillover into human populations, with significant impacts on human health (9).

Viruses of the genus *Ranavirus* and fungi of the genus *Batrachochytrium* have had a significant impact on amphibian populations in recent years [e.g., (10, 12)]. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), is a generalist pathogen that has driven declines and extinctions across a broad range of amphibian host species (13, 14). *Bd* is able to infect over 50% of all tested amphibian species, with over 1,000 confirmed host species in at least 86 countries (15). Viral infections caused by ranaviruses (hereafter *Rv*), an emerging group of pathogens with a host range spanning all ectothermic vertebrates, have also become more prevalent and are increasingly associated with mass amphibian die-offs (16, 17). Since *Bd* was described in 1998 (13) the distribution of the fungus has been recorded to include all continents on which amphibians occur (18, 19). Similarly, *Rv* are now found on all continents but Antarctica, although the known geographic distribution remains patchy and, along with the host range of *Rv*, is likely underestimated (20).

The effects of both pathogens vary both inter- and intra-specifically, with impacts ranging from seemingly no effect to population declines (10, 16, 17, 21, 22). Whereas, chytridiomycosis, the disease that may result from *Bd* infection, was quickly recognized as a significant threat to biodiversity, ranavirosis has been slower to gain attention as a threat to amphibian populations. The heterogeneity in individual and population-level response to pathogen exposure means that it is very difficult to ascertain whether the presence of a pathogen, or pathogens, will lead to ill-effects for host-species.

The temporal scale over which disease emergence may affect host populations must be taken into account when attempting to better understand host-pathogen(s) dynamics. Unfortunately, whereas highly visual disease outbreaks in naïve amphibian populations have been recorded, the long-term disease impacts have received less attention, leaving a gap in our knowledge of how infectious disease affects amphibian hosts in the long-term.

The amphibians of Picos de Europa National Park (PNPE) have a long-history of exposure to both *Rv* and *Bd*, and exhibit a great deal of heterogeneity in their response to the pathogens (10). Outbreaks of ranavirosis were first observed in the park in 2005 and resulted in severe and dramatic declines in some species at several locations (10). *Bd* was also found to be present in PNPE shortly after the first mortality incidents associated with ranavirus, although no evidence of chytridiomycosis emergence has been observed (10). Two of the most common species in the park, the common midwife toad (*Alytes obstetricans*) and the alpine newt (*Ichthyosaura alpestris*), can act as drivers of persistence and spread of infection within the community (23). However, although both pathogens are present and life stages of some species harbor infection at high levels, the long-term effects on the host populations remain unclear.

Here, we report the results of long-term monitoring of 24 amphibian populations at 15 sites in a protected area in northern Spain. The monitoring took place for 14 years after an initial disease outbreak of both pathogens (2007–2020). To our knowledge, these analyses represent the first attempt to monitor the long-term effects of *Rv* and *Bd* concurrently in multiple amphibian species and their populations at multiple sites. We used amphibian population data and disease surveillance to identify whether the distribution of pathogens and host population statuses are consistent with long-term disease-related amphibian declines at PNPE.

To do so we aim to answer the following specific questions:

- What are the 14-year trends of the 24 populations in this study?
- Are populations under decline more likely to have experienced mass mortality events, or have *Bd* or *Rv* present compared to populations that are stable?
- Are the prevalence of *Bd* or *Rv* infection higher in sites where mass mortalities were recorded, or in sites containing populations under decline?
- Is the population trend of a population associated with prevalence of pathogen infections?

For these questions we worked with the hypotheses that the prevalence of infection with the pathogens, and the presence of mass mortalities, would be negatively associated with the population status [e.g., (12, 24)].

METHODS

The Picos de Europa National Park (hereafter PNPE) is a protected wilderness area comprised of limestone mountains in the north of Spain. It is an important wildlife area but is also used for recreational activities and stockbreeding. There are nine species of amphibian that can be found in the park, but two of them, the golden-striped salamander (*Chioglossa lusitanica*) and the marbled newt (*Triturus marmoraturs*) occur at only a very small number of locations within the park boundary. This study therefore focused on the remaining seven, more commonly found, species: the fire salamander (*Salamandra salamandra*), the alpine newt (*I. alpestris*), the palmate newt (*Lissotriton helveticus*), the midwife toad (*A. obstetricans*), the spiny toad (*Bufo spinosus*), the Iberian frog (*Rana iberica*) and the common frog (*Rana temporaria*).

Population Estimates

We comprehensively surveyed 15 sites containing 24 amphibian populations during the amphibian reproductive and development period (May to September) each year from 2007 to 2020 (**Table 1**). For each species, population monitoring focused on the sites and life-stages that would maximize the probability of obtaining consistently reliable population estimates that would act as a robust measure of population size for that species. For *A. obstetricans* we counted larvae at water bodies instead of terrestrial adults that are difficult to locate. For *S. salamandra* we counted adults crossing roads and/or larvae at

TABLE 1 | Results of TRIM models analyzing count data generated in 24 amphibian populations located at 15 sites during a 14 year period.

						AICc		Linear model			Additive change							
Site	**Altitude (m), habitat**	**Species**	**Live stage surveillance**	**Number of surveys**	**Serial r**	**Null**	**Linear**	**Slope**	**SE**	**p**	**Parameter**	**SE**	**p**	**Population trend**	***Bd* pos/n**	***Bd* prevalence**	***Rv* pos/n**	***Rv* prevalence**
Bajero	1,865, small lake	*Alytes obstetricans*	Larvae	68	−0.087	31,789.3	13,142.6	−0.0808	0.0245	0.0010	−0.1126	0.0011	<0.0001	Decline	0/69	0.0000–0.0521	8/54	0.0662–0.2712
		Ichthyosaura alpestris	Adults	74	0.247	3,198.9	2,732.9	−0.0340	0.0724	0.6391	−0.0704	0.0054	<0.0001	Stable	–	–	–	–
Pozo Llau	1,860, small lake	*Alytes obstetricans*	Larvae	62	−0.126	7,430.1	5,383.5	−0.1815	0.0937	0.0526	−0.1146	0.0044	<0.0001	Decline	2/43	0.0057–0.1581	29/35	0.6635–0.9344
		Ichthyosaura alpestris	–	–	–	–	–	–	–	–	–	–	–	–	0/3	0.0000–0.7076	0/3	0.0000–0.7076
		Rana temporaria	Egg clutches	50	−0.211	312.98	359.81	0.0150	0.0633	0.8131	−0.0389	0.0148	<0.0001	Stable	2/5	0.0527–0.8534	–	–
		Salamandra salamandra	Larvae	59	−0.328	30.1	20.3	−0.1970	0.0804	0.0142	–	–	–	Decline	0/1	–	1/1	–
Lloroza	1,860, small lake	*Alytes obstetricans*	Larvae	72	0.007	11,631.6	3,461.2	−0.3839	0.1081	0.0004	−0.3839	0.1081	<0.0001	Decline	3/48	0.0131–0.1720	21/54	0.2592–0.5312
		Bufo spinosus	–	–	–	–	–	–	–	–	–	–	–	–	2/69	0.0035–0.1008	48/76	0.5131–0.7394
		Ichthyosaura alpestris	Adults	74	0.371	3795.8	2201.78	−0.1489	0.1099	0.1754	–	–	–	Stable	25/223	0.0739–0.1610	113/226	0.4330–0.5670
		Rana temporaria	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/2	0.0000–0.8419
Andara	1,750, small lake	*Alytes obstetricans*	–	–	–	–	–	–	–	–	–	–	–	–	9/18	0.2602–0.7398	4/12	0.0992–0.6511
		Ichthyosaura alpestris	Adults	101	−0.277	119.1	109.25	−0.0521	0.0366	0.1542	−0.0514	0.0157	<0.0001	Stable	–	–	1/1	–
		Lissotriton helveticus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Moñetas	1,730, small lake	*Alytes obstetricans*	Larvae	69	−0.165	47,797.7	8,975.4	−0.4923	0.0847	<0.0001	−0.4618	0.0103	<0.0001	Decline	0/6	0.0000–0.4593	5/8	0.2449–0.9148
		Ichthyosaura alpestris	Adults	67	−0.227	496.6	478.7	−0.0233	0.0292	0.4251	−0.0065	0.0065	<0.0001	Stable	1/22	0.0012–0.2284	5/24	0.0713–0.4215
Cable	1,730, group of ponds	*Ichthyosaura alpestris*	Adults	66	0.153	222.5	946.3	0.0780	0.0949	0.0538	0.0843	0.0085	<0.0001	Stable	0/18	0.0000–0.1853	0/18	0.0000–0.1853
		Lissotriton helveticus	Adults	64	0.074	329.2	240.4	0.0869	0.0604	0.1503	0.0225	0.0154	<0.0001	Stable	0/1	–	0/1	–
		Rana temporaria	Egg clutches	62	0.001	135.2	133.3	0.0005	0.0253	0.9841	0.0047	0.0068	<0.0001	Stable	0/1	–	0/1	–
Vega Salambre	1,300, pond	*Rana temporaria*	Egg clutches	64	0.019	662.3	931.31	0.0073	0.0413	0.8602	−0.0158	0.0066	<0.0001	Stable	–	–	–	–
Vau los lobos	1,130, cattle tank	*Alytes obstetricans*	–	–	–	–	–	–	–	–	–	–	–	–	0/11	0.0000–0.2849	0/11	0.0000–0.2849
		Ichthyosaura alpestris	Adults	39	−0.115	2.7	−3.0	0.0801	0.029	0.0072	0.0775	0.0291	0.2463	Increase	–	–	–	–
		Lissotriton helveticus	Adults	38	0.109	113.4	114.0	−0.0106	0.0485	0.8275	−0.0115	0.0124	<0.0001	Stable	–	–	–	–
Rasa Pandecarmen	1,117, pond	*Rana temporaria*	Egg clutches	45	0.425	816.8	3,617.6	0.0985	0.1128	0.3823	–	–	–	Stable	–	–	–	–
Ercina	1,110, lake	*Alytes obstetricans*	–	–	–	–	–	–	–	–	–	–	–	–	72/102	0.6075–0.7920	49/183	0.2051–0.3381
		Bufo spinosus	Adults	74	0.104	758.2	79.5	−0.2452	0.0422	<0.0001	−0.2084	0.1096	<0.0001	Decline	2/42	0.0058–0.1616	14/47	0.1734–0.4489
		Ichthyosaura alpestris	–	–	–	–	–	–	–	–	–	–	–	–	2/43	0.0057–0.1581	6/43	0.0530–0.2793
		Lissotriton helveticus	–	–	–	–	–	–	–	–	–	–	–	–	2/2	0.1581–1.0000	0/2	0.0000–0.8419
		Rana temporaria	–	–	–	–	–	–	–	–	–	–	–	–	8/38	0.0955–0.3732	6/39	0.0586–0.3053
La Güelga	1,050, cattle tank and stream pond	*Alytes obstetricans*	–	–	–	–	–	–	–	–	–	–	–	–	33/87	0.2774–0.4897	1/34	0.0000–0.1533
		Ichthyosaura alpestris	Adults	42	−0.089	34.8	18.4	−0.173	0.0983	0.0786	–	–	–	Stable	–	–	–	–
		Lissotriton helveticus	Adults	63	0.270	824.0	666.0	−0.0723	0.1072	0.4996	−0.0127	0.0093	<0.0001	Stable	–	–	1/1	–
Allende Cabañes	790, stream	*Rana iberica*	Larvae	33	−0.342	556.1	490.6	−0.1995	0.2096	0.3411	–	–	–	Stable	0/2	0.0000–0.8419	0/2	0.0000–0.8419
		Alytes obstetricans	–	–	–	–	–	–	–	–	–	–	–	–	0/10	0.0000–0.3085	0/14	0.0000–0.2316
Pontón-Oseja	1,260–750, road	*Salamandra salamandra*	Adults	67	−0.131	426.9	377.4	−0.0558	0.0614	0.3632	−0.0764	0.0137	<0.0001	Stable	0/19	0.0000–0.1765	0/19	0.0000–0.1765
La Guxana	200, stream	*Rana iberica*	Larvae	29	0.068	1,085.9	880.3	−0.1125	0.1520	0.4592	–	–	–	Stable	0/3	0.0000–0.7076	0/7	0.0000–0.4096
		Bufo spinosus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/13	0.0000–0.2471
Soto Cangas-Covadonga	200, road	*Bufo spinosus*	Adults	40	0.020	2,232.5	209.1	−0.5096	0.0820	<0.0001	–	–	–	Decline	0/20	0.0000–0.1684	17/26	0.4433–0.8279

Two different models were constructed for each population; a no time-effects models (null; testing for the absence of population trends) and linear trend (assuming an increasing or decreasing monotonic trend). Serial r: serial correlation measuring the association of the counts in year t with year t−1. The AIC figures are provided for each of these models, as is the slope of the linear trend model, its standard error and significance (against Ho: slope = 0). The additive parameter defines the overall additive change in abundance for each species (SE = standard error and the significance of deviation from the null hypothesis of 0 –p–). Population counts remained constant if the additive parameter equaled zero; an additive figure of −0.1126 denotes that from the first to the last year of amphibian counts the population has decreased at an average inter-annual rate of 11.3%. All significant tests remain significant after Bonferroni sequential correction. For each population and pathogen, the number of positive and total samples analyzed, as well the Clopper-Pearson confidence intervals, are shown.

water bodies. For alpine and palmate newts (*I. alpestris* and *L. helveticus*) we counted adults at water bodies. For *R. temporaria* we counted egg clutches at small and shallow ponds whereas for *R. iberica* we counted larvae in selected, accessible stream sections. Finally, for *B. spinosus* we counted egg clutches at water bodies and/or adults on roads.

In order to sample this diverse set of species and different life-history stages at 15 heterogeneous sites a range of survey techniques were required. Surveys included transect walks ($n = 11$), walks along fixed sections of streams ($n = 2$) and car transects along specified sections of roads ($n = 2$). Road surveys for adult amphibians were conducted by a single observer driving at very slow speed at twilight. Water body surveys were completed during the day by a pair of observers traveling on foot at a consistent speed to maintain a standardized sampling effort and counting every animal or clutch. Both observers independently counted every observation resulting in a total estimate of abundance. If one of the counts was more than twice the other, the survey was repeated, otherwise the mean count was used as the abundance. We estimated population abundances up to 12 times per year (mean = 4.7; **Table 1**). Thus, the methodology and life history stage targeted varied with the characteristics of the study species and the locality but remained consistent for each population within and between years.

A mass mortality incident was defined as an unusually high number (more than five) of dead animals of a single species being recorded at a given site on more than one occasion during a single year.

Pathogen Diagnostic Sampling

Tail or toe-clips were used for diagnosis of both *Rv* and *Bd*, except for *Bd* in anuran larvae. The latter only become infected in their oral disc, and so for *A. obstetricans* larvae an oral disc swab was taken for *Bd* diagnosis. Since *B. spinosus*, *R. iberica*, and *R. temporaria* have smaller larvae, oral swabs are not a reliable method of sampling, and tail clips would compromise animal welfare. Individuals were therefore humanely euthanized using an overdose of buffered MS222 (5 g/l), and the whole oral disc was used for detection of *Bd*. All tissue samples were fixed in 70% ethanol in the field. Swabs were kept dry and refrigerated prior to processing in the laboratory.

In terms of sample sizes, for each species individuals were sampled for *Bd*/*Rv* infection exhaustively for those sampled via swabs, and up to a maximum of 20 per population and year in the case of tissue samples of live individuals. Most samples used in this study were collected during the past 5 years. All research was performed in accordance with relevant guidelines and regulations and under license from the PNPE. All experimental protocols were approved by Comisión Ética de Experimentación Animal MNCN-CSIC (reference 666/2018).

DNA was extracted from tissue samples using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA was obtained from swabs with PrepMan Ultra following Hyatt et al. (25). qPCR for *Bd* and *Rv* was performed following Boyle et al. (26) and Leung et al. (27), respectively, on a MyGo Pro PCR machine. Negative controls and standards with known concentrations of *Bd*/*Rv* were used in each plate.

Statistical Analyses

To obtain population trends through time for further analyses, population monitoring data generated as described above were used in time-effects model. Modeling was performed using TRIM, software developed specifically for time series of animal counts from monitoring programs (28). Log-linear Poisson regression models were used on the species' count data from 2007 to 2020, using the maximum value obtained for each species' population as the best estimate of the population size for that year. The population estimate was included as the continuous predictor and the number or surveys performed each year for a particular population as weights. These models accounted for over-dispersion and serial autocorrelation in the data to obtain population trends (±1 SE) as the slope of the regression of the logarithms of the yearly indices. Standard errors of the trends were estimated as a measure of uncertainty in average linear population trends. We did not use non-linear models as our objective was to investigate overall change during the course of the sampling period and not annual, or other short-term fluctuations in abundance [see also (12, 29) for a similar approach]. Linear trend TRIM models were compared with their corresponding null TRIM models (not including the linear effect of year) using Akaike's Information Criterion (AIC). We estimated the overall additive change for each species by measuring the average inter-annual rate from the first to the last year of the study.

One-tail Fisher's exact tests were used to check whether population trends were associated with observation of mass mortality events, and if the status of amphibian populations were associated with the presence of *Bd*, *Rv*, or both at the site. To do this contingency tables were constructed using the count of declining and non-declining sites against the count of sites ± mass mortality; ± *Bd* presence; and ± *Rv* presence respectively.

One-tail *t*-tests were used to determine whether there was a significant association between the prevalence of *Bd* and *Rv* and the occurrence of mass mortalities, and also between the pathogen prevalence and the presence of declining populations at a site. To do this, for each site, the prevalence of each pathogen was averaged across all sampled species located in each site. Those sites were then categorized depending on whether one of their constituent species had experienced a mass mortality or not and whether one of their constituent species exhibits a declining population or not.

Finally, we used a general linear mixed model to analyze if the slope of the linear trend models for the 24 studied populations, across species and sites (as random factors), was related to the log-transformed *Bd* and *Rv* obtained values of prevalence. The number of surveys was introduced as a covariate into the analysis, and species was considered as a random factor because different species were surveyed using different life-stages. For populations of species in which the prevalence of *Bd*/*Rv* was not obtained, a value of 0 was assigned if a co-existing population of the higher sensitive species *A. obstetricans* in that site tested free for *Bd*/*Rv*.

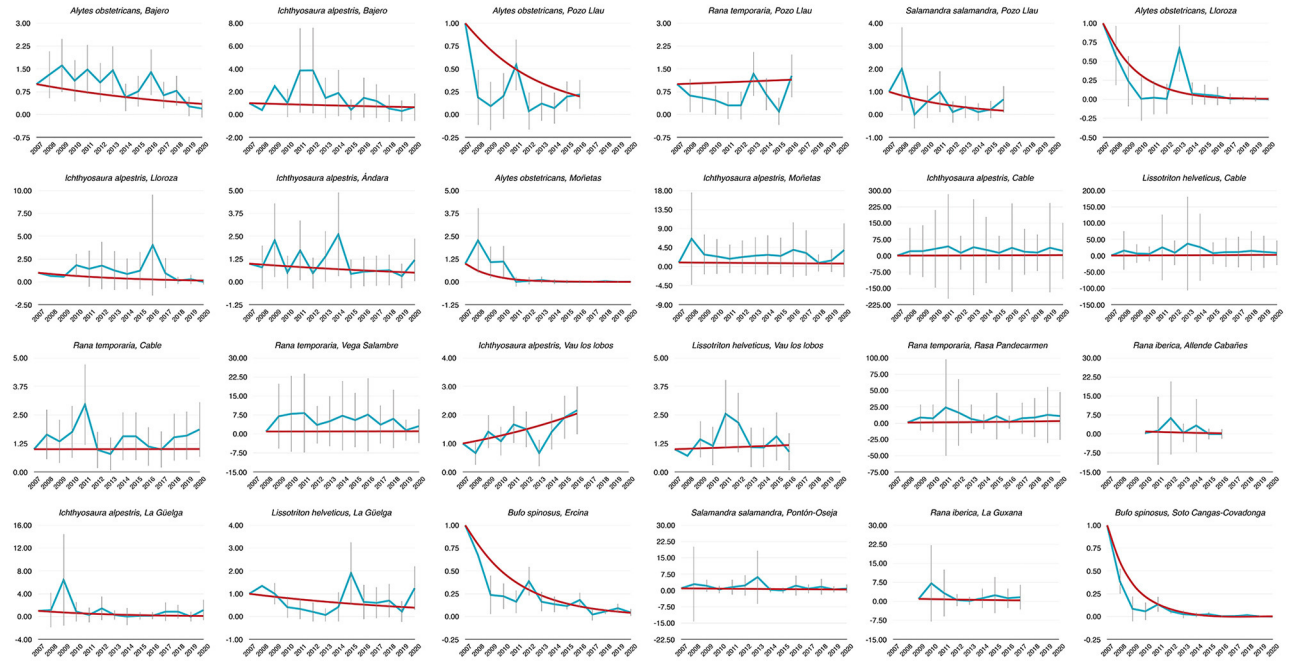


FIGURE 1 | Dynamics of abundance of 24 populations of PNPE estimated from 2007 to 2020. Time index 1 refers to the amphibian counts measured in 2007 (i.e., the baseline in first sampling year). Blue lines are annual abundance estimates, while red lines were established by means of TRIM models.

FIGURE 2 | Map of PNPE with sampling sites and signs of ranavirosis in three species. *Alytes obstetricans* adults with severe limb necrosis **(A)** or erythema **(B)**, metamorph with erythema and swelling of the body and legs **(C)**, and larvae with systemic hemorrhage **(D, E)**. *Ichthyosaura alpestris* adults with missing eyes **(F)** or cachexia **(G)** and larvae with systemic hemorrhaging **(H)**. *Bufo spinosus* adults with erythema **(I)** and with open lesion on the feet **(J)**. Study locations of Ercina **(K)**, Lloroza **(L)**, Moñetas **(M)** and Pozo Llau **(N)**, and signage to inform visitors about the presence of *Rv* in PNPE **(O)**.

In other cases the averaged prevalence of *Bd*/*Rv* per species at that particular site was used.

RESULTS

Population trends over the period 2007–2020 were obtained from TRIM analysis and are outlined in **Table 1** and shown in **Figure 1**. In total data for 24 amphibian populations at 15 sites were collected during this time period, during which 60% of sites held non-declining populations, the remaining 40% of sites held a declining population of at least one species. Declining population trends were obtained for *A. obstetricans* (4/4), *B. spinosus* (2/2), and *S. salamandra* (1/2). Non-declining population trends were obtained for *I. alpestris* (7/7), *L. helveticus* (3/3), *R. iberica* (2/2), and *R. temporaria* (4/4). Mass mortalities were recorded in 47% of the study sites. *Bd* and *Rv* were present in 46 and 62% of sites, respectively. During the time-frame of the study we have consistently recorded mass mortality incidents consistent with ranavirosis in four species: *A. obstetricans*, *I. alpestris*, *S. salamandra*, and *B. spinosus* (**Figure 2**).

There was a significant association between the occurrence of mass mortality and whether or not a site held a population of species in decline: 86% of the sites in which mass mortalities were recorded were home to declining populations (Fisher's exact test, $p = 0.0014$). There were contrasting patterns in relation to whether sites with declining populations had associations with the presence of amphibian pathogens: there was no significant difference in the status of sites with and without declining populations and the presence of *Bd* (declining and *Bd*-positive = 67%; declining and *Bd*-negative = 33%; Fisher's exact test, $p = 0.2086$). On the other hand, none of the *Rv*-free sites were home to declining populations, whereas just 25% of the sites where *Rv* is present are not home to populations which have declined (Fisher's exact test, $p = 0.0163$). These results suggest that sites that have experienced mass mortality are more likely to have declining populations, and that *Rv*, but not *Bd*, is associated with declining populations within a site.

There was no association between the average *Bd* prevalence at a site and the occurrence of a mass mortality in one of its species (0.1542 vs. 0.0632, $t_{11} = 0.9032$, $p = 0.1929$), whereas the prevalence of *Rv* was significantly higher at sites in which a mass mortality was recorded (0.4309 vs. 0.0630, $t_{11} = 4.5438$, $p = 0.0004$). Sites with populations under decline exhibit similar *Bd* prevalence to sites supporting non-declining populations (0.0967 vs. 0.1256, $t_{11} = 0.2774$, $p = 0.3933$), whereas those sites with populations under decline present higher values of averaged *Rv* prevalence (0.4385 vs. 0.063, $t_{11} = 3.4364$, $p = 0.0028$).

The general linear mixed model explained 88% of the variation in the slopes of the 24 studied populations. The slopes are not related to the number of surveys ($F_{1,13} = 1.9320$, $p = 0.1874$) nor the prevalence of *Bd* ($F_{1,16} = 2.6965$, $p = 0.1199$). On the other hand, the prevalence of *Rv* is a key factor in the variation of the population slopes ($F_{1,18} = 10.5318$, $p = 0.0046$): those populations with higher values of *Rv* prevalence present slopes with the largest negative coefficients.

DISCUSSION

Analyses of 14 years of amphibian population data at multiple sites for multiple species suggest that population declines in this community of amphibians are consistently associated with the presence of ranavirosis. *Bd*, the causal agent of chytridiomycosis, does not seem to be associated with disease emergence or population impacts, which contrasts with the situation in another montane region in Spain, Guadarrama National Park, in which two of nine species exhibited severe population-level effects of chytridiomycosis (12).

It is unclear why *Bd*, which has consistently affected montane communities of similar species in other parts of Europe has not had major population-level impacts at PNPE. One possibility is that *Bd* and *Rv* (which were first detected in 2005) caused declines in the most susceptible species before systematic monitoring of populations began (monitoring started in 2007). The common midwife toad, *A. obstetricans*, is known to be important in the persistence and spread of both *Bd* and *Rv* in amphibian communities (23, 30). If their populations were reduced significantly before 2007, their role in the transmission of *Bd* infection to other species could have been greatly reduced, and hence the impact of *Bd* on sympatric species could have significantly reduced, which is consistent with the lack of strong association between the presence of *Bd* and mass mortalities or population decline. In contrast *Rv*, which can be spread more readily by other species, could continue to drive population reductions even in the absence of *Alytes obstetricans*. Alternatively, and since it has been proved that susceptibility to an emerging pathogen is related to population genetic diversity [e.g., (31)], *A. obstetricans* populations of northern Spain may be more protected than others on the Iberian Peninsula because of their broad distribution and abundance (32).

Three species in the PNPE, *A. obstetricans*, *B. spinosus*, and *S. salamandra* exhibited declining population trends over the 14 years of the study. All study populations of *A. obstetricans* (4/4) were declining, with some having been practically extirpated. The one population of *S. salamandra* infected with *Bd* and *Rv* has been reduced to very low abundance, whereas the other population—apparently free of infection by either pathogen—exhibited a stable population size over the 14-year period. Both populations of *B. spinosus* experienced sharp declines when infected with *Rv* or with both pathogens. These three species represent the most heavily *Bd*-affected species in Guadarrama National Park (12). *Bufo spinosus* and *S. salamandra*, have been severely affected in PNPE and in Guadarrama NP and appear to be sensitive to both *Bd* and *Rv*. These two species present a broad distribution in the Iberian Peninsula, and have experienced a remarkable decline during the last few decades (33), the drivers of which are poorly understood. However, given their susceptibility to infectious disease it remains a possibility that those declines and extirpations were driven by pathogens.

Four species at PNPE had stable population trends between 2007 and 2020, highlighting the heterogeneity in population-level effects of infectious disease. The palmate newt, *L. helveticus*, and common frog, *R. temporaria*, look to be unaffected by both pathogens at the population-level. Most populations remain free

of both pathogens, and when infected, their population trends are stable. This finding in respect of *R. temporaria* is somewhat surprising given that ranavirosis in this species has been observed in this species and the severe impact it has had on the species in England (11, 21). However, common frogs in England are mostly infected by frog virus 3-like ranaviruses (34, 35) whilst common midwife toad ranaviruses circulate in PNPE (10). In addition, the severe effects of ranavirosis in English frogs have generally been observed in populations in residential garden ponds (35, 36), which provide a very different environment to that observed in the protected area of the PNPE. It is therefore unclear whether viral genetics, environmental effects or some other factor explains the different outcome for *R. temporaria* in the PNPE. The two study populations of *R. iberica*, even when they are present in very small numbers, also remain stable and free of both pathogens.

The final species monitored in this study seems to have had a more complex history of dynamics with these pathogens. The alpine newt, *I. alpestris*, which experienced recurrent mass mortalities and sharp declines during the initial period following *Rv* emergence (10), is apparently recovering at sites housing infected individuals. At the population-level, *I. alpestris* has remained stable over 14 years, and populations are increasing in some locations that are free of pathogens. These results are broadly similar to those recorded at Guadarrama National Park (12): even when most larvae and adults are infected with *Bd* (37), the species continues to expand its distribution and population size soon after its introduction in the area (38). Again, an explanation for the apparent rebound of *I. alpestris* during the last years could be the sharp decline of *A. obstetricans*. Adult *I. alpestris* were observed often in the area feeding on moribund and heavily *Rv* infected larvae of *A. obstetricans* during the first years after ranavirosis emerged. These animals soon presented facial hemorrhaging, swollen skin around the mouth, problems opening jaws and even loss of eyes. In recent years, when no moribund *Alytes* tadpoles were present in the water, adult *I. alpestris* exhibited less severe and less frequent signs of ranavirosis (J. Bosch, personal observations) despite *Rv* infection being persistent in the species. Over-wintering larvae are known to be an important host for many infectious pathogens, including *Bd* and *Rv* (23, 39–41), and so perhaps with a reduction in their abundance other species can benefit from more breaks in the chain of initial transmission and subsequent re-infections.

The long-term outlook for amphibian populations in PNPE remains unclear; our results suggest that *Rv* and not *Bd* is more closely associated with population declines in the past 13 years. Our results are similar to those obtained in other, similar communities in the Iberian Peninsula (e.g., the species exhibiting the highest prevalence levels and susceptibility to decline), but vary in others (the importance of the pathogens in question). The effects of both pathogens can be highly context-dependent, with declines perhaps being driven by interactions with other important factors including climate (11, 42); ozone levels (43); and microbiome (44, 45) of hosts; and the specific ecological context in which the host community lives (23). All of these factors are likely to interact with pathogens as a driver of population-level effects and warrant careful attention both alone and in combination with each other.

Altitude appears to be the key factor driving *Bd* infections in Iberia [e.g., (46)] and dictating chytridiomycosis outbreaks (47, 48), but in our study area mass mortalities occurred across a broad range of altitudes (from 200 to 1,865 m). Within this range the accessibility of the sites to human traffic varies greatly, which could affect the likelihood of pathogen introduction and spread: those study sites with low visitor numbers appear to be the ones free of one or both pathogens. PNPE receives more than two millions visitors per year, with a great number of visitors coming from overseas. Unfortunately, no biosecurity measures have been adopted to avoid pathogen or pest introductions. Among other things, encouraging visitors to undertake biosecurity hygiene practices such as cleaning footwear is desirable, and possibility of installing physical barriers at some sites to avoid visitors coming into direct contact with water and moving animals between sites could also be a positive step in preventing the spread of pathogens.

This study highlights the long-term, persistent effects of pathogens of the genus *Ranavirus* on amphibian populations and communities. Over a 14 year period *Rv* was consistently associated with declines, whereas *Bd* appears to have a weaker, if any association. Long-term datasets and their analyses provide a means to identify and highlight host-pathogen dynamics that are not immediately obvious, or may even be missed entirely from shorter-term studies. While *Bd* is still heralded as the major disease threat to amphibians [e.g., (49)], long-term site-level surveillance is often not included in the related research. Our results suggest that, in addition to large-scale comparative analyses, longer-term, more detailed studies encompassing a broader range of pathogens may prove informative for planning conservation action.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité de Ética, CSIC.

AUTHOR CONTRIBUTIONS

JBo: conceptualization, funding acquisition, investigation, methodology, data curation, formal analysis, writing - initial draft and review and editing. AM-C and SM: data curation and writing - review and editing. SP: writing - review and editing. BT: formal analysis and writing review and editing. JBi: writing - initial draft and review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Spanish Ministry for Science, Innovation and Universities (CGL2015-70070-R, PI: JBo and

RTI2018-095023-B-I00, PI: Luz Boyero) and by Organismo Autónomo Parques Nacionales of Spain (2399/2017, PI: JBo).

ACKNOWLEDGMENTS

This work was possible thanks to the support from people working at Picos de Europa NP, especially Dolores Hórreo, Ramón González, José Luis Mosquera, Manuel Díaz, Ángel Tejedor, Pedro A. Fernández, Víctor Delgado, Fernando Rey, José Enrique Díez, Julio M. Martínez, Elías Ignacio Díez, Marcelino Fernández, Félix Rojo, Miguel Ángel Bermejo, José Manuel Castrillo, Rubén Varona, Saturnino González, Iván Alonso, Sergio Casares, Sara González and César Obeso. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

REFERENCES

1. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. *Science.* (2004) 306:1783–6. doi: 10.1126/science.1103538
2. Stuart SN, Hoffmann M, Chanson JS, Cox NA, Berridge RJ, Ramani P, et al. *Threatened Amphibians of the World*. Barcelona, Spain: Lynx, Editions (2008) 132 p.
3. Adams MJ, Miller DA, Muths E, Corn PS, Grant EHC, Bailey LL, et al. Trends in amphibian occupancy in the United States. *PLoS ONE.* (2013) 8:e64347. doi: 10.1371/journal.pone.0064347
4. Petrovan SO, Schmidt BR. Volunteer conservation action data reveals large-scale and long-term negative population trends of a widespread amphibian, the common toad (Bufo bufo). *PLoS ONE.* (2016) 11:e0161943. doi: 10.1371/journal.pone.0161943
5. Daszak P, Cunningham AA, Hyatt A. Emerging infectious diseases of wildlife- threats to biodiversity and human health. *Science.* (2000) 287:443–9. doi: 10.1126/science.287.5452.443
6. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, et al. Emerging fungal threats to animal, plant and ecosystem health. *Nature.* (2012) 484:186–94. doi: 10.1038/nature10947
7. Frick WF, Puechmaille SJ, Willis CKR. White-nose syndrome in bats. In: Voigt C, Kingston T, editors. *Bats in the Anthropocene: Conservation of Bats in a Changing World*. Cham: Springer (2016). p. 245–62.
8. 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 USA*. (2013) 110:15325–9. doi: 10.1073/pnas.1307356110
9. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
10. Price SJ, Garner TWJ, Nichols RA, Balloux F, Ayres C, Mora-Cabello de Alba A, et al. Collapse of amphibian communities due to an introduced ranavirus. *Curr Biol.* (2014) 24:2586–91. doi: 10.1016/j.cub.2014.09.028
11. Price SJ, Leung WTM, Owen CJ, Puschendorf R, Sergeant C, Cunningham AA, et al. Effects of historic and projected climate change on the range and impacts of an emerging wildlife disease. *Global Change Biol.* (2019) 25:2648–60. doi: 10.1111/gcb.14651
12. Bosch J, Fernández-Beaskoetxea S, Garner TWJ, Carrascal LM. Long-term monitoring of an amphibian community after a climate change- and infectious disease-driven species extirpation. *Glob Change Biol.* (2018) 24:2622–32. doi: 10.1111/gcb.14092
13. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci USA*. (1998) 95:9031–6. doi: 10.1073/pnas.95.15.9031
14. Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, et al. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Natl Acad Sci USA*. (2006) 103:3165–70. doi: 10.1073/pnas.0506889103
15. Castro Monzon F, Rödel MO, Jeschke JM. Tracking *Batrachochytrium dendrobatidis* infection across the globe. *Ecohealth.* (2020) 17:270–9. doi: 10.1007/s10393-020-01504-w
16. Green DE, Converse KA, Schrader AK. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. *Ann NY Acad Sci.* (2002) 969:323–39. doi: 10.1111/j.1749-6632.2002.tb04400.x
17. Price SJ, Ariel E, Maclaine A, Rosa GM, Gray MJ, Brunner JL, et al. From fish to frogs and beyond: impact and host range of emergent ranaviruses. *Virology.* (2017) 511:272–9. doi: 10.1016/j.virol.2017.08.001
18. Fisher MC, Garner TW, Walker SF. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annu Rev Microbiol.* (2009) 63:291–310. doi: 10.1146/annurev.micro.091208.073435
19. Lips KR. Overview of chytrid emergence and impacts on amphibians. *Phil Trans R Soc B.* (2016) 371:20150465. doi: 10.1098/rstb.2015.0465
20. Duffus ALJ, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, et al. Distribution and host range of ranaviruses. In: Gray M, Chinchar V, editors. *Ranaviruses*. Cham: Springer (2015). p. 9–57.
21. Teacher AGF, Cunningham AA, Garner TWJ. Assessing the long-term impact of Ranavirus infection in wild common frog populations. *Anim Conserv.* (2010) 13:514–22. doi: 10.1111/j.1469-1795.2010.00373.x
22. Rosa GM, Sabino-Pinto J, Laurentino TG, Martel A, Pasmans F, Rebelo R, et al. Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. *Sci Rep.* (2017) 7:43260. doi: 10.1038/srep43260
23. Bielby J, Price SJ, Monsalve-Carcaño C, Bosch J. Host contribution to parasite persistence is consistent between parasites and over time, but varies spatially. *Ecol Appl.* (2021) 31:e02256. doi: 10.1002/eap.2256
24. Briggs CR, Vredenburg VT, Knapp RA, Rachowicz LJ. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology.* (2005) 86:3149–59. doi: 10.1890/04-1428
25. 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*. *Dis Aquat Org.* (2007) 73:175–92. doi: 10.3354/dao073175
26. Boyle DG, Boyle D, Olsen V, Morgan J, Hyatt A. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis Aquat Organ.* (2004) 60:141–8. doi: 10.3354/dao060141
27. Leung WT, Thomas-Walters L, Garner TWJ, Balloux F, Durrant C, Price S. A quantitative-PCR based method to estimate ranavirus viral load following normalisation by reference to an ultraconserved vertebrate target. *J Virol Methods.* (2017) 249:147–55. doi: 10.1016/j.jviromet.2017.08.016
28. Pannekoek J, van Strien AJ. *TRIM 3 manual*. Voorburg, The Netherlands: Statistics Netherlands (2005).
29. Heldbjerg H, Fox AD. Long-term population declines in Danish trans-Saharan migrant birds. *Bird Study.* (2008) 55:267–79. doi: 10.1080/00063650809461532
30. Fernández-Beaskoetxea S, Bosch J, Bielby J. Heterogeneity in infection and transmission of a multi-host pathogen within a community of amphibians. *Dis Aquat Organ.* (2016) 118:11–20. doi: 10.3354/dao02963
31. Pearman PB, Garner TWJ. Susceptibility of Italian agile frog populations to an emerging strain of Ranavirus parallels population genetic diversity. *Ecol Lett.* (2005) 8:401–8. doi: 10.1111/j.1461-0248.2005.00735.x
32. Bosch J. Sapo partero común (*Alytes obstetricans*). In: Márquez R, Lizana M, editors. *Atlas y Libro Rojo de los Anfibios y Reptiles Españoles*. Madrid: Ministerio de Medio Ambiente (2002).
33. Pleguezuelos JM, Márquez R, Lizana M. *Atlas y Libro Rojo de los Anfibios y Reptiles de España*. Madrid: Ministerio de Medio Ambiente - Asociación Herpetológica Española (2002).
34. Price SJ, Garner TWJ, Cunningham AA, Langton TES, Nichols RA. Reconstructing the emergence of a lethal infectious disease of wildlife supports

a key role for spread through translocations by humans. *Proc R Soc B.* (2016) 283:20160952. doi: 10.1098/rspb.2016.0952
35. Price SJ, Wadia A, Wright ON, Leung WTM, Cunningham AA, Lawson B. Screening of a long-term sample set reveals two Ranavirus lineages in British herpetofauna. *PLoS ONE.* (2017) 12:e0184768. doi: 10.1371/journal.pone.0184768
36. Cunningham AA, Langton TES, Bennett, PM, Lewin JF, Drury SEN, Gough RE, et al. Pathological and microbiological findings from incidents of unusual mortality of the common frog (Rana temporaria). *Phil Trans R Soc Lond B.* (1996) 351:1539–57. doi: 10.1098/rstb.1996.0140
37. Daversa D, Manica A, Bosch J, Jolles J, Garner TWJ. Routine habitat switching alters the likelihood and persistence of infection with a pathogenic parasite. *Funct Ecol.* (2018) 32:1262–70. doi: 10.1111/1365-2435.13038
38. Palomar G, Vörös J, Bosch J. Tracking the introduction history of Ichthyosaura alpestris in a protected area of Central Spain. *Conserv Genet.* (2017) 18:867–76. doi: 10.1007/s10592-017-0934-x
39. Brunner JL, Schock DM, Davidson EW, Collins JP. Intraspecific reservoirs: complex life history and the persistence of a lethal Ranavirus. *Ecology.* (2004) 85:560–6. doi: 10.1890/02-0374
40. Narayan EJ, Graham C, McCallum H, Hero JM. Over-wintering tadpoles of *Mixophyes fasciolatus* act as reservoir host for *Batrachochytrium dendrobatidis*. *PLoS ONE.* (2014) 9:e92499. doi: 10.1371/journal.pone.0092499
41. Medina D, Garner TWJ, Carrascal LM, Bosch J. Delayed metamorphosis of amphibian larvae facilitates *Batrachochytrium dendrobatidis* transmission and persistence. *Dis Aquat Org.* (2015) 117:85–92. doi: 10.3354/dao02934
42. Clare FC, Halder JB, Daniel O, Bielby J, Semenov MA, Jombart T, et al. Climate forcing of an emerging pathogenic fungus across a montane multihost community. *Philos Trans R Soc Lond B.* (2016) 371:20150454. doi: 10.1098/rstb.2015.0454
43. Bosch B, Elvira S, Sausor C, Bielby J, González-Fernández I, Alonso R, et al. Increased tropospheric ozone levels enhance pathogen infection levels of amphibians. *Sci Total Environ.* (2020) 759:143461. doi: 10.1016/j.scitotenv.2020.143461
44. Bates KA, Clare FC, O'Hanlon S, Bosch J, Brookes L, Hopkins K, et al. Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. *Nat Commun.* (2018) 9:693. doi: 10.1038/s41467-018-02967-w
45. Harrison XA, Price SJ, Hopkins K, Leung WTM, Sergeant C, Garner TWJ. Diversity-stability dynamics of the amphibian skin microbiome and susceptibility to a lethal viral pathogen. *Front Microbiol.* (2009) 10:2883. doi: 10.3389/fmicb.2019.02883
46. Fernández-Beaskoetxea S, Carrascal LM, Fernández-Loras A, Fisher MC, Bosch J. Short term minimum water temperatures determine levels of infection by the amphibian chytrid fungus in *Alytes obstetricans* tadpoles. *PLoS ONE.* (2015) 10:e0120237. doi: 10.1371/journal.pone.0120237
47. Walker SF, Bosch J, Gomez V, Garner TWJ, Cunningham AA, Schmeller DS, et al. Factors driving pathogenicity versus prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecol Lett.* (2010) 13:372–382. doi: 10.1111/j.1461-0248.2009.01434.x
48. Rosa GM, Anza I, Moreira PL, Conde J, Martins F, Fisher MC, et al. Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal. *Anim Conserv.* (2013) 16:306–15. doi: 10.1111/j.1469-1795.2012.00602.x
49. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel AN, Beukema W, et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science.* (2019) 363:1459–63. doi: 10.1126/science.aav0379

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 June 2021
doi: 10.3389/fvets.2021.687084

Thermal Performance Curves of Multiple Isolates of *Batrachochytrium dendrobatidis*, a Lethal Pathogen of Amphibians

Ciara N. Sheets[1]*, *Deena R. Schmidt*[2], *Paul J. Hurtado*[2], *Allison Q. Byrne*[3,4], *Erica Bree Rosenblum*[3,4], *Corinne L. Richards-Zawacki*[5] and *Jamie Voyles*[1]

[1] Department of Biology, University of Nevada, Reno, NV, United States, [2] Department of Mathematics and Statistics, University of Nevada, Reno, NV, United States, [3] Department of Environmental Science, Policy, and Management, University of California, Berkeley, Berkeley, CA, United States, [4] Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, CA, United States, [5] Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, United States

OPEN ACCESS

Edited by:
Amanda Linda Jean Duffus, Gordon State College, United States

Reviewed by:
Reid Harris, James Madison University, United States
Mohanned Naif Alhussien, Technical University of Munich, Germany
Brittany McHale, University of Georgia, United States

***Correspondence:**
Ciara N. Sheets
csheets@nevada.unr.edu

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 28 March 2021
Accepted: 20 May 2021
Published: 22 June 2021

Citation:
Sheets CN, Schmidt DR, Hurtado PJ, Byrne AQ, Rosenblum EB, Richards-Zawacki CL and Voyles J (2021) Thermal Performance Curves of Multiple Isolates of Batrachochytrium dendrobatidis, a Lethal Pathogen of Amphibians. Front. Vet. Sci. 8:687084. doi: 10.3389/fvets.2021.687084

Emerging infectious disease is a key factor in the loss of amphibian diversity. In particular, the disease chytridiomycosis has caused severe declines around the world. The lethal fungal pathogen that causes chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*), has affected amphibians in many different environments. One primary question for researchers grappling with disease-induced losses of amphibian biodiversity is what abiotic factors drive *Bd* pathogenicity in different environments. To study environmental influences on *Bd* pathogenicity, we quantified responses of *Bd* phenotypic traits (e.g., viability, zoospore densities, growth rates, and carrying capacities) over a range of environmental temperatures to generate thermal performance curves. We selected multiple *Bd* isolates that belong to a single genetic lineage but that were collected across a latitudinal gradient. For the population viability, we found that the isolates had similar thermal optima at 21°C, but there was considerable variation among the isolates in maximum viability at that temperature. Additionally, we found the densities of infectious zoospores varied among isolates across all temperatures. Our results suggest that temperatures across geographic point of origin (latitude) may explain some of the variation in *Bd* viability through vertical shifts in maximal performance. However, the same pattern was not evident for other reproductive parameters (zoospore densities, growth rates, fecundity), underscoring the importance of measuring multiple traits to understand variation in pathogen responses to environmental conditions. We suggest that variation among *Bd* genetic variants due to environmental factors may be an important determinant of disease dynamics for amphibians across a range of diverse environments.

Keywords: amphibian declines, chytridiomycosis, *Batrachochytrium dendrobatidis*, thermal performance curves, climate, latitudinal gradient

INTRODUCTION

Emerging infectious diseases are a primary driver of global amphibian declines (1). Disease outbreaks from ranaviruses, chytrid fungi, and bacterial pathogens have contributed to an unprecedented loss of global amphibian diversity (2–4). Therefore, understanding what factors influence the emergence, spread, pathogenicity, and ecology of these pathogens is important for amphibian conservation (5). Many of these pathogens are strongly influenced by their local environments, and corresponding shifts in pathogen phenotypic traits (e.g., reproductive rates, pathogen persistence in the environment) can alter disease risks for susceptible amphibian host species (6–8). By investigating how a pathogen responds to its environment, as well as the genotypic and phenotypic variation that underpins those responses, we can begin to unravel the disease dynamics that threaten amphibians (9, 10).

Chytridiomycosis is one such infectious disease that is lethal to many amphibian species and has caused global declines in susceptible species (1, 11). The disease is caused by the fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) (12) and *Batrachochytrium salamandrivorans* (*Bsal*) (13). However, *Bd* has spread globally and impacted far more amphibian host species than *Bsal*, making it a priority pathogen for study (1). Since its discovery in 1999, *Bd* has spread rapidly through multiple naïve amphibian communities, causing mass mortality events, and even the complete extinction of amphibian species (11). No other pathogen is known to have had such a ubiquitous effect on such a broad range of host species and in so many different environments (1, 14, 15). As a result, *Bd*-related declines have been called, "the most spectacular loss of biodiversity due to disease in recorded history" (11).

Bd has a two-stage life cycle that consists of a substrate-dependent immobile sporangium and a free-living uniflagellated, motile zoospore (12, 16). Infection occurs during the motile zoospore stage of the pathogen's life cycle (12, 16). The motile zoospores encyst on a substrate, such as the keratinized tissue found in amphibian larval mouthparts or on adult epidermis, and then mature into a zoosporangium (12, 17, 18). Zoosporangia produce motile zoospores and then release the new zoospores into the environment to re-infect the same host or transmit to another individual host (18). Once infection is established within a host, increases in infection intensity (or pathogen load) in amphibian skin is a key feature of pathogenesis (19, 20). As such, understanding the factors that regulate *Bd* growth and reproductive rates is integral to resolving questions concerning pathogenesis and the disease ecology of this lethal disease system (21, 22).

Recent phylogenetic analyses indicate that there are several major lineages of *Bd* that are genetically distinct (23–25). One lineage that has garnered considerable attention from the scientific community, due to its high lethality, is the Global Panzootic Lineage (*Bd*GPL) (23, 24, 26). Genomic sequencing of many *Bd* isolates within this lineage has shown that it contains substantial genetic diversity, including two genetic clades (*Bd*GPL1 and *Bd*GPL2) (26–28). With a global distribution, *Bd*GPL occurs in a wide range of amphibian habitats and causes disease in diverse microclimates and thermal environments (2, 29). As such, researchers have focused on resolving the factors that determine variation among *Bd*GPL isolates to understand how temperature may mediate disease dynamics (21, 22, 30, 31). To date, no clear patterns have emerged that can explain the extent of variation among and within *Bd*GPL isolates across diverse thermal environments. This outstanding question may be most appropriately investigated by generating thermal performance curves, which would allow for comparative investigations within the *Bd*GPL lineage.

Thermal performance curves (TPCs) are widely used to measure an organism's performance across a range of temperatures, estimate the thermal sensitivity of different traits, and facilitate an understanding of ecological and evolutionary processes that may explain an organism's success within a given environment (32–34). TPCs include measures of thermal optimum [i.e., temperature optimum (T_{opt})], critical thermal minimum (CT_{min}), critical thermal maximum (CT_{max}), and thermal tolerance range (also known as thermal breadth; T_{br}) (**Figure 1**). Temperature sensitive parameters that determine an organism's TPC frequently vary with geographic clines (e.g., latitude), reflecting local adaptation (34, 35). TPC models (e.g., vertical or horizontal shifts) offer a framework to consider the adaptive potential for temperature-sensitive organisms (36, 37). For example, horizontal shifts toward a higher T_{opt} would provide evidence in support of the "hotter is better" hypothesis, which predicts that organisms will adapt to thermal conditions according to thermodynamic constraints (e.g., with higher T_{opt} in latitudes where mean temperatures are higher) (38–40) (**Figure 2**).

It is generally thought that *Bd* has a thermal tolerance range of 2–28 °C (42), with a T_{opt} of 17–25 °C (8, 43), and CT_{min}, and CT_{max} of 2–5 and 25–28 °C, respectively (42, 44). Mounting evidence suggests that *Bd* isolates differ in their thermal optima (8, 42, 44), but experimental approaches have not yet explored this idea by comparing isolates collected across a latitudinal gradient (45). We predicted that the TPCs of *Bd* isolates collected along a latitudinal gradient would differ due to thermal constraints in each region. More specifically, we expected that isolates from northern latitudes would have a lower T_{opt} and exhibit a lower maximum performance at that temperature. In contrast, we expected isolates from southern latitudes to have a higher T_{opt} and higher performance at that temperature (**Figure 2**). To test these predictions, we generated TPCs for five *Bd* isolates collected across a latitudinal gradient.

MATERIALS AND METHODS

Bd Isolate Collection and Maintenance

We used five different *Bd* isolates that originated from amphibians in the United States (**Table 1**). The collection of *Bd* isolates from the United States provides an ideal repertoire for investigating phenotypic variation and differences in TPCs for multiple reasons. First, the collection of *Bd* isolates that originate from amphibians in the United States is large, with numerous isolates from across the country, spanning a latitudinal gradient. Second, previous work using a microfluidic PCR genotyping

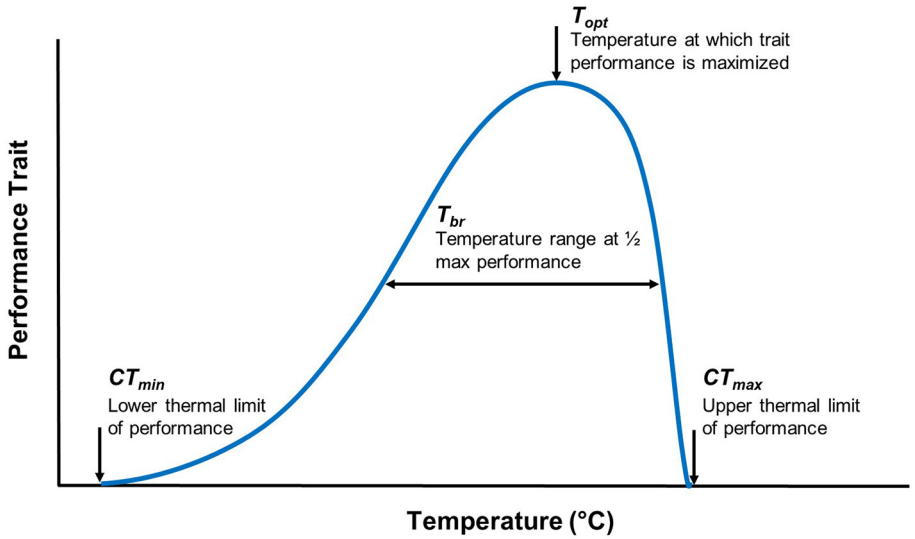


FIGURE 1 | Traditional thermal performance curve parameters for a given trait. When a performance curve is generated, the performance of a trait is plotted against a temperature range. The thermal breadth (T_{br}), also referred to as the thermal tolerance range, is the temperature range at which a level of performance is achieved. A thermal optimum (T_{opt}) is the temperature at which trait performance is maximized. The critical thermal minimum (CT_{min}) and maximum (CT_{max}) are the lower and upper thermal limits of a trait's performance, respectively.

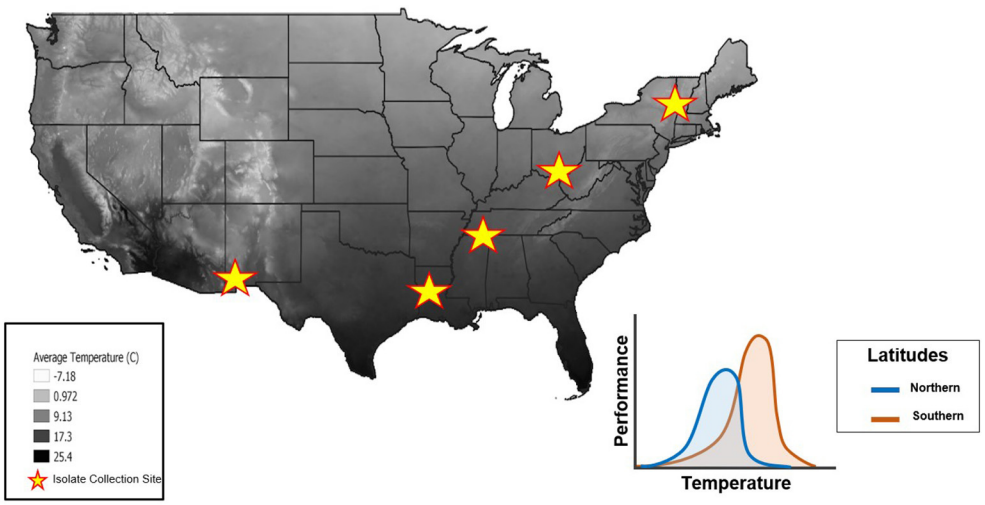


FIGURE 2 | Mean annual air temperatures throughout the United States (highest temperatures in black, lowest temperatures in white). Stars represent the locations where isolates were collected from across a latitudinal gradient. The thermal constraint hypothesis ("hotter is better") predicts that isolates from Northern latitudes will have lower maximal performance at a lower T_{opt} (blue curve) whereas isolates from Southern latitudes will have a higher maximal performance at a higher T_{opt} due to adaptations from local temperature regimes (orange curve). Temperature data from the National Forest Climate Change Maps website to generate this figure in QGIS software (41).

method (one that targets ∼200 loci) suggested that *Bd*GPL is the primary lineage found in North America (25).

All isolates were cryoarchived and subsequently revived according to standard protocols (46) prior to the beginning of the experiment. Following isolate revival, we cultured the *Bd* isolates in tryptone/gelatin hydrolysate/lactose (TGhL) liquid growth media in 75 cm^2 tissue culture flasks (47). We incubated each isolate at 21°C and monitored them through the *Bd* life cycle until the point of peak zoospore densities (42). Once each culture flask reached peak zoospore density, 2 mL of culture was transferred to a new culture flask containing 13 mL of fresh TGhL media for standard passage. We used a biosafety cabinet for all laboratory work involving these isolates (e.g., passaging, experimental setup).

TABLE 1 | Genotyping for isolates of *Batrachochytrium dendrobatidis*, amphibian host, and geographic origins.

Genotype	Isolate	Location	Host species name	Latitude
GPL1	Louisiana (LA)	New Orleans, LA	*Acris crepitans*	29.9511 °N
GPL1	Tennessee (TN)	Memphis, TN	*Lithobates sphenocephala*	35.1495 °N
GPL1	Vermont (VT)	VT	*Lithobates clamitans*	44.5588 °N
GPL2	New Mexico (NM)	Beaver Creek, NM	*Lithobates catesbeianus*	34.5199 °N
GPL2	Ohio (OH)	Toledo, OH	*Lithobates pipiens*	41.6528 °N

Generating Thermal Performance Curves

We filtered each of the five cultures using sterile filter paper (Whatman Qualitative Filter Papers, Grade 3) and used a vacuum filtration pump to remove zoosporangia (47). With the remaining filtrate, we quantified zoospores using a hemocytometer and diluted each culture with TGhL to a concentration of $\sim 50 \times 10^4$ zoospores/mL (48). We inoculated the cultures of each isolate containing only zoospores into 96-well-plates. We then added 50 μL of additional TGhL media to each well. We included five negative control wells with 50 μL of 50×10^4 zoospores/mL heat-killed zoospores and 50 μL of TGhL media for each isolate (48). We filled the perimeter wells of the plate with 150 μL TGhL media to provide a buffer against culture evaporation (45).

To establish a thermal profile for each respective isolate, we incubated all isolates at multiple stable temperatures (4, 12, 17, 21, 25, 26, and 27 °C). Because we used only zoospores to start the growth experiments, we were able to track and quantify several parts of the *Bd* life cycle as they occurred at different time points in these different temperature conditions. Specifically, by tracking cultures for multiple successive days, we were able to measure the change in population growth, time to maximum zoospore densities, zoospore densities, and calculate fecundity (49). At multiple time points following experimental set up (Day 0), we randomly selected five wells ($N = 5$) for each of two destructive measures: zoospore counts and viability assays (49).

To quantify zoospore densities, we manually withdrew 20 μL of culture and counted live zoospores using a hemocytometer (48). Following these counts, we omitted those wells for the remainder of the experiment (48). To measure population growth, we conducted a standard viability assay (45). The MTT viability assay is a standard microbiological technique where a yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to purple MTT-formazan crystals in metabolically active cells (50). These crystals can be solubilized, and the color change can be quantified by reading culture absorbance at 570 nm (45). We added 20 μL of MTT to each experimental and negative control wells of the plate selected for that day and incubated the plate at 21°C for 2 h (45). After incubation, we added 140 μL of the stop-reagent to stop the reaction and solubilize the MTT-formazan crystals (45). We then read culture absorbance at 570 nm using a Biotek EL x 800 Absorbance Reader.

Bd Isolate Genotyping

We genotyped the isolates using an amplicon sequencing approach according to published protocols (51). Briefly, we extracted DNA following the manufacturer's protocol for the Qiagen DNeasy Blood and Tissue kit. Next, to prepare raw DNA extracts for sequencing, we cleaned each using an isopropanol precipitation and preamplified each in two separate PCR reactions, each containing 96 primer pairs. Primers were designed to target 150–200 base pair regions of the *Bd* nuclear and mitochondrial genome (51). After preamplification, samples were cleaned using EXOSap-it™ (ThermoFisher Scientific) and diluted 1:5 in water. Finally, we cleaned and diluted products from the two preamplification reactions, combined in equal proportions, and sent to the University of Idaho IBEST Genomics Resources Core, where they were loaded into a Fluidigm June LP 192.24 IFC (Fluidigm Inc.) for amplification and barcoding. Amplified products were pooled and sequenced on an Illumina MiSeq.

Raw sequences were processed as previously described (25, 51). Raw reads were joined via FLASH [(52); v.1.2.11] and consensus sequences for each sample/amplicon combination were called using the reduce amplicons R script (https://github.com/msettles/dbcAmplicons/blob/master/scripts/R/reduce_amplicons.R). Here, consensus sequences use IUPAC ambiguity codes to indicate multiple alleles at a locus. We compared the consensus sequences of each of our five isolates to 21 previously published *Bd* samples using a phylogenetic approach. We selected previously published reference sequences to represent every known major *Bd* lineage (25). To create a phylogeny, we used a gene tree to species tree approach: first aligning all sequences for each amplicon using MUSCLE [(53); v.3.32], then creating a tree for each amplicon using RAxML [(54); v.8.2.11] to search for the best scoring ML tree from 100 bootstrap replicates. Afterwards, we used newick utils [(55); v.1.6] to collapse all nodes in each amplicon tree with <10 bootstrap support. We then input a total of 190 amplicon trees with collapsed branches into Astral-III [(56); v.5.5.9], which estimates an unrooted species tree given a set of unrooted gene trees using the multispecies coalescent model.

Statistical Analysis

For all statistical analyses, we used R version 3.4.3 (57). We used QGIS software and the "ggplot2" package within R to generate figures. Summary statistics reported in the figures and the tables include means ± standard error (SE) of the viability, zoospore densities, or fecundity measure among isolates or between genotypes. We analyzed the performance of each isolate when grouped by genetic variant and independently to compare for differences among isolates at T_{opt}, CT_{min}, and

CT_{max} temperature treatments. We used Analysis of Variance (ANOVA) and Games Howell *post-hoc* tests to make comparisons in mean maximum viability (OD following the MTT assay), mean maximum zoospore densities, mean fecundity, and time to maximum zoospore densities. We used a non-parametric *post-hoc* test when there was a violation of the homogenous variance assumption for each of the traits compared among isolates. To calculate mean maximum viability and maximum zoospore densities, we used the measures from within the 2–6 day period at which cultures exhibited maximum viability or zoospore densities in each temperature condition.

To make comparisons of fecundity, we calculated the ratio of zoospores densities to mean culture viability. Within the fecundity calculations, all viability measurements that were <0.005 were considered zeros to ensure that fecundity ratios were not artificially inflated. For statistical analyses, we log-transformed the fecundity metric and added a correction factor of 1 to accommodate for the wells that had zero zoospores. For comparing genetic variants in viability, zoospore densities, fecundity, and time to maximum zoospore densities, we used Welch's *t*-test because we had unequal variance after grouping by genotype. We used a Bonferroni correction after running the *t*-test at each temperature experiment for comparisons of MTT across the thermal range to reduce the likelihood of a type-1 error.

To further quantify the differences across temperatures, we fit a logistic growth curve to the normalized optical density measurements (i.e., *Bd* viability) data time series for each isolate-temperature combination. This approach allowed us to estimate the intrinsic growth rate (r) and carrying capacity (K). We used the resulting estimates for r and K to quantify differences among isolates over the range of temperatures considered. To calculate 95% confidence intervals for these estimates, we used likelihood profile-based methods (58, 59). We attempted to constrain r estimates to follow a Johnson–Lewin (J–L) curve as a function of temperature to characterize the thermal breadth of each isolate (59). See the **Supplementary Material** for details.

RESULTS

Differential Responses to Temperature Between Genetic Variants

Our genetic sequencing revealed that these isolates belong to the *Bd*GPL clades 1 or 2 (**Table 1**). When we grouped the isolates by genetic lineage, we found no differences in viability between *Bd*GPL1 and *Bd*GPL2 lineages at the T_{opt}, 21°C [$t_{(57.51)} = 0.91$, $p = 0.37$; **Figure 3A**]. Furthermore, there were no significant differences in viability between *Bd*GPL1 and *Bd*GPL2, except at the low temperature of 4 °C and the high temperature at 27 °C (**Figure 3A**, **Table 2**).

We also measured zoospore densities for the two genetic lineages in all temperatures because the capacity to generate high zoospore densities is thought to be a critical factor for disease development (21). We found patterns in our measures of zoospore densities that differed from those in our viability assays (**Figure 3B**). There were significant differences between *Bd*GPL1 and *Bd*GPL2 in zoospore densities at every temperature where zoospores were produced (**Figure 3B**, **Table 3**). Specifically, *Bd*GPL1 had higher zoospore densities than *Bd*GPL2 at all temperatures except 4 °C (**Table 3**). We found that fecundity was significantly different between *Bd*GPL1 and *Bd*GPL2 at three temperatures: 4 °C [$t_{(182.81)} = -3.2$, $p = 0.002$], 17 °C [$t_{(162.63)} = 6.039$, $p \leq 0.001$], and 21 °C [$t_{(131.85)} = 6.9127$, $p \leq 0.001$] (**Figure 3C**). There were no significant differences between *Bd*GPL1 and *Bd*GPL2 in the time to maximum zoospore densities at any temperature except 21 °C [$t_{(22.29)} = -2.7584$, $p = 0.01$].

Differential Responses to Temperature Among the *Bd* Isolates

All isolates exhibited maximum viability at 21°C. However, there were differences among isolates in their mean viability at the T_{opt} of 21°C [ANOVA, $F_{(4,63)} = 15.94$, $P < 0.001$, **Table 2**, **Figure 3D**]. The isolate from Louisiana exhibited the greatest mean viability in the T_{opt}, 21°C (**Table 2**, **Figure 3D**), as well as at every other temperature treatment except 27 °C (**Table 2**, **Figure 3D**). The isolate from Vermont exhibited the lowest viability except in the low temperature treatments of 4 and 12 °C (**Table 2**, **Figure 3D**).

We found that there were differences among the *Bd* isolates in their viability in both low and high temperature conditions (**Table 2**, **Figure 3D**). For the lowest temperature treatment, all isolates exhibited minimal growth at 4 °C but there were differences among the isolates in viability at that temperature [**Table 2**; ANOVA, $F_{(4,45)} = 146.6$, $P < 0.001$]. The isolates also differed in their responses to high temperature treatments (**Table 2**, **Figure 3D**). The isolate from Ohio exhibited significantly greater viability at the highest temperature treatment of 27 °C [ANOVA, $F_{(4,70)} = 25.82$, $P < 0.001$; Game's Howell, $P < 0.01$], whereas the isolate from Vermont had low viability at 26 °C and was not viable at 27 °C (**Figure 3D**).

The patterns found in zoospore densities among isolates also differed from viability results (**Figure 3E**). Specifically, two of the isolates produced their maximum zoospore densities at the low temperatures of 4 and 12 °C (**Figure 3E**, **Table 4**). Notably, for the New Mexico isolate, zoospore densities were highest at 4 °C and were dramatically lower at all other temperatures (**Table 4**, **Figure 3E**). Accordingly, the New Mexico isolate exhibited the highest fecundity (zoospores per viability measure) at 4 °C (**Figure 3F**). All isolates exhibited a similar pattern, with higher fecundity in lower temperatures, but it was most pronounced in the New Mexico isolate at 4 °C. In addition, we found that the time to maximum zoospore densities differed among isolates at 4 °C [ANOVA, $F_{(4,70)} = 250.9$, $P < 0.001$] and 21°C [ANOVA, $F_{(4,70)} = 48.36$, $P < 0.001$]. We also found that, although the cultures were viable and growth measurements increased at the higher temperatures of 25, 26, and 27 °C, none of the isolates produced zoospores at these high temperatures (**Figure 3E**).

We then assessed how these r and K estimates varied with temperature for each isolate. The overall trend for all isolates is r estimates that increase and then plateau for temperatures up to 21 °C (**Figure 4A**). However, for higher temperatures (25–27 °C), the r estimates are larger and more variable both

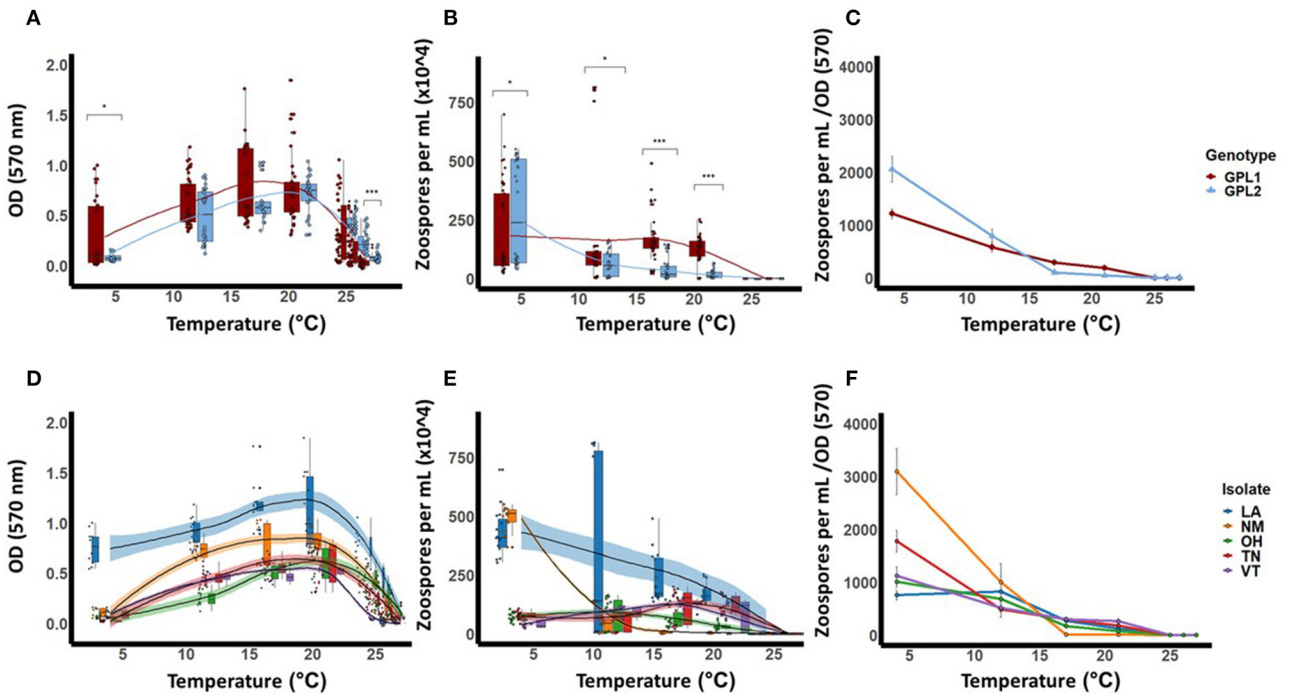


FIGURE 3 | Thermal performance curves for isolates of *Batrachochytrium dendrobatidis* (*Bd*). Five isolates were collected from across a latitudinal gradient in the United States, genotyped, and tested for these responses across the known thermal range for *Bd* (4–27 °C). Data show means (±SE) for *Bd* viability **(A)**, zoospore densities **(B)**, and fecundity (calculated as optical density/zoospore densities) **(C)**, for two *Bd* genotypes, Global Panzootic Lineage 1 and Global Panzootic Lineage 2 (*Bd*GPL1 and *Bd*GPL2; top row, red and blue). Additionally, data show means (±SE) for *Bd* viability **(D)**, zoospore densities **(E)**, and fecundity **(F)**, for each of the five isolates that were collected in Louisiana (LA, blue), New Mexico (NM, organe), Ohio (OH, green), Tennessee (TN, red), and Vermont (VT, purple). Significance levels are indicated by the asterisks as such: * < 0.05, ** < 0.01, *** < 0.001.

across isolates and in terms of having larger confidence intervals. The intrinsic growth rates at the higher temperatures, however, do not yield much long-term growth. The corresponding *K* estimates also increase and plateau at ∼21°C, but then markedly decline at the higher temperatures (**Figure 4B**). The combined effect is a short-lived exponential growth phase that quickly reaches a relatively low upper bound at these high temperatures (**Supplementary Materials**).

DISCUSSION

Chytridiomycosis is a disease that has impacted amphibians in a wide range of environmental conditions (21, 60). Past studies have attempted to link *Bd* phenotypic patterns with environmental factors in order to understand how abiotic factors might mitigate or exacerbate disease (5, 30, 43, 61). For example, both Becker et al. (30) and Greener et al. (21) documented considerable phenotypic variation for isolates within the *Bd*GPL that was associated with differential pathogenicity in common susceptible host species (*Lithobates sylvaticus* and *Alytes obstetricans*, respectively). In addition, Lambertini et al. (22) and Muletz-Wolz et al. (31) demonstrated phenotypic variation in morphological characteristics (e.g., zoosporangia size) in multiple isolates from within the *Bd*GPL lineage. However, to date, studies that have tried to link pathogen traits to environmental predictors have not been able to account for the extent of phenotypic variation among *Bd* isolates across different environments [e.g., *Bd* growth has not been linked to any environmental parameters such as mean annual temperature, mean annual precipitation, elevation, etc., (22, 31)].

We predicted that quantifying *Bd* growth and reproductive traits from isolates of the same genotype, but collected across a latitudinal gradient (representing different mean annual air temperature regimes), might show distinct TPCs. We conducted temperature experiments to measure traits related to growth, reproduction, and fitness across the known thermal range of *Bd* and generated TPCs for five isolates from within the *Bd*GPL lineage. Our results reveal informative similarities and differences in several of the measured traits between two genetic lineages (*Bd*GPL1 and *Bd*GPL2) and among five *Bd* isolates.

We found that there was no obvious geographic pattern that could explain the distribution of genetic variants of *Bd*GPL collected across a latitudinal gradient within the United States. Three of our isolates nested within the *Bd*GPL1 clade and each originated from a different latitude (**Table 1**). Two of the isolates nested within the *Bd*GPL2 clade and similarly originated from different latitudes. Both genetic variants had the same T_{opt} of 21 °C, but the maximum viability differed between *Bd*GPL1 and

TABLE 2 | Population growth viability (MTT) across temperatures (means, SE, *r*, and *K*).

Isolate ID		4 °C			12 °C			17 °C			21 °C			25 °C			26 °C			27 °C		
		Mean (±SE)	*r*	*K*	Mean (±SE)	*r*	*K*	Mean (±SE)	*r*	*K*	Mean (±SE)	*r*	*K*	Mean (±SE)	*r*	*K*	Mean (±SE)	*r*	*K*	Mean (±SE)	*r*	*K*
GPL1	LA	0.75 ± 0.053	0.2591	0.6246	0.91 ± 0.036	0.5867	0.8337	1.19 ± 0.050	0.8546	1.275	1.12 ± 0.104	1.504	1.048	0.78 ± 0.046	0.9155	0.9878	0.40 ± 0.049	0.482	107300	0.02 ± 0.007	0.4817	0.0462
	VT	0.09 ± 0.013	0.1644	2.022	0.46 ± 0.016	0.2312	0.4757	0.47 ± 0.021	0.4321	0.6248	0.53 ± 0.011	0.3991	0.602	0.04 ± 0.003	0.9506	0.0716	0.02 ± 0.004	5.431	0.0035	0.00 ± 0.001	1.433	5.725
	TN	0.02 ± 0.002	0.07248	3.661	0.45 ± 0.019	0.8495	0.4357	0.57 ± 0.027	0.7361	0.6317	0.63 ± 0.052	0.8277	0.6702	0.23 ± 0.019	1.017	0.3259	0.14 ± 0.018	1.314	0.1533	0.06 ± 0.005	1.874	0.0533
GPL2	NM	0.10 ± 0.015	0.1939	0.1047	0.74 ± 0.023	0.6297	0.5608	0.76 ± 0.076	0.8781	0.8241	0.82 ± 0.029	0.808	0.882	0.40 ± 0.018	1.324	0.4455	0.21 ± 0.021	1.187	0.2709	0.07 ± 0.007	1.063	0.075
	OH	0.06 ± 0.005	0.1305	0.0996	0.25 ± 0.019	0.3071	0.2313	0.52 ± 0.029	0.9106	0.5445	0.60 ± 0.046	0.8264	0.6487	0.44 ± 0.036	1.498	0.3906	0.21 ± 0.032	1.451	0.2994	0.10 ± 0.012	0.9965	0.1123

*Bd*GPL2. In addition, while both genetic variants had maximum zoospores densities and fecundity at low temperatures (4 °C), there were differences between *Bd*GPL1 and *Bd*GPL2 in these key reproductive traits. These findings corroborate previous studies that suggest considerable variation exists even within a single *Bd* lineage (21, 30). We suggest that there are likely numerous factors contributing to variation within *Bd*GPL in addition to thermal conditions. For example, each isolate for this study was collected from a unique host species (**Table 1**), with each host species occupying habitats that differ in a multitude of factors, including water pH, drying periods, microbiome composition, and other seasonality effects that likely have a large impact on *Bd* (5, 30, 43). Although it is impractical for *Bd* researchers to eliminate all confounding variables for *Bd* isolate origin, we should nevertheless make efforts to treats isolates identically following isolation (e.g., during laboratory maintenance) and acknowledge these limitations for resolving questions concerning differential pathogenicity.

We found intriguing patterns in the responses of *Bd* to temperature when assessing differences among all five isolates. To begin with, we found that the overall patterns of viability were similar and exhibited a T_{opt} at the intermediate temperature of 21°C. However, within each temperature, the isolates frequently differed from each other in their maximal viability, zoospore densities, fecundity, growth rates, and carrying capacities. These differences were pronounced at either end of the thermal spectrum, at low (4 °C) and high (26 and 27 °C) temperatures. For example, the temperature of the T_{opt} for zoospores densities is lower than 21°C, with far more zoospores produced in low temperatures (4 and 12°C), for a subset of the isolates. Furthermore, the fecundity of *Bd* was highest in low temperatures for every isolate. These findings are in line with those from previous studies that suggest understanding *Bd* responses (particularly zoospore production) in low temperatures is important to resolving the complexities of the fundamental niche and the disease ecology of *Bd* (42, 49, 62).

Additionally, we observed interesting patterns of *Bd* viability, growth rates, and carrying capacities at both extremes of the thermal range, making it difficult to determine the true CT_{max} and CT_{min}. Notably, we found the greatest complexity in thermal responses at the CT_{max}; most of the *Bd* isolates (all except Vermont) exhibited at least some zoosporangia development, and early exponential growth (*r*), in the high temperature treatments (25, 26, and even 27 °C). Yet none of the isolates produced any zoospores and growth could not be sustained for the duration of the experiment. In addition, we found that for the higher temperatures (>21°C) considered in this experiment, the *r* estimates did not decline as one might expect. Rather, it was the *K* estimates that seemed to decline over the upper temperatures. As a result, the *r* estimates (constrained to follow a J–L curve) were overfit to the mid-range temperature data, causing unrealistically high CT_{max} and T_{opt} estimates, and poor *r* estimates, for high and low temperatures. Our findings for the higher temperature treatments differ from some previous studies that found no *Bd* growth at temperatures above 24 °C (31, 43, 62). Thus, our findings that *Bd* can remain viable at high temperatures, but fail to produce zoospores, underscore the

TABLE 3 | Zoospore density descriptive difference of means using *t*-tests between genotypes.

	4 °C			12 °C			17 °C			21 °C			25 °C			26 °C			27 °C		
	t	df	*p*	*t*	df	*p*	*t*	df	*p*	*t*	df	*p*	*t*	df	*p*	*t*	df	*p*	*t*	df	*p*
Genotype	−2.0148	56	0.049	2.4708	51	0.017	8.035	67.4	<0.001	11.56	54.3	<0.001	NA	NA	NA	NA	NA	NA	NA	NA	NA

TABLE 4 | Zoospore densities across temperatures (means, SE, *r*, and *K*).

	Isolate ID	4 °C Mean (±SE)	12 °C Mean (±SE)	17 °C Mean (±SE)	21 °C Mean (±SE)	25 °C Mean (±SE)	26 °C Mean (±SE)	27 °C Mean (±SE)
GPL1	LA	431.9 ± 27.141	314.7 ± 92.641	235.7 ± 27.795	172.8 ± 10.806	NA	0	0
	VT	38.9 ± 3.998	89.1 ± 5.996	127.3 ± 4.525	84.6 ± 16.238	NA	0	0
	TN	78.4 ± 4.296	56.1 ± 10.333	119.7 ± 17.980	120.6 ± 9.395	NA	0	0
GPL2	NM	492.1 ± 13.133	37.4 ± 6.995	4.5 ± 0.885	2.8 ± 0.433	NA	0	0
	OH	70.3 ± 4.853	89.2 ± 16.559	65.7 ± 11.233	30.7 ± 3.596	NA	0	0

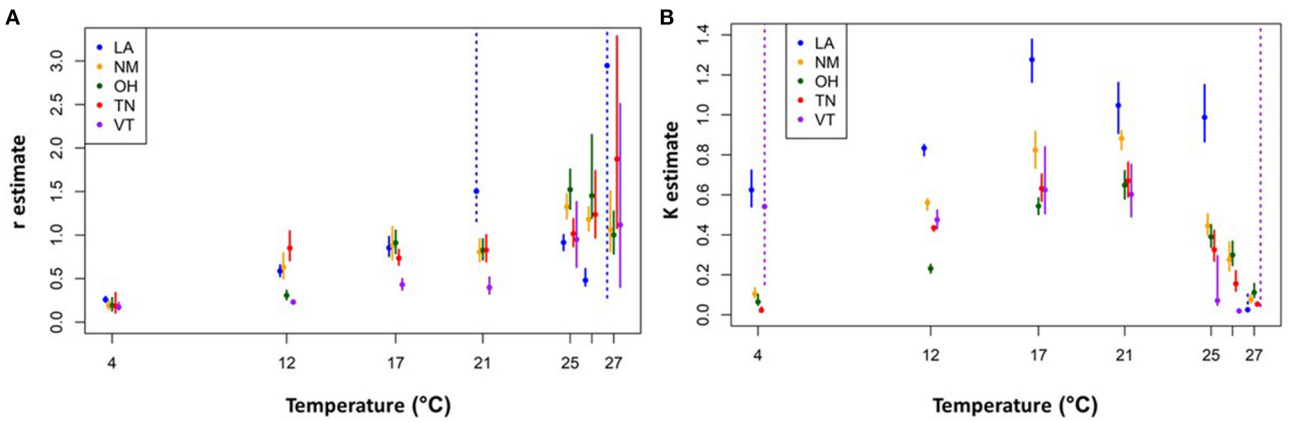


FIGURE 4 | Logistic growth curve parameter estimates for each isolate and temperature combination. Intrinsic exponential growth rate (*r*) estimates **(A)** carrying capacity (*K*) estimates **(B)**. Solid circles indicate the best fit parameter values and vertical bars show the 95% confidence intervals. The dashed vertical bars indicate situations in which the confidence interval for the parameter of interest had a poorly resolved upper end point due to parameter identifiability issues (e.g., for time series data in the exponential growth phase, all values of *K* above a certain threshold will give equally good fits). Not shown, for clarity: VT-26 °C *r* estimate is 8.58, CI = (4.61, 14.12). See **Supplementary Material** for further details.

importance of using a viability assay to investigate additional questions concerning *Bd* responses to temperature (45).

Taken together, the variation in TPCs of the maximum viability of *Bd* isolates collected across a latitudinal gradient did not fit a pattern that could be explained by the "hotter is better" hypothesis; all isolates had the same T_{opt} for viability at 21°C. Instead, our viability results suggest that a vertical shift model may better explain the patterns for the TPCs of all five isolates. Namely, our viability measurements, and results from carrying capacities (*K*) among isolates, provide some evidence that mean temperatures across latitudes may influence the maximal performance of *Bd*. The isolates from northern latitudes (i.e., Vermont & Ohio), where mean temperatures are generally lower (∼4–12 °C; 61), exhibited lower viability and carrying capacities across temperatures, including at their T_{opt}. In contrast, the isolates from Louisiana, New Mexico, and Tennessee, in more southern latitudes where mean temperatures are generally higher (∼14–25 °C; 61), exhibited increased viability and carrying capacities across temperatures, including at their T_{opt}. As such, our evidence indicating a vertical shift in TPCs suggest that the mean temperatures experienced by amphibians across a latitudinal gradient may influence maximal viability—but not the T_{opt} or CT_{max}—of *Bd*. We note, however, that our results for our other reproductive parameters, including zoospore densities and fecundity, did not exhibit a similar pattern, underscoring the importance of measuring multiple traits to gain a full understanding of the complexities of *Bd* responses to temperature (37, 38).

Disease ecologists are concerned with how changes in environmental factors, such as temperature gradients, may influence disease dynamics through alterations in the biology of pathogens such as *Bd* (63, 64). Environmental influences

on *Bd* traits such as growth and reproduction may ultimately influence the disease outcomes of chytridiomycosis (42, 44). For example, temperature conditions within local environments may increase viability, zoospores densities, fecundity, growth rates, or carrying capacities of *Bd*, leading to higher infectivity, and greater threat of disease for vulnerable amphibians (49). The threat of biodiversity loss for amphibian communities may be exacerbated from diseases like chytridiomycosis in the coming decades (63). To intervene in the continued population declines of amphibians, we must understand how pathogen biology is mediated across different environments, and within and among genetic lineages. We must also determine what environmental factors are driving the disease dynamics responsible for the disease-induced losses of amphibian biodiversity.

DATA AVAILABILITY STATEMENT

The data are deposited in the Figshare repository, doi: 10.6084/m9.figshare.14714865, doi: 10.6084/m9.figshare.14714856, and doi: 10.6084/m9.figshare.14714841.

AUTHOR CONTRIBUTIONS

CS and JV conceived and executed the study, analyzed results, and wrote the paper. DS and PH conducted several statistical analyses and data visualizations. AB and ER conducted the sequencing and provided genetic information for the isolates. CR-Z provided input on the study design and editorial assistance. All authors contributed to the writing of the manuscript and assisted in the resulting finished product.

FUNDING

This study was funded by the National Science Foundation (DEB 1846403 to JV) and by the US. Department of Defense (SERDP: RC-2638 to CR-Z, ER, and JV).

ACKNOWLEDGMENTS

We thank T. Disbow, R. Godkin, and B. Christman for assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.687084/full#supplementary-material

REFERENCES

1. 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.* (2019) 363:1459–63. doi: 10.1126/science.aav0379
2. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci USA.* (1998) 95:9031–6. doi: 10.1073/pnas.95.15.9031
3. Bradford DF. Mass mortality and extinction in a high-elevation population of rana muscosa. *J Herpetol.* (1991) 25:174–77. doi: 10.2307/1564645
4. Miller D, Gray M, Storfer A. Ecopathology of ranaviruses infecting amphibians. *Viruses.* (2011) 3:2351–73. doi: 10.3390/v3112351
5. Sonn JM, Utz RM, Richards-Zawacki CL. Effects of latitudinal, seasonal, and daily temperature variations on chytrid fungal infections in a North American frog. *Ecosphere.* (2019) 10:2892. doi: 10.1002/ecs2.2892
6. Hamilton PT, Richardson JML, Govindarajulu P, Anholt BR. Higher temperature variability increases the impact of *Batrachochytrium dendrobatidis* and shifts interspecific interactions in tadpole mesocosms. *Ecol Evol.* (2012) 2:2450–9. doi: 10.1002/ece3.369
7. Berger L, Speare R, Hines HB, Marantelli G, Hyatt AD, McDonald KR, et al. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust Vet J.* (2004) 82:434–9. doi: 10.1111/j.1751-0813.2004.tb11137.x
8. Bradley PW, Brawner MD, Raffel TR, Rohr JR, Olson DH, Blaustein AR. Shifts in temperature influence how *Batrachochytrium dendrobatidis* infects amphibian larvae. *PLoS ONE.* (2019) 14:e0222237. doi: 10.1371/journal.pone.0222237
9. Altizer S, Ostfeld RS, Johnson PTJ, Kutz S, Harvell CD. Climate change and infectious diseases: from evidence to a predictive framework. *Science.* (2013) 341:514–9. doi: 10.1126/science.1239401
10. Daskin JH, Alford RA, Puschendorf R. Short-term exposure to warm microhabitats could explain amphibian persistence with *Batrachochytrium dendrobatidis*. *PLoS ONE.* (2011) 6:e26215. doi: 10.1371/journal.pone.0026215
11. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, et al. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth.* (2007) 4:125. doi: 10.1007/s10393-007-0093-5
12. Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. *et sp. nov., a* chytrid pathogenic to amphibians. *Mycologia.* (1999) 91:219–27. doi: 10.2307/3761366
13. 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 USA.* (2013) 110:15325–9. doi: 10.1073/pnas.1307356110
14. Lambert MR, Womack MC, Byrne AQ, Hernández-Gómez O, Noss CF, Rothstein AP, et al. Comment on "Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity." *Science.* (2020) 367:eaay1838. doi: 10.1126/science.aay1838
15. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, et al. Response to comment on "Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity." *Science.* (2020) 367:eaay2905. doi: 10.1126/science.aay2905
16. Berger L, Hyatt AD, Speare R, Longcore JE. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Dis Aquat Organ.* (2005) 68:51–63. doi: 10.3354/dao068051
17. Berger L, Speare R, Skerratt L. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs Litoria caerulea with severe chytridiomycosis. *Dis Aquat Organ.* (2005) 68:65–70. doi: 10.3354/dao068065
18. Van Rooij P, Martel A, D'Herde K, Brutyn M, Croubels S, Ducatelle R, et al. Germ tube mediated invasion of *Batrachochytrium*

dendrobatidis in amphibian skin is host dependent. *PLoS ONE.* (2012) 7:e41481. doi: 10.1371/journal.pone.0041481
19. Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, et al. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science.* (2009) 326:582–5. doi: 10.1126/science.1176765
20. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc Natl Acad Sci USA.* (2010) 107:9689–94. doi: 10.1073/pnas.0914111107
21. Greener MS, Verbrugghe E, Kelly M, Blooi M, Beukema W, Canessa S, et al. Presence of low virulence chytrid fungi could protect European amphibians from more deadly strains. *Nat Commun.* (2020) 11:5393. doi: 10.1038/s41467-020-19241-7
22. Lambertini C, Becker CG, Jenkinson TS, Rodriguez D, da Silva Leite D, James TY, et al. Local phenotypic variation in amphibian-killing fungus predicts infection dynamics. *Fungal Ecol.* (2016) 20:15–21. doi: 10.1016/j.funeco.2015.09.014
23. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc Natl Acad Sci USA.* (2011) 108:18732–6. doi: 10.1073/pnas.1111915108
24. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D, Rodriguez D, et al. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proc Natl Acad Sci USA.* (2013) 110:9385–90. doi: 10.1073/pnas.1300130110
25. Byrne AQ, Vredenburg VT, Martel A, Pasmans F, Bell RC, Blackburn DC, et al. Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation. *Proc Natl Acad Sci USA.* (2019) 116:20382–7. doi: 10.1073/pnas.1908289116
26. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, et al. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade: Chytrid Genotypes and the Bullfrog Trade. *Mol Ecol.* (2012) 21:5162–77. doi: 10.1111/j.1365-294X.2012.05710.x
27. James TY, Toledo LF, Rödder D, Silva Leite D, Belasen AM, Betancourt-Román CM, et al. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecol Evol.* (2015) 5:4079–97. doi: 10.1002/ece3.1672
28. Basanta MD, Byrne AQ, Rosenblum EB, Piovia-Scott J, Parra-Olea G. Early presence of *Batrachochytrium dendrobatidis* in Mexico with a contemporary dominance of the global panzootic lineage. *Mol Ecol.* (2021) 30:424–37. doi: 10.1111/mec.15733 .
29. Briggs CJ, Knapp RA, Vredenburg VT. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc Natl Acad Sci USA.* (2010) 107:9695–700. doi: 10.1073/pnas.0912886107
30. Becker CG, Greenspan SE, Tracy KE, Dash JA, Lambertini C, Jenkinson TS, et al. Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. *Fungal Ecol.* (2017) 26:45–50. doi: 10.1016/j.funeco.2016.11.007
31. Muletz-Wolz CR, Barnett SE, DiRenzo GV., Zamudio KR, Toledo LF, James TY, et al. Diverse genotypes of the amphibian-killing fungus produce distinct phenotypes through plastic responses to temperature. *J Evol Biol.* (2019) 32:287–98. doi: 10.1111/jeb.13413
32. Angilletta MJ. Estimating and comparing thermal performance curves. *J Therm Biol.* (2006) 31:541–5. doi: 10.1016/j.jtherbio.2006.06.002
33. Huey RB, Kingsolver JG. Evolution of thermal sensitivity of ectotherm performance. *Trends Ecol Evol.* (1989) 4:131–5. doi: 10.1016/0169-5347(89)90211-5
34. Schulte PM, Healy TM, Fangue NA. Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr Comp Biol.* (2011) 51:691–702. doi: 10.1093/icb/icr097
35. Khelifa R, Blanckenhorn WU, Roy J, Rohner PT, Mahdjoub H. Usefulness and limitations of thermal performance curves in predicting ectotherm development under climatic variability. *J Anim Ecol.* (2019) 88:1901–12. doi: 10.1111/1365-2656.13077
36. Knies JL, Izem R, Supler KL, Kingsolver JG, Burch CL. The genetic basis of thermal reaction norm evolution in lab and natural phage populations. *PLoS Biol.* (2006) 4:40201. doi: 10.1371/journal.pbio.0040201
37. Kingsolver JG. The well-temperatured biologist. *Am Nat.* (2009) 174:755–68. doi: 10.1086/648310
38. Angilletta MJ, Huey RB, Frazier MR. Thermodynamic effects on organismal performance: is hotter better? *Physiol Biochem Zool.* (2010) 83:197–206. doi: 10.1086/648567
39. Frazier MR, Huey RB, Berrigan D. Thermodynamics constrains the evolution of insect population growth rates: "warmer is better." *Am Nat.* (2006) 168:512–20. doi: 10.1086/506977
40. Gaitán-Espitia JD, Belén Arias M, Lardies MA, Nespolo RF. Variation in thermal sensitivity and thermal tolerances in an invasive species across a climatic gradient: lessons from the land snail cornu aspersum. *PLoS ONE.* (2013) 8:70662. doi: 10.1371/journal.pone.0070662
41. U.S. Forest Service. *National Forest Climate Change Maps* (2019).
42. Voyles J, Johnson LR, Rohr J, Kelly R, Barron C, Miller D, et al. Diversity in growth patterns among strains of the lethal fungal pathogen *Batrachochytrium dendrobatidis* across extended thermal optima. *Oecologia.* (2017) 18:363–73. doi: 10.1007/s00442-017-3866-8
43. Piotrowski JS, Annis SL, Longcore JE. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia.* (2004) 96:9–15. doi: 10.2307/3761981
44. Stevenson LA, Alford RA, Bell SC, Roznik EA, Berger L, Pike DA. Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *PLoS ONE.* (2013) 8:e73830. doi: 10.1371/journal.pone.0073830
45. Lindauer A, May T, Rios-Sotelo G, Sheets C, Voyles J. Quantifying *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* viability. *Ecohealth.* (2019) 16:346–50. doi: 10.1007/s10393-019-01414-6
46. 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) 60:141–8. doi: 10.3354/dao060141
47. Voyles J. Phenotypic profiling of *Batrachochytrium dendrobatidis*, a lethal fungal pathogen of amphibians. *Fungal Ecol.* (2011) 4:196–200. doi: 10.1016/j.funeco.2010.12.003
48. Voyles J, Johnson LR, Briggs CJ, Cashins SD, Alford RA, Berger L, et al. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. *Ecol Evol.* (2012) 2:2241–9. doi: 10.1002/ece3.334
49. Lindauer AL, Maier PA, Voyles J. Daily fluctuating temperatures decrease growth and reproduction rate of a lethal amphibian fungal pathogen in culture. *BMC Ecol.* (2020) 20:18. doi: 10.1186/s12898-020-00286-7
50. Levitz SM, Diamond RD. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. *J Infect Dis.* (1985) 152:938–45. doi: 10.1093/infdis/152.5.938
51. Byrne AQ, Rothstein AP, Poorten TJ, Erens J, Settles ML, Rosenblum EB. Unlocking the story in the swab: a new genotyping assay for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Mol Ecol Resour.* (2017) 17:1283–92. doi: 10.1111/1755-0998.12675
52. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics.* (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507
53. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* (2004) 32:1792–7. doi: 10.1093/nar/gkh340
54. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics.* (2014) 30:1312–3. doi: 10.1093/bioinformatics/btu033
55. Junier T, Zdobnov EM. The Newick utilities: high-throughput phylogenetic tree processing in the UNIX shell. *Bioinformatics.* (2010) 26:1669–70. doi: 10.1093/bioinformatics/btq243
56. Zhang C, Rabiee M, Sayyari E, Mirarab S. ASTRAL-III: polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinform.* (2018) 19:153. doi: 10.1186/s12859-018-2129-y

57. R-Core-Team. R: *A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing (2019).
58. Eisenberg MC, Hayashi MAL. Determining identifiable parameter combinations using subset profiling. *Math Biosci.* (2014) 256:116–26. doi: 10.1016/j.mbs.2014.08.008
59. Venzon DJ, Moolgavkar SH. A method for computing profile-likelihood-based confidence intervals. *Appl Stat.* (1988) 37:87. doi: 10.2307/2347496
60. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. Mapping the Global Emergence of *Batrachochytrium dendrobatidis*, the Amphibian Chytrid Fungus. *PLoS ONE.* (2013) 8:e56802. doi: 10.1371/journal.pone.0056802
61. Kärvemo S, Meurling S, Berger D, Höglund J, Laurila A. Effects of host species and environmental factors on the prevalence of *Batrachochytrium dendrobatidis* in northern Europe. *PLoS ONE.* (2018) 13:199852. doi: 10.1371/journal.pone.0199852
62. Woodhams DC, Alford RA, Briggs CJ, Johnson M, Rollins-Smith LA. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology.* (2008) 89:1627–39. doi: 10.1890/06-1842.1
63. Rohr JR, Dobson AP, Johnson PTJ, Kilpatrick AM, Paull SH, Raffel TR, et al. Frontiers in climate change–disease research. *Trends Ecol Evol.* (2011) 26:270–7. doi: 10.1016/j.tree.2011.03.002
64. Rohr JR, Raffel TR, Romansic JM, McCallum H, Hudson PJ. Evaluating the links between climate, disease spread, and amphibian declines. *Proc Natl Acad Sci USA.* (2008) 105:17436–41. doi: 10.1073/pnas.0806368105

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 July 2021
doi: 10.3389/fvets.2021.685877

Global Patterns of the Fungal Pathogen *Batrachochytrium dendrobatidis* Support Conservation Urgency

Deanna H. Olson[1]*, *Kathryn L. Ronnenberg*[1], *Caroline K. Glidden*[2], *Kelly R. Christiansen*[1] *and Andrew R. Blaustein*[3]

[1] *Pacific Northwest Research Station, United States Department of Agriculture (USDA) Forest Service, Corvallis, OR, United States,* [2] *Department of Biology, Stanford University, Stanford, CA, United States,* [3] *Department of Integrative Biology, Oregon State University, Corvallis, OR, United States*

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a skin pathogen that can cause the emerging infectious disease chytridiomycosis in susceptible species. It has been considered one of the most severe threats to amphibian biodiversity. We aimed to provide an updated compilation of global *Bd* occurrences by host taxon and geography, and with the larger global *Bd* dataset we reanalyzed *Bd* associations with environmental metrics at the world and regional scales. We also compared our *Bd* data compilation with a recent independent assessment to provide a more comprehensive count of species and countries with *Bd* occurrences. *Bd* has been detected in 1,375 of 2,525 (55%) species sampled, more than doubling known species infections since 2013. *Bd* occurrence is known from 93 of 134 (69%) countries at this writing; this compares to known occurrences in 56 of 82 (68%) countries in 2013. Climate-niche space is highly associated with *Bd* detection, with different climate metrics emerging as key predictors of *Bd* occurrence at regional scales; this warrants further assessment relative to climate-change projections. The accretion of *Bd* occurrence reports points to the common aims of worldwide investigators to understand the conservation concerns for amphibian biodiversity in the face of potential disease threat. Renewed calls for better mitigation of amphibian disease threats resonate across continents with amphibians, especially outside Asia. As *Bd* appears to be able to infect about half of amphibian taxa and sites, there is considerable room for biosecurity actions to forestall its spread using both bottom-up community-run efforts and top-down national-to-international policies. Conservation safeguards for sensitive species and biodiversity refugia are continuing priorities.

Keywords: amphibian chytrid, *Bd*, climate associations, emerging infectious disease, fungal pathogen

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Reviewed by:
Hudson Alves Pinto, Minas Gerais State University, Brazil
Gonçalo Rosa, Zoological Society of London, United Kingdom

***Correspondence:**
Deanna H. Olson
deanna.olson@usda.gov

Specialty section:
This article was submitted to Zoological Medicine, a section of the journal Frontiers in Veterinary Science

Received: *26 March 2021*
Accepted: *15 June 2021*
Published: *16 July 2021*

Citation:
Olson DH, Ronnenberg KL, Glidden CK, Christiansen KR and Blaustein AR (2021) Global Patterns of the Fungal Pathogen Batrachochytrium dendrobatidis *Support Conservation Urgency. Front. Vet. Sci. 8:685877. doi: 10.3389/fvets.2021.685877*

INTRODUCTION

The Earth is undergoing a "biodiversity crisis," with population losses and species extinctions occurring at unprecedented rates (1–6). Contributing factors to biodiversity losses are multifaceted and are complicated by species, population- and site-specific differences. Anthropogenic stressors such as habitat loss and fragmentation, chemical contamination, introduced species, and climate

change are key factors influencing losses across taxonomic groups. Furthermore, there is increasing recognition of health concerns as species are exposed to emerging infectious diseases (EIDs) [e.g., coral disease outbreaks (7, 8); sea star wasting disease (9); bat white-nose syndrome (10, 11); avian West Nile virus (12); >50 United States (US) wildlife disease factsheets (13)].

For the especially vulnerable vertebrate class Amphibia, the Global Amphibian Assessment first reported 32.5% of species as threatened with extinction (14) and that estimate has since risen to 40% of species (5, 6), with an increase in concern for disease impacts (2, 15–20). Amphibian diseases span both lethal and sublethal multiple-host species infections by microparasites such as trematodes (21), bacteria (22), fungi (15, 23, 24), protists (25, 26), and viruses (16, 24). Information on amphibian disease-causing pathogens has increased substantially in the last two decades, especially relative to field surveillance of taxonomic and geographic patterns of pathogen occurrences at global scales (27–31) and experimental research that illustrates species-specific vulnerabilities and interacting factors (24, 32). Keeping informed about rapid advances in research and monitoring of amphibian diseases is challenging. Further complicating the challenge of tracking host-pathogen patterns of disease threats, anthropogenic processes are linked with amphibian disease dynamics. For example, human-mediated translocation of amphibian EIDs is an increasing concern, especially for chytridiomycosis, the disease caused by the chytrid fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). These two pathogens are associated with amphibian infections across continents and disease-caused mortality resulting in population losses (14, 17–20, 33, 34).

Amphibian chytridiomycosis research has transitioned from initial pathogen identification associated with amphibian mortality [*Bd* (35); *Bsal* (23)] to understanding pathogen occurrences and patterns of amphibian losses as knowledge of host-susceptibility, pathogen strain virulence, and transmission scenarios has unfolded (15, 17, 19, 24, 33, 34). Several geographic origins of *Bd* have been proposed, spanning Asia, Africa, and North and South America (36–40). A recent genetic analysis reported east Asia to be a *Bd* biodiversity hotspot, where the source of *Bd* was traced to the Korean Peninsula and one lineage showed the signature of an ancestral population tied to global emergence in the early twentieth century (34). Scheele et al. (17) estimated that *Bd* chytridiomycosis has contributed to the declines of 6.5% of amphibian species, and categorized *Bd* as one of the most destructive invasive species. Lambert et al. (18) concurred that *Bd* chytridiomycosis irrefutably harmed amphibians but because their re-analysis could not reproduce the specific results of Scheele et al. (17), they called for a more comprehensive approach to quantify the complexities of interacting amphibian threat factors.

The globalization of amphibian diseases and increasing need for both researchers and natural-resource stewards to understand EIDs and their incremental science advances has been aided by the advent of amphibian pathogen databases at world-accessible web portals. The Global *Bd* Mapping Project began in 2007, with its database of *Bd* occurrences by host taxon and geographic location going online at the web portal *Bd*-Maps.net in 2008, hosted by Imperial College, UK. This exportable database and mapping application provided the first global-scale visualization of an amphibian panzootic (19, 28). The broad use of *Bd*-Maps.net led to the development of an analogous but more comprehensive online database for Ranavirus, the Global Ranavirus Reporting System [GRRS (31)]. The GRRS inspired the development of the more sophisticated amphibian chytrid disease portal for both *Bd* and *Bsal*, AmphibianDisease.org, hosted by AmphibiaWeb and the University of California at Berkeley (41). The *Bd*-Maps.net dataset is currently in transition to AmphibianDisease.org, providing continuity of and a means to archive the Global *Bd* Mapping project from 2007 to present.

The importance of globally accessible online databases for discerning pathogen occurrence patterns is multifold. First, gaps in knowledge are readily apparent by species and location and can guide subsequent inventory and monitoring efforts; there is support that taxonomic and geographic gaps have been filled over time (28, 42, 43). Second, world occurrence maps of amphibian pathogens [e.g., *Bd* (28, 42, 43)] have been widely used for education and outreach across disciplines, raising awareness of potential emerging threat factors and informing conservation efforts. For example, global *Bd* maps have appeared in textbooks (44), museum and zoo exhibits (e.g., Panama exhibit by Smithsonian Institution; US National Zoo exhibit, Washington, DC; *Fungi and Their Diversity* exhibit, Hesse Museum, Wiesbaden, Germany), and other multimedia venues [(45, 46); e.g., ArgoFilms 2009 film for the Public Broadcasting System's *Nature* TV show, *Frogs: The Thin Green Line*]. Third, knowledge of pathogen occurrences can inform biosecurity procedures to forestall human-mediated translocation (47–49). In addition, global datasets can enable novel metadata analyses of specific hypotheses; the global *Bd* database has contributed to a variety of analyses of host-pathogen and disease-threat dynamics [e.g., (50–53)]. However, the initial *Bd* online database had some constraints. Occurrences of the disease chytridiomycosis were not tracked, as *Bd* occurrence studies often do not report the development of disease signs in sampled animals. Additionally, we now understand that disease emergence varies with *Bd* strain (34, 54), and as of this writing, the *Bd* database has not recorded *Bd* lineages with surveillance data, nor have most published sources isolated or reported the strain(s) surveyed. Furthermore, the initial *Bd* database was not set up to report zoospore loads for samples—these were not being reported in 2007, and even today, not all publications report *Bd* zoospore loads. Owing to continuing requests for the world *Bd* database, renewed calls for comprehensive analyses of world-scale data of amphibian disease threat patterns (18), and significantly increasing reports of *Bd* research and surveillance (24, 29, 30, 32), the global *Bd* database and web portal warrant maintenance and improved capacity. The new web portal AmphibianDisease.org (41) is developing to enable broader chytrid data reporting (e.g., strains, captive hosts, eDNA, zoospore loads) and user-friendly data import and export functions.

Our aim in this paper is to provide updated summaries of taxonomic and geographic detections and non-detections from newly compiled world *Bd* data through 2019. We use a format for quick comparison to *Bd* occurrence patterns previously reported

(28, 42, 43). We summarize *Bd* detection and no-detection data from wild and captive specimens, inclusive of wild-caught museum specimens that have been tested for *Bd*. Geographic patterns of *Bd* detection are assessed for countries, sites, and US 5th-field hydrologic unit code (HUC) watersheds (42) which have been used in some land-management decisions to forestall inadvertent *Bd* translocation during water draws for fire-fighting (55, 56). Furthermore, we examine environmental correlates with *Bd* site-level occurrence data compiled through 2019. Several studies have investigated the importance of temperature and moisture regimes for *Bd* occurrence, growth, and host-infection dynamics using laboratory and local- to landscape-scale analyses (57–71). Here, we examine elevation and climate parameters analyzed previously with the *Bd*-Maps.net dataset compiled through 2013 (28) and June 2014 (43) to investigate whether there is stability in *Bd* predictors (e.g., temperature range at a site). Owing to an abundance of new occurrence data and the potential climate-change implications for significant temperature and precipitation metrics with *Bd* occurrences, we conduct downscaled analyses of environmental associations with *Bd* occurrence for North America, South America, Europe, Africa, eastern Asia, and Australia.

Lastly, we compare our *Bd* database tallies by taxon and countries through 2019 with the 2020 results reported by Castro Monzon et al. (30) who examined the peer-reviewed literature of *Bd* occurrences aggregated by a web-search engine. We combine unique taxonomic and country data from Castro Monzon et al. (30) with our findings for an overarching summary of the taxonomic and geographic scope of *Bd* knowledge to date.

MATERIALS AND METHODS

Bd occurrence data management was based on methods reported previously, whereas analyses conducted here were intended to complement previous assessments (28, 43). To standardize methods among years, *Bd* occurrence database oversight including limited data quality assurance and quality control were conducted by the 2007–2019 *Bd* database manager (KLR).

Data Compilation

Bd occurrence data were compiled primarily by four methods. First, an initial dataset was compiled by regional data coordinators who submitted project data or reports for their regions for the 2007 Global *Bd* Mapping Project, presented in the first *Bd* map at the International *Bd* Conference, Tempe, Arizona, USA, in November 2007; this Global *Bd* Mapping Project database initiated the development of the *Bd*-Maps.net web portal (28). Second, *Bd* surveillance data were directly uploaded to *Bd*-Maps.net by principal investigators, 2007–2014. Third, web-based literature searches were conducted of the main international and regional journals reporting on *Bd* studies (**Supplementary Appendix 1**). Fourth, published or unpublished reports were sent directly to us (DHO, KLR) for import to the *Bd*-Maps database. We quantified the number of data sources in our 2019 database by five types: (1) peer-reviewed journal articles; (2) reports; (3) theses and dissertations; (4) online sources (newspapers, newsletters, online compilations); and (5) unpublished contributed datasets.

Amphibian taxonomy and geographic locations of *Bd* sampling per report were examined for reporting consistency (**Supplementary Appendix 1**). Taxonomy used herein followed Frost [(72); **Supplementary Appendix 1**]. Geographic locations with detectable errors (e.g., coordinates clearly outside the study area) were corrected by consultation with principal investigators, or based on other location information provided in the report. Laboratory results of *Bd* analyses were not examined for scientific integrity, including analytical sensitivity or accuracy (e.g., sample size analyzed; histological or PCR analyses). Hence, we caution that "no detection" is not synonymous with *Bd* absence in a sample, as likelihood of detection can vary with population size, sample size, *Bd* prevalence, and analysis method [e.g., (47, 73)]. Data duplication was assessed for studies imported to the *Bd*-Maps database prior to publication that were later identified in literature searches of published papers.

Bd Occurrence Categorization by Taxa and Geography

Bd data were compiled for species, records, sites, watersheds (USA only), and at the region or country level when precise coordinates or locations were not available. To assess whether *Bd* had ever been detected in an amphibian taxon, the composite data records were compiled and the taxon was labeled as "*Bd* detected" if there had ever been a single *Bd*-positive report. "*Bd* not detected" was the usual alternative, however a few reports have been challenged in the literature due to diagnostics concerns, and as a precaution those were labeled as "uncertain," as were cases where the authors themselves reported an uncertain result of a diagnostics test. A "record" was a database entry for a species at a particular location for a study (28). There were multiple records for a location if *Bd* sampling occurred for multiple species, sampling occasions, or studies.

Site-level data compilations were composite records for a common latitude/longitude coordinate, or a specific locality description (28). Site-level *Bd* occurrence was assigned to one of three categories: *Bd* detected; *Bd* not detected; *Bd* detection uncertain. Thus, even if multiple species were sampled for *Bd* at a unique geographic coordinate, or the location was sampled over multiple years, the site was designated "*Bd* detected" if *Bd* had ever been detected at that location for any species in any year. Site-level *Bd* detected and not-detected data were included in geospatial analyses described below. Countries were designated as "*Bd* detected" based on field or museum specimens sampled or collected from the wild. If the only positive sample for a country came from a captive sample, the country was not designated as "*Bd* detected." An analysis of continental USA watershed-scale *Bd* occurrence was conducted. As for sites, an individual watershed was designated as "*Bd* detected" if *Bd* had ever been detected in samples of any species in any year. If a watershed had been sampled but *Bd* had never been detected there, it was labeled as "*Bd* not detected." For country-scale patterns, a country was labeled as *Bd* detected or not detected based on the composite data in the database for that nation.

TABLE 1 | Environmental attributes analyzed for associations with *Batrachochytrium dendrobatidis* (*Bd*) occurrence (detection, no detection) across world sites with *Bd* sampling compiled through 2019.

Attribute (units)	Description
Elevation (m)	Altitude above sea level
Mean annual precipitation (mm)	10-year mean annual precipitation
Low average monthly precipitation (mm)	10-year average of lowest monthly precipitation
Mean average monthly precipitation (mm)	10-year mean of average monthly precipitation
High average monthly precipitation (mm)	10-year average of highest monthly precipitation
Temperature range (°C)	10-year monthly average daily maximum temperature (tmax) minus 10-year monthly average daily minimum temperature (tmin)
Low average monthly temperature (°C)	10-year average of lowest monthly temperature
Mean average monthly temperature (°C)	10-year mean of average monthly temperature
High average monthly temperature (°C)	10-year average of highest monthly temperature
Low average monthly minimum temp. (°C)	10-year average of lowest monthly minimum temperature
Mean average monthly minimum temp. (°C)	10-year mean of average monthly minimum temperature
High average monthly minimum temp. (°C)	10-year average of highest monthly minimum temperature
Low average monthly maximum temp. (°C)	10-year average of lowest monthly maximum temperature
Mean average monthly maximum temp. (°C)	10-year mean of average monthly maximum temperature
High average monthly maximum temp. (°C)	10-year average of highest monthly maximum temperature

Environmental Predictors of *Bd* Occurrence

Analyses of *Bd* occurrence associations with environmental attributes focused on elevation and 14 climate metrics (**Table 1**) of world sites with *Bd* sampling compiled through 2019. Elevation and climate data were derived from online global geographic models (**Supplementary Appendix 1**). World climate data were available for 0.5-degree latitude/longitude grid cells, hence *Bd* site-level occurrences were consolidated per grid cell for consistency with climate data (i.e., per grid cell, *Bd* was either detected or not). This consolidation likely reduces potential spatial autocorrelation, data collection biases among sampling events, and geographic- and population-level redundancy considerations of the reported source data.

To avoid collinearity issues, we removed highly correlated predictor variables (74). Consequently, we refined elevation and climate data to six parameters for analyses. Three elevation metrics were determined per 0.5-degree latitude and longitude grid cell and used in analyses: mean elevation; minimum elevation; and maximum elevation. For climate metrics, 10-year mean annual precipitation was highly correlated (>0.7) with: 10-year average of lowest monthly precipitation, 10-year average of highest monthly precipitation, and 10-year average of average monthly precipitation. Thus, only 10-year mean annual precipitation was used in analyses. Similarly, 10-year mean annual daily temperature was highly correlated (>0.7) with all other temperature variables (10-year lowest mean temperature, 10-year highest mean temperature, 10-year mean low temperature, 10-year lowest mean low temperature, 10-year highest mean low temperature, 10-year mean high temperature, 10-year lowest mean high temperature, 10-year highest mean high temperature). Hence, only 10-year mean annual daily temperature was used in analyses. The final six covariates used in the models of environmental associations with *Bd* occurrence were: 10-year mean annual precipitation, 10-year mean annual daily temperature, 10-year average temperature range, mean elevation, minimum elevation within the cell, and maximum elevation within the cell.

Both presence-only and presence-and-absence (i.e., absence = no detection) Species Distribution Models (SDMs) were evaluated. Given the uncertain nature of true absences, presence-only models have been considered more robust (75), whereas presence-absence data include the broader dataset assembled for *Bd* and can be compared with previous models. With global and regional subsets of data, using presence-only (detections-only) data, a maximum-entropy SDM was used to estimate the effect of environmental covariates on relative odds of *Bd* occurrence. With global and regional datasets, using both detection and no-detection data, a logistic regression SDM was used to estimate the effect of environmental covariates on odds of *Bd* occurrence. As we expect non-linear relationships between environmental covariates and probability of *Bd* occurrence, we transformed each covariate (linear, monotonous, deviation, forward hinge, reverse hinge, threshold) and used forward selection to select the transformations that best-explained variation in *Bd* occurrence. After variable transformation, a subset selection procedure was used to determine the best-fit model (i.e., select the final environmental covariates). As we expected interactions among covariates (e.g., the effect of mean temperature depends on annual precipitation), we allowed for interactions among all covariates. To visualize the form of the relationship between final model covariates and probability of *Bd* occurrence (e.g., unimodal), we plotted model predictions for a range of the environmental covariate while holding all other environmental covariates at their mean. To visualize the form of interactions among covariates, we plotted model predictions for a range of the environmental covariate while holding the interacting covariate at the 0.25% percentile, mean, and 0.75%, and all other covariates at the mean.

Per SDM, we determined the fraction of total variation accounted (FTVA) for by main parameters in the best-fit model [i.e., measure of the parameter contribution to explain variation in *Bd* occurrence (76)]. We evaluated model performance by calculating the area under the curve (AUC) which provides an aggregate measure of model sensitivity (i.e., ability to correctly classify grid cells with *Bd* detection) and specificity (i.e., ability to correctly classify grid cells with no *Bd* detection). At AUC = 1.0, the model can perfectly categorize true negatives and

positives, whereas if AUC = 0, it incorrectly categorizes all true negatives and positives. If AUC = 0.5, the model makes predictions equivalent to random guesses. For each SDM, we trained the model using 75% of the data and tested the model with the remaining 25%. The data were randomly split into a training and test set using the R package *caTools* (77). Final models were fit with the entire dataset. FTVA and AUC were calculated using *MIAMaxent*.

Finally, to visualize habitat suitability of *Bd* using analyzed environmental parameters, we calculated model predictions for each grid location within a global or regional map and plotted using model predictions. Importantly, predictions for the presence-only models were scaled to the probability ratio output [PRO (78)] and can be interpreted as relative habitat suitability of *Bd* occurrence (79), whereas predictions from the presence-absence models represented absolute probability of *Bd* occurrence. The probability ratio output was $\log_2$ (log base 2) transformed to improve visualization. We excluded data from Madagascar when fitting the African regional models owing to some uncertain results for the area in the literature (see **Supplementary Table 1** footnote), but we projected the African regional models to Madagascar to show potential *Bd* occurrence. We also excluded data from Papua New Guinea when fitting the regional model because the amphibian fauna has similarities to Australia, whereas habitat may be more reflective of Southeast Asia; we projected the Asian model to Papua New Guinea to predict potential *Bd* occurrence probability based on Asian *Bd* environmental associations. SDM model predictions were calculated in the R package *MIAMaxent* (80) and global and regional predictions were plotted in the R package *ggplot2* (81).

RESULTS

Our *Bd* occurrence data compilation through 2019 included 773 sources: 661 peer-reviewed journal articles; 16 reports or proceedings; 13 theses and dissertations; 5 online sources; and 78 unpublished contributed datasets. Worldwide *Bd* surveillance across amphibian taxa and geographies through 2019 showed advancing knowledge of *Bd* occurrences, with geographic knowledge gaps filled compared to June 2014 (**Figures 1**–**3**). *Bd* data were summarized across 33,753 overall sampling records (e.g., sampling effort for a species for a project location in a year) with *Bd* detections and no-detections for wild (including museum specimens of wild-caught animals) and captive animals (**Supplementary Table 1**).

Taxonomic Patterns

Through 2019, our world *Bd* data compilation showed that *Bd* had been detected in 1,294 of 2,412 (54%) amphibian species sampled, and that sampling had been conducted in 29% of all amphibian species (**Table 2** and **Supplementary Table 2**). Anurans (frogs and toads) had the highest species-level prevalence of infection (54.7%) compared to caudates (newts and salamanders: 49.2%), and gymnophionans (caecilians: 29.2%). Through 2019, there were *Bd* detections in 86% of amphibian families. *Bd* surveys have been reported for all amphibian families except one anuran family (Nasikabatrachidae, 2 spp.: Western Ghats, India); one caudate family (Rhyacotritonidae, 4 spp.: Pacific Northwest USA); and one gymnophionan family (Chikilidae, 4 spp.: Northeast India) (**Tables 2**, **3**). However, we are aware that in ongoing experiments of *Bsal* susceptibility in USA salamanders, wild-caught members of Rhyacotritonidae have been screened for *Bd* prior to use in laboratory trials, and *Bd* has not been detected (J. Piovia-Scott, Washington State University, Vancouver, WA, USA; pers. commun.). Species-level *Bd* prevalence among families was highly variable (**Table 3**). Through 2019, 6 of 55 (11%) Anura families and 3 of 9 (33%) Gymnophiona families had no *Bd* detections among sampled species (**Tables 2**, **3**).

Geographic Patterns

Geographically, our compilation of studies detected *Bd* in the wild in 88 of 124 (71%) countries sampled through 2019 (**Figures 1**, **2** and **Supplementary Table 3**); these 124 countries included 6 countries for which only *Bd*-negative (no-detection) samples with a "country-centroid coordinate" were reported (i.e., no location reported: Armenia, Barbados, Central African Republic, Gambia, Iran, Latvia). We recognize that some country names and boundaries have been dynamic, our intention here is to include recognizable principalities over time. For example, the record of *Bd* occurrence in North Korea is from the analysis of a museum specimen reported in 2015 (82) yet the animal had been collected in the year 1911 when the Korean Peninsula was a single political entity; Hong Kong is included separately here although it is now part of China. Our limited quality assurance and quality control of reported data resulted in correction of a small minority of location coordinates (**Supplementary Appendix 1**).

We examined our data by sites (i.e., locations having a common latitude/longitude coordinate for sampling of one or more amphibian species for *Bd* infection), compiling *Bd* sampling across 14,647 discrete sites worldwide, inclusive of both wild and captive animals but not including 67 reports from regions lacking geographic specificity (**Supplementary Table 1**). We could not know if captive animals were infected with *Bd* in the wild or during captivity, so captive sites were not used in subsequent analyses of environmental associations. Excluding captive animals and a small number of results with diagnostic uncertainties, *Bd* in wild-caught amphibians was detected at 5,550 of 14,413 (38.5%) sites. These 14,413 site-level *Bd* detection and no-detection data were used in analyses of climatic and geographic correlates (below). We mapped *Bd* detections and no-detections for continental-USA 5th-field HUC watersheds: *Bd* detections were reported for 916 of 1,874 (49%) sampled watersheds (**Figure 3**).

Environmental Associations With *Bd* Occurrence

Consolidation of site-level *Bd* occurrence data compiled through December 2019 into 0.5-degree grid cells resulted in 3,777 grid cells used in global SDMs analyzed with both detection and no-detection data. Using only the detection data in global SDMs (presence-only models), 2,012 grid cells were analyzed.

Using only detection data, the best-fit global model included four environmental parameters and their interactions per grid

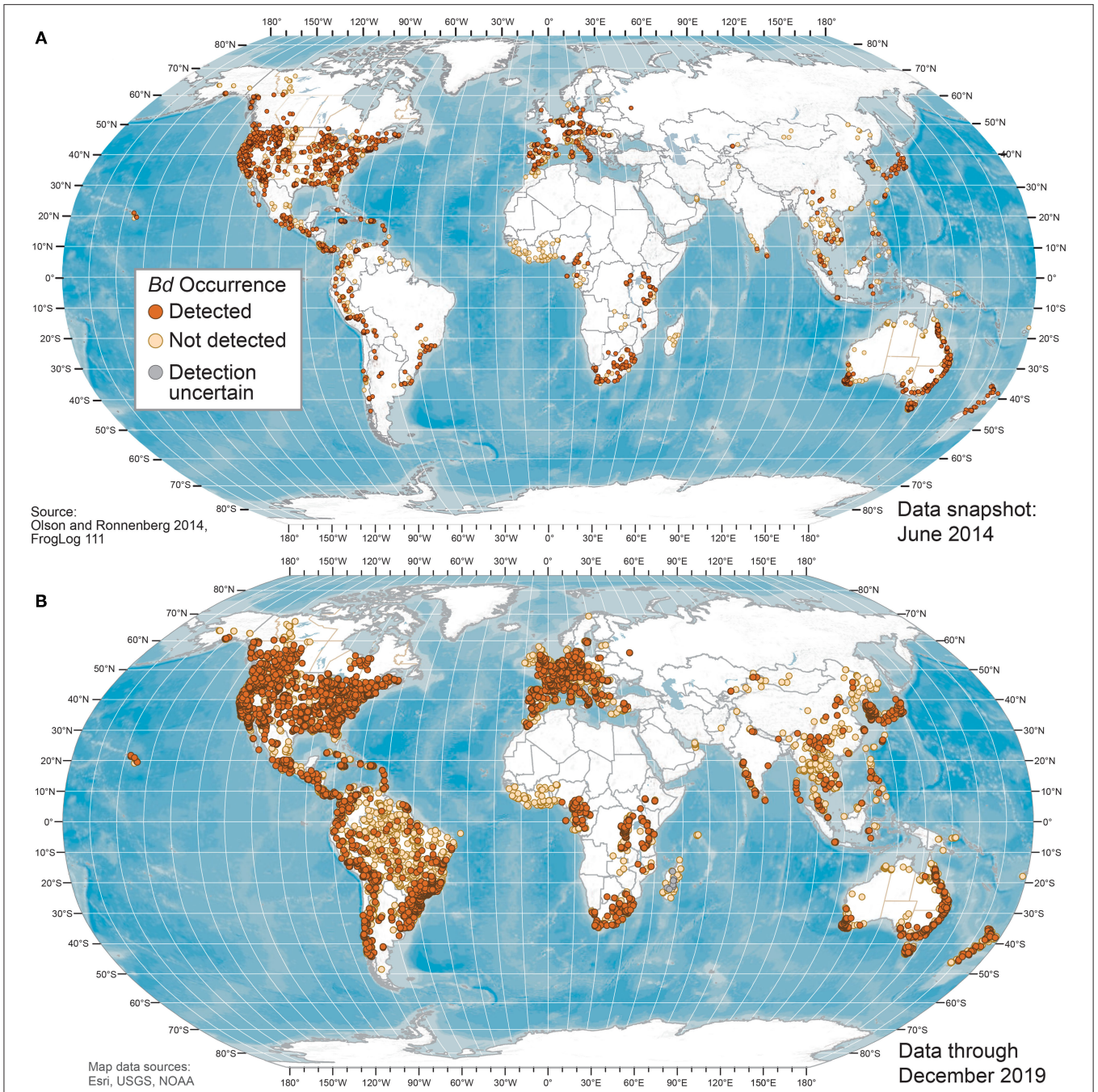


FIGURE 1 | World maps of *Batrachochytrium dendrobatidis* (*Bd*) occurrences at unique sites from data compiled for the Global *Bd* Mapping project through: **(A)** June 2014 (42); and **(B)** December 2019. Sites shown with *Bd* detections also may have sampling results with no detection; records with only country-level coordinates are not mapped.

cell: 10-year mean annual daily temperature (mean temp); 10-year mean annual precipitation (annual precipitation); 10-year average temperature range (temp range); and maximum elevation within the grid cell (elevation max). The relative probability of *Bd* occurrence was a function of mean temp + annual precipitation + temp range + elevation max + (annual precipitation * elevation max) + (annual precipitation * temp range) + (mean temp * elevation max) + (mean temp * temp range) + (mean temp * annual precipitation) + (elevation max * temp range). Mean annual daily temperature accounted for the highest fraction of total variation in probability of *Bd* occurrence (0.713), with annual precipitation accounting for the second highest fraction (0.218; **Table 4**).

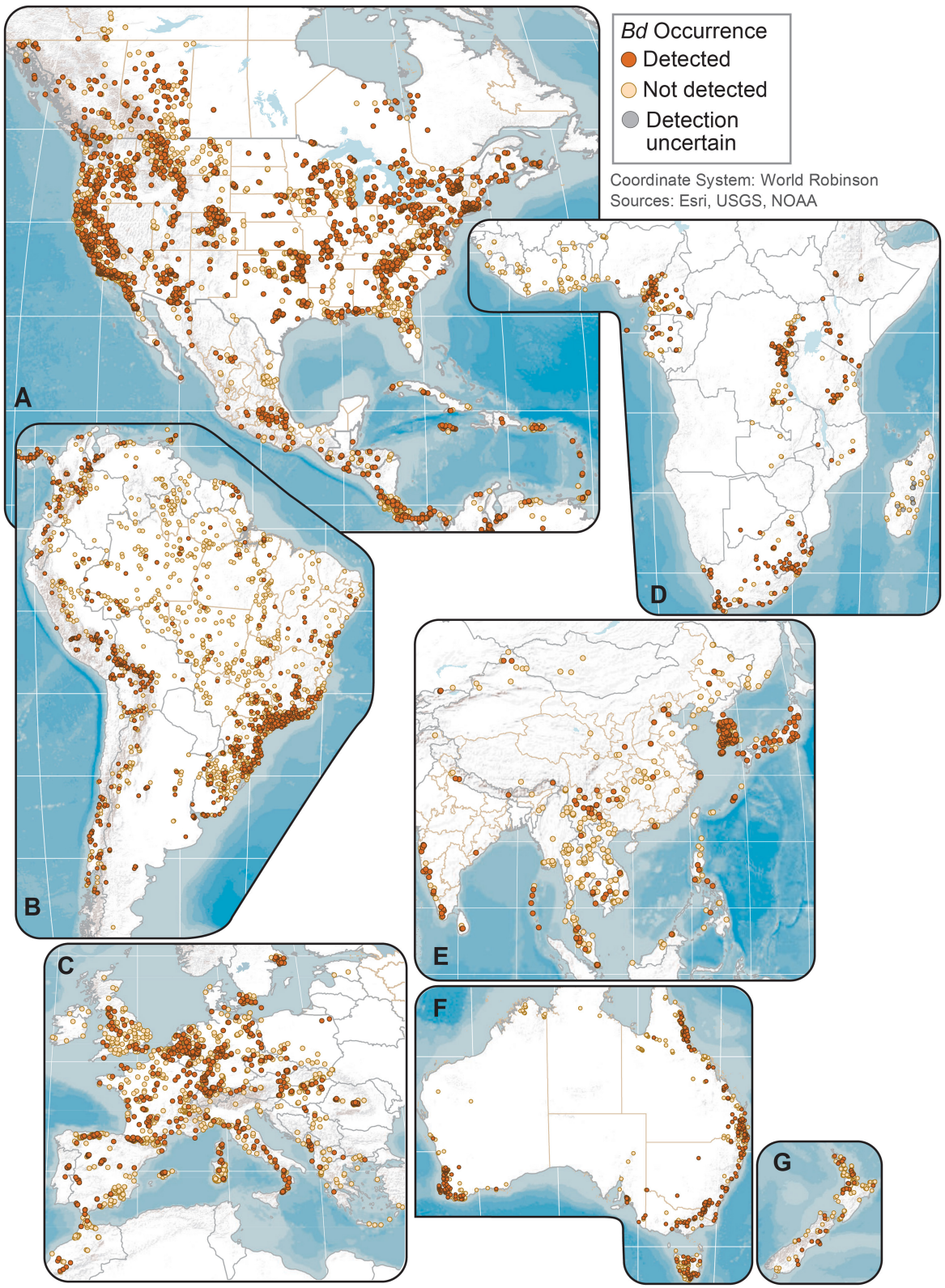


FIGURE 2 | Regional maps of *Batrachochytrium dendrobatidis* (*Bd*) occurrences at unique sites from data compiled for the Global *Bd* Mapping project: **(A)** North America; **(B)** South America; **(C)** Europe; **(D)** Africa; **(E)** Eastern Asia; **(F)** Australia; and **(G)** New Zealand. Sites shown with *Bd* detections also may have sampling results with no detection; records with only country-level coordinates are not mapped.

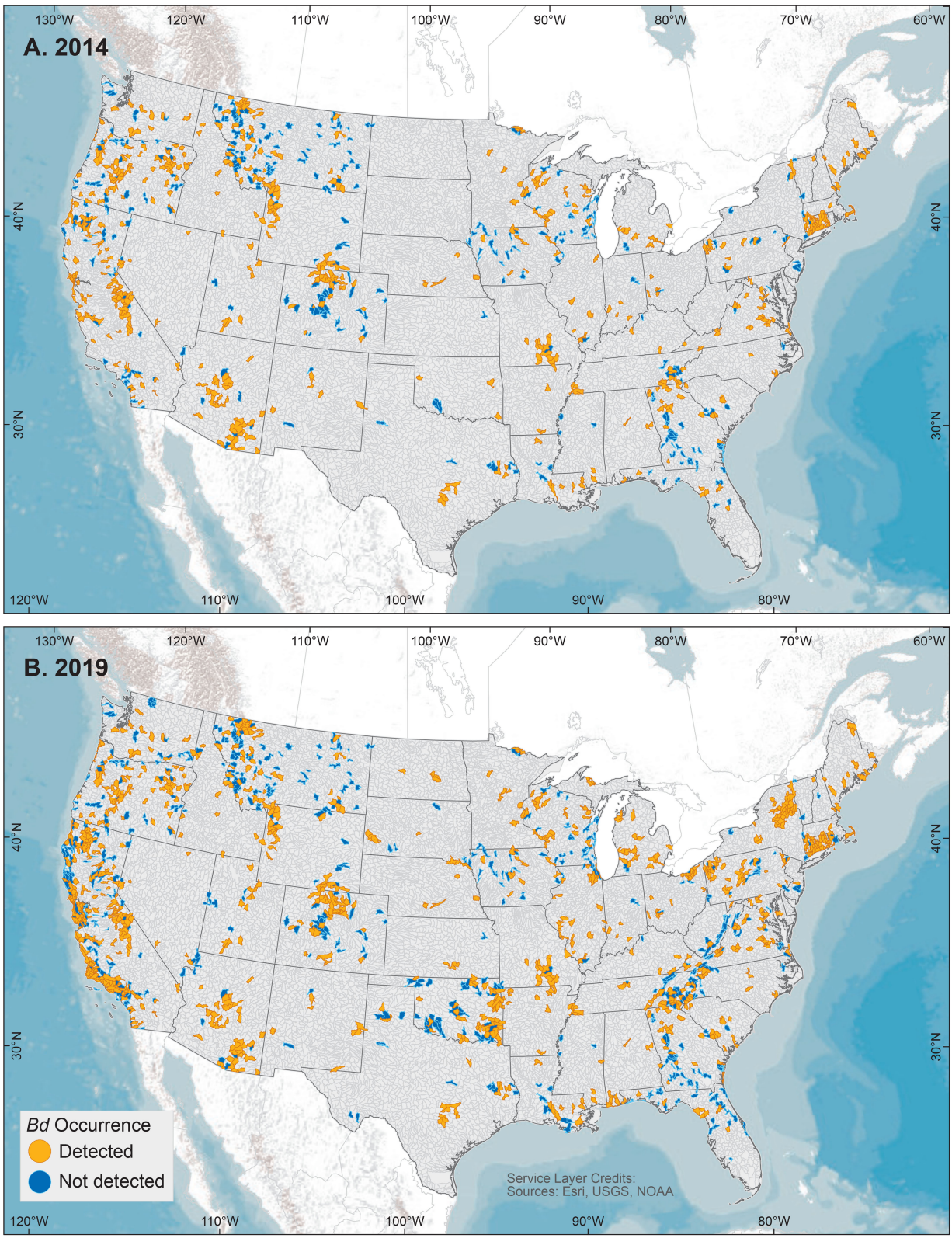


FIGURE 3 | United States 5th-field hydrologic unit code watershed maps of *Batrachochytrium dendrobatidis* (*Bd*) occurrences from data compiled for the Global *Bd* Mapping project through: **(A)** June 2014 (42); and **(B)** December 2019. Watersheds shown with *Bd* detections also may have sampling results with no detection.

TABLE 2 | Global *Batrachochytrium dendrobatidis* (*Bd*) detections in amphibians as compiled through December 2019.

	Species				Families			
	***Bd* detected**	**Tested**	**Prevalence (%)**	**Total species**	***Bd* detected**	**Tested**	**Prevalence (%)**	**Total families**
Anura	1,153	2,106	54.7	7,311	49	55	89.1	56
Caudata	127	258	49.2	762	8	8	100	9
Gymnophiona	14	48	29.2	210	6	9	66.7	10
Total	1,294	2,412	53.6	8,283	63	72	85.9	75

In the SDM derived from both detection and no-detection data at the global scale, the best-fit model included two environmental parameters and their interactions: mean temp and temp range. Probability of *Bd* occurrence was a function of mean temp + temp range + mean temp * temp range. Variation in probability of *Bd* occurrence was primarily described by mean temperature (0.969; **Table 4**).

In the global SDMs, the relative probability (presence-only model) or absolute probability (presence-absence model) of *Bd* occurrence responded non-linearly to each environmental covariate (**Figures 4**, **5**). A more detailed representation of these figures showing frequency of observations for each environmental covariate and kernel-estimated data density showing the sampling effort (no. grid cells) are depicted in **Supplementary Figures 1**, **2**.

In the SDM derived from detection-only data, there was a unimodal relationship between 10-year mean annual daily temperature and relative probability of *Bd* occurrence, peaking at ∼12–13°C, when holding all other covariates at their mean (**Figure 4A**). Relative probability of *Bd* occurrence had a more complex relationship with mean annual precipitation, showing an initial modal maximum at ∼1,200–1,400 mm, a dip at ∼3,000 mm, before increasing around 4,000 mm, when holding all other covariates at their mean (**Figure 4B**). Relative probability of *Bd* also formed a unimodal relationship with maximum elevation, peaking at ∼4,000 m, when holding all other covariates at their mean (**Figure 4C**). The relationship between temperature range and *Bd* occurrence plateaued between ∼8 and 21°C, with a stark decrease in relative probability of *Bd* occurrence at both cooler and warmer temperatures, when holding all other covariates at their mean (**Figure 4D**). To visualize interactions among covariates, model predictions were plotted with interacting covariates held at the 0.25% percentile, mean, and 0.75% percentile (**Supplementary Figures 3A,B**). The AUC for the global presence-only model was 0.86, indicating a model with high sensitivity and specificity.

In the presence-absence model, at the average temperature range, probability of *Bd* detection increased once mean temperature increased past 0°C and decreased when mean temperature exceeded ∼20°C (**Figure 5A**). At the mean temp, probability of *Bd* detection tended to increase as temperature range increased, with a peak around 18°C (**Figure 5B**). Interactions between mean temperature and temperature range are plotted in **Supplementary Figure 4**. The AUC for the presence-absence global model was 0.63, indicating a model with less sensitivity and specificity than the presence-only model.

The maps of *Bd* habitat suitability (presence-only model) and probability of *Bd* occurrence (presence-absence model) from our best-fit global models (**Figure 6**) were reflective of our dot distribution of *Bd* occurrences (**Figure 1**). Areas of heightened likelihoods of *Bd* occurrence included mesic mid-latitude and coastal influences, especially when considering the presence-only model. North-temperate, interior-continental, and arid zones had lowest *Bd* probabilities.

In regional SDMs, relative probability (using detection-only data) and absolute probability of *Bd* (using detection and no-detection data) also responded non-linearly to environmental covariates. For regional SDMs using detection-only data, 10-year mean annual daily temperature (mean temp) was retained in all final models (**Table 5**), with mean temperature capturing the most variation in relative probability of *Bd* occurrence in North America, South America, and Europe (**Table 5**). In all three regional models, shape of the response of *Bd* occurrence reflected that of the regional model (unimodal with a peak around ∼12–13°C). Notably, in North America and South America, the effect of mean temperature changed with temperature range and max elevation, respectively. In Africa, max elevation in a ∼55-km grid cell (max elevation) and annual precipitation contributed the most variation to relative probability of *Bd* occurrence (0.413 and 0.339, respectively; **Table 5**). When all other covariates were held at their mean, relative probability of *Bd* occurrence increased with max elevation until a plateau around 2,000 m and linearly increased with annual precipitation. However, the effect of precipitation was dependent upon mean temperature. In Asia, annual precipitation contributed the most variation in relative probability of *Bd* occurrence followed by mean temperature (0.626 and 0.374, respectively; **Table 5**). The relative probability of *Bd* occurrence increased with annual precipitation, whereas model response to mean temperature followed a unimodal pattern reflective of the global presence only model. In Australia, temperature range contributed the most variation to relative probability of *Bd* occurrence (0.675, **Table 5**). When all other covariates were held at their mean, relative probability of *Bd* followed a unimodal response to temperature range, with relative probability of *Bd* occurrence peaking at a temperature range of ∼10°C. Model predictions from the presence-only models (PRO of *Bd* occurrence) were projected in regional maps (**Figure 7**). AUC for regional presence-only models ranged from 0.79 to 0.93 (**Table 5**).

TABLE 3 | Family-level summary of *Batrachochytrium dendrobatidis* (*Bd*) detections among species sampled for *Bd*.

Family	No. spp. *Bd* detected	No. spp. tested	Spp. prevalence	Total spp. in family
Anura[a]				
Allophrynidae	0	1	0.00	3
Alsodidae	8	17	0.47	26
Alytidae	7	9	0.78	12
Aromobatidae	17	25	0.68	128
Arthroleptidae	52	96	0.54	149
Ascaphidae	1	2	0.50	2
Batrachylidae	6	7	0.86	12
Bombinatoridae	4	5	0.80	8
Brachycephalidae	9	12	0.75	74
Brevicipitidae	1	7	0.14	37
Bufonidae	102	204	0.50	630
Calyptocephalellidae	2	2	1.00	5
Centrolenidae	18	33	0.54	156
Ceratobatrachidae	2	14	0.14	102
Ceratophryidae	7	7	1.00	12
Conrauidae	2	5	0.40	6
Craugastoridae[b]	97	185	0.52	874
Cycloramphidae[c]	13	19	0.68	36
Dendrobatidae	33	49	0.67	203
Dicroglossidae	17	46	0.37	215
Eleutherodactylidae	36	68	0.53	230
Heleophrynidae	5	5	1.00	7
Hemiphractidae	20	225	0.80	118
Hemisotidae	0	2	0.00	9
Hylidae[d]	190	255	0.74	734
Hylodidae	23	29	0.79	47
Hyperoliidae	68	107	0.64	228
Leiopelmatidae	1	4	0.25	4
Leptodactylidae	49	87	0.56	231
Limnodynastidae	14	23	0.61	43
Mantellidae	1[e]	77	0.01	233
Megophryidae	5	31	0.16	280
Micrixalidae	1	2	0.50	24
Microhylidae	26	96	0.27	703
Myobatrachidae	20	37	0.54	89
Nyctibatrachidae	2	2	1.00	39
Odontobatrachidae	0	1	0.00	5
Odontophrynidae[f]	7	11	0.64	50
Pelobatidae	3	4	0.75	5
Pelodryadidae[g]	35	69	0.51	219
Pelodytidae	0	1	0.00	5
Petropedetidae	6	11	0.54	13
Phrynobatrachidae	20	40	0.50	95
Phyllomedusidae	18	25	0.72	67
Pipidae	21	26	0.81	41
Ptychadenidae	12	23	0.52	64
Pyxicephalidae	20	28	0.71	85
Ranidae	96	166	0.58	419
Ranixalidae	2	6	0.33	18
Rhacophoridae	27	62	0.44	434
Rhinodermatidae[h]	2	2	1.00	3
Rhinophrynidae	0	1	0.00	1
Scaphiopodidae	4	7	0.57	7
Sooglossidae	0	3	0.00	4
Telmatobiidae	21	25	0.84	63
Total Anura	1,153	2106	0.55	7,311
Caudata[i]				
Ambystomatidae	20	24	0.83	37
Amphiumidae	2	3	0.67	3
Cryptobranchidae	3	3[j]	1.00	4
Hynobiidae	3	20	0.15	85
Plethodontidae	70	150	0.47	491
Proteidae	4	4	1.00	9
Salamandridae	22	50	0.44	128
Sirenidae	3	4	0.75	5
Total Caudata	127	258	0.49	762
Gymnophiona[k]				
Caeciliidae	0	7	0.00	43
Dermophiidae	2	5	0.40	14
Herpelidae	2	3	0.67	10
Ichthyophiidae	0	5	0.00	57
Indotyphlidae	1	7	0.14	24
Rhinatrematidae	0	2	0.00	14
Scolecomorphidae	2	3	0.67	6
Siphonopidae	3	7	0.43	26
Typhlonectidae	4	9	0.44	14
Total gymnophiona	14	48	0.29	210

Total species in family as of November 2020 based on Frost 2020 (72).

[a] *Family Nasikabatrachidae (Western Ghats of India, with 2 species) not yet sampled.*

[b] *Includes former family Strabomantidae.*

[c] *Not including family Rhinodermatidae, listed separately below; genus* Proceratophrys *moved to Odontophrynidae.*

[d] *Not including species split off into new family Pelodryadidae.*

[e] *Twelve species with uncertain positive tests in Madagascar, 1 positive captive animal in the USA, see* ***Supplementary Material*** *and discussion of Madagascar results in the text.*

[f] *Genus* Proceratophrys *moved from Cycloramphidae to Odontophrynidae.*

[g] *New family split from Hylidae.*

[h] *New family split from Cyclorhamphidae.*

[i] *Family Rhyacotritonidae (Pacific Northwest United States, 4 spp.) may have been sampled, but results have not yet been reported.*

[j] *Prior versions of this table treated* Cryptobranchus alleganiensis alleganiensis *and* C. a. bishopi *as separate species, but the current taxonomy regards them as one species.*

[k] *Family Chikilidae (Northeast India, 4 spp.) has not been sampled.*

Like regional models using detection-only data, for regional SDMs using detection and no-detection data, mean temperature was retained in all final models (**Table 6**), with mean temperature capturing the majority of variation in relative probability of *Bd* detection in North America, South America, and Europe (**Table 6**). Furthermore, mean temperature accounted for the majority (or all) of variation in *Bd* detection in Africa, Asia, and

TABLE 4 | Fraction of total variation accounted for (FTVA) by each variable in best-fit global species distribution models (SDMs) of *Batrachochytrium dendrobatidis* (*Bd*) occurrence from data compiled through December 2019.

Model	Variable	FTVA
Presence-only global (AUC = 0.86)	10-year mean annual daily temperature	0.713
	10-year mean annual precipitation	0.218
	maximum elevation in a 55-km grid cell	0.046
	10-year average temperature range	0.023
Presence-absence global (AUC = 0.63)	10-year mean annual daily temperature	0.969
	10-year average temperature range	0.031

The presence-only model used Bd detection data and the presence-absence model used Bd detection and no-detection data. AUC, area under the curve, measure of model sensitivity (ability to correctly classify 0.5-degree latitude/longitude grid cells with Bd detection).

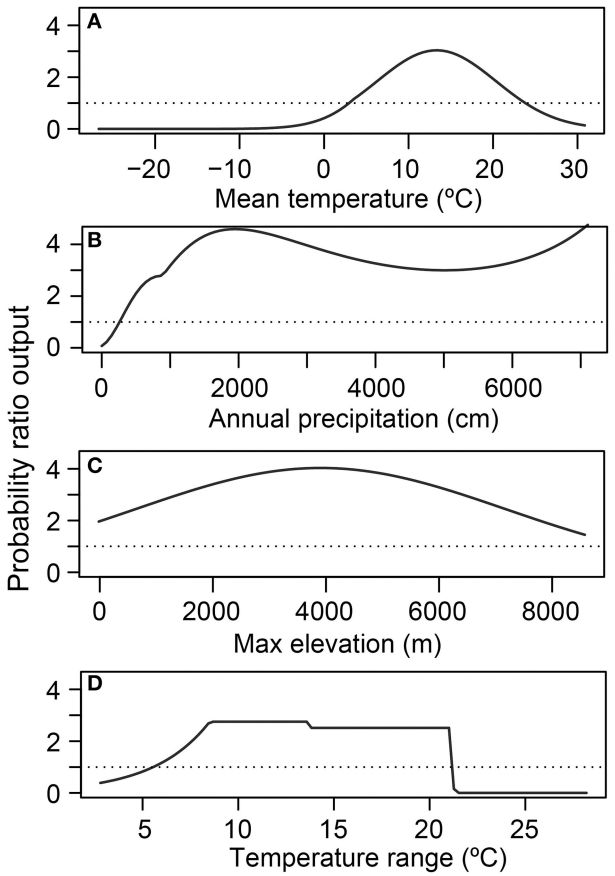


FIGURE 4 | Relative habitat suitability of *Batrachochytrium dendrobatidis* (*Bd*) occurrence (probability ratio output) for each environmental attribute [**(A)** mean temperature; **(B)** annual precipitation; **(C)** maximum elevation; **(D)** temperature range] from the global presence-only best-fit species distribution model. Each environmental attribute marginal-response plot is calculated while holding all other covariates at the mean.

Australia (**Table 6**). In North America, South America, Africa and Australia, response of probability of *Bd* detection to mean temperature followed a hinge-type pattern similar to the global

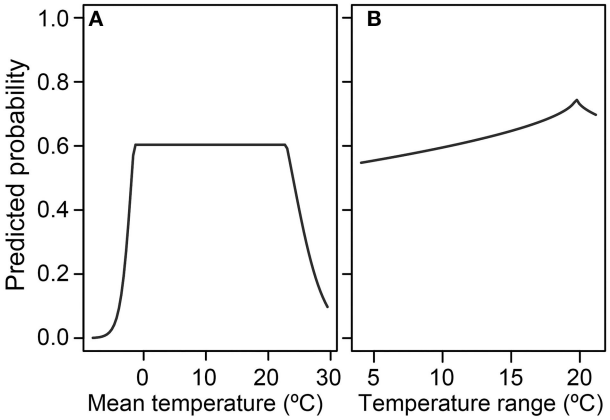


FIGURE 5 | Absolute probability of *Batrachochytrium dendrobatidis* (*Bd*) occurrence for the environmental attributes **(A)** mean temperature and **(B)** temperature range from the global presence-absence best-fit species distribution model.

presence-absence model, where probability of *Bd* increased until ∼0°C, plateaued, and then decreased after ∼20°C. In Europe and Asia, probability of *Bd* detection to mean temperature was more typically unimodal and similar in shape to the global presence-only model: probability of occurrence increased up to ∼12–13°C and then decreased. In North America, temperature range contributed a substantial portion of variation to probability of *Bd* detection (0.355; **Table 6**). In South America, max elevation also contributed a small portion of variation to probability of *Bd* detection (0.079; **Table 6**). In Australia, annual precipitation accounted for almost half of variation in probability of *Bd* detection (0.433; **Table 6**). Regional model predictions (probability of *Bd* detection) from the *Bd* detection and no-detection model were projected in maps (**Figure 8**). AUC for regional presence-absence models ranged from 0.495 to 0.724 (**Table 6**).

DISCUSSION

Our results provide new insights into a pathogen that has emerged as one of the most severe threats to amphibian biodiversity (2, 34), representing a "paradigm shift in our understanding of how emerging infectious diseases contribute to global patterns of biodiversity loss" (19). Our compilation of *Bd* sampling shows increasing taxonomic scope of sampled families and species, increased incidence of species infection, and increased geographic occurrence. Below, we combine our findings with those of Castro Monzon et al. (30) to yield a more comprehensive tally of total *Bd* occurrence patterns. In addition, our analyses support emergence of new key climate predictors of *Bd* occurrence and geographic variance in climate metrics associated with the occurrence of *Bd*. These support new hypotheses for downscaled analyses of regional contexts associated with pathogen occurrence patterns and renewed efforts for species and microrefugia

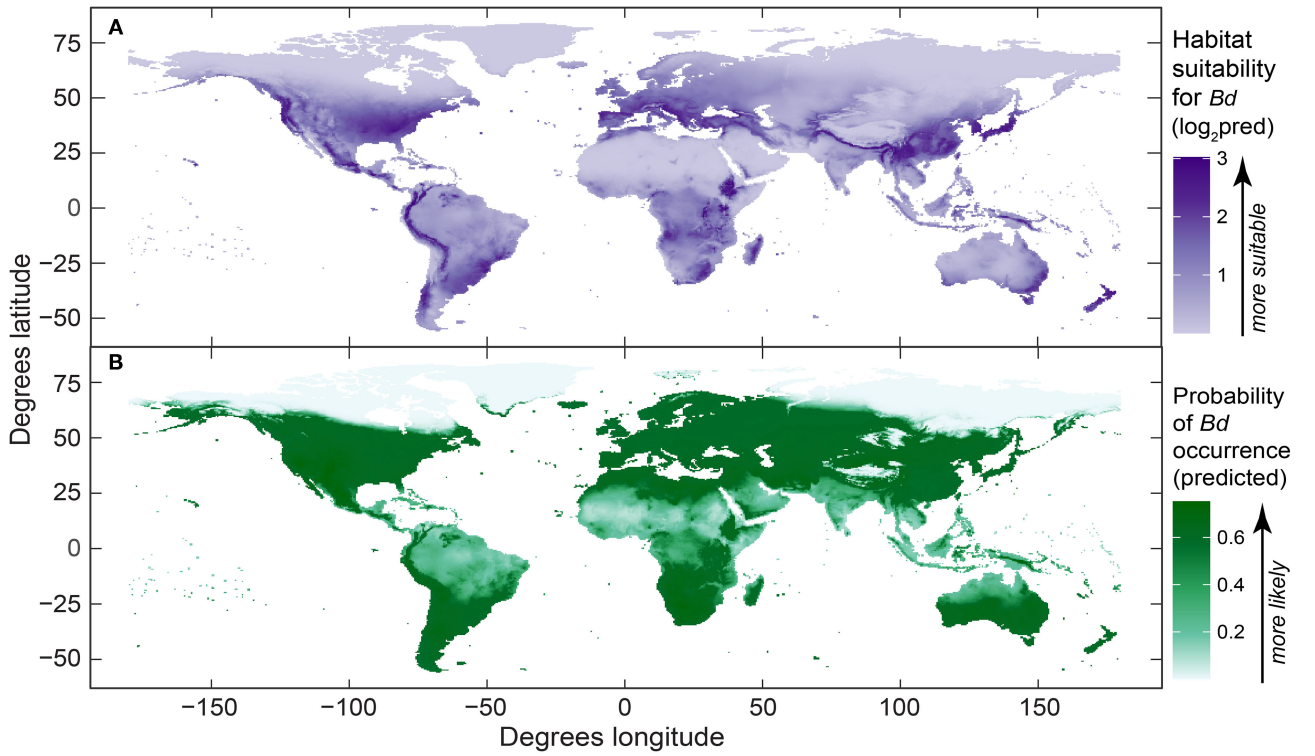


FIGURE 6 | Global maps of predicted *Batrachochytrium dendrobatidis* (*Bd*): **(A)** habitat suitability derived from the best-fit presence-only species distribution model (log_2-transformed [log base 2] probability ratio output, area under curve [AUC] = 0.86); and **(B)** probability of *Bd* occurrence from the best-fit presence-absence species distribution model (AUC = 0.62). Both maps were derived using *Bd* data compiled through December 2019.

identification and management for more effective conservation. The large number of *Bd* occurrence reports accruing over time (**Table 2** and **Supplementary Table 2**) points to the common aims of worldwide investigators to understand the taxonomic and geographic scope of *Bd* infections and the underlying global conservation concerns for amphibian biodiversity in the face of potential disease threat. A better understanding of linkages between these pathogen occurrence patterns and amphibian disease threats are needed across continents.

Global *Bd* Occurrence

To gain a more comprehensive summary of *Bd* occurrence by taxonomy and geography, we compared our *Bd* data compilation through 2019 with the web search conducted by Castro Monzon et al. (30), who independently compiled *Bd* occurrences from the published literature through mid-2020. We summed unique reports from our analyses and Castro Monzon et al. (30) to calculate the total numbers of families, species, and countries with *Bd detections* from these combined datasets. Our two data compilation approaches differed in data sources. Castro Monzon et al. (30) cited 554 papers produced by their web search. In comparison, our data compilation through 2019 included 773 total sources, including sources outside the peer-reviewed literature. Together, a more comprehensive perspective is gained by combining our two approaches, but we acknowledge that even together, the compilation is incomplete; past reports that have not yet been included in these composite summaries are continually brought to our attention. Below, we also compared our compilation by taxonomy and geography to the earlier *Bd*-Maps database (28, 42) to estimate accretion of knowledge over time.

Castro Monzon et al. (30) reported *Bd* sampling across 71 amphibian families; these families were included in our compilation, although in our data compilation we added an unpublished captive report of a detection in Mantellidae (*Mantella* sp.; **Supplementary Table 2**), bringing the total to 72 families. Also, if the caudate family Rhyacotritonidae is included (Piovia-Scott, pers. commun.), then *Bd* is now known to have been sampled in a total of 73 amphibian families. Since 2014, 6 additional families (including Rhyacotritonidae, plus Pelodryadidae which has since split off from Hylidae) have been sampled for *Bd* (42). Compared to amphibian family tallies of *Bd* occurrence from 2014 (42), family patterns changed slightly over the ∼5 years. For example, for species-level prevalence in families with over 100 species sampled, in 2014, *Bd* occurrence was highest in hylids (60%), ranids (59%), craugastorids (57%), and bufonids (44%), whereas in 2019, *Bd* occurrence was higher in hylids + pelodryadids (67%) and hyperoliids (64%), followed by ranids (58%), craugastorids (52%), bufonids (50%), and plethodontids (47%). Species tested nearly doubled for four relatively under-sampled families in 2014

(Microhylidae, Rhacophoridae, Hynobiidae, Typhlonectidae). Overall, knowledge of previously under-sampled species and families grew, suggesting there have been focal efforts targeting taxonomic knowledge gaps. Previously, amphibian family has been reported to be a strong predictor of *Bd* infection status, including severity of infection and development of chytridiomycosis (51, 83).

Castro Monzon et al. (30) reported 1,062 of 1,966 (54%) *Bd*-infected species from their web search. We report on *Bd* occurrences in 1,286 of 2,389 (54%) amphibian species, an additional 423 species but a comparable rate of infection. Upon closer comparison of these two datasets, we found that Castro Monzon et al. (30) included *Bd* sampling in 126 different, additional species (excluding hybrid species and uncertain species designations, i.e., *Genus* sp.: **Supplementary Appendix 2**) which were not in our data compilation. Adding these species to our total, *Bd* has been detected in 1,375 of 2,525 (55%) species sampled and compiled from both datasets. Our knowledge of world *Bd* surveillance across species has more than doubled since the 2013 paper by Olson et al. (28) where *Bd* detection was reported in 516 of 1,240 (42%) sampled species. The incidence of known species infection has increased by 13% over this relatively short time period, 2013 to 2020.

Geographically, we compared our *Bd*-in-the-wild occurrence results by country with the web search conducted by Castro Monzon et al. (30) (**Supplementary Appendix 2**). In comparison to our reported *Bd* sampling in 124 countries through 2019 (88 with detections), they reported sampling in 119 countries (86 with detections) through early May 2020. Our country lists differed (**Supplementary Appendix 2**) in that we reported *Bd* sampling in 13 countries that they did not include, and they reported *Bd* sampling from 9 countries that we did not include; hence, our datasets compiled different reports for 22 countries. Adding their 9 additional countries with 4 additional *Bd* detections to our sample (124 countries) yields 133 countries with known *Bd* sampling, with *Bd* detected in a total of 92 countries. From a very recent publication, we became aware of *Bd* sampling in one additional country that had not been included in either compilation, the Kingdom of Bhutan [*Bd* not detected (84)]. Adding this to the grand total, *Bd* has been detected in wild samples from 93 of 134 (69%) countries to our knowledge at this writing. This compares to *Bd* detections in 71 of 105 (68%) sampled countries in 2014 (42) and in 56 of 82 (68%) countries in 2013 (28).

For comparisons of site-level knowledge gain over time, using similar methods, Olson et al. (28) reported compilation of *Bd* sampling data at 4,281 sites, Xie et al. (43) reported 5,166 site-level records through June 2014, and herein we report 14,647 total sites. Site-level *Bd* data more than tripled since our initial report in 2013. In data compiled through June 2014, *Bd* sampling had occurred in 923 total USA 5th-field HUC watersheds, with 560 (60%) watershed having *Bd* detections (42). In comparison, by December 2019, our knowledge of *Bd* sampling had doubled across US watersheds, with 1,874 watersheds sampled, and *Bd* detections were reported for 916 (49%) watersheds.

TABLE 5 | Final best-fit model covariates of regional presence-only species distribution models (North America, South America, Europe, Africa, Asia, Australia), including regional model area under the curve (AUC), and fraction of total variation accounted for (FTVA) for each variable.

Region and final best-fit model	Variable	FTVA
North America (AUC = 0.89)		
*Mean temp + annual precipitation + elevation max + mean temp*elevation max*	Mean temp	0.910
	Annual precipitation	0.034
	Elevation max	0.056
South America (AUC = 0.91)		
*Mean temp + elevation max + temp range + mean temp*elevation max*	Mean temp	0.667
	Elevation max	0.292
	Temp range	0.042
Europe (AUC = 0.79)		
Mean temp + annual precipitation	Mean temp	0.833
	Annual precipitation	0.167
Africa (AUC = 0.90)		
*Elevation max + annual precipitation + mean temp + temp range + annual precipitation*mean temp*	Elevation max	0.413
	Annual precipitation	0.339
	Mean temp	0.183
	Temp range	0.064
Asia (AUC = 0.91)		
Annual precipitation + mean temp	Annual precipitation	0.626
	Mean temp	0.374
Australia (AUC = 0.93)		
Temp range + elevation max + mean temp + annual precipitation	Temp range	0.675
	Elevation max	0.211
	Annual precipitation	0.066
	Mean temp	0.048

Environmental Associations of *Bd* Occurrences

Our analyses of environmental associations with *Bd* occurrence through 2019 further support the importance of climate-niche space for this pathogenic aquatic fungus [e.g., (28, 43, 57, 59–61, 63–66)]. In our global SDMs using the largest dataset to date with both detection and no-detection data, mean temperature was the most important environmental correlate of *Bd* occurrence, and accounted for 97% of the variation in *Bd* occurrence. In the more predictive global model using *Bd* detection-only data, mean temperature accounted for 71% of the variation in *Bd* occurrence whereas annual precipitation accounted for 22%. Although the relationship between probability of *Bd* occurrence and mean temperature (**Figure 4A**) is consistent with our knowledge of temperature constraints on *Bd* growth [e.g., (57)], the pattern of *Bd* occurrence with annual precipitation is not easily reconciled (**Figure 4B**) and may result from: a sampling artifact of *Bd* occurrence patterns in our dataset—perhaps relating to underlying, complex host-pathogen interactions with temperature; an artifact of our 0.5-degree latitude and longitude grid cells being the unit of analysis, within which heterogeneous precipitation patterns are likely; regional diversity in *Bd* environmental associations; or *Bd* lineage effects. Sampling

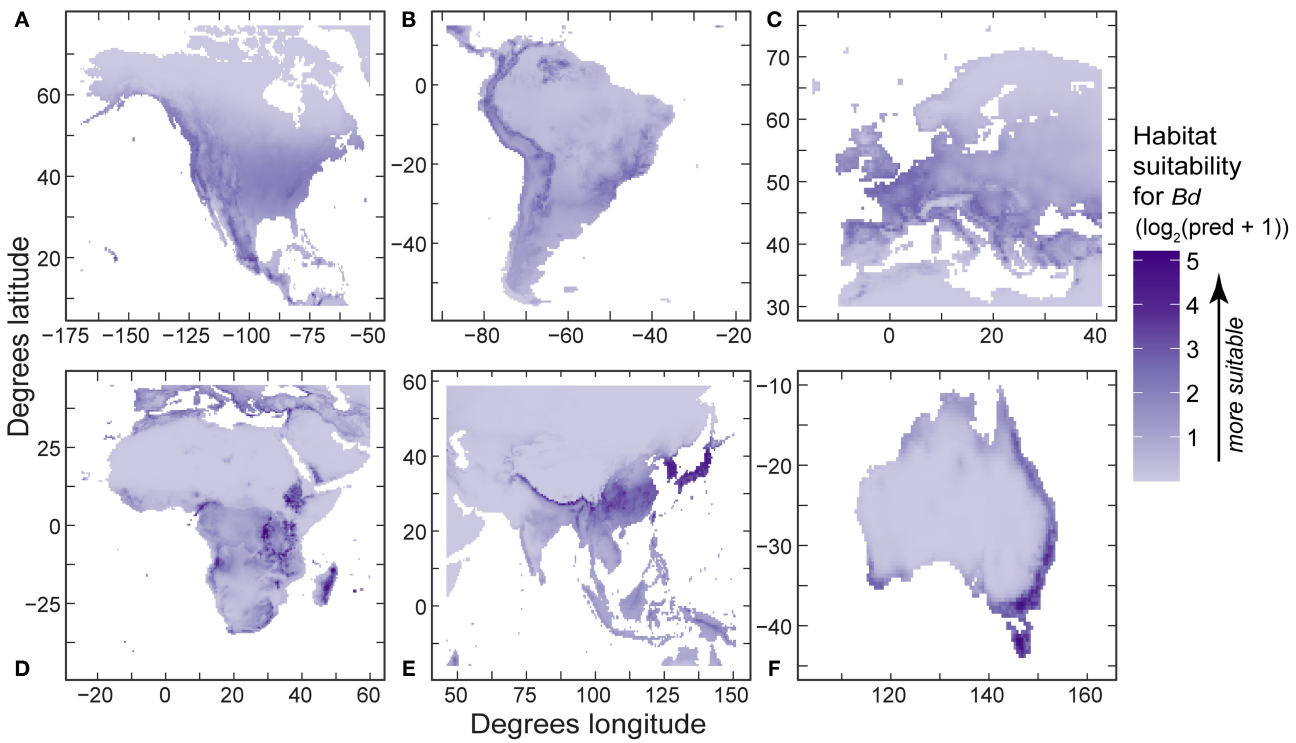


FIGURE 7 | Regional predictions of *Batrachochytrium dendrobatidis* (*Bd*) habitat suitability from our best-fit presence-only species distribution models (log_2-transformed [log base 2] probability ration output, area under curve values in **Table 5**) using *Bd* data compiled through December 2019 for: **(A)** North America; **(B)** South America; **(C)** Europe; **(D)** Africa; **(E)** eastern Asia; and **(F)** Australia.

intensity across each covariate (**Supplementary Figure 1**) and interactions among covariates are important considerations to fully understand the role of the different parameters across their range extents (**Supplementary Figures 3A,B**). Mean temperature was not the top predictor in previous presence-absence models of globally compiled data, as temperature range had previously emerged as a highly predictive covariate (28, 43). Our approach of examining correlations among similar climate metrics and consolidating to fewer potential covariates prior to SDM analyses may have contributed to this difference. Additionally, the change in surveillance patterns geographically, 2014 to 2019 (**Figures 1**, **3**), may have led to emergence of different predictor covariates at the global level. Previously, sampling bias favoring species or locations in the United States, for example, may have led to skewed environmental associations during global assessments. With many former data gaps filled by the time of our 2019 data snapshot, this single-region bias is a lesser concern. However, the different covariates that emerged in our regional SDMs support the unique role that different climate metrics in each area may have on emerging *Bd* patterns.

Differences among regional SDMs with our more robust 2019 dataset support the importance of additional downscaled analyses to understand potential geographic context-specific patterns of *Bd* emergence. In the presence-only regional models (**Table 2**), the models with highest sensitivity: (1) mean temperature dominated *Bd* predictors in North America (0.91), Europe (0.83), and South America (0.67), and was a close-second predictor in Asia (0.37); (2) annual precipitation was a top predictor or close-second predictor in Asia (0.63) and Africa (0.34); (3) maximum elevation was a top predictor or close-second predictor in Africa (0.41) and Australia (0.21);

TABLE 6 | Final best-fit model covariates of regional presence-absence species distribution models (North America, South America, Europe, Africa, Asia, Australia), including regional model area under the curve (AUC), and fraction of total variation accounted for (FTVA) for each variable.

Region and final best-fit model	Variable	FTVA
North America (AUC = 0.545)		
Mean temp + temp range	Mean temp	0.645
	Temp range	0.355
South America (AUC = 0.714)		
Mean temp + temp range	Mean temp	0.921
	Elevation max	0.079
Europe (AUC = 0.495)		
Mean temp	Mean temp	1.00
Africa (AUC = 0.724)		
Mean temp	Mean temp	1.00
Asia (AUC = 0.664)		
Mean temp	Mean temp	1.00
Australia (AUC = 0.664)		
Mean temp + annual precipitation	Mean temp	0.57
	Annual precipitation	0.433

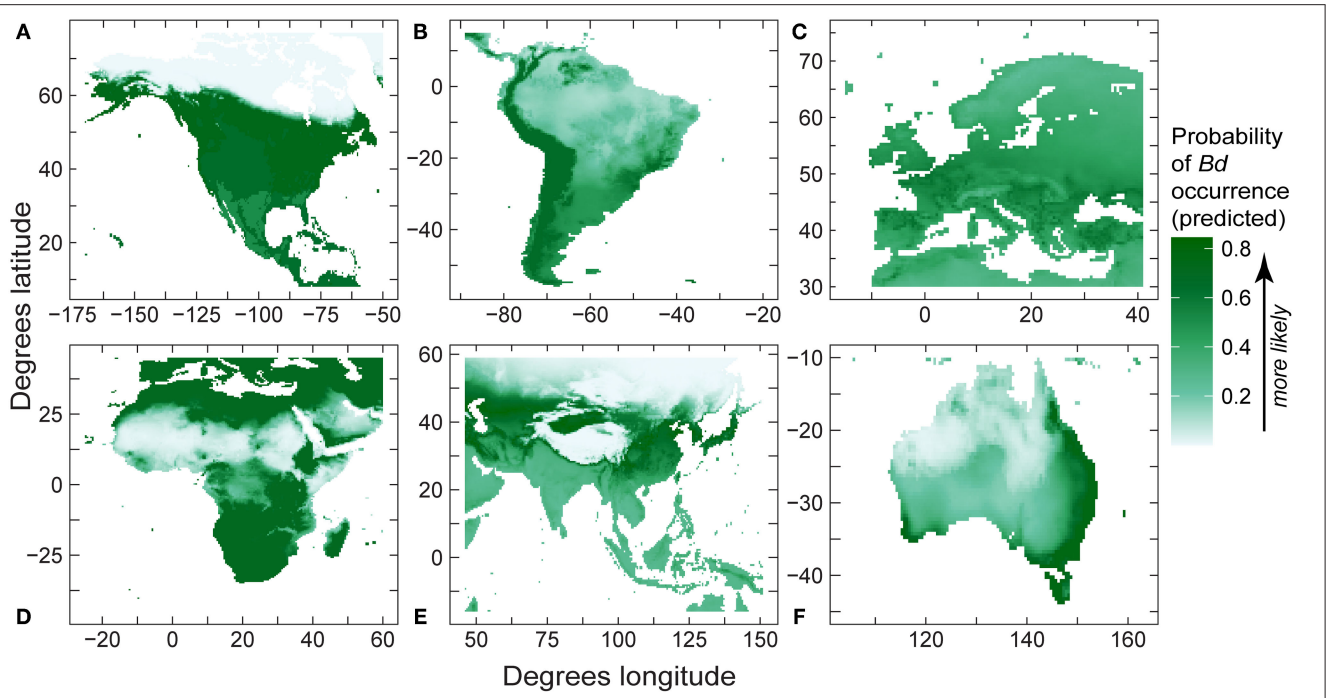


FIGURE 8 | Regional predictions of *Batrachochytrium dendrobatidis* (*Bd*) absolute probability of *Bd* occurrence from our best-fit presence-absence species distribution models (area under curve values in **Table 6**) using *Bd* data compiled through December 2019 for: **(A)** North America; **(B)** South America; **(C)** Europe; **(D)** Africa; **(E)** eastern Asia; and **(F)** Australia.

and (4) temperature range was a top predictor in Australia (0.67). Per continent, scenarios could be developed to add more specificity to these regional patterns and explore their complexities. For example, the high gradients in temperature ranges across coastal-to-interior Australia were a significant contributing factor to the downscaled models already developed by Murray et al. (65). Regional patterns warrant additional study at smaller spatial scales, and relative to additional interactions among environmental covariates. At smaller scales, relationships between temperature and the pathogen biology, host biology, and their interplay could be further explored (85). Spatially downscaled approaches could have ramifications for the direction of regionally specific conservation actions to forestall disease threat, such as site-specific efforts to manage microclimate conditions (86).

Research and Management Implications

Although *Bd* is globally distributed, occurring on every continent with amphibians, support is growing for *Bd* to have expanded its global range relatively recently (34, 36–39). Our analyses do not quantify recent spread but are an update of knowledge of *Bd* occurrence. Despite its present broad occurrence, *Bd* is clearly not ubiquitous across amphibian taxa or geographies, likely owing to a complex combination of transmission dynamics, host susceptibilities to infection, and pathogen environmental associations. Our updated *Bd*-occurrence data (downloadable from the AmphibiaWeb portal, AmphibianDisease.org: previously released public data to 2014, DOI = https://n2t.net/ark:/21547/DsA2; updated public data 2015 to 2019, DOI = https://gcc02.safelinks.protection.outlook.com/?url=https%3A%2F%2Fn2t.net%2Fark%3A%2F21547%2FDsM2&data=04%7C01%7C%7Cd307ffc12a5e407dad1e08d92ea75cce%7Ced5b36e701ee4ebc867ee03cfa0d4697%7C0%7C0%7C637592118618600952%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoi V2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C1000&sdata=hBkSl%2FScrh83TNo1HeQkG7QTovBwrI3OJ8E8RblAXFY%3D&reserved=0), showed significant *Bd*-knowledge gaps have been filled across amphibian families and countries, with updated world occurrence patterns likely to inform novel research investigations and conservation actions. In combination with additional more-recent data including the independent *Bd*-data compilation by Castro Monzon et al. (30), these composite surveillance efforts are an unparalleled accomplishment by a vast global community of natural-resource managers and amphibian scientists. With about half of sampled amphibian species being infected and *Bd* occurring at <40% of sites sampled, the need for effective pathogen biosecurity-and-mitigation is paramount to reduce further *Bd* transmission and losses of vulnerable hosts.

Management actions to curtail *Bd* can take several paths. Sampled taxa and geographies without *Bd* detections warrant continued assessments for novel pathogen detection and disease threat, and consideration for heightened biosecurity to forestall human-mediated pathogen translocation pathways [e.g., global trade markets (19, 20, 34)]. Priorities for *Bd* monitoring and exclusion include sensitive species habitat strongholds, both

micro- or macro-scale refugia, and broader *Bd*-free geographies that are habitat for high- or unique-diversity communities (49). Biodiverse locations of Africa, Asia, and South America with fragmented *Bd* occurrences warrant attention for elevated biosecurity to protect rich endemic fauna from potential disease threats. In more highly sampled areas, such as the USA, similar patterns of patchy *Bd* occurrences are evident at finer geographic scales (**Figures 2**, **3**), supporting the value of downscaled biosecurity efforts to reduce human-mediated spread into current apparent host macro- or micro-refugia from the pathogen, such as *Bd*-free watersheds (**Figure 3**). Watersheds could be a practical spatial unit for aquatic-pathogen management (28), as a variety of water resources are often managed by watershed boundaries.

As supported by O'Hanlon et al. (34), listing *Bd* as a notifiable disease by the World Organization for Animal Health (OIE) in 2008 has had little effect on human-mediated translocation. Instead, reliance on local, regional, and national jurisdictions with *Bd*-clean trade, transportation, and fieldwork procedures is a more effective biosecurity strategy. Pathogen biosecurity approaches for wild amphibians include species- and geographic-specific risk assessment and prioritization of between-site measures that are applicable for public and natural-resource manager implementation (49, 87, 88). Enforceable regulations protecting the national heritage of non-game wildlife in separate jurisdictions could be considered for development with a focus on wildlife health, clean trade, and management of injurious invasive species [e.g., salamander import restrictions to forestall *Bsal* transmission to the USA and Canada (89, 90); inclusion of *Bd* on aquatic invasive species lists of injurious species, with hygiene measures promoted across geographic boundaries]. Biosecurity guidance is available for some types of field activities that could have broad implementation [e.g., large equipment use at field sites (48); water draws for wildfire management (55, 56)], as well as for amphibian research where within-site methods are applicable (47). These biosecurity measures transcend application to *Bd* and are relevant for cross-taxonomic pathogens, parasites, and invasive species. These actions are consistent with One Health approaches, to recognize the interconnections among people, species, and our shared environment, and to work collaboratively to optimize health of each component [e.g., CDC (91)]. Building upon the increasing public awareness of the linkages between human and wildlife pathogens and their diseases resulting from the recent coronavirus pandemic could bolster biosecurity implementation for broader One Health aims.

The value of forestalling human-mediated spread of *Bd* is several-fold. First, we are still learning about the pathogen and its complex context-dependent interactions with hosts, environmental conditions, and other threats factors [e.g., (24, 32)]. *Bd*-strain differences have emerged as a key element in host-pathogen dynamics [e.g., (34, 54, 92)]. Our current *Bd* dataset does not record *Bd* strains, which sets a new challenge for the global community conducting surveillance to contribute strain data to the next phase of the *Bd* web portal (41). Also, there is increasing information about the role of microbiotic community interactions on amphibian skin, with some bacteria having antifungal properties that afford protection to host amphibians from adverse effects of *Bd* infections (93–96). Despite advances in understanding amphibian immune responses, this is an active area of ongoing research that is likely to offer new insights to *Bd* control (97–100). Such ongoing research interleaves with novel *Bd*-management opportunities (e.g., microbiota vaccines) and could help forestall mass-mortality events in susceptible species. In situations where at-risk taxa appear threatened, the ability to develop rescue measures and learn from their efficacy can inform later efforts [e.g., (101, 102)]. Furthermore, relative efficacy of additional conceptual field mitigations for amphibian EIDs have been qualitatively evaluated but warrant field trials to see if they can alter site-scale *Bd* infection dynamics [e.g., habitat attributes such as shading and water temperature management—essentially microclimate manipulations—could alter site-scale *Bd* infection dynamics (86)]. Adaptive management and learning from such field trials is needed to advance effective mitigations with knowledge of the risks and benefits they may entail. Each intervention that might safeguard species from severe infection merits study for efficacy and practicality as part of research and conservation trials, while biosecurity measures could stall inadvertent spread.

Next Steps

Our newly updated dataset points to the broad human dimensions of *Bd* surveillance, and specifically the contributions to our current understanding of global *Bd* occurrences from a broad world community. Our 769 data sources show international partnerships have been established between local faunal and land-management experts and personnel from numerous universities and institutions to pursue *Bd* surveillance (**Supplementary Table 2**). Such co-production underscores the local and global interest in *Bd* occurrence, spread, and threat. Although published data from peer-reviewed journals dominate our compilation of data sources (86%), incentives are needed to improve this rate to ensure data quality assurance of sampling and analytical procedures. Publishing could be promoted prior to graduate student defenses, or with permitting procedures.

Communication of the global *Bd* database move to AmphibianDisease.org and encouragement of ongoing project plans as well as data imports is important going forward, as metadata analyses of global data compilations can be important for hypothesis testing and pattern revelations [e.g., (50–53)]. The consistency of the results presented here from joint web-portal data imports compilation and literature searches was comparable to a more technical web search of journals (30). Clearly, the combined approaches yield a more comprehensive picture, and may be useful for a more complete *Bd* dataset at AmphibianDisease.org into the future.

With initiation of the new web portal AmphibianDisease.org, we anticipate phases of *Bd* database updates over the near term. First, as novel *Bd*-data imports have been made already to the new portal from early users and local projects, cross-checking between the *Bd*-Maps database upload and these datasets will be needed to reconcile redundancies. Data gaps discovered from 2019 and earlier should be addressed as they are identified, including data sources uncovered by Castro Monzon et al. (30)

that we had not included in our data compilation for this paper. Ongoing efforts to educate researchers on new procedures to archive their data will be critical. The Amphibian Disease database may include records of captive animals and museum specimens, allowing unpublished data to be added. Additionally, *Bd* occurrence patterns derived from eDNA or fomite samples provide additional data sources requiring special attention for compilation, especially as new multi-taxonomic community-based analyses are conducted [e.g., (103)]. Analyses of *Bd* samples for genetic variants is a needed technological bridge, expanding basic *Bd* surveillance and monitoring objectives to another level of specificity. Innovative collaborations among diagnostics laboratories and amphibian researchers in a variety of subdisciplines is likely needed to meet this objective. In addition to pathogen strains, virulence parameters could be included in the updated database, addressing new research priorities to understand pathogen demographics, pathogenicity, and disease dynamics (19, 34, 92). Most importantly, expanding the new portal to identify chytridiomycosis occurrences, rather than simply the pathogen *Bd*, is a key goal.

The Amphibian Disease database has new web applications compared with *Bd*-Maps.net (41). Importantly, user-friendly import and export capacities have been a priority in portal development. Data can be uploaded by "projects" for both *Bd* and *Bsal* studies. Data for a project can be assigned a DOI. Koo and Olson (41) explain further how AmphibianDisease.org can tap into additional online databases via their network partnerships, such as genetic and genomic public databases.

As *Bd* data accumulation accelerates, a corresponding increase in the depth of knowledge of species status and threat occurs, echoing calls for conservation urgency. As *Bd* chytridiomycosis appears to be about a half-Earth pandemic across amphibian taxa and sites, there is considerable room for action from both bottom-up community-run efforts and top-down national-to-international policies having importance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no live animals were used for this research, we only summarized existing datasets.

AUTHOR CONTRIBUTIONS

DO designed research and led manuscript development. KR compiled and summarized data, drafted figures and tables, and developed **Supplementary Material**. CG conducted statistical analyses. KC developed geographic information for analyses and drafted figures. AB assisted with logistical support and manuscript development. All authors provided critical feedback and contributed to the final manuscript.

FUNDING

This project was supported by the US Department of Agriculture, Forest Service, Pacific Northwest Research Station.

ACKNOWLEDGMENTS

We thank Matt Gregory (Oregon State University) for his assistance with geographic analytical procedures for the compilation of environmental metrics for world *Bd* sampling sites, Michelle Koo for providing comments on an earlier draft of this paper and creating the two *Bd* database DOIs in amphibiandisease.org, and two reviewers for their comments on our submitted manuscript. We thank Mat Fisher and David Aanensen for their pivotal roles in the initiation of the *Bd*-Maps.net web portal for world *Bd* data, and Imperial College, UK for hosting that initial website. We thank the world amphibian science and management community for contributing data to this project. The use of trade or firm names is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.685877/full#supplementary-material

Supplementary Appendix 1 | Detailed methods.

Supplementary Appendix 2 | Comparing the current study with results from Castro Monzon et al. (30).

Supplementary Figure 1 | Relative habitat suitability of *Batrachochytrium dendrobatidis* (*Bd*) occurrence (probability ratio output) for each environmental attribute from the global presence-only best-fit species distribution model. Each environmental attribute [**(A)** mean temperature; **(B)** annual precipitation; **(C)** temperature range; **(D)** maximum elevation] marginal-response plot is calculated while holding all other covariates at the mean. The frequency of observed presence (FOP) plots (black dots, orange line estimating trend) show the number of sites with *Bd* occurrence across the range of the explanatory variable (e.g., the frequency of *Bd* occurrence points increases as max elevation increases, until about 4,000 m). The kernel estimated data density (light gray background) shows the sampling effort.

Supplementary Figure 2 | Absolute probability of *Batrachochytrium dendrobatidis* (*Bd*) occurrence for the one environmental attribute (mean temperature, °C) from the global presence-absence best-fit species distribution model. The frequency of observed presence (FOP) plot (black dots, orange line estimating trend) shows the number of sites with *Bd* occurrence across the range of the variable. The kernel estimated data density (light gray background) shows the sampling effort.

Supplementary Figure 3 | Marginal-response plots [$N = 24$; 18 shown in **(A)**, 12 shown in **(B)**] depicting interactions of covariates from the best-fit presence-only global species distribution model of *Batrachochytrium dendrobatidis* (*Bd*) occurrence using detection-only data for 0.5-degree latitude/longitude grid cells. The probability ratio output (PRO, model predictions) is calculated for a range of the environmental covariate (named below plot) while holding the interacting

covariate (named above plot) at the 0.25 percentile (left), mean (center), and 0.75 percentile (right). Non-interacting covariates per plot are held at their mean.

Supplementary Figure 4 | Marginal-response plots ($N = 6$) depicting interactions of covariates from the best-fit presence-absence global species distribution model of *Batrachochytrium dendrobatidis* (*Bd*) occurrence, using detection and no-detection data for 0.5-degree latitude/longitude grid cells. The predicted probability of *Bd* occurrence is calculated for a range of the environmental covariate (named below plot) while holding the interacting covariate (named above plot) at the 0.25 percentile **(Left)**, mean **(Center)**, and 0.75 percentile **(Right)**.

Supplementary Table 1 | Summary of *Batrachochytrium dendrobatidis* (*Bd*) detection (+) and no-detection (–) records and sites in the Global Bd Mapping Project (*Bd*-Maps) database through December 2019.

Supplementary Table 2 | *Batrachochytrium dendrobatidis* detections by species, with references and countries of detection.

Supplementary Table 3 | *Batrachochytrium dendrobatidis* (*Bd*) detections by country, with source citations (see Data References following **Supplementary Table 2**).

REFERENCES

1. Dirzo R, Raven PH. Global state of biodiversity and loss. *Annu Rev Env Resour.* (2003) 28:137–67. doi: 10.1146/annurev.energy.28.050302.105532
2. Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci USA.* (2008) 105(Suppl. 1):11466–73. doi: 10.1073/pnas.0801921105
3. Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, et al. Has the earth's sixth mass extinction already arrived? *Nature.* (2011) 471:51–7. doi: 10.1038/nature09678
4. Ceballos G, Ehrlich PR, Barnosky AD, Garcia A, Pringle RM, Palmer TM. Accelerated modern human-induced species losses: entering the sixth mass extinction. *Sci Adv.* (2015) 1:e1400253. doi: 10.1126/sciadv.1400253
5. International Union for the Conservation of Nature. *IUCN Red List of Threatened Species*. Version 2020-3 (2021). Available online at: https://www.iucnredlist.org (accessed February 1, 2021).
6. International Union for the Conservation of Nature. *IUCN Species Survival Commission (SSC) Amphibian Specialist Group.* (2021). Available online at: https://www.iucn-amphibians.org/ (accessed December 1, 2020).
7. Kuta KG, Richardson LL. Ecological aspects of black band disease of corals: relationships between disease incidence and environmental factors. *Coral Reefs.* (2002) 21:393–398. doi: 10.1007/s00338-002-0261-6
8. Howells E, Vaughan G, Work TM, Burt J, Abrego D. Annual outbreaks of coral disease coincide with extreme seasonal warming. *Coral Reefs.* (2020) 29:771–81. doi: 10.1007/s00338-020-01946-2
9. Aquino CA, Besemer RM, DeRito CM, Kocian J, Porter IR, Raimondi PT, et al. Evidence that microorganisms at the animal-water interface drive sea star wasting disease. *Front Microbiol.* (2021) 11:610009. doi: 10.3389/fmicb.2020.610009
10. Frick WF, Pollock JF, Hicks AC, Langwig KE, Reynolds DS, Turner GG, et al. An emerging disease causes regional population collapse of a common North American bat species. *Science.* (2010) 329:679–82. doi: 10.1126/science.1188594
11. Verant ML, Boyles JG Waldrep W Jr., Wibbelt G, Blehert DS. Temperature-dependent growth of *Geomyces destructans*, the fungus that causes bat white-nose syndrome. *PLoS ONE.* (2012) 7:e46280. doi: 10.1371/journal.pone.0046280
12. LaDeau SL, Kilpatrick AM, Marra PP. West Nile virus emergence and large-scale declines of North American bird populations. *Nature.* (2007) 447:710. doi: 10.1038/nature05829
13. Northeast Wildlife Disease Cooperative. *Disease Fact Sheets*. Northeast USA Wildlife Disease Cooperative (2020). Available online at: https://www.northeastwildlife.org/disease-fact-sheets (accessed May 19, 2021).
14. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. *Science.* (2004) 306:1783–6. doi: 10.1126/science.1103538
15. 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.* (2014) 346:630–1. doi: 10.1126/science.1258268
16. Gray MJ, Chinchar VG, editors. *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Switzerland: Springer International Publishing (2015). p. 246.
17. 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.* (2019) 363:1459–63. doi: 10.1126/science.aav0379
18. Lambert MR, Womack MC, Byrne AQ, Hernandez-Gomez O, Noss CF, Rothstein AP, et al. Comment on "amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity". *Science.* (2020) 367:eaay1838. doi: 10.1126/science.aay1838
19. Fisher MC, Garner TWJ. Chytrid fungi and global amphibian declines. *Nat Rev Microbiol.* (2020) 18:332–43. doi: 10.1038/s41579-020-0335-x
20. Kerby J, Berger L. Global trade in frogs has led to catastrophic amphibian declines. In: Tsing A, Deger J, Saxena AK, Zhou F, editors. *Feral Atlas: The More-than-Human Anthropocene. Bd chytrid fungus*. Redwood City, CA: Stanford University Press (2020). Available online at: feralatlas.org (accessed October 22, 2020).
21. Calhoun DM, Leslie KL, Riepe TB, Achatz TJ, McDevitt-Galles T, Tkach VV, et al. Patterns of *Clinostomum marginatum* infection in fishes and amphibians: integration of field genetic, and experimental approaches. *J Helminthol.* (2020) 94:e44. doi: 10.1017/S0022149X18001244
22. Pessier AP. Hopping over red leg: the metamorphosis of amphibian pathology. *Vet Pathol.* (2017) 54:355–7. doi: 10.1177/0300985817699861
23. 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 USA.* (2013) 110:15325–29. doi: 10.1073/pnas.1307356110
24. Blaustein AR, Urbina J, Snyder PW, Reynolds E, Dang T, Hoverman JT, et al. Effects of emerging infectious diseases on amphibians: a review of experimental studies. *Diversity.* (2018) 10:1–49. doi: 10.3390/d10030081
25. Isidoro-Ayza M, Grear DA, Chambouvet A. Pathology and case definition of severe perkinsea infection of frogs. *Vet Pathol.* (2019) 56:133–42. doi: 10.1177/0300985818798132
26. Chambouvet A, Smilansky V, Jirku M, Isidoro-Ayza M, Itoïz S, Derelle E, et al. Diverse alveolate infections of tadpoles, a new threat to frogs? *PLoS Pathogens.* (2020) 16:e1008107. doi: 10.1371/journal.ppat.1008107
27. Duffus A, Olson D. *The Establishment of a Global Ranavirus Reporting System*. Vol. 96. FrogLog (2011). p. 37. Available online at: https://www.amphibians.org/wp-content/uploads/2018/12/Froglog96.pdf (accessed March 22, 2021).
28. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLoS ONE.* (2013) 8:e56802. doi: 10.1371/journal.pone.0056802
29. Olson DH. A decade of herpetological disease papers: puzzle pieces of a bigger picture. *Herpetol Rev.* (2019) 50:37–40. Available online at: https://www.fs.usda.gov/treesearch/pubs/57951 (accessed June 24, 2021).
30. Castro Monzon F, Rödel MO, Jeschke JM. Tracking *Batrachochytrium dendrobatidis.* infection across the globe. *EcoHealth.* (2020) 17:270–9. doi: 10.1007/s10393-020-01504-w
31. Brunner JL, Olson DH, Gray MJ, Miller DL, Duffus ALJ. Global patterns of Ranavirus detections. Collection: Ranavirus research: 10 years of global collaboration. *FACETS.* (2021) 6:912–24. doi: 10.1139/facets-2020-0013
32. Blaustein AR, Han BA, Relyea RA, Johnson TJ, Buck JC, Gervasi SS, et al. The complexity of amphibian declines: understanding the role of cofactors in driving amphibian losses. *Ann NY Acad Sci.* (2011) 1223:108–19. doi: 10.1111/j.1749-6632.2010.05909.x
33. Laking A, Ngo HN, Pasmans F, Martel A, Nguyen TT. *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Sci Rep.* (2017) 7:44443. doi: 10.1038/srep44443

34. O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, et al. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*. (2018) 360:621–7. doi: 10.1126/science.aar1965
35. Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. et sp. nov. a chytrid pathogenic to amphibians. *Mycologia.* (1999) 91:219–27. doi: 10.1080/00275514.1999.12061011
36. Weldon C, Du Preez L, Hyatt A, Muller R, Speare R. Origin of the amphibian chytrid fungus. *Emerg Infect Dis.* (2004) 10:2100–5. doi: 10.3201/eid1012.030804
37. Goka K, Yokoyama JUN, Une Y, Kuroki T, Suzuki K, Nakahara M, et al. Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Mol Ecol.* (2009) 18:4757–74. doi: 10.1111/j.1365-294X.2009.04384.x
38. Bataille A, Fong JJ, Cha M, Wogan GOU, Baek HJ, Lee H, et al. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in Wild Asian amphibians. *Mol Ecol.* (2013) 22:4196–209. doi: 10.1111/mec.12385
39. Rodriguez D, Becker CG, Pupin NC, Haddad CFB, Zamudio KR. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic forest of Brazil. *Mol Ecol.* (2014) 23:774–87. doi: 10.1111/mec.12615
40. Talley BL, Muletz CR, Vredenburg VT, Fleischer RC, Lips KR. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biol Conserv.* (2015) 182:254–61. doi: 10.1016/j.biocon.2014.12.007
41. Koo MS, Olson DH. Data management working group. In: *North American Bsal Task Force. A North American Strategic Plan to Control Invasions of the Lethal Salamander Pathogen Batrachochytrium salamandrivorans.* (2020). p. 47–52. Available online at: https://www.salamanderfungus.org/wp-content/uploads/2020/04/Bsal-Strategic-Plan-2020-Posted-OM-edits.pdf (accessed May 19, 2021).
42. Olson DH, Ronnenberg KL. *Global Bd Mapping Project: 2014 Update*. Vol. 111. FrogLog (2014). p. 17–21. Available online at: https://www.iucn-amphibians.org/wp-content/uploads/2018/10/froglog110_lowres.pdf (accessed March 22, 2021).
43. Xie GY, Olson DH, Blaustein AR. Projecting the global distribution of the emerging amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, based on IPCC climate futures. *PLoS ONE.* (2016) 11:e0160746. doi: 10.1371/journal.pone.0160746
44. Wessner D, Dupont C, Charles T. *Microbiology*. New York, NY: John Wiley & Sons (2013).
45. Holland JS. *Bd* map, In: *The Vanishing. National Geographic Magazine*. Washington, DC: National Geographic Society (2009). p. 145.
46. Tsing A, Deger J, Saxena AK, Zhou F, editors. *Bd* Chytrid Fungus. In: *Feral Atlas: The More-than-Human Anthropocene*. Redwood City, CA: Stanford University Press (2020). Available online at: feralatlas.org (accessed October 22, 2020). doi: 10.21627/2020fa
47. Gray MJ, Duffus ALJ, Haman KH, Harris RN, Allender MC, Thompson TA, et al. Pathogen surveillance in herpetofaunal populations: guidance on study design, sample collection, biosecurity, and intervention strategies. *Herpetol Rev.* (2017) 48:334–51. Available online at: https://parcplace.org/wp-content/uploads/2017/08/Grayetal2017.pdf (accessed June 24, 2021).
48. Julian JT, Henry PFP, Drasher JM, Jewell SD, Michell K, Oxenrider KJ, et al. Minimizing the spread of herpetofaunal pathogens in aquatic habitats by decontaminating construction equipment. *Herpetol Rev.* (2020) 51:472–83. Available online at: https://parcplace.org/wp-content/uploads/2020/11/Julian-2020-Decontamination-for-Herps-for-large-equipment.pdf (accessed June 24, 2021).
49. Olson DH, Haman KH, Gray MJ, Harris R, Thompson TA, Iredale M, et al. Enhanced between-site biosecurity to minimize herpetofaunal disease-causing pathogen transmission. *Herpetol Rev*. (2021) 52:36–46. Available online at: https://www.fs.usda.gov/treesearch/pubs/62316 (accessed June 24, 2021).
50. Grant ECH, Miller DAW, Schmidt BR, Adams MJ, Amburgey SM, Chambert T, et al. Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *Nature.* (2016) 6:25625. doi: 10.1038/srep25625
51. Gervasi SS, Stephens PR, Hua J, Searle CL, Urbina J, Olson DH, et al. Linking ecology and epidemiology to understand predictors of multi-host responses to an emerging pathogen, the amphibian chytrid fungus. *PLoS ONE.* (2017) 12:e0167882. doi: 10.1371/journal.pone.0167882
52. Yap TA, Koo MS, Ambrose RF, Vredenburg VT. Introduced bullfrog facilitates pathogen invasion in the Western United States. *PLoS ONE.* (2018) 13:e0188384. doi: 10.1371/journal.pone.0188384
53. Jairam R. A historical overview of *Batrachochytrium dendrobatidis* infection from specimens at the national zoological collection Suriname. *PLoS ONE.* (2020) 15:e0239220. doi: 10.1371/journal.pone.0239220
54. Dang TD, Searle CL, Blaustein AR. Virulence variation among strains of the emerging infectious fungus *Batrachochytrium dendrobatidis* (*Bd*) in multiple amphibian host species. *Dis Aquat Organ.* (2017) 124:233–9. doi: 10.3354/dao03125
55. National Wildfire Coordinating Group. *Guide to Preventing Aquatic Invasive Species Transport by Wildland Fire Operations*. Invasive species subcommittee, Equipment Technology Committee, National Wildfire Coordinating Group, United States. PMS 444. (2017). p. 64. Available online at: https://www.nwcg.gov/sites/default/files/publications/pms444.pdf (accessed August 26, 2020).
56. National Wildfire Coordinating Group. *Invasive Species Mitigation for Ground Resources*. Invasive species subcommittee, Equipment Technology Committee, National Wildfire Coordinating Group, United States. Operations video (2020). Available online at: https://www.nwcg.gov/publications/training-courses/rt-130/operations/op819 (accessed August 26, 2020).
57. Piotrowski JS, Annis SL, Longcore JE. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia.* (2004) 96:9–15. doi: 10.1080/15572536.2005.11832990
58. Berger L, Speare R, Hines HB, Marantelli G, Hyatt AD, McDonald KR, et al. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Austr Vet J.* (2004) 82:434–9. doi: 10.1111/j.1751-0813.2004.tb11137.x
59. Ron SR. Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the new world. *Biotropica.* (2005) 37:209–21. doi: 10.1111/j.1744-7429.2005.00028.x
60. Pounds JA, Bustamante MR, Coloma LA, Consuegra JA, Fogden MPL, Foster PN, et al. Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature.* (2006) 439:161–7. doi: 10.1038/nature04246
61. Bosch J, Carrascal LM, Duran L, Walker S, Fisher MC. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *P R Soc Lond B Bio.* (2007) 274:253–60. doi: 10.1098/rspb.2006.3713
62. Kriger KM, Hero J-M. Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *J Zool.* (2007) 271:352–9. doi: 10.1111/j.1469-7998.2006.00220.x
63. Rödder D, Kielgast J, Lötters S. Future potential distribution of the emerging amphibian chytrid fungus under anthropogenic climate change. *Dis Aquat Organ.* (2010) 92:201–7. doi: 10.3354/dao02197
64. Rohr JR, Raffel TR. Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proc Natl Acad Sci USA.* (2010) 107:8269–74. doi: 10.1073/pnas.0912883107
65. Murray KA, Retallick RWR, Puschendorf R, Skerratt LF, Rosauer D, McCallum HI, et al. Assessing spatial patterns of disease risk to biodiversity: implications for the management of the amphibian pathogen, *Batrachochytrium dendrobatidis*. *J Appl Ecol.* (2011) 48:163–73. doi: 10.1111/j.1365-2664.2010.01890.x
66. Puschendorf R, Carnaval AC, VanDerWal J, Zumbado-Ulate H, Chaves G, Bolanos F, et al. Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. *Divers Distrib.* (2009) 15:401–8. doi: 10.1111/j.1472-4642.2008.00548.x
67. Voyles J, Johnson LR, Briggs CJ, Cashins SD, Alford RA, Berger L, et al. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. *Ecol Evol.* (2012) 2:2241–9. doi: 10.1002/ece3.334
68. Voyles J, Johnson LR, Rohr J, Kelly R, Barron C, Miller D, et al. Diversity in growth patterns among strains of the lethal fungal pathogen *Batrachochytrium dendrobatidis* across extended thermal optima. *Oecologia.* (2017) 184:363–373. doi: 10.1007/s00442-017-3866-8

69. Raffel TR, Romansic JM, Halstead NT, McMahon TA, Venesky MD, Rohr JR. Disease and thermal acclimation in a more variable and unpredictable climate. *Nat Clim Change.* (2013) 3:146–51. doi: 10.1038/nclimate1659
70. Chestnut T, Anderson C, Popa R, Blaustein AR, Voytek M, Olson DH, et al. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. *PLoS ONE.* (2014) 9:e106790. doi: 10.1371/journal.pone.0106790
71. Bradley PW, Brawner MD, Raffell TR, Rohr JR, Olson DH, Blaustein AR. Shifts in temperature influence how *Batrachochytrium dendrobatidis* Infects amphibian larvae. *PLoS ONE.* (2019) 14:e0222237. doi: 10.1371/journal.pone.0222237
72. Frost DR. *Amphibian Species of the World: An Online Reference. Version 6.1.* New York, NY: American Museum of Natural History (2020).
73. Skerratt LF, Berger L, Hines HB, McDonald KR, Mendez D, Speare R. Survey protocol for detecting chytridiomycosis in all Australian frog populations. *Dis Aquat Organ.* (2008) 80: 85–94. doi: 10.3354/dao01923
74. Merow C, Smith MJ, Silander JA Jr. A Practical guide to MaxEnt for modelling species' distributions: what it does, and why inputs and settings matter. *Ecography.* (2013) 36:1058–69. doi: 10.1111/j.1600-0587.2013.07872.x
75. Pearce JL, Boyce MS. Modelling distribution and abundance with presence-only data. *J Appl Ecol.* (2006) 43:405–12. doi: 10.1111/j.1365-2664.2005.01112.x
76. Halvorsen R, Mazzoni S, Bryn A, Bakkestuen V. Opportunities for improved distribution modelling practice via a strict maximum likelihood interpretation of MaxEnt. *Ecography.* (2015) 38:172–83. doi: 10.1111/ecog.00565
77. Tuszynski J. *caTools: Tools: Moving Window Statistics, GIF, Base64, ROC AUC, etc. R Package, Version 1.18.0.* (2020). Available online at: https://CRAN.R-project.org/package=caTools (accessed December 1, 2020).
78. Halvorsen R. A strict maximum likelihood explanation of MaxEnt, and some implications for distribution modelling. *Sommerfeltia.* (2013) 36:1–32. doi: 10.2478/v10208-011-0016-2
79. Elith J, Phillips SJ, Hastie T, Dudík M, Chee YE, Yates CJ. A statistical explanation of MaxEnt for ecologists. *Divers Distrib.* (2011) 17:43–57. doi: 10.1111/j.1472-4642.2010.00725.x
80. Vollering J, Halvorsen R, Mazzoni S. The MIAmaxent R package: variable transformation and model selection for species distribution models. *Ecol Evol.* (2019) 9:12051–68. doi: 10.1002/ece3.5654
81. Wickham H. *ggplot2: Elegant Graphics for Data Analysis.* New York, NY: Springer-Verlag (2016). doi: 10.1007/978-3-319-24277-4_9
82. Fong JJ, Cheng TJ, Battaile A, Pessier AP, Waldman B, Vredenburg VT. Early 1900s detection of *Batrachochytrium dendrobatidis* in Korean amphibians. *PLoS ONE.* (2015) 10: e0115656. doi: 10.1371/journal.pone.0115656
83. Bancroft B, Han B, Searle C, Michael L, Olson DH, Lawler J, et al. Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the United States. *Biodivers Conserv.* (2011) 20:1911–20. doi: 10.1007/s10531-011-0066-4
84. Streicher JW, Mahony S, Kamei RG, Nidup T, Jervis P, Fisher MC. Preliminary survey reveals no evidence of *Batrachochytrium dendrobatidis* in the Kingdom of Bhutan. *Herpetol Rev.* (2020) 51:494–7. Available online at: https://ssarherps.org/herpetological-review-pdfs/ (accessed June 24, 2021).
85. Kirk D, O'Connor MI, Mordecai EA. Temperature effects on individual-level parasitism translate into predictable effects on parasitism in populations. *bioRxiv.* (2020). doi: 10.1101/2020.12.01.406884. Available online at: https://www.biorxiv.org/content/10.1101/2020.12.01.406884v2.full.pdf (accessed June 24, 2021).
86. Bernard RF, Grant EHC. Rapid assessment indicates context-dependent mitigation for amphibian disease risk. *Wildlife Soc B.* (2021). Available online at: https://wildlife.onlinelibrary.wiley.com/journal/23285540 (accessed June 24, 2021).
87. Phillott AD, Speare R, Hines HB, Skerratt LF, Meyer E, McDonald KR, et al. Minimising exposure of amphibians to pathogens during field studies. *Dis Aquat Organ.* (2010) 92:175–85. doi: 10.3354/dao02162
88. More S, Miranda MA, Bicout D, Bøtner A, Butterworth A, Calistri P, et al. Risk of survival, establishment and spread of *Batrachochytrium salamandrivorans* (*Bsal*) in the EU. *EFSA J.* (2018) 16: e05259. doi: 10.2903/j.efsa.2018.5259
89. United States Fish and Wildlife Service. *Listing Salamanders as Injurious Due to Risk of Salamander Chytrid Fungus.* (2016). Available online at: https://www.fws.gov/injuriouswildlife/salamanders.html (accessed October 12, 2020).
90. Canada Border Services Agency. *Environment and Climate Change Canada (ECCC)'s Import Restrictions on Salamanders. Customs Notice 17-17.* (2018). Available online at: https://www.cbsa-asfc.gc.ca/publications/cn-ad/cn17-17-eng.html (accessed October 12, 2020).
91. Center for Disease Control and Prevention. *One Health.* United States Department of Health and Human Services (2020). Available online at: https://www.cdc.gov/onehealth/index.html (accessed December 29, 2020).
92. Byrne AQ, Vredenburg VT, Martel A, Pasmans F, Bell RC, Blackburn DC, et al. Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation. *Proc Natl Acad Sci USA.* (2019) 116:20382–7. doi: 10.1073/pnas.1908289116
93. Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, Flaherty DC, et al. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* (2009) 3:818–24. doi: 10.1038/ismej.2009.27
94. Harris RN, Lauer A, Simon MA, Banning JL, Alford RA. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis Aquat Organ.* (2009) 83:11–16. doi: 10.3354/dao02004
95. Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, Minbiole KPC, et al. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol Lett.* (2013) 16:807–20. doi: 10.1111/ele.12099
96. Bletz MC, Kelly M, Sabino-Pinto J, Bales E, Van Praet S, Bert W, et al. Disruption of skin microbiota contributes to salamander disease. *Proc R Soc Lond B Bio.* (2018) 285:20180758. doi: 10.1098/rspb.2018.0758
97. Knapp RA, Fellers GM, Kleeman PM, Miller DAW, Vredenburg VT, Rosenblum EB, et al. Large-scale recovery of an endangered amphibian despite ongoing exposure to multiple stressors. *Proc Natl Acad Sci USA.* (2016) 113:11889–94. doi: 10.1073/pnas.1600983113
98. Wilbur MQ, Knapp RA, Toothman M, Briggs CJ. Resistance, tolerance and environmental transmission dynamics determine host extinction risk in a load-dependent amphibian disease. *Ecol Lett.* (2017) 20:1169–81. doi: 10.1111/ele.12814
99. Rodriguez KM, Voyles J. The amphibian complement system and chytridiomycosis. *J Exper Zool.* (2020) 333:706–19. doi: 10.1002/jez.2419
100. Rollins-Smith LA. Global amphibian declines, disease, and the ongoing battle between *Batrachochytrium* fungi and the immune system. *Herpetologica.* (2020) 76:178–88. doi: 10.1655/0018-0831-76.2.178
101. Bosch J, Sanchez-Tomé E, Fernández-Loras A, Oliver JA, Fisher MC, Garner TWJ. Successful elimination of a lethal wildlife infectious disease in nature. *Biol Lett.* (2015) 11:20150874. doi: 10.1098/rsbl.2015.0874
102. Martel A, Vila-Escale M, Fernandez-Giberteau D, Martinez-Silvestre A, Canessa S, Van Praet S, et al. Integral chain management of wildlife diseases. *Conserv Lett.* (2020) 13:212707. doi: 10.1111/conl.12707
103. Hauck LL, Weitemier KA, Penaluna BE, Garcia TS, Cronn R. Casting a broader net: using microfluidic metagenomics to capture aquatic biodiversity data from diverse taxonomic targets. *Environ DNA.* (2019) 1:251–67. doi: 10.1002/edn3.26

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fvets.2021.733939

Molecular Confirmation of Ranavirus Infection in Amphibians From Chad, Africa

Erin K. Box[1*†], *Christopher A. Cleveland*[1*†], *Kuttichantran Subramaniam*[2,3], *Thomas B. Waltzek*[2,3] *and Michael J. Yabsley*[1,4*]

[1] *Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA, United States,* [2] *Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL, United States,* [3] *Emerging Pathogens Institute, University of Florida, Gainesville, FL, United States,* [4] *Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA, United States*

OPEN ACCESS

Edited by:
Amanda Linda Jean Duffus, Gordon State College, United States

Reviewed by:
Leon Grayfer, George Washington University, United States
Rachel Goodman, Hampden–Sydney College, United States

***Correspondence:**
Erin K. Box
erin.box25@uga.edu
Christopher A. Cleveland
ccleve@uga.edu
Michael J. Yabsley
myabsley@uga.edu

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *30 June 2021*
Accepted: *16 August 2021*
Published: *16 September 2021*

Citation:
Box EK, Cleveland CA, Subramaniam K, Waltzek TB and Yabsley MJ (2021) Molecular Confirmation of Ranavirus Infection in Amphibians From Chad, Africa. Front. Vet. Sci. 8:733939. doi: 10.3389/fvets.2021.733939

Ranaviruses are DNA viruses (Family *Iridoviridae*; Subfamily *Alphairidovirinae*) and ranaviral disease is considered an emerging infectious disease of ectothermic vertebrates. Ranavirus infection can have varying pathological effects on infected amphibians, reptiles, and fish, most notably causing significant mortality events and population declines. Despite having a broad global range with reports from six continents, only a single incidental finding in *Xenopus longipes* from mainland Africa (Cameroon) is known and lacks molecular confirmation. Thus, there is a considerable knowledge gap concerning ranaviruses in Africa. We opportunistically obtained tissue samples from 160 amphibians representing five genera (*Hoplobatrachus*, *Hylarana*, *Ptychadena*, *Pyxicephalus,* and *Xenopus*) and two turtles (*Pelomedusa* sp.) from Chad, Africa. Samples were tested for ranavirus infection using a conventional PCR assay targeting the major capsid protein (MCP). A total of 25/160 (16%) frogs tested positive including 15/87 (17%) *Hoplobatrachus occipitalis*, 10/58 (17%) *Ptychadena* spp., 0/3 *Pyxicephalus* spp., 0/9 *Xenopus* spp., and 0/3 *Hylarana* spp. One of two turtles tested positive. Partial MCP gene sequences indicated all samples were >98% similar to several frog virus 3 (FV3)-like sequences. Additional gene targets (DNA polymerase [DNApol], ribonucleotide reductase alpha [RNR- α], ribonucleotide reductase beta subunit [RNR- β]) were sequenced to provide further detailed classification of the virus. Sequences of individual gene targets indicate that the ranavirus detected in frogs in Chad is most similar to tiger frog virus (TFV), a FV3-like virus previously isolated from diseased amphibians cultured in China and Thailand. Full genome sequencing of one sample indicates that the Chad frog virus (CFV) is a well-supported sister group to the TFVs previously determined from Asia. This work represents the first molecular confirmation of ranaviruses from Africa and is a first step in comparing ranavirus phylogeography on a local and global scale.

Keywords: ranavirus, amphibian, reptile, phylogeography, Africa

INTRODUCTION

Members of the genus *Ranavirus* are double-stranded DNA viruses (Family *Iridoviridae*; Subfamily *Alphairidovirinae*) that can infect fish, amphibians, and reptiles (1). Transmission of some ranaviruses can occur between hosts of these taxonomic classes (2). Although asymptomatic infections may occur, in recent decades it has been recognized that ranaviruses can cause epizootics (3, 4). Ranaviruses induce systemic infections with variable presentation of disease (5). Clinical signs in amphibians include buoyancy problems, anorexia, swelling of the legs and body, redness, hemorrhages, cutaneous erosions, and ulcerations (6). In reptiles, common clinical signs include swelling of the head and extremities, skin ulcerations, and ocular discharge (5).

Ranavirus infection has been reported on every continent except Antarctica and is cited as a cause of amphibian, reptile, and fish die-offs (6–8). Epizootic hematopoietic necrosis virus (EHNV) in fish and ranaviruses in amphibians are reportable to the Office International des Epizooties (OIE) (7, 9, 10). Although some new reports are due to enhanced surveillance and testing, it is likely that both host and geographic range of infection is also increasing (7). The introduction of ranavirus into new regions may be due to numerous factors, including the exotic pet trade, commercialization of amphibians as food, and the import of animals for research (11).

As amphibian populations worldwide experience increasing threats to their stability, it is important that causes of mortality events are thoroughly investigated. To date, there is a single report of ranavirus from mainland Africa (Cameroon), and it was an incidental finding in *Xenopus longipes* that lacked molecular confirmation (12). Ranavirus has also been detected *via* qPCR in amphibians in Madagascar, although results were not confirmed with sequence analysis (13). Because of the high diversity of fish, amphibian, and reptile species in Africa, it is important to assess the distribution and host range of ranaviruses in Africa.

The aim of this study was to utilize opportunistically collected frog and turtle tissue samples to screen for the presence of ranavirus in Chadian amphibian populations. Assessing the ranavirus infection status of Chadian amphibian populations will aid in developing a better understanding of ranavirus phylogeography on a local and global scale. In doing this, we aim to inform existing knowledge gaps concerning the presence of ranaviruses among amphibian populations in Africa.

MATERIALS AND METHODS

Tissue Collection

Tissue samples were opportunistically obtained from animals captured for an unrelated study being conducted in several fishing villages along the Chari River Basin in Chad, Africa between June 2016 and July 2018 (**Figure 1**) (14). Local villagers obtained frogs either by hand or through the use of submersible nets baited with fish tissue (14). Two African helmeted turtles (*Pelomedusa* sp.) were incidentally collected and included in this study (14). Captured animals had no apparent clinical signs of disease upon routine observation by C. A. C. Animals were euthanized following American Veterinary Medical Association guidelines (15) and tissue samples (<1 cm toe clips) were acquired from each animal and preserved in 70% ethanol. All animal procedures were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee (protocol no. A2016 07–024).

DNA Extraction and PCR

DNA was extracted from tissue samples using a commercial DNA extraction kit (DNeasy Blood and Tissue Extraction Kit, QIAGEN, Germantown, MD). Samples were screened for ranavirus using conventional and real-time polymerase chain reaction (PCR). Initial determination of ranavirus infection status was done using conventional PCR primers targeting a ∼500bp region of the ranavirus major capsid protein (MCP) gene (**Table 1**). Amplicons were purified from a 0.8% agarose gel stained with gel red (Biotium Inc., Hayward, California, USA) using a commercial gel-purification kit (QIAGEN). Bi-directional Sanger sequencing of amplicons was conducted by Georgia Genomics and Bioinformatics Core (Athens, GA) or GeneWiz (South Plainfield, New Jersey). The sequencing reads were edited and assembled in Geneious 10.2.6 software (Biomatters Limited, Auckland, New Zealand). The consensus sequences were then used as queries for BLASTN searches against the National Center for Biotechnology Information (NCBI) GenBank non-redundant (nr) nucleotide sequence database. Additional PCR analyses were performed targeting DNA polymerase (DNApol), ribonucleotide reductase alpha subunit (RNR-α) and ribonucleotide reductase beta subunit (RNR-β) gene targets (**Table 1**) of a select number of samples to determine genetic diversity of ranaviruses in Chad by geographic location and host species.

Real-Time PCR

Real-time PCR was performed to determine which samples had the highest viral DNA concentration and would be best for full viral genome sequencing. Real-time primers targeted the MCP gene and a FAM fluorescent probe was used for detection of the amplified product (**Table 1**). All samples were run in duplicate. The DNA sample with the lowest cycle threshold (Ct) value (A-21) was used for full ranaviral genome sequencing.

Complete Genome Sequencing and Genome Annotation

The DNA extracted from a crowned bullfrog (*Hoplobatrachus occipitalis*; sample A-21) was used to prepare a DNA sequencing library with a NEBNext® Ultra™ II DNA Library Prep Kit according to the manufacturer's instruction. Sequencing was performed on an Illumina MiSeq sequencer using a v3 chemistry 600-cycle kit. *De novo* assembly of the paired-end reads was performed using SPAdes 3.10.1 (20). The quality of the genome assembly was verified by mapping the reads back to the consensus sequence in Bowtie 2 and visually inspecting the alignments in Tablet (21, 22). The genome was annotated using the Genome Annotation Transfer Utility with the tiger frog virus isolate D2008 (TFV-D2008; GenBank Accession No. MT512502.1) used as the reference genome (23). Additional putative open reading frames (ORFs) were identified or removed by comparison to

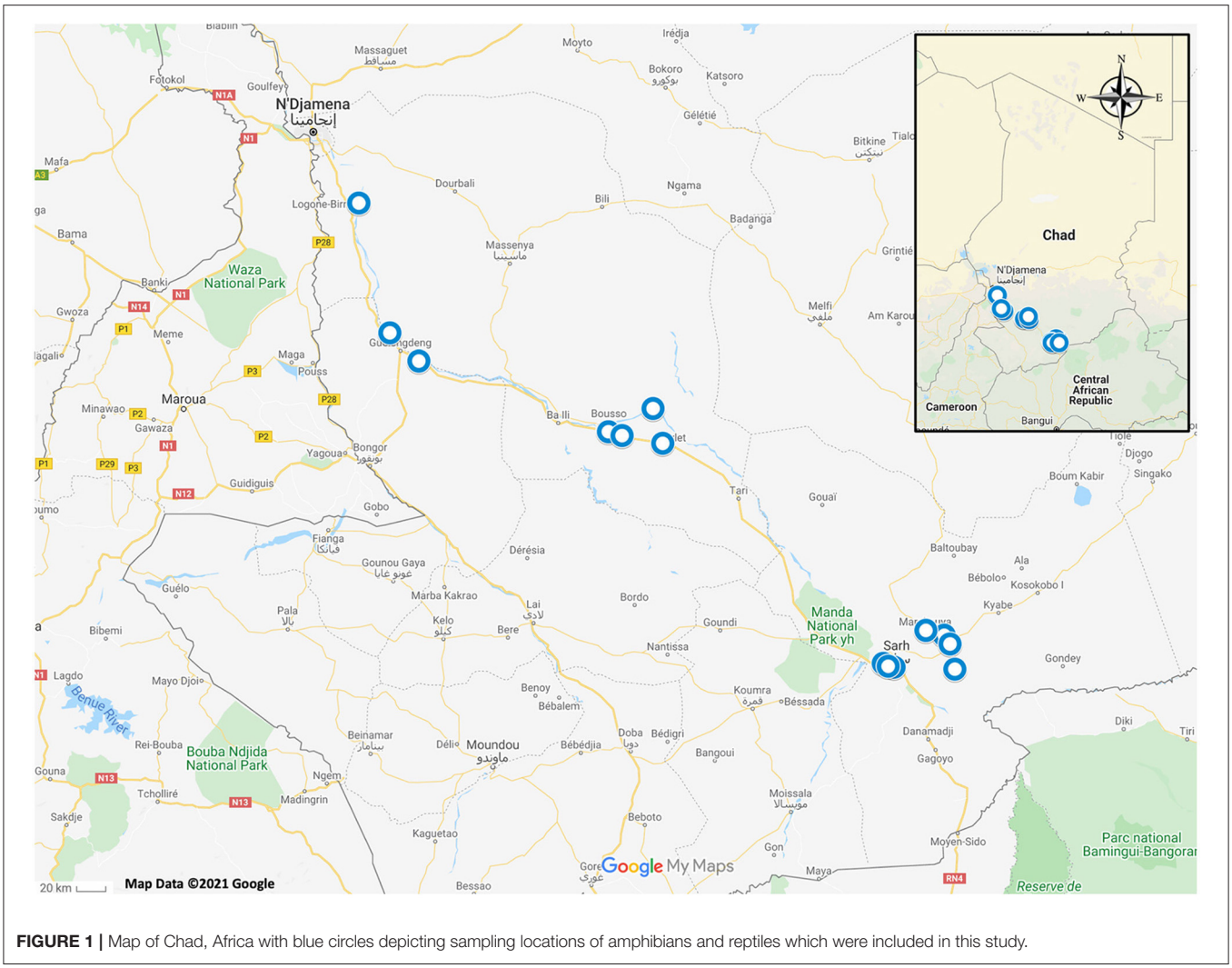


FIGURE 1 | Map of Chad, Africa with blue circles depicting sampling locations of amphibians and reptiles which were included in this study.

FV3 and TFV genome annotations (24, 25). The functions of the open reading frames (ORFs) were predicted based on BLASTP searches against the NCBI GenBank nr protein sequence database.

Phylogenetic and Genetic Analyses

Supplementary Table 1 summarizes the ranaviruses, genes, and corresponding NCBI GenBank accession numbers used to generate five datasets for the phylogenetic and genetic analyses. Dataset 1 (DS1) contained the Chad ranavirus genome (sample A-21) and 44 fully sequenced ranavirus genomes retrieved from NCBI GenBank. These sequences were aligned using Mauve 2.4 software to visualize genomic inversions and obtain the locally collinear block (LCB) alignments (26). The LCB alignments were then concatenated in Geneious 10.2.6 and contained 144,720 nucleotide (nt) characters (including gaps) (27). MEGA X was used to determine best-fit models and perform Maximum Likelihood (ML) phylogenetic analyses (28).

To elucidate the relationship of Chad ranaviruses to other ranaviruses for which the full genome sequences have not been determined (e.g., the blood phyton ranavirus [BPRV], Dopasia gracilis ranavirus [DGRV], and Wamena virus [WV]), additional phylogenetic and genetic analyses were performed using four conserved genes encoding MCP, RNR-α, RNR-β, and DNApol. These gene alignments included partial sequences and missing data were coded as questions marks (?). Dataset 2 (DS2) contained 1,392 nt characters (including gaps) and consisted of seven partial (DGRV, BPRV, Chad ranavirus A-62, Chad ranavirus A-61, Chad ranavirus T17-01, Chad ranavirus 033c, and the consensus sequence of 18 identical Chad ranaviruses [i.e., A-19, A-20, A-21, A-22, A-24, A-25, A-27, A-35, A-50, A-51, A-54, A-58, A-60, A-64, A-73, A17-07, A17-08, 053]) and 45 complete MCP sequences. Dataset 3 (DS3) contained 3,042 nt characters (including gaps) and consisted of four partial (DGRV, BPRV, WV, and the consensus sequence of nine identical Chad ranaviruses [i.e., A-21, A-24, A-27, A-35, A-36, A-50, A-55, A17-07, 053]) and 44 complete DNApol sequences. Dataset 4 (DS4) contained 1,698 nt characters (including gaps) and consisted of three partial (DGRV, BPRV, and the consensus sequence of four identical Chad ranaviruses [i.e., A-21, A-50,

TABLE 1 | Primers used for molecular diagnosis and differentiation of ranavirus from amphibian samples in Chad, Africa.

Target Gene	Primer Name	Nucleotide Sequence (5′ – 3′)	Reference
major capsid protein (MCP)	MCP 4R	GACTTGGCCACTTATGAC	(16)
	MCP 5	GTCTCTGGAGAAGAAGAA	
DNA polymerase (DNApol)	DNApol-F	GTGTAYCAGTGGTTTTGCGAC	(17)
	DNApol-R	TCGTCTCCGGGYCTGTCTTT	
ribonucleotide reductase alpha subunit (RNR-α)	RNR-AF	CTGCCCATCTCKTGCTTTCT	(18)
	RNR-AR	CTGGCCCASCCCATKGCGCCCA	
ribonucleotide reductase beta subunit (RNR-β)	RNR-BF	AGGTGTRCCRGGGYCGTA	(18)
	RNR-BR	GACGCTCCAYTCGACCACTT	
major capsid protein (MCP)	rtMCP-for	ACACCACCGCCCAAAAGTAC	(19)
	rtMCP-rev	CCGTTCATGATGCGGATAATG	
	MCP FAM Probe	6FAM CCTCATCGTTCTGGCCATCAACCA BHQ1	

A17-07, 053]) and 45 complete RNR- α ranavirus sequences. Dataset 5 (DS5) contained 1,164 nt characters (including gaps) and consisted of four partial (DGRV, BPRV, WV, and the consensus sequence of six identical Chad ranaviruses [i.e., A-21, A-27, A-36, A-50, A17-07, 053]) and 44 complete RNR- β sequences. ML phylogenetic analyses of DS2, DS3, DS4, and DS5 were performed as described above and genetic analyses were conducted using the Sequence Demarcation Tool Version 1.2 with the MAFFT option implemented (29).

RESULTS

Prevalence Determined by PCR

Tissue samples from 160 frogs were tested, representing five genera (*Hoplobatrachus, Hylarana, Ptychadena, Pyxicephalus, Xenopus*). A total of 25/160 (16%) samples were confirmed positive for ranavirus using conventional PCR and Sanger sequencing. The prevalence for both *Hoplobatrachus occipitalis* (15/87) and *Ptychadena* spp. (10/58) was 17%. All three *Pyxicephalus* spp., nine *Xenopus* spp., and three *Hylarana* spp. were negative. Additionally, one of two (50%) African helmeted turtles was confirmed positive for ranavirus. Sequences from all MCP amplicons (∼500 bp) were most similar (>98%) to several FV3-like virus sequences.

Real-Time PCR

The lowest cycle threshold (Ct) value obtained from a real-time PCR sample was 17.94 from sample A-21, so this sample was used for full genomic sequencing and analysis.

Complete Genome Sequencing and Genome Annotation

The *de novo* assembly of the 50,786,552 paired-end reads recovered a contiguous sequence of 106,120 bp. The G+C content of the genome was 54% with an average coverage of nine reads/nucleotides. The Chad ranavirus genome is predicted to encode 96 open reading frames (ORFs; **Supplementary Table 2**) and possesses a FV3-like ranavirus genome arrangement as observed among the members of the tiger frog virus (i.e., Chinese TFV and Thai ranaviruses) subclade (data not shown) (25, 30). Comparative genomic analyses revealed that the Chad ranavirus possesses a hypothetical protein (TFV ORF 28), which is only encoded by the Chinese TFV and Thai ranaviruses, but absent in all other ranaviruses. Chad ranavirus possesses a FV3 30R equivalent ORF (ORF 31 in Chad ranavirus; hypothetical protein), which is absent in the Chinese TFV and all Thai ranaviruses. Three hypothetical proteins (TFV ORFs 38, 43, 71), a L-protein-like protein (TFV 36), a thymidylate synthase (TFV 87), and an integrase-like protein (TFV 17) encoded by the Chinese TFV and all Thai ranaviruses were absent in the Chad ranavirus. TFV ORFs 6.5 and 59, encoding hypothetical proteins, were present in the Chad ranavirus, Chinese TFV and all Thai ranaviruses except Oxyeleotris marmorata ranavirus (OMRV) and Asian grass frog ranavirus (AGFRV). An ORF encoding a putative nuclear calmodulin-binding protein (TFV ORF 54) is only present in the Chad ranavirus, Chinese TFV, OMRV, and AGFRV. TFV ORF 97, encoding a hypothetical protein, is present in the Chad ranavirus, Chinese TFV, and all Thai ranaviruses except AGFRV. TFV ORFs 26 and 63, encoding hypothetical proteins, were present in the Chinese TFV, all Thai ranaviruses except OMRV, and the Chad ranavirus. The complete genome of the Chad ranavirus has been deposited in GenBank under accession number MW727505.

Phylogenetic and Genetic Analyses

The Maximum Likelihood (ML) analysis based on the DS1 generated a well-supported tree with a topology similar to a recent analysis (**Figure 2**) (25). The ML tree supported the Chad ranavirus as the most basal branch of the TFV subclade within the larger FV3 clade. The ML analyses based on the DS2 demonstrated the monophyly of Chad ranaviruses within the TFV subclade (**Supplementary Figure 1**). The ML analyses based on the DS3, DS4, and DS5 supported the Chad ranaviruses and three squamate reptile ranavirus strains (BPRV, DGRV, and WV) as members of the TFV subclade (**Supplementary Figures 2–4**). Sequences from the MCP gene

grouped separately from other ranaviruses, and all samples, except four, grouped together (**Supplementary Figure 1**). Sequences from the DNApol gene grouped together, as the sister clade to DGRV (**Supplementary Figure 2**). Analysis of the RNR-α and RNR- β genes produced similar topologies to the other genes (**Supplementary Figures 3**, **4**).

Genetic analysis of the DS2 revealed the nucleotide identity of the Chad ranaviruses ranged from 98.5–99.8% when compared to each other, 97.5–98.5% when compared to members of the TFV subclade, and 94.0–99.3% when compared to other ranaviruses (**Supplementary Table 3**). Genetic analysis of the DS3 revealed the nucleotide identity of the Chad ranaviruses ranged from 98.4 to 99.6% when compared to other TFVs, and 95.9–98.9% when compared to other ranaviruses (**Supplementary Table 4**). Genetic analysis of the DS4 revealed the nucleotide identity of the Chad ranaviruses ranged from 99.1 to 99.6% when compared to other TFVs, and 94.6–99% when compared to other ranaviruses (**Supplementary Table 5**). Genetic analysis of the DS5 revealed the nucleotide identity of the Chad ranaviruses ranged from 98.5 to 99.2% when compared to other TFVs, and 96.1–98.6% when compared to other ranaviruses (**Supplementary Table 6**). Finally, the Mauve 2.4 analysis revealed that the Chad ranavirus genome possessed a FV3-like Ranaviral genome arrangement (**Supplementary Figure 5**) (30).

DISCUSSION

The objective of this study was to investigate the presence of ranavirus in the Chari River Basin of Chad, Africa using opportunistically collected tissue samples. Our results provide the first sequence confirmed detection of ranavirus in mainland Africa. Infections were detected in two anuran genera (*Hoplobatrachus* and *Ptychadena*) and one turtle species (*Pelomedusa* sp.) across the geographic range of our sampling efforts. Phylogenetic and genetic analyses, based on partial and complete genome datasets, supported the Chad frog ranaviruses as the sister group to the TFVs, described from Asian fish, amphibians, and reptiles [reviewed in Sriwanayos et al. (25)].

During this study, ranavirus was found in samples from crowned bullfrogs (*H. occipitalis*). Although they are referred to as bullfrogs, crowned bullfrogs are not closely related to other African, European, or North American bullfrog species (31). Generally, the *Hoplobatrachus* species (Family Dicroglossidae) are found throughout Southeast Asia; the crowned bullfrog is the only African member of the genus *Hoplobatrachus*, which are also referred to as tiger frogs (31). Tiger frog virus, the ranavirus strain with which our samples grouped most closely during analysis of the full genome, was first isolated in 2000 from *Hoplobatrachus tigerinus* (previously, *Rana tigrina*) in Chinese aquaculture facilities following severe mortality events (32, 33). Strains of TFV have also been associated with disease in cultured fish and frogs in Thailand (25). Interestingly, variation in presentation has been noted between some infected hosts in China and Thailand with some having cutaneous lesions and no internal gross lesions and others having classic internal lesions (e.g., enlargement of internal organs and petechial hemorrhages) (25). This contrasts with the lack of apparent disease in ranavirus-infected Chadian frogs, including *H. occipitalis*. Further surveillance is required to determine whether amphibian morbidity or mortality is being caused by ranaviruses in Chad and may have been missed in initial sampling, or whether these populations may be more resistant to ranaviral disease than Asian *Hoplobatrachus* species.

Samples from *Ptychadena* spp. also tested positive for ranavirus during this study. There is great species diversity within genus *Ptychadena* (Family Ptychadenidae) in Sub-Saharan Africa (34). Species are still being discovered and hotspots of endemism may be at risk if threatened with spreading range of ranavirus infections. Further testing of these species, especially in areas of high endemism may benefit conservation efforts and understanding of these amphibian populations.

Although no *Xenopus* tested positive for ranavirus in this study, the sample size was very small (nine individuals). It has been suggested that *Xenopus* may serve as viral reservoirs for ranaviruses, as asymptomatic individuals have been found to harbor low-level infections (35). Asymptomatic ranaviral infections have been detected in *Xenopus* sourced from multiple laboratory animal suppliers (35). Ranavirus has also been detected from feral, invasive *Xenopus* sampled in Chile during an amphibian health survey (36). These studies suggest that *Xenopus* may be resistant to clinical disease when infected with ranavirus and may contribute to expansion of ranavirus distribution. Whether this resistance to ranaviral disease exhibited by *Xenopus* is a consequence of coevolution with ranaviruses in Africa is yet unknown. It is also unknown whether this disease resistance occurs in wild *Xenopus* populations, as wild African *Xenopus* have not been widely surveyed for ranavirus infection or disease.

This detection of ranavirus in an African helmeted turtle, *Pelomedusa* sp., marked the first detection of ranavirus in a wild African reptile. Like all animals included in this study, this turtle showed no apparent clinical signs of disease upon sampling. This finding highlights the importance of wildlife health and disease monitoring, even when a positive result may be unexpected. Ranaviruses have been associated with disease in other species of aquatic turtles from Asia, Europe and North America (37–40) but there is also evidence that some species of aquatic turtles can have asymptomatic ranavirus infections (41, 42). As we detected only a single positive animal, and sampled only two, additional understanding of Chadian turtle populations and surveillance for ranavirus may elucidate what impact, if any, they have on these animals and animal populations.

Our analysis detected at least one ranavirus strain in the sampled individuals. It is also possible that the samples which grouped separately from the majority of our samples (033-C, T17-01, A-62, and A-62) during analysis of MCP gene sequences could represent additional species. Additional full-genome sequencing and analysis of these samples could lead to additional insight into relatedness of these ranavirus samples. We did not detect variation within the sequenced gene targets between the two ranavirus-positive amphibian species or across our sampling sites. Similarly, whole genome sequencing of the Chad ranavirus and subsequent phylogenomic analysis with other fully sequenced ranaviruses revealed a high similarity

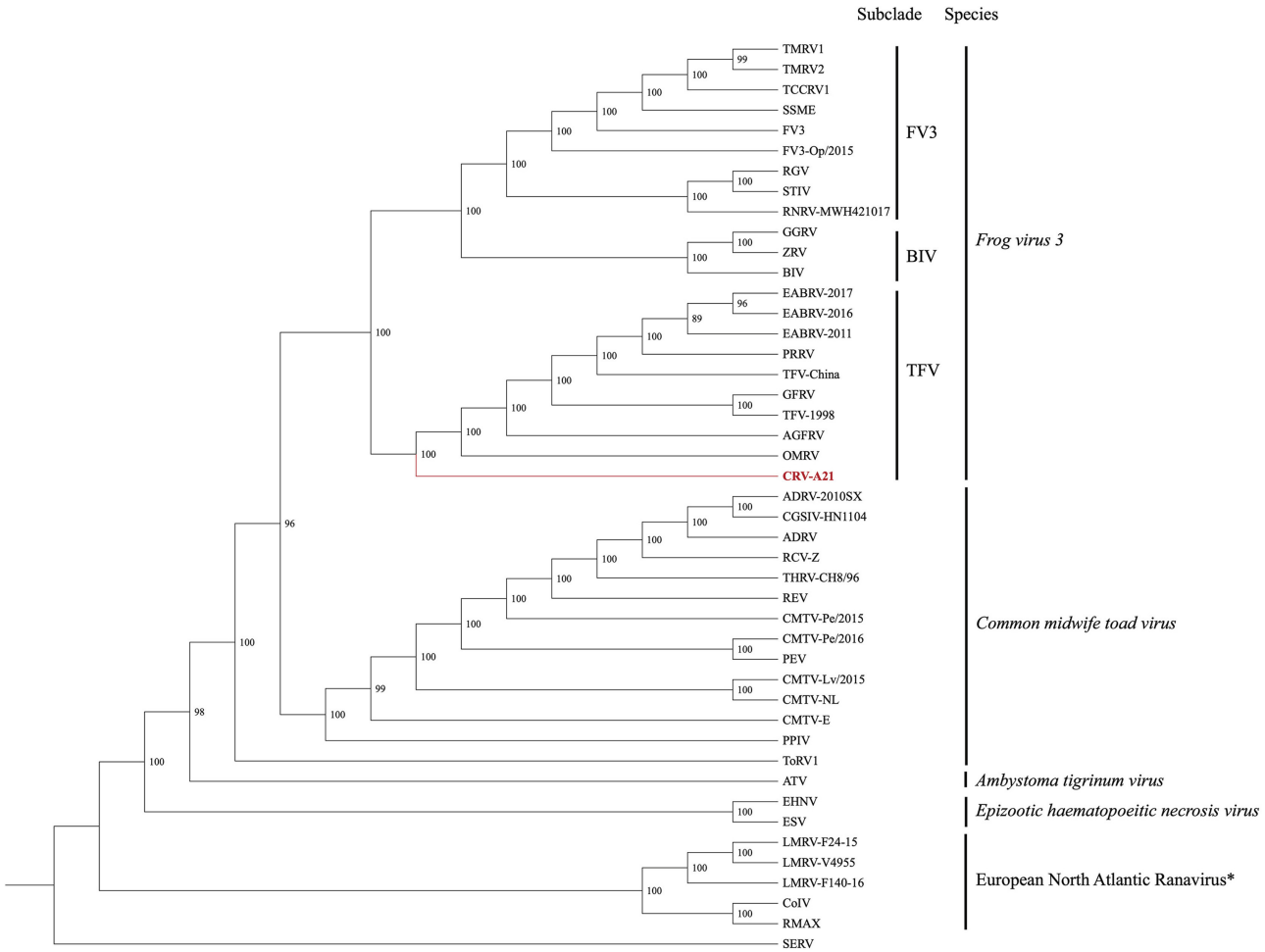


FIGURE 2 | Cladogram illustrating the relationship of the newly sequenced Chad ranavirus (CRV-A21) to members of the genus *Ranavirus* (Family *Iridoviridae*; Subfamily *Alphairidovirinae*). The Maximum Likelihood (ML) phylogenetic analysis included the aligned genomes (concatenated locally collinear blocks) of 44 ranaviruses. Clade support was assessed by running 1,000 bootstrap replicates with values presented at each node. See **Supplementary Table 1** for virus abbreviations. *Note: European North Atlantic Ranavirus has not been approved as a ranavirus species by the ICTV.

between Chad ranavirus and TFVs. The formation of a well-supported and distinct clade between Chad ranavirus and other TFVs, from two different continents, reveals that there is more diversity to this group of ranaviruses infecting a wide range of hosts.

It is possible that some of the animals screened with conventional PCR and considered negative during this study had ranavirus infections that were not detected, but may have been detected via other means, such as qPCR. It is also possible that viral load was considerably lower in the tissue type we tested (toe clips) and that virus may have been detected if tissues such as liver or kidney were sampled. Although this is a potential limitation of this study, samples were not acquired with ranavirus screening in mind. Future studies could be improved by targeting multiple tissue types which would aid in direct comparison with findings of other studies and create a standardized approach to ranavirus screening in wild animals.

Central African amphibian populations are greatly understudied (43). Frogs often play a significant role in local communities, highlighting a need for long-term studies in Central Africa to further understand frog population health (44). To local communities, frogs hold economic, medicinal, pest control, and nutritional value (44). The edible *Hoplobatrachus occipitalis* (one of the species positive for ranavirus) is extensively traded for food (44, 45). As amphibian trade is often cited as a factor in global ranavirus spread (6, 11), it is possible that amphibian trade could contribute to the spread of ranavirus on the African continent. A better understanding of the impact of ranaviruses on these amphibian populations may aid in conservation of these valuable amphibian populations.

Our results confirm the presence of ranavirus on mainland Africa. Genetic analysis of the Chad Ranavirus has allowed us to develop a better understanding of the global phylogeography of ranaviruses and is the beginning of an exploration of ranaviruses

in Africa. Further investigation is necessary to better understand the geographic and host range of ranavirus on the continent, as well as how they may be impacting animal health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Georgia IACUC.

AUTHOR CONTRIBUTIONS

EB and CC compiled samples and performed testing and initial molecular analysis. KS and TW performed phylogenetic and genetic analyses. MY was the principal investigator for the experiment. All authors made substantial contribution to the conception, design, sample acquisition, analysis, and/or interpretation of the data for this study, agree to be personally accountable for their contributions, drafted, and reviewed the manuscript.

FUNDING

Samples were collected during surveillance studies conducted in collaboration with the Carter Center in an effort to further the mission to eradicate Guinea worm disease. These efforts were made possible by financial and in-kind contributions from many donors to the Carter Center. A full listing of supporters is available at http://www.cartercenter.org/donate/corporate-government-foundation-partners/index.html. Additional support was provided by the wildlife management agencies of the Southeastern Cooperative Wildlife Disease Study member states through the Federal Aid to Wildlife Restoration Act (50 Stat. 917) and by a U.S. Department of the Interior Cooperative Agreement.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.733939/full#supplementary-material

REFERENCES

1. Chinchar VG, Hick P, Ince IA, Jancovich JK, Marschang R, Qin Q, et al. ICTV virus taxonomy profile: Iridoviridae. *J General Virol.* (2017) 98:890–1. doi: 10.1099/jgv.0.000818
2. Brenes R, Gray MJ, Waltzek TB, Wilkes RP, Miller DL. Transmission of ranavirus between ectothermic vertebrate hosts. *PLoS ONE.* (2014) 9:e92476. doi: 10.1371/journal.pone.0092476
3. Jancovich JK, Davidson EW, Morado JF, Jacobs BL, Collins JP. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. *Dis Aquat Organ.* (1997) 31:161–7. doi: 10.3354/dao031161
4. Chinchar V. Ranaviruses (family Iridoviridae): emerging cold-blooded killers. *Arch Virol.* (2002) 147:447–70. doi: 10.1007/s007050200000
5. Miller DL, Pessier AP, Hick P, Whittington RJ. Comparative pathology of ranaviruses and diagnostic techniques. *Ranaviruses*. Springer, Cham. (2015). p. 171–208. doi: 10.1007/978-3-319-13755-1_7
6. Miller D, Gray M, Storfer A. Ecopathology of ranaviruses infecting amphibians. *Viruses.* (2011) 3:2351–73. doi: 10.3390/v3112351
7. Duffus AL, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, et al. Distribution and host range of ranaviruses. *Ranaviruses*. Springer, Cham (2015). p. 9–57. doi: 10.1007/978-3-319-13755-1_2
8. Gray MJ, Chinchar VG. Introduction: History and future of ranaviruses. *Ranaviruses*. Springer (2015). p. 1–7. doi: 10.1007/978-3-319-13755-1_1
9. Office International des Epizooties (OIE) - Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.1.2. - Infection with Ranavirus. Paris: World Organization for Animal Health (2019).
10. Schloegel LM, Daszak P, Cunningham AA, Speare R, Hill B. Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. *Dis Aquat Organ.* (2010) 92:101–8. doi: 10.3354/dao02140
11. Picco AM, Collins JP. Amphibian commerce as a likely source of pathogen pollution. *Conservation Biology*. (2008) 22:1582–9. doi: 10.1111/j.1523-1739.2008.01025.x
12. Doherty-Bone T, Ndifon R, Nyingchia O, Landrie F, Yonghabi F, Duffus A, et al. Morbidity and mortality of the critically endangered Lake Oku clawed frog *Xenopus longipes*. *Endanger Species Res.* (2013) 21:115–28. doi: 10.3354/esr00514
13. Kolby JE, Smith KM, Ramirez SD, Rabemananjara F, Pessier AP, Brunner JL, et al. Rapid response to evaluate the presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in wild amphibian populations in Madagascar. *PLoS ONE.* (2015) 10:e0125330. doi: 10.1371/journal.pone.0125330
14. Cleveland CA, Eberhard ML, Thompson AT, Garrett KB, Swanepoel L, Zirimwabagabo H, et al. A search for tiny dragons (*Dracunculus medinensis* third-stage larvae) in aquatic animals in Chad, Africa. *Sci Rep.* (2019) 9:1–7. doi: 10.1038/s41598-018-37567-7
15. Association AVM. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Schaumburg, IL,: American Veterinary Medical Association (2013).
16. Mao J, Hedrick R, Chinchar V. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. *Virology.* (1997) 229:212–20. doi: 10.1006/viro.1996.8435
17. Holopainen R, Ohlemeyer S, Schütze H, Bergmann S, Tapiovaara H. Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Dis Aquat Organ.* (2009) 85:81–91. doi: 10.3354/dao02074
18. Ariel E, Holopainen R, Olesen NJ, Tapiovaara H. Comparative study of ranavirus isolates from cod (*Gadus morhua*) and turbot (*Psetta maxima*) with reference to other ranaviruses. *Arch Virol.* (2010) 155:1261–71. doi: 10.1007/s00705-010-0715-z
19. Brunner J. Ecology of an amphibian pathogen: Transmission, persistence, and virulence. Arizona: Arizona State University. (2004).
20. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Computational Biol.* (2012) 19:455–77. doi: 10.1089/cmb.2012.0021
21. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* (2012) 9:357. doi: 10.1038/nmeth.1923
22. Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, Cardle L, et al. Using Tablet for visual exploration of second-generation sequencing data. *Brief Bioinform.* (2013) 14:193–202. doi: 10.1093/bib/bbs012
23. Tcherepanov V, Ehlers A, Upton C. Genome Annotation Transfer Utility (GATU): rapid annotation of viral genomes using a closely related reference genome. *BMC Genomics.* (2006) 7:1–10. doi: 10.1186/1471-2164-7-150

24. Tan WG, Barkman TJ, Chinchar VG, Essani K. Comparative genomic analyses of frog virus 3, type species of the genus Ranavirus (family Iridoviridae). *Virology.* (2004) 323:70–84. doi: 10.1016/j.virol.2004.02.019
25. Sriwanayos P, Subramaniam K, Stilwell NK, Imnoi K, Popov VL, Kanchanakhan S, et al. Phylogenomic characterization of ranaviruses isolated from cultured fish and amphibians in Thailand. *Facets.* (2020) 5:963–79. doi: 10.1139/facets-2020-0043
26. Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* (2004) 14:1394–403. doi: 10.1101/gr.2289704
27. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics.* (2012) 28:1647–9. doi: 10.1093/bioinformatics/bts199
28. Kumar S, Stecher G, Li M, Knyaz C, Tamura K, MEGA X. molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol.* (2018) 35:1547. doi: 10.1093/molbev/msy096
29. Muhire BM, Varsani A, Martin DP, SDT. a virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS ONE.* (2014) 9:e108277. doi: 10.1371/journal.pone.0108277
30. Claytor SC, Subramaniam K, Landrau-Giovannetti N, Chinchar VG, Gray MJ, Miller DL, et al. Ranavirus phylogenomics: signatures of recombination and inversions among bullfrog ranaculture isolates. *Virology.* (2017) 511:330–43. doi: 10.1016/j.virol.2017.07.028
31. Kosuch J, Vences M, Dubois A, Ohler A, Böhme W. Out of Asia: mitochondrial DNA evidence for an oriental origin of tiger frogs, genus *Hoplobatrachus*. *Mol Phylogenet Evol.* (2001) 21:398–407. doi: 10.1006/mpev.2001.1034
32. He JG, Lü L, Deng M, He HH, Weng SP, Wang XH, et al. Sequence analysis of the complete genome of an iridovirus isolated from the tiger frog. *Virology.* (2002) 292:185–97. doi: 10.1006/viro.2001.1245
33. Weng S, He J, Wang X, Lü L, Deng M, Chan SM. Outbreaks of an iridovirus disease in cultured tiger frog, *Rana tigrina rugulosa*, in southern China. *J Fish Dis.* (2002) 25:423–7. doi: 10.1046/j.1365-2761.2002.00386.x
34. Smith ML, Noonan BP, Colston TJ. The role of climatic and geological events in generating diversity in Ethiopian grass frogs (genus *Ptychadena*). *R Soc Open Sci.* (2017) 4:170021. doi: 10.1098/rsos.170021
35. Robert J, Abramowitz L, Gantress J, Morales HD. *Xenopus laevis*: a possible vector of Ranavirus infection? *J Wildl Dis.* (2007) 43:645–52. doi: 10.7589/0090-3558-43.4.645
36. Soto-Azat C, Peñafiel-Ricaurte A, Price SJ, Sallaberry-Pincheira N, García MP, Alvarado-Rybak M, et al. *Xenopus laevis* and emerging amphibian pathogens in Chile. *Eco Health.* (2016) 13:775–83. doi: 10.1007/s10393-016-1186-9
37. Chen Z-x, Zheng J-c, Jiang Y-l. A new iridovirus isolated from soft-shelled turtle. *Virus Res.* (1999) 63(1-2):147-51. doi: 10.1016/S0168-1702(99)00069-6
38. Huang Y, Huang X, Liu H, Gong J, Ouyang Z, Cui H, et al. Complete sequence determination of a novel reptile iridovirus isolated from soft-shelled turtle and evolutionary analysis of Iridoviridae. *BMC Genomics.* (2009) 10:1–14. doi: 10.1186/1471-2164-10-224
39. Winzeler ME, Hamilton MT, Tuberville TD, Lance SL. First case of ranavirus and associated morbidity and mortality in an eastern mud turtle *Kinosternon subrubrum* in South Carolina. *Dis Aquat Organ.* (2015) 114:77–81. doi: 10.3354/dao02849
40. Borzym E, Stachnik M, Reichert M, Rzezutka A, Jasik A, Waltzek TB, et al. Genome sequence of a ranavirus isolated from a red-eared slider (*Trachemys scripta elegans*) in Poland. *Microbiology Resource Announcements.* (2020) 9:e00781–20. doi: 10.1128/MRA.00781-20
41. Ariel E, Elliott E, Meddings J, Miller J, Santos M, Owens L. Serological survey of Australian native reptiles for exposure to ranavirus. *Dis Aquat Organ.* (2017) 126:173–83. doi: 10.3354/dao03172
42. Carstairs SJ, Kyle CJ, Vilaça ST. High prevalence of subclinical frog virus 3 infection in freshwater turtles of Ontario, Canada. *Virology.* (2020) 543:76–83. doi: 10.1016/j.virol.2020.01.016
43. Jackson K, Zassi-Boulou A-G, Mavoungou L-B, Pangou S. Amphibians and reptiles of the Lac Télé community reserve, Likouala region, Republic of Congo (Brazzaville). *Herpetol Conserv Biol.* (2007) 2:75–86.
44. Mohneke M. *(Un) sustainable use of frogs in West Africa and resulting consequences for the ecosystem (dissertation)*. Humboldt-Universität, Berlin (2011).
45. Penner J, Adum GB, McElroy MT, Doherty-Bone T, Hirschfeld M, Sandberger L, et al. West Africa-A safe haven for frogs? A Sub-continental assessment of the chytrid fungus (Batrachochytrium dendrobatidis). *PLoS ONE.* (2013) 8:e56236. doi: 10.1371/journal.pone.0056236

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with several of the authors TW and KS.

frontiers in Veterinary Science

REVIEW
published: 21 September 2021
doi: 10.3389/fvets.2021.733404

Nidoviruses in Reptiles: A Review

Kate Parrish[1,2]*, *Peter D. Kirkland*[1,2], *Lee F. Skerratt*[3] *and Ellen Ariel*[2]

[1] *Virology Laboratory, Elizabeth Macarthur Agricultural Institute, New South Wales (NSW) Department of Primary Industries, Menangle, NSW, Australia,* [2] *College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia,* [3] *Faculty of Veterinary and Agricultural Sciences, Melbourne Veterinary School, University of Melbourne, Melbourne, VIC, Australia*

Since their discovery in 2014, reptile nidoviruses (also known as serpentoviruses) have emerged as significant pathogens worldwide. They are known for causing severe and often fatal respiratory disease in various captive snake species, especially pythons. Related viruses have been detected in other reptiles with and without respiratory disease, including captive and wild populations of lizards, and wild populations of freshwater turtles. There are many opportunities to better understand the viral diversity, species susceptibility, and clinical presentation in different species in this relatively new field of research. In captive snake collections, reptile nidoviruses can spread quickly and be associated with high morbidity and mortality, yet the potential disease risk to wild reptile populations remains largely unknown, despite reptile species declining on a global scale. Experimental studies or investigations of disease outbreaks in wild reptile populations are scarce, leaving the available literature limited mostly to exploring findings of naturally infected animals in captivity. Further studies into the pathogenesis of different reptile nidoviruses in a variety of reptile species is required to explore the complexity of disease and routes of transmission. This review focuses on the biology of these viruses, hosts and geographic distribution, clinical signs and pathology, laboratory diagnosis and management of reptile nidovirus infections to better understand nidovirus infections in reptiles.

Keywords: reptile, nidovirus, taxonomy, serpentovirus, respiratory disease, infectious disease

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Reviewed by:
Tim Hyndman, Murdoch University, Australia
Laura Lizbeth Hoon-Hanks, Colorado State University, United States

***Correspondence:**
Kate Parrish
kate.parrish@my.jcu.edu.au

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *30 June 2021*
Accepted: *19 August 2021*
Published: *21 September 2021*

Citation:
Parrish K, Kirkland PD, Skerratt LF and Ariel E (2021) Nidoviruses in Reptiles: A Review. Front. Vet. Sci. 8:733404. doi: 10.3389/fvets.2021.733404

INTRODUCTION

The order *Nidovirales* is a large group of diverse enveloped positive-strand RNA viruses (1). Nidoviruses are known to infect a range of vertebrate and invertebrate hosts, several of which have caused serious diseases in both humans and animals. In humans, prominent nidoviruses belong to the family *Coronaviridae* and infections can result in a wide range of presentations from asymptomatic infections to significant morbidity and mortality associated with severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome (MERS-CoV) (2, 3). This family also includes the virus responsible for the current COVID-19 global pandemic, severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) (4).

Following the emergence of these viruses in humans from animal sources, there is a renewed interest in animal nidoviruses including understanding the risk of cross species transmission from wildlife reservoirs. Novel viruses originating in wildlife reservoirs, especially bats, have also caused significant mortality and morbidity in animal populations, including swine acute diarrhoea syndrome coronavirus (SADS-CoV). This virus was implicated in the death of nearly 25,000 piglets (5). Other nidoviruses in animals associated with significant economic losses include

infections with equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhoea virus (PEDV) and infectious bursal disease virus (IBDV) (6–8). Although most well-known nidoviruses are associated with terrestrial hosts, they also infect and cause significant disease in fish (white bream virus, fathead minnow virus, chinook salmon bafinivirus), shrimp (yellow head virus, gill-associated virus), and several lesser known nidoviruses infect sea hares, freshwater free-living flatworms, crabs, and marine mammals (9–12). Due to their increasing importance and recent association with morbidity and mortality, nidoviruses are also of interest in reptiles.

The number of viruses in the order *Nidovirales* continues to expand rapidly with the introduction of next generation sequencing (NGS) and metagenomics studies (13, 14). Such technology allows for an unbiased approach to pathogen detection when classical methods of diagnosis are unsuccessful. This is how nidoviruses in reptiles were first discovered and subsequently reported in 2014 (15–17). Respiratory disease in captive ball pythons (*Python regius*) is not a new phenomenon, with some groups reporting a syndrome of unknown cause being observed by veterinarians since the late 1990's (16). Following the exclusion of known pathogens, several research groups simultaneously used NGS to identify novel nidovirus sequences in captive pythons with respiratory disease (15–17).

Since 2014, additional reptile nidoviruses in snakes have been discovered globally in a wide range of predominantly Pythonidae species (13, 14, 18–20). They have also been found in lizards and turtles (21–23). Like the captive ball pythons, a respiratory syndrome has been reported in wild Australian shingleback lizards (*Tiliqua rugosa*) since the 1990's. In 2016, the first nidovirus in a lizard was reported in this species, both with and without respiratory disease (21). More recently another novel lizard associated nidovirus was discovered following respiratory disease-associated mortalities in a captive collection of veiled chameleons (*Chamaeleo calyptratus*) (23). In freshwater turtles, the only reported nidovirus was discovered following a mortality event in the sole extant wild population of the freshwater Bellinger River snapping turtle (*Myuchelys georgesi*). In contrast to the infections in snakes and lizards, respiratory disease was not the predominant syndrome observed, but rather the most significant pathological changes were in the kidneys (22).

As the number of nidovirus detections in reptiles continues to grow, for the most part, studies are confined to captive collections, especially of pythons. Consequently, the disease risk that reptile nidoviruses pose to wild populations of reptiles is largely unknown. Worldwide the number of reptile species listed as threatened continues to increase (24) and emerging or reemerging infectious diseases including various fungi (25–27), bacteria (28) and viruses (29–31) are cause for concern. Infectious disease is rarely a single contributing factor in known plant and animal extinctions, with the exception being amphibian panzootics caused by chytrid fungus (*Batrachochytrium dendrobatidis*) (32–34), yet understanding the risk they pose may be critical in preventing ongoing population declines. This relatively new field of research offers unique opportunities to explore major gaps in knowledge. This review summarises the key findings to date from the published literature and offers recommendations for the direction of future research.

MATERIALS AND METHODS

A literature search was conducted between January 2018 and June 2021 using the following databases: Web of Science (Clarivate Analytics), PubMed, Google Scholar to find peer-reviewed articles as well as Trove for thesis manuscripts. Search terms included "reptile AND nidovirus," "python AND nidovirus," "turtle AND nidovirus," "lizard AND nidovirus," and "*Serpentovirinae*" OR "serpentovirus." Searches were conducted without limits on publication dates or geographical location. A total of 455 articles were identified and 391 remained once duplicates were removed. Additional articles were excluded if the study was not available in English, a reptile was not referred to throughout the article (only an author surname or Python software© or programming language), unable to access or non-relevant articles leaving 213 articles to be reviewed. Additional articles were found manually from the citations of relevant articles. A summary of the search terms and results can be found in **Figure 1**.

To identify key published *Serpentovirinae* spp. sequences, viruses identified by the International Committee on Taxonomy of Viruses (ICTV) ($n = 8$) were included (**Table 1**) as well as sequences where a substantial proportion of genome has been sequenced. Sequences were included if they had more than 10,000 base pairs (bp) and were sequenced from a reptile or reptile-associated sample ($n = 41$). The following search terms were used to identify these sequences; "*Tobaniviridae,*" "*Serpentovirinae,*" "unclassified *Nidovirales,*" "unclassified *Torovirinae,*" and "unclassified *Serpentovirinae*" (**Supplementary Table 1**). A phylogenetic tree of reptile nidovirus sequences ($n = 49$) and a remotovirus (bovine nidovirus) from the family *Tobaniviridae* can be found in **Figure 2**. The entirety of ORF 1b amino acids were aligned using Geneious Prime® (Version 2021.1.1) and based on these alignments maximum likelihood trees (PhyML) were calculated using the HKY85 substitution model and 1,000 bootstrap replicates. Following initial alignment, three sequences were removed (MK182569, MK722379, and MK722377) where they had 100% similarity to corresponding sequences that were included (MK182566, MK722366, and MK722376) leaving 47 sequences including bovine nidovirus (NC_027199) in the alignment (**Figure 2**).

TAXONOMY

Prior to 2019, the order *Nidovirales* was composed of four families: *Arteriviridae, Coronaviridae, Mesoniviridae,* and *Roniviridae* (36). At the time of their discovery, the group of reptilian nidoviruses clustered within a proposed new genus within the family *Coronaviridae* and subfamily *Torovirinae* (16, 18, 22). Recently, a new taxonomic nomenclature was approved by the ICTV. This revised taxonomy has resulted in 8 suborders, 14 families, 25 subfamilies, 39 genera, 65 subgenera and 109 species (35, 37). This reclassification has

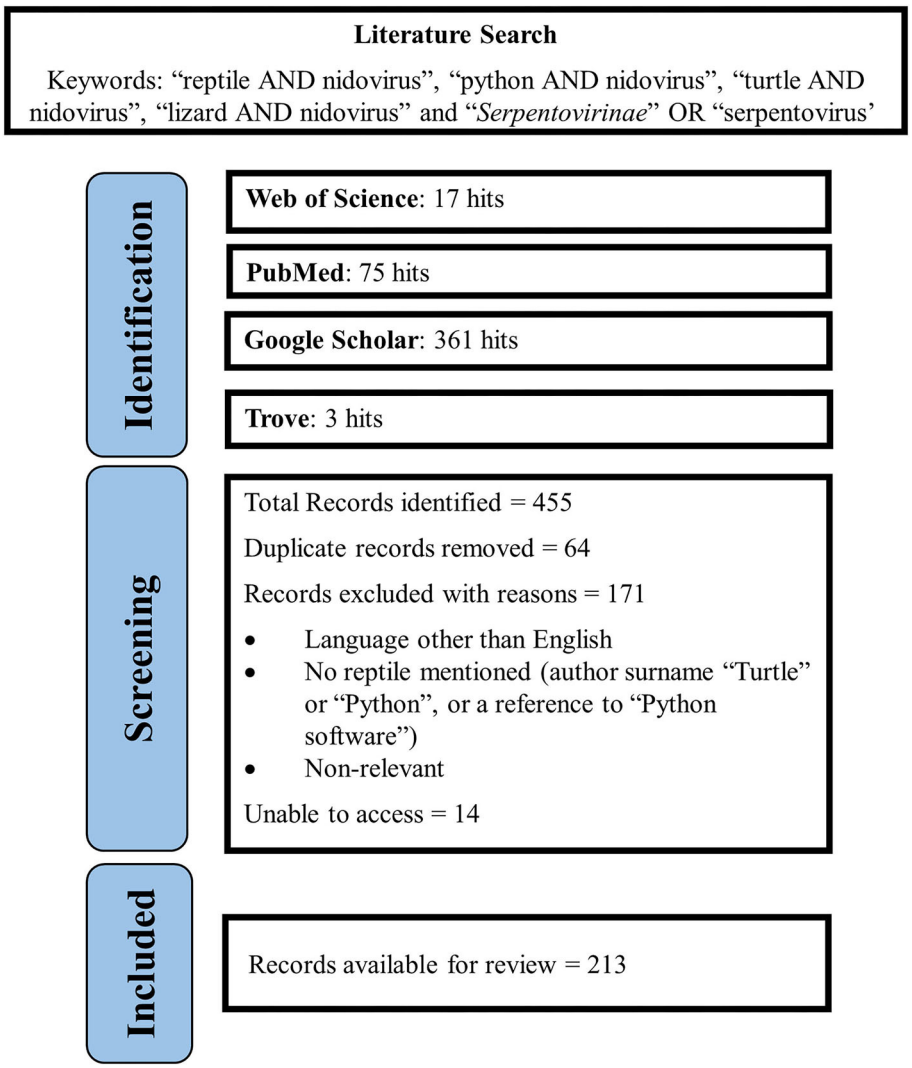


FIGURE 1 | Literature search methodology.

now placed reptile-associated nidoviruses within the suborder *Tornidovirineae* and the family *Tobaniviridae*. The nidoviruses discovered in snakes, turtles and lizards have been grouped into the subfamily *Serpentovirinae* (**Table 1**) (35). This has led to reptile-associated nidoviruses, potentially misleadingly, being referred to as "serpentoviruses" (20). Derived from the Latin word "serpēns," the term "serpent" is synonymous with "snake" for many people and it is important not to categorise these viruses as only "snake" viruses. With an expected increase in the discovery of novel reptile nidoviruses, there will likely be additional changes to classification in the future.

BIOLOGY OF THE VIRUS

Virus Morphology and Size

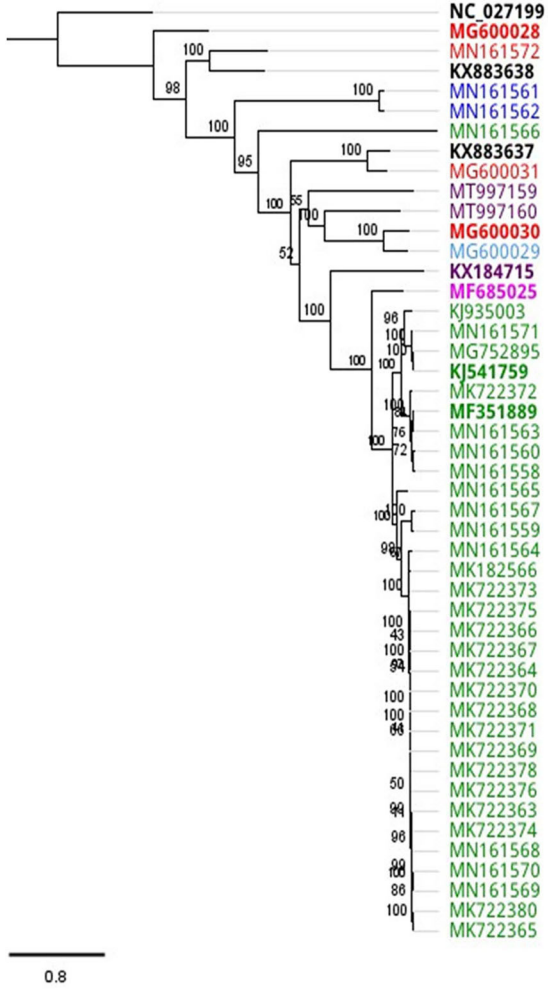
Nidovirales exhibit significant diversity in their morphology. The recently revised family *Tobaniviridae* includes both of the previously known genera *Torovirus* and *Bafinivirus*. When examined by electron microscopy, toroviruses are known to be pleomorphic with bacilliform (rod-shaped), kidney shaped, and spherical virions often observed, while bafiniviruses have a rod-like or bacilliform appearance. A distinctive feature of both the previously known toroviruses and bafiniviruses are the surface projections that correspond to the club or petal shaped projections found on the surface of coronaviruses (36, 38).

Within the *Serpentovirinae* subfamily, Stenglein et al. (16) identified ball python nidovirus (BPNV) particles within the pneumocytes of affected lung tissue. They were able to capture the pleomorphic appearance of the virus representing the various stages of viral replication. Mature bacillary virions measured 180 × 50 nm while the uncoated intracellular viral capsids measured 10–12 nm. The bacillary nucleocapsids contained a lucent core that was surrounded by fine granular cytoplasmic material presumed to be a component of the envelope. A cross section of a mature virion demonstrated a clear lipid

TABLE 1 | Viruses within the subfamily *Serpentovirinae* (35).

Genus	Subgenus	Species	Virus name	Accession	Genome coverage	Host	Country	Size (nt)	References
Pregotovirus	*Roypretovirus*	*Ball python nidovirus 1*	Ball python nidovirus (BPNV)	KJ541759	Complete genome	Ball python (*P.regius*)	USA, CHE	33, 452	(16)
		Morelia tobanivirus 1	Morelia viridis nidovirus (MVNV)	MF351889	Complete genome	Green tree python (*M. viridis*)		32, 399	(18)
	Snaturtovirus	*Berisnavirus 1*	Bellinger River virus (BRV)	MF685025	Complete genome	Bellinger River snapping turtle (*M. georgesi*)	AUS	30, 742	(22)
	Tilitovirus	*Shingleback nidovirus 1*	Shingleback nidovirus (SBNV)	KX184715	Partial genome	Shingleback lizard (*T. rugosa*)	AUS	23, 832	(21)
Sectovirus	*Sanematovirus*	*Sectovirus 1*	Xinzhou nematode virus 6	KX883637	Partial genome	Snake-associated nematodes mix Xinzhou [Nematoda spp. (14), Ascarididae spp. (2)]	CHN	25, 960	(13)
Infratovirus	*Hepoptovirus*	*Hebius tobanivirus 1*	Hainan hebius popei torovirus	MG600028	Complete coding genome	Pope's keelback (*H. popei*)	CHN	29, 409	(14)
	Xintolivirus	*Infratovirus 1*	Xinzhou toro-like virus	KX883638	Complete coding genome	Snake-associated nematodes mix Xinzhou [Nematoda spp. (14), *Ascarididae* spp. (2)]	CHN	30, 353	(13)
Lyctovirus	*Rebatovirus*	*Lycodon tobanivirus 1*	Guangdong red-banded snake-Lycodon rufozonatus-torovirus	MG600030	Complete coding genome	Red banded snake (*L. rufozonatus*)	CHN	30, 859	(14)

Country of origin: United States of America (USA), Switzerland (CHE), Australia (AUS), China (CHN).



	Host	Disease present	Country	Terrestrial or Aquatic	Population	Reference
NC_027199 ◄	***Bos taurus (Bovine nidovirus outgroup)***	Y	United States of America	Terrestrial	Captive	(Tokarz et al., 2015)
MG600028 ◄	***Pope's keelback (H. popei)***	NE	China	Semi-Aquatic	Wild	(Shi et al., 2018)
MN161572	*Honduran milk snake (L. t. hondurensis)*	N	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
KX883638 ◄	***Snake-associated nematodes***	NE	China	Terrestrial	Wild	(Shi et al., 2016)
MN161561	*Emerald tree boa (C. caninus)*	N	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161562	*Emerald tree boa (C. caninus)*	N	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161566	*Reticulated python (M. reticulatus)*	N	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
KX883637 ◄	***Snake-associated nematodes***	NE	China	Terrestrial	Wild	(Shi et al., 2016)
MG600031	*Mandarin rat snake (E. mandarinus)*	NE	China	Terrestrial	Wild	(Shi et al., 2018)
MT997159	*Veiled chameleon (C. calyptratus)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2020)
MT997160	*Veiled chameleon (C. calyptratus)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2020)
MG600030 ◄	***Red banded snake (L. rufozonatus)***	NE	China	Terrestrial	Wild	(Shi et al., 2018)
MG600029	*Chinese water snake (M. chinensis)*	NE	China	Semi-Aquatic	Wild	(Shi et al., 2018)
KX184715 ◄	***Shingleback lizard (T. rugosa)***	Y	Australia	Terrestrial	Wild	(O'Dea et al., 2016)
MF685025 ◄	***Bellinger River snapping turtle (M. georgesi)***	Y	Australia	Aquatic	Wild	(Zhang et al., 2018)
KJ935003	*Indian python (P.molorus)*	Y	Germany	Terrestrial	Captive	(Bodewes et al., 2014)
MN161571	*Woma python (A. ramsayi)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MG752895	*Ball python (P. regius)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2018)
KJ541759 ◄	***Ball python (P.regius)***	Y	United States of America	Terrestrial	Captive	(Stenglein et al., 2014)
MK722372	*Green tree python (M. viridis)*	Y	Spain	Terrestrial	Captive	(Dervas et al., 2020)
MF351889 ◄	***Green tree python (M. viridis)***	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2017)
MN161563	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161560	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161558	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161565	*Blood python (P. brongersmai)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161567	*Ball python (P. regius)*	N	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161559	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161564	*Blood python (P. brongersmai)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MK182566	*Green tree python (M. viridis)*	Y	Germany	Terrestrial	Captive	(Blahak et al., 2020)
MK722373	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722375	*Ball python (P. regius)*	NE	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722366	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722367	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722364	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722370	*Angolan python (P. anchietae)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722368	*Carpet python (M. spilota)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722371	*Ball python (P. regius)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722369	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722378	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722376	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722363	*Carpet python (M. spilota)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722374	*Ball python (P. regius)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MN161568	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161570	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MN161569	*Green tree python (M. viridis)*	Y	United States of America	Terrestrial	Captive	(Hoon-Hanks et al., 2019)
MK722380	*Woma python (A. ramsayi)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)
MK722365	*Green tree python (M. viridis)*	Y	Switzerland	Terrestrial	Captive	(Dervas et al., 2020)

FIGURE 2 | Phylogenetic tree of reptile nidovirus sequences with >10,000bp. The entirety of ORF 1b amino acid sequences were aligned. Following initial alignment three sequences were removed (MK182569, MK722379, and MK722377) where they had 100% similarity to corresponding sequences that were included (MK18256, MK722366, and MK722376), leaving 47 sequences (including bovine nidovirus (NC_027199) in the alignment. Genbank accession numbers are included in the tree alongside key features in the table. The current ICTV approved members of subfamily ***Serpentovirinae*** are **BOLD** with a "◄" to highlight them. The associated host is also in **BOLD**. The presence of disease is reported as Y = Yes, N = No, NE = Not Examined. For snakes, host families are indicated in the tree: Pythons (Pythonidae, green), Boas (Boidae, blue), Colubrids (Colubridae, red), and Homalopsid (Homalopsidae, light blue). The remaining sequences from reptiles including lizards (Chamaeleonidae and Scincidae, purple) and turtles (Chelidae, pink) are also coloured. The nematode associated sequences and the bovine nidovirus sequence remain uncoloured.

envelope and surface spikes within cytoplasmic vesicles (16). In cell culture supernatant, Bellinger River virus (BRV) virions were bacilliform in appearance (22). They measured 170 nm long while Morelia viridis nidovirus (MVNV) had both a rod and kidney shaped appearance, measuring ∼120 nm in length (100–150 nm) (18, 22). Both BPNV and MVNV bacillary particles have been observed as abundant tubular structures arranged in a stack formation within the cytoplasm of affected cells (16, 18). In contrast to the morphological descriptions available for the broader order *Nidovirales*, descriptions within the *Serpentovirinae* subfamily are similar, but limited to a few publications.

Genome (Size), Structure, and Protein Expression

Historically nidoviruses were grouped into small and large, with the small nidoviruses originating from a single family, *Arteriviridae*, with genomes of 12.7–15.7 kb in length. With the revised taxonomy, there are now three other families that have genomes <15 kb (35). Viruses belonging to the family *Tobaniviridae*, in the order *Nidovirales*, continue to be some of the largest RNA genomes known (1, 11). At the time of discovery, BPNV was the largest known RNA virus with a 33.5 kb viral genome, however in 2018, planarian secretory cell nidovirus (PSCNV), about 25% larger with a 41.1 kb genome, was discovered in a free-living flatworm (11). Despite the wide variation in genome size, the nidoviruses generally share a similar genome structure. They are typically made up of multiple open reading frames (ORFs), including overlapping ORF 1a and ORF 1b, and multiple ORFs at the 3′-end, which are flanked by both the 5′-end untranslated region (UTR) and the 3′-end UTR (1).

Nidoviruses in reptiles are known to share this genome structure, with two large overlapping 5′-end ORFs, replicase ORF 1a and 1b, with a ribosomal frameshift signal (-1, AAAAAC) (15–18, 21, 22). These encode two polyproteins: pp1a and pp1ab that are known to be involved in viral genome replication, expression and modulation of host cell activities (1, 39). The production of the large pp1ab is a key feature of nidoviruses, as is a set of functional subunits within this protein. Depending on the study and the amount of genome sequenced (partial or full), the pp1ab and most of the associated functional subunits have been identified in reptile nidoviruses (15, 16, 18, 21–23).

Reptile nidoviruses share the distinct replication strategy for viruses within the order *Nidovirales*. Named from the Latin word "nidus" for nest, the order is characterised by a nested set of viral subgenomic messenger RNA's that are produced during infection (1, 3, 40). In reptile nidoviruses, are several ORF's at the 3′-end coding for the "S" or spike glycoprotein (ORF 2), and other structural proteins including the transmembrane glycoproteins, matrix protein and nucleocapsid protein. These proteins are expressed from the subgenomic messenger RNA's (15–17, 22). With ongoing research into reptile nidoviruses we anticipate more studies will be published on the impact of variations of these proteins on viral pathogenesis or virulence.

Viral Diversity

Divergent nidoviruses have been discovered in different snake species. The initial reptile nidovirus sequences found in the ball python, *P. regius* (16, 17) and the Indian python, *P. molorus* (15) were relatively similar. Subsequent partial and full-length sequences that were detected in the python genus *Morelia spp.* were genetically different (18, 19). In 2017, a complete genome of MVNV was sequenced from a green tree python (*M. viridis*) in Switzerland. This sequence was <85% identical to BPNV (18). Additional sequences from *M. viridis* have continued to show diversity, with two complete genomes from Germany that share a 99.7% nucleotide identity with each other, but only 66.8 and 66.9% overall similarity to the original MVNV isolate from Switzerland (41).

Generally, virus sequences from Pythonidae (pythons) tend to cluster (**Figure 2**). To date, a clear host species-specific lineage is not evident. In one collection, virtually identical sequences (≥99.8% pairwise identity) were identified in three different snake genera within the family Pythonidae: *Morelia* spp., *Antaresia* spp., and *Python* spp. (20) suggesting similar viruses are capable of infecting multiple python species. Sequences found in Boidae (boas), Colubridae (colubrids), and Homalopsidae (mud snakes) are genetically different when compared to those found in Pythonidae (14, 19, 20). The exception are sequences found in reticulated pythons (*M. reticulatus*) that clustered with sequences found in boas, colubrids and mud snakes (**Figure 2**) (20).

In contrast, our knowledge of the reptile nidovirus diversity in lizards and turtles is limited to key publications with viruses detected from several reptile families including Scincidae (skinks), Chamaeleonidae (chameleons), and Chelidae (Austro-South American side-neck turtles) (21–23). Despite the apparent clustering of similar viruses in certain snake families, further research is required to determine if there are clear host specific nidovirus lineages, especially in the lesser studied species and wild reptile populations. It is likely our discovery of reptile nidovirus diversity is just beginning.

HOSTS AND GEOGRAPHIC DISTRIBUTION

Reptile nidoviruses have been detected worldwide. This includes detections on four continents, including North America, Europe, Asia, and Australia. They have also been found in multiple hosts from the orders Squamata (lizards and snakes) and Testudines (turtle, tortoise and terrapin).

Squamata (Lizards and Snakes)

The frequency of nidoviral detections in snake species has increased dramatically in recent years. This is largely a result of polymerase chain reaction (PCR) based surveys of captive snake species (19, 20, 41–43). To date, viruses have been discovered in several snake families including Pythonidae, Boidae, Colubridae, and Homalopsidae (14, 19, 20, 41). Nidoviral prevalence in captive python species has been reported to be as high as 27.4% (19), 30.7% (41) and 37.7% (20). Others have also reported differences in prevalence between species, with detections occurring more frequently in the green tree python (*M. viridis*) (32.2, 41.2, 75.8%) when compared with the ball

python (*P. regius*) (22.2, 22.1, 5.1%) (20, 41, 42). Infections are more frequent in pythons, however, sampling has been weighted toward Pythonidae species.

In contrast to Pythonidae, the prevalence in snakes from the Boidae and Colubridae families appears to be significantly less with 2.4, 10.1, and 0.8% reported in captive Boidae species (19, 20, 41) and 0.9% reported in captive Colubridae species (20). However, much lower sample numbers have been used to generate these values. It is also possible that genetically diverse nidoviruses in Boidae and Colubriae families may have been missed with the current assay designs. Metagenomic sequencing of negative samples could minimise this possibility (44). Only limited sampling of wild snake species has been undertaken and only as part of a large scale meta-transcriptomics survey to detect vertebrate associated RNA viruses (14). This survey identified key reptile nidovirus sequences found in snakes from the families Colubridae and Homalopsidae in China but did not report on prevalence or presence of disease. In the future, surveys to obtain an unbiased estimate of prevalence in captive and wild snake populations are required.

O'Dea et al. (21) first reported shingleback nidovirus 1 (SBNV) in Australian shingleback lizards (*Tiliqua rugosa*) admitted to a wildlife rehabilitation centre. The virus was present in lizards with and without respiratory disease. Ongoing surveillance at two wildlife rehabilitation centres found 58.1% of *T. rugosa* admitted to this facility (for a variety of reasons) gave positive results in an SBNV PCR (45). Surveys of wild shingleback populations (not those submitted to a care facility) have not been undertaken to date. Two novel nidoviruses have recently been described in a collection of veiled chameleons (*Chamaeleo calyptratus*) experiencing respiratory disease associated mortalities (23). The two genotypically distinct viruses were named veiled chameleon serpentovirus A (VCSV-A) and B (VCSV-B). Additional lizard species were housed in the same facility, including bearded dragons (*Pogona vitticeps*), common leopard geckos (*Eublepharis macularius*), and ocelot geckos (*Paroedura pictus*). These animals were clinically healthy throughout this period and gave negative results for VCSV by PCR (23). This could be due to a lack of exposure or resistance to VCSV infection. Opportunistic PCR based surveys of wild or captive lizards could provide insight into the range of lizard species susceptible to infection with reptile nidoviruses. Following on from this, similarly to snakes, random surveys to obtain unbiased estimates of prevalence in captive and wild snake populations are required.

Testudines (Turtles, Tortoises, and Terrapins)

The first report of a nidovirus, BRV, in a turtle was in 2015. It was detected in a wild population of freshwater turtles (*M. georgesi*), in a single river system, and was implicated in the mortality of over 400 turtles (22). This river system is home to many other species including reptiles, amphibians, arthropods, and fish. No morbidity or mortality was reported in any other species at the time, including the sympatric Murray River turtle (*Emydura macquarii*). Since 2015, there have been no confirmed clinical cases of BRV in wild *M. georgesi*, yet BRV was detected on conjunctival swabs from a small number of clinically normal *M. georgesi* (9 of 31 sampled) and *E. macquarii* (2 of 49 sampled) in a follow-up survey 6 months after the cessation of the outbreak (22). In addition, swabs ($n = 360$) from many reptiles, amphibians, arthropods, and fish failed to detect other species infected with BRV (22). Ongoing PCR based surveys to provide insight into the prevalence, incidence, and clinical outcomes of apparently asymptomatic individuals following this mortality event are required. Opportunistic sample collection associated with population monitoring surveys presents a cost-effective option for infectious disease detection and monitoring and could be considered for other reptile species (46, 47).

Other Species

Closely related nidoviruses have been detected in snake-associated nematodes as part of a large scale metagenomic screening of vertebrate and invertebrate samples (13, 14). The significance of these detections remains uncertain. The nematodes may have ingested or been contaminated with a nidovirus infecting the snake. While unlikely, the possibility of a recent horizontal transfer between species cannot be excluded (48). Additional testing of nematodes is required to clarify their susceptibility to nidovirus infection.

CLINICAL SIGNS, PATHOLOGY, AND TISSUE TROPISM

Clinical Signs

Nidoviruses are known for causing respiratory and enteric disease in terrestrial vertebrates (49). This is true for viruses infecting cattle, horses, chickens, and pigs (6, 50–52). In aquatic animals, while not as well-studied only some nidoviruses appear to follow this trend (12, 53, 54). To provide an overview, the clinical signs reported to be associated with reptile nidovirus infections are summarised in **Table 2**. Clinical signs appear in order from the most reported to the least reported across species from key reptile nidovirus publications.

In reptiles, clinical signs associated with infection of the respiratory tract appear to be the most common feature of nidovirus infection (15, 16, 18, 21, 41, 55). Initial clinical signs in captive pythons include increased amounts of clear or mucoid material in the nose and mouth and oral inflammation (stomatitis). This proceeds to wheezing, open mouth breathing, increased respiratory rate, or coughing. Additional clinical signs include inappetence, weight loss, lethargy, dehydration, inappropriate skin shedding, difficulty perching in arboreal snakes, and speculitis (20, 56). In some cases, a respiratory syndrome characterised by severe acute pneumonia and sudden death has occurred (15–18, 58). In a PCR based nidovirus survey of captive snakes, clinical signs of respiratory disease were more common in infected pythons (85 of 144) compared to boas (1 of 8) (20). This may suggest differences in species susceptibility or differences in nidovirus virulence.

A single study has confirmed Koch's postulates using a reptile nidovirus and described the clinical signs observed following infection. In 2018, three captive bred ball python (*P. regius*) juveniles (∼6 weeks old) were exposed orally and intratracheally

TABLE 2 | Clinical signs associated with nidoviruses in reptiles.

Clinical sign	Squamata									Testudines	References
Secretion from oral cavity of clear, foamy, mucoid or mucopurulent material	✓	✓	✓	✓	✓	✓	✓	✓	✓		(16, 18, 20, 21, 23, 41, 55–57)
Found dead or sudden death	✓	✓	✓	✓	✓	✓		✓		✓	(15–18, 22, 23, 57, 58)
Dyspnea or open mouth breathing including increased respiratory rate (respiratory distress)	✓	✓	✓	✓	✓	✓		✓			(16, 18, 20, 23, 41, 55, 57)
Hyperaemia or inflammation of mucous membranes in the oral cavity	✓	✓	✓	✓	✓	✓					(16, 17, 20, 41, 55, 59)
Anorexia or inappetence	✓	✓	✓	✓	✓			✓			(16, 18, 20, 23, 55, 57)
Audible breathing or wheezing	✓	✓	✓					✓			(16, 20, 23, 56)
Secretion of clear, foamy, mucoid or mucopurulent material from nasal passages	✓	✓						✓		✓	(20–22, 57, 60)
Poor body condition or weight loss	✓							✓	✓	✓	(21–23, 56)
Notable “cough-like” forced expiration	✓	✓									(16, 20)
Ocular discharge (clear to mucopurulent)								✓	✓		(21, 45)
Lethargy or depression								✓		✓	(21, 22)
Expulsion of mucus	✓										(18)
Excessive swallowing	✓										(55)
Ventral oral swelling	✓										(55)
Spectaculitis	✓										(20)

(Continued)

TABLE 2 | Continued

Clinical sign	Squamata	Testudines	References
Petechiations (small oral mucosal haemorrhages)			(55)
Opisthotonos (star gazing)			(16)
Emaciation			(18)
Inappropriate shedding			(20)
Difficulty perching in arboreal snakes			(20)
Bilateral crusting of the eyes			(23)
Sunken eyes			(23)
Pale mucous membranes			(21)
Sneezing			(21)
Vertical head tilt			(23)
Reduced water intake			(23)
Severe bilateral ocular inflammation			(22)
Hindlimb paresis			(60)
Tan foci on skin of ventral thighs			(22)

The clinical signs associated with nidovirus infected reptiles reported from key reptile nidovirus publications are included. Included publications discovered or conducted research on reptile nidoviruses specifically. Host species are classified as Squamata; snakes (), lizards (), and Testudines; turtles (). A single publication where "pneumonia" was described as a clinical sign, has been included under "Dyspnea or open mouth breathing including increased respiratory rate (respiratory distress)" which likely would have been observed clinically (41). Clinical signs appear in order from the most reported across species to the least reported.

with a cell culture grown BPNV. Clinical signs were observed 4 weeks after exposure and included mucosal hyperaemia and profuse mucus secretion, followed by a progression to the appearance of petechial mucosal haemorrhages, open mouth breathing, and anorexia by 10–12 weeks post exposure. The presence of infectious virus was confirmed using virus isolation from oroesophageal swabs taken on the day of euthanasia; 5, 10, and 12 weeks post exposure (55).

Australian shingleback lizards (*T. rugosa*) infected with SBNV can also present with respiratory disease. Similarly to *P. regius*, a respiratory syndrome has been observed since the 1990's that is characterised by excess mucous in the oral cavity, sneezing, serous to mucopurulent discharge from the eyes and nose, lethargy, inappetence, pale mucous membranes, depression, and emaciation (21). In captive bred chameleons (*C. calyptratus*) infected with VCSV, clinical signs included wheezing, vertical head tilting with gasping, increased mucus in the oral cavity, anorexia, and reduced water intake (23). To date, experimental infection studies to confirm the role of nidovirus in the development of respiratory disease has not been described in lizards.

Bellinger River snapping turtles (*M. georgesi*) infected with BRV were largely found as dead or moribund animals with bilateral ocular inflammation, poor body condition, and some had tan foci on the skin of the ventral thighs or hind limb paresis (22). Many also had a slight clear nasal discharge, and some animals had hindlimb paresis (60). In contrast to other species, respiratory disease was not the dominant syndrome observed. As with lizards, experimental infection trials have not yet been completed.

Pathology and Tissue Tropism

Most pathology associated with nidoviral infection in snakes has been associated with the respiratory tract, and to a lesser extent the oral cavity and upper alimentary tract. The tropism for respiratory epithelium has also been confirmed using *in situ* hybridisation (ISH) (15, 18). Experimental infection of *P. regius* resulted in histological findings consistent with a chronic-active mucinous rhinitis, stomatitis, tracheitis, oesophagitis, and proliferative interstitial pneumonia (55). The proliferative interstitial pneumonia has been a consistent finding in clinical cases of nidovirus infection in snakes and has more recently been called "nidovirus associated proliferative disease—NPD" (16, 57).

Consistent with this pathology, the viral load is often highest in the lung tissue. However, the viral load in the intestine has also been reported at similar levels (16). This finding is consistent with other closely related viruses, namely coronaviruses and toroviruses, where respiratory and enteric tropism has been well-established (4, 61, 62). In green tree pythons (*M. viridis*) high viral loads have also been confirmed in the lung, but in contrast to the findings of Stenglein et al. (16) intestinal samples were mostly negative by PCR or negative using ISH (18, 41). This finding could be reflective of differences in tropism between reptile nidoviruses, susceptibility of different host species, or a function of the time of exposure and disease progression.

Since their discovery, nidoviruses have been reported in tissues other than the respiratory tract but their presence and pathology have been inconsistently examined and reported. Stenglein et al. (16) identified virus in liver, kidney, heart, spleen, and brain, but largely at levels 3–5 orders of magnitude lower than the lung. More recently, during multiple necropsies ($n = 30$) Dervas et al. (57) identified pyogranulomatous and fibrinonecrotic lesions in organ systems aside from the respiratory tract suggesting a much broader cell and tissue tropism. Virus was also detected in epithelial cells (alimentary, hepatic, renal, pancreatic), intravascular monocytes, intralesional macrophages, and endothelial cells (57). Dervas et al. (57) also identified animals with evidence of disseminated granulomatous and/or fibrinonecrotic lesions, vascular and perivascular lesions, and infected monocytes. These lesions were more predominant in *Morelia* spp. (57).

The suggestion of a broad cell and tissue tropism is consistent with the pathology caused by BRV in *M. georgesi*. There was histological evidence of fibrinonecrotising splenitis and nephritis with multisystemic fibrinoid vasculopathy (22, 60). Using ISH, BRV was detected in glandular epithelial cells, in areas of necrotising inflammation within the lacrimal gland, in degenerate or necrotic renal tubule epithelial cells, and in foci of vasculitis. Virus was also detected in necrotizing lesions in the urinary bladder, scattered granulocytes in the oedematous urothelium, and occasional granulocytes within the myocardial interstitium (22).

To date, no histology has been reported following infection of shingleback lizards (*T. rugosa*) with SBNV. However, a single veiled chameleon (*C. calyptratus*) coinfected with VCSV-A and VCSV-B had both respiratory symptoms and histological lung lesions like those identified in snakes. This included an interstitial proliferative and catarrhal pneumonia, rhinitis, and tracheitis (23). Interestingly, in this collection other VCSV infected veiled chameleons examined had no histologic lesions ($n = 3$) or mild non-specific lesions ($n = 3$) including focal xanthomatous mural enteritis with coelomic foreign body, severe heterophilic enteritis with mural granulomas, splenic lymphoid hyperplasia, mild lymphocytic portal hepatitis, rare mineralization of the tunica intima of large cardiac vessels, and hepatocellular vacuolization (23).

Transmission studies with reptile nidoviruses are scarce, with only a single experimental infection trial using BPNV and a small number of juvenile ball pythons (*P. regius*) (55). Subsequent studies have identified a statistically significant association between age and infection status, reporting that in a survey of captive snakes, older snakes were more likely to be infected, but that increasing age did not increase the likelihood of disease (20). This finding highlights the need for additional transmission trials to explore the "triad" of disease determinants: host, agent, and environment (63). For example, these trials could examine the impact of viruses from different backgrounds (cell culture amplified, passage level, strains), various host factors (age, species), routes of exposure, environmental temperatures, and the duration of the trial. This will allow for further assessment of the general pathology associated with nidovirus infections in reptiles. In the absence of this, we are left to make judgements of pathogenesis based on antemortem (clinical signs) and post-mortem findings of naturally infected animals.

Asymptomatic Infection

The detection of a virus or nucleic acid in an animal without clinical signs could be a result of testing during the incubation period, the animal having recovered but continuing to be a carrier, or that some individuals remain asymptomatic following infection. The possibility of superficial contamination, ingestion, or inhalation of nucleic acid from the environment without active virus replication must also be considered. There is evidence that reptiles can be asymptomatic when infected with nidoviruses. Detections have been reported in snakes, lizards and turtles in the absence of clinical disease. In one survey of captive snakes, signs of respiratory disease were only identified in 59% (85 of 144) of infected pythons, 12.5% (1 of 8) of infected boas, and were absent in a single infected colubrid (20). Another survey that examined a large number of pythons that had given positive results in a PCR found only 17.8% (67 of 377) had stomatitis and/or respiratory disease (41) and a smaller survey in Poland found only 23.1% (3 of 13) of nidovirus positive pythons had respiratory disease at the time of testing (43). There is also some evidence that animals may remain infected and asymptomatic for prolonged periods. Five pythons (*Morelia spp.*) that were nidovirus PCR positive for over 2 years with serial testing at ∼4 month intervals remained asymptomatic for the duration (20).

Asymptomatic infection has also been observed in shingleback lizards (*T. rugosa*) infected with SBNV. In animals presenting to a wildlife care facility, SBNV was detected by qRT-PCR in 12% of apparently healthy individuals (4 of 33) (21). Similarly, virus was detected on oral/choanal swabs (5 of 6) from "healthy" adult and subadult VCSV PCR positive chameleons (*C. calyptratus*) that did not develop respiratory disease over a 3 month period of monitoring prior to euthanasia (23). In freshwater turtles (*M. georgesi*) an intensive field survey was undertaken 6 months after the cessation of the initial BRV outbreak and BRV was detected by qRT-PCR in 29% (9 of 31) of apparently healthy individuals (22). It is likely as a result of ongoing development of targeted diagnostics, increased accessibility and affordability of NGS and a "virus exploration" approach (14) that additional nidoviruses will be detected in reptile species without clinical disease. The ongoing challenge will be to determine the clinical significance of these viruses unless detections are associated with investigations of natural outbreaks of disease or experimental infection trials are undertaken.

CO-INFECTIONS

A co-infection, defined as more than one pathogen infecting an individual, is not uncommon in reptiles (64). To date coinfections with bacteria (15, 17, 55, 59), parasites (21, 45), and other viruses with nidoviral infection including snake retroviruses (18, 41) and an orthoreovirus (23) have been reported. Reptiles are known to harbour a wide range of normal resident microflora that can vary with the reptile species and the anatomical area of interest (65, 66). Therefore, interpretation of culture results, especially of the upper respiratory tract, must consider both the sampling methods, the clinical condition of the individual and evidence of associated pathology. Gramme-negative bacteria are commonly cultured from reptiles with acute or chronic respiratory disease but also from healthy animals (67). However, bacterial colonisation of the lower respiratory tract would be generally be considered a finding of significance (68). There is some evidence that secondary bacterial infections can contribute to the severity and clinical progression of reptile nidovirus infections (20). However, the interaction between nidoviral infection and other opportunistic pathogens, and the potential impact on morbidity and mortality, is an area for further investigation.

Genetically divergent reptile nidoviruses have also been identified in a single animal. Two reptile nidovirus sequences that shared only 71% global nucleotide identity have been reported in a python (20). A similar finding has also been reported in a veiled chameleon (*C. calyptratus*). These viruses only shared 53% nucleotide identity of ORF 1b, which is considered the most conserved region of the genome (23). Both the python and chameleon infected with more than one nidovirus died while exhibiting signs of respiratory disease. Nevertheless, in general, the clinical significance of infection with more than one nidovirus in reptiles remains largely uncertain.

TRANSMISSION

The natural route(s) of transmission of nidoviruses in reptiles remains unclear. The successful infection of several *P. regius* with BPNV was achieved following oral and upper respiratory tract exposure (55). Subsequently, virus was detected in oral secretions and faeces of exposed animals. Multiple transmission routes are possible, including faecal-oral, fomite, and aerosolization. This is supported by detection of virus in respiratory epithelium, tissues of the gastrointestinal tract and on various antemortem swabs (18, 22). In reptiles generally, additional transmission trials are needed to provide further insights into the possible mechanisms of horizontal transmission.

Vertical transmission has been reported with nidoviruses in other animal species including porcine reproductive and respiratory syndrome (PRRS), equine arteritis virus (EAV), and gill-associated virus (GAV) (69–71). In a limited capacity, vertical transmission has been investigated by testing the eggs ($n = 26$) and hatchlings ($n = 18$) of nidovirus positive python mating pairs. Eggs were "cleaned" by exposure to UV irradiation or a quaternary ammonium disinfectant and artificially incubated. Despite virus being detected in/on most eggs following hatching only a single offspring became infected. This infection was detected when the offspring was 8–12 months of age and had been sampled at 4-monthly intervals. The viral sequence was more similar to the infected male (>98%), than the female (84%) of the breeding pair raising the question of whether there had been true vertical transmission (20). To date, vertical transmission has not been explored under "natural" conditions.

LABORATORY DIAGNOSIS

The diagnostic options currently available for the detection of reptile nidovirus infections largely reflect the methodology

used for their discovery. To date, most methods of detection are directed at the detection of virus or its components with next generation sequencing (NGS) laying the foundation for the development of both real time and conventional reverse transcription polymerase chain reaction (RT-PCR) assays. Such molecular assays are the predominant diagnostic tool available. Other diagnostic tools include transmission electron microscopy (TEM), virus isolation in cell culture, immunohistochemistry (IHC), and *in situ* hybridisation (ISH). There are currently no assays available for the detection of nidovirus specific antibodies. Given the recent discovery of nidoviruses in reptiles there is limited validation, or standardisation of diagnostic methods, offering opportunities for future research.

Next Generation Sequencing

NGS and viral metagenomics have been used to detect novel reptile nidoviruses from a range of samples including fresh tissues (lung, trachea, oral mucosa, oesophagus, spleen, liver, kidney, gastrointestinal tract, whole snake associated nematodes) and swabs in viral transport media (oral swabs). With advances in technology, partly as a result of the difficulties when undertaking virus isolation, most sequencing occurs on nucleic acid extracted directly from a tissue sample or swab (13, 15–17), with few instances where the sequencing was undertaken on tissue culture fluid following successful virus isolation (18, 22). NGS results have been infrequently confirmed with additional sequencing as a method of validating sequence assembly (16, 22).

Polymerase Chain Reaction

Both conventional and quantitative reverse-transcription (qRT-PCR) assays have been used to detect reptile nidoviruses. To detect virus, PCR offers several advantages when compared to TEM, virus isolation in cell culture and ISH. This includes fast results and a high analytical sensitivity and, usually, specificity. One of the advantages of NGS and molecular assays such as PCR is that new assays can be designed and first evaluated *in silico* to optimise performance before being applied to routine testing. In addition to qualitative (positive/negative) results, quantitative PCR assays also provide insights into the amount of virus present which can be used to develop associations between the virus detected and a possible role as the cause of a disease process (16, 18, 22). For juvenile pythons experimentally infected with BPNV, viral loads monitored by qRT-PCR increased for the duration of the trial with a more significant increase observed at 4 weeks post exposure (55), confirming active virus replication.

Most PCR assays target the most conserved region of the virus, ORF 1a or ORF 1b, or the more variable region encoding for the spike protein (16, 19, 20, 22, 41, 55, 59). As we improve our understanding of reptile nidoviral diversity, the design of PCR assays may move toward being more broadly reactive to ensure the spectrum of different reptile nidoviruses is initially detected. Subsequently, these should be followed by the use of more specific assays to detect individual viruses. Conversely, such broadly reactive assays can sometimes have reduced sensitivity when compared to virus specific assays. Therefore, assay selection will be influenced by the question at hand, and screening with both broad and specific assays may be necessary.

Several ante-mortem sample types including swabs, tracheal washes, faeces, and blood samples have been successfully used for PCR (41, 55). For snakes, swabs include choanal, oral/oesophageal, and cloacal (55). For lizards, oral swabs have been used from shinglebacks and chameleons (21, 23). For turtles conjunctival, oral, and cloacal swabs have been used (22). Little is known regarding the occurrence, onset, and duration of viraemia in reptiles infected with nidoviruses. Initial largescale antemortem surveys in snakes suggest that detecting virus in blood samples is not as sensitive as detecting virus on antemortem swabs. Consequently, blood should not be used as a preferred PCR sample type (19). A number of fresh tissues collected at post-mortem have been used to detect nidoviruses including the trachea, oesophagus, lung, liver, kidney, heart, spleen, stomach, small and large intestine, bladder, brain, eye, and ovary (17, 22, 55, 56).

The long duration of infection and viral shedding that is apparent in reptiles infected with nidoviruses facilitates the application of PCR based virus detection and surveillance. A longitudinal survey of pythons in a single collection over 28 months revealed that infection with a nidovirus can be chronic and definitive evidence of viral clearance was not observed (20). However, negative results should be considered carefully as they may reflect poor swabbing technique, sample handling, a period of low or interrupted viral shedding, the limit of detection of the assay or clearance of the virus. To control for the negative impacts of suboptimal swabbing technique and reduced efficiency of RNA extraction, an internal control to detect host DNA could be considered. However, there can be challenges associated with the selection of a host DNA target when testing a diversity of animal species. Alternative and perhaps preferred options that control for sample processing and PCR workflow issues include spiking of extraction solutions with an irrelevant/exogenous internal positive control RNA (72, 73). In practical terms, generating confidence in an individual animal's status can be effectively improved with serial sampling, which can be readily achieved by using qRT-PCR.

Transmission Electron Microscopy

This technology has been responsible for the detection of many viruses either in suspension or in sections of tissue. Although it has been in use for many decades, TEM finds merit in situations where currently available assays do not allow visualisation of virus morphology, tissue tropism, intracellular events associated with virus replication, assembly and release from cells. Consequently, TEM is a valuable tool for pathogenesis studies (74, 75). Transmission electron microscopy has been used to identify the unique morphology of nidoviruses within pulmonary epithelial cells (16) and in cell culture supernatants (18, 22). Visualisation of the size and shape of virion particles, including the presence of a lipid envelope decorated with spikes, can also guide the subsequent selection of diagnostic assays, but the technology does lack sensitivity. Many opportunities remain to visualise and describe other reptile nidoviruses.

Virus Isolation

Although considered a "gold standard" for the laboratory diagnosis of viral diseases, when compared to options available for the testing of mammalian species, the choice of established continuous reptile cell lines is limited. Furthermore, consideration must be given to the cultural requirements, especially temperature, for both cell lines and virus replication due to the poikilothermic nature of reptiles (76). However, for reptile nidoviruses, a range of both continuous cell lines and primary cell cultures have been successfully used to isolate viruses.

Stenglein et al. (16) attempted unsuccessfully to isolate BPNV from frozen infected tissues in several snake cell lines including the boa constrictor kidney (boa constrictor JK) and viper heart (viper VSW and VH-2) cell lines (16) but Hoon-Hanks et al. (55) were subsequently able to isolate BPNV in a primary diamond python (*Morelia spilota spilota)* cell culture. Cultures were inoculated with viral transport media from an oral swab collected from a *P. regius* and maintained at 30°C (55). Dervas et al. (18) successfully isolated MVNV using primary cultures of green tree python (*M. viridis*) liver and brain cells. Cultures were inoculated with homogenates of lung tissue from diseased snakes and maintained at 30°C (18). This isolate was then inoculated onto selected brain, kidney and lung cell cultures of a *Boa constrictor* to assess susceptibility and obtain an isolate free of a retrovirus contaminant. Convincing virus replication was identified in the kidney and lung cell cultures. The infected cells were also stained using anti-MVNV nucleoprotein (N protein) anti-serum at 3 days post inoculation, with all cell types except brain cells shown to be permissive for MVNV (18). Blahak et al. (41) attempted unsuccessfully to isolate virus from green tree pythons (*M. viridis*) using suspensions of liver, lung, kidney and intestine inoculated onto viper heart cells (VH-2) at 29°C (41).

Isolation of reptile nidoviruses from lizards was attempted unsuccessfully using *Boa constrictor* kidney and diamond python (*Morelia spilota spilota)* heart cell cultures, and two cell lines: iguana heart (IgH2) and viper heart (VH-2). Cultures were inoculated with fresh-frozen tissue homogenate (oral mucosa, lung, trachea) from a single chameleon (C. *calyptratus*) and maintained at 30°C (23). In freshwater turtles BRV was successfully isolated from pooled homogenates of spleen and lung tissue from freshwater turtles. Despite attempts using hamster lung (HmLu-1), avian (CEF), fish (SB, FHM, SSN-1), reptile (VH-2), and mosquito (C6/36) cell lines, BRV was successfully isolated, perhaps unexpectedly, using monkey kidney cells (CV-1, BGM, and Vero) maintained at 25°C (22).

The successful use of both primary and continuous cell cultures to isolate reptile nidoviruses highlights the opportunities to explore the susceptibility various cell cultures to infection with reptile nidoviruses. The benefits of producing an isolate are numerous, including easier identification and characterisation of a virus, differentiation between viable and non-viable virus, and production of high concentrations of material to facilitate nucleic acid sequencing and to underpin transmission studies. Unfortunately, generating primary cell cultures is going to depend on the capacity and interest of individual researchers to develop primary cell cultures for their species of interest or explore the suitability of a wide range of established cell lines.

Immunohistochemistry and *in situ* Hybridisation

IHC and ISH offer unique opportunities to demonstrate viral proteins or RNA within the observed pathology or tissues of interest. IHC has been successfully used to visualise reptile nidoviral proteins in affected tissues. Dervas et al. (18) produced a polyclonal rabbit antibody by immunising a rabbit with a purified recombinant nucleoprotein of MVNV to demonstrate the nidovirus N protein in tissue sections of lung and trachea (18).

ISH has also been used to confirm the presence of nidoviral RNA in lung lesions (15, 18). Bodowes et al. (15) targeted the RNA-directed RNA polymerase (RdRp) gene to detect virus in viable and degenerate respiratory epithelial cells of the trachea and pharynx but not any other tissues from the python (15). Dervas et al. (18) performed ISH on the lungs of all affected snakes plus all major organs or tissues from five affected animals. In respiratory tissues virus was detected in the cytoplasm of pneumocytes lining the faveolar space and degenerated tracheal and nasopharyngeal epithelium. Viral RNA was also found within a few macrophages in the focal granulomatous-necrotising nephritis of a single snake but was not detected in other tissues including the stomach or intestines of infected snakes (18). In freshwater turtles, ISH was used to detect the gene encoding for the membrane protein (M) of BRV on a selection of tissues including in areas of necrotizing inflammation within the lacrimal gland, residual glandular epithelial cells, degenerate, or necrotic renal tubule epithelial cells and a foci of vasculitis. Viral RNA was also found in a dense focus of necrotizing cystitis, in scattered granulocytes in the urothelium and occasional granulocytes in the myocardial interstitium (22). When investigating disease outbreaks or undertaking pathogenesis studies the direct detection of viral RNA or antigens adds weight toward establishing the role of a new virus in the pathology observed (77). However, these methods depend on the availability or production of specific reagents, particularly antisera and labelled probes. In the absence of a virus isolate, to develop an IHC capability, nucleic acid sequence data is needed firstly for cloning to produce antigens using recombinant DNA technology and then the immunisation of animals to produce either polyclonal or monoclonal antibodies. ISH also depends entirely on the availability of nucleic sequence data but the development and synthesis of probes is much more prescriptive than the development of reagents for IHC.

Serology

Serology often provides the missing link in disease investigations or establishing epidemiological patterns of disease. However, suitable tests are dependent on the nature and quality of the host immune response. Despite the diversity in reptiles, the study of the reptile immune system has generated one common conclusion: each taxa's immune response consists of a strong, broad, innate response, followed by a moderate specific immune response (78–80). Serological assays rely on the capacity of the host to develop and produce a detectable antibody

response which may vary with a specific pathogen and reptile species (81). It can also be affected by other factors including temperature, reproductive status, seasonality, and stress or cortisone levels (78, 80). Serological assays have been developed for various tobaniviruses affecting cattle, pigs and horses (82, 83). However, there are no published serological assays for reptile nidoviruses. Serological assays have been developed for several other pathogens in reptiles with mixed success. Commonly used assay designs include virus neutralisation tests (VNTs) (84, 85), haemagglutination inhibition assays (HI) (86, 87), and enzyme-linked immunosorbent assays (ELISAs) (88, 89) although other designs have also been used.

A common difficulty in the development ELISA and similar assays is the limited availability of either broadly reactive or species-specific anti-reptile immunoglobulins, leaving researchers to develop reagents for their species of interest (90, 91) or design assays to avoid the requirement of such reagents. HI tests and VNTs do not suffer from these limitations but usually require whole virus and for VNTs appropriate cell cultures as well as an isolate. Current laboratory diagnosis of reptile nidoviruses relies heavily on PCR based detection, which is useful in detecting acute, chronic, or persistently infected animals, yet detecting antibodies as a means of indicating previous exposure remains a significant knowledge gap. The development of a serological assay can also complement a transmission trial through the detection of a humoral immune response to the virus. Intermittent viral shedding and varying viral loads in naturally infected animals (20) also highlights the need for a serological assay, despite the likely challenges, to provide alternative diagnostic approaches, especially for live animals.

MANAGEMENT OF REPTILE NIDOVIRUS INFECTION

Mortality rates in nidovirus infected reptiles can be significant. In a single collection of captive pythons, 75% (30 of 40) of infected animals died over a 28 month period (20) and there is strong indirect evidence BRV was associated with a significant population decline in an endangered turtle population (22). This decline is estimated to be more than 90% using population estimates generated years prior to the mortality event (47, 92, 93), however the population size of *M. georgesi* immediately prior to the outbreak is unknown. Specific antiviral treatments for nidovirus infected reptiles have not yet been reported and evidence to support other therapeutic treatments is limited. Reptiles are known to harbour a large range of normal microflora (64, 65, 94), and their role as a primary or secondary pathogen as part of a multifactorial respiratory syndrome can provide a target for supportive treatments (95). Antimicrobials, antifungals, antiprotozoals, anti-inflammatories, immunomodulators (e.g., parapox ovis virus immunomodulator) and supportive care (hydration, nebulization) have been used, however definitive peer-reviewed studies on the efficacy of various treatments has not been undertaken to date (16, 20, 21, 41, 56). Adequate light and heat are also fundamental aspects of supportive care known to impact on the overall health and immune response of reptiles (81). Furthermore, the factors that contribute to disease and the long-term survival following recovery from acute infection offers an opportunity for further investigation in both captive and wild reptile populations.

Management practises that limit the introduction and transmission of a reptile nidovirus in captive collections are consistent with general recommendations for hygiene and biosecurity in all facilities that house reptiles. Quarantining new animals from collection for a designated period to enable appropriate health cheques and screening of pathogens is strongly recommended (96). The duration of this period is often debated and is influenced by the knowledge of pathogens for that species (96). In light of the apparent long duration of reptile nidovirus infection in snakes (20) and as research continues into the field of reptile nidoviruses infecting lizards and turtles recommendations for different species may need to be revised. To date, effective strategies that have successfully prevented infection rates rising in a captive snake collection include quarantine of new or infected individuals and a separate caretaker, clothes, equipment, and separate ventilation for infected snakes. Additional measures included shower-out procedures, one-way flow of food and bedding, changing of disposable gloves between groups or species, hand sanitizer disinfection of gloves between breed rotations in racks, and disinfection of all surfaces and instruments following use (20). Management of reptile nidovirus infections in wild reptile populations has not yet been explored. In the absence of such specific data or proven recommendations, appropriate general biosecurity practises at national and international levels should be implemented (97, 98).

DISCUSSION

A review of nidoviruses in reptiles reveals a relatively new field of research. The literature is dominated by the detection of novel nucleic acid sequences that broaden our understanding of viral diversity in reptiles. Opportunities exist to further our understanding of this diversity, especially in the lesser studied species by opportunistic screening of samples from disease investigations, or PCR based surveys of wild reptile species. There also remains exciting knowledge gaps to fill, especially in the linking of novel sequences to clinical disease and pathology through transmission trials and fulfilment of Koch's postulates in various species. This will also explore the complexities of the likely routes of transmission. As more sequences are detected further research into determining if there are clear host-specific lineages, and the apparent resistance of different reptile species to infection will provide additional insights into host susceptibility. Opportunities to explore the impact of reptile nidovirus genotypes and co-infections on pathogenesis or virulence are also numerous.

Improvements in sequencing technology and analysis underpin the rapid development of targeted PCR assays. However, given the diversity of viruses detected in different species there may be significant advantages in moving toward the initial use of assays that are more broadly reactive to ensure different reptile nidoviruses or strains with minor genetic

variation are not missed. The apparent long duration of nidoviral infection and shedding in snake species facilitates the use of PCR based monitoring. Conversely, asymptomatic infections, intermittent shedding, and varying viral loads, highlight the need for a serological assay to provide alternative diagnostic and surveillance approaches, especially for live animals. However, the development of such assays may prove challenging.

The study of nidoviruses in reptile populations has been focused on research in captive populations. The nature of captive reptile collections (the acquisition of wild and exchange of captive reptiles) can provide an opportunity for the introduction of both known and unknown pathogens. High holding densities and high rates of transfer between collections can potentially increase pathogen exposure and lower barriers to transmission (16). Furthermore, the escape or intentional release from captivity, especially of invasive species, could provide the opportunity for a pathogen to enter a naïve wild reptile population and cause significant mortality and morbidity. The illegal international trade of rare, unique and/or range restricted species also risks the introduction of both known or unknown pathogens into naïve reptile populations (99).

Unfortunately, the prevalence and distribution of nidoviruses in wild reptile populations is still largely unknown. In the midst of a global SARS-CoV-2 pandemic, the importance of understanding wildlife disease not only for the species involved but also for the potential public health implications, is readily apparent. By their nature, detecting emerging or novel pathogens is difficult, yet with advancing detection methods it is imperative that research into the extent and distribution of reptile nidoviruses continues so that we preserve the priceless biodiversity and critical reptile populations worldwide.

AUTHOR CONTRIBUTIONS

KP and LS conceived and designed this review. KP wrote the manuscript, analysed the data, and prepared figures and tables. EA, PK, and LS contributed to the concept and reviewed drafts of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This review was supported by the College of Public Health, Medical, and Veterinary Sciences Higher Degree Research Enhancement Scheme, James Cook University, Australia.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Wytamma Wirth and Andrew Read for providing valuable feedback on this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.733404/full#supplementary-material

REFERENCES

1. Gorbalenya AE, Enjuanes L, Ziebuhr J, Snijder EJ. Nidovirales: evolving the largest RNA virus genome. *Virus Res.* (2006) 117:17–37. doi: 10.1016/j.virusres.2006.01.017
2. Tyrrell DA, Bynoe ML. Cultivation of a novel type of common-cold virus in organ cultures. *Br Med J.* (1965) 1:1467470. doi: 10.1136/bmj.1.5448.1467
3. Pringle C. Virus taxonomy 1996—a bulletin from the Xth international congress of virology in jerusalem. *Arch Virol.* (1996) 141:2251–6. doi: 10.1007/BF01718231
4. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017
5. Zhou P, Fan H, Lan T, Yang, XL, Shi F, Zhang W, et al. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature.* (2018) 556:255–8. doi: 10.1038/s41586-018-0010-9
6. Müller H, Islam MR, Raue R. Research on infectious bursal disease—the past, the present and the future. *Vet Microbiol.* (2003) 97:153–65. doi: 10.1016/j.vetmic.2003.08.005
7. Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, et al. Emergence of porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. *J Vet Diagnost Invest.* (2013) 25:649–54. doi: 10.1177/1040638713501675
8. Balasuriya UBR, Carossino M, Timoney PJ. Equine viral arteritis: a respiratory and reproductive disease of significant economic importance to the equine industry. *Equine Vet Educ.* (2018) 30:497–512. doi: 10.1111/eve.12672
9. Bukhari K, Mulley G, Gulyaeva AA, Zhao L, Shu G, Jiang J, et al. Description and initial characterization of metatranscriptomic nidovirus-like genomes from the proposed new family abyssoviridae, and from a sister group to the Coronavirinae, the proposed genus alphaletovirus. *Virology.* (2018) 524:160–71. doi: 10.1016/j.virol.2018.08.010
10. Debat HJ. Expanding the size limit of RNA viruses: evidence of a novel divergent nidovirus in California sea hare, with a ∼35.9 kb virus genome. *bioRxiv*. (2018) 307678. doi: 10.1101/307678
11. Saberi A, Gulyaeva AA, Brubacher JL, Newmark PA, Gorbalenya AE. A planarian nidovirus expands the limits of RNA genome size. *PLoS Pathog.* (2018) 14:e1007314. doi: 10.1371/journal.ppat.1007314
12. Buchatsky LP, Makarov VV. Nidoviruses associated with aquatic animals. *Vet Sci Today.* (2020) 33:115–21. doi: 10.29326/2304-196X-2020-2-33-115-121
13. Shi M, Lin XD, Tian JH, Chen J, Chen X, Li CX, et al. Redefining the invertebrate RNA virosphere. *Nature.* (2016) 540:539–43. doi: 10.1038/nature20167
14. Shi M, Lin XD, Chen X, Tian JH, Chen LJ, Li K, et al. The evolutionary history of vertebrate RNA viruses. *Nature.* (2018) 556:197–202. doi: 10.1038/s41586-018-0012-7
15. Bodewes R, Lempp C, Schürch AC, Habierski A, Hahn K, Lamers M, et al. Novel divergent nidovirus in a python with pneumonia. *J Gen Virol.* (2014) 95 (Pt. 11):2480–5. doi: 10.1099/vir.0.068700-0
16. Stenglein MD, Jacobson ER, Wozniak EJ, Wellehan JFX, Kincaid A, Gordon M, et al. Ball python nidovirus: a candidate etiologic agent for severe respiratory disease in python regius. *MBio.* (2014) 5:e01484–14. doi: 10.1128/mBio.01484-14
17. Uccellini L, Ossiboff RJ, de Matos REC, Morrisey JK, Petrosov A, Navarrete-Macias I, et al. Identification of a novel nidovirus in an outbreak of fatal respiratory disease in ball pythons (python regius). *Virol J.* (2014) 11:144. doi: 10.1186/1743-422X-11-144
18. Dervas E, Hepojoki J, Laimbacher A, Romero-Palomo F, Jelinek C, Keller S, et al. Nidovirus-associated proliferative pneumonia in the green tree python (morelia viridis). *J Virol.* (2017) 91:e00718–17. doi: 10.1128/JVI.00718-17

19. Marschang RE, Kolesnik E. Detection of nidoviruses in live pythons and boas. *Tierarztl Prax Ausg K Kleintiere Heimtiere.* (2017) 45:22–6. doi: 10.15654/TPK-151067
20. Hoon-Hanks LL, Ossiboff RJ, Bartolini P, Fogelson SB, Perry SM, Stöhr AC, et al. Longitudinal and cross-sectional sampling of serpentovirus (nidovirus) infection in captive snakes reveals high prevalence, persistent infection, and increased mortality in pythons and divergent serpentovirus infection in boas and colubrids. *Front Vet Sci.* (2019) 6:338. doi: 10.3389/fvets.2019.00338
21. O'Dea MA, Jackson B, Jackson C, Xavier P, Warren K. Discovery and partial genomic characterisation of a novel nidovirus associated with respiratory disease in wild shingleback lizards (tiliqua rugosa). *PLoS ONE.* (2016) 11:e0165209. doi: 10.1371/journal.pone.0165209
22. Zhang J, Finlaison DS, Frost MJ, Gestier S, Gu X, Hall J, et al. Identification of a novel nidovirus as a potential cause of large scale mortalities in the endangered Bellinger River snapping turtle (Myuchelys georgesi). *PLoS ONE.* (2018) 13:e0205209. doi: 10.1371/journal.pone.0205209
23. Hoon-Hanks LL, Stöhr AC, Anderson AJ, Evans DE, Nevarez JG, Díaz RE, et al. Serpentovirus (nidovirus) and orthoreovirus coinfection in captive veiled chameleons (*Chamaeleo calyptratus*) with respiratory disease. *Viruses.* (2020) 12:1329. doi: 10.3390/v12111329
24. International Union for Conservation of Nature. *The IUCN Red List of Threatened Species. Version 2021-1.* (2021). Available online at: https://www.iucnredlist.org (accessed June 1, 2021).
25. Cabañes FJ, Sutton DA, Guarro J. Chrysosporium-related fungi and reptiles: a fatal attraction. *PLoS Pathog.* (2014) 10:e1004367. doi: 10.1371/journal.ppat.1004367
26. Tetzlaff SJ, Ravesi MJ, Allender MC, Carter ET, DeGregorio BA, Josimovich JM, et al. Snake fungal disease affects behavior of free-ranging massasauga rattlesnakes (*Sistrurus catenatus*). *Herpetol Conserv Biol.* (2017) 12:624–34.
27. Peterson NR, Rose K, Shaw S, Hyndman TH, Sigler L, Kurtböke DI, et al. Cross-continental emergence of *Nannizziopsis barbatae* disease may threaten wild Australian lizards. *Sci Rep.* (2020) 10:20976. doi: 10.1038/s41598-020-77865-7
28. Rose K, Agius J, Hall J, Thompson P, Eden JS, Srivastava M, et al. Emergent multisystemic enterococcus infection threatens endangered Christmas Island reptile populations. *PLoS One.* (2017) 12:e0181240. doi: 10.1371/journal.pone.0181240
29. Liu L, Cao Z, Lin F, Ye XP, Xu Y. Partial sequence of a novel virus isolated from pelodiscus sinensis hemorrhagic disease. *Intervirology.* (2015) 58:197–204. doi: 10.1159/000437354
30. Ossiboff RJ, Raphael BL, Ammazzalorso AD, Seimon TA, Newton AL, Chang TY, et al. Three novel herpesviruses of endangered clemmys and glyptemys turtles. *PLoS ONE.* (2015) 10:e0122901. doi: 10.1371/journal.pone.0122901
31. Liu L, Cao Z, Lin F, Ye X, Lu S, Lyv S. The histopathological characteristics caused by trionyx sinensis hemorrhagic syndrome virus (TSHSV) and comparative proteomic analysis of liver tissue in TSHSV-infected Chinese soft-shelled turtles (*Pelodiscus sinensis*). *Intervirology.* (2017) 60:19–27. doi: 10.1159/000479795
32. Smith KF, Sax DF, Lafferty KD. Evidence for the role of infectious disease in species extinction and endangerment. *Conservat Biol.* (2006) 20:1349–57. doi: 10.1111/j.1523-1739.2006.00524.x
33. Hudson MA, Young RP, D'Urban Jackson J, Orozco-terWengel P, Martin L, James A, et al. Dynamics and genetics of a disease-driven species decline to near extinction: lessons for conservation. *Sci Rep.* (2016) 6:30772. doi: 10.1038/srep30772
34. 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.* (2019) 363:1459–63. doi: 10.1126/science.aav0379
35. International Committee on Taxonomy of Viruses. Virus taxonomy: 2020.v1 release - order nidovirales. In: *EC 52, Online Meeting, October 2020. Email ratification March 2021 (MSL #36).* Elsevier (2021).
36. King AM, Lefkowitz E, Adams MJ, Carstens EB. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses - Order Nidovirales.* St. Louis, MO: Elsevier (2011).
37. Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Dempsey DM, Dutilh BE, et al. Changes to virus taxonomy and the by the atifInternational committee on taxonomy of viruses (international code of virus classification and nomenclature ried 2019). *Arch Virol.* (2019) 164:2417–29. doi: 10.1007/s00705-019-04306-w
38. Hoet AE, Horzinek MC. Torovirus. In: Mahy BWJ, Van Regenmortel MGV, editors. *Encyclopedia of Virology, 3rd Edn.* Oxford: Academic Press (2008). p. 151–7. doi: 10.1016/B978-012374410-4.00516-1
39. Nga PT, Parquet M, d.C., Lauber C, Parida M, Nabeshima T, et al. Discovery of the first insect nidovirus, a missing evolutionary link in the emergence of the largest RNA virus genomes. *PLoS Pathog.* (2011) 7:e1002215–e1002215. doi: 10.1371/journal.ppat.1002215
40. Pasternak AO, Spaan WJ, Snijder EJ. Nidovirus transcription: how to make sense.? *J Gen Virol.* (2006) 87(Pt. 6), 1403–21. doi: 10.1099/vir.0.81611-0
41. Blahak S, Jenckel M, Höper D, Beer M, Hoffmann B, Schlottau K. Investigations into the presence of nidoviruses in pythons. *Virol J.* (2020) 17:6. doi: 10.1186/s12985-020-1279-5
42. Marschang RE, Kolesnik E, Müller E. Nidoviruses in snakes in Europe. In: *BSAVA Congress Proceedings 2019.* Birmingham: British Small Animal Veterinary Association (2019). doi: 10.22233/9781910443699.68.3
43. Pasterny J, Skomorucha Ł, Stanicki K, Marschang RE. Detection of infectious agents in samples from reptiles presented at veterinary clinics in Poland. *J Herpetol Med Surg.* (2021) 31:64–72. doi: 10.5818/12-2020.1
44. Hoon-Hanks LL. *The use of metagenomic sequencing as a tool for pathogen discovery with further investigation of novel reptilian serpentoviruses* (Doctor of Philosophy). Colorado State University, Fort Collins, CO, United States (2019).
45. Martinez J. *Associations Between Gastrointestinal Parasites and Nidovirus infection in Western Australian Shingleback Lizards (Tiliqua rugosa).* Murdoch, WA: Murdoch University (2019).
46. Berry KH, Yee JL, Shields TA, Stockton L. The catastrophic decline of tortoises at a fenced natural area. *Wildlife Monogr.* (2020) 205:1–53. doi: 10.1002/wmon.1052
47. Chessman BC, McGilvray G, Ruming S, Jones HA, Petrov K, Fielder DP, et al. On a razor's edge: status and prospects of the critically endangered Bellinger River snapping turtle, *Myuchelys georgesi*. *Aquat Conserv Mar Freshw Ecosyst.* (2020) 30:586–600. doi: 10.1002/aqc.3258
48. Dolja VV, Koonin EV. Metagenomics reshapes the concepts of RNA virus evolution by revealing extensive horizontal virus transfer. *Virus Res.* (2018) 244:36–52. doi: 10.1016/j.virusres.2017.10.020
49. Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev.* (2005) 69:635–64. doi: 10.1128/MMBR.69.4.635-664.2005
50. Draker R, Roper RL, Petric M, Tellier R. The complete sequence of the bovine torovirus genome. *Virus Res.* (2006) 115:56–68. doi: 10.1016/j.virusres.2005.07.005
51. Sun H, Lan D, Lu L, Chen M, Wang C, Hua X. Molecular characterization and phylogenetic analysis of the genome of porcine torovirus. *Arch Virol.* (2014) 159:773–8. doi: 10.1007/s00705-013-1861-x
52. Tokarz R, Sameroff S, Hesse RA, Hause BM, Desai A, Jain K, et al. Discovery of a novel nidovirus in cattle with respiratory disease. *J Gen Virol.* (2015) 96:2188–93. doi: 10.1099/vir.0.000166
53. Schütze H. Chapter 20 - coronaviruses in aquatic organisms. In: Kibenge FSB, Godoy MG, editors. *Aquaculture Virology.* San Diego, CA: Academic Press (2016). p. 327–35.
54. Mordecai GJ, Hewson I. Coronaviruses in the sea. *Front Microbiol.* (2020) 11:1795. doi: 10.3389/fmicb.2020.01795
55. Hoon-Hanks LL, Layton ML, Ossiboff RJ, Parker JSL, Dubovi EJ, Stenglein MD. Respiratory disease in ball pythons (*Python regius*) experimentally infected with ball python nidovirus. *Virology.* (2018) 517:77–87. doi: 10.1016/j.virol.2017.12.008
56. Li WT, Lee MS, Tseng YC, Yang NY. A case report of reptile-associated nidovirus (serpentovirus) in a ball python (*Python regius*) in Taiwan. *J Vet Med Sci.* (2020) 82:788–92. doi: 10.1292/jvms.20-0166
57. Dervas E, Hepojoki J, Smura T, Prähauser B, Windbichler K, Blümich S, et al. Serpentoviruses: more than respiratory pathogens. *J Virol.* (2020) 94:e00649–20. doi: 10.1128/JVI.00649-20

58. Lempp C, Bodewes R, Habierski A, Hahn K, Wohlsein P, Schürch AC, et al. Detection of a novel nidovirus in an indian python (Python molurus). *J Comp Pathol.* (2015) 152:91. doi: 10.1016/j.jcpa.2014.10.203
59. Rampacci E, Masi M, Origgi FC, Stefanetti V, Bottinelli M, Selleri P, et al. First molecular detection of ball python nidovirus in Italy–Short communication. *Acta Vet Hung.* (2019) 67:127–34. doi: 10.1556/004.2019.014
60. Moloney B, Britton S, Matthews S. *Bellinger River Snapping Turtle Mortality Event 2015 - Epidemiology Report*. Orange, NSW: NSW Department of Primary Industries (2015).
61. Schütze H, Ulferts R, Schelle B, Bayer S, Granzow H, Hoffmann B, et al. Characterization of white bream virus reveals a novel genetic cluster of nidoviruses. *J Virol.* (2006) 80:11598–609. doi: 10.1128/JVI.01758-06
62. Batts WN, Goodwin AE, Winton JR. Genetic analysis of a novel nidovirus from fathead minnows. *J Gen Virol.* (2012) 93 (Pt. 6):1247–52. doi: 10.1099/vir.0.041210-0
63. Thrusfield MV. *Veterinary Epidemiology.* Chicester: Wiley (2013).
64. Jacobson ER. *Infectious Diseases and Pathology of Reptiles: Color Atlas and Text.* Boca Raton, FL: CRC Press (2007). doi: 10.1201/9781420004038
65. Cushing A, Pinborough M, Stanford M. Review of bacterial and fungal culture and sensitivity results from reptilian samples submitted to a UK laboratory. *Veterinary Record.* (2011) 169:390. doi: 10.1136/vr.d4636
66. McKnight DT, Zenger KR, Alford RA, Huerlimann R. Microbiome diversity and composition varies across body areas in a freshwater turtle. *Microbiology.* (2020) 166:440–52. doi: 10.1099/mic.0.000904
67. Schumacher J. Reptile respiratory medicine. *Vet Clin Exotic Anim Pract.* (2003) 6:213–31. doi: 10.1016/S1094-9194(02)00020-8
68. Murray MJ. Chapter 65 - pneumonia and lower respiratory tract disease. In: Mader DR, editor. *Reptile Medicine and Surgery*. 2nd Edn. Saint Louis, MO: W.B. Saunders (2006). p. 865–77. doi: 10.1016/B0-72-169327-X/50069-9
69. Balasuriya UBR, Hedges JF, Nadler SA, McCollum WH, Timoney PJ, MacLachlan NJ. Genetic stability of equine arteritis virus during horizontal and vertical transmission in an outbreak of equine viral arteritis. *J Gen Virol.* (1999) 80:1949–58. doi: 10.1099/0022-1317-80-8-1949
70. Cowley JA, Hall MR, Cadogan LC, Spann KM, Walker PJ. Vertical transmission of gill-associated virus (GAV) in the black tiger prawn penaeus monodon. *Dis Aquat Organ.* (2002) 50:95–104. doi: 10.3354/dao050095
71. Harding JCS, Ladinig A, Novakovic P, Detmer SE, Wilkinson JM, Yang T, et al. Novel insights into host responses and reproductive pathophysiology of porcine reproductive and respiratory syndrome caused by PRRSV-2. *Vet Microbiol.* (2017) 209:114–23. doi: 10.1016/j.vetmic.2017.02.019
72. Hoffmann B, Depner K, Schirrmeier H, Beer M. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *J Virol Methods.* (2006) 136:200–9. doi: 10.1016/j.jviromet.2006.05.020
73. Schroeder ME, Bounpheng MA, Rodgers S, Baker RJ, Black W, Naikare H, et al. Development and performance evaluation of calf diarrhea pathogen nucleic acid purification and detection workflow. *J Vet Diagn Invest.* (2012) 24:945–53. doi: 10.1177/1040638712456976
74. Goldsmith CS, Miller SE. Modern uses of electron microscopy for detection of viruses. *Clin Microbiol Rev.* (2009) 22:552–63. doi: 10.1128/CMR.00027-09
75. Roingeard P, Raynal PI, Eymieux S, Blanchard E. Virus detection by transmission electron microscopy: still useful for diagnosis and a plus for biosafety. *Rev Med Virol.* (2019) 29:e2019. doi: 10.1002/rmv.2019
76. Ariel E. Viruses in reptiles. *Vet Res.* (2011) 42:100. doi: 10.1186/1297-9716-42-100
77. Fredericks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microbiol Rev.* (1996) 9:18–33. doi: 10.1128/CMR.9.1.18
78. Zimmerman LM, Vogel LA, Bowden RM. Understanding the vertebrate immune system: insights from the reptilian perspective. *J Exp Biol.* (2010) 213:661–71. doi: 10.1242/jeb.038315
79. Rios FM, Zimmerman LM. *Immunology of Reptiles.* Chichester: John Wiley & Sons, Ltd (2015). doi: 10.1002/9780470015902.a0026260
80. Zimmerman LM. Reptilia: humoral immunity in reptiles. In: Cooper EL, editor. *Advances in Comparative Immunology.* Cham: Springer International Publishing (2018). p. 751–72. doi: 10.1007/978-3-319-76768-0_20
81. Zimmerman LM. The reptilian perspective on vertebrate immunity: 10 years of progress. *J Exp Biol.* (2020) 223:jeb214171. doi: 10.1242/jeb.214171
82. Brown DW, Selvakumar R, Daniel DJ, Mathan VI. Prevalence of neutralising antibodies to berne virus in animals and humans in Vellore, South India. *Brief Rep Arch Virol.* (1988) 98:267–9. doi: 10.1007/BF01322174
83. Koopmans M, Van Den Boom U, Woode G, Horzinek MC. Seroepidemiology of Breda virus in cattle using ELISA. *Vet Microbiol.* (1989) 19:233–43. doi: 10.1016/0378-1135(89)90069-2
84. Origgi FC, Romero CH, Bloom DC, Klein PA, Gaskin JM, Tucker SJ, et al. Experimental transmission of a herpesvirus in greek tortoises (*Testudo graeca*). *Vet Pathol.* (2004) 41:50–61. doi: 10.1354/vp.41-1-50
85. Martel A, Blahak S, Vissenaekens H, Pasmans F. Reintroduction of clinically healthy tortoises: the herpesvirus trojan horse. *J Wildl Dis.* (2009) 45:218–20. doi: 10.7589/0090-3558-45.1.218
86. Allender MC, Mitchell MA, Dreslik MJ, Phillips CA, Beasley VR. Measuring agreement and discord among hemagglutination inhibition assays against different ophidian paramyxovirus strains in the eastern massasauga (*Sistrurus catenatus catenatus*). *J Zoo Wildlife Med.* (2008) 39:358–61. doi: 10.1638/2007-0111.1
87. Rösler R, Abbas MD, Papp T, Marschang RE, Mikrobiologie F, Reptilien ZB. Detection of antibodies against paramyxoviruses in tortoises. *J Zoo Wildlife Med.* (2013) 44:333–9. doi: 10.1638/2012-0149R.1
88. Johnson AJ, Wendland L, Norton TM, Belzer B, Jacobson ER. Development and use of an indirect enzyme-linked immunosorbent assay for detection of iridovirus exposure in gopher tortoises (*Gopherus polyphemus*) and eastern box turtles (*Terrapene carolina carolina*). *Vet Microbiol.* (2010) 142:160–7. doi: 10.1016/j.vetmic.2009.09.059
89. Ariel E, Elliott E, Meddings JI, Miller J, Santos MB, Owens L. Serological survey of Australian native reptiles for exposure to ranavirus. *Dis Aquat Organ.* (2017) 126:173–83. doi: 10.3354/dao03172
90. Korzyukov Y, Hetzel U, Kipar A, Vapalahti O, Hepojoki J. Generation of anti-boa immunoglobulin antibodies for serodiagnostic applications, and their use to detect anti-reptarenavirus antibodies in boa constrictor. *PLoS ONE.* (2016) 11:e0158417. doi: 10.1371/journal.pone.0158417
91. Shaik Abdool F, Coetzer THT, Goldring JPD. Isolation of nile crocodile (*Crocodylus niloticus*) serum immunoglobulin M and Y (IgM and IgY). *J Immunol Methods.* (2020) 478:112724. doi: 10.1016/j.jim.2019.112724
92. Blamires SJ, Spencer RJ, King P, Thompson MB. Population parameters and life-table analysis of two coexisting freshwater turtles: are the Bellinger River turtle populations threatened? *Wildlife Res.* (2005) 32:339–47. doi: 10.1071/WR04083
93. Spencer RJ, Van Dyke J, Petrov K, Ferronato B, McDougall F, Austin M, et al. Profiling a possible rapid extinction event in a long-lived species. *Biol Conserv.* (2018) 221:190–7. doi: 10.1016/j.biocon.2018.03.009
94. Hilf M, Wagner R, Yu V. A prospective study of upper airway flora in healthy boid snakes and snakes with pneumonia. *J Zoo Wildlife Med.* (1990) 21:318–25.
95. Sonntag FD, Rüschoff B, Troll C, Heckers KO, Marschang RE. Bacteria associated with clinically suspected respiratory disease in snakes and effective antimicrobial treatment options. *J Herpetol Med Surg.* (2021) 30:254–60, 57. doi: 10.5818/19-07-205.1
96. Pasmans F, Blahak S, Martel A, Pantchev N. Introducing reptiles into a captive collection: the role of the veterinarian. *Vet J.* (2008) 175:53–68. doi: 10.1016/j.tvjl.2006.12.009
97. World Organisation for Animal Health. Chapter 4.3 disinfection of aquaculture establishments and equipment. In: *Aquatic Animal Health Code*. World Organisation for Animal Health. (2017). Available online at:

http://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_disinfection.htm (accessed July 2, 2019).
98. Wildlife Health Australia. *National Wildlife Biosecurity Guidelines*. Sydney, NSW: Wildlife Health Australia (2018).
99. Heinrich S, Toomes A, Shepherd CR, Stringham OC, Swan M, Cassey P. Strengthening protection of endemic wildlife threatened by the international pet trade: the case of the Australian shingleback lizard. *Anim Conserv*. (2021). doi: 10.1111/acv.12721

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors, EA.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 24 September 2021
doi: 10.3389/fvets.2021.733357

Chytridiomycosis Outbreak in a Chilean Giant Frog (*Calyptocephalella gayi*) Captive Breeding Program: Genomic Characterization and Pathological Findings

Mario Alvarado-Rybak[1,2,3], Paz Acuña[4], Alexandra Peñafiel-Ricaurte[1], Thomas R. Sewell[5], Simon J. O'Hanlon[5], Matthew C. Fisher[5], Andres Valenzuela-Sánchez[1,6,7], Andrew A. Cunningham[2] and Claudio Azat[1]**

[1] Sustainability Research Centre & PhD Program in Conservation Medicine, Faculty of Life Sciences, Universidad Andres Bello, Santiago, Chile, [2] Institute of Zoology, Zoological Society of London, London, United Kingdom, [3] Núcleo de Ciencias Aplicadas en Ciencias Veterinarias y Agronómicas, Universidad de las Américas, Santiago, Chile, [4] Criadero y Centro de Exhibición de la Rana Chilena Calyptocephalella gayi, Santiago, Chile, [5] Department of Infectious Disease Epidemiology, MRC Centre for Global Infectious Disease Analysis, School of Public Health, Imperial College London, London, United Kingdom, [6] ONG Ranita de Darwin, Valdivia, Chile, [7] Instituto de Conservación, Biodiversidad y Territorio, Facultad de Ciencias Forestales y Recursos Naturales, Universidad Austral de Chile, Valdivia, Chile

Emerging infectious diseases in wildlife are increasingly associated with animal mortality and species declines, but their source and genetic characterization often remains elusive. Amphibian chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been associated with catastrophic and well-documented amphibian population declines and extinctions at the global scale. We used histology and whole-genome sequencing to describe the lesions caused by, and the genetic variability of, two *Bd* isolates obtained from a mass mortality event in a captive population of the threatened Chilean giant frog (*Calyptocephalella gayi*). This was the first time an association between *Bd* and high mortality had been detected in this charismatic and declining frog species. Pathological examinations revealed that 30 dead metamorphosed frogs presented agnathia or brachygnathia, a condition that is reported for the first time in association with chytridiomycosis. Phylogenomic analyses revealed that *Bd* isolates (PA1 and PA2) from captive *C. gayi* group with other *Bd* isolates (AVS2, AVS4, and AVS7) forming a single highly supported Chilean *Bd* clade within the global panzootic lineage of *Bd* (*Bd*GPL). These findings are important to inform the strengthening of biosecurity measures to prevent the impacts of chytridiomycosis in captive breeding programs elsewhere.

Keywords: agnathia, amphibians, *Batrachochytrium dendrobatidis*, BdGPL, brachygnathia, Chile, emerging infectious disease, whole-genome sequencing

OPEN ACCESS

Edited by:
Amanda Linda Jean Duffus, Gordon State College, United States

Reviewed by:
Rebecca Hardman, The University of Tennessee, Knoxville, United States
An Martel, Ghent University, Belgium

****Correspondence:***
Mario Alvarado-Rybak
maalry@gmail.com
Claudio Azat
claudio.azat@unab.cl

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *30 June 2021*
Accepted: *27 August 2021*
Published: *24 September 2021*

Citation:
Alvarado-Rybak M, Acuña P, Peñafiel-Ricaurte A, Sewell TR, O'Hanlon SJ, Fisher MC, Valenzuela-Sánchez A, Cunningham AA and Azat C (2021) Chytridiomycosis Outbreak in a Chilean Giant Frog (Calyptocephalella gayi) Captive Breeding Program: Genomic Characterization and Pathological Findings. Front. Vet. Sci. 8:733357. doi: 10.3389/fvets.2021.733357

INTRODUCTION

Although amphibian enigmatic declines had been identified by herpetologists as early as the 1970s, they were only recognized two decades later as a global phenomenon that in some cases could not be explained by environmental changes or other expected anthropogenic factors alone (1–3). The discovery of the amphibian-killing fungus *Batrachochytrium dendrobatidis* [hereafter *Bd* (4, 5)] was a turning point in understanding why many amphibian species have been in steep decline. The emergence of *Bd*, which causes the lethal disease, amphibian chytridiomycosis, has been associated with amphibian population declines of more than 500 species, including the presumed extinction of at least 90 species (6). Evidence suggests that *Bd* recently spread across the globe from an endemic focus, with East Asia as the most likely source from where it expanded to other continents during the past century (7–10). Its global spread has been mainly facilitated by the international trade of amphibians, particularly the North American bullfrog (*Lithobates catesbeianus*), the most intensively farmed frog worldwide (11–15).

Novel genomic techniques, including whole-genome sequencing and multilocus sequence typing have shown the existence of at least five major lineages of *Bd*: *Bd*GPL, *Bd*CAPE, *Bd*ASIA-1 (including *Bd*CH), *Bd*ASIA-2/*Bd*BRAZIL and *Bd*ASIA-3 (9, 10, 16). Of these, the global panzootic lineage (*Bd*GPL) is the most widespread variant of *Bd* and is responsible for most known cases of amphibian population declines due to chytridiomycosis (9). Although *Bd*GPL is highly virulent, its impacts are context-dependent (17), and under some conditions other lineages may be responsible for lethal disease and population declines (18). Additionally, multiple introductions of *Bd* have led to different lineages of *Bd* coming into contact, resulting in the formation of interlineage recombinants (e.g., through the co-infection of amphibians) that may have higher pathogenicity or transmissibility (9, 10, 14, 16, 19, 20). For instance, interlineage recombinants have been reported for *Bd*GPL with *Bd*ASIA-2/*Bd*BRAZIL, and for *Bd*GPL with *Bd*CAPE (9, 10, 14, 19).

Despite South America being the region with the greatest loss of biodiversity due to *Bd* (6), only low numbers of *Bd* isolates from this region have been genetically characterized (14, 21–26). This limits our capacity to adequately understand the epidemiological processes that have led to impacts of chytridiomycosis on South American native amphibians. Our focus here, the Chilean giant frog (*Calyptocephalella gayi*), is endemic to Chile and is considered as a living fossil since its family represents an old neobatrachian clade that diverged during the Cretaceous around 100–120 Mya (27). With females reaching up to 2 kg, this is the second largest anuran species worldwide, which has led this species to be of economic interest as a food source (28). This highly aquatic species is considered Vulnerable by the IUCN Red List, and is threatened by overconsumption, habitat loss due to agriculture, and invasive species including several introduced fish and the African clawed frog [*Xenopus laevis* (29)]. Chytridiomycosis has been suspected to be a contributing factor in its steep decline (30), but to date, no evidence has been found linking *Bd* to lethal effects in *C. gayi*. Based on histology and whole-genome sequencing, the aim of this study is to describe the lesions of chytridiomycosis in *C. gayi* and the genetic characterization of *Bd* isolates obtained from a chytridiomycosis outbreak that occurred in a *C. gayi* captive breeding program in Chile. In addition, the genomics of *Bd* isolates from captive *C. gayi* together with previously obtained Chilean *Bd* isolates are compared with a global panel of *Bd*.

MATERIALS AND METHODS

Chilean Giant Frog Captive Breeding Center

The Chilean giant frog captive breeding center (Resolution N°2358/2013 by the Chilean Agriculture and Livestock Service) in Santiago has been functioning since 2013, with its objective to generate reproductive knowledge and to support the conservation of *C. gayi*. The center was built in an area of 70 m^2 and was composed of 10 large and 10 small tanks for tadpoles (100 and 30 L each, respectively), 10 small tanks for recently metamorphosed frogs (30 L) and 20 medium size tanks for adult frogs (50 L). Water used in tanks was from the mains supply but had been left to stand for 2 days to allow chlorine evaporation. Around 50% of water in the tanks was changed twice a week. Tadpoles were fed on spirulina algae supplemented with lettuce given *ad libitum*, while postmetamorphs and adults were fed twice a day with dried amphipod crustaceans (*Orchistoidea* spp.) supplemented with chicken protein, vitamins, and minerals. By August 2016, the captive breeding program comprised ∼400 1-year-old tadpoles, six juveniles, and 86 breeding adults (43 females and 43 males).

Mortality and Pathological Analyses

In September 2016, the program received new individuals from a separate *C. gayi* captive breeding program that had been terminated. The newly incoming individuals consisted of 800 2-year-old tadpoles and 18 breeding adults (9 females, 9 males), which were maintained in separate tanks from the resident animals. By early November 2016, 40 of the new tadpoles completed metamorphosis. From December 2016 to January 2017, 75 of the new individuals died: 37 tadpoles, 35 postmetamorphs and three reproductive adults. All dead postmetamorphs had not consumed any food after metamorphosis, and some of the tadpoles were observed to be lethargic prior to death and exhibiting partial depigmentation of the mouthparts, a finding consistent with amphibian chytridiomycosis (31). Freshly dead animals (4 tadpoles and 29 postmetamorphs) were transported in refrigerated conditions to the laboratory for postmortem examination and *Bd* isolation. Necropsies of the 29 postmetamorphs were performed according to standard protocol (32). Tissue sections of postmetamorphs were collected in neutral-buffered 10% formaldehyde from any organ displaying gross lesions and from lung, liver, spleen, kidney, skeletal muscle, heart, skin, stomach, small and large intestines. For histopathological analyses, tissues were embedded in paraffin wax, sectioned (4–5 μm) and stained with hematoxylin and eosin (H&E).

Bd Sampling and qPCR Assay

Non-invasive skin swabs (MW100, Medical & Wire Equipment Co.) were obtained from postmetamorphic amphibians ($n = 29$) by firmly running the swabs five times each over the ventral abdomen and pelvis, each ventral hind limb (femur and tibia) and the plantar surface of each hind foot (33). Also, from dead tadpoles ($n = 4$), samples were obtained from the oral disc by rotating the swab 10 times around the mouth opening. Swabs were kept in a cool box until freezing at −80°C once back at the laboratory. Briefly, DNA extraction from skin and oral swabs and subsequent detection of *Bd* DNA using a specific real time qPCR assay was done following Soto-Azat et al. (34). For each sample, diagnostic assays were performed in duplicate, and standards of known zoospore concentration (obtained from a previous *Bd* culture) were included within each PCR plate as positive controls. We assumed that a *Bd*-positive swab was indicative of *Bd* infection. By including known concentrations of *Bd* DNA in serial diluted positive control wells on each PCR plate, we were able to quantify infection intensity, which we defined as the number of zoospore equivalents/swab (ZE). To quantify and correct the infection intensity per swab, each genomic value obtained from the qPCR assay was multiplied by 120 to account for sample dilution (35).

Bd Isolation

Freshly dead tadpoles with suspected *Bd* infection ($n = 4$) were used for *Bd* isolation following Longcore et al. (5) and Fisher et al. (36). Subsequent confirmation of *Bd* infection status and load by qPCR, served to guide *Bd* isolation efforts. Within 8 h. after death, the whole mouthparts of *Bd*-positive dead tadpoles were removed and sectioned into small pieces and deposited in a fungal growth TGhL medium (8 g. tryptone, 2 g. gelatin hydrolysate, 4 g. lactose, 10 g. agar). Cultured sections were first cleaned using an agar plate with antibiotics (200 mg/L penicillin-G and 400 mg/L streptomycin sulfate), and then placed singly into TGhL agar plate with antibiotics incubated at 15–20°C. Because zoospore release may occur immediately, especially from tadpole mouthparts, cultures were examined with an inverted microscope for the presence of active zoospores every day for up to 1 week. Once growth of zoospores and/or zoosporangia was observed, part of the agar was transferred to a new TGhL agar plate without antibiotics and incubated at 15–20°C up to 1 week. Isolates were then passaged no more than three times in order to lessen the chance of genomic change due to prolonged laboratory culture (37).

DNA Extraction, Sequence Library Preparation and Phylogenomic Analyses

We performed DNA extraction using the MasterPure™ Yeast DNA Purification Kit (Epicentre, Wisconsin, USA) from all obtained purified *Bd* cultures. DNA extractions were first quantified using a Tapestation 2200 (Agilent Technologies, California, USA) and Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Massachusetts, USA), and then sequenced using an Illumina HiSeq 2000 (Illumina, California, USA). Subsequently, TruSeq Nano 350 gel-free sequencing libraries were prepared for 125 + 125 bp paired-end sequencing using Illumina HiSeq high output v4 chemistry, and sequencing reads cleaned of adapter sequences and quality trimmed using cutadapt v1.10 (38). Reads of the JEL423 reference genome (GenBank assembly accession: GCA_000149865.1) were mapped using Burrows-Wheeler Aligner v0.7.8 (39).

We processed the resulting sequence alignment map (SAM) files using SAMtools v1.3.1 with the "fixmate" and "sort" programs to read the files for variants discovery. We performed the variant detection in a two-step process using Freebayes version dbb6160 (40). First, sorted SAM files for each isolate in the phylogeny were independently called to find variant positions and merged into a single variant call format (VCF) file. Second, each of the samples was re-called using the positions in VCF file, to produce a squared-off call set (a genotype call was made at every locus for each isolate, including for missing data). All VCF files were processed using vcflib (41) to break complex variants into allelic primitives and vt (42) to normalize short insertion and deletion sequences. VCF files were then quality filtered using bcftools v1.3.1 (43) to accept only variants with sufficient supporting evidence. Potentially polymorphic sites were filtered using the settings in the bcbio.variation.recall squaring-off pipeline (44), with sites not passing these filters set to homozygous reference (there was not enough evidence to call a variant at that position). Then, the processed VCF files were merged into a single multisample VCF and extracted a FASTA file of the SNP variant calls. Phylogenomic analyses were conducted using RAxML v8.2.9 with GTRCAT model with 500 bootstrap runs. Weir and Cockerham's estimator was performed using a sliding-window comparison of F_{ST} of Chilean *Bd* isolates against a global panel of representative global diversity of *Bd* in vcftools. Single nucleotide polymorphisms (SNPs) that were in high linkage disequilibrium were pruned from the dataset using the SNPRelate package version 1.10.2 in R v3.4.0 (45). After pruning using a sliding-window based analysis and a linkage disequilibrium threshold of 0.125, 3,900 SNPs positions remained which were analyzed using SNPRelate and plotted with ggplot2 (46). Finally, the clustering of Chilean *Bd* isolates against a global panel of *Bd* was investigated using principal component analysis (PCA) with adegenet package (47) and plotted with ggplot2 (46).

RESULTS

Pathology and *Bd* qPCR Assay

During December 2016 and January 2017, we observed a mass mortality event in a *C. gayi* captive breeding program, killing 87.5% (35/40) of metamorphosed frogs in the newly acquired group of animals. Of these, 30 individuals presented: jaw deformation ($n = 21$) or absence ($n = 9$) of oral structures, dying a few weeks after completing metamorphosis as they could not feed properly (**Figure 1A**). PCR *Bd*-positive samples were detected in 100% of sampled tadpoles, and postmetamorphic individuals ($n = 33$). The infection load in *Bd*-positive amphibians ranged from 95 to 147,366 ZE (median = 9,842). Of the total number of infected frogs, 33.3% (11/33) had more than 10,000 ZE. At gross necropsy a distended gallbladder (with bile) and the absence of gastrointestinal content was observed in all individuals with jaw absence/deformation

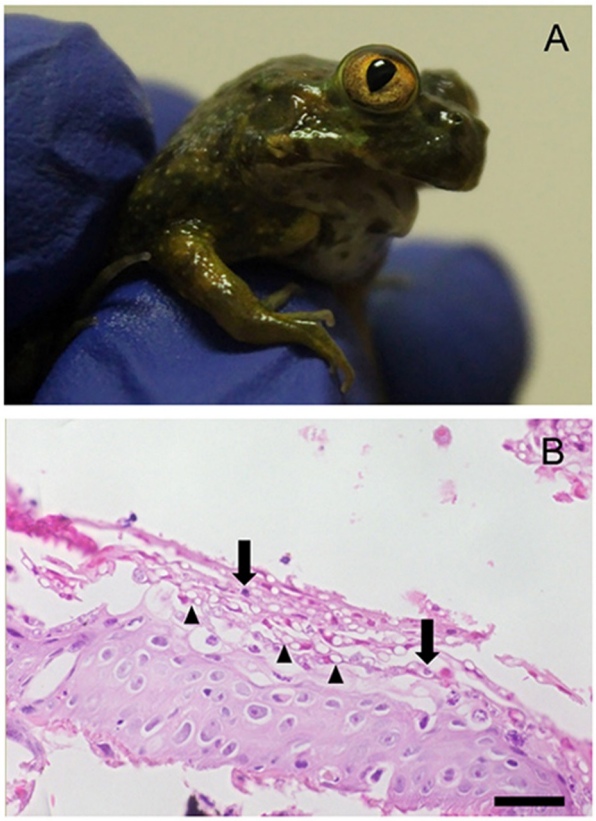


FIGURE 1 | A Chilean giant frog (*Calyptocephalella gayi*) postmetamorph showing signs of disease. **(A)** Absence of jaw (agnathia). **(B)** Histological section of hind limb skin. Note distinct stages of developing zoosporangia (arrows) and multiple empty spaces (arrowheads) within the superficial keratinized layer, morphologically typical of *Batrachochytrium dendrobatidis* infection. Stained with hematoxylin and eosin. Bar = 24 μm (bottom).

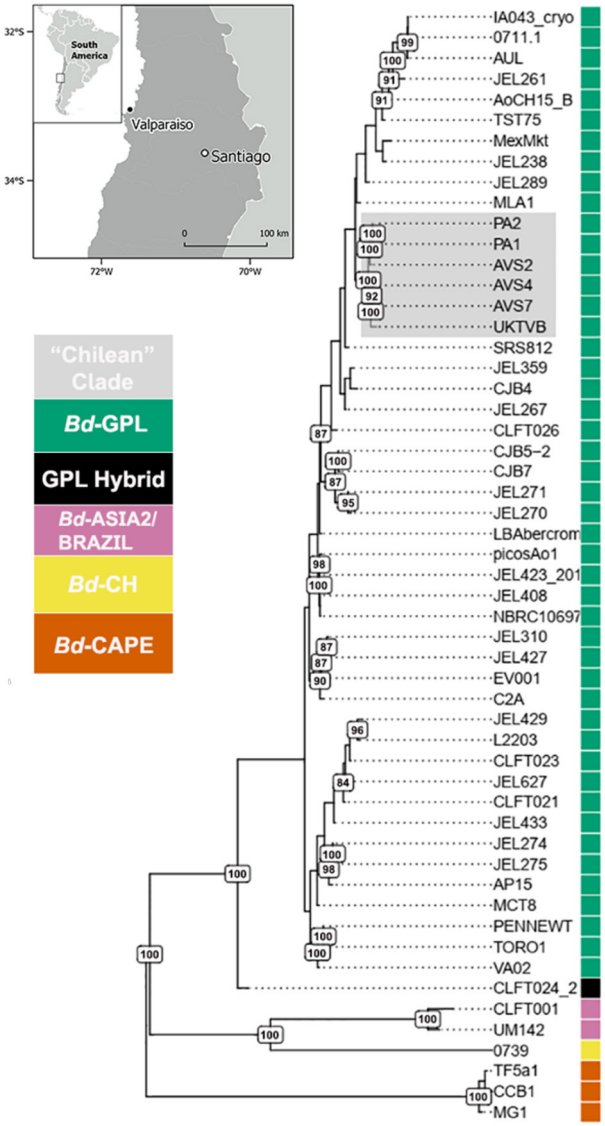


FIGURE 2 | Global phylogeny of 54 isolates of *Batrachochytrium dendrobatidis* (Bd) based on 363,497 segregating sites obtained by whole genome sequencing. The clade containing the Bd isolates PA1 and PA2 from captive *C. gayi* grouping with other Bd isolates from Chile and the United Kingdom is highlighted in gray. The branches of the tree are weighted (thickness) by bootstrap support (500 replicates), with branches with 80% of support and above labeled.

(30/35). We confirmed *Bd* infection microscopically, as we observed hyperkeratosis within the superficial layer of the skin with distinct stages of developing zoosporangia that are morphologically typical of *Bd* (**Figure 1B**). No other macroscopic or microscopic findings were observed in the other analyzed tissues.

Bd Isolation and Phylogenomic Analyses

From our attempts to culture *Bd* from four freshly dead *C. gayi* tadpoles, we obtained two isolates (PA1 and PA2, WGS read data available at the NCBI Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra under accession numbers SRS8215364 and SRS8216816, respectively). Phylogenomic analyses showed that our *C. gayi* isolates grouped within *Bd*GPL forming a single and highly supported clade (100% bootstrap support; **Figure 2**). The *Bd* isolates were fixed for 99.4% of the segregating sites that were observed in *Bd*GPL after filtering for missing positions. There were only 2,257 variable sites exclusive to the *C. gayi Bd* isolates. Although we compared the genomes of *C. gayi* isolates (PA1 and PA2) with an extensive global panel of *Bd*, they were shown to be highly divergent from the only known regional potentially endemic lineage in South America (*Bd*Asia2/*Bd*Brazil). Within *Bd*GPL, the *Bd* isolates from captive *C. gayi* clustered with other isolates obtained from wild amphibians in Chile (AVS2 from *Batrachyla antartandica*, AVS4 from *Xenopus laevis* and AVS7 from *C. gayi*) and an isolate from the UK (UKTvB), collected from a smooth newt (*Lissotriton vulgaris*) in 2009 in Kent, United Kingdom, to form a well-supported clade (100% bootstrap support; **Figure 2**).

We used the Weir and Cockerham's estimator to perform a sliding-window comparison of F_{ST} of *Bd* isolates (PA1 and PA1) against all the other *Bd*GPL isolates. In this analysis, we identified several stretches of genome where the F_{ST} estimator was more than two standard deviations greater than the mean of all F_{ST} values, indicating differentiation due to positive selection or reduced rates of recombination (**Figure 3**). Finally, we analyzed isolate clustering using PCA on a filtered subset of 3,900 SNPs in linkage equilibrium, revealing an overall population structure that is consistent with our phylogenetic analyses (**Figure 4**).

DISCUSSION

The now globalized *Bd* has caused the greatest loss of biodiversity known due to a single pathogen (6). The impacts of chytridiomycosis have likely been underestimated, as the affected amphibian species are often difficult to study, particularly in endangered cryptic species that occur in remote locations (48). In addition, not all *Bd* lineages have the same impact on infected amphibian populations and species, therefore a better understanding of the genetic diversity of *Bd* is critical to understanding the risk presented by this pathogen and to informing mitigation actions (16). In this study, we describe a mass mortality event due to chytridiomycosis in an endangered species of amphibian in a captive breeding program. We genetically characterized two *Bd* isolates from this outbreak, showing that they nested within the *Bd*GPL clade and were highly related to *Bd* genotypes previously isolated from wild amphibians in Chile.

The presence of agnathia and brachygnathia associated with *Bd* infection in postmetamorphic amphibians has not been reported before. Tadpole oral malformations have been associated with low temperatures (49), water contamination (50), nutrition (51), or ecological factors (52, 53). Although, this malformation might have been due to an unknown environmental or other cause, it is likely associated with *Bd* infection of the oral discs of tadpoles (54–56). Absence or reduction in development of the lower jaw may have had a profound impact on the ability of postmetamorphic amphibians (and tadpoles) to acquire food, contributing to death along with chytridiomycosis. The presence of distended gall bladders and the absence of gastrointestinal content in all the animals examined suggests a lack of feeding. Although oral deformations in postmetamorphic amphibians have not been used before as an indication of chytridiomycosis, they might be still important and may indicate an unknown sequela of *Bd i*nfection that may have been overlooked previously. This study highlights the need to use accurate diagnostic techniques such as qPCR or histology to be able to complement these observations (57).

Captive breeding has increasingly been used as a tool for amphibian conservation, but for these initiatives to be successful, several aspects must be considered, including genetic management and biosecurity protocols (58, 59). In our case, newly admitted individuals of *C. gayi* came from a semi-open captive breeding program that was supplied with water from an agricultural canal, in which *X. laevis* had previously been recorded (34). Despite the implementation of quarantine, this was not enough to prevent the introduction of *Bd* to the captive breeding program, causing mortality in the newly admitted animals. This highlights the importance of implementing strict biosecurity protocols against *Bd* (and other pathogens), such as *Bd* testing prior to admittance, preventive antifungal treatment or disinfection of water and materials (59).

Susceptibility to *Bd* is also influenced by environmental factors, such as climate (20). Immune function in amphibians is closely dependent on environmental temperature (60). For instance, low temperature has been associated with a lower survival in *Bd*-exposed frogs under laboratory conditions (60, 61) and chytridiomycosis die-offs have been often associated with higher elevation, lower temperature and winter season (62–64). It is possible that in our case, stress associated with transportation might have induced immunosuppression, facilitating the development of chytridiomycosis. Ramsey et al. (65) demonstrated that natural resistance to *Bd* in *X. laevis* can be reversed with the implementation of sub-lethal immunosuppressive treatments, such as exposure to X-irradiation or norepinephrine injections.

The *Bd* lineage that we isolated from infected individuals in this chytridiomycosis outbreak, *Bd*GPL, has been associated with catastrophic mass mortalities and population declines in multiple continents (22, 48, 66, 67). Over the last four decades, a severe population decline of two species of Darwin's frogs (*Rhinoderma darwinii* and *R. rufum*) populations has occurred across central and south Chile. Retrospective and cross-sectional *Bd* studies and population monitoring data suggest that chytridiomycosis has contributed to these extirpations (33, 48) and that *Bd* has been present in Chile at least since 1970 (33). Although characterization of *Bd* isolates infecting *R. darwinii* has not been achieved so far, the identification of *Bd*GPL over a large area of Chile, provides support to the hypothesis that BdGPL is causing these declines (25).

In our phylogenomic whole-genome analysis, all the Chilean *Bd* isolates (including the two isolates from captive *C. gayi*) grouped together with a genotype isolated in 2009 from the United Kingdom (UKTvB). A similar phylogenetic relationship was observed when restricting the analysis to a subset of the genome spanning a heterozygosity loss event shared by all *Bd*GPL isolates, but in this case, isolates from other European countries and a Canadian isolate also group with the Chilean isolates (25). While there has been no report of mortality in the wild caused by UKTvB, a challenge with this isolate in the Mallorcan midwife toad (*Alytes muletensis*) under laboratory conditions caused a 73% mortality rate (18). It is likely, therefore, that the *Bd*GPL genotypes in Chile are virulent and have caused amphibian mortalities in nature (25). However, detecting mortalities in the wild is often difficult, particularly in cryptic species, for which better surveillance is needed. In addition, we also evaluated the presence of *Frog virus 3* (FV3) ranavirus in the same individuals as a possible cause of mortality, however all tested negative using a qPCR assay. Previously, a mass mortality of adult *C. gayi*, allegedly due to a drought, was described in central Chile, however whether *Bd* was involved in this mortality event is unknown as no fresh carcasses were available for necropsy, *Bd* detection or other diagnostics to be performed (68).

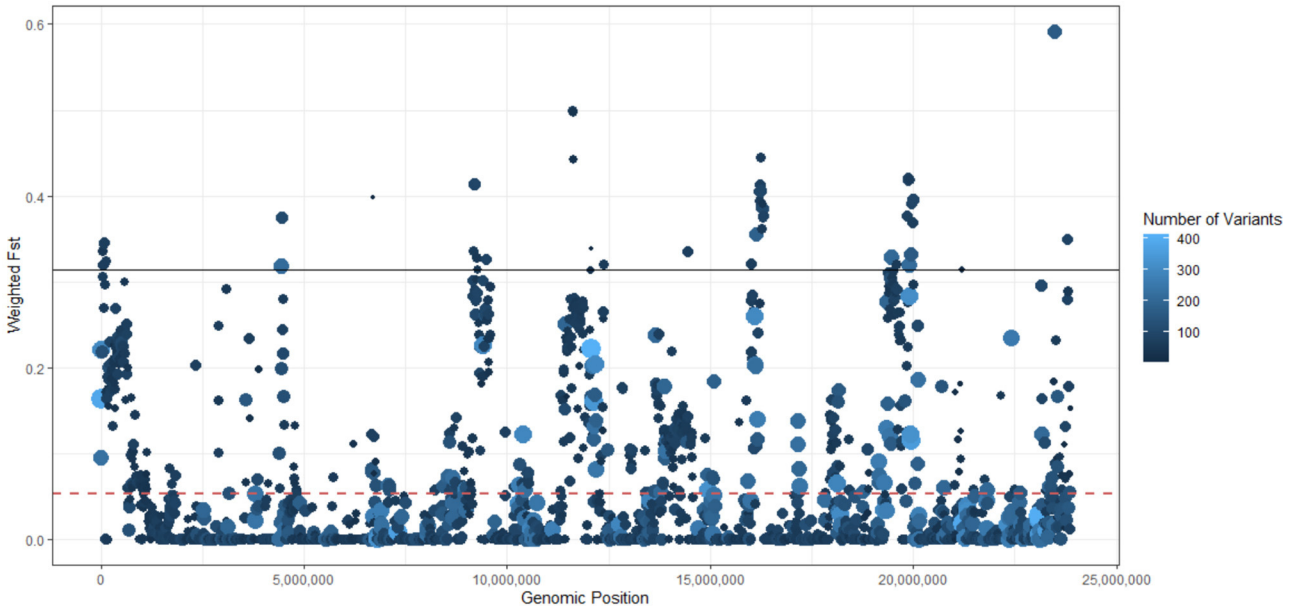


FIGURE 3 | Sliding window analysis of population differentiation of *Batrachochytrium dendrobatidis* (*Bd*) isolates (PA1 and PA2) against other 45 global panzootic lineage of *Bd* (*Bd*GPL) isolates using Weir and Cockerham's F_{ST} estimator. Each point represents a 10 Kb genomic window, with a 5 kb step-size. The dashed red line represents the mean F_{ST} (0.0538). The solid black line represents the 95% quantile threshold of the F_{ST} estimator (0.3141). Each point is sized and colored on a log scale by the number of variants in each window. The legend indicates the color scale (the number of SNPs included in each window varied from 1 to 364, with a median of 31). Point size from small to large is scaled from low to high numbers of variants.

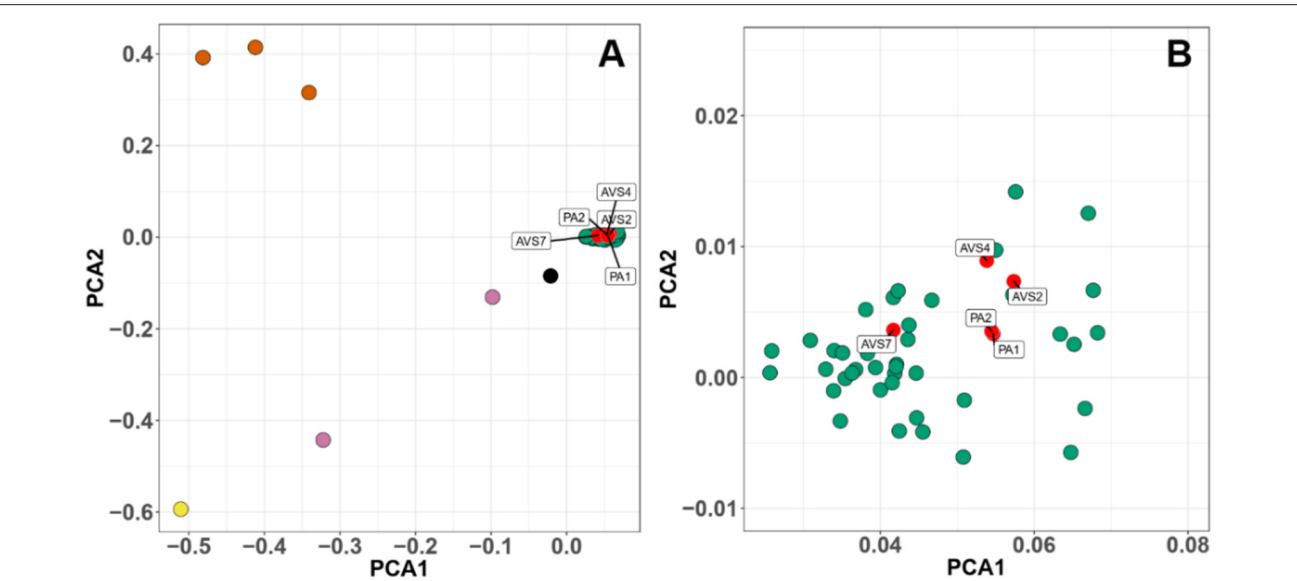


FIGURE 4 | Principal components analysis (PCA) of 3,900 SNPs in linkage equilibrium of a global panel of 54 *Batrachochytrium dendrobatidis* (Bd) isolates. Each point represents an isolate, colored by phylogenetic lineage. **(A)** Bd isolates separate into clearly defined clusters. **(B)** Higher resolution of the global panzootic lineage of Bd (BdGPL) cluster showing the position of the Chilean isolates within the cluster. The axes plot the first and second principal components, PCA1 and PCA2. Chilean isolates are clustered within BdGPL. Chilean isolates (red), other BdGPL (green), BdASIA2/BdBRAZIL (pink), hybrids between the previous two groups (black), BdCH (yellow), BdCAPE (orange).

The low number of segregating sites exclusive to the Chilean *Bd* isolates, compared to the total number of sites where the *Bd*GPL isolates are polymorphic, suggests a single and recent introduction of *Bd* into Chile, possibly through the international movement of amphibians, other aquatic animals, fomites, or tourism (9, 14, 25, 66). Molecular characterization

of further isolates from non-surveyed areas in Chile and neighboring countries (e.g., Argentina and Peru), along with the calibration of a genome-wide molecular clock, is required to confirm this hypothesis. The existence of an apparently unique and recently introduced lineage of *Bd* in Chile differs with the known history of this pathogen in Brazil, where both *Bd*GPL and *Bd*Asia2/*Bd*Brazil coexist, with evidence of multiple interlineage recombination events between them (9, 24). Therefore, together with the potential introduction of novel *Bd* genotypes, interlineage recombination can potentially arise facilitated by the globalization of human and animal transport (16). This highlights the importance of biosecurity measures at the national and local level, to prevent the introduction and establishment of further pathogenic *Bd* lineages, as this pathogen has the capability to increase its genomic diversity through the exchange of haplotypes among lineages (25).

Our work describes for the first time a mass mortality event in the endangered giant Chilean frog from a captive breeding program. We also provide new data on the potential susceptibility of *C. gayi* to the impacts of chytridiomycosis, a species that has been declining fast across its distribution in Chile. The high mortality observed in *C. gayi* with postmetamorphs exhibiting agnathia or brachygnathia as a possible consequence of oral infection with *Bd* in tadpoles has not been described previously and is a condition that can be considered in the monitoring of amphibians maintained in captivity, such as farms, zoos and *ex situ* conservation programs. We described two new isolates of *Bd* in Chile, belonging to *Bd*GPL and clustering in a single group with another three previously isolated *Bd* isolates from central and south Chile (9, 25), for which evidence as a cause of amphibian mortality and population declines is growing.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI SRA; SRS8215364 and SRS8216816.

ETHICS STATEMENT

The animal study was reviewed and approved by Bioethics Committee Universidad Andres Bello and by the Zoological Society of London's Ethics Committee.

AUTHOR CONTRIBUTIONS

MA-R and CA led the manuscript writing. MA-R, PA, and AP-R collected the data. MA-R and AP-R completed the PCR analysis, post-mortem investigation, and Bd isolation. TS, SO'H, and MF competed the whole genome analyses. AV-S, AC, and CA supported data analyses and pathological research. All authors contributed to the manuscript.

FUNDING

This study was funded by FONDECYT Regular number 1211587 (to CA), and the Dirección General de Investigación y Doctorados, Universidad Andres Bello, grant N ○ DI-582-14/I (to MA-R). TS and MF were supported by a grant from Natural Environmental Research Council (NERC; NE/S000844/1) and the UK Medical Research Council (MRC; MR/R015600/1). MF is a fellow in the CIFAR Fungal Kingdoms program.

REFERENCES

1. Blaustein AR, Wake DB. Declining amphibian populations: a global phenomenon? *Trends Ecol. Evol.* (1990) 5:203–4. doi: 10.1016/0169-5347(90)90129-2
2. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues A, Fishman D, et al. Status and Trends of amphibian declines and extinctions worldwide. *Science.* (2004) 306:1783–6. doi: 10.1126/science.1103538
3. Collins JP, Crump ML. *Extinction in Our Times: Global Amphibian Decline*. New York, NY: Oxford University Press (2009).
4. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *PNAS.* (1998) 95:9031–6. doi: 10.1073/pnas.95.15.9031
5. Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. et sp nov, a chytrid pathogenic to amphibians. *Mycologia.* (1999) 91:219–27. doi: 10.1080/00275514.1999.12061011
6. 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.* (2019) 363:1459–63. doi: 10.1126/science.aav0379
7. Goka K, Yokoyama JUN, Une Y, Kuroki T, Suzuki K, Nakahara M, et al. Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Mol Ecol.* (2009) 18:4757–74. doi: 10.1111/j.1365-294X.2009.04384.x
8. Bataille A, Fong JJ, Cha M, Wogan GO, Baek HJ, Lee H, et al. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Mol Ecol.* (2013) 22:4196–209. doi: 10.1111/mec.12385
9. O'hanlon S, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, et al. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science.* (2018) 360:621–7. doi: 10.1126/science.aar1965
10. Byrne AQ, Vredenburg VT, Martel AN, Pasmans F, Bell RC, Blackburn DC, et al. Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation. *PNAS.* (2019) 116:20382–7. doi: 10.1073/pnas.1908289116
11. Garner TW, Perkins MW, Govindarajulu P, Seglie D, Walker S, Cunningham A, et al. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biol Lett.* (2006) 2:455–9. doi: 10.1098/rsbl.2006.0494
12. Fisher MC, Garner TW. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biol Rev.* (2007) 21:2–98. doi: 10.1016/j.fbr.2007.02.002
13. Schloegel LM, Picco AM, Kilpatrick AM, Davies AJ, Hyatt AD, Daszak P. Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). *Biol Conserv.* (2009) 142:1420–6. doi: 10.1016/j.biocon.2009.02.007
14. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, et al. Novel, panzootic and hybrid genotypes of amphibia chytridiomycosis associated with the bullfrog trade. *Mol Ecol.* (2012) 21:5162–77. doi: 10.1111/j.1365-294X.2012.05710.x

15. Kolby JE, Smith KM, Berger L, Karesh WB, Preston A, Pessier A, et al. First evidence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Hong Kong amphibian trade. *PLoS ONE.* (2014) 9:e90750. doi: 10.1371/journal.pone.0090750
16. Azat C. Not just a pathogen: the importance of recognizing genetic variability to mitigate a wildlife pandemic. *Mol Ecol Resour*. (2021) 21:1410–2. doi: 10.1111/1755-0998.13348
17. Bates KA, Clare FC, O'Hanlon S, Bosch J, Brookes L, Hopkins K, et al. Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. *Nat Commun.* (2018) 9:693. doi: 10.1038/s41467-018-02967-w
18. Doddington BJ, Bosch J, Oliver JA, Grassly NC, Garcia G, Schmidt BR, et al. Context-dependent amphibian host population response to an invading pathogen. *Ecology.* (2013) 94:1795–804. doi: 10.1890/12-1270.1
19. Greenspan SE, Lambertini C, Carvalho T, James TY, Toledo LF, Haddad CFB, et al. Hybrids of amphibian chytrid show high virulence in native hosts. *Sci Rep.* (2018) 8:1–10. doi: 10.1038/s41598-018-27828-w
20. Fisher MC, Garner TW. Chytrid fungi and global amphibian declines. *Nat Rev Microbiol.* (2020) 18:332–43. doi: 10.1038/s41579-020-0335-x
21. Flechas SV, Medina EM, Crawford AJ, Sarmiento C, Cárdenas ME, Amézquita A, et al. Characterization of the first Batrachochytrium dendrobatidis isolate from the Colombian Andes, an amphibian biodiversity hotspot. *Ecohealth.* (2013) 10:72–6. doi: 10.1007/s10393-013-0823-9
22. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D, Rodriguez D, et al. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *PNAS.* (2013) 110:9385–90. doi: 10.1073/pnas.1300130110
23. James T, Toledo F, Rödder D, da Silva D, Belasen A, Betancourt-Roman C, et al. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecol Evol.* (2015) 5:4079–97. doi: 10.1002/ece3.1672
24. Jenkinson TS, Betancourt CM, Lambertini C, Valencia-Aguilar A, Rodriguez D, Nunes-de-Almeida CH, et al. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Mol Ecol.* (2016) 25:2978–96. doi: 10.1111/mec.13599
25. Valenzuela-Sánchez A, O'Hanlon SJ, Alvarado-Rybak M, Uribe-Rivera DE, Cunningham AA, Fisher MC, et al. Genomic epidemiology of the emerging pathogen *Batrachochytrium dendrobatidis* from native and invasive amphibian species in Chile. *Transbound Emerg Dis.* (2018) 65:309–14. doi: 10.1111/tbed.12775
26. Burrowes PA, James TY, Jenkinson TS, De la Riva I. Genetic analysis of post-epizootic amphibian chytrid strains in Bolivia: adding a piece to the puzzle. *Transb Emerg Dis.* (2020) 67:2163–71. doi: 10.1111/tbed.13568
27. Feng Y-J, Blackburn DC, Liang D, Hillis DM, Wake DB, Cannatella DC, et al. Phylogenomics reveals rapid, simultaneous diversification of three major clades of Gondwanan frogs at the Cretaceous-Paleogene boundary. *PNAS.* (2017). 114:E5864E5870. doi: 10.1073/pnas.1704632114
28. Vélez CM. *Manejo en cautiverio de la Rana Grande Chilena Capytocephalella gayi (Duméril and Bibron, 1841)*. Santiago: Ediciones Universidad Santo Tomás (2014).
29. IUCNSSC Amphibian Specialist Group. *Calyptocephalella gayi*. *The IUCN Red List of Threatened Species* 2019:e.T4055A85633603. Downloaded on 21 May 2021 (2019).
30. Bacigalupe LD, Soto-Azat C, García-Vera C, Barría-Oyarzo I, Rezende EL. Effects of amphibian phylogeny, climate and human impact on the occurrence of the amphibian-killing chytrid fungus. *Glob Change Biol.* (2017) 23:3543–53. doi: 10.1111/gcb.13610
31. Knapp RA, Morgan JAT. Tadpole mouthpart depigmentation as an accurate indicator of chytridiomycosis, an emerging disease of amphibians. *Copeia.* (2006) 2:188–97. doi: 10.1643/0045-8511(2006)6[188:TMDAAA]2.0.CO;2
32. Pessier AP, Pinkerton M. Practical gross necropsy of amphibians. *Semin Avian Exot Pet Med.* (2003) 12:81–8. doi: 10.1053/saep.2003.127884
33. Soto-Azat C, Valenzuela-Sánchez A, Clarke BT, Busse K, Ortiz JC, Barrientos C, et al. Is chytridiomycosis driving Darwin's frogs to extinction? *PLoS ONE.* (2013) 8:e79862. doi: 10.1371/journal.pone.0079862
34. Soto-Azat C, Peñafiel-Ricaurte A, Price SJ, Sallaberry-Pincheira N, García MP, Alvarado-Rybak M, et al. *Xenopus laevis* and emerging amphibian pathogens in Chile. *Ecohealth.* (2016) 13:775. doi: 10.1007/s10393-016-1186-9
35. Hudson M, Young R, Jackson J, Orozco-terWengel P, Martin L, James A, et al. Dynamics and genetics of a disease-driven species decline to near extinction: lessons for conservation. *Sci Rep*. (2016) 6:30772. doi: 10.1038/srep30772
36. Fisher M, Ghosh P, Shelton J, Bates K, Brookes L, Wierzbicki C, et al. Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Sci Rep.* (2018) 8:7772. doi: 10.1038/s41598-018-24472-2
37. 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) 4:3633–41. doi: 10.1002/ece3.1199
38. Martin M. Cutadapt removes adapter sequences from highthroughput sequencing reads. *EMBnet J.* (2011) 17:10–2. doi: 10.14806/ej.17.1.200
39. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics.* (2009) 25:1754–60. doi: 10.1093/bioinformatics/btp324
40. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. *arXiv*. 1207.3907v2 [q-bio.GN]. (2012).
41. Garrison E. *vcflib: A C++ Library for Parsing and Manipulating VCF Files*. Retrieved from: https://github.com/vcflib/vcflib (accessed November 12, 2016) (2016).
42. Tan A, Abecasis GR, Kang HM. Unified representation of genetic variants. *Bioinformatics.* (2015) 31:2202–4. doi: 10.1093/bioinformatics/btv112
43. Li H, Handsaker B, Wysoker A, Fennell T, Rua J, Homer N, et al. The sequence alignment/map format and SAMtools. *Bioinformatics.* (2009) 25:1–2. doi: 10.1093/bioinformatics/btp352
44. Chapman B. *v0.1.7: Avoid FreeBayes Error*. Retrieved from: https://github.com/chapmanb/bcbio.variation.recall/blob/master/src/bcbio/variation/recall/square (accessed November 12, 2016) (2016).
45. Zheng X, Levine D, Shen J, Gogarten S, Laurie C, Weir B. A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics.* (2012) 28:3326–8. doi: 10.1093/bioinformatics/bts606
46. Wickham H. *ggplot2: Elegant Graphics for Data Analysis. Use R!*.New York, NY: Springer (2009).
47. Jombart T. Adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics.* (2008) 24:1403–5. doi: 10.1093/bioinformatics/btn129
48. Valenzuela-Sánchez A, Schmidt BR, Uribe-Rivera DE, Costas F, Cunningham AA, Soto-Azat C. Cryptic disease-induced mortality may cause host extinction in an apparently stable host–parasite system. *Proc Roy Soc B.* (2017) 284:20171176. doi: 10.1098/rspb.2017.1176
49. Rachowicz LJ. Mouthpart pigmentation in *Rana muscosa* tadpoles: seasonal changes without chytridiomycosis. *Herpetol Rev.* (2002) 33:263–5.
50. Rowe CL, Kinney OM, Congdon JD. Oral deformities in tadpoles of the bullfrog (*Rana catesbeiana*) caused by conditions in a polluted habitat. *Copeia.* (1998) 1:244–6. doi: 10.2307/1447729
51. McDiarmid RW, Altig R. *Tadpoles: The Biology of Anuran Larvae*. Chicago, IL: The University of Chicago Press (1999).
52. Brett MA, Gouchie GM, Wassersug RJ. Can visual stimulation alone induce phenotypically plastic responses in *Rana sylvatica* tadpole oral structures? *J. Herpetol.* (2009) 43:165–8. doi: 10.1670/07-197R3.1
53. Relyea RA, Auld JR. Predator and competitor induced plasticity: how changes in foraging morphology affect phenotypic trade-offs. *Ecology.* (2005) 86:1723–9. doi: 10.1890/04-1920
54. Drake DL, Altig R, Grace JB, Walls SC. Occurrence of oral deformities in larval anurans. *Copeia.* (2007) 2007:449–58. doi: 10.1643/0045-8511(2007)7[449:OOODIL]2.0.CO;2
55. Venesky MD, Parris MJ, Storfer A. Impacts of *Batrachochytrium dendrobatidis* infection on tadpole foraging performance. *Ecohealth.* (2009) 6:565–75. doi: 10.1007/s10393-009-0272-7

56. Patel YA, Cavin JN, Moore MK. Morphological anomalies as indicators of chytrid infection in *Bufo marinus* from Trinidad, West Indies. *Bios.* (2012) 83:75–80. doi: 10.1893/0005-3155-83.3.75
57. Navarro-Lozano A, Sánchez-Domene D, Rossa-Feres DC, Bosch J, Sawaya RJ. Are oral deformities in tadpoles accurate indicators of anuran chytridiomycosis?. *PloS ONE.* (2018) 13:e0190955. doi: 10.1371/journal.pone.0190955
58. Harding G, Griffiths RA, Pavajeau L. Developments in amphibian captive breeding and reintroduction programs. *Conserv Biol.* (2016) 30:340–9. doi: 10.1111/cobi.12612
59. Linhoff LJ, Soorae PS, Harding G, Donnelly MA, Germano JM, Hunter DA, et al. *IUCN Guidelines for Amphibian Reintroductions and Other Conservation Translocations.* 1st ed. Gland: IUCN (2021).
60. Murphy PJ, St-Hilaire S, Corn PS. Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads. *Dis Aquat Organ.* (2011) 95:31–42. doi: 10.3354/dao02336
61. Andre SE, Parker J, Briggs CJ. Effect of temperature on host response to *Batrachochytrium dendrobatidis* infection in the mountain yellow-legged frog (*Rana muscosa*). *J Wildl Dis.* (2008) 44:716–20. doi: 10.7589/0090-3558-44.3.716
62. Berger L, Speare R, Hines H, Marantelli G, Hyatt AD, Skerratt LF, et al. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust Vet J.* (2004) 82:31–6. doi: 10.1111/j.1751-0813.2004.tb11137.x
63. Kriger KM, Pereoglou F, Hero JM. Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. *Conserv Biol.* (2007) 21:1280–90. doi: 10.1111/j.1523-1739.2007.00777.x
64. Savage AE, Sredl MJ, Zamudio KR. Disease dynamics vary spatially and temporally in a North American amphibian. *Biol Conserv.* (2011) 144:1910–5. doi: 10.1016/j.biocon.2011.03.018
65. Ramsey JP, Reinert LK, Harper LK, Woodhams DC, Rollins-Smith LA. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infect Immun.* (2010) 78:3981–92. doi: 10.1128/IAI.00402-10
66. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *PNAS.* (2011) 108:18732–6. doi: 10.1073/pnas.1111915108
67. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *PNAS.* (2010) 107:9689–94. doi: 10.1073/pnas.0914111107
68. Acuña P, Vélez,-R. C, Mizobe C, Bustos-López C, Contreras-López M. Mortalidad de la población de rana grande chilena, *Calyptocephalella gayi* (Calyptocephalellidae), en la Laguna Matanzas, del Humedal el Yali, en Chile central. *Anales MHN27 V*. (2014) 27:35–50.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

TECHNOLOGY AND CODE
published: 04 October 2021
doi: 10.3389/fvets.2021.728232

Tracking, Synthesizing, and Sharing Global *Batrachochytrium* Data at AmphibianDisease.org

Michelle S. Koo[1]*, *Vance T. Vredenburg*[1,2], *John B. Deck*[3], *Deanna H. Olson*[4], *Kathryn L. Ronnenberg*[4] and *David B. Wake*[1]

[1] *Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, CA, United States,* [2] *Department of Biology, San Francisco State University, San Francisco, CA, United States,* [3] *Berkeley Natural History Museums, University of California, Berkeley, Berkeley, CA, United States,* [4] *US Department of Agriculture, Forest Service, Pacific Northwest Research Station, Corvallis, OR, United States*

Emerging infectious diseases have been especially devastating to amphibians, the most endangered class of vertebrates. For amphibians, the greatest disease threat is chytridiomycosis, caused by one of two chytridiomycete fungal pathogens *Batrachochytrium dendrobatidis (Bd)* and *Batrachochytrium salamandrivorans* (*Bsal*). Research over the last two decades has shown that susceptibility to this disease varies greatly with respect to a suite of host and pathogen factors such as phylogeny, geography (including abiotic factors), host community composition, and historical exposure to pathogens; yet, despite a growing body of research, a comprehensive understanding of global chytridiomycosis incidence remains elusive. In a large collaborative effort, *Bd*-Maps was launched in 2007 to increase multidisciplinary investigations and understanding using compiled global *Bd* occurrence data (*Bsal* was not discovered until 2013). As its database functions aged and became unsustainable, we sought to address critical needs utilizing new technologies to meet the challenges of aggregating data to facilitate research on both *Bd* and *Bsal*. Here, we introduce an advanced central online repository to archive, aggregate, and share *Bd* and *Bsal* data collected from around the world. The Amphibian Disease Portal (https://amphibiandisease.org) addresses several critical community needs while also helping to build basic biological knowledge of chytridiomycosis. This portal could be useful for other amphibian diseases and could also be replicated for uses with other wildlife diseases. We show how the Amphibian Disease Portal provides: (1) a new repository for the legacy *Bd*-Maps data; (2) a repository for sample-level data to archive datasets and host published data with permanent DOIs; (3) a flexible framework to adapt to advances in field, laboratory, and informatics technologies; and (4) a global aggregation of *Bd* and *Bsal* infection data to enable and accelerate research and conservation. The new framework for this project is built using biodiversity informatics best practices and metadata standards to ensure scientific reproducibility and linkages across other biological and biodiversity repositories.

Keywords: DarwinCore standards, amphibia, chytridiomycosis, MIxS standards, *Bd*, biodiversity informatics infrastructure, *Bsal*

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH Co. KG, Germany

Reviewed by:
Louise A. Rollins-Smith, Vanderbilt University, United States
David Lesbarreres, Laurentian University, Canada

***Correspondence:**
Michelle S. Koo
mkoo@berkeley.edu

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *21 June 2021*
Accepted: *02 September 2021*
Published: *04 October 2021*

Citation:
Koo MS, Vredenburg VT, Deck JB, Olson DH, Ronnenberg KL and Wake DB (2021) Tracking, Synthesizing, and Sharing Global Batrachochytrium Data at AmphibianDisease.org. Front. Vet. Sci. 8:728232. doi: 10.3389/fvets.2021.728232

INTRODUCTION

The amphibian vertebrate lineage evolved over 360 million years ago, and has survived multiple mass extinction events, yet today amphibians are the most endangered class of vertebrates and may be harbingers of a new sixth mass-extinction event (1). Emerging infectious diseases have been especially devastating to amphibians (1–3). Chytridiomycosis is a potentially lethal amphibian skin disease caused by one of two chytridiomycete fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd)* and *Batrachochytrium salamandrivorans (Bsal*). *Bd* chytridiomycosis, was first discovered over two decades ago (4–6), and later in 2013, *Bsal* chytridiomycosis was detected (7). A growing body of research has shown that amphibian susceptibility to these diseases varies phylogenetically, geographically, and is influenced by synergisms with abiotic and biotic factors (8, 9). However, a comprehensive understanding of the lethal, sublethal, and benign effects of these fungal pathogens and their long-term effects on vertebrates in class Amphibia is still incomplete. Chytridiomycosis has raised alarms and the World Organization of Animal Health (OIE) has listed *Bd* and *Bsal* as reportable pathogens (10) on the global stage. *Bd*, which includes multiple genetic lineages (11, 12), has spread across continents likely by human actions, and in some regions it has invaded naive host populations causing epizootics (epidemics in wildlife) that affect hundreds of species (13–15). The discovery of chytridiomycosis and the documentation of its impacts on amphibians have fundamentally altered the way scientists view emerging infectious diseases, their contributions to global biodiversity losses, and biodiversity conservation approaches to emerging disease threats (3, 8, 16).

We are facing a rapidly changing scientific knowledge landscape for amphibian emerging infectious diseases in the 21st Century (7), which has increased the challenges for reporting and tracking advances (6). Indeed, the relatively recent discovery of the *Bsal* pathogen (7) has shown that we must be nimble in our approach to data management and analysis and adapt to new diseases and new technologies. Can we predict the data management needs of the next emerging disease that will infect amphibians or other wildlife species? What we do know is that sharing data and responding rapidly is essential to disease mitigation. As a scientific community we can heed lessons learned from our collective experience in *Bd* research for the last two decades. For example, we know that *Bd* can devastate entire amphibian populations (13) and entire amphibian communities quickly (14, 17). In outbreaks in Panama, 50% of local amphibian species were extirpated (17), and in Peru, 40% of species were extirpated (14). The advent of the discovery of *Bsal* motivated the formation of the North American *Bsal* Task Force (18), which included representatives from governmental, academic, and advocacy organizations in a broad coalition across the USA, Canada, and Mexico. Two of us (MK, DO) lead the *Bsal* Task Force Data Management Working Group. Our *Bsal* Task Force discussions clearly showed consensus for coordinated efforts in planning for when, not if, *Bsal* would eventually be detected in North America (19, 20). All parties agreed it is essential to track, archive, and quickly share sampling efforts for *Bsal*.

The first effort to compile *Batrachochytrium* occurrences for online access and mapping, beginning in 2007, resulted from a collaboration between the USDA Forest Service and Imperial College London known as *Bd*-Maps (21). The chytridiomycosis research community soon turned to *Bd*-Maps as the main source for compiled global data on *Bd* (21). This has been a labor-intensive aggregation of global *Bd* sampling efforts including >33,000 data records at >14,000 unique site coordinates to date (22). Based on both the *Bd*-Maps data and unique records recently summarized by Castro Monzon et al. (23), *Bd* has been detected in 1,375 of 2,525 (55%) species (composed of 88% of frog families, 100% of salamander families, and 70% of caecilian families) and 93 of 134 (69%) countries sampled to date [see (22) for details]. Over time, the labor-intensive methodology and unfunded infrastructure of the *Bd*-Maps became unsustainable. A new urgency arose when *Bsal* was discovered (7), and we embraced the challenge of how best to share data as quickly as it was produced and verified. Aggregating disease data can immediately address fundamental questions about where sampling effort has been applied, where disease occurrences are documented, and which species are affected, in addition to identifying active researchers to facilitate collaboration. In particular, it has become increasingly important to not only document known occurrences of *Bd* and *Bsal*, but also known instances of negative data (i.e., samples tested for *Bd* and/or *Bsal* that did not find evidence of infection), which by themselves may not be suitable for publication in many peer-reviewed journals. These data are critically important, however, in predictive distribution modeling [e.g., (22, 24)], and in examining host species traits such as phylogeny, habitat use, or behavior that may explain host infection or disease susceptibility [e.g., (25, 26)], and help understand the synergisms of co-occurring chytrid fungi [e.g., (27)]. If samples have linked genetic and genomic data, pathogenic fungal migration and evolution can be examined, revealing new insights on virulence, novel introductions, and origins of pathogens using phylodynamics [e.g., (11, 28–30)]. In addition, negative data can help describe the timing of pathogen invasion, which can also help in our understanding of present-day dynamics (31).

We introduce here an open-access repository and archive for *Batrachochytrium* data called the Amphibian Disease Portal (https://amphibiandisease.org) that addresses two urgent needs: (1) to create a sustainable, modernized repository to aggregate and rapidly share data on the fungal pathogens of amphibians *Bd* and *Bsal*; and (2) to upgrade and migrate the *Bd*-Maps datasets to a new repository that can continue to grow.

METHODS AND MATERIALS

In the creation of this online resource, we considered a broad range of users, facilitated by discussions with the *Bsal* Task Force, the AmphibiaWeb steering committee, and members of natural history museums and other institutions that provide biodiversity informatics data to users online. When planning how to store, structure, and share the data we considered the needs of conservation biologists, disease ecologists, evolutionary

biologists, resource managers, and many others. We aimed to follow several principles to achieve these goals. The Amphibian Disease Portal was developed to: (1) prioritize structured data, data quality, and online accessibility, maximizing its usefulness and accessibility with modern web technologies and standards; (2) offer tangible benefits to users who are contributing their data (for the purposes of this paper, we refer to researchers who submit data as "contributors," and those who access data for summaries or downloading for analysis as "portal users"); (3) be sustainable and cost-efficient for both maintenance and users (contributors and portal users will not be charged); and (4) support reproducible and replicable (i.e., repeatable methods with the same or comparable data, respectively, to produce the same results) data-driven science.

We initiated development in 2015, and the final architecture for the portal is described here. The overall architecture can be divided as a database "backend" and a user interface "frontend." Many of our core goals align with an established metadata repository, the Genomic Observatories Metadatabase or GEOME (32), especially with respect to biological data management and structured, internet-accessible data. The GEOME repository offers a suite of user-friendly tools to manage and aggregate standardized biological sample data with its derivative genetic data, including associated geospatial, diagnostic, and publication context data [see [32] for core architecture]. We adopted the GEOME platform as the database and validation service for the ADP's "backend." To meet the needs of research access and data access, we developed a dedicated "frontend," a user-friendly website where all users can interact, visualize, and discover data. In addition, we created a middleware application programming interface (API) that communicates with the GEOME "backend." Together, they comprise of the frontend, API, and backend components forming the Amphibian Disease Portal (**Figure 1**); all code is open-source and available on Github in two code repositories licensed as GNU General Public License (33), one for its frontend website (https://github.com/BNHM/AmphibiaWebDiseasePortal) and one for the API (https://github.com/BNHM/AmphibiaWebDiseasePortalAPI). We describe how we address and meet the principles and goals of the project with respect to its infrastructure.

The Amphibian Disease Portal has four interwoven Goals. (1) A key aim of the repository is to provide structured data, data quality, and online accessibility. GEOME is founded on a principle of providing interlinked and machine-readable data over the Internet, while drawing on standardized vocabularies from the scientific community. We built the repository with a focus on biological samples, and recognized the need to integrate data that may be housed in separate databases, especially gene-sequence data, which is critical in furthering our understanding of *Bd* and *Bsal* disease dynamics utilizing the field of phylodynamics (30). To achieve this goal, the portal uses DarwinCore (34) and MIxS (35) metadata standards, now common among biodiversity data repositories. The GEOME validation process checks data requirements such as appropriate diagnostic fields for *Bd* and *Bsal*, compliance with the AmphibiaWeb taxonomy (36), and controlled vocabularies for country, disease type, sample type, and basis of record.

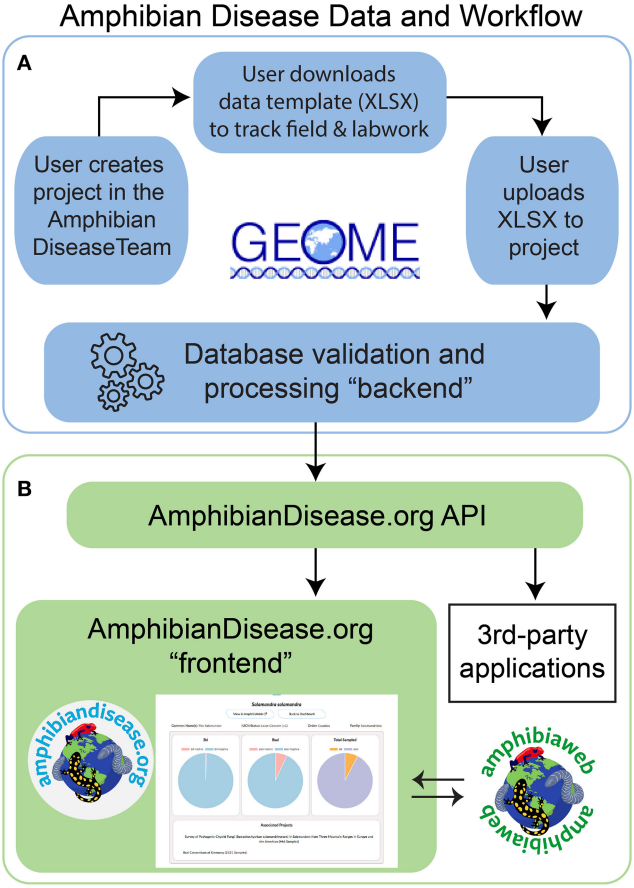


FIGURE 1 | Amphibian Disease Portal data and work flow. **(A)** Users begin the process of contributing data by creating a project in Geome under the Amphibian Disease Team; user can download a data template for field and laboratory work. Once data gathering is complete, user uploads to the project for processing at Geome, and data are now available via the AmphibianDisease API. **(B)** The "frontend" includes the website AmphibianDisease.org where aggregated data can be graphed, mapped, queried, and downloaded. The AmphibianDisease API allows data to be accessible by any third-party application that can integrate web services like R Statistics. AmphibiaWeb site reciprocally links to Amphibian Disease species charts when data are available for a given species.

(Examples of controlled vocabularies are included in **Table 1** and are lists of terms from which users can choose). Datasets with the DarwinCore fields of institutionID, collectionID, and catalogNumber constitute a global unique identifier for a cataloged specimen in a natural history museum collection, and may be linked to a biodiversity aggregator (e.g., Global Biodiversity Information Facility, GBIF); likewise, contributors may provide a Uniform Resource Identifier (or URI, e.g., a unique web address) for the field "associatedSequences" to link to external genetic repositories (i.e., National Center for Biotechnology Information, NCBI), for genomic Sequence Read Archive (SRA) or other molecular data. Contributors can choose to submit data directly to SRA through GEOME using its "FASTQ" module, in which case the sample is

automatically linked between GEOME and SRA using the material Sample Identifier.

(2) We prioritized tangible benefits to the user to incentivize the contributions of researchers to the repository. Therefore, we considered the needs of the contributor foremost, including how and when to make a dataset publicly accessible. Many datasets are created for publication, and may have pre-publication restrictions (e.g., a graduate student's dissertation); hence, contributors can make newly uploaded datasets private and thus not searchable or accessible until a later date (e.g., when the study is published).

Even before uploading data, contributors may benefit from project planning and data-management tools provided by the portal. In GEOME, the Amphibian Disease Portal is identified as a "Team," and all *Bd* and *Bsal* projects that are part of the team adhered to the database rules of the portal. These projects share not only database rules for *Bd* and *Bsal* samples but customized and customizable data templates. Contributors are encouraged to download and use these templates in Microsoft Excel format (XLSX) for data collection in the field and lab (See **Supplementary Material** for example templates). Instructions are included in each template file where fields and controlled vocabulary are defined and are clearly labeled. Once completed, the same file can be used to upload directly to the database without complicated formatting requirements. The portal has a minimum set of required fields and many optional fields from which users can customize their own template. Currently the Amphibian Disease Portal provides two different template configurations to any participating project: one designed for samples from museum specimens, and another addressing catch-and-release specimens from the field. We will continue to add new templates as new use cases arise, such as one for environmental samples and for laboratory or captive specimens. Along with an online guideline and instructions, we aimed to make data management easier during the data-collection phase.

Closing the cycle of a project for contributors is usually the publication step; journals are increasingly requiring authors to provide their data in an accessible archive. Data uploaded to the portal are provided with archival resource keys (ARK), a form of digital object identifier (DOI), generated by GEOME using California Digital Library's EZID service (37), and have satisfied data access requirements for journals to ensure that the data are citable, accessible, and credited.

(3) We designed the portal for flexibility, accessibility, and sustainability. By using representational state transfer (REST) web services (or the application programming interface, API), we display database-supplied data in easy-to-use formats, which appear on the Amphibian Disease Portal website via open access JavaScript libraries. Having data accessible through web services also allows third-party applications (e.g., R Statistics software) and other programming tools (e.g., Python) access to data without requiring secured database access. The portal's programming interface scripts are written in Python and allow for specialized processing such as matching taxonomy and synonyms from

TABLE 1 | Comparison of terms in legacy and current database.

Bd-Maps (legacy terms)	**Amphibian disease (DarwinCore compliant terms)**
• Record Number	• MaterialSampleID (unique identifier)
• SpecimenID	• otherCatalogNumber
• StartDay, StartMonth, StartYear, EndDay, EndMonth, EndYear	• dayCollected, monthCollected, yearCollected required For range of dates, these are noted in occurrenceRemarks
Location – (these generally had a DarwinCore counterpart)	
• Continent, Country, Region	• continentOcean, country, state_province
• Location	• locality
• Location Number	• locationID
• Latitude, Longitude	• decimalLatitiude, decimalLongitude
• Elevation (in meters)	• minimum ElevationInMeters, maximum ElevationInMeters
• CoordinateSource	• georeferenceProtocol (method and sources for determining coordinates)
• CoordinateAccuracy (free text)	• coordinateUncertaintyInMeters • georeferenceRemarks (derivation of coordinates, assumptions, and notes if coordinates are centroid-based)
• Order, Family, Genus, Species	• Order, Family, Genus, Species (validated using AmphibiaWeb taxonomy; synonyms applied)
• Species cf/aff/kl, Synonyms	• taxonRemarks (for additional specific epithet notes or remarks)
• WildCapt	• organismRemarks Controlled vocabulary of "wild," "captive"
• MusSpec	• basisOfRecord Controlled vocabulary of "PreservedSpecimen" "LivingSpecimen," "MaterialSample" etc. The term "Event" is used to distinguish Bd-Maps project data
• Test P/N/Q or U	• diseaseDetected Controlled vocabulary of "TRUE," "FALSE"
Bd Sampling Fields Fields are tallies of lab results per location	
• BdDet – number of amphibians that had a positive Bd test	To map "BdDet": • diseaseTestedPositiveCount "BdNeg" is not mapped.
• BdNeg - number of amphibians that had a negative Bd test	To map "Tested": • individualCount
• Tested - number of amphibians that were tested for Bd	To map "Test Positive/Negative": • diseaseDetected
• Test Positive/Negative – binary of P or N to facilitate filtering	Allowed values of "TRUE," "FALSE"
Site-level Summary Fields are used for spatial metadata-analysis [see Olson et al. (22)], and are tallies for locations that share a locationNumber	Given the specific methodology used to compile these counts, these fields are concatenated to be included as a record note, with reference to Olson et al.

(Continued)

TABLE 1 | Continued

Bd-Maps (legacy terms)	Amphibian disease (DarwinCore compliant terms)
• Site Detected/Not Detected (D/ND) – tally of records for positive and negative samples • Species with Detection – number of species with positive Bd detection • Species Tested – number of species tested	• eventRemarks
• LifeStage, Sex	• lifeStage • Sex Both are controlled vocabulary fields
• Method Free text for diagnostic test method	• testMethod Controlled vocabulary for diagnostic test method; if more than one, note added to measurementRemarks
• Publication (also general provenance field if from unpublished source) • Data Source Type	Concatenated into: • associatedReferences
• Contact • Lab	• principalInvestigator • diagnosticLab
Other observations • Morbidity, mortality	If binary, • Fatal Allowed values of "TRUE," "FALSE" If free text, then concatenated into: • occurrenceRemarks

the AmphibiaWeb database. Using web services or APIs for the website has other development advantages, as well. We can deploy web developers as they are available from other projects for maintenance or let multiple developers work on new features for the site without relying solely on a database administrator, who has different skills and more strict access requirements. Thus, the architecture of the Amphibian Disease Portal allows for nimble management and enhancements as funding and needs change, providing a more sustainable project.

(4) Lastly, we aimed to support reproducible and replicable data-driven science. Reproducibility in science, under which independent researchers can repeat study results, requires access to the original sample data, yet published papers rarely contain those data and instead provide summarized data. Often the burden of providing the original data falls on scientists who have neither the time nor capacity to adequately store and retrieve data on request [e.g., (38)]. Replication in science, when applying comparable datasets to methods to test outcomes, requires access to datasets collated in a comparable manner, which also requires access to the original data. Submitting data to discipline-specific repositories compliant with well-known metadata standards, such as this portal, will reduce the future burden on the scientists who produced the data, and will provide aggregated data for re-use in potentially novel studies.

RESULTS

The outcome of this needs-based effort is the Amphibian Disease Portal, an online site with a user-friendly interface created in partnership with the GEOME repository. We established a user workflow that is easy to follow, cyber-secure, and makes data discoverable and accessible in a single site (**Figure 1**). Data and workflow include these steps: (1) registered contributors initiate projects based on their study and can use customized data templates in MS Excel format for project management (the same template, when completed after field and lab work is done, can be uploaded to their project); (2) uploaded data are validated against the portal's database rules ("backend"), which are defined in the template (feedback during this process is designed to help contributors correct and successfully load their data); (3) when the data are made public by the contributor, either immediately on upload or subsequent to publication, they will then be harvested and processed by the portal's web service; and 4) the data from public projects become output to the Amphibian Disease Portal ("frontend") and other third-party applications (e.g., R program analyses).

The main website (https://amphibiandisease.org) is comprised of: (1) a basic map-based query interface allowing for filtered or spatial searches and mapping; (2) a dashboard of summary statistics and dynamic charts by country and by species; (3) search interfaces for projects, species, and datasets; (4) various other information pages such as how to contribute data and a blog (**Figure 2**). Each species in the portal has a dedicated dashboard page to display aggregated *Bd* and *Bsal* samples with links to contributing projects. Graphs of species data (**Figure 2B**) are dynamic data-driven and reciprocally linked by URL to the respective species account page in AmphibiaWeb (https://amphibiaweb.org). The scripts enabling these reciprocal links are adaptable to other external websites as well.

With respect to the integration of the legacy Bd-maps dataset into the portal, our challenge in modernizing and migrating the *Bd*-Maps data is to overcome the differences in their original conception and data structure. Instead of a biological samples-based approach, *Bd*-Maps database compiles locality-based summaries by species as reported in diverse ways in the literature. Entries generally were not submitted by authors but compiled from literature queries or sent to the data manager as unpublished observations (21, 22). We elucidate the transition with an important migration step: matching all *Bd*-Maps fields to the DarwinCore standard equivalent used in the Amphibian Disease Portal. **Table 1** compares the two respective metadata schemas.

Fields for date, taxonomy, and geography such as latitude and longitude, for example, were relatively straightforward to map. Three *Bd*-Maps fields that tally counts of total positive *Bd* samples ("BdDet"), total samples ("Tested") and total number of samples with low zoospore levels which made them questionable ("BdQues" or "BdUnc") required different accommodations, and are important to *Bd*-Maps based analyses [e.g., (21, 22)]. These fields were dependent on the "Location Number" field, which is matched to the portal field "locationID." To make these data more usable in future studies, "BdDet" is mapped to a

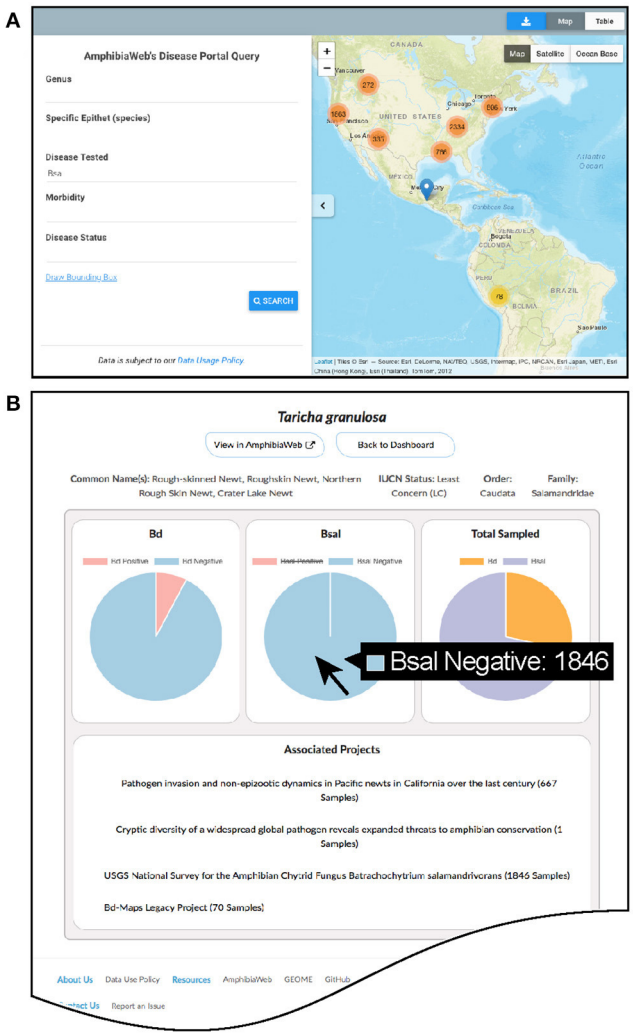


FIGURE 2 | Examples of the Amphibian Disease portal's user-friendly pages. **(A)** Basic query and interactive mapping interface showing zoom-dependent clustering of data points with counts. **(B)** Dynamic graphing page for species showing proportion and counts of *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) data with relevant links to projects (example species *Taricha granulosa*). Hovering over pie chart will display the specific counts of samples. All graphs in the Data Dashboard section of the website are enabled by the Amphibian Disease web services.

new field "diseaseTestedPositiveCount" and "Tested" is mapped to "individualCount." Together they can be used to estimate prevalence ("diseaseTestedPositiveCount"/"individualCount") for any given spatial aggregation or help recreate *Bd*-Maps by summarizing results for all records sharing a given "locationID." Many of the other *Bd*-Maps data, especially those specialized for previous analysis are not abandoned but shifted to remarks fields. The Amphibian Disease Portal field "occurrenceRemarks" contains observations on morbidity and mortality; if mortality was noted, the portal field "fatal" was marked as "TRUE." Details on the source and derivation for a *Bd*-Maps locality are captured in "georeferenceProtocol" and "georeferenceRemarks." Other *Bd*-Maps notes on data sources (e.g., museum vs. field) or record type (species records as opposed to full records; 22) are concatenated into "occurrenceRemarks." Finally, meta-analysis observations on number of *Bd* positive and negative species at a location are compiled into "eventRemarks" such that each record indicates whether a site has the disease Detected (D) or Not Detected (ND), number of species with positive *Bd* detection and the total number of species tested, separated by pipes for subsequent parsing by users; for example, "D | 2 | 4" indicates "*Bd* Detected, 2 positive out of 4 tested". (For details of the Bd-Maps legacy database fields, refer to **Appendix S1** of 22.)

Although usage, interest, and records in the portal are in their early growth stages, we observe trends from early user submissions, which may reflect broader research priorities and reveal useful patterns and gaps in effort. A total of 62,045 samples from 2,760 taxa and 128 countries are shown in 5-degree bins in **Figure 3**, including 34,267 records from the legacy *Bd*-Maps database (22) and excluding private datasets. Of these initial samples, about a quarter (26%) of the samples are derived from voucher specimens deposited in natural history museum collections, whereas the majority of records come from field surveillance and sampling of live amphibians globally. The repository includes the results from the first US-wide survey for *Bsal* (from May 2014 through August 2017) conducted by the US Geological Survey (39); more than 11,000 amphibians from reserves across the US were tested for *Bsa*l and all were *Bsal*-negative, providing a valuable baseline for future *Bsal* monitoring in North America. Lastly, one contributor has submitted pathogen sampling data from an ongoing monitoring program that uses environmental DNA (eDNA) analyses, in which organismal DNA either from shed skin cells or directly from microbes is found in terrestrial or aquatic habitats [e.g., (40, 41)]. The portal is flexible in its structure to accommodate these types of samples while adhering to metadata standards. The dynamic displays will be adapted to differentiate these records, which undoubtedly will grow in usage especially as a means of early detection.

DISCUSSION

The great amphibian epizootic, caused by *Bd* and *Bsal*, is on track to becoming a significant factor in an unfolding mass extinction event as extensive as the previous five recorded in Earth's geologic history (1, 43). Unlike previous extinction events, however, this one is unusual in that it is occurring over a significantly shorter time period (43), and at least for amphibians, disease is a major factor (1, 9, 23). Globally, amphibian chytridiomycosis has changed the way that we think about and understand wildlife disease (8, 44). For example, when amphibians were first reported to be mysteriously declining and disappearing in the late 1970s and early 1980s in Australia, Central America, and western North America (45), few imagined so much of the decline could be driven by epizootics caused by a single pathogen [(46, 47) but see (48)]. At the time, our relatively poor understanding of the biology of Chytridiomycete fungi and a general perception that a fungal pathogen could not drive populations or species

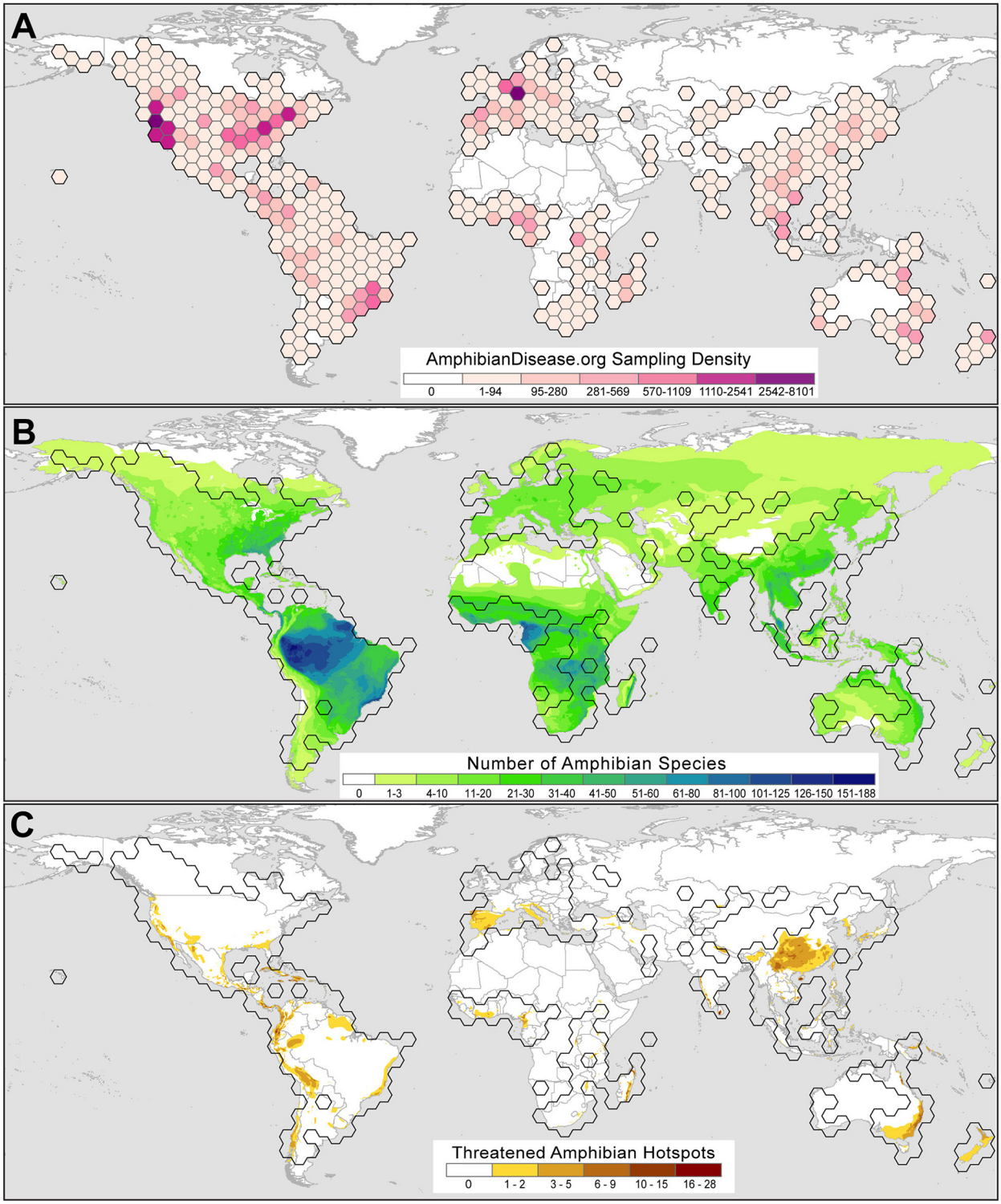


FIGURE 3 | Amphibian Disease sampling point data for the chytrid fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) summarized by 5-degree latitude and longitude bins as of June 2021. **(A)** *Bd* and *Bsal* sampling is worldwide and reflects prominent sampling efforts for *Bsal* in the USA by the US Geological Survey (39) and in Germany by the *Bsal* Consortium Germany (42). Sampling strength is classified using natural breaks with Jenks optimization where darker colors denote greater numbers of samples per bin. **(B)** *Bd* and *Bsal* global sampling with respect to amphibian species alpha-diversity, where darker gradient denotes greater number of amphibian species. **(C)** *Bd* and *Bsal* global sampling with respect to threatened amphibian species, where darker gradient denotes greater number of threatened amphibian species. Range map sources: AmphibiaWeb (2021; https://amphibiaweb.org) and IUCN Red List (2021; https://www.iucnredlist.org). Threatened species status: IUCN Red List (IUCN 2021, accessed June 10, 2021).

to extinction, likely contributed to the fact that it took nearly 20 years to identify *Bd*, discovered in 1998 (4) and described in 1999 (5), as a proximal cause of amphibian declines and extinctions.

The study of chytridiomycosis has led to significant breakthroughs in our understanding of the factors that can lead to outbreaks (epizootics) and population collapse, or non-fatal pathogen infections of hosts (9, 49). Yet, our ability to predict infection outcomes has been hampered because of the great variation in the timing of when this pathogen invaded different continents, the array of species traits in the numerous amphibian hosts, the biotic conditions under which it can infect amphibians, and the multiple genetic lineages differing significantly in virulence (8, 11, 12). Studies have described widely divergent host-pathogen dynamics in this system. Some characterize *Bd* host dynamics as stable enzootics where hosts do not succumb (50), whereas in other settings, even though they may consist of the same host species, living in the same nearby environment, *Bd*-host dynamics are characterized as epizootic, and hosts suffer mass die-offs (13). It has become evident that our ability to understand this disease and predict the outcome of infection relies on many complex variables including but not limited to the timing of pathogen invasion (31). Thus, understanding present-day disease dynamics may require describing the past. The Amphibian Disease Portal contains critical archival *Bd* data that provide unique historic insights. Cheng et al. (51) demonstrated that amphibian museum specimens can be successfully tested for the presence of *Bd*, and thus opened the door to studies of *Bd* temporal dynamics over long timescales (over a century). Other studies followed using museum specimens to help describe *Bd* distributions in the past (31, 52–65), revealing the unique value of museum specimen collections to disease ecology, and the importance of negative data. This realization has set next priorities to develop a voucher specimen lookup service, which will facilitate incorporation of collection records in data templates and increase data quality.

Currently, the data from the growing base of portal users show that global amphibian disease sampling efforts are uneven (**Figure 3A**); baseline *Bd* and *Bsal* sample data cover some amphibian hotspots but only lightly sample others (**Figure 3B**). For example, the Amazon basin is the most species-rich region for amphibians and yet sampling is sparse. Likewise, there are few samples from the range of the Western Ghats (India), which has high beta-diversity. However, a baseline of *Bd* and *Bsal* data for the Appalachian region in southeastern North America is forming, which is a global hotspot for salamander diversity (24, 66). The portal can visualize *Bd* and *Bsal* sampling in areas where threatened amphibian species occur [species with IUCN Red List status of Critically Endangered, Endangered or Vulnerable, (67)] and may help prioritize monitoring and surveillance efforts for the *Bd* and *Bsal* pathogens (**Figure 3C**). Regions where an accumulation of threatened species are known and baseline *Bd* and *Bsal* data have been collected include Central America south through the Andean cordillera (S. America), regions in which studies have increased our understanding of chytrid disease dynamics [e.g., (13, 51, 68, 69)].

Outcomes of chytridiomycosis also are influenced by host species (70, 71), pathogen lineage (72, 73), host community (74, 75), host microbiome (49, 76–79), abiotic conditions (75, 80), and host and pathogen population genetics (11, 12, 72). This complex reality requires collaboration between research groups allowing for sharing and visualization of original, raw data (not summarized data), in ways that are not currently possible with the peer-review-based, publication-based science that is currently the norm. The Amphibian Disease Portal provides a platform to share data on pathogen lineage, host traits, and a suite of metadata associated with where the samples were collected (various abiotic and biotic factors) as well as other potential cofactors that may yet be identified.

Disease ecology, in particular the study of emerging infectious diseases, is decidedly hampered by the standard peer-reviewed science approach because negative data are not typically published. For emerging infectious diseases, knowing that a host population was negative for a pathogen before pathogen invasion and emergence is critically important. Researchers need to be able to share pre-publication data with trusted collaborators (password protected). In conservation emergencies, they should also be able to share data in a completely open format (when appropriate) in ways that many current peer-review-based, scientific publications do not allow. Knowing whether a host population is naïve to an invading pathogen completely alters the prediction of disease outcome (50). If hosts are naïve to a pathogen, they are predicted to be much more vulnerable to epizootic dynamics and host mortality (50) than if they have previous experience with the pathogen (50, 81). The Amphibian Disease Portal provides that missing scientific platform to archive host/pathogen data by providing unique and citable digital object identifiers (DOI), making them available for the benefit of science and for the conservation of species. The uploaded data in the portal must follow strict, yet simple, rules that ensure compatibility of data across studies. Researchers must upload specific details that do not vary across projects but are given the opportunity to provide additional data (e.g., host size and weight) that might prove to be important after further analysis. The portal also gives researchers the option of storing their data in a private, password-protected environment when necessary; however, if the situation is deemed an emergency, researchers can quickly make the data available to the public. With the creation of the Amphibian Disease Portal, we show that we can harness new technologies to increase collaboration and communication among scientists globally using a workflow that is simple, sustainable, and low-cost. We provide a place to rapidly access data not only from published papers, but also from researchers who are willing to share data not yet published. The Amphibian Disease Portal enables digital object identifier assignments for these data regardless of whether they are published in a journal and allows scientists to cite their data on a stable platform that is permanently accessible. Because the portal stores original data and not summarized data, researchers are able to access the raw sample data and potentially analyze them in novel ways. This is significant because it means that data will be more easily compiled and compared across different studies. For research that is global in scope, such as studies of emerging infectious

diseases (e.g., the great amphibian panzootic), being able to access and share verified data across studies is essential. In addition, the portal provides a flexible framework that allows for new research results to be added quickly and efficiently. For example, new studies are showing that eDNA methods (82) can detect the presence of *Bd* in aquatic systems, and this could greatly increase sampling across geographic space and could help direct limited resources toward organismal surveys in key areas. Additionally, the portal can link to existing biodiversity datasets to integrate access to genomic/genetic raw sequence data that could facilitate research on lineages of *Bd* pathogens (11, 12, 83–85). Thus, the portal utilizes critical functions of modern biodiversity data repositories and promotes open science practices. The lessons of the past decades of emerging infectious disease studies in amphibians have focused our attention on a few focal pathogens with the recognition that we need to expand our temporal and genomic investigations. The Amphibian Disease Portal is designed to maximize compatibility, access, and value of data to understand the current disease dynamics and to predict and adapt to future disease threats.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://amphibiandisease.org and https://geome-db.org/workbench/project-overview?projectId=291.

AUTHOR CONTRIBUTIONS

MK conceived, designed, led development, and writing of the study. VV conceived, consulted, and wrote the study. JD designed and wrote the study. DO, KR, and DW consulted and wrote the study. All authors approved the final version of this manuscript.

FUNDING

Funding for initial development was provided by a USDA Forest Service Cooperative Agreement #15-CR-11261953-098 (University of California Berkeley) and a Belmont Forum Project NSF #1633948 to Vredenburg. The use of trade or firm names is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

ACKNOWLEDGMENTS

We thank developer Philip Kahn and web developer Diana Lovette, who brought creative enthusiasm. We are indebted to early adopters of the Amphibian Disease portal: David Blackburn, Allison Byrne, Alessandro Catenazzi, and Greg Jongsma. We dedicate this paper to David Wake who passed away while we were finalizing the manuscript; his commitment to open-access data to better inform decisions and facilitate research to save amphibians has inspired generations of students and projects like this.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.728232/full#supplementary-material

REFERENCES

1. Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci USA.* (2008) 105 (Supp 1):11466–73. doi: 10.1073/pnas.0801921105
2. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. *Science.* (2004) 306:1783–6. doi: 10.1126/science.1103538
3. IPBES. Workshop Report on Biodiversity and Pandemics of the Intergovernmental Platform on Biodiversity and Ecosystem Services. Daszak P, das Neves C, Amuasi J, Hayman D, Kuiken T, Roche B, et al. Bonn, Germany (2020).
4. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of australia and central america. *Proc Natl Acad Sci USA.* (1998) 95:9031–6. doi: 10.1073/pnas.95.15.9031
5. Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. et sp. nov. a Chytrid Pathogenic to Amphibians. *Mycologia.* (1999) 91:219–27. doi: 10.1080/00275514.1999.12061011
6. Olson DH. A decade of herpetological disease papers: puzzle pieces of a bigger picture. *Herpetol Rev.* (2019) 50:37–40.
7. 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 USA.* (2013) 110:15325–9. doi: 10.1073/pnas.1307356110
8. Blaustein AR, Urbina J, Snyder PW, Reynolds E, Dang T. Hoverman JT, et al. Effects of emerging infectious diseases on amphibians: a review of experimental studies. *Diversity.* (2018) 10:1–49. doi: 10.3390/d10030081
9. Fisher MC, Garner TWJ. Chytrid fungi and global amphibian declines. *Nat Rev Microbiol.* (2020) 18:332–43. doi: 10.1038/s41579-020-0335-x
10. OIE [World Organization of Animal Health]. *Aquatic Animal Health Code, Disease Listed by the OIE, Chapter 1.3.* (2019). Available online at: https://www.oie.int/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access (accessed June 15, 2021).
11. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc Natl Acad Sci USA.* (2011) 108:18732–6. doi: 10.1073/pnas.1111915108
12. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D, Eastman JM, et al. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proc Natl Acad Sci USA.* (2013) 110:9385–90. doi: 10.1073/pnas.1300130110
13. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc Natl Acad Sci USA.* (2010) 107:9689–94. doi: 10.1073/pnas.0914111107
14. Catenazzi A, Lehr E, Rodriguez LO, Vredenburg VT. *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper manu national park, southeastern Peru. *Conserv Biol.* (2011) 25:382–91. doi: 10.1111/j.1523-1739.2010.01604.x
15. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, et al. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth.* (2007) 4:125–34. doi: 10.1007/s10393-007-0093-5

16. Garner TWJ, Schmidt BR, Martel A, Pasmans F, Muths E, Cunningham AA, et al. Mitigating amphibian chytridiomycoses in nature. *Phil Trans R Soc B.* (2021) 371:20160207. doi: 10.1098/rstb.2016.0207
17. Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, et al. Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proc Natl Acad Sci USA.* (2006) 103:3165–70. doi: 10.1073/pnas.0506889103
18. North American *Bsal* Task Force [NABTF]. Annual Report. (2016). Available online at: https://www.salamanderfungus.org/task-force/ (accessed November 1, 2020).
19. 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) 11:e1005251. doi: 10.1371/journal.ppat.1005251
20. Yap TA, Nguyen NT, Serr M, Shepack A, Vredenburg VT. *Batrachochytrium salamandrivorans* and the risk of a second amphibian pandemic. *EcoHealth.* (2017) 14:851–64. doi: 10.1007/s10393-017-1278-1
21. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLoS ONE.* (2013) 8:e56802. doi: 10.1371/journal.pone.0056802
22. Olson DH, Ronnenberg KL, Glidden CK, Christiansen KR, Blaustein AR. Global patterns of the fungal pathogen *Batrachochytrium dendrobatidis* support conservation urgency. Duffus ALJ, Marschang RE, editors. Emerging Infections and Diseases of Herpetofauna. *Front Vet Sci Zool Med.* (2021) 8:685877. doi: 10.3389/fvets.2021.685877
23. Castro Monzon F, Rödel MO, Jeschke JM. Tracking *Batrachochytrium dendrobatidis* infection across the globe. *EcoHealth.* (2020) 17:270–9. doi: 10.1007/s10393-020-01504-w
24. Yap TA, MS Koo, RF Ambrose, DB Wake, Vredenburg VT. Averting a North American biodiversity crisis. *Science.* (2015) 349:481–2. doi: 10.1126/science.aab1052
25. Gervasi SS, Stephens PR, Hua J, Searle CL, Urbina J, Olson DH, et al. Linking ecology and epidemiology to understand predictors of multi-host responses to an emerging pathogen, the amphibian chytrid fungus. *PLoS ONE.* (2017) 12:e0167882. doi: 10.1371/journal.pone.0167882
26. Greenberg DA, Palen WJ, Mooers AØ. Amphibian species traits, evolutionary history and environment predict *Batrachochytrium dendrobatidis* infection patterns, but not extinction risk. *Evol Appl.* (2017) 10:1130–45. doi: 10.1111/eva.12520
27. Longo AV, Fleischer RC, Lips KR. Double trouble: co-infections of chytrid fungi will severely impact widely distributed newts. *Biol Invasions.* (2019) 21:2233–45. doi: 10.1007/s10530-019-01973-3
28. Basanta DM, Byrne AQ, Rosenblum EB, Piovia-Scott J, Parra-Olea G. Early presence of *Batrachochytrium dendrobatidis* in Mexico with a contemporary dominance of the Global Panzootic Lineage. *Mol Ecol.* (2020) 30:424–37. doi: 10.1111/mec.15733
29. Becker CG, Greenspan SE, Tracy KE, Dash JA, Lambertini C, Jenkinson TS, et al. Variation in Phenotype and Virulence Among Enzootic and Panzootic Amphibian Chytrid Lineages. *Fungal Ecol.* (2017) 26:45–50. doi: 10.1016/j.funeco.2016.11.007
30. Rife BD, Mavian C, Chen X, et al. Phylodynamic Applications in 21st Century Global Infectious Disease Research. *Glob Health Res PolGHeRP.* (2017) 2:13. doi: 10.1186/s41256-017-0034-y
31. Vredenburg VT, McNally SVG, Sulaeman H, Butler HM, Yap T, Koo MS, et al. Pathogen Invasion History Elucidates Contemporary Host Pathogen Dynamics. *PLoS ONE.* (2019) 14:e0219981. doi: 10.1371/journal.pone.0219981
32. Deck J, Gaither MR, Ewing R, Bird CE, Davies N, Meyer C, et al. The genomic observatories metadatabase (GEOME): a new repository for field and sampling event metadata associated with genetic samples. *PLoS Biol.* (2017) 15:e2002925. doi: 10.1371/journal.pbio.2002925
33. Free Software Foundation. The GNU General Public License v3.0 - GNU Project - Free Software Foundation. (n.d.) Available online at: https://www.gnu.org/licenses/gpl-3.0.html (accessed January 10, 2021).
34. Wieczorek J, Bloom D, Guralnick R, Blum S, Döring M, Giovanni R, et al. Darwin core: an evolving community-developed biodiversity data standard. *PLoS ONE.* (2012) 7:e29715. doi: 10.1371/journal.pone.0029715
35. Yilmaz P, Kottmann R, Field D, Knight R, Cole JR, Amaral-Zettler L, et al. Minimum information about a marker gene sequence (MIMARKS) and minimum information about any (x) sequence (MIxS) specifications. *Nat Biotechnol.* (2011) 29:415–20. doi: 10.1038/nbt.1823
36. AmphibiaWeb. (2020) Taxonomy and Synonym Daily List. Available online at: https://amphibiaweb.org/taxonomy/AWtaxonomy.html (accessed November 1, 2020).
37. EZID California Digital Library. (2020). Available online at: https://ezid.cdlib.org (accessed November 1, 2020).
38. Vision TJ. Open data and the social contract of scientific publishing. *BioScience.* (2010) 60:330–1. doi: 10.1525/bio.2010.60.5.2
39. Waddle JH, Grear DA, Mosher BA, Campbell Grant EH, Adams MJ, et al. *Batrachochytrium salamandrivorans* (*Bsal*) not detected in an intensive survey of wild North American amphibians. *Sci Rep.* (2020) 10:13012. doi: 10.1038/s41598-020-69486-x
40. Goldberg CS, Pilliod DS, Arkle RS, Waits LP. Molecular detection of vertebrates in stream water: a demonstration using rocky mountain tailed frogs and Idaho giant salamanders. *PLoS ONE.* (2011) 6:e22746. doi: 10.1371/journal.pone.0022746
41. Chestnut T, Anderson C, Popa R. Blaustein AR, Voytek M, Olson DH, et al. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. *PLoS ONE.* (2014) 9:e106790. doi: 10.1371/journal.pone.0106790
42. Vences M, Lötters S. The salamander plague in Europe—a German perspective. *Salamandra.* (2020) 56:169–71.
43. Barnosky AD, Matzke N, Tomiya S, Wogan G, Swartz B, Quental T, et al. Has the earth's sixth mass extinction already arrived? *Nature.* (2011) 471:51–7. doi: 10.1038/nature09678
44. Yong E. The Worst Disease Ever Recorded. The Atlantic (2019). Available online at: https://www.theatlantic.com/science/archive/2019/03/bd-frogs-apocalypse-disease/585862/ (accessed March 28, 2019).
45. Wake DB. Declining amphibian populations. *Science.* (1991) 253:860. doi: 10.1126/science.253.5022.860
46. Blaustein AR, Wake DB. The puzzle of declining amphibian populations. *Sci Am.* (1995) 272:52–7. doi: 10.1038/scientificamerican0495-52
47. Hero J-M, Gillespie GR. Epidemic disease and amphibian declines in Australia. *Conserv Biol.* (1997) 11:1023–5. doi: 10.1046/j.1523-1739.1997.96291.x
48. Laurance WF, McDonald KR, Speare R. Epidemic disease and the catastrophic decline of australian rain forest frogs. *Conserv Biol.* (1996) 10:406–13. doi: 10.1046/j.1523-1739.1996.10020406.x
49. Bernardo-Cravo AP, Schmeller DS, Chatzinotas A, Vredenburg VT, Loyau A. Environmental factors and host microbiomes shape host–pathogen dynamics. *Trends Parasitol.* (2020) 36:616–33 doi: 10.1016/j.pt.2020.04.010
50. Briggs CJ, Knapp RA, Vredenburg VT. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc Natl Acad Sci USA.* (2010) 107:9695–700. doi: 10.1073/pnas.0912886107
51. Cheng TL, Rovito SM, Wake DB, Vredenburg VT. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc Natl Acad Sci USA.* (2011) 108:9502–7. doi: 10.1073/pnas.1105538108
52. Vredenburg VT, Felt SA, Morgan EA, McNally SVG, Wilson S, Green SL. Prevalence of *Batrachochytrium dendrobatidis* in *Xenopus* Collected in Africa (1871–2000) and in California (2001–2010). *PLoS ONE.* (2013) 8:e63791. doi: 10.1371/journal.pone.0063791
53. Huss M, Huntley L, Vredenburg V, Johns J, Green S. Presence of *Batrachochytrium dendrobatidis* in 120 archived specimens of *Lithobates catesbeianus* (American Bullfrog) collected in California, 1924–2007. *EcoHealth.* (2013) 10:339–43. doi: 10.1007/s10393-013-0895-6
54. Zhu W, Bai C, Wang S, Soto-Azat C, Li X, Liu X, et al. Retrospective survey of museum specimens reveals historically widespread presence of *Batrachochytrium dendrobatidis* in China. *EcoHealth.* (2014) 11:241–50. doi: 10.1007/s10393-013-0894-7
55. Talley BL, Muletz CR, Vredenburg VT, Fleischer RC, Lips KR. A century of *Batrachochytrium dendrobatidis* in Illinois Amphibians (1888–1989). *Biol Conserv.* (2015) 182:254–61. doi: 10.1016/j.biocon.2014.12.007
56. Fong JJ, Cheng TJ, Battaile A, Pessier AP, Waldman B, Vredenburg VT. Early 1900s detection of *Batrachochytrium dendrobatidis* in Korean

Amphibians. *PLoS ONE.* (2015) 10:e0115656. doi: 10.1371/journal.pone.0115656
57. Sette CM, Vredenburg VT, Zink AG. Reconstructing historical and contemporary disease dynamics: a case study using the california slender salamander. *Biol Conserv.* (2015) 192:20–9. doi: 10.1016/j.biocon.2015.08.039
58. Yap TA, Gillespie L, Ellison S, Flechas SV, Koo MS, Martinez Vredenburg VT. Invasion of the fungal pathogen *Batrachochytrium dendrobatidis* on California Islands. *EcoHealth.* (2016) 13:145–50. doi: 10.1007/s10393-015-1071-y
59. Adams AJ, Pessier AP, Briggs CJ. Rapid extirpation of a north american frog coincides with an increase in fungal pathogen prevalence: historical analysis and implications for reintroduction. *Ecol Evol.* (2017) 7:10216–32. doi: 10.1002/ece3.3468
60. De Léon ME, Vredenburg VT, Piovia-Scott J. Recent emergence of a chytrid fungal pathogen in California cascades frogs (*Rana cascadae*). *EcoHealth.* (2017) 14:155–61. doi: 10.1007/s10393-016-1201-1
61. Yap TA, Koo MS, Ambrose RF, Vredenburg VT. Introduced bullfrog facilitates pathogen invasion in the Western United States. *PLoS ONE.* (2018) 13:e0188384. doi: 10.1371/journal.pone.0188384
62. Chaukulkar S, Sulaeman H, Zink AG, Vredenburg VT. Pathogen invasion and non-epizootic dynamics in Pacific newts in California over the last century. *PLoS ONE.* (2018) 13:e0197710. doi: 10.1371/journal.pone.0197710
63. Rios-Sotelo G, Figueroa-Valenzuela R, Vredenburg VT. Retrospective survey reveals extreme rarity of amphibian fungal pathogen *Batrachochytrium dendrobatidis* in Japanese amphibians from 1890–1990s. *Herpetol Rev.* (2018) 49:247–52.
64. De León ME, Zumbado-Ulate H, García-Rodríguez A, Alvarado G, Sulaeman H, Bolaños F, et al. *Batrachochytrium dendrobatidis* infection in amphibians predates first known epizootic in costa rica. *PLoS ONE.* (2019) 14:e0208969. doi: 10.1371/journal.pone.0208969
65. Sette CM, Vredenburg VT, Zink AG. Differences in fungal disease dynamics in co-occurring terrestrial and aquatic amphibians. *EcoHealth.* (2020) 17:302–14. doi: 10.1007/s10393-020-01501-z
66. AmphibiaWeb. (2021). Database Application: species range maps. Available online at: https://amphibiaweb.org (accessed May 1, 2021).
67. Mace GM, Collar NJ, Gaston KJ, Hilton-Taylor C, Akçakaya HR, Leader-Williams N, et al. Quantification of extinction risk: IUCN's system for classifying threatened species. *Conserv Biol.* (2008) 22:1424–42. doi: 10.1111/j.1523-1739.2008.01044.x
68. Catenazzi A, von May R, Vredenburg VT. High prevalence of infection in tadpoles increases vulnerability to fungal pathogen in high-andean amphibians. *Biol Conserv.* (2013) 159:413–21. doi: 10.1016/j.biocon.2012.11.023
69. Catenazzi A, Lehr E, Vredenburg VT. Thermal physiology, disease, and amphibian declines on the eastern slopes of the andes. *Conserv Biol.* (2014) 28:509–17. doi: 10.1111/cobi.12194
70. Gahl MK, Longcore JE, Houlahan JE. Varying responses of northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. *Conserv Biol.* (2012) 26:135–41. doi: 10.1111/j.1523-1739.2011.01801.x
71. Reeder NMM, Pessier AP, Vredenburg VT. A Reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. *PLoS ONE.* (2012) 7:e33567. doi: 10.1371/journal.pone.0033567
72. Morgan JAT, Vredenburg VT, Rachowicz LJ, Knapp RA, Stice MJ, Tunstall T, et al. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proc Natl Acad Sci USA.* (2007) 104:13845–50. doi: 10.1073/pnas.0701838104
73. Retallick RW, Miera V. Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Dis Aquat Org.* (2007) 75:201–7. doi: 10.3354/dao075201
74. Searle CL, Gervasi SS, Hua J, Hammond JI, Relyea RA, Olson DH, et al. Differential host susceptibility to *batrachochytrium dendrobatidis*, an emerging amphibian pathogen. *Conserv Biol.* (2011) 25:965–74. doi: 10.1111/j.1523-1739.2011.01708.x
75. Lambertini C, Becker CG, Belasen AM, Valencia-Aguilar A, Nunes-de-Almeida CHL, Betancourt-Román CM, et al. Biotic and abiotic determinants of *Batrachochytrium dendrobatidis* infections in amphibians of the Brazilian Atlantic forest. *Fungal Ecol.* (2021) 49:100995. doi: 10.1016/j.funeco.2020.100995
76. Woodhams DC, Vredenburg VT, Simon M, Billheimer D, Shakhtour B, Shyr Y, et al. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol Conserv.* (2007) 138:390–8. doi: 10.1016/j.biocon.2007.05.004
77. Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, Flaherty DC. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* (2009) 3:818–24 doi: 10.1038/ismej.2009.27
78. Lam BA, Walke JB, Vredenburg VT, Harris RN. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol Conserv.* (2010) 143:529–31. doi: 10.1016/j.biocon.2009.11.015
79. Vredenburg VT, Briggs CJ, Harris RN. Host-pathogen dynamics of amphibian chytridiomycosis: the role of the skin microbiome in health and disease. In: Olsen L, Choffnes ER, Relman DA, Pray L, editors. *Fungal Diseases: An Emerging Threat to Human, Animal and Plant Health*. Washington, DC: The National Academies Press IOM (Institute of Medicine) (2011). p. 342–55.
80. Murrieta-Galindo R, Parra-Olea G, González-Romero A, López-Barrera F, Vredenburg VT. Detection of *Batrachochytrium dendrobatidis* in amphibians inhabiting cloud forests and coffee agroecosystems in central Veracruz, Mexico. *Eur J Wildlife Res.* (2014) 60:431–9. doi: 10.1007/s10344-014-0800-9
81. Rachowicz LJ, Hero J-M, Alford RA, Taylor JW, Morgan JA, Vredenburg VT, et al. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conserv Biol.* (2005) 19:1441–8. doi: 10.1111/j.1523-1739.2005.00255.x
82. Barnes MA, Brown AD, Daum MN, de la Garza KA, Driskill J, Garrett K, et al. Detection of the amphibian pathogens chytrid fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in West Texas, USA, using environmental DNA. *J Wildlife Dis.* (2020) 56:702–6 doi: 10.7589/2019-08-212
83. Byrne AQ, Rothstein AP, Poorten TJ, Erens J, Settles ML, Rosenblum EB. Unlocking the story in the swab: a new genotyping assay for the amphibian Chytrid fungus *Batrachochytrium dendrobatidis*. *Mol Ecol Resour.* (2017) 17:1283–92. doi: 10.1111/1755-0998.12675
84. O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, et al. Recent Asian origin of Chytrid fungi causing global amphibian declines. *Science.* (2018) 360:621–7. doi: 10.1126/science.aar1965
85. Rodriguez D, Becker CG, Pupin NC, Haddad CFB, Zamudio KR. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the atlantic forest of Brazil. *Mol Ecol.* (2014) 23:774–87. doi: 10.1111/mec.12615

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 13 October 2021
doi: 10.3389/fvets.2021.733299

Herpesviruses in Captive Chelonians in Europe Between 2016 and 2020

*Christoph Leineweber, Elisabeth Müller and Rachel E. Marschang**

Laboklin GmbH & Co. KG, Bad Kissingen, Germany

Herpesviruses are important pathogens in tortoises and turtles, yet little is known about the epidemiology of these viruses. We analyzed herpesviruses detected by PCR in samples from captive chelonians in Europe according to virus strain, host species, year and season in which the animal was tested, and country in which the animal was kept. A total of 4,797 samples submitted to a diagnostic laboratory in Europe between January 2016 and December 2020 were evaluated. Of these, 312 (6.50%) were positive for herpesviruses. The types most commonly found were testudinid herpesvirus (TeHV)1 (143 positive, 45.83%) and TeHV3 (153 positive, 49.04%), but also included TeHV2 (1 positive, 0.32%), TeHV4 (3 positive, 0.96%), Terrapene herpesvirus 1 (7 positive, 2.24%), Trachemys herpesvirus 1 (2 positive, 0.64%), and three previously undescribed herpesviruses (0.96%). Herpesviruses were detected in chelonians in the families Testudinidae, Emydidae, Geoemydidae, and in the suborder Pleurodira. Among the species for which 100 samples or more were available, the highest proportions of positive samples (positivity rates) were found in samples from Horsfield's tortoises (*Testudo horsfieldii*) (14.96%), and radiated tortoises (*Astrochelys radiata*) (14.05%). Among tortoises (Testudinidae), viruses were most often detected in the spring, while in emydid turtles (Emydidae) they were most often detected in the summer. A comparison of the positivity rates according to country showed significant differences, with the highest rate in samples from Italy (16.01%). This study indicated possible differences in herpesvirus positivity rates depending on host species, virus strain, year of sampling, season, and country of origin. It provides useful information in further understanding fluctuations in infection rates as well as in helping to guide decision making for herpesvirus diagnostics in chelonian patients. It also provides evidence for the international dispersal of herpesviruses with their hosts through international trade.

Keywords: Emydidae, season, testudinid herpesvirus, Testudinidae, *Testudo*, *Terrapene*, tortoise, turtle

OPEN ACCESS

Edited by:
Arvo Viltrop, Estonian University of Life Sciences, Estonia

Reviewed by:
Benjamin G. Dewals, University of Liège, Belgium
Francesco Carlo Origgi, University of Bern, Switzerland

****Correspondence:***
Rachel E. Marschang
rachel.marschang@gmail.com

Specialty section:
This article was submitted to Zoological Medicine, a section of the journal Frontiers in Veterinary Science

Received: *30 June 2021*
Accepted: *16 September 2021*
Published: *13 October 2021*

Citation:
Leineweber C, Müller E and Marschang RE (2021) Herpesviruses in Captive Chelonians in Europe Between 2016 and 2020. Front. Vet. Sci. 8:733299. doi: 10.3389/fvets.2021.733299

INTRODUCTION

Herpesviruses are among the most commonly described viruses in chelonians. Herpesviruses found in these animals that have been genetically analyzed have clustered together in the subfamily *Alphaherpesvirinae*, in the genus *Scutavirus* (1–3). That genus currently contains two species: *Chelonid alphaherpevirus 5* and *Testudinid alphaherpesvirus 3* (4). Additional herpesviruses have been described in multiple species of chelonian and have been shown to cause significant disease in many cases.

Herpesviruses found in sea turtles (family Cheloniidae) include *Chelonid alphaherpesvirus 5* which is associated with fibropapillomatosis (3, 5, 6). Lung-eye-trachea-disease-associated virus (LETV) was originally described in green turtles (*Chelonia mydas*) with respiratory disease (7). It is categorized in the species *Chelonid alphaherpesvirus 6* (4), and is related to the members of the genus *Scutavirus*, but has not been assigned to a genus yet. Other herpesviruses described in sea turtles include gray patch disease virus found in juvenile green turtles with skin lesions (8). No information on the genome of that virus is available. Loggerhead genital-respiratory herpesvirus (LGRV) and loggerhead orocutaneous herpesvirus (LOCV) were both found in wild-caught loggerhead turtles (*Caretta caretta*). Analysis of sequences from a part of the DNA-dependent DNA-polymerase gene showed that both clustered with members of the genus *Scutavirus* (9).

A number of herpesviruses have been described in tortoises in the family Testudinidae. The best characterized of these is *Testudinid alphaherpesvirus 3* (TeHV3), the genome of which has been fully sequenced (2, 10). Some genetic diversity has been found in members of this species (2, 10). Transmission studies with TeHV3 isolates in spur-thighed tortoises (*Testudo graeca*) and Hermann's tortoises (*T. hermanni*) have demonstrated a connection between infection and disease, including apathy, anorexia, nasal discharge and oral lesions (2, 11). TeHV3 has been shown to infect a wide range of testudinid tortoise species (3). Several other genetically distinct herpesviruses have also been described in tortoises. These have commonly been referred to as testudinid herpesvirus 1, 2, and 4 (TeHV1, 2, and 4). These have all been less well-investigated than TeHV3. TeHV1 was originally described in Japan in imported pancake (*Malacochersus tornieri*) and Horsfield's (*Testud horsfieldii*) tortoises (12). This virus has been most frequently found in Horsfield's tortoises in Europe, although it can also infect other tortoise species (13). TeHV2 was first described in a captive desert tortoise (*Gopherus agassizii*) in California, USA (14). TeHV4 was found by chance during a quarantine examination of a clinically healthy Bowsprit tortoise (*Chersina angulata*) at a zoo in the USA (15). It has also been found in a leopard tortoise (*Stigmochelys pardalis*) in Europe (16). Reported prevalence of testudinid herpesviruses in diagnostic samples from captive tortoises has ranged between 8.2 and 25% (13, 17–20).

A variety of herpesviruses have also been detected in wild and captive turtles of other families in recent years. Most of these reports have been from animal in the families Emydidae and Geoemydidae. While reports of herpesvirus infections in these species were originally based on histological and electronmicroscopic findings (21–23), recent detections have been based more often on PCR and sequencing. These viruses have been divided into different strains based on partial sequencing data and have generally been designated based on the host species in which they were detected. Emydid herpesvirus 1 has been detected in several species of emydid turtles, including northern map turtles (*Graptemys geographica*), painted turtles (*Chrysemys picta*), and eastern river cooters (*Pseudemys concinna concinna*) in the United States and Germany (24, 25) as well as in a mixed collection including *Trachemys* spp., *Graptemys* spp., and *Pseudemys* spp. in Germany (3). Emydid herpesvirus 2 was detected in asymptomatic bog turtles (*Glyptemys muhlenbergii*) and spotted turtles (*Clemmys guttata*) (26). Glyptemys herpesvirus 1 and glyptemys herpesvirus 2 were both detected in asymptomatic bog and wood turtles (*Glyptemys insculpta*) (26). Trachemys herpesvirus 1 has been detected in asymptomatic free ranging red-eared sliders (*Trachemys scripta elegans*) in the United States (27). Emydoidea herpesvirus 1 was detected in free ranging Blanding's turtles (*Emydoidea blandingii*) in the United States (28), while Emydoidea herpesvirus 2 was detected in a squamous cell carcinoma in the oral cavity of a free ranging Blanding's turtle (29). Terrapene herpesvirus 1 and 2 have each been described in Eastern box turtles (*Terrapene carolina carolina*). Terrapene herpesvirus 1 has been reported associated with disease outbreaks in captive box turtles (30, 31). It has also been detected at high prevalence among free ranging eastern box turtles in the United States (32). Terrapene HV2 was detected in an eastern box turtle with recurrent papillomatous skin lesions (33).

Herpesviruses have also been detected in turtles in several other families in individual cases. Chelydra herpesvirus 1 was found in free ranging common snapping turtles (*Chelydra serpentina*) in the United States (27). Pelomedusid herpesvirus 1 was detected in clinically healthy captive West African mud turtles (*Pelusios castaneus*) in Europe (34). Another, unnamed herpesvirus was reported in a William's mud turtle (*Pelusios williamsi*) with papillomatous skin lesions in Europe (35).

Molecular detection and differentiation of herpesviruses in chelonians has most often been carried out using a pan-herpesvirus PCR targeting a conserved portion of the DNA-dependent DNA-polymerase gene (36). In some cases, additional PCRs have been developed targeting specific chelonid herpesviruses, e.g., for TeHV1 and 3 (11–13, 37, 38). Real-time PCRs have been described for the detection of several strains of herpesviruses found in emydid turtles (28, 39, 40).

There is only limited data available on the seasonality of herpesvirus infections in turtles and tortoises. In surveys of free ranging eastern box turtles for a variety of pathogens, Terrapene HV 1 has been detected significantly more often in summer (40) or fall (32) than in other seasons. Season has been hypothesized to play a role in disease development and virus shedding in tortoises (13, 41). However, no long term studies of patterns of herpesvirus infection are currently available.

The purpose of this study was to analyse detection of herpesviruses in samples from chelonians submitted to a diagnostic laboratory in Europe. It was hypothesized that herpesvirus detection would differ depending on herpesvirus type, season, and year and that the type of herpesvirus detected would depend on the host species. In addition, the country of origin of the sample was hypothesized to impact the prevalence of herpesvirus detection.

MATERIALS AND METHODS

Samples submitted to Laboklin GmbH & Co. KG (Bad Kissingen, Germany), a veterinary diagnostic laboratory, over the course

of a 5 year period (2016–2020) were evaluated. Samples were submitted by veterinarians and pet owners. Only samples identified as having been collected from a chelonian were included in the study, although the host species was not provided in all cases. The vast majority of samples tested were oral swabs. Reasons for testing were not provided, and background information on the animals was not generally available. No samples were solicited for this study. Repeat or multiple samples from individual animals were counted as single samples.

All samples were processed within 24 h of arrival at the laboratory. DNA was extracted using a commercial kit (MagNA Pure 96 DNA and viral NA small volume kit, Roche, Penzberg, Germany) according to the manufacturer's instructions. Three different PCRs were performed for the detection of herpesviral DNA as described previously (13). These included a consensus PCR targeting a small region of the DNA-dependent DNA-polymerase gene that is able to detect a wide range of herpesviruses in the family *Herpesviridae* (36), a second PCR targeting a portion of the DNA polymerase gene able to detect TeHV1 (12, 38), and a PCR targeting a part of the *UL5* gene able to detect TeHV3 (37, 38). For all samples in which only one of these PCRs was positive or in which a herpesvirus or the herpesvirus type identified had not been previously reported in that host species, the PCR product was sequenced by Sanger sequencing. In these cases, PCR products were purified (MinElute purification kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed using a Big-Dye Terminator v3.1 cycle sequencing kit (Life Technologies, Carlsbad, CA, USA) and analyzed on an ABI 3130 sequencer (Applied Biosystems, Waltham, MA, USA). Sequences were manually edited and primer sequences were removed for further analysis. Sequences were compared to those in GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analyses were carried out using the statistical analysis software (SAS) (SAS Institute, Cary, NC, USA) for the calculation of the proportion of positive samples, refered to as positivity rates in this text. The 95% binomial confidence intervals were calculated based on the Wilson procedure (42). The Pearson chi-squared test was used with a type I error α of 0.05 to test the independence of compared positivity rates. Fisher exact test was used for small sample sizes for the specific calculations on Testudinidae and Emydidae. A p-value < 0.05 was considered significant.

RESULTS

Herpesviruses Detected

From the beginning of 2016 to the end of 2020 4,797 samples from chelonians were tested for herpesviruses by PCR. Overall, 312 (6.50%; 95% CI 5.84–7.23%) were positive for herpesviruses. Of these positive samples, 143 were positive for TeHV1 (45.83%; 95% CI 40.39–51.38%), one for TeHV2 (0.32%; 95% CI 0.06–1.79%), 153 for TeHV3 (49.04%; 95% CI 43.54–54.56%), 3 for TeHV4 (0.96%; 95% CI 0.33–2.79%), 7 for Terrapene herpesvirus 1 (2.24%; 95% CI 1.09–4.55%), 2 for Trachemys herpesvirus 1 (0.64%; 95% CI 0.18–2.31%), and 3 for other alphaherpesviruses (0.96%; 95% CI 0.33–2.79%) (**Table 1**). Of the 3 that were positive for other alphaherpesviruses, one was from an oral swab from a Siebenrock's snake-necked turtle (*Chelodina rugosa*) collected in Italy in March 2018. The sequence obtained from that sample was 100% identical to that from a herpesvirus found in a panther chameleon (*Furcifer pardalis*) (GenBank accession No. MW015088.1) and did not cluster with members of the *Scutavirus* genus (43). One was from an oral swab from a Chinese striped-necked turtle (*Mauremys sinensis*) collected in France in August 2019. The sequence obtained from that sample had the highest identity (76.4%) to a herpesvirus detected in a Williams' mud turtle (*Pelusios williamsi*) (GenBank accession No. KX374559.1) and clustered with other herpesviruses from chelonians in the *Scutavirus* genus. The third was from an oral swab from a European pond turtle (*Emys orbicularis*) collected in Italy in November 2020. The sequence from this sample had the highest identity (94.4%) to Terrapene herpesvirus 1 (GenBank accession No. KJ004665.1). The sequences from the TeHV2, TeHV4, and other alphaherpesviruses not described previously in chelonians are provided in **Supplementary Table 1**.

Herpesvirus Detection According to Host Family, Year of Sampling, and Country of Origin

The vast majority of samples tested were from tortoises in the family Testudinidae (3,475 samples), 216 of these (6.22%; 95% CI 5.46–7.07%) were positive for herpesviruses. In the other families tested, 10 of 176 samples from Emydidae were positive (5.68%; 95% CI 3.11–10.14%) and no significant ($p = 0.7743$) difference between the positivity rates were noted between these two families. One sample (2.04%; 95% CI 0.36–10.69%) from the family Geoemydidae ($n = 49$) and one (12.50%; 95% CI 2.24–47.09%) from the suborder Pleurodira ($n = 8$) were positive. All other samples from Cheloniidae ($n = 10$), Kinosternidae ($n = 8$), Platysternidae ($n = 4$), Carettochelyidae ($n = 2$), and Chelydridae ($n = 3$) tested were negative. 1,062 samples could not be assigned a specific family, 84 of these were tested positive (7.91%; CI 6.43–9.69%) (**Table 1**). A comparison between the different tested species showed significant ($p < 0.0001$) differences.

The herpes virus positivity rates varied significantly ($p = 0.0002$) between 4.78 and 9.47% depending on the year of sampling (**Table 2**). A significant variation ($p < 0.0001$) was also found between the seasons in which the samples were submitted, with the highest positivity rate in spring (9.48%) and the lowest in fall (2.98%) (**Table 3**).

The tested samples originated from different countries and showed significant differences ($p < 0.0001$) in positivity rates between the different countries. **Figure 1** and **Supplementary Table 2** show the positivity rates in countries from which 50 samples or more were received.

Herpesviruses in *Testudo* spp.

A comparison of positivity rates in specific species showed differences among the various *Testudo* spp. tested (Hermann's tortoises, spur-thighed tortoises, marginated tortoises, *Testudo marginata*, Horsfield's tortoises, Egyptian tortoises, *Testudo*

TABLE 1 | Chelonian species tested and herpesvirus positivity rate depending on species and virus strain.

Family	Species	Total (*n*)	HV positive	TeHV 1	TeHV 2	TeHV 3	TeHV 4	Terrapene HV 1	Trachemys herpesvirus 1	Other alphaherpes viruses
Testudinidae	Tortoise	363	14 (3.86%; CI 2.31–6.37%)	6 (1.65%; CI 0.76–3.55%)	0 (0%; CI 0–1.05%)	8 (2.20%; CI 1.12–4.28%)	0 (0%; CI 0–1.05%)	0 (0%; CI 0–1.05%)	0 (0%; CI 0–1.05%)	0 (0%; CI 0–1.05%)
	Testudo spp.	33	8 (24.24%; CI 12.83–41.02%)	2 (6.06%; CI 1.68–19.61%)	0 (0%; CI 0–10.43%)	6 (18.18%; CI 8.61–34.39%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)
	Testudo hermanni	1,072	50 (4.66%; CI 3.55–6.09%	7 (0.65%; CI 0.31–1.34%)	0 (0%; CI 0–0.36%)	43 (4.01%; CI 2.99–5.36%)	0 (0%; CI 0–0.36%)	0 (0%; CI 0–0.36%)	0 (0%; CI 0–0.36%)	0 (0%; CI 0–0.36%)
	Testudo graeca	464	29 (6.25%; CI 4.39–8.83%)	4 (0.86%; CI 0.33–2.19%)	0 (0%; CI 0–0.82%)	25 (5.39%; CI 3.68–7.84%)	0 (0%; CI 0–0.82%)	0 (0%; CI 0–0.82%)	0 (0%; CI 0–0.82%)	0 (0%; CI 0–0.82%)
	Testudo marginata	171	10 (5.85%; CI 3.21–10.43%)	3 (1.75%; CI 0.60–5.02%)	0 (0%; CI 0–2.20%)	7 (4.09%; CI 1.99–8.20%)	0 (0%; CI 0–2.20%)	0 (0%; CI 0–2.20%)	0 (0%; CI 0–2.20%)	0 (0%; CI 0–2.20%)
	Testudo horsfieldii	361	54 (14.96%; CI 11.65–19.01%)	53 (14.68%; CI 11.40–18.70%)	0 (0%; CI 0–1.05%)	1 (0.28%; CI 0.05–1.56%)	0 (0%; CI 0–1.05%)	0 (0%; CI 0–1.05%)	0 (0%; CI 0–1.05%)	0 (0%; CI 0–1.05%)
	Testudo kleinmani	51	2 (3.92%; CI 1.08–13.21%	2 (3.92%; CI 1.08–13.21%)	0 (0%; CI 0–0.70%)	0 (0%; CI 0–0.70%)	0 (0%; CI 0–0.70%)	0 (0%; CI 0–0.70%)	0 (0%; CI 0–0.70%)	0 (0%; CI 0–0.70%)
	Centrochelys sulcata	181	6 (3.31%; CI 1.52–7.4%)	5 (2.76%; CI 1.18–6.30%)	0 (0%; CI 0–2.08%)	1 (0.55%; CI 0.10–3.06%)	0 (0%; CI 0–2.08%)	0 (0%; CI 0–2.08%)	0 (0%; CI 0–2.08%)	0 (0%; CI 0–2.08%)
	Astrochelys radiata	121	17 (14.05%; CI 8.96–21.35%)	0 (0%; CI 0–3.08%)	0 (0%; CI 0–3.08%)	17 (14.05%; CI 8.96–21.35%)	0 (0%; CI 0–3.08%)	0 (0%; CI 0–3.08%)	0 (0%; CI 0–3.08%)	0 (0%; CI 0–3.08%)
	Stigmochelys pardalis	231	13 (5.63%; CI 3.32–9.39%)	6 (2.60%; CI 1.20–5.55%)	0 (0%; CI 0–1.64%)	5 (2.16%; CI 0.93–4.96%)	2 (0.87%; CI 0.24–3.11%)	0 (0%; CI 0–1.64%)	0 (0%; CI 0–1.64%)	0 (0%; CI 0–1.64%)
	Aldabrachelys gigantea	98	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)	0 (0%; CI 0–3.77%)
	Chelonoidis nigra	26	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)	0 (0%; CI 0–12.87%)
	Chelonoidis carbonarius	86	1 (1.16%; CI 0.20–6.29%)	0 (0%; CI 0–4.28%)	0 (0%; CI 0–4.28%)	1 (1.16%; CI 0.20–6.29%)	0 (0%; CI 0–4.28%)	0 (0%; CI 0–4.28%)	0 (0%; CI 0–4.28%)	0 (0%; CI 0–4.28%)
	Chelonoidis chilensis	35	10 (28.57%; CI 16.33–45.05%)	9 (25.71%; CI 14.16–42.06%)	0 (0%; CI 0–9.89%)	1 (2.86%; CI 0.51–14.54%)	0 (0%; CI 0–9.89%)	0 (0%; CI 0–9.89%)	0 (0%; CI 0–9.89%)	0 (0%; CI 0–9.89%)
	Chelonoidis denticulata	14	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)
	Geochelone elegans	73	1 (1.37%; CI 0.24–7.36%)	1 (1.37%; CI 0.24–7.36%)	0 (0.0%; CI 0–5.0%)	0 (0%; CI 0–5.0%)	0 (0%; CI 0–5.0%)	0 (0%; CI 0–5.0%)	0 (0%; CI 0–5.0%)	0 (0%; CI 0–5.0%)
	Kinixys sp.	3	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)
	Indotestudo elongata	12	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)	0 (0%; CI 0–24.25%)
	Gopherus berlandien	8	1 (12.50%; CI 2.24–47.09%)	0 (0%; CI 0–32.44%)	1 (12.50%; CI 2.24–47.09%)	0 (0%; CI 0–32.44%)	0 (0%; CI 0–32.44%)	0 (0%; CI 0–32.44%)	0 (0%; CI 0–32.44%)	0 (0%; CI 0–32.44%)

(Continued)

TABLE 1 | Continued

Family	Species	Total (*n*)	HV positive	TeHV 1	TeHV 2	TeHV 3	TeHV 4	Terrapene HV 1	Trachemys herpesvirus 1	Other alphaherpesviruses
	Malacochersus tornieri	14	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)	0 (0%; CI 0–21.53%)
	Homopus ssp.	11	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)
	Manouria spp.	13	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)	0 (0%; CI 0–22.81%)
	Pyxis spp.	19	0 (0% CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%: CI 0–16.82%)	0 (0%; CI 0–16.82%)
	Geochelone platynota	6	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)	0 (0%; CI 0–39.03%)
	Psammobates spp.	4	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)
	Astrochelys yniphora	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)
	Chersina angulata	1	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)
	Chersobius signatus	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)
Emydidae	Turtles	16	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)	0 (0%; CI 0–19.36%)
	Emys orbicularis	33	1 (3.03%; CI 0.54–15.32%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	0 (0%; CI 0–10.43%)	1 (3.03%; CI 0.54–15.32%)
	Trachemys spp.	18	0 (0%; CI 0–17.59%)	0 (0%; CI 0–17.59%)	0 (0%; CI 0–17.59%)	0 (0%; CI 0–17.59%)	0 (0%; CI 0–17.59%)	0 (0%; CI 0–17.59%)	0 (0%; CI 17.59%)	0 (0%; CI 0–17.59%)
	Trachemy scripta elegans	48	1 (2.08%; CI 0.37–10.89%	0 (0%; CI 0–7.41%)	0 (0%; CI 0–7.41%)	0 (0%; CI 0–7.41%)	0 (0%; CI 0–7.41%)	0 (0%; CI 0–7.41%)	1 (2.08%; CI 0.37–10.89%)	0 (0%; CI 0–7.41%)
	Trachemys scripta scripta	19	1 (5.26%; CI 0.93–24.63%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	0 (0%; CI 0–16.82%)	1 (5.26%; CI 0.93–24.63%)	0 (0%; CI 0–16.82%)
	Pseudemys spp.	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)
	Graptemys spp.	7	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)	0 (0%; CI 0–35.43%)
	Terrapene spp.	30	7 (23.33%; CI 11.79–40.92%)	0 (0%; CI 0–11.35%)	0 (0%; CI 0–11.35%)	0 (0%; CI 0–11.35%)	0 (0%; CI 0–11.35%)	7 (23.33%; CI 11.79–40.92%)	0 (0%; CI 0–11.35%)	0 (0%; CI 0–11.35%)
	Chrysemys spp.	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)

(Continued)

TABLE 1 | Continued

Family	Species	Total (*n*)	HV positive	TeHV 1	TeHV 2	TeHV 3	TeHV 4	Terrapene HV 1	Trachemys herpesvirus 1	Other alphaherpes viruses
	Clemmys guttata	1	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)
Geoemydidae	*Mauremys* spp.	15	1 (6.67%; CI 1.19–29.82%)	0 (0%; CI 0–20.39%)	0 (0%; CI 0–20.39%)	0 (0%; CI 0–20.39%)	0 (0%; CI 0–20.39%)	0 (0%; CI 0–20.39%)	0 (0%; CI 0–20.39%)	1 (6.67%; CI 1.19–29.82%)
	Geoemyda spengleri	5	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)	0 (0%; CI 0–43.45%)
	Batagur affinis	4	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)
	Curora spp.	11	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)	0 (0%; CI 0–25.88%)
	Rhinoclemmys pucherrima	10	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)
	Heosemys grandis	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)
	Orlitia borneensis	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)
Cheloniidae	Sea turtle	10	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)	0 (0%; CI 0–27.75%)
Kinostenidae	*Kinosternon leucostomum*	4	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)
	Sternotherus spp.	4	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)
Platysternidae	*Platysternon megacephalum*	4	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)	0 (0%; CI 0–48.99%)
Carettochelydidae	*Carettochelys insculpta*	2	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)	0 (0%; CI 0–65.76%)
Chelydridae	*Chelydra serpentina*	3	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)
Suborder Pleurodira	*Podocnemis* ssp.	3	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)
	Phrynops hilarii	1	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.3%)	0 (0%; CI 0–79.35%)
	Chelodina longicollis	1	1 (100%; CI 20.65–100%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	0 (0%; CI 0–79.35%)	1 (100%; CI 20.65–100%)
	Chelus fimbriata	3	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)	0 (0%; CI 0–56.15%)
Unknown Family	Unknown Species	1,062	84 (7.91%; CI 6.43–9.69%)	45 (4.24%; CI 3.18–5.63%)	0 (0%; CI 0–0.36%)	38 (3.58%; CI 2.62–4.88%)	1 (0.09%; CI 0.02–0.52%)	0 (0%; CI 0–0.36%)	0 (0%; CI 0–0.36%)	0 (0%; CI 0–0.36%)

Results shown as: Number positive [% positive of total tested from each individual species; 95% confidence interval (CI)].

TABLE 2 | Herpesvirus positivity rate according to virus strain and year of sampling.

Year	Total (*n*)	Herpesvirus positive	TeHV 1	TeHV 2	TeHV 3	TeHV 4	Terrapene HV 1	Trachemys herpesvirus 1	Other alphaherpes viruses
2016	681	58 (8.52%; CI 6.65–10.86%)	31 (4.55% CI 3.22–6.39%)	0 (0%; CI 0–0.56%)	22 (3.24%; CI 2.14–4.84%)	2 (0.29%; CI 0.08–1.06%)	1 (0.15%; CI 0.03–0.83%)	2 (0.29%; CI 0.08–1.06%)	0 (0%, CI 0–0.56%)
2017	581	55 (9.47%; CI 7.35–12.12%)	25 (4.30%; CI 2.93–6.27%)	1 (0.17%; CI 0.03–0.96%)	28 (4.82%; CI 3.36–6.88%)	0 (0%; CI 0–0.66%)	1 (0.17%; CI 0.03–0.96%)	0 (0%; CI 0–0.66%)	0 (0%; CI 0–0.66%)
2018	1,178	68 (5.77%; CI 4.58–7.25%)	32 (2.72%; CI 1.93–3.81%)	0 (0%; CI 0–0.33%)	29 (2.46%; CI 1.72–3.51%)	1 (0.08%; CI 0.01–0.47%)	5 (0.42%; CI 0.18–0.98%)	0 (0%; CI 0–0.33%)	1 (0.08%; CI 0.01–0.47%)
2019	1,235	59 (4.78%; CI 3.72–6.12%)	27 (2.19%; CI 1.51–3.17%)	0 (0%; CI 0–0.31%)	31 (2.51%; CI 1.77–3.54%)	0 (0%; CI 0–0.31%)	0 (0%; CI 0–0.31%)	0 (0%; CI 0–0.31%)	1 (0.08%; CI 0.01–0.46%)
2020	1,122	72 (6.42%; CI 5.13–8.01%)	28 (2.50%; CI 1.74–3.59%)	0 (0%; CI 0–0.34%)	43 (3.83%; CI 2.86–5.12%)	0 (0%; CI 0–0.34%)	0 (0%; CI 0–0.34%)	0 (0%; CI 0–0.34%)	1 (0.09%; CI 0.02–0.50%)

Results shown as: Number positive [% positive out of total tested in that year; 95% confidence interval (CI)].

TABLE 3 | Herpesvirus positivity rate according to virus strains and season of sampling.

Season	Total (*n*)	Herpesvirus positive	TeHV 1	TeHV 2	TeHV 3	TeHV 4	Terrapene HV 1	Trachemys herpesvirus 1	Other alphaherpes viruses
Spring	1,666	158 (9.48%; CI 8.17–10.98%)	90 (5.40%; CI 4.41–6.59%)	0 (0%; CI 0–0.23%)	66 (3.96%; CI 3.12–5.01%)	1 (0.06%; CI 0.01–0.34%)	0 (0%; CI 0–0.23%)	0 (0%; CI 0–0.23%)	1 (0.06%; CI 0.01–0.34%)
Summer	1,368	78 (5.70%; CI 4.59–7.06%)	23 (1.68%; CI 1.12–2.51%)	0 (0%; CI 0–0.28%)	49 (3.58%; CI 2.72–4.70%)	0 (0%; CI 0–0.28%)	5 (0.37%; CI 0.16–0.86%)	0 (0%; CI 0–0.28%)	1 (0.07%; CI 0.01–0.41%)
Fall	1,075	32 (2.98%; CI 2.12–4.18%)	12 (1.12%; CI 0.64–1.95%)	1 (0.09%; CI 0.02–0.52%)	15 (1.40%; CI 0.85–2.30%)	0 (0%; CI 0–0.36%)	1 (0.09%; CI 0.02–0.52%)	2 (0.19%; CI 0.05–0.68%)	1 (0.09%; CI 0.02–0.52%)
Winter	688	44 (6.40%; CI 4.80–8.48%)	18 (2.62%; CI 1.66–4.10%)	0 (0%; CI 0–0.56%)	23 (3.34%; CI 2.24–4.96%)	2 (0.29%; CI 0.08–1.05%)	1 (0.15%; CI 0.03–0.83%)	0 (0%; CI 0–0.56%)	0 (0%; CI 0–0.56%)

Results shown as: Number positive [% positive out of total tested in each season; 95% confidence interval (CI)].

kleinmanni) (**Supplementary Table 3**). Herpesviruses were detected significantly more often in Horsfield's tortoises (14.96%) than in the other *Testudo* spp. tested (between 3.92 and 6.25%) ($p < 0.0001$). For all of these species, except the Egyptian tortoises ($p = 0.1341$), the positivity rate was significantly impacted by the year ($p < 0.0001$) with the highest rates measured in 2017 (11.36%) and the lowest measured in 2018 (3.45%) (**Figure 2**). Positivity rates also differed significantly depending on the season ($p < 0.0001$ for Hermann', spur-thighed, and Horsfield's tortoises, $p = 0.0012$ for marginated tortoises), except in the Egyptian tortoises ($p = 0.1412$) (**Figure 3**). The distribution of herpesvirus types detected also differed significantly ($p < 0.0001$) depending on the host species among the *Testudo* spp. tested. In Hermann's tortoises, TeHV3 (86.0%) was significantly more commonly detected than TeHV1 (14.0%). A similar distribution was found in spur-thighed tortoises (TeHV3 86.2% and TeHV1 13.8%) and marginated tortoises (TeHV3 70.0%, TeHV1 30.0%). In contrast, TeHV1 (98.15%) was more common than TeHV3 (1.85%) in Horsfield's and in Egyptian tortoises (TeHV1 100%). The distribution of TeHV1 and TeHV3 in four of the *Testudo* ssp. tested also differed significantly ($p = 0.0002$) between the eight countries from which the largest numbers of samples were tested (**Figure 4**). The data from the Egyptian tortoises were excluded from these analyses because only one of the 10 samples from this species from Spain and the only sample from this species from Italy were TeHV1 positive. The highest positivity rate for both TeHV1 and TeHV3 was found in samples submitted from Italy, while the lowest rates were found in Austria and Great Britain for TeHV1 and in Austria for TeHV3 (**Figure 4**).

Herpesviruses in Emydidae

A comparison of positivity rates among samples from turtles in the family Emydidae also showed significant differences ($p < 0.0001$) depending on the host species (**Table 1**). A comparison of the three genera in the Emydidae in which herpesviruses were detected (*Emys*, *Terrapene*, and *Trachemys*) showed a significantly ($p = 0.0002$) higher positivity rate in box turtles (*Terrapene* spp.) than in sliders (*Trachemys* spp.) or European pond turtles (*Emys* spp.). Similar to the *Testudo* species, herpesvirus detection in Emydidae varied significantly ($p = 0.0009$) over the test period, with the positivity rate increasing from 2016 (3 of 53; 5.66%; 95% CI 1.94–15.37%) over 2017 (1 of 16; 6.25%; 95% CI 1.11–28.33%) to 2018 (5 of 37; 13.51%; 95% CI 5.91–27.97%), while no herpesviruses were detected in any of the samples tested in 2019 ($n = 36$; 0%; 95% CI 0–9.64%) and only one of 34 samples tested in 2020 was positive (2.94%; 95% CI 0.52–14.91%). A significant ($p = 0.0065$) seasonal variation was also noticed with no positive samples in spring ($n = 36$ tested;

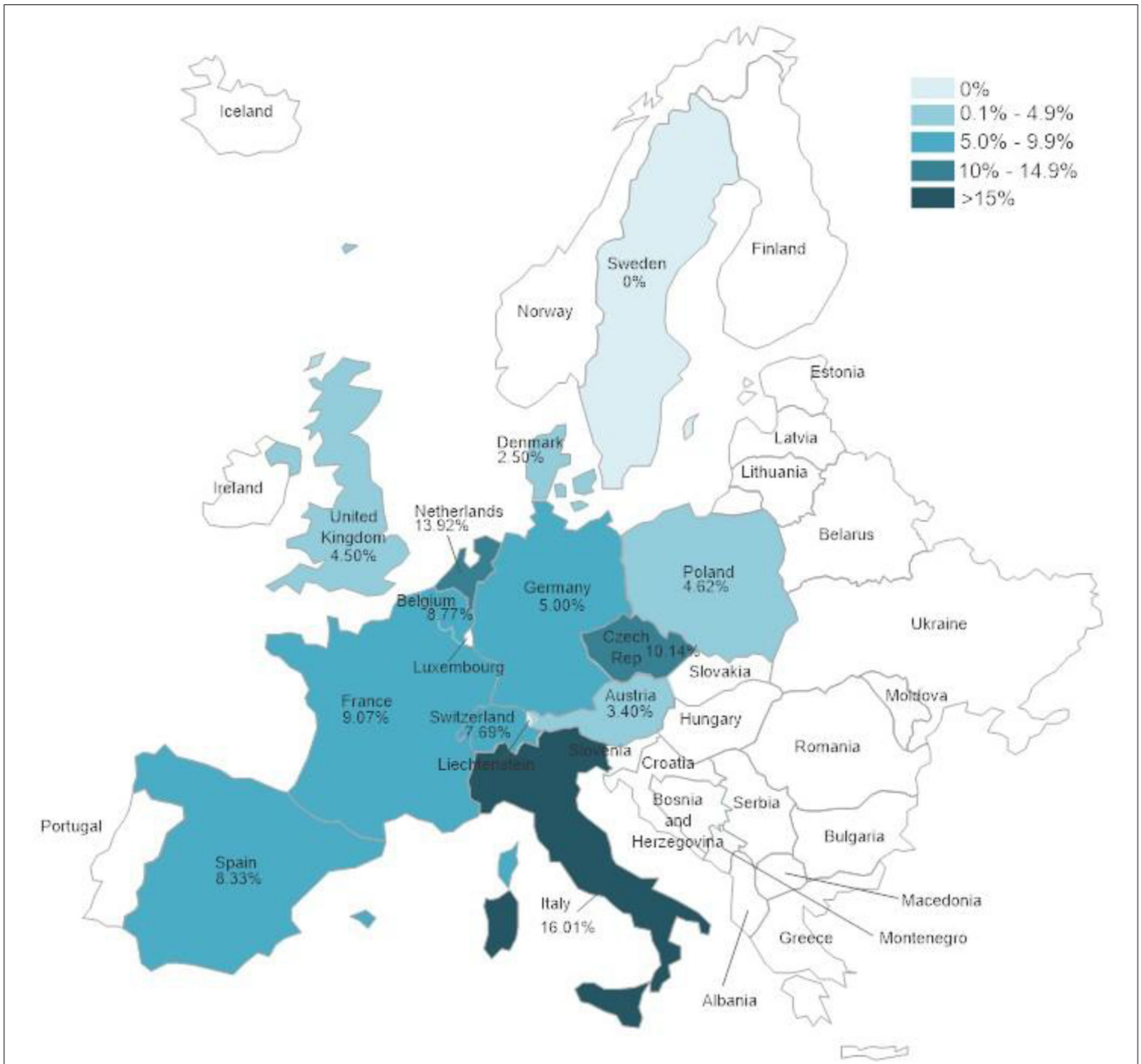


FIGURE 1 | Herpesvirus positivity rates according to country in which samples were collected. Only countries from which 50 samples or more were submitted were included in the evaluation.

0%; 95% CI 0–9.64%); 5 of 51 positive in summer (9.80%; 95% CI 4.26–20.97%), 4 of 67 positive in fall (5.97%; 95% CI 2.35–14.37%) and only one of 22 positive in the winter season (4.55%; 95% CI 0.81–21.80%).

DISCUSSION

Herpesviruses have frequently been described in various chelonian species and have been shown to be important pathogens in many of these animals (3). Previous studies screening samples from captive chelonians in Europe have reported positivity rates of 25% in various species of tortoises in Spain (19), 17% in mixed species from various collections in Belgium (18), 13.7% in captive tortoises from several European countries (17), 8.2% in various tortoise species in the United Kingdom (20), and 8.0% in a wide variety of turtle and tortoise species in various countries (13). The highest positivity rate was in tortoises specifically showing clinical signs considered typical of herpesvirus infection (19), but the other reports included both clinically ill and inapparently infected animals. The overall positivity rate reported here of 6.50% is lower than these previously reported numbers. However, the results of this study

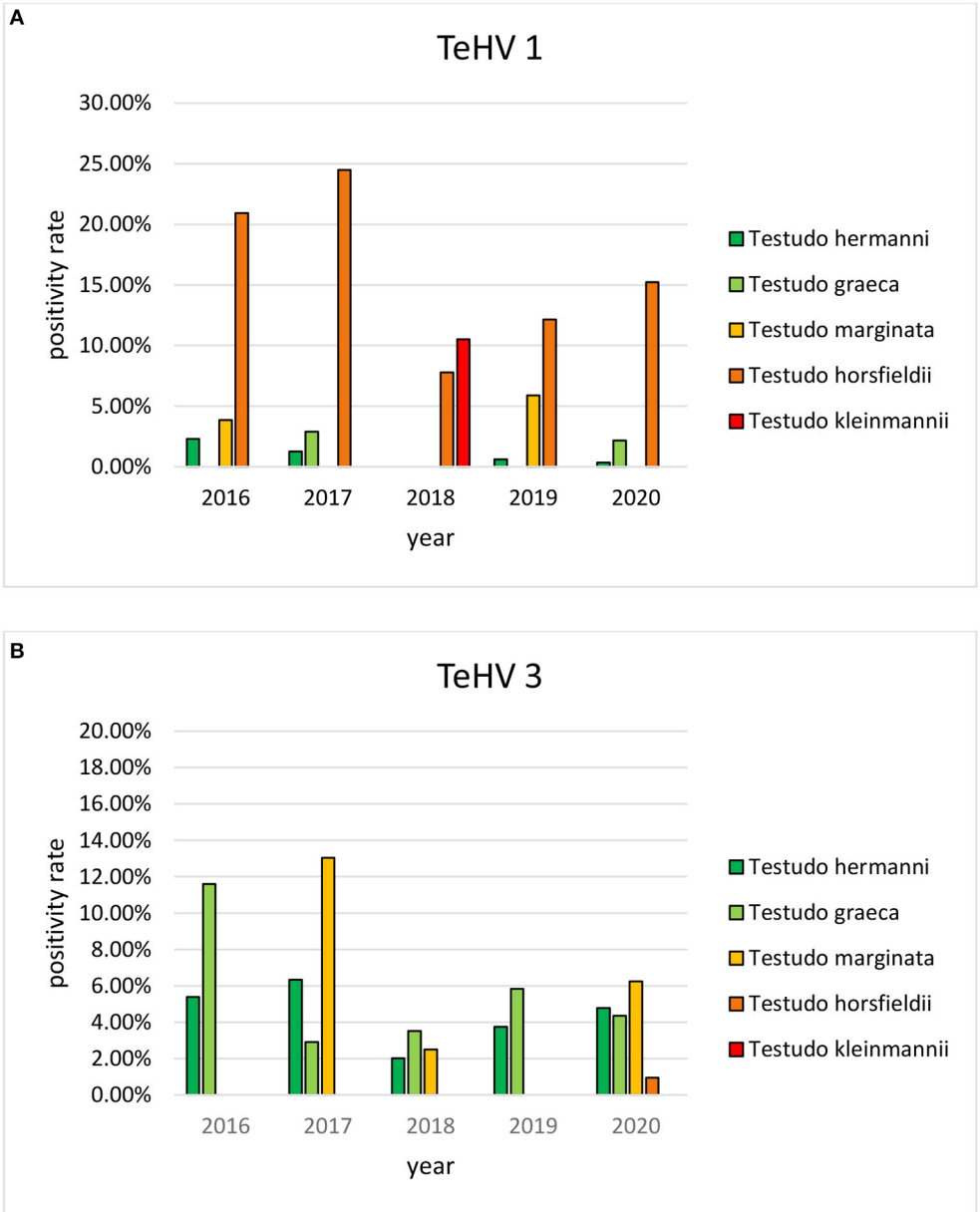


FIGURE 2 | Herpesvirus positivity rates in *Testudo* species (Hermann's tortoises, *T. hermanni*, spur-thighed tortoises, *T. graeca*, marginated tortoises, *T. marginata*, Horsfield's tortoises, *T. horsfieldii*, and Egyptian tortoises, *T. kleinmanni)* for the different years of sampling (**A** TeHV 1 and **B** TeHV 3).

indicate that positivity rate may depend on year, season, and host species, so differences in the testing period and the range of species included influence the overall herpesvirus positivity rate. This is the first report describing herpesvirus detection in captive chelonians over a period of several years and in such a large number of samples. It is interesting to note that detection varied over the course of the 5 year period examined, with an apparent undulating pattern. Other studies reporting on prevalence of herpesvirus infections in groups or populations of chelonians have also found differences in positivity rates between individual years (32, 44), although no previous studies have stretched over more than two separate years. A factor in the numbers of positive samples detected per year could be the numbers of samples tested. This increased from 2016 and 2017 (681 resp. 581 samples) to 2018 through 2020, with over 1,000 samples per year. Reasons for this increase are not known, but changes in willingness of owners to test animals with or without clinical signs of disease could influence positivity rates. Additional study is necessary to better understand possible temporal effects on herpesvirus infection and shedding in captive chelonians. This is likely a complex issue involving multiple factors including host species, virus strain, breeding practices, and the pet trade, as well as possible

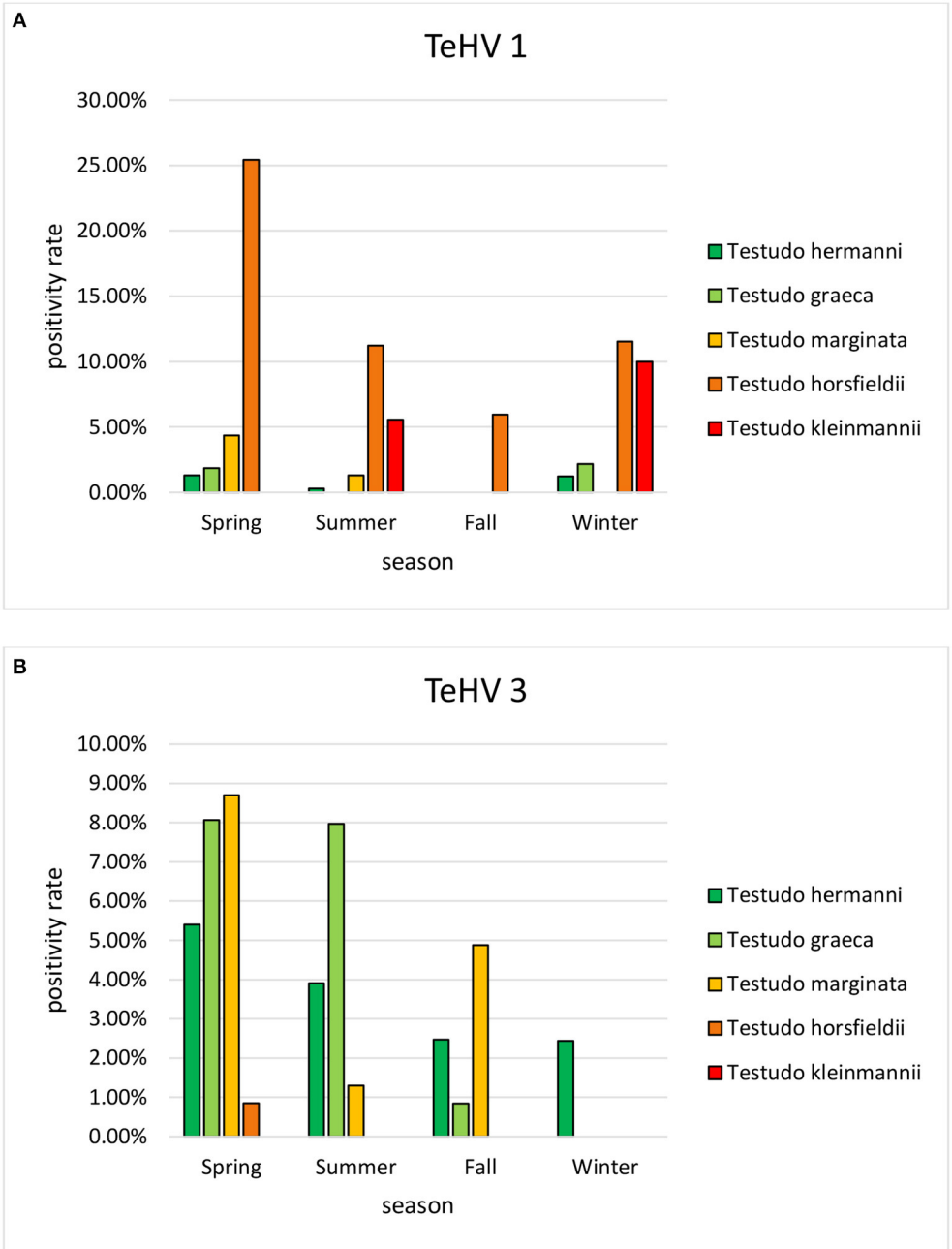


FIGURE 3 | Herpesvirus positivity rates in *Testudo* species (Hermann's tortoises, *T. hermanni*, spur-thighed tortoises, *T. graeca*, marginated tortoises, *T. marginata*, Horsfield's tortoises, *T. horsfieldii*, and Egyptian tortoises, *T. kleinmanni)* according to season (**A** TeHV 1 and **B** TeHV 3).

additional factors such as weather, husbandry, and additional infectious agents.

The types of herpesviruses detected during the course of this study included mostly previously described types, including the two most commonly described in tortoises, TeHV1 and TeHV3 (3, 13) as well as the type most often reported in box turtles, Terrapene herpesvirus 1 (30–32, 44). In addition, this includes the first report of a TeHV2 from a tortoise in Europe. This virus has previously only been described in *Gopherus* spp. in North America (14, 45). The single TeHV2 detected was also found in a tortoise in the same genus (a Texas tortoise, *Gopherus berlandieri*) kept in Spain. This is a reminder of the possible role of the pet trade in the transport of pathogens throughout the world, as it is most likely that the virus was imported into Europe with a tortoise host. TeHV4 was also detected in a total of three tortoises tested in this study, two leopard tortoises and one tortoise of unknown species. This strain has previously been described in a leopard tortoise in Europe (16) and in a Bowsprit

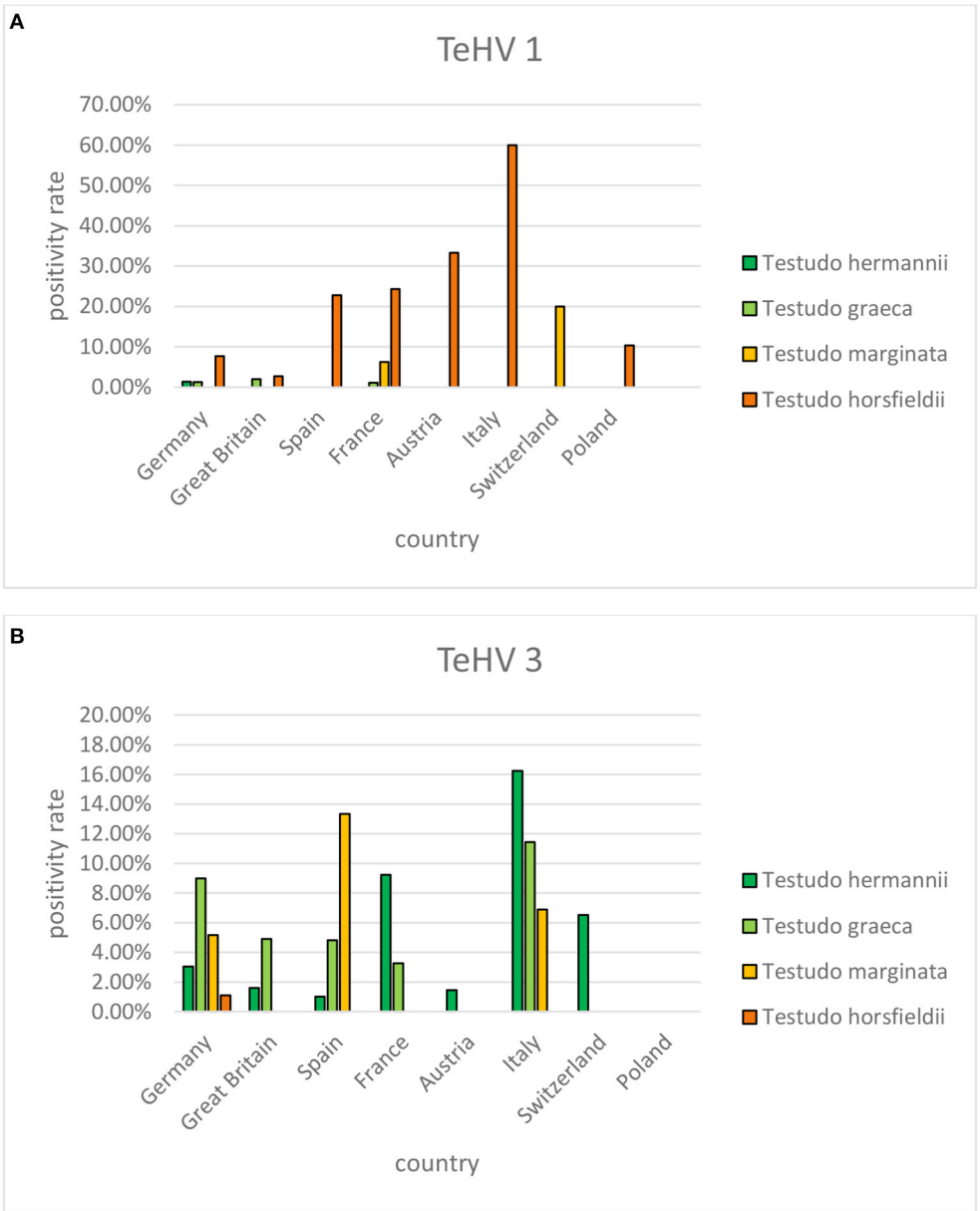


FIGURE 4 | Herpesvirus positivity rates in four *Testudo* species (Hermann's tortoises, *T. hermanni*, spur-thighed tortoises, *T. graeca*, marginated tortoises, *T. marginata*, and Horsfield's tortoises, *T. horsfieldii)* according to country for countries from which more than 100 samples were submitted (**A** TeHV 1 and **B** TeHV 3).

tortoise in the USA (15). It has been hypothesized that it may be specific to African tortoises (16, 46). This hypothesis is supported by the findings in the present study. Trachemys herpesvirus 1, which was detected in one red- and one yellow-eared (*Trachemys scripta scripta*) slider in the present study, has previously been described in a free-ranging red-eared slider in the United States (27). As for TeHV2, this is another example of international movement of viruses with their hosts, although in both cases there was no evidence from our findings of transmission of these two viruses from their presumed original North American host species into European species.

In addition to the previously described herpesviruses found in this study, three previously undescribed herpesviruses were identified in individual cases. While two of these, from a European pond turtle and a Chinese stripe-necked turtle, clustered with previously described putative members of the genus *Scutavirus*, one, from a Siebenrock's snake-necked turtle, did not. This virus was detected in a turtle from a pet store. Since the herpesvirus detected in that animal was from an oral swab and clustered with a herpesvirus from a squamate reptile (a chameleon), it is unclear whether it represents a real infection in the turtle or a possible contaminant from e.g., another reptile

in the pet store. Unfortunately, the animal was lost to follow up, but the question of whether herpesviruses from reptiles in orders other than Testudines might be able to also infect chelonians requires further study. The finding of previously undescribed scutaviruses in individual cases in this study is not unexpected. As of December 2020 there were 361 species of Testudines listed in the reptile database (http://www.reptile-database.org/db-info/SpeciesStat.html). Since viruses in the genus *Scutavirus* have been hypothesized to have co-evolved with chelonian hosts, it is likely that there are many more chelonian herpesviruses yet to be discovered (1, 26).

The majority of the samples examined in this study were from tortoises in the genus *Testudo*. The distribution of herpesvirus strains found in these animals confirmed some of the previously reported data regarding species specificity of TeHV1 and 3, with TeHV1 found much more commonly in Horsfield's tortoises than in most other *Testudo* spp., while TeHV3 was more common in Hermann's, spur-thighed, and marginated tortoises. TeHV1 was, however, also found in a variety of other species, including Hermann's, spur-thighed, marginated, Egyptian, African spurred (*Centrochelys sulcata*), leopard (*Stigmochelys pardalis*), Argentine (*Chelonoidis chilensis*), and an Indian star (*Geochelone elegans*) tortoise. This extends the list of species in which this virus has been detected (3) by several species, including the marginated, African spurred, Argentine, Egyptian, and Indian star tortoises. TeHV3 was also detected in a wide variety of species in addition to the *Testudo* spp. listed above, including Horsfield's, African spurred, radiated (*Astrochelys radiata*), leopard, and a red-footed tortoise (*Chelonoidis carbonarius*). Although there were clear differences in the species in which each of these viruses was most often found, the wide range of hosts emphasizes the ability of each of these viruses to infect a wide range of tortoises in the family Testudinidae. Since the animals tested in this study were captive, findings of specific virus strains in various species also reflect circulation of strains in various holdings.

Among the non-*Testudo* tortoise species tested, there were also clear differences in types of viruses found in different species as well as in positivity rates (**Table 1**). Although there were smaller numbers tested in each of these compared to the *Testudo* spp., there were some interesting findings. Of the 121 radiated tortoises tested, 17 (14.05%) were positive, all with TeHV3. A similar high infection rate was reported in a previous study, but with a smaller number of animals tested (13). Whether this represents an increased susceptibility of this species for this virus or sampling bias deserves further study. Among tortoises in the genus *Chelonoidis* (**Table 1**), highly significant differences ($p < 0.0001$) were found in overall positivity and for the detected virus types between the different species, with a remarkably high positivity rate in Argentine tortoises (28.57%, 90% of which were TeHV1) compared to all of the other *Chelonoidis* spp. tested. In the Emydidae tested, box turtles were significantly more often herpesvirus positive than other turtles. Similar or higher positivity rates have been reported in studies of wild box turtles in the USA (32, 44).

There were significant differences between positivity rates in samples from different European countries. It is expected that trade in animals between these countries would lead to similar disease trends in all of them. The volume of trade in reptiles has been shown to be high in Europe (47–49) but it is difficult to determine differences between different countries within Europe. The country with the highest herpesvirus positivity rate, Italy, also had the highest percentages of both TeHV1 and TeHV3 detections when taken individually. A factor that could influence the numbers of herpesvirus positive results is the distribution of species kept in captivity within the different countries. However, data on the species distribution in captivity was not available. In a study screening reptiles presented to specialized veterinarians within Poland for infectious diseases, Horsfield's tortoises were the chelonian species most often seen (50). In that study, no herpesviruses were found in any of the tortoises tested. However, a possible bias toward Horsfield's tortoises over other species as pets in Poland could explain the detection of TeHV1 but no other testudinid herpesvirus types in samples from that country.

Season was shown to affect the positivity rate of herpesviruses in general as well as of TeHV1 and TeHV3 individually and for herpesviruses in Emydidae. Herpesviruses in general, as well as both TeHV1 and TeHV3 were all most often detected in samples submitted in the spring. Increased virus detection and increased herpesvirus-associated disease in tortoises in the spring has been described before (13, 41). The influence of season on susceptibility to infection, recrudescence of infection, and disease development is not yet understood, but several authors have hypothesized that seasonal differences in behavior as well as environmental temperature could influence all of these elements. The majority of species sampled in this study hibernate in the winter. Hibernation is associated with various changes in the immune system (51), and has been hypothesized to increase herpesvirus recrudescence (41). Studies screening wild-caught Blanding's turtles (*Emydoidea blandingii*) in North America for Emydoidea herpesvirus 1 have shown higher positivity rates in May than in other months (52, 53). The authors pointed out that this corresponded to the start of nesting season and increased viral shedding could be associated with the corresponding increased physiologic demands. A study of an outbreak of TeHV3 infection and associated disease in a breeding facility with a mixed collection of turtles and tortoises in Italy showed that the disease outbreak began in spring, although it persisted over several months (41). Surviving animals were most likely to shed virus in the spring, post hibernation. In contrast to the findings in tortoises, herpesviruses in turtles in the family Emydidae were most often found in the summer, followed by the fall. This corresponds to findings in free-ranging Eastern box turtles in the USA. In one such study, the highest prevalence of Terrapene herpesvirus 1 was found in July compared to September and May (44). In that case, the authors discussed increased activity and differences in behaviors, including a possible increase in contacts between animals during foraging, as possible reasons for increased detection rates in the summer. Another study screening eastern box turtles in the same states in the USA found the highest prevalence of Terrapene herpesvirus 1 in the fall (32). Influences of seasonality of herpesvirus infections may therefore be complex and requires further study.

The vast majority of samples included in this study were oral swabs. Oral swabs have been shown to be a good and relatively uninvasive sample for the detection of herpesviruses in tortoises (54). In aquatic turtles, oral swabs (28, 30, 44) or combined oral and cloacal swabs (26, 52, 53) have most often been used for the detection of herpesviruses in live animals. However, it is possible that alternative samples could have been more appropriate in some cases. The quality of sample collection was also not standardized in this study. Samples were submitted in almost all cases by veterinarians, but it is likely that there were differences in sampling technique between the different individuals that submitted samples for testing. The time between sample collection and arrival in the laboratory was also not standardized. These factors could have contributed to false negative results in some cases, so that the actual infection rate among the sampled chelonians could have been higher than detected.

The methods used for herpesvirus detection have all been described previously for use in the detection of herpesviruses in chelonians (13). The pan-herpesvirus PCR described by VanDevanter et al. (36) specifically has been very useful in detecting previously unknown herpesviruses in multiple species (9, 15, 24–26, 30) and was key to the identification of several of the viruses described here. In many cases, more specific and often more sensitive PCRs have been developed for the detection of individual herpesvirus strains in specific species and populations (28, 39, 40, 55, 56). However, these methods are not expected to be able to detect strains other than their target strains, limiting their use in the situation described here in which samples from a very wide range of species were submitted and where in numerous cases the exact species from which the sample was obtained was not identified to the laboratory.

Unfortunately, there were only very few cases in which more in-depth data on the animals from which the samples were collected was available. It was therefore not possible to evaluate the results based on clinical signs reported in the animals or on other characteristics such as sex or age of the animals tested.

It is important to note that the sample population examined in this study was biased as it relied on samples submitted by veterinarians or owners willing to pay for diagnostic testing. It is likely that testing was carried out more often in animals that were clinically ill or had known contact with clinically ill animals. The reported herpesvirus positivity rates therefore likely do not reflect the actual prevalence among captive chelonians in Europe. This bias is likely to lead to a higher estimated prevalence of herpesvirus infection than the actual prevalence. However, it is also important to note that diagnosis in this study relied on PCR detection, so that in some cases, latently infected animals not actively shedding virus might have tested false negative. The numbers of latently infected animals that would not have tested positive using the methods described in this study is not known, but likely depends on various factors, especially host species and virus strain.

The results of this study provide important information on both the diversity and relative importance of various herpesvirus strains in a wide range of captive chelonian species as well as some information on variation in prevalence of infection and shedding depending on a number of different factors. It is important to continue to monitor herpesvirus positivity rates and herpesvirus strains in captive reptiles over time to better understand their importance and impacts on reptiles in captivity. Additional studies on herpesviruses in the reptile trade as well as interactions between infections in captive reptiles, reptiles in trade, and wild reptiles would be of interest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CL participated in the conceptualization, compiled, summarized, and analyzed the data and drafted figures and tables. EM participated in the conceptualization and planning of the project. RM participated in the conceptualization of the project and data analysis and led manuscript development. All authors provided critical feedback and contributed to the final manuscript.

ACKNOWLEDGMENTS

The authors thank Amanda L. J. Duffus, Gordon State College, GA, USA for her assistance with data presentation. We thank Ekaterina Salzmann, Lisa Schüler, and Ellen Schöner Laboklin GmbH & C. KG for technical assistance. We also thank the many veterinarians who submitted samples and provided background data on the animals from which the samples were collected.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.733299/full#supplementary-material

REFERENCES

1. McGeoch DJ, Gatherer D. Integrating reptilian herpesviruses into the family Herpesviridae. *J Virol.* (2005) 79:725–31. doi: 10.1128/JVI.79.2.725-731.2005
2. Gandar F, Wilkie GS, Gatherer D, Kerr K, Marlier D, Diez M, et al. The genome of a tortoise herpesvirus (testudinid herpesvirus 3) has a novel structure and contains a large region that is not required for replication in vitro or virulence in vivo. *J Virol.* (2015) 89:11438–56. doi: 10.1128/JVI.01794-15
3. Marschang RE, Origgi FC, Stenglein MD, Hyndman TH, Wellehan JFX, Jacobson ER. Viruses and viral diseases of reptiles. In: Jacobson ER, Garner MM, editors. *Infectious Diseases and Pathology of Reptiles Volume 1*: C*olor Atlas and Text*. 2nd ed. Boca Raton, LA: CRC Press (2021). p. 575–703. doi: 10.1201/9780429155567-10

4. International Committee on Taxonomy of Viruses. *Virus Taxonomy*. (2019). Available online at: https://talk.ictvonline.org/taxonomy/ (accessed September 28, 2021).
5. Herbst LH, Jacobson ER, Moretti R, Brown T, Sundberg JP, Klein PA. Experimental transmission of green turtle fibropapillomatosis using cell-free extracts. *Dis Aquat Org.* (1995) 22:1–12. doi: 10.3354/dao022001
6. Ackermann M, Koriabine M, Hartmann-Fritsch F, de Jong PJ, Lewis TD, Schetle N, et al. The genome of chelonid herpesvirus 5 harbors atypical genes. *PLoS ONE.* (2012) 7:e46623. doi: 10.1371/journal.pone.0046623
7. Jacobson ER, Gaskin JM, Roelke M, Greiner EC, Allen J. Conjunctivitis, tracheitis, and pneumonia associated with herpesvirus infection in green sea turtles. *J Amer Vet Med Assoc.* (1986) 189:1020–3.
8. Rebell G, Rywlin A, Haines H. A herpesvirus agent associated with skin lesions of green sea turtles in aquaculture. *Am J Vet Res.* (1975) 36:1221–4.
9. Stacy BA, Wellehan JFX, Foley AM, Coberley SS, Herbst LH, Manire CA, et al. Two herpesviruses associated with disease in wild Atlantic loggerhead sea turtles (Caretta caretta). *Vet Microbiol.* (2008) 126:63–73. doi: 10.1016/j.vetmic.2007.07.002
10. Origgi FC, Tecilla M, Pilo P, Aloisio F, Otten P, Aguilar-Bultet L, et al. A genomic approach to unravel host-pathogen interaction in chelonians: The example of testudinid herpesvirus 3. *PLoS ONE.* (2015) 10:e0134897. doi: 10.1371/journal.pone.0134897
11. Origgi FC, Romero CH, Bloom DC, Klein PA, Gaskin JM, Tucker SJ, et al. Experimental transmission of a herpesvirus in Greek tortoises (Testudo graeca). *Vet Path.* (2004) 41:50–61. doi: 10.1354/vp.41-1-50
12. Une Y, Murakami M, Uemura K, Fujitani H, Ishibashi T, Nomura Y. Polymerase chain reaction (PCR) for the detection of herpesvirus in tortoises. *J Vet Med Sci.* (2000) 62:905–7. doi: 10.1292/jvms.62.905
13. Kolesnik E, Obiegala A, Marschang RE. Detection of Mycoplasma spp., herpesviruses, topiviruses, and ferlaviruses in samples from chelonians in Europe. *J Vet Diagn Invest.* (2017) 29:820–32. doi: 10.1177/1040638717722387
14. Johnson AJ, Pessier AP, Wellehan JF-X, Brown R, Jacobson ER. Identification of a novel herpesvirus from a California desert tortoise (Gopherus agassizii). *J Vet Micro.* (2005) 111:107–16. doi: 10.1016/j.vetmic.2005.09.008
15. Bicknese EJ, Childress AL, Wellehan JFX. A novel herpesvirus of the proposed genus Chelonivirus from an asymptomatic bowsprit tortoise (Chersina angulata). *J Zoo Wildl Med.* (2010) 41:353–8. doi: 10.1638/2009-0214R.1
16. Kolesnik E, Mittenzwei F, Marschang RE. Detection of testudinid herpesvirus type 4 in a leopard tortoise (Stigmochelys pardalis). *Tierärztl Praxis Kleintiere.* (2016) 44:283–6. doi: 10.15654/TPK-150843
17. Marschang RE. *Isolation and characterization of irido-, herpes-, and reoviruses from tortoises and description of an uncharacterized cytopathogenic agent*. DrMedVet Dissertation, Giessen, Justus Leibig Univsersitat (2000).
18. Martel A, Blahak S, Vissenaekens H, Pasmans F. Reintroduction of clinically healthy tortoises: the herpesvirus Trojan horse. *J Wildl Dis.* (2009) 45:218–20. doi: 10.7589/0090-3558-45.1.218
19. Salinas M, Francino O, Sánchez A, Altet L. Mycoplasma and herpesvirus PCR detection in tortoises with rhinitis-stomatitis complex in Spain. *J Wildl Dis.* (2011) 47:195–200. doi: 10.7589/0090-3558-47.1.195
20. Soares JF, Chalker VJ, Erles K, Holtby S, Waters M, McArthur S. Prevalence of Mycoplasma agassizii and chelonian herpesvirus in captive tortoises (Testudo sp.) in the United Kingdom. *J Zoo Wildl Med.* (2004) 35:25–33. doi: 10.1638/02-092
21. Frye FL, Oshiro LS, Dutra FR, Carnet JD. Herpesvirus-like infection in two Pacific pond turtles. *J Amer Vet Med Assoc.* (1977) 171:882–4.
22. Cox WR, Rapley WA, Barker IK. Herpesvirus infection in a painted turtle. *J Wildl Dis.* (1980) 16:445–9. doi: 10.7589/0090-3558-16.3.445
23. Jacobson ER, Gaskin JM, Wahlquist H. Herpesvirus-like infection in map turtles. *J Amer Vet Med Assoc.* (1982) 181:1322–4.
24. Jungwirth N, Bodewes R, Osterhaus ADME, Baumgartner W, Wohlsein P. First report of a new alphaherpesvirus in a freshwater turtle (Pseudemys concinna concinna) kept in Germany. *Vet Microbiol.* (2014) 170:403–7. doi: 10.1016/j.vetmic.2014.02.029
25. Ossiboff RJ, Newton AL, Seimon TA, Moore RP, McAloose D. Emydid herpesvirus 1 infection in northern map turtles (Graptemys geographica) and painted turtles (Chrysemys picta). *J Vet Diagn Invest.* (2015) 27:392–5. doi: 10.1177/1040638715584793
26. Ossiboff RJ, Raphael BL, Ammazzalorso AD, Seimon TA, Newton AL, Chang TV, et al. Three novel herpesviruses of endangered Clemmys and Glyptemys turtles. *PLoS ONE.* (2015) 10:e0122901. doi: 10.1371/journal.pone.0122901
27. Aplasca AC, Titus V, Ossiboff RJ, Murphy L, Seimon TA, Ingerman K, et al. Health assessment of free-ranging chelonians in an urban section of the Bronx River, New York. *J Wildl Dis.* (2019) 55:352–62. doi: 10.7589/2017-12-304
28. Lindemann DM, Allender MC, Thompson D, Adamovicz L, Dzhaman E. Development and validation of a quantitative PCR assay for detection of Emydoidea herpesvirus 1 in free-ranging Blanding's turtles (Emydoidea blandingii). *J Virol Methods.* (2018) 254:40–5. doi: 10.1016/j.jviromet.2018.01.006
29. Andersson KE, Adamovicz L, Mumm LE, Winter JM, Glowacki G, Teixeira-Neto R, et al. Detection of a novel herpesvirus associated with squamous cell carcinoma in a free-ranging Blanding's turtle. *J Vet Diagn Invest.* (2021) 33:348–51. doi: 10.1177/1040638721989302
30. Sim RR, Norton TM, Bronson E, Allender MC, Stedman N, Childress AL, et al. Identification of a novel herpesvirus in a captive Eastern box turtles (Terrapene carolina carolina). *Vet Microbiol.* (2015) 175:218–33. doi: 10.1016/j.vetmic.2014.11.029
31. Sim RR, Allender MC, Crawford LK, Wack AN, Murphy KJ, Mankowski J, et al. Ranavirus epizootic in captive eastern box turtles (Terrapene carolina carolina) with concurrent herpesvirus and mycoplasma infection: Management and monitoring. *J Zoo Wildl Med.* (2016) 47:256–70. doi: 10.1638/2015-0048.1
32. Archer GA, Phillips CA, Adamovicz L, Band M, Byrd J, Allender MC. Detection of copathogens in free-ranging eastern box turtles (Terrapene carolina carolina) in Illinois and Tennessee. *J Zoo Wildl Med.* (2017) 48:1127–34. doi: 10.1638/2017-0148R.1
33. Yonkers SB, Schneider R, Reavill DR, Archer LL, Childress AL, Wellehan, JFX, Jr. Coinfection with a novel fibropapilloma-associated herpesvirus and a novel Spirorchis sp. in an eastern box turtle (Terrapene carolina) in Florida. *J Vet Diagn Invest.* (2015) 27:408–13. doi: 10.1177/1040638715589612
34. Marschang RE, Heckers KO, Heynol V, Weider K, Behncke H. Nachweis eines Herpesvirus bei klinisch gesunden Klappbrust-Pelomedusenschildkroeten (Pelusios castaneous). *Tieraerztl Prax Kleintiere.* (2015) 43:166–9. doi: 10.15654/TPK-140575
35. Široký P, Frye FL, Dvoráková N, Hostovský M, Prokop H, Kulich P. Herpesvirus associated dermal papillomatosis in Williams' mud turtle Pelusios williamsi with effects of autogenous vaccine therapy. *J Vet Med Sci.* (2018) 80:1248–54. doi: 10.1292/jvms.18-0126
36. VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, et al. Detection and analysis of diverse herpesviral species by consensus primer PCR. *J Clin Microbiol.* (1996) 34:1666–71. doi: 10.1128/jcm.34.7.1666-1671.1996
37. Teifke JP, Löhr CV, Marschang RE, Osterrieder N, Posthaus H. Detection of chelonid herpesvirus DNA by nonradioactive in situ hybridization in tissues from tortoises suffering from stomatitis–rhinitis complex in Europe and North America. *Vet Pathol.* (2000) 37:377–85. doi: 10.1354/vp.37-5-377
38. Marschang RE, Gleiser CB, Papp T, Pfitzner AJP, Böhm R, Roth BN. Comparison of eleven herpesvirus isolates from tortoises using partial sequences from three conserved genes. *Vet Microbiol.* (2006) 117:258–66. doi: 10.1016/j.vetmic.2006.06.009
39. Engel AI, Adamovicz L, Wellehan, JFX, Jr, Allender MC. Development and validation of a quantitative PCR assay for detection of Terrapene herpesvirus 2 in eastern box turtles (Terrapene carolina carolina). *J Virol Methods.* (2020) 286:113968. doi: 10.1016/j.jviromet.2020.113968
40. Kane LP, Bunick D, Abd-Eldaim M, Dzhaman E, Allender MC. Development and validation of quantitative PCR for detection of Terrapene herpesvirus 1 utilizing free-ranging eastern box turtles (Terrapene carolina carolina). *J Virol Methods.* (2016) 232:57–61. doi: 10.1016/j.jviromet.2016.02.002
41. Marenzoni ML, Santoni L, Felici A, Maresca C, Stefanetti V, Sforna M, et al. Clinical, virological epidemiological characterization of an outbreak of Testudinid Herpesvirus 3 in a chelonian captive breeding facility: Lessons learned first evidence of TeHV3 vertical transmission. *PLoS ONE.* (2018) 13:e0197169. doi: 10.1371/journal.pone.0197169
42. Wilson EB. Probable inference, the law of succession, statistical inference. *J Am Stat Assoc.* (1927) 22:209–12. doi: 10.1080/01621459.1927.10502953

43. Hellebuyck T, Couck L, Ducatelle R, Van den Broeck W, Marschang RE. Cheilitis associated with a novel herpesvirus in two panther chameleons (*Furcifer pardalis*). *J Comp Pathol.* (2021) 182:58–66. doi: 10.1016/j.jcpa.2020.12.004
44. Kane LP, Allender MC, Archer G, Dzhaman E, Pauley J, Moore AR, et al. Prevalence of terrapene herpesvirus 1 in free-ranging eastern box turtles (Terrapene carolina carolina) in Tennessee and Illinois, USA. *J Wildl Dis.* (2017) 53:285–95. doi: 10.7589/2016-06-138
45. Jacobson ER, Berry KH, Wellehan JFX, Origgi F, Childress AL, Braun J, et al. Serologic and molecular evidence for Testudinid herpesvirus 2 infection in wild Agassiz's desert tortoises, Gopherus agassizii. *J Wildl Dis.* (2012) 48:747–57. doi: 10.7589/0090-3558-48.3.747
46. Origgi FC. Testudinid herpesviruses: a review. *J Herpetol Med Surg.* (2012) 22:42–54. doi: 10.5818/1529-9651-22.1-2.42
47. Auliya M, Altherr S, Ariano-Sanchez D, Baard EH, Brown C, Brown RM, et al. Trade in live reptiles, its impact on wild populations, and the role of the European market. *Biol Conserv.* (2016) 204:103–19. doi: 10.1016/j.biocon.2016.05.017
48. Bush ER, Baker SE, Macdonald DW. Global trade in exotic pets 2006-2012. *Conserv Biol.* (2014) 28:663–76. doi: 10.1111/cobi.12240
49. Robinson JE, Criffiths RA, St. John FAV, Roberts DL. Dynamics of the global trade in live reptiles: Shifting trends in production and consequences for sustainability. *Biol Conserv.* (2015) 184:42–50. doi: 10.1016/j.biocon.2014.12.019
50. Pasterny J, Skomorucha L, Stanicki K, Marschang RE. Detection of infectious agents in samples from reptiles presented at veterinary clinics in Poland. *J Herpetol Med Surg.* (2021) 31:64–72. doi: 10.5818/12-2020.1
51. Marschang RE, Meddings J, Ariel E. Viruses of reptiles. In: Hurst CJ, editor. *Studies in Viral Ecology*. 2nd ed. New York, NY: Wiley (2021). p. 449–510. doi: 10.1002/9781119608370.ch13
52. Lindemann DM, Allender MC, Thompson D, Glowacki GA, Newman EM, Adamovicz LA, et al. Epidemiology of Emydoidea herpesvirus 1 in free-ranging Blanding's turtles (Emydoidea blandingii) from Illinois. *J Zoo Wildl Med.* (2019) 50:547–56. doi: 10.1638/2018-0074
53. Winter JM, Mumm L, Adamovicz LA, Andersson KE, Glowacki GA, Allender MC. Characterizating the epidemiology of historic and novel pathogens in Blanding's turtles (Emydoidea blandingii). *J Zoo Wildl Med.* (2020) 51:606–17. doi: 10.1638/2019-0154
54. Marschang RE. Virology. In: Divers SJ, Stahl SJ, editors. *Mader's Reptile and Amphibian Medicine and Surgery*. 3rd ed. St. Louis, MO: Elsevier (2019). p. 247–69. doi: 10.1016/B978-0-323-48253-0.00030-1
55. Alfaro-Núñez A, Gilbert MT. Validation of a sensitive PCR assay for the detection of Chelonid fibropapilloma-associated herpesvirus in latent turtle infections. *J Virol Methods.* (2014) 206:38–41. doi: 10.1016/j.jviromet.2014.05.019
56. Braun J, Schrenzel M, Witte C, Gokool L, Burchell J, Rideout BA. Molecular methods to detect Mycoplasma spp. and testudinid herpesvirus 2 in desert tortoises (Gopherus agassizii) and implications for disease managements. *J Wildl Dis.* (2014) 50:757–66. doi: 10.7589/2013-09-231

Conflict of Interest: CL, EM, and RM are employed by a private laboratory that offers diagnostic testing for veterinarians. This employment did not influence the study design, data interpretation, or writing of this manuscript.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 29 October 2021
doi: 10.3389/fvets.2021.732993

Direct and Latent Effects of Pathogen Exposure Across Native and Invasive Amphibian Life Stages

Jenny Urbina[1]*, *Evan M. Bredeweg*[2], *Andrew R. Blaustein*[3] and *Tiffany S. Garcia*[2]

[1] Environmental Sciences Graduate Program, Oregon State University, Corvallis, OR, United States, [2] Department of Fisheries, Wildlife and Conservation Sciences, Oregon State University, Corvallis, OR, United States, [3] Department of Integrative Biology, Oregon State University, Corvallis, OR, United States

OPEN ACCESS

Edited by:
Rachel E. Marschang,
Laboklin GmbH & Co. KG, Germany

Reviewed by:
Gabriela Parra-Olea,
National Autonomous University of Mexico, Mexico
John Abramyan,
University of Michigan–Dearborn, United States
Susan C. Walls,
United States Geological Survey (USGS), United States

***Correspondence:**
Jenny Urbina
jennyurbina@gmail.com

Specialty section:
This article was submitted to Zoological Medicine, a section of the journal Frontiers in Veterinary Science

Received: 29 June 2021
Accepted: 28 September 2021
Published: 29 October 2021

Citation:
Urbina J, Bredeweg EM, Blaustein AR and Garcia TS (2021) Direct and Latent Effects of Pathogen Exposure Across Native and Invasive Amphibian Life Stages. Front. Vet. Sci. 8:732993. doi: 10.3389/fvets.2021.732993

Emerging infectious diseases are one of the multiple factors contributing to the current "biodiversity crisis". As part of the worldwide biodiversity crisis, amphibian populations are declining globally. Chytridiomycosis, an emerging infectious disease, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is a major cause of amphibian population declines. This fungus primarily affects keratinized structures in larval, juvenile, and adult amphibians as well as heart function. However, we know little about how *Bd* can impact embryos as well as potential latent effects of *Bd* exposure over ontogeny. Using two different *Bd* strains and multiple exposure times, we examined the effects of *Bd* exposure in Pacific chorus frog (*Pseudacris regilla*), Western toad (*Anaxyrus boreas*) and American bullfrog (*Lithobates catesbeianus*) life stages. Using a factorial experimental design, embryos of these three species were exposed to *Bd* at early and late embryonic stages, with some individuals re-exposed after hatching. Embryonic *Bd* exposure resulted in differential survival as a function of host species, *Bd* strain and timing of exposure. *P. regilla* experienced embryonic mortality when exposed during later developmental stages to one *Bd* strain. There were no differences across the treatments in embryonic mortality of *A. boreas* and embryonic mortality of *L. catesbeianus* occurred in all *Bd* exposure treatments. We detected latent effects in *A. boreas* and *L. catesbeianus* larvae, as mortality increased when individuals had been exposed to any of the *Bd* strains during the embryonic stage. We also detected direct effects on larval mortality in all three anuran species as a function of *Bd* strain, and when individuals were double exposed (late in the embryonic stage and again as larvae). Our results suggest that exposure to *Bd* can directly affect embryo survival and has direct and latent effects on larvae survival of both native and invasive species. However, these impacts were highly context dependent, with timing of exposure and *Bd* strain influencing the severity of the effects.

Keywords: pathogen exposure, development, carry-over, embryos, larvae

INTRODUCTION

In many organisms, exposure to stressors during embryonic or prenatal stages can result in both direct and latent effects on subsequent developmental stages. For example, in birds, conditions experienced at early life are determinants of fitness in adults (1); in snapping turtles (*Chelydra serpentina*), hatchling from eggs incubated on a wet substrate have an improved locomotor performance in comparison to hatchlings from drier substrates (2) and in fishes, embryos of pink salmon (*Oncorhynchus gorbuscha*) exposed to oil have a reduction in juvenile growth and survival (3). In amphibians, these effects can be on individual growth rates, behavior, locomotion or immunology (4–7). Thus, exposure to predator cues in the pinewoods tree frog *Hyla femoralis* slowed larval growth and development, resulting in metamorphs with relatively smaller body sizes (8). In amphibians, repeated exposure at early life stages to other environmental stressors, such as contaminants, predator cues, and pathogens, can produce latent effects in juvenile and adult amphibians (4, 9, 10). Particularly in amphibian embryos, environmental cues can cause significant changes in hatching traits (11–14). Time of hatching can change in response to risks and opportunities. For example, embryos can hatch early to escape predators and pathogens, and this life history shift can have effects that persist through later life stages (15–17). Additionally, embryos infected with water molds can suffer differential mortality rates relative to the timing of exposure to the pathogen (18). As such, the timing of pathogen exposure might play a critical role on host susceptibility to infection (19).

Host ontogeny is a key factor for examining or predicting disease dynamics. There is precedence in different systems than minimum changes in the history of exposure to pathogens during a particular developmental stage can drastically change host life history. Changes in individual susceptibility to pathogens occur throughout ontogeny in many organisms, including plants (20), insects (21), birds (22), reptiles (23), mammals (24) and amphibians (25, 26). The key, however, to understanding temporal association between pathogens and susceptibility is to empirically discern latent and direct effects within and across life history stages. We posit that amphibians can be model systems for testing these questions as they are a taxon of conservation concern, have complex life histories, and are susceptible to multiple emerging infectious diseases.

One of the most researched amphibian pathogens is the fungus *Batrachochytrium dendrobatidis* (*Bd*), which has been implicated in the decline of numerous amphibian species worldwide (27–29). Differential susceptibility to *Bd* has been documented across species (30–35), populations (36, 37), life stages (38–42), and *Bd* strains (29, 33, 43–46). However, how exposure to *Bd* in one developmental stage can produce latent affects in a later life stage is unclear. Information regarding direct *Bd* impacts on embryos is also lacking as *Bd* mainly affects keratinized structures, which are absent in embryos. Further, the importance of evolutionary relationships between *Bd* strain and the embryonic host may also have significant implications.

We explored the direct and latent effects of *Bd* exposure on both the embryonic and larval stages using three amphibian species with differential susceptibility to native and invasive *Bd* strains (33, 34, 46, 47). We posit that amphibian embryos will be susceptible to the chytrid fungus as *Bd* can produce enzymes that can destroy tissue (48–50). Further, the release of fungal toxin (30, 51) could impact embryos by delaying growth, triggering key transitions resulting in ontogenetic shifts or latent effects on life history trajectories. Finally, we hypothesized that *Bd* can impact embryos by depletion of oxygen. The lack of dissolved oxygen slows down the development in *Bufo bufo* (52) and hypoxia can kill early life stages (13). Direct or latent effects may also vary with *Bd* strain and with host species, therefore evaluating different strains is critical to disentangle intrinsic aspects of the pathogen as virulence and how it changes among hosts. We also examined the influence of *Bd* exposure on larval survival predicting that repeated exposure to *Bd* across the embryonic/larval transition would result in decreased survival.

MATERIALS AND METHODS

We studied three anuran species found in the US Pacific Northwest (PNW). These are the Pacific chorus frog (*Pseudacris regilla*) a common species throughout its PNW range found in a variety of habitats from sea level to montane regions, the Western toad (*Anaxyrus boreas*) whose populations have experienced declines across much of its historic range and American bullfrogs (*Lithobates catesbeianus*) an introduced species in the PNW (53–55). Twenty clutches of *P. regilla* were collected from Little Three creeks on 19 June 2014 (44°06′03.5″ N, 121°38′34.7″ WGS84 Deschutes County, OR, elevation = 2,000 m) and 600 eggs of *A. boreas* were collected from 20 different egg masses at Todd Lake (44°01′44.5″ N, 121°41′07.6″W WGS84 Deschutes County, OR, elevation = 1,870 m) on 29 May 2015. We collected 600 newly laid eggs from six distinct *L. catesbeianus* egg masses from William L. Finley National Wildlife Refuge on 20 May 2014 (44°25′23.6″ N, 123°18′41.8″W WGS84 Benton County, OR, elevation = 276 m). After collection, eggs were immediately transported to a climate-controlled environment at Oregon State University and held under constant temperature (14°–15.5°C) and photoperiod (12L: 12D) conditions. Less than 6 h after arrival, every clutch of *P. regilla* or group of eggs of *A. boreas* and *L. catesbeianus* were divided into three groups and each group for *P. regilla* and *A. boreas* contained ∼10 eggs (±1.95 eggs), and 20 eggs for *L. catesbeianus*.

Pre-hatch Exposure Regime

Bd exposure treatments were administered in either the early embryonic developmental stages or closer to hatching. Early exposure (early) corresponded to the late gastrula stages, or Gosner Developmental Stage 12 (56) while closer to hatching exposure (late) corresponded to embryos capable of muscular response, or Gosner Developmental Stage 18 (56). *Bd* strains (i.e., the isolate of the fungus used for the inoculation) included an endemic *Bd* strain (JEL 630, hereafter "West," isolated from *L. catesbeianus* in Oregon), and a novel *Bd* strain to Oregon

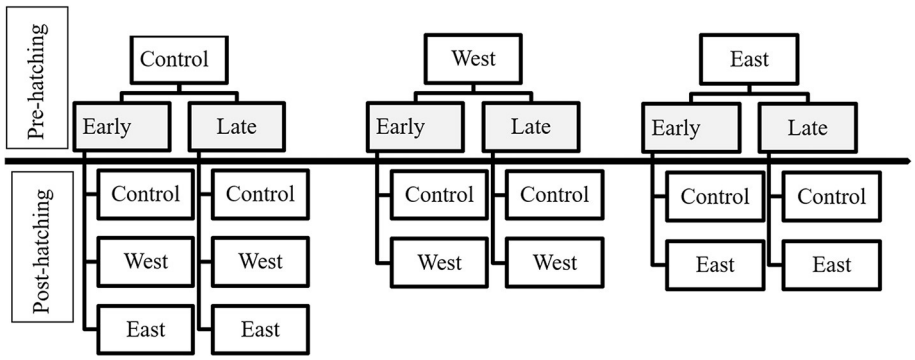


FIGURE 1 | Exposure treatments of egg masses and tadpoles according to the time of exposure and chytrid strain. Pre-hatching treatments are indicated in gray and above the separation line. Treatments for tadpoles (post-hatching) are listed below the separation line.

freshwater habitats (JEL 627, hereafter "East," isolated from *L. catesbeianus* in Maine USA). These strains were identified as part of the North American clade (*Bd*-GPL-1) (57). We obtained cryogenically preserved culture plates from J. Longcore to prepare 1% sterile tryptone—agar media plates with 0.5 ml of stock *Bd* broth coming from each particular strain of the fungus (31). *Bd* cultures were allowed to grow for 5–8 days at 20°C before used in the experiment (31).

Using a hemocytometer, we quantified the zoospores from a pooled inoculation broth (8–12 plates per *Bd* strain). Five ml inoculations of the zoospore broth (30 K zoospores/ml) were then administered to experimental units (18 cm H × 10 cm OD high-density polyethylene graduated beakers) containing 800 ml of dechlorinated water. A similar dose was previously tested in larvae of *P. regilla* (47), *A. boreas* (33, 58, 59), and *L. catesbeianus* (32, 60). Controls were inoculated with a sham inoculum created by rinsing the same number of sterile agar plates with 5 ml of dechlorinated water.

Using a factorial experimental design, each group of eggs was assigned to a time of exposure treatment (early, late) and a *Bd* strain treatment (West, East, Control) (**Figure 1**, Pre-Hatching). Sixty experimental units (581 total eggs) were assigned for *P. regilla* (10 replicates per Early and Late treatment groups), 51 experimental units (506 total eggs) for *A. boreas* (8 replicates per Early exposure treatment, 9 replicates per Late exposure treatment), and 30 experimental units (600 total eggs) for *L. catesbeianus* (5 replicates for all groups, except for the East and West/Late exposure groups, which had 6 and 4 replicates, respectively) (**Table 1**). The length of the pre-hatching phase varied by species, lasting 19 days for *P. regilla* and *A. boreas*, and 22 days for *L. catesbeianus*. Embryos that died were preserved individually in 2.0 ml Eppendorf tubes with 95 % ethanol. No water changes were performed during the pre-hatching phase as movement associated with water changes can induce hatching, thus influencing our results. Upon hatching at Gosner stage 21, water changes were conducted weekly. We quantified the time of hatching by direct observation, and hatchling events and survival were recorded twice per day.

To analyze differences in hatching time, we compared proportions between treatments of eggs being exposed to *Bd* and control treatments (no exposure to the pathogen) using quasibinomial generalized linear models (GLM) performed independently per species. All analyses were run in R (61). To evaluate differences among strains and controls we calculated pairwise comparison using a Tukey HSD test.

Post-hatch Exposure Regime

Upon hatching, survivors were pooled within pre-hatch treatment groups (Early or Late; East, West, Control) to standardize sample sizes for the post-hatch phase of the experiment. In this phase, larvae were either re-exposed to the same pre-hatch *Bd* strain or held as controls to estimate latent effects (**Figure 1**, Post-Hatch). For *P. regilla*, we had a total of 328 surviving hatchlings distributed across the larval exposure treatments for a total of 82 experimental units, resulting in 33 control replicates, 21 East strain replicates, and 28 West strain replicates. Less than 10% of eggs hatched from the East/Late pre-hatch exposure treatment group; as such, there was no continuation of this treatment in the post-hatch phase. For *A. boreas*, we ran 42 control replicates, 28 East strain replicates, and 26 West strain that contained a total of 384 surviving hatchlings for a total of 96 experimental units. For *L. catesbeianus*, we ran 23 control replicates, 17 East strain replicates, and 16 West strain using a total of 228 surviving hatchlings with a total of 56 experimental units. Due to complete mortality in the East/Early and the West/Early pre-hatch phase, these treatments were not continued in the post-hatch phase (**Table 2**).

Larvae were held individually and those that were re-exposed to *Bd* were re-inoculated once a week (every 7 d) for the duration of the experiment. Individuals were held in rectangular plastic containers (31 × 18 × 8 cm) filled with 2,000 ml dechlorinated water. Water changes occurred concurrently with re-inoculation using 5 ml of 50 K zoospores/ml. Animals that died during the experiment were preserved in 95% ethanol. At the end of the experiment, animals remaining alive were humanely euthanized in accordance with institutional animal care protocol in MS-222 (Tricaine methanesulfonate) and then preserved in 95% ethanol. The experimental trials for each species lasted until individuals reached Gosner stage 30–31 (distinctive rear limb bud) or death.

TABLE 1 | Number of replicates per treatment per species followed by total number of eggs per treatment between parentheses.

	Pre-hatch exposure regime *Bd* strain × Time exposure treatments					
	Control		East		West	
Host species	**Early**	**Late**	**Early**	**Late**	**Early**	**Late**
Pseudacris regilla	10 (101)	10 (96)	10 (97)	10 (94)	10 (98)	10 (95)
Anaxyrus boreas	8 (80)	9 (85)	8 (84)	9 (86)	8 (84)	9 (87)
Lithobates catesbeianus	5 (100)	5 (100)	5 (100)	6 (120)	5 (100)	4 (80)

TABLE 2 | Number of replicated groups exposed in the different treatments per species as larvae.

		Pre-hatching treatments *Bd* strain × Time					
		Control		East		West	
		Early	**Late**	**Early**	**Late**	**Early**	**Late**
Pseudacris regilla							
Post-hatch	Control	8 (32)	7 (28)	5 (20)	0 (0)	7 (28)	6 (24)
Bd treatment	East	8 (32)	7 (28)	6 (24)	0 (0)	–	–
	West	8 (32)	7 (28)	–	–	7 (28)	6 (24)
Anaxyrus boreas							
Post-hatch	Control	5 (20)	3 (12)	8 (32)	8 (32)	9 (36)	9 (36)
Bd treatment	East	6 (24)	5 (20)	9 (36)	8 (32)	–	–
	West	5 (20)	4 (16)	–	–	9 (36)	8 (32)
Lithobates catesbeianus							
Post-hatch	Control	6 (24)	6 (24)	0 (0)	7 (28)	0 (0)	4 (16)
Bd treatment	East	5 (20)	5 (20)	0 (0)	7 (28)	–	–
	West	6 (24)	6 (24)	–	–	0 (0)	4 (16)

In parentheses total number of individuals per treatment including all replicates; (–) no treatment.

Total duration for the experiment was 65 days for *P. regilla*, 59 days for *A. boreas* and 19 days for *L. catesbeianus*.

We monitored survival twice per day and quantified developmental differences through time by staging all larvae (Gosner stage) every week during water changes. At the end of the post-hatch phase, we sampled a subset of all *Bd*-exposed animals of each species and also randomly sampled 5 control animals of each species to confirm no contamination happened. To assess infection load at the termination of the experiment, we dissected larvae mouthparts for *P. regilla* individuals, and we swabbed mouthparts using fine tipped sterile rayon swabs (Medical Wire and Equipment MW&E 113) for *A. boreas and L. catesbeianus*. Both protocols, swabbing and cutting mouthparts, are recommended as adequate protocols for assessing infection loads. Excised mouthparts and swabbing are similar in the likelihood of detecting *Bd* infection regardless of developmental stage and larval size (62–64).

Each sample was analyzed using quantitative polymerase chain reaction (qPCR) following the methods of (65). A small modification of the amount of Prepman Ultra (Applied Biosystems®, Life Technologies) was used to extract the DNA; we used 60 μL instead of 40 μL (66). Our extractions were diluted 1:10 and each sample was analyzed in triplicate to quantify the average number of genome equivalents per animal (7,500 real-time PCR Applied Biosystems instrument). To analyze infection loads, we log transformed the qPCR results as log (genome equivalents per individual + 1) to normalize data.

Effect of exposure on survivorship was analyzed independently by species using odds ratios calculated with a generalized linear mixed model, family: binomial (logit). The values of the ratios represent the likelihood or the risk of mortality due to exposure to the pathogen in comparison to the controls. Therefore, odds ratios higher than 1 represent an increased risk after exposure, odds ratios equal to one represent no difference in the risk, and odds ratios lower than 1 represent a lower risk of the exposed group. All analyses were run in R (version 4.0.3).

RESULTS

Pre-hatching Phase

Pseudacris regilla embryos exposed to both the East and West *Bd* strains in the Early exposure groups had a lower proportion of hatchlings relative to controls (East strain: $t = -4.40$, $p < 0.001$; West strain: $t = 1.99$, $p = 0.04$). A *post hoc* Tukey test showed that this proportion was different in embryos exposed to the East strain in contrast to the control ($z = -4.45$, $p < 0.001$) and the West strain ($z = 6.14$, $p < 0.001$), with approximately a 50% hatching rate (**Figure 2A**). Reduced hatching was also found in the Late/East treatment group ($t = -11.03$, $p < 0.001$) relative to the Late/West treatment ($t = -1.29$, $p = 0.19$). In fact, <10% of embryos hatched after being exposed late in development to the non-native East strain (**Figure 2B**). In *A. boreas*, the proportion of embryos that hatched was similar across both strains in comparison to controls across the Early (East strain: $t = -0.49$, $p = 0.62$; West strain: $t = 0.62$, $p = 0.53$) (**Figure 2C**) and Late exposure treatments (East strain: $t = 1.31$, $p = 0.19$; West strain: $t = 0.73$, $p = 0.46$) (**Figure 2D**).

The proportion of *L. catesbeianus* embryos that hatched was low when embryos were exposed early in development, with lower survival in the West *Bd* strain treatment relative to controls (West strain: $t = 3.58$, $p < 0.001$) (**Figure 2E**). There were no survivors in the East strain exposure treatment. The estimate of *Bd* strain as a factor in our model was high (5,329), potentially due to the 100% mortality, making the t and p-value not significant ($t = 0.003$, $p = 0.99$). The proportion of embryos that hatched in the Late exposure treatment was lower across both *Bd* strains in comparison to the controls (East strain: $t = 2.89$, $p < 0.01$; West strain: $t = 2.13$, $p = 0.03$) (**Figure 2F**). A *post hoc* Tukey test showed that this proportion was different in embryos exposed to the East strain in contrast to the control ($z = 2.89$, $p < 0.01$), but it was not different for embryos exposed to the West strain ($z = 2.13$, $p = 0.08$).

Post-hatching Phase

Our generalized linear mixed model quantified as odds ratios (OR) the effects of exposure to a particular strain on larvae

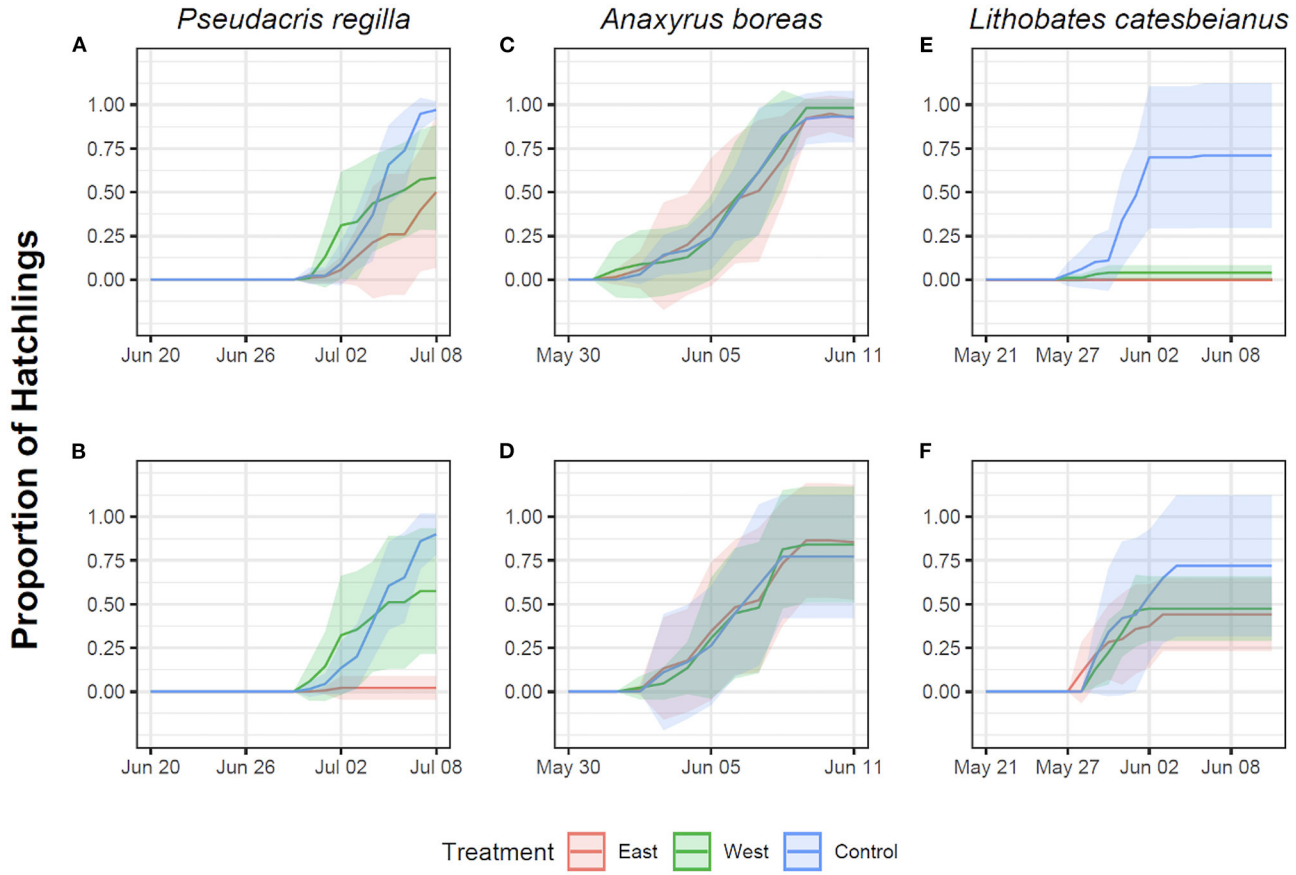


FIGURE 2 | Proportion of hatchlings in *Pseudacris regilla*, *Anaxyrus boreas*, and *Lithobates catesbeianus* after exposure of eggs to different chytrid treatments. Red represents exposure to the East strain, green represents exposure to the West strain, and controls are indicated in blue. First row represents Early exposure (Gosner stage 12) for *Pseudacris regilla* **(A)**, *Anaxyrus boreas* **(C)** and *Lithobates catesbeianus* **(E)**. Second row represents Late exposure (Gosner stage 18) for *Pseudacris regilla* **(B)**, *Anaxyrus boreas* **(D)** and *Lithobates catesbeianus* **(F)**.

mortality in comparison to the controls given their history of exposure as embryos. As such, results are reported as an increase or decrease in odds of mortality.

Direct Effects on Larvae -Only Exposed to *Bd* as Larvae (Control-*Bd*)

We found evidence for direct effects of *Bd* exposure on larval mortality for the three species. In *P. regilla*, post-hatching exposure to the East strain increased the odds of mortality (OR $_{\text{Early/Control−East}}$ 8.88, $p = 0.01$, CI. 1.43–54.85, **Figure 3**, Left panel). For *A. boreas*, we found that individuals exposed during the post-hatch phase to the West strain had lower odds of mortality relative to controls (OR $_{\text{Early/Control−West}}$ 0.12, $p = 0.03$, CI: 0.018–0.84, **Figure 4**, Left panel). In contrast, larvae coming from the Late control group and exposed post-hatch to East or West had higher odds of mortality than controls (OR $_{\text{Late/Control−East}}$ 14.38, $p = 0.03$, CI: 1.19–173.65; OR $_{\text{Late/Control−West}}$ 19.56, $p = 0.03$, CI: 1.32–288, **Figure 4**, Right panel). Larvae of *L. catesbeianus* increased their odds of mortality when exposed to either East or West strain (OR $_{\text{East}}$ 9.9, $p = 0.04$, CI: 1.06–92; OR $_{\text{West}}$ 539, $p < 0.001$, CI: 29.64–9,801) (**Figure 5**).

Latent Effects on Larvae -Only Exposed to *Bd* as Embryos (*Bd*-Control)

We did not find evidence for latent effects in *P. regilla*. In *A. boreas* odds of mortality changed according to the time of exposure and *Bd* strain. Odds of mortality for larvae decreased when embryos were exposed early in development to the West strain of *Bd* (OR $_{\text{Early/West−control}}$ 0.14, $p = 0.02$, CI: 0.026–0.73, **Figure 4**, Left panel). On the contrary, individuals exposed Late as embryos to the East strain had higher odds of mortality than controls (OR $_{\text{Late/East−control}}$ 10.62, $p = 0.04$, CI: 1.07–105, **Figure 4**, Right panel). In *L. catesbeianus,* we found higher odds of mortality than controls for both *Bd* strains (OR $_{\text{Late/East−control}}$ 31, $p = 0.001$, CI: 3.5–272, OR $_{\text{Late/West−control}}$ 23.21, $p = 0.006$, CI: 2.42–222.14, **Figure 5**).

Double Exposed Treatments -Exposed to *Bd* as Both Embryos and Larvae (*Bd*-*Bd*)

We found evidence that exposure to *Bd* in both the embryonic and larval stages affect the larval odds of mortality in all three species. We found *P. regilla* that were re-exposed to the West

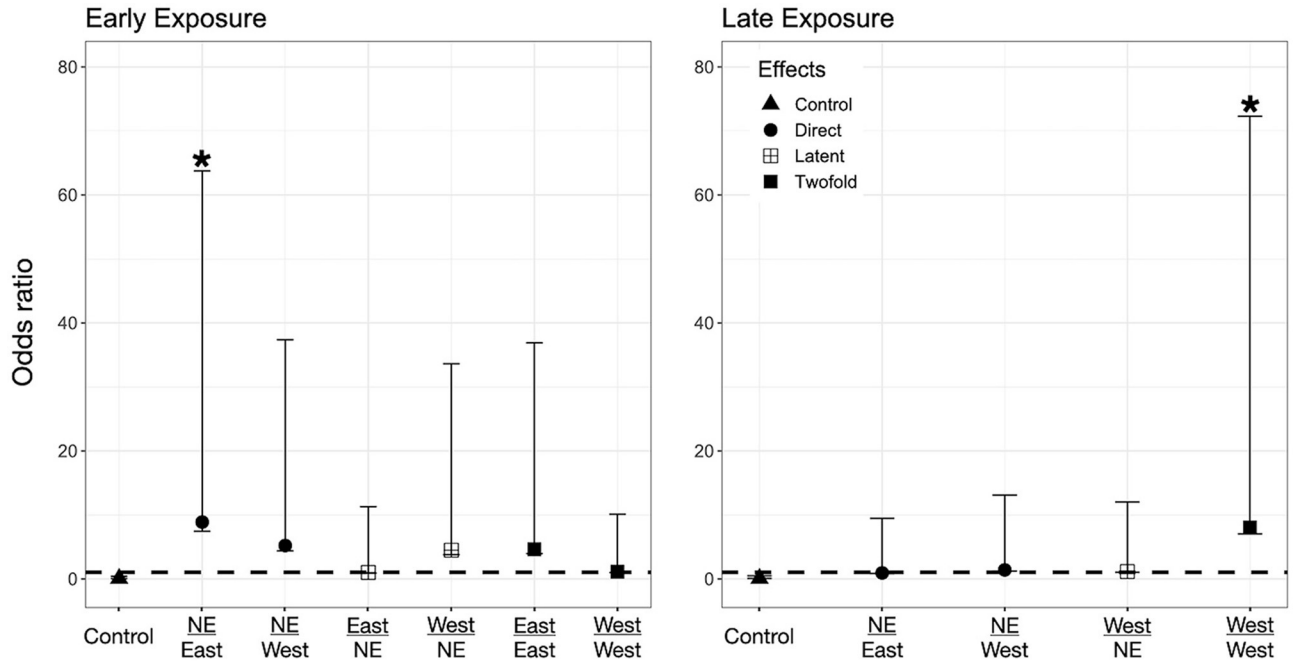


FIGURE 3 | Odds ratio (OR) for *P. regilla* tadpoles according to their exposure regimes. Label of x-axis includes a fraction that indicates in the numerator the exposure regime as embryo and in the denominator exposure regime post-hatching (no exposure = NE, East or West for *Bd* chytrid strain). A dashed line indicates value 1. OR > 1 higher risk after exposure, OR = 1 no risk difference, OR < 1 lower risk after exposure. A star (*) indicates treatments with significant odds ratios. Effects are represented as follows: Direct effects –only exposed to *Bd* as larvae– are represented by circles, latent effects –only exposed as embryos– are represented as four squares, and 2-fold effects –exposed as embryos and larvae– are represented with filled squares; controls are represented by triangles.

strain, (Late exposure group) increased the odds of mortality (OR $_{\text{Late/West−West}}$ 8.05, $p = 0.04$, CI 1.01–64.22, **Figure 3**, Right panel). In *A. boreas* odds of mortality increased in re-exposed individuals to both the East and West strains (Late exposure groups) (OR $_{\text{Late/East−East}}$ 9.37, $p = 0.05$, CI: 0.92–95; OR $_{\text{Late/West−West}}$ 9.12, $p = 0.05$, CI: 0.91–91.32) (**Figure 4**, Right panel). In *L. catesbeianus*, odds of mortality were high for re-exposed animals to either *Bd* strain (OR $_{\text{East/Late−East}}$ 58.3, $p = 0.0003$, CI: 6.35–534.6, OR $_{\text{West/Late−West}}$ 101.29, $p < 0.001$, CI: 8.9–1,145) (**Figure 5**).

Infection Loads

We confirmed *Bd* infection using real-time PCR analyses of tadpole mouthparts for *P. regilla* and swabs for *A. boreas* and *L. catesbeianus*. All tadpoles from control treatments tested negative for *Bd*. Individuals from the three species only exposed as larvae (direct effects) were positive for the East strain. From individuals only exposed as embryos (latent effects), we found that A. *boreas* tested positive for *Bd* when exposed late as embryos with the East strain. In *L. catesbeianus*, individuals tested positive when exposed late to either of the strains (**Table 3**). In the group of re-exposed individuals (2-fold effects), *P. regilla* was reported *Bd* positive for both East and West strain when exposed early as embryos. In *A. boreas*, individuals were positive for *Bd* when exposed early to the West strain and late when exposed to the East strain (**Table 3**).

DISCUSSION

Life stage, time of exposure, and *Bd* strain influenced susceptibility to *Bd* in the embryo-larvae life history transition for three anuran species: *P. regilla*, *A. boreas*, and *L. catesbeianus*. We detected direct effects of *Bd* on embryonic and larval mortality, latent effects across the embryo/larval transition, and additive effects when double-exposed to *Bd* across both life stages. Exposure of embryos to *Bd* resulted in direct impacts on hatchling survivorship. We found direct, negative impacts of *Bd* strain and time of exposure on embryonic survival and proportion of hatching success for *P. regilla* and *L. catesbeianus*. Embryos of *P. regilla* were drastically affected by the non-native East *Bd* strain, resulting in more than 90% mortality when exposed later in embryonic development. Interestingly, embryos of invasive *L. catesbeianus* died when exposed to either *Bd* strain (East or West). When exposed early in embryonic development to the East strain, the number of viable hatchlings was zero and we detected a mortality of 90% in hatchlings after early exposure to the West *Bd* strain. When exposed later in development (East or West strains), only 50% of embryos hatched. The influence of time of exposure may be explained by changes in the thickness of the jelly layer surrounding the embryo through development. This jelly layer becomes thinner over development, thus late-stage embryos may be more affected by exposure to a pathogen (15, 18). Amphibian species-specific egg deposition forms (films, strings, clusters),

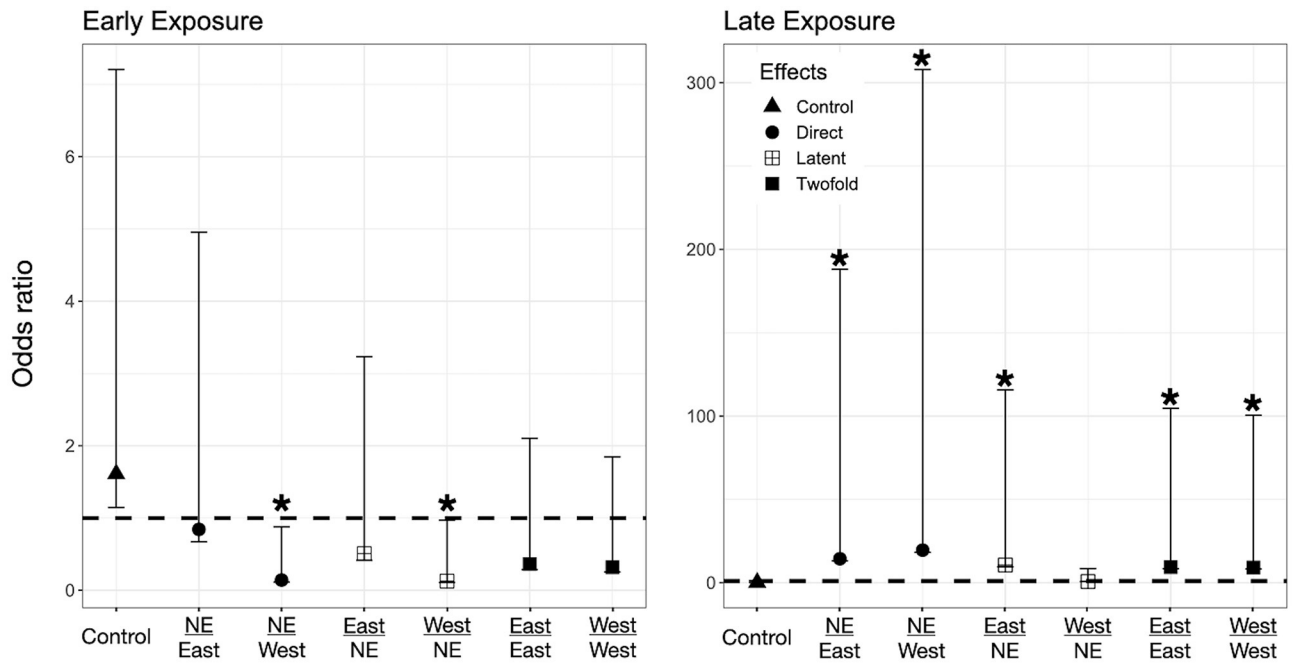


FIGURE 4 | Odds ratio (OR) for *A. boreas* tadpoles according to their exposure regimes. Label of x-axis includes a fraction that indicates in the numerator the exposure regime as embryo and in the denominator exposure regime post-hatching (no exposure = NE, East or West for *Bd* chytrid strain). A dashed line indicates value 1. OR > 1 higher risk after exposure, OR = 1 no risk difference, OR < 1 lower risk after exposure. A star (*) indicates treatments with significant odds ratios. Effects are represented as follow: Direct effects –only exposed to *Bd* as larvae– are represented by circles, latent effects –only exposed as embryos– are represented as four squares, and 2-fold effects –exposed as embryos and larvae– are represented with filled squares; controls are represented by triangles.

as well as morphology of egg structure, provide differential protection from pathogens (67).

Post-hatching exposure resulted in both direct and latent impacts on larval survivorship. Direct effects on larvae are reported mainly as an increase in the odds of mortality for all three anuran species. *P. regilla* was negatively affected by exposure to the non-native East *Bd* strain, while *A. boreas* and *L. catesbeianus* were affected by both strains (East and West). Odds of mortality in *A. boreas* were higher when exposed to the West strain (19.56) than when exposed to the East strain (14.38). On the contrary, the odds of mortality in *L. catesbeianus* were higher when exposed to the East strain (9.9) than when exposed to the West strain (5.39). This result was not wholly unexpected as larvae mortality has been reported in experimental studies exposing these same species to *Bd*. *A. boreas* has been identified as particularly susceptible to *Bd* (30, 46) while *P. regilla* and *L. catesbeianus* larvae have relatively high survivorship (30, 33, 46, 68). In this study, we found a direct effect of *Bd* on larval survivorship for all three species. The increase in the odds of mortality in *P. regilla* and *L. catesbeianus* larvae can be explained by the origin and characteristics of the East strain. Isolated from *L. catesbeianus* in Maine (USA), this strain has been identified as hypervirulent (57, 69, 70) and categorized as part of the North American clade in the Global Pandemic Lineage (GPL) (57). As such, we anticipated an increase in larval mortality due to a lack of evolutionary relationship with this strain. However, *L. catesbeianus* larvae were also susceptible to the East strain even though it was isolated from their conspecifics within their native range.

We detected an increase in the odds of mortality in *P. regilla* when exposed early in embryonic development to *Bd* while the odds ratio for larval mortality were lower for later exposure. We could not evaluate potential latent effects after late exposure of *P. regilla* embryos to the East strain. Effects of *Bd* differed in embryos according to the timing of exposure. However, these effects of timing of exposure and duration of stressors such as pathogens and its relation to critical window and disease is not broadly tested (71, 72). We found an increase in the odds of larval mortality of both *A. boreas* and *L. catesbeianus* as a function of *Bd* strain and timing of embryonic exposure. In *A. boreas*, embryos exposed early to the West strain showed a decrease in the odds of mortality. Conversely, when *A. boreas* were exposed to the East strain late in embryonic development, larvae were almost 10 times more likely to die than control individuals. There was a similar increase in the odds of larval mortality in *L. catesbeianus* when exposed as embryos to any of the *Bd* strains. The high mortality rates in *L. catesbeianus* when exposed early to *Bd* prevented us from understanding potential latent effects for this invasive species. Our results support the hypothesis that timing of pathogen exposure is a major factor that influences host survivorship. Exposures of amphibian embryos to stressors such as pathogens at early stages of development can trigger effects over ontogeny (73). This exposure can later modify other characteristics, such as growth

rates (74), mortality rates (75), mass (5), and development of immune response (76).

We also found effects of double *Bd* exposure (exposed in both the embryonic and larval stages) in all three anuran species. All species showed an increase in the odds of larval mortality when the first *Bd* exposure occurred at a later embryonic developmental stage (Gosner stage 18). In *P. regilla*, odds of mortality increased after double exposure to the West strain. Exposure to either strain (East or West) increased the odds of mortality in *A. boreas* and *L. catesbeianus*. Double exposure effects have been reported in experiments examining the larval/metamorph transition (77), thus our experiment provides additional information concerning other life history transitions providing a comprehensive view of the jeopardy through life.

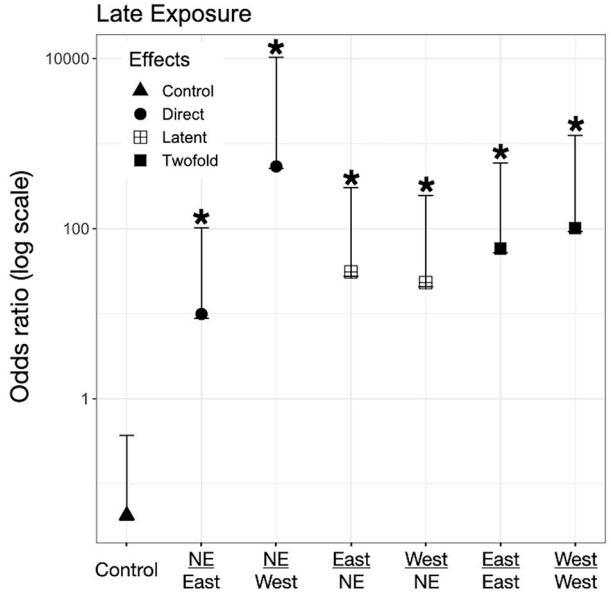


FIGURE 5 | Odds ratio (OR) for *L. catesbeianus* tadpoles according to their exposure regimes. Label of x-axis includes a fraction that indicates in the numerator the exposure regime as embryo and in the denominator exposure regime post-hatching. A star (*) indicates treatments with significant odds ratios. Effects are represented as follow: Direct effects –only exposed to *Bd* as larvae– are represented by circles, latent effects –only exposed as embryos– are represented as four squares, and 2-fold effects –exposed as embryos and larvae– are represented with filled squares; controls are represented by triangles.

The differential response of *A. boreas* to Early/Late and East/West *Bd* treatments may be explained by the presence of a potential critical window of vulnerability for this species and by the virulence of the *Bd* strain. Late exposure of *A. boreas* embryos to the East strain increased the odds of larval mortality of this species. Fernandez-Beneitez et al. (18) found that embryos of toads (*Bufo calamita* and *Pelobates cultripes*) exposed to *Saprolegnia* spp. at an early developmental stage (Gosner stage 12) suffered no increase in mortality, while embryos challenged at later stages of embryonic development (Gosner stages 15 and 19) were sensitive to the pathogen dying 72 h after exposure. Understanding which species experience latent effects will help target management efforts by identifying how exposure in particular life history stages can change host response. If a species is identified as being particularly susceptible to exposure to *Bd* as embryos, actions such as ex *situ* protection could be useful for its conservation.

Our findings complement the information on susceptibility of *P. regilla* to *Bd* as larvae of this species had previously been reported to be tolerant to certain *Bd* strains (30, 46). Interestingly, we found that this tolerance can change with an individual's previous exposure regime to non-native strains. Our experimental evaluation revealed that *Bd* strains from an invasive species can have harmful consequences on native and even invasive conspecific hosts. Our findings for *A. boreas* support previous work showing species as being susceptible to both the East and West strains of *Bd* (30, 46, 47). In *L. catesbeianus*, larvae and adults have been reported as able to withstand infection loads of the chytrid in different regions (78) and this species is suggested as an asymptomatic carrier or reservoir of *Bd* (79, 80). Our results indicate that larvae can also be susceptible to *Bd* but this response will be mediated by previous exposure in an early life stage. Individuals that were re-exposed to *Bd* were about 50 times more likely to die than individuals kept as controls.

TABLE 3 | Mean *Bd* loads (genome equivalents ± SD) at the end of experiment for *P. regilla*, *A. boreas* and *L. catesbeianus* exposed early (Gosner developmental stage 12) and late (Gosner developmental stage 18) during embryonic development.

Species	Exposure timing	Direct effects		Latent effects		2-fold effects	
		CONTROL		EAST	WEST	EAST	WEST
		East	West	Control		East	West
P. regilla	Early	1.25*±1.009	1.86 ± 1.24	0	0	0.005 ± 0.009	1.45 ± 1.77
	Late	0.74 ± 1.24	3.39 ± 1.05	NA	0	NA	1.62* ± 1.17
A. boreas	Early	0.008 ± 0.008	0*	0	0*	0	0.012 ± 0.02
	Late	0.019* ± 0.01	0*	0.00016* ± 0.0003	0	0.005* ± 0.006	0
L. catesbeianus	Early	0.004 ± 0.006	0.022 ± 0.022	NA	NA	NA	NA
	Late	0.53* ± 0.62	0	0.03** ± 0.05	0.59** ± 0.76	0	0

*A star * indicates significant effects in the odds of mortality of larvae. NA indicates treatments without samples due to mortality in the pre-hatching exposure part.*

This contrasts with previous experimental studies reporting this species as a carrier of *Bd* (30, 32, 60). Generally, those studies directly exposed individuals in the larval stage [Gosner stage (26–30)] without considering previous exposure regimes. In our study, *L. catesbeianus* were vulnerable to *Bd* exposure in response to direct exposure and across life history transitions.

We found species-specific embryonic mortality after exposure to *Bd*. Many pathogens impact anuran embryos, including ranavirus (76), oomycetes (81–84), filamentous ascomycetes (85) and microsporidia (86). The mechanisms of protection offered by the vitelline membrane against ranavirus are unknown. However, bacteriostatic activity of the egg membrane or the capsule (87) or just its role as structural barrier when exposed to contaminants have been proposed (88, 89). In the case of Oomycetes, zoospores are chemotactic and move toward suitable substrates where they can germinate and grow. Similarly, ascomycetes are able to grow through the jelly. Few studies have suggested how *Bd* affects anuran embryos. *Bd* enzymatic action is one mechanism that could explain this result, as it can cause damage in skin tissue of hosts after exposure (30, 49, 90, 91). A complex mix of proteolytic and hydrolyzing enzymes (esterases) that degrade amphibian tissue have been described from different *Bd* isolates (90–92). In addition, many hatching anurans release enzymes to assist with degradation of the egg capsule at the moment of hatching (93, 94); this could potentially facilitate the enzymatic action of *Bd* to degrade tissues. Recently, dose-dependent mortality and proliferation in zebrafish (*Danio rerio*) tissue was reported with toxins secreted after the establishment of *Bd* sporangia (95).

The present study offers useful information about the complexity of host response to a pathogen, particularly with multiple exposures across life stages. Our study provides information about direct effects of *Bd* on anuran embryos, with significant impacts on mortality and the proportion of hatching success. Our results also quantified latent effects of *Bd* exposure over ontogeny (96). Despite being a relatively brief period, exposure to *Bd* in the egg led to increased mortality after hatching and species-specific differences were due to the timing of embryonic exposure and re-exposure in the larval stage. As we increase our understanding of how *Bd* impacts amphibians through direct and even latent effects, we are recognizing that the effects *Bd* may have on population dynamics and conservation of amphibians have been underestimated in wild populations.

Additional research exploring the mechanisms protecting the embryos is needed to better understand the susceptibility of this developmental stage to disease. Characteristics such as jelly thickness and composition, or size of the capsule, can be involved in resistance to chytrid. As eggs received material from their parents during oviposition, evaluating the role of parents in the immune response of their offspring can help us to understand more about embryonic immunity. As continued survey efforts have located *Bd* in populations of amphibians around the world, there is growing evidence that the risk of *Bd* cannot be simplified to species susceptibility. Instead, *Bd* risk includes strain, host life-stage, and specific exposure scenarios. Further studies are also required to better understand how variation in other environmental and biological parameters can affect the outcome of repeated *Bd* exposure in anuran species. Our results add information to the growing body of evidence concerning differential susceptibility to pathogens among amphibian species and across life stages.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use committee Oregon State University.

AUTHOR CONTRIBUTIONS

JU, AB, and TG conceptualized the manuscript. JU and EB performed the field work, experiments and data analysis. JU, EB, AB, and TG have made a significant, direct and intellectual contribution to the work. All authors contributed to the article and approved the submitted version.

FUNDING

Publication of this paper was supported, in part, by the Henry Mastin Graduate Student Fund (Department of Fisheries, Wildlife and Conservation Sciences, Oregon State University) and the US Department of Agriculture, Forest Service, Pacific Northwest Research Station through Deanna H. Olson.

ACKNOWLEDGMENTS

We thank N. Soria, L. Londono, and G. Mayorga for assistance measuring and staging tadpoles and assistance with animal care. D. Olson, C. Harjoe, and L. Thurman for help during egg masses collection, Spatafora Lab., Tanguay Lab., and the Center for Genome Research and Biocomputing at Oregon State University for providing laboratory resources for analysis.

REFERENCES

1. Merilä J, Svensson E. Are fat reserves in migratory birds affected by condition in early life? *J Avian Biol.* (1997) 28:279–86. doi: 10.2307/3676940
2. Miller K, Packard GC, Packard MJ. Hydric conditions during incubation influence locomotor performance of hatchling snapping turtles. *J Exp Biol.* (1987) 127:401–12. doi: 10.1242/jeb.127.1.401
3. Heintz RA, Rice SD, Wertheimer AC, Bradshaw RF, Thrower FP, Joyce JE, et al. Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. *Mar Ecol Prog Ser.* (2000) 208:205–16. doi: 10.3354/meps208205
4. Pechenik JA. Larval experience and latent effects—metamorphosis is not a new beginning. *Integr Comp Biol.* (2006) 46:323–33. doi: 10.1093/icb/icj028

5. Uller T, Sagvik J, Olsson M. Pre-hatching exposure to water mold reduces size at metamorphosis in the moor frog. *Oecologia*. (2009) 160:9–14. doi: 10.1007/s00442-009-1280-6
6. Murillo-Rincón AP, Laurila A, Orizaola G. Compensating for delayed hatching reduces offspring immune response and increases life-history costs. *Oikos*. (2017) 126:565–71. doi: 10.1111/oik.04014
7. Sniegula S, Janssens L, Stoks R. Integrating multiple stressors across life stages and latitudes: combined and delayed effects of an egg heat wave and larval pesticide exposure in a damselfly. *Aquatic Toxicology*. (2017) 186:113–22. doi: 10.1016/j.aquatox.2017.02.029
8. LaFiandra EM, Babbitt KJ. Predator induced phenotypic plasticity in the pinewoods tree frog, *Hyla femoralis*: necessary cues and the cost of development. *Oecologia*. (2004) 138:350–9. doi: 10.1007/s00442-003-1412-3
9. Richter-Boix A, Orizaola G, Laurila A. Transgenerational phenotypic plasticity links breeding phenology with offspring life-history. *Ecology*. (2014) 95:2715–22. doi: 10.1890/13-1996.1
10. Garcia TS, Urbina J, Bredeweg E, Ferrari MCO. Embryonic learning and developmental carry-over effects in an invasive anuran. *Oecologia*. (2017) 184:623–31 doi: 10.1007/s00442-017-3905-5
11. Sih A, Moore RD. Delayed hatching of salamander eggs in response to enhanced larval predation risk. *Am Nat*. (1993) 142:947–60. doi: 10.1086/285583
12. Warkentin KM. Adaptive plasticity in hatching age: a response to predation risk trade-offs. *Proc Nat Acad Sci*. (1995) 92:3507–10. doi: 10.1073/pnas.92.8.3507
13. Warkentin KM. Plasticity of hatching in amphibians: evolution, trade-offs, cues and mechanisms. *Integr Comp Biol*. (2011) 51:111–27. doi: 10.1093/icb/icr046
14. Chivers DP, Kiesecker JM, Marco A, Devito J, Anderson MT, Blaustein AR. Predator-induced life history changes in amphibians: egg predation induces hatching. *Oikos*. (2001) 92:135–42. doi: 10.1034/j.1600-0706.2001.920116.x
15. Gomez-Mestre I, Touchon JC, Warkentin KM. Amphibian embryo and parental defenses and a larval predator reduce egg mortality from water mold. *Ecology*. (2006) 87:2570–81. doi: 10.1890/0012-9658(2006)87[2570:AEAPDA]2.0.CO;2
16. Touchon JTJ, Gomez-Mestre IG-MI, Warkentin KWK. Hatching plasticity in two temperate anurans: responses to a pathogen and predation cues. *Can J Zool*. (2006) 84:556–63. doi: 10.1139/z06-058
17. Touchon JC, McCoy MW, Vonesh JR, Warkentin KM. Effects of plastic hatching timing carry over through metamorphosis in red-eyed treefrogs. *Ecology*. (2013) 94:850–60. doi: 10.1890/12-0194.1
18. Fernández-Benéitez M, Ortiz-Santaliestra M, Lizana M, Diéguez-Uribeondo J. Differences in susceptibility to Saprolegnia infections among embryonic stages of two anuran species. *Oecologia*. (2011) 165:819–26. doi: 10.1007/s00442-010-1889-5
19. Rumschlag SL, Boone MD. How time of exposure to the amphibian chytrid fungus affects *Hyla chrysoscelis* in the presence of an insecticide. *Herpetologica*. (2015) 71:169–76. doi: 10.1655/HERPETOLOGICA-D-13-00070
20. Develey-Rivière M-P, Galiana E. Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom. *New Phytol*. (2007) 175:405–16. doi: 10.1111/j.1469-8137.2007.02130.x
21. Brutscher LM, Daughenbaugh KF, Flenniken ML. Antiviral defense mechanisms in honey bees. *Curr Opin Insect Sci*. (2015) 10:71–82. doi: 10.1016/j.cois.2015.04.016
22. Mast J, Goddeeris BM. Development of immunocompetence of broiler chickens. *Vet Immunol Immunopathol*. (1999) 70:245–56. doi: 10.1016/S0165-2427(99)00079-3
23. Holgersson MCN, Nichols WA, Paitz RT, Bowden RM. How important is the eggshell as a source for initial acquisition of Salmonella in hatchling turtles? *J Exp Zool*. (2016) 325:142–8. doi: 10.1002/jez.2004
24. Valkenburg SA, Venturi V, Dang THY, Bird NL, Doherty PC, Turner SJ, et al. Early priming minimizes the age-related immune compromise of $CD8^+$ T cell diversity and function. *PLoS Pathog*. (2012) 8:e1002544. doi: 10.1371/journal.ppat.1002544
25. Rohr JR, Raffel TR, Hall CA. Developmental variation in resistance and tolerance in a multi-host–parasite system. *Functional Ecology*. (2010) 24:1110–21. doi: 10.1111/j.1365-2435.2010.01709.x
26. Echaubard P, Pauli BD, Trudeau VL, Lesbarrères D. Ranavirus infection in northern leopard frogs: the timing and number of exposures matter. *J Zool*. (2016) 298:30–6. doi: 10.1111/jzo.12281
27. Hatcher MJ, Dick JTA, Dunn AM. Disease emergence and invasions. *Funct Ecol*. (2012) 26:1275–87. doi: 10.1111/j.1365-2435.2012.02031.x
28. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. The *Bd* mapping group. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLoS ONE*. (2013) 8:e56802. doi: 10.1371/journal.pone.0056802
29. Berger L, Roberts AA, Voyles J, Longcore JE, Murray KA, Skerratt LF. History and recent progress on chytridiomycosis in amphibians. *Fungal Ecol*. (2016) 19:89–99. doi: 10.1016/j.funeco.2015.09.007
30. Blaustein AR, Romansic JM, Scheessele EA, Han BA, Pessier AP, Longcore JE. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conservation Biology*. (2005) 19:1460–8. doi: 10.1111/j.1523-1739.2005.00195.x
31. Searle CL, Gervasi SS, Hua J, Hammond JI, Relyea RA, Olson DH, et al. Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. *Conservation Biology*. (2011) 25:965–74. doi: 10.1111/j.1523-1739.2011.01708.x
32. Gahl MK, Longcore JE, Houlahan JE. Varying responses of northeastern north american amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. *Conservation Biology*. (2012) 26:135–41. doi: 10.1111/j.1523-1739.2011.01801.x
33. Gervasi S, Gondhalekar C, Olson DH, Blaustein AR. Host identity matters in the amphibian-*Batrachochytrium dendrobatidis* system: fine-scale patterns of variation in responses to a multi-host pathogen. *PLoS ONE*. (2013) 8:e54490. doi: 10.1371/journal.pone.0054490
34. Gervasi SS, Stephens PR, Hua J, Searle CL, Xie GY, Urbina J, et al. Linking ecology and epidemiology to understand predictors of multi-host responses to an emerging pathogen, the amphibian chytrid fungus. *PLoS ONE*. (2017) 12:e0167882. doi: 10.1371/journal.pone.0167882
35. Bielby J, Fisher MC, Clare FC, Rosa GM, Garner TWJ. Host species vary in infection probability, sub-lethal effects, and costs of immune response when exposed to an amphibian parasite. *Sci Rep*. (2015) 5:10828. doi: 10.1038/srep10828
36. Tobler U, Schmidt BR. Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE*. (2010) 5:e10927. doi: 10.1371/journal.pone.0010927
37. Bradley PW, Gervasi SS, Hua J, Cothran RD, Relyea RA, Olson DH, et al. Differences in sensitivity to the fungal pathogen *Batrachochytrium dendrobatidis* among amphibian populations: pathogen effects across populations. *Conservation Biology*. (2015) 29:1347–56. doi: 10.1111/cobi.12566
38. Briggs CJ, Vredenburg VT, Knapp RA, Rachowicz LJ. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology*. (2005) 86:3149–59. doi: 10.1890/04-1428
39. Briggs CJ, Knapp RA, Vredenburg VT. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc Nat Acad Sci*. (2010) 107:9695–700. doi: 10.1073/pnas.0912886107
40. Garner TWJ, Walker S, Bosch J, Leech S, Marcus Rowcliffe J, Cunningham AA, et al. Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*. (2009) 118:783–91. doi: 10.1111/j.1600-0706.2008.17202.x
41. Piovia-Scott J, Pope KL, Lawler SP, Cole EM, Foley JE. Factors related to the distribution and prevalence of the fungal pathogen *Batrachochytrium dendrobatidis* in *Rana cascadae* and other amphibians in the Klamath Mountains. *Biol Conserv*. (2011) 144:2913–21. doi: 10.1016/j.biocon.2011.08.008
42. Ortiz-Santaliestra ME, Rittenhouse TAG, Cary TL, Karasov WH. Interspecific and postmetamorphic variation in susceptibility of three north american anurans to *Batrachochytrium dendrobatidis*. *J Herpetol*. (2013) 47:286–92. doi: 10.1670/11-134
43. Retallick RWR, Miera V. Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Dis Aquat Org*. (2007) 75:201–7. doi: 10.3354/dao075201

44. Doddington BJ, Bosch J, Oliver JA, Grassly NC, Garcia G, Schmidt BR, et al. Context-dependent amphibian host population response to an invading pathogen. *Ecology.* (2013) 94:1795–804. doi: 10.1890/12-1270.1
45. Piovia-Scott J, Pope K, Joy Worth S, Rosenblum EB, Poorten T, Refsnider J, et al. Correlates of virulence in a frog-killing fungal pathogen: evidence from a California amphibian decline. *ISME J.* (2015) 9:1570–8. doi: 10.1038/ismej.2014.241
46. Dang T, Searle CL, Blaustein AR. Virulence variation among strains of the emerging infectious fungus *Batrachochytrium dendrobatidis* (Bd) in multiple amphibian host species. *Dis Aquat Org.* (2017) 124:233–9. doi: 10.3354/dao03125
47. Gervasi SS, Urbina J, Hua J, Chestnut T, Relyea RA, Blaustein AR. Experimental evidence for American bullfrog (*Lithobates catesbeianus*) susceptibility to chytrid fungus (*Batrachochytrium dendrobatidis*). *Ecohealth.* (2013) 10:166–71. doi: 10.1007/s10393-013-0832-8
48. Rosenblum EB, Fisher MC, James TY, Stajich JE, Longcore JE, Gentry LR, et al. Molecular perspective: biology of the emerging pathogen *Batrachochytrium dendrobatidis*. *Dis Aquat Organ.* (2010) 92:131–47. doi: 10.3354/dao02179
49. McMahon TA, Brannelly LA, Chatfield MWH, Johnson PTJ, Joseph MB, McKenzie VJ, et al. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proc Nat Acad Sci.* (2013) 110:210–5. doi: 10.1073/pnas.1200592110
50. Fites JS, Ramsey JP, Holden WM, Collier SP, Sutherland DM, Reinert LK, et al. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science.* (2013) 342:366. doi: 10.1126/science.1243316
51. Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, et al. pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science.* (2009) 326:582–5. doi: 10.1126/science.1176765
52. Dmitrieva EV. Influence of the concentration of dissolved oxygen on embryonic development of the common toad (*Bufo bufo*). *Russ J Dev Biol.* (2015) 46:368–80. doi: 10.1134/S1062360415060041
53. Blaustein A, Beatty J, Olson D, Storm R. The biology of amphibians and reptiles in old-growth forests in the Pacific Northwest. In: *General Technical Report (GTR).* Portland, OR: Pacific Northwest Research Station (1995). p.98. doi: 10.2737/PNW-GTR-337
54. Muths E, Corn PS, Pessier AP, Green DE. Evidence for disease-related amphibian decline in Colorado. *Biol Conserv.* (2003) 110:357–65. doi: 10.1016/S0006-3207(02)00239-2
55. Jones LLC, Leonard WP, Olson DH. *Amphibians of the Pacific Northwest*. Seattle, WA: Seattle Audubon Society (2005). p. 227
56. Gosner KL, A. simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica.* (1960) 16:183–90.
57. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, et al. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Mol Ecol.* (2012) 21:5162–77. doi: 10.1111/j.1365-294X.2012.05710.x
58. Marcum R, St-Hilaire S, Murphy P, Rodnick K. Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus* (Bufo) *boreas boreas*. *Dis Aquat Org.* (2010) 91:17–21. doi: 10.3354/dao02235
59. Searle CL, Belden LK, Du P, Blaustein AR. Stress and chytridiomycosis: exogenous exposure to corticosterone does not alter amphibian susceptibility to a fungal pathogen. *J Exp Zool.* (2014) 321:243–53. doi: 10.1002/jez.1855
60. Eskew EA, Worth SJ, Foley JE, Todd BD. American bullfrogs (*Lithobates catesbeianus*) resist infection by multiple isolates of *Batrachochytrium dendrobatidis*, including one implicated in wild mass mortality. *Ecohealth.* (2015) 12:513–8. doi: 10.1007/s10393-015-1035-2
61. R Core Team. *R: A language and environment for statistical computing.* Vienna, Au: R Foundation for Statistical Computing (2016). Available online at: http://www.R-project.org/
62. Retallick RWR, Miera V, Richards KL, Field KJ, Collins JP, A. non-lethal technique for detecting the chytrid fungus *Batrachochytrium dendrobatidis* on tadpoles. *Dis Aquat Org.* (2006) 72:77–85. doi: 10.3354/dao072077
63. 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*. *Dis Aquat Organ.* (2007) 73:175–92. doi: 10.3354/dao073175
64. Kadekaru S, Une Y. Comparison of methods for detection of chytrid fungus (*Batrachochytrium dendrobatidis*) in bullfrog tadpole mouthparts. *J Vet Med Sci.* (2018) 80:260–2. doi: 10.1292/jvms.17-0071
65. Boyle D, Boyle D, Olsen V, Morgan J, Hyatt A. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis Aquat Organ.* (2004) 60:141–8. doi: 10.3354/dao060141
66. Searle CL, Xie GY, Blaustein AR. Development and infectious disease in hosts with complex life cycles. *PLoS ONE.* (2013) 8:e60920. doi: 10.1371/journal.pone.0060920
67. Altig R, McDiarmid RW. Morphological diversity and evolution of egg and clutch structure in amphibians. *Herpetol Monogr.* (2007) 21:1–32. doi: 10.1655/06-005.1
68. Reeder NM, Pessier AP, Vredenburg VT. A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. *PLoS ONE.* (2012) 7:e33567. doi: 10.1371/journal.pone.0033567
69. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc Nat Acad Sci.* (2011) 108:18732–6. doi: 10.1073/pnas.1111915108
70. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D, Rodriguez D, et al. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proc Nat Acad Sci.* (2013) 110:9385–90. doi: 10.1073/pnas.1300130110
71. Johnson PTJ, Kellermanns E, Bowerman J. Critical windows of disease risk: amphibian pathology driven by developmental changes in host resistance and tolerance. *Funct Ecol.* (2011) 25:726–34. doi: 10.1111/j.1365-2435.2010.01830.x
72. Kirschman LJ, Crespi EJ, Warne RW. Critical disease windows shaped by stress exposure alter allocation trade-offs between development and immunity. *J Anim Ecol.* (2018) 87:235–46. doi: 10.1111/1365-2656.12778
73. Rohr JR, Raffel TR, Halstead NT, McMahon TA, Johnson SA, Boughton RK, Martin LB. Early-life exposure to a herbicide has enduring effects on pathogen-induced mortality. *Proc Biol Soc B.* (2013) 280:20131502. doi: 10.1098/rspb.2013.1502
74. Capellán E, Nicieza A. Trade-offs across life stages: does predator induced hatching plasticity reduce anuran post-metamorphic performance? *Evol Ecol.* (2007) 21:445–58. doi: 10.1007/s10682-006-9133-9
75. Vonesh JR, Bolker BM. Compensatory larval responses shift trade-offs associated with predator-induced hatching plasticity. *Ecology.* (2005) 86:1580–91. doi: 10.1890/04-0535
76. Haislip NA, Gray MJ, Hoverman JT, Miller DL. Development and disease: how susceptibility to an emerging pathogen changes through anuran development. *PLoS ONE.* (2011) 6:e22307. doi: 10.1371/journal.pone.0022307
77. Fernández-Loras A, Fernández-Beaskoetxea S, Arriero E, Fisher MC, Bosch J. Early exposure to *Batrachochytrium dendrobatidis* causes profound immunosuppression in amphibians. *Eur J Wildl Res.* (2017) 63:99. doi: 10.1007/s10344-017-1161-y
78. Hanselmann R, Rodriguez A, Lampo M, Fajardo-Ramos L, Aguirre A, Kilpatrick AM, et al. Presence of an emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela. *Biol Conserv.* (2004) 120:115–9. doi: 10.1016/j.biocon.2004.02.013
79. Daszak P, Strieby A, Cunningham AA, Longcore J, Brown C, Porter D. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetol J.* (2004) 14:201–7. Available online at: https://www.thebhs.org/publications/the-herpetological-journal/volume-14-number-4-october-2004/1773-06-experimental-evidence-that-the-bullfrog-rana-catesbeiana-is-a-potential-carrier-of-chytridiomycosis-an-emerging-fungal-disease-of-amphibians/file
80. Garner TWJ, Perkins MW, Govindarajulu P, Seglie D, Walker S, Cunningham AA, et al. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biol Lett.* (2006) 2:455. doi: 10.1098/rsbl.2006.0494
81. Kiesecker JM, Blaustein AR. Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. *Proc Natl Acad Sci USA.* (1995) 92:11049–52. doi: 10.1073/pnas.92.24.11049

82. Fernández-Benéitez MJ, Ortiz-Santaliestra ME, Lizana M, Diéguez-Uribeondo J. *Saprolegnia diclina*: another species responsible for the emergent disease 'Saprolegnia infections' in amphibians. *FEMS Microbiol Lett.* (2008) 279:23–9. doi: 10.1111/j.1574-6968.2007.01002.x
83. Ruthig GR. The influence of temperature and spatial distribution on the susceptibility of southern leopard frog eggs to disease. *Oecologia.* (2008) 156:895–903. doi: 10.1007/s00442-008-1026-x
84. Ruthig G. Water molds of the genera Saprolegnia and Leptolegnia are pathogenic to the North American frogs *Rana catesbeiana* and *Pseudacris crucifer*, respectively. *Dis Aquat Org.* (2009) 84:173–8. doi: 10.3354/dao02042
85. Warkentin KM, Currie CR, Rehner SA. Egg-killing fungus induces early hatching of red-eyed treefrog eggs. *Ecology.* (2001) 82:2860–9. doi: 10.1890/0012-9658(2001)082[2860:EKFIEH]2.0.CO;2
86. Green DE, Converse KA. "Diseases of amphibian eggs and embryos." In: Majumdar SK, Huffman JE, Brenner FJ, Panah AI, editors. *Wildlife Diseases: Landscape Epidemiology, Spatial Distribution and Utilization of Remote Sensing Technology*. Easton, PA: The Pennsylvania Academy of Science (2005). p. 62–71. Available online at: http://pubs.er.usgs.gov/publication/85665
87. Han Y, Yu H, Yang X, Rees HH, Liu J, Lai R, et al. serine proteinase inhibitor from frog eggs with bacteriostatic activity. *Comp Biochem Physiol B Biochem Mol Biol.* (2008) 149:58–62. doi: 10.1016/j.cbpb.2007.08.003
88. Berrill M, Coulson D, McGillivray L, Pauli B. Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environ Toxicol Chem.* (1998) 17:1738–44. doi: 10.1002/etc.5620170914
89. Pauli BD, Coulson DR, Berrill M. Sensitivity of amphibian embryos and tadpoles to Mimic® 240 LV insecticide following single or double exposures. *Environ Toxicol Chem.* (1999) 18:2538–44. doi: 10.1002/etc.5620181122
90. Symonds EP, Trott DJ, Bird PS, Mills P. Growth characteristics and enzyme activity in *Batrachochytrium dendrobatidis* isolates. *Mycopathologia.* (2008) 166:143–7. doi: 10.1007/s11046-008-9135-y
91. Moss A, Carty N, San Francisco M. Identification and partial characterization of an elastolytic protease in the amphibian pathogen *Batrachochytrium dendrobatidis*. *Dis Aquat Org.* (2010) 92:149–58. doi: 10.3354/dao02223
92. Brutyn M, D'Herde K, Dhaenens M, Rooij PV, Verbrugghe E, Hyatt AD, et al. *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. *Fungal Genet Biol.* (2012) 49:830–7. doi: 10.1016/j.fgb.2012.07.002
93. Carroll EJ, Hedrick JL. Hatching in the toad *Xenopus laevis*: morphological events and evidence for a hatching enzyme. *Dev Biol.* (1974) 38:1–13. doi: 10.1016/0012-1606(74)90254-1
94. Cohen KL, Seid MA, Warkentin KM. How embryos escape from danger: the mechanism of rapid, plastic hatching in red-eyed treefrogs. *J Exp Biol.* (2016) 219:1875. doi: 10.1242/jeb.139519
95. Liew N, Mazon Moya MJ, Wierzbicki CJ, Hollinshead M, Dillon MJ, Thornton CR, et al. Chytrid fungus infection in zebrafish demonstrates that the pathogen can parasitize non-amphibian vertebrate hosts. *Nat Commun.* (2017) 8:15048. doi: 10.1038/ncomms15048
96. Hamdoun A, Epel D. Embryo stability and vulnerability in an always changing world. *Proc Nat Acad Sci.* (2007) 104:1745–50. doi: 10.1073/pnas.0610108104

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 29 November 2021
doi: 10.3389/fvets.2021.742288

Social Behavior, Community Composition, Pathogen Strain, and Host Symbionts Influence Fungal Disease Dynamics in Salamanders

*Mae Cowgill[1], Andrew G. Zink[1], Wesley Sparagon[2], Tiffany A. Yap[3], Hasan Sulaeman[1], Michelle S. Koo[4] and Vance T. Vredenburg[1,4]**

[1] Department of Biology, San Francisco State University, San Francisco, CA, United States, [2] Daniel K. Inouye Center for Microbial Oceanography: Research and Education, Department of Oceanography and Sea Grant College Program, UUniversity of Hawai'i at Mānoa, HI, United States, [3] Center for Biological Diversity, Oakland, CA, United States, [4] Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, CA, United States

The emerging fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), which can cause a fatal disease called chytridiomycosis, is implicated in the collapse of hundreds of host amphibian species. We describe chytridiomycosis dynamics in two co-occurring terrestrial salamander species, the Santa Lucia Mountains slender salamander, *Batrachoseps luciae*, and the arboreal salamander, *Aneides lugubris*. We (1) conduct a retrospective *Bd*-infection survey of specimens collected over the last century, (2) estimate present-day *Bd* infections in wild populations, (3) use generalized linear models (GLM) to identify biotic and abiotic correlates of infection risk, (4) investigate susceptibility of hosts exposed to *Bd* in laboratory trials, and (5) examine the ability of host skin bacteria to inhibit *Bd* in culture. Our historical survey of 2,866 specimens revealed that for most of the early 20th century (∼1920–1969), *Bd* was not detected in either species. By the 1990s the proportion of infected specimens was 29 and 17% (*B. luciae* and *A. lugubris*, respectively), and in the 2010s it was 10 and 17%. This was similar to the number of infected samples from contemporary populations (2014–2015) at 10 and 18%. We found that both hosts experience signs of chytridiomycosis and suffered high *Bd*-caused mortality (88 and 71% for *B. luciae* and *A. lugubris*, respectively). Our GLM revealed that *Bd*-infection probability was positively correlated with intraspecific group size and proximity to heterospecifics but not to abiotic factors such as precipitation, minimum temperature, maximum temperature, mean temperature, and elevation, or to the size of the hosts. Finally, we found that both host species contain symbiotic skin-bacteria that inhibit growth of *Bd* in laboratory trials. Our results provide new evidence consistent with other studies showing a relatively recent *Bd* invasion of amphibian host populations in western North America and suggest that the spread of the pathogen may be enabled both through conspecific and heterospecific host interactions. Our results suggest that wildlife disease studies should assess host-pathogen dynamics that consider the interactions and effects of multiple hosts, as well as the historical context of pathogen invasion, establishment, and epizootic to enzootic transitions to better understand and predict disease dynamics.

Keywords: chytridiomycosis, sociality, symbiotic bacteria, historical prevalence, microbiome, *Batrachochytrium dendrobatidis*, *Aneides lugubris*, *Batrachoseps luciae*

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Reviewed by:
Deanna Olson, USDA Forest Service, United States
Kenneth James Genovese, United States Department of Agriculture, United States

***Correspondence:**
Vance T. Vredenburg
vancev@sfsu.edu

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *16 July 2021*
Accepted: *06 October 2021*
Published: *29 November 2021*

Citation:
Cowgill M, Zink AG, Sparagon W, Yap TA, Sulaeman H, Koo MS and Vredenburg VT (2021) Social Behavior, Community Composition, Pathogen Strain, and Host Symbionts Influence Fungal Disease Dynamics in Salamanders. Front. Vet. Sci. 8:742288. doi: 10.3389/fvets.2021.742288

INTRODUCTION

Amphibians are an ancient and diverse lineage of vertebrates [∼370 mya; currently 8,345 species; (1, 2)] that have survived the last four global mass extinction events (3–5). Their biphasic lifestyle links aquatic and terrestrial productivity (6), and they fill key positions in food webs because of their large population sizes and the fact that they serve as abundant consumers, and as prey to other species (7). However, amphibians have recently experienced extinctions and severe global population declines due to a combination of factors, including habitat loss and fragmentation, environmental contaminants, disease, and climate change (3, 5, 8, 9).

An emerging infectious fungal disease, chytridiomycosis, has captured the attention of biologists and the general public because it has driven die-offs in hundreds of amphibian species around the world (3, 9–13). This disease is caused by the chytridiomycete fungi, *Batrachochytrium dendrobatidis* (*Bd*) (14) and *Batrachochytrium salamandrivorans* (*Bsal*) (15), which infect and disrupt skin function, including osmoregulation, and can lead to death (15–18). While less is known about *Bsal*, the impact of the *Bd* pathogen on amphibians represents the worst known case of disease among vertebrates in recorded history (9, 12). To predict the effects that *Bd* chytridiomycosis will continue to have on host species, it is essential to understand longitudinal patterns of *Bd* infection prevalence [e.g., temporal pattern of pathogen invasion, epizootic outbreak, and either establishment or loss; (19, 20)], host species susceptibility patterns (influenced by skin microbiomes, social behavior and interactions with heterospecifics), and host-*Bd* dynamics so that models can be used to understand and predict the interactions across a wide range of circumstances (20).

Though *Bd* chytridiomycosis has mostly been studied in anurans (Order Anura: frogs and toads), salamanders and newts (Order Caudata) are also known to be affected by *Bd* (12, 21–25). In the North America salamander biodiversity hotspot which contains ∼50% of all known species (1, 26), very little is known about which salamander species have declined and if *Bd* is the cause of such declines (12). The west coast of North America has the second most diverse assemblage of salamander species in North America (after Appalachia region), and 64% (49/77) of its extant amphibian species are salamanders (1). Whereas, the emergence of *Bd* is closely linked to mass die-offs and local extinctions in anurans in California (17, 19, 27, 28), the relationship between *Bd* emergence and population declines is not well-characterized in salamanders (21, 22, 25). Lack of attention to salamander disease dynamics in California may be attributed to the fact that most salamander species are fossorial or semi-fossorial (living under leaf litter and woody debris), nocturnal, and solitary breeders; as such, many abundance estimates suffer from ascertainment bias (29). Furthermore, in California, a lack of consistent historical population sampling [relative to anurans; (27)] makes it difficult to confidently assess whether salamander populations have declined.

In California, recent studies have shown a consistent pattern of no or almost no *Bd* early in the 20th century, followed by *Bd* invasion and emergence in amphibians in the late 1960s and 1970s (19, 21, 22, 25, 28, 30–32). The effects of this pathogen on salamanders, which in California represent a majority of the diversity of amphibians, are poorly understood. A better understanding of host pathogen dynamics requires contextual information such as spatial and temporal pathogen data (i.e., longitudinal pathogen data), host susceptibility, host behavior, immune responses, as well as other biotic and abiotic factors that may influence host survival (33–36). Another important factor is *Bd* lineage. Molecular studies reveal a complex evolutionary history in *Bd* (37), with significant differences in pathogenicity and in spatial patterns of spread between lineages [e.g., (12, 38–40)]. The pathogen is believed to have originated in Asia, and there are at least five major *Bd* lineages which vary in pathogenicity (12, 24, 38). The "global panzootic lineage" (*Bd*-GPL; used in this study) is associated with mass mortalities in wild amphibian populations (41), and to date is the only *Bd* lineage found in California (12, 39), but there is currently little or no information available on different populations of *Bd*, and their effects on hosts, in California.

We investigate the proportion of individuals infected with the *Bd* pathogen in wild salamander populations and combine both field sampling and lab-based (i.e., experimental) studies to investigate pathogen host susceptibility and historical *Bd* occurrence in two species of co-occurring terrestrial salamanders: the Santa Lucia Mountains slender salamander (*Batrachoseps luciae*), and the arboreal salamander (*Aneides lugubris*). The arboreal salamander has a wide range that overlaps with much of the amphibian diversity in California, whereas this species of slender salamander has a very limited geographic range (42) (**Figure 1**), making them potentially more vulnerable to extinction (43, 44). In particular, in this study, we provide in-depth host/pathogen information on two sympatric terrestrial salamander host species: we (1) conduct a 90-year retrospective survey to document the proportion of *Bd*-infected hosts through time using specimens from natural history museum collections, (2) non-intrusively document the proportion of *Bd*-infected animals in contemporary wild populations, (3) use modeling to identify biotic (e.g., species overlap, group size) and abiotic (e.g., precipitation. temperature) infection correlates, (4) investigate host susceptibility in laboratory trials where hosts were exposed to either one or two populations of the *Bd* pathogen (one from a *Bd*-epizootic that led to frog population collapse, and another from focal hosts that were infected upon capture), and (5) examine the ability of naturally occurring amphibian host skin bacteria from our focal host species to inhibit *Bd* in culture.

We describe longitudinal patterns of *Bd*-infection in order to determine if they are consistent with previous studies on other *Bd* host species that occur in western North America, where several frog species have experienced *Bd* epizootics followed by mass die offs and population extinctions [e.g., *Rana muscosa*, *Rana sierrae*; (17)]. The prevailing hypothesis to explain disease dynamics in these species is that *Bd* invaded novel host populations causing epizootics, and surviving host populations eventually transitioned to more stable pathogen/host enzootic dynamics (19, 20, 28, 45). Theoretically, infection prevalence will peak during the epizootic and then decrease as the dynamics between the host and the pathogen shift toward an enzootic state (17, 20), but the

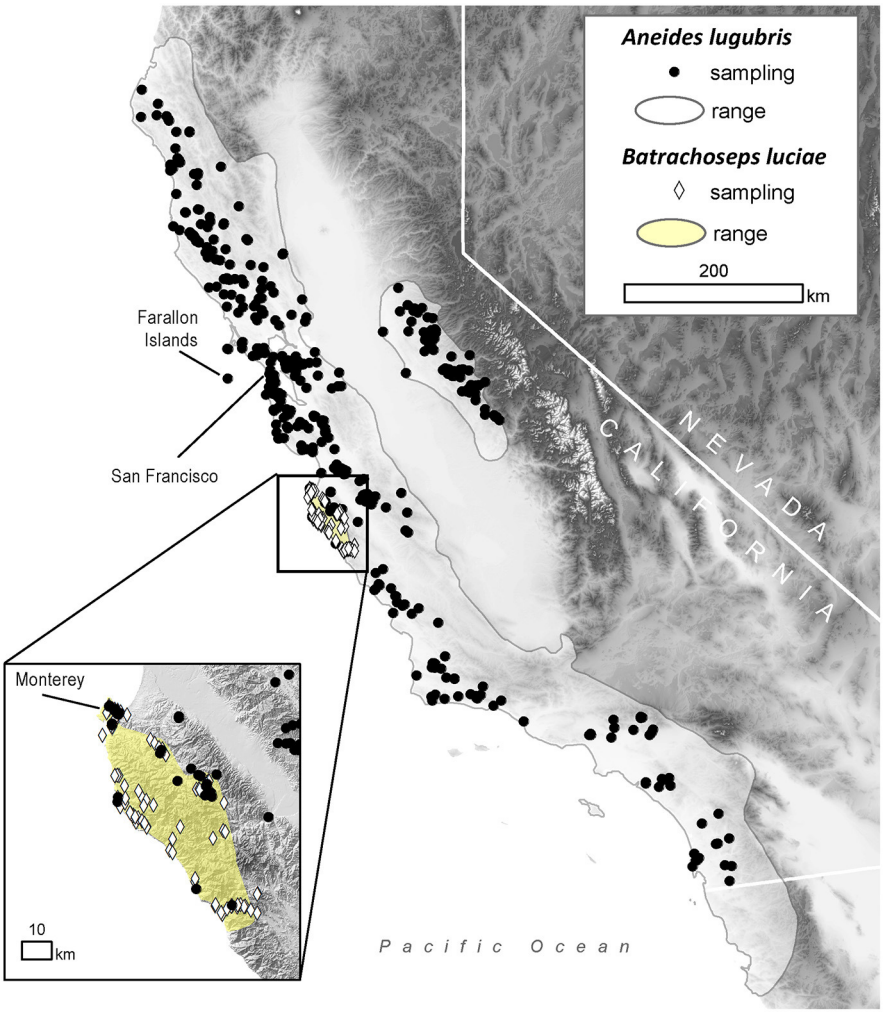


FIGURE 1 | Distribution of sampling locations within the range of the arboreal salamander (*Aneides lugubris*; solid gray line) and the Santa Lucia Mountains slender salamander (*Batrachoseps luciae*; yellow area) in California, USA.

effects of *Bd* on remaining populations could still be significant (46). Our approach combines a retrospective and contemporary view of host pathogen dynamics as we also test host *Bd*-infection susceptibility and look for correlates of *Bd* infection of individual hosts in wild populations. Finally, our skin bacterial cultures and *Bd* inhibition trials allow us to test whether these hosts might be protected from *Bd* infection by their skin microbiota. Thus, we use a combination of field and laboratory techniques to investigate the role of the *Bd* pathogen in populations of a widely occurring terrestrial salamander and a narrowly endemic terrestrial salamander in western North America, where *Bd* epizootics have severely affected other amphibian host species (3, 17, 19, 46, 47).

METHODS AND MATERIALS

Historical Infection Study (1920–2015)

To estimate historical infection prevalence of *Bd* in populations of *B. luciae* and *A. lugubris*, we analyzed *Bd* occurrence in a total of 2,459 museum specimens (1,522 *B. luciae*, 936 *A. lugubris*) from natural history collections at the Museum of Vertebrate Zoology (MVZ), University of California Berkeley, and the California Academy of Sciences (CAS), San Francisco, California. For *B. luciae*, we aimed to sample all available specimens (1,522 specimens were sampled from MVZ; none were available at CAS). The salamander *A. lugubris* has a much wider distribution (**Figure 1**) and has many more available specimens (MVZ plus CAS = 3,882; VertNet.org); thus, to minimize costs, we used a stratified (by decade) random sampling design to select 936 specimens as a subsample. We randomly selected 40 specimens per decade (collection years include 1940–2015). If there were fewer than 40 available in a decade, we selected all available specimens for that decade. In addition, we also selected all available *A. lugubris* collected within the area of overlap with *B. luciae* ($n = 78$; Monterey County, California; **Figure 1**) to enable us to determine possible disease-related relationships between these co-occurring species.

In museum specimens, we used a standard skin swabbing technique to test for the presence of *Bd* (48–50). Specimens were stroked 30 times with a MW113 dry swab (Medical Wire and Equipment Company)-−10 times both dorsally and ventrally, and five times on each laterum, spanning the majority of individual's body length. To decrease the chance of cross contamination between specimens, each specimen was rinsed in 70% ethanol before swabbing and gloves were changed between handling every specimen (50). Swabs were stored in 1.5-mL microcentrifuge tubes at 4°C. We used standard *Bd* DNA extraction and real-time quantitative polymerase chain reaction methods to detect *Bd* from swabs collected [described in (48, 49)]. These methods have been validated both in live specimens and formalin-fixed museum specimens stored in 70% ethanol (50, 51). Considering the relatively small difference in *Bd* detection rate using qPCR for samples run in singlicate and triplicate (50), we ran a larger number of samples in singlicate rather than a smaller number of samples in triplicate to both minimize costs and increase the sampling power and the geographic and temporal spread of the samples. Genomic equivalent results were multiplied by 80, the dilution factor in qPCR sample preparation, to estimate the number of *Bd* zoospores on the entire swab [Zswab; *Bd*-infection intensity; (17, 20). Samples with a Zswab score > zero were defined as *Bd*-positive.

Contemporary Field Study and Generalized Linear Model

Field surveys for contemporary disease prevalence were conducted in Monterey County, California, from May 2014 to March 2015 at the following locations: Don Dahvee Park (Monterey, CA), Veterans Memorial Park (Monterey, CA), Mission Trails Park (Carmel, CA), and Lynn "Rip" Van Winkle Open Space (Pacific Grove, CA). In this area, mixed pine and oak woodlands are dominant, and *B. luciae* and *A. lugubris* are found in close proximity to each other; often under the same cover objects (e.g., decomposing woody debris, downed logs, bark, sticks). *B. luciae* has a very limited distribution, occurring solely in the central coast region, primarily Monterey County (42), while *A. lugubris* has a wide distribution across coastal California and the Sierra Nevada foothills (52) (**Figure 1**). However, we focused our field sampling of *A. lugubris* to the sites where it is sympatric with *B. luciae*, but also collected outside the area of sympatry. Sites were surveyed by turning logs, rocks and other debris to locate salamanders beneath them.

All *B. luciae* and *A. lugubris* encountered were captured by hand and measured (snout-vent length; tail length, weight). From each individual, we collected one or two non-invasive skin swabs, in a method identical to those described above for museum specimens [but without an ethanol wash; (48); following (17)] before being released. If two skin swabs were taken, the first swab was used to culture skin bacterial symbionts (see "*Bd* inhibition" section below), the second skin swab was used to detect *Bd* using a qPCR Bd assay (48). If only one swab was taken, it was used to detect *Bd*. For each cover item where a salamander was found, the number of intra- and interspecific individuals found within 15 m of the focal individual were recorded. Individual animals were captured, sampled, and released at the site of capture within a 10-min period.

Laboratory Host Susceptibility Trials

In addition to the catch-and-release animals, we collected 31 *B. luciae* and 18 *A. lugubris* and transported them alive to the laboratory for susceptibility trials. All *B. luciae* and 12 *A. lugubris* were collected in Monterey County, California, from July 2014 to March 2015. Severe drought made salamander fieldwork difficult, thus we expanded our collection locations for *A. lugubris* and collected 6 additional individuals outside of the range of *B. luciae* (from San Mateo and Calaveras counties, California) in January and March 2015. At the site of capture, each salamander was placed individually in a plastic container lined with moist paper towels and transported to the animal care facility at San Francisco State University, where they were kept in individual standard plastic mouse cages (19 × 29.2 × 2.7 cm) lined with moist paper towels for the duration of the experiment. Salamanders were fed twice a week and received 5–10 crickets or ∼10 wingless *Drosophila* (*B. luciae* only) at each feeding. Clean cages were provided 1–2 times per week and temperature was maintained between 17 and 20°C. Each animal was checked visually every day and kept on a 12-h light and 12-h dark daily schedule.

Ten of the 31 *B. luciae* collected were found to be *Bd*-infected at the time of collection, verified using qPCR from field swabs. We called this experimental group "wild strain (WS) field-infected" and we followed the infection throughout the study period, testing weekly for *Bd* (as described below). All 10 of the WS field-infected animals were individually housed for the duration of the experiment. For the remaining uninfected *B. luciae* individuals collected from the field ($n = 21$), we split them into three experimental groups: two experimental inoculation groups using two different *Bd* strains ("GPL lab-inoculated" and "wild strain lab-inoculated") and a sham-inoculated control group ("uninfected controls"). The first group included eight randomly chosen individuals (of the 21 uninfected) and was named "GPL lab-inoculated." Here we used the global panzootic lineage, *Bd*-GPL [strain id # CJB57-(4)-p6; (39)], cultured from Southern Mountain yellow-legged frog (*Rana muscosa*) epizootics in the Sierra Nevada, California (17). Individuals were inoculated with 2 × 106 zoospores (confirmed via hemacytometer) suspended in 15 mL of sterile water. These salamanders were placed in petri dishes and exposed to the solution individually for 20 min per day for 5 consecutive days, using the protocol from a previous study (53).

The second experimental group involved eight randomly chosen *B. luciae* and was termed "wild strain (WS) lab-inoculated." For the zoospore source, we placed a known wild-infected *A. lugubris* from the same population as the *B. luciae* in a small container with 30 mL of sterile water for 20 min allowing time for *Bd* zoospores to be shed into the water. This "*Bd* water" was then divided equally among eight 50-mL falcon tubes. Sterile water was added to total 5 mL of solution in each falcon tube. A single *B. luciae* individual was placed in each falcon tube with the liquid and left for 1 h. The process was repeated each day for a total of 10 consecutive days.

For the third experimental group, termed "uninfected controls," the five remaining individuals received sham inoculations. For the uninfected control group, we followed the steps described for the second experimental group (WS lab-inoculated) with the only difference being that we used an uninfected *A. lugubris* (as determined by qPCR) instead of an infected *A. lugubris* to produce the sham "*Bd* water." The process was repeated each day for 10 consecutive days, as described above.

For susceptibility trials with *A. lugubris*, we had fewer field-collected animals available. Therefore, we had fewer trials and did not use the *Bd*-GPL strain (i.e., there was no "GPL lab-inoculated" treatment). Of the 18 *A. lugubris* collected in the wild, 5 showed an initial infection and we labeled them "WS field-infected." Of the 13 uninfected individuals, seven were inoculated using the same protocol as the WS lab-inoculated *B. luciae* experiment. The 30 mL of "*Bd* water" from soaking a known, wild-infected *A. lugubris* was evenly divided into 7 small plastic containers (150- mL volume); however, since these salamanders are much larger than *B. luciae*, we added more sterile water to total 30 mL in each container. This was repeated for 10 consecutive days. The remaining six uninfected *A. lugubris* were exposed to sham "*Bd* water" and kept as controls in the same manner as described above.

The initial post-inoculation swab (for all groups and both species including sham controls) was taken 48 h after the last exposure period, and swabs were subsequently taken weekly for 14 weeks. For all experiments, individuals were euthanized using MS-222 if they exhibited loss of righting reflex, leg-locking, lethargy, very high *Bd*-infection intensity [i.e., Vredenburg's 10,000 Zoospore Rule; (17, 54)], or other signs indicative of severe chytridiomycosis (55).

Host Skin Bacteria *Bd* Inhibition Trials

Bacteria were collected from the skin of 48 salamanders (40 *B. luciae* and 8 *A. lugubris*) and one clutch of *A. lugubris* eggs in the field. All individuals in this trial were captured in Don Dahvee Park, Monterey, CA and were handled with sterile nitrile gloves and sterile plastic bags. Animals were rinsed thoroughly with 50 mL of sterile water to remove particulates and transient bacteria before we collected a skin swab for bacterial culture. For the bacterial cultures, all *B. luciae* were released within 10 min of capture, but the eight *A lugubris*, were first sampled for bacteria and then transported to the laboratory for use in the susceptibility trial. Each swab was suspended in a microfuge tube with 1 mL of DS solution, a salt solution resembling pond water (56) and transported to the laboratory for bacterial culturing.

A cell free supernatant (CFS) inhibition assay was used to determine whether cultured bacteria produced anti-fungal activity (57). The cultured *Bd*-GPL (strain id#CJB57-(4)-p6) used in the susceptibility trials was also used in this assay (17). Microfuge tubes containing DS solution and bacterial swabs from the field were vortexed within 24 h after field collection. Bacterial isolates in DS solution were incubated on R2A media and morphologically distinct bacteria were placed in axenic culture. *Bd* zoospore growth was then challenged against the various bacterial isolate supernatants (in replicates of five) in an adapted version of a previously published protocol (57). The optical density at 490 nm in each well was measured every 24 h using a SpectraMax 190 Microplate Reader and SoftMax Pro software. The total change in optical density after 7 days was used as a proxy for *Bd* growth. The average percent growth, normalized to the positive control, was calculated for each isolate using a previously published equation (58). Isolates that exhibited significantly lower % Growth values than the positive control were labeled as "Inhibitors" and isolates that did not differ significantly from the positive control were labeled as having "No Effect."

In order to identify bacterial isolates, the 16S rDNA region of each isolate was sequenced at the San Francisco State University Genomics/Transcriptomics Analysis Core (GTAC) facility laboratory. Bacterial DNA was extracted using either Chelex or direct colony lifts into PCR reactions. The 16S rDNA region was amplified in PCR reactions using the 515F and 1492R 16S primers. The 16S regions were sequenced using standard protocol for chain termination sequencing, including an ExoSAPit PCR product purification, followed by cycle sequencing with BigDye 3.1 and the same 515F and 1492R primers as the initial PCR reaction.

Sequencher version 4.9 was used to assemble contigs from forward and reverse reads. Sequence data was then used to identify closest species match through BLAST, using default parameters for megablast searches. In order to confirm the genus of each isolate, sequence data was bootstrapped in MEGA version 6.06 against reference sequences belonging to the same family as the isolate's closest species match in BLAST. 16S rDNA reference sequences were selected from The NCBI Reference Sequence Database (RefSeq). Following a bootstrap sensitivity test, a tree was constructed for each isolate using the neighbor joining method and bootstrapped 500 times.

Statistical Analyses

All statistical analyses were performed using the software R (version 3.5.0) in RStudio (version 1.1.447). All of the code used are freely available on Zenodo (https://zenodo.org/badge/latestdoi/137778706). To characterize the temporal distribution of *Bd* in *B. luciae* and *A. lugubris* within our historical data, we calculated 95% credible intervals for *Bd* prevalence in each decade sampled based on the binomial probability distribution, using sample size and the number of *Bd* positive individuals (28, 59). To test the likelihood of detecting a *Bd*-positive individual in each decade sampled, we calculated the probability of detecting zero positives using the binomial distribution. Two previous studies that used an identical qPCR assay found an enzootic level of ∼11% prevalence in amphibian populations in Illinois, USA (60), and in another California slender salamander *Batrachoseps attenuatus* (21); therefore, we used 0.11 as the probability of detecting a *Bd* positive individual.

We performed a stepwise binomial logistic regression on field-collected individuals of both species using *Bd* infection status as the response variable with the R stats and MASS packages (version 3.5.0, version 7.3-50; respectively). We used the following variable as our explanatory variables: precipitation, minimum temperature, maximum temperature,

mean temperature, snout-to-vent length (i.e., the size of the animal as measured from the nares to the cloaca), elevation (meters above sea level), the number of conspecific hosts found with the individual, and the number of heterospecific hosts found within a 15 m radius of the individual. Using the skin swab collection date of every individual host (January 2014–December 2015), we matched the month and year for the climate variables using monthly averaged data from the PRISM Climate Group, Oregon State University (http://prism.oregonstate.edu, created 25 May 2018). To validate the model, we performed k-fold cross validation, subsetting our data into 100-folds to minimize bias in producing the folds using the package DAAG [version 1.22; (61)]. Survival estimates for our laboratory infection trials were performed using the R package "survival" [version 2.42-3; (62)]. In the bacterial *Bd* inhibition trials, percent growth values of each isolate were compared to the positive control using a two-tailed Student's *t*-test, with significant *p*-value cutoffs ($p < 0.005$ for isolates from *A. lugubris* eggs [$n = 9$], $p < 0.003$ for isolates from *A. lugubris* [$n = 16$], and $p < 0.003$ for isolates from *B. luciae* [$n = 15$]) determined using the Bonferroni correction to minimize false-positives (63).

RESULTS

Historical Infection Study (1920–2015)

The historical samples spanned the entire range of *B. luciae* and nearly the entire range of *A. lugubris* (**Figure 1**). All *B. luciae* specimens in the decades of the 1920s−1960s ($n = 123$) were negative for *Bd* (**Table 1**). The first *Bd*-positive individuals of *B. luciae* were collected in 1972, and after that time, prevalence began to steadily increase (**Figure 2A**). *A. lugubris* was found to have a similar pattern; the first *Bd*-positive individual was from 1968, after more than 300 specimens tested negative (1940–1972; **Table 1**), and after that *Bd* prevalence increased (**Figure 2B**). For both species, *Bd* prevalence appears to trend higher in the 1980s and 1990s (**Figures 2A,B**). In *B. luciae*, *Bd* prevalence peaked at 29.23% in the 1990s and then subsided to 9.82% in 2010–2015. In *A. lugubris*, *Bd* prevalence increased to 16.92% in the 1990s and remained at 16.67% in 2010–2015. Both species show an upward trend in infection intensity through the 1990s, though few individuals surpassed Vredenburg's 10,000 Zoospore Rule, the expected lethal infection intensity for *Bd* in anurans (17, 54). When we compared the *Bd* prevalence from the decade with the highest prevalence (1990s for both species) in the museum specimens to the prevalence in contemporary field samples (collected 2014–2015; **Table 2**), we found that *Bd* prevalence had decreased significantly from 29.23 to 9.87% in *B. luciae* ($p < 0.01$; $X^2 = 18.8$), but showed no significant difference in *A. lugubris* (16.92% compared to 18.18%; $p = 0.87$; $X^2 = 0.03$).

Contemporary Field Study and Generalized Linear Model

For *B. luciae* swabbed in the field between 2014 and 2015, 38 of 385 (9.87%) were *Bd* positive (**Table 2**); in contrast, 6 of 34 (18%) field-swabbed *A. lugubris* were *Bd* positive (**Table 2**). Of the 34 *A. lugubris* individuals sampled, 21 were located in Monterey County and 13 were located in San Mateo, Alameda and Calaveras counties. All georeferenced data (excluding the laboratory trial data) from the contemporary populations (catch and release animals) and from the museum specimens sampled are freely available for download at AmphibiaWeb's Amphibian Disease Portal (AmphibianDisease.org; https://amphibiandisease.org/projects/?id=251; doi's available at: https://n2t.net/ark:/21547/DTN2 - field based samples; https://n2t.net/ark:/21547/DoZ2 - museum based samples), a global archive for chytrid sampling data (see Koo et al., this issue).

The binary logistic regression model with the best (lowest) AIC (AIC = 225.4; **Table 3**), *Bd* infection status had a positive relationship with the following variables: elevation, number of heterospecifics, and number of conspecifics ($p = 0.01$, $p < 0.01$, $p < 0.01$; respectively). Precipitation had a negative relationship with *Bd* infection status ($p = 0.01$), and mean temperature had no significant relationship with *Bd* infection status ($p = 0.09$). Our k-fold cross-validation of the best model showed an estimate accuracy of 90% for the best model.

In addition to revealing an effect of conspecifics in the binary logistic regression model (**Table 3**) we separated all field sampled individuals for *B. luciae* into their respective social group sizes, as determined by presence together under the same cover item. There was a clear pattern where the probability of infection increased 2x to 3x in social groups relative to individuals who were found to be solitary (**Table 4**). Interestingly, there was no effect of social group size (2 vs. 3 or 4+) on the prevalence of *Bd*, revealing that sociality itself rather than size of social group is the best indicator of *Bd* risk (**Table 4**).

Laboratory Host Susceptibility Trials

Both *B. luciae* and *A. lugubris* suffered high mortality from chytridiomycosis in the laboratory experiments (**Figures 3A**, **4A**), yet all of the uninfected controls for both species survived the entire experiment with no mortality and no positive *Bd* test results from the qPCR assay. None of the *B. luciae* exposed to *Bd*-GPL (GPL lab-inoculated; *Bd*-GPL, strain id#CJB57-(4)-p6) died (**Figure 3A**), however they did become infected (**Figure 3B**). High mortality was observed among *B. luciae* in the WS field-infected and WS lab-inoculated groups, with 60 and 87.5% mortality, respectively. When mortality between the two groups (WS field-infected and WS lab-inoculated) was compared, we found no significant difference between them ($p = 0.08$). However, there was a significant difference in mortality rates between WS field-infected and uninfected control groups ($p < 0.05$; **Figure 3A**), and between WS lab-inoculated and uninfected controls ($p < 0.001$; **Figure 3A**). In *A. lugubris*, we also found a significant difference in survival ($p < 0.02$; **Figure 4A**) between WS lab-inoculated *A. lugubris* (71.4% mortality) compared to the uninfected controls (**Figure 4A**). Although mortality was observed in WS field-infected individuals (40% mortality), there was no significant difference in mortality rates between the WS field-infected and uninfected control groups ($p = 0.3$; **Figure 4A**). When we compared the *Bd*-infection intensity of individuals in our different groups (**Figures 3B**, **4B**; uninfected values not included), we consistently found higher levels of infection in groups that suffered mortality, but the differences,

TABLE 1 | *Batrachochytrium dendrobatidis* (*Bd*) prevalence in the Santa Lucia Mountains slender salamander (*Batrachoseps luciae*) and the arboreal salamander (*Aneides lugubris*) museum specimens collected in California.

Species	Decade	Sample size	*Bd* positive	% Prevalence	95% CI		
					Lower	*Upper*	*Pr (no Bd)*
B. luciae	1920–29	1	0	0	0	0	0.89
	1930–39	30	0	0	0	0	0.03
	1940–49	53	0	0	0	0	< 0.01
	1950–59	0	0	0	0	0	1
	1960–69	40	0	0	0	0	< 0.01
	1970–79	1,292	34	2.63	1.829	3.658	< 0.01
	1980–89	14	1	7.14	0.181	33.87	0.19
	1990–99	65	19	29.23	18.6	41.82	< 0.01
	2000–09	27	4	14.81	4.189	33.73	0.04
	2010–15	387	38	9.82	7.043	13.23	< 0.01
	Total	**1,909**	**96**				
A. lugubris	1920–29	0	0	0	0	0	1
	1930–39	0	0	0	0	0	1
	1940–49	157	0	0	0	2.322	< 0.01
	1950–59	152	0	0	0	2.397	< 0.01
	1960–69	155	2	1.29	0.156	4.583	< 0.01
	1970–79	173	4	2.31	0.633	5.814	< 0.01
	1980–89	190	13	6.84	3.693	11.416	< 0.01
	1990–99	65	11	16.92	8.762	28.266	< 0.01
	2000–09	38	4	10.52	2.943	24.805	0.01
	2010–15	27	6	16.67	8.621	42.258	0.04
	Total	**957**	**40**				

TABLE 2 | *Batrachoseps luciae* and *Aneides lugubris* field *Bd* prevalence by location, May 2014-March 2015.

Species	Location	Sample size	Bd positive	% Prevalence	95% Credible Intervals	
					Lower	*Upper*
B. luciae	Don Dahvee	188	19	10.11%	0.07	0.15
	Veterans Memorial	16	1	6.25%	0.01	0.29
	Van Winkle	137	15	10.94%	0.68	0.17
	Mission Trails	36	1	2.78%	0.01	0.14
	Other	8	2	25%	0.07	0.60
	Total	**385**	**38**	**9.87%**	–	–
A. lugubris	Monterey Co.	21	5	23.81%	0.11	0.45
	Other	12	1	8.33%	0.02	0.36
	Total	**33**	**6**	**18.18%**	–	–

compared as averages compiled for each group, were not significantly different.

Host Skin Bacteria *Bd* Inhibition Trials

A total of 32 bacterial samples were identified using 16S rDNA sequencing (**Supplementary Table 1**). The majority of bacterial isolated demonstrated significant *Bd* inhibitory activity against *Bd*-GPL, strain id#CJB57-(4)-p6. Thirteen isolates were identified from five *B. luciae* hosts' skin microbiome; and, all of the isolates demonstrated significant levels of *Bd* inhibition. The *A. lugubris* skin microbiome from three hosts yielded 13 isolates (**Supplementary Table 1**) and 10 of them demonstrated *Bd* inhibition. We also identified 6 bacterial isolates from the *A. lugubris* egg mass, and half of the isolates demonstrated similar *Bd* inhibitory activity. Isolates represented 4 bacterial phyla, Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Isolates from *B. luciae* skin represented Proteobacteria (61.5%), Firmicutes (23.1%), and Actinobacteria (15.4%). *A. lugubris* skin bacterial isolates mirrored these phyla representations; Proteobacteria (76.9%)

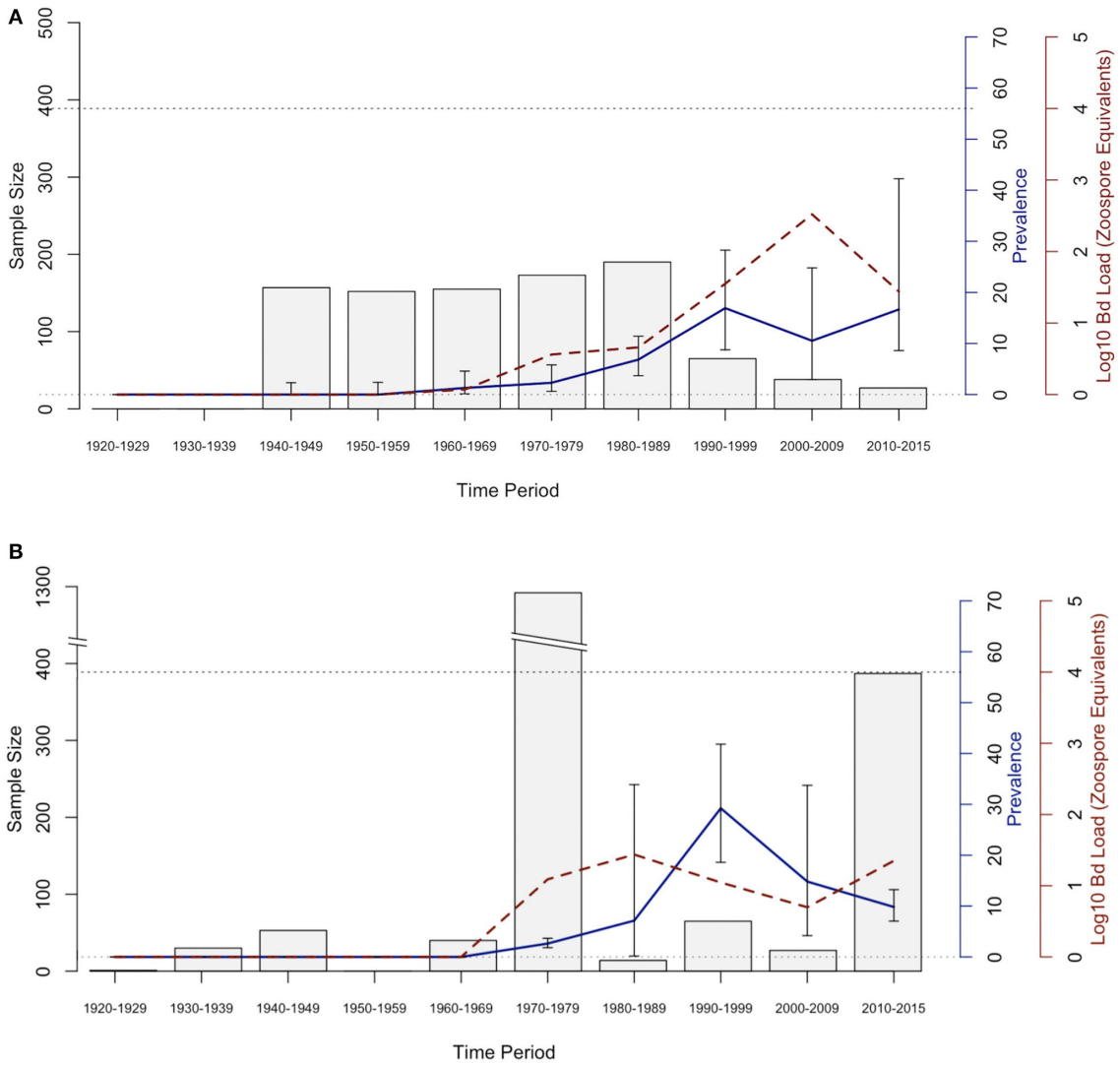


FIGURE 2 | *Bd* prevalence and infection intensity by decade in museum specimens of **(A)** *Aneides lugubris* and **(B)** *Batrachoseps luciae*. From left to right, 1st Y-axis and bars represents sample size, 2nd Y-axis axis and blue line graph represents *Bd* infection prevalence, and the 3rd Y-axis and dotted red line represents *Bd* infection intensity (Log10 zoospore equivalents). Error bars represent credible intervals of *Bd* infection prevalence based on a binomial distribution and a 0.11 expected *Bd* prevalence from an area where *Bd* is assumed to be endemic (21, 60).

was the dominant phylum, followed by Firmicutes (15.4%) and Actinobacteria (7.7%). Isolates from *A. lugubris* eggs also had Proteobacteria (50%), Bacteroidetes (33.6%), and Firmicutes as the most abundant Phyla (16.7%). Inhibitory isolates were found to span all 4 phyla, with only Proteobacteria and Firmicutes containing isolates that had no effect on *Bd* growth.

DISCUSSION

The results from our 90-year retrospective museum study suggest that *Bd* emerged in *B. luciae* and *A. lugubris* in the late 1960s and early 1970s, with prevalence and infection intensity steadily increasing into the 1990s. In *B. luciae*, the proportion of *Bd*-infected specimens tested decreased from a high near 30% *Bd*-positive in the 1990s to below 10% *Bd*-positive in more recent time periods. A similar temporal pattern has been documented in previous retrospective studies of amphibians in California (19, 21, 22, 25, 28, 30, 32). Thus, our study provides further support for the hypothesis that *Bd* invaded California, but establishment, emergence and spread occurred several decades after *Bd* was first detected. This emergence is roughly coincident with two of the earliest documented mass-die off events for anurans (frogs) in North America, both in 1978 (32, 64, 65). In addition, we suggest that the temporal patterns of *Bd* that we describe may signal a shift from epizootic dynamics in the 1970–1990's to more stable enzootic dynamics in the years since

TABLE 3 | Top 4 models of the stepwise binary logistic regression for *B. luciae* and *A. lugubris* with *Bd* infection status as the dependent variable.

Variables	Model 1	Model 2	Model 3	Model 4
Precipitation (–)	X	X	X	X
Mean temperature	X	X	X	X
Elevation (+)	X	X	X	X
Number of conspecifics (+)	X	X	X	X
Number of heterospecifics (+)	X	X	X	X
Species	X	X	X	
Maximum temperature	X	X		
Snout-to-vent Length	X			
AIC	229	228	226	225
ΔAIC	NA	1	2	1

(+) Indicates a significant positive relationship with Bd infection status while (–) indicates a negative relationship with Bd infection status in the best model (model 4, AIC = 225).

TABLE 4 | Effects of *B. luciae* group size on *Bd* infection prevalence: "Group size" = number individuals under cover item; "No. groups" = number of replicate groups found of each size; "No. individuals" = total individuals observed of each group size category; "No. sampled" = number of individuals observed that were swabbed; "No. infected" = number of sampled individuals that were *Bd* positive.

Group size	No. groups	No. individuals	No. sampled	No. infected	% sampled infected
1	164	164	164	8	4.9%
2	41	82	76	8	10.5%
3	17	51	50	7	14%
4 or more	20	148	95	15	15.8%

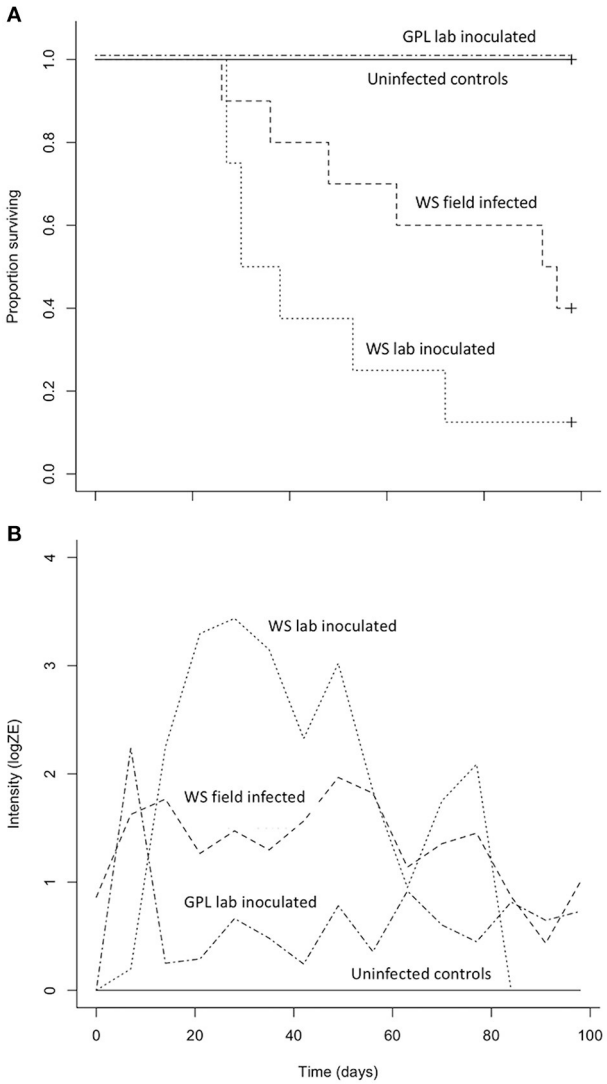


FIGURE 3 | Results of *Batrachoseps luciae Bd* susceptibility trial [four treatment groups; wild-strain (WS) lab-inoculated ($n = 8$; dotted line), wild-strain (WS) field infected ($n = 10$; dashed line), GPL (global panzootic lineage) lab-inoculated ($n = 8$; solid line), and uninfected control group ($n = 5$; dash and dot line)] showing Kaplan-Meier survival estimate **(A)** illustrating proportion of individuals surviving over time, and **(B)** average *Bd* infection intensity (zoospore equivalents, log scale) over time.

2000 (20, 45). This pattern is consistent with the one proposed for the Sierra Nevada yellow-legged frog, *Rana sierrae* (66), that suffered a major decline and appears to be recovering after nearly four decades despite *Bd* infections remaining in host populations (45). However, *Bd* infections may still slow population growth even in species that may have survived epizootics (20, 46).

We find no indication of a unidirectional spread of *Bd* across a large geographic range, which is consistent with previous studies on historical *Bd* invasion in California (19, 21, 25, 28, 30). Previous studies found evidence that *Bd* may have invaded California earlier than we detected in our study (67, 68), but as new evidence comes to light, it seems clear that those early invasions of *Bd* did not become established or spread widely until several (∼4–5) decades later (19, 21, 22, 30). Our results are consistent with a regional pattern (western North America) showing a geographic expansion of collection localities for *Bd*-positive samples, as well as a large increase in the number of *Bd*-positive samples detected beginning ∼1969–1972, and is consistent with previous work (19, 21, 25, 28, 30). What caused the invasion of *Bd* to California remains unclear, but the introduction of non-native amphibians is one likely factor (26, 32, 67, 68), and there are indications this also could be important globally (24, 69–71). The invasion of *Bd* in western North America and elsewhere was unnoticed because the first epizootics [e.g., frogs in the Sierra Nevada; (19)] occurred two or three decades before *Bd* was first discovered (10, 14). For cryptic, understudied species like terrestrial salamanders in California, we hypothesize that it is possible they experienced un-documented *Bd*-epizootics in the past ["ghost of epidemics past"; (23)], but also escaped notice due to their cryptic life history, behavior, and relatively unstudied populations compared to frogs [e.g., unlike salamanders, many frogs have large, often noticeable populations, schools of larvae-tadpoles, and vocalize during mating; (72)].

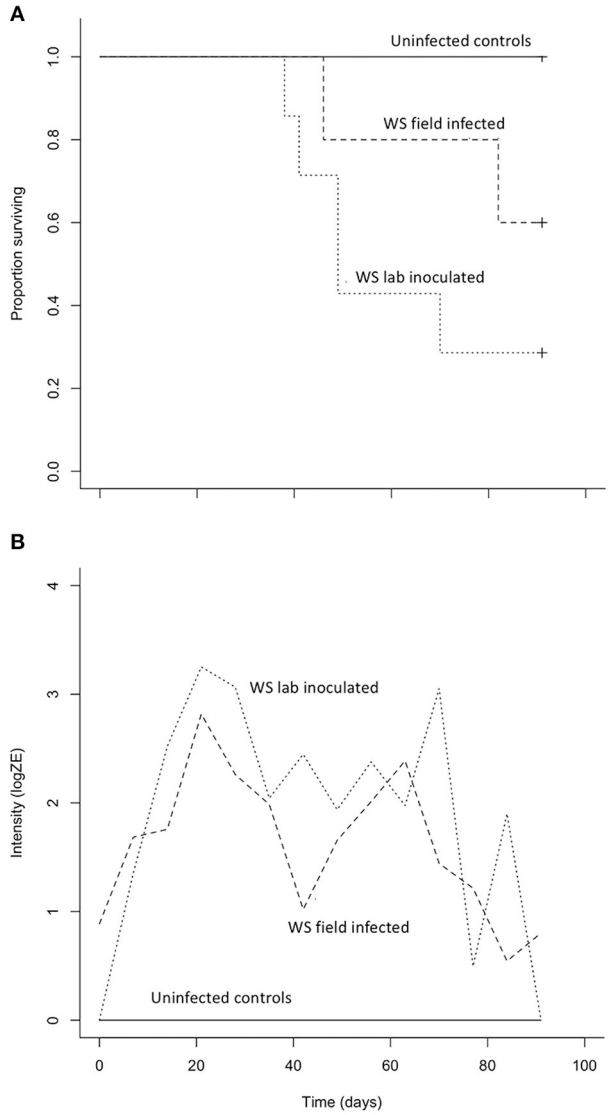


FIGURE 4 | Results of *Aneides lugubris Bd* susceptibility trial [three treatment groups; wild-strain (WS) lab-inoculated ($n = 7$; dotted line), wild-strain (WS) field infected ($n = 5$; dashed line), and uninfected control group ($n = 6$; solid line)] with Kaplan-Meier survival estimate **(A)** illustrating proportion of individuals surviving over time, and **(B)** average *Bd* infection intensity (zoospore equivalents, log scale) over time.

Our field study indicates that both *B. luciae* and *A. lugubris* populations in the Monterey Bay Area, California, currently sustain *Bd* infection prevalence at or slightly below 20% without visible epizootics (we found no carcasses and infected individuals appeared healthy when captured, only to die later in captivity from chytridiomycosis). Because both *B. luciae* and *A. lugubris* contract *Bd* in the wild and overlap in both diet and microhabitat, it is likely that interspecific contact plays a role in disease transmission. Our logistic regression found that the number of *A. lugubris* in proximity to *B. luciae* does, in fact, correlate positively with higher disease prevalence and infection intensity in *B. luciae*. The same is not true, however, when considering the proximity of *B. luciae* to *A. lugubris*, which may be a sample size issue because we did not find as many *A. lugubris*. It is interesting to note that *A. lugubris* have a much broader distribution, and have almost twice the *Bd* infection prevalence, which may suggest that *A. lububris* could be a source of *Bd* infection in *B. luciae* and perhaps other species. Nevertheless, the mechanism behind any asymmetric effect of co-infection remains unclear. One possibility is that *A. lugubris* is a more effective vector of *Bd* than *B. luciae;* for example, *A. lugubris* are much larger and as such, may shed more infective zoospores than a much smaller *B. luciae*. The shedding rate of *Bd* zoospores is thought to directly affect infection dynamics (53, 55, 73). It is possible that predation attempts by *A. lugubris* on *B. luciae* (rather than vice versa) may provide opportunities for individual contact between the two species or increase stress in the prey, which could facilitate the unidirectional transfer of zoospores or increased susceptibility of infection from predator to prey, increasing the transmission rate and ultimately the infection prevalence of the prey species, *B. luciae*.

Our results showing a positive relationship between sociality and *Bd* prevalence suggest that, in *B. luciae*, attraction to conspecifics is likely to facilitate disease spread. It is well-known that both the overall size of social groups as well as their intrinsic structure can facilitate parasite transmission (34, 35). A social structure involving high rates of behavioral interaction among hosts within a group can result in rapid and sustained pathogen spread, especially when individuals are susceptible to re-infection (74, 75). Individual *B. luciae* in social groups were often found in direct skin-to-skin contact, similar to other species studied within the same genus (21, 25). Under these conditions of frequent contact, social organisms are known to experience higher rates of infection and pathogen spread in a population (R^0), even when infection arises from a single initially infected individual (76, 77). Despite the costs of group living that relate to parasites and pathogens, it has also been shown that social behavior can benefit hosts by spreading beneficial microbes important in disease resistance (78, 79). While our study found a behavioral effect, with larger groups of *B. luciae* having higher infection prevalence, it is possible that these social groups also harbored more protective microbes, which could mitigate the effect of the fungal pathogen [as we found in our skin bacterial cultures; (80)]. However, there are several alternative explanations. For example, *Bd*-infected *B. luciae* may prefer to group, while uninfected individuals prefer to be solitary, or there could be some environmental factors, not included in this study, that could make some locations more favorable for the hosts and lead to host aggregation. A retrospective study in *Batrachoseps attenuatus,* a closely related and similar species, found a *Bd* infection pattern consistent with our study: *Bd* infection was positively associated with host group size (21). This study also revealed that host populations with longer *Bd* exposure histories (over several decades) were significantly less social (i.e., had smaller average group sizes) than host populations with either a short history of infection (months to a few years) or populations very recently infected by *Bd* (21). These combined results suggest that sociality may increase *Bd* transmission rate while also leading

populations with long-term exposure to evolve away from the proposed ancestral mode of sociality.

When uninfected wild collected *B. luciae* and *A. lugubris* were exposed to wild strain *Bd* (WS lab-inoculated) they were highly susceptible and experienced high mortality, ∼90%, and >70%, respectively. In both species, individuals that succumbed to the disease exhibited similarly high levels of *Bd* infection intensity consistent with signs of chytridiomycosis as described by the Vredenburg 10,000 Zoospore Rule (17, 54). Interestingly, *B. luciae* were not susceptible when exposed to *Bd*-GPL. This *Bd* genotype has been sequenced and described as the "Global Panzootic Lineage" [*Bd*-GPL (39)], and is the same lineage found in *Bd* epizootics that were responsible for species extinction events in Central and South America and Australia (12, 23). Different genotypes of *Bd* have varying levels of virulence on hosts (12, 24, 38, 81). Of the most widespread and well-known *Bd* lineages, *Bd*-GPL is the most virulent on adult hosts (38). The fact that we found no mortality of *B. luciae* when exposed to *Bd*-GPL may indicate that hosts are adapted to this lineage, or that the culture had decreased in virulence in captivity. Other studies have proposed similar ideas regarding host adaptation to *Bd* lineages [(40); reviewed in (12)]. The cultured *Bd*-GPL we used to expose *B. luciae* was collected from *Rana muscosa* populations experiencing *Bd* epizootics that subsequently drove populations to extinction (17). For this study, the *Bd*-GPL culture was revived from cryopreserved culture before the trials; we did not use a culture that was continually kept active. Cryopreservation decreases passage rates, which are known to decrease virulence in many pathogens kept cultured in laboratory conditions (82). The *Bd*-GPL culture used here was lethal to frogs in other *Bd* laboratory susceptibility experiments using the exact same exposure protocol we used (45, 50, 53). Along with previous studies, we conclude that pathogen lineage should be carefully considered when designing host susceptibility trials and should be linked with contemporary field studies whenever possible (83, 84).

For this study, we also inoculated individuals from both salamander species with *Bd* from live, collected wild *Bd*-infected salamanders. Laboratory conditions vary greatly from the wild, and we suggest that laboratory susceptibility trials are best interpreted in conjunction with other data (e.g., spatio-temporal infection prevalence, host behavior, and other biotic and abiotic factors) that could help describe the particular context of the host/pathogen dynamics. Despite extensive searches, we did not find sick or dying animals in our field surveys, and population declines have not been reported for these two species; however, there are also no population surveys available that could be used to detect declines for these species. In addition, the absence of sick or dying individuals in the field alone is not enough evidence to suggest that the pathogen is not affecting populations in the wild (23, 46). Salamanders are known to quickly decompose in the wild after death, and thus ascertainment bias may occur. This is especially important to consider given the fact that the wild strain *Bd* caused high mortality in both *B. luciae* and *A. lugubris* individuals in our laboratory susceptibility trials (**Figures 3A**, **4A**). In fact, to our knowledge, most population extinctions caused by *Bd* epizootics revealed few sick animals or carcasses [but see (17, 85)] before populations disappeared, perhaps because amphibian carcasses are rapidly consumed by predators, scavengers, and saprophytic microbes. Finally, it is important to recognize that for terrestrial salamanders, dead individuals are not expected to be conspicuous since these species live under cover items and/or underneath the soil surface. Unlike previous studies mainly on frogs where hosts are exposed to *Bd* while in aquatic habitats, our host species are exposed to *Bd* in terrestrial habitats and never go to aquatic habitats. How this impacts chytridiomycosis dynamics is poorly understood.

There is a growing body of evidence that host skin microbiome communities affect host health. Several studies have shown that *Bd*-inhibiting skin bacteria from hosts can inhibit *Bd* growth and limit the effects of *Bd* on susceptible amphibians (36, 80, 86, 87), and *Bd* inhibiting skin bacteria occur on many amphibian species (58, 88, 89), including terrestrial salamanders that live in California (90, 91). In this study we found *Bd* inhibiting bacteria on both focal salamander species, with a high percentage of culturable species exhibiting *Bd* inhibition (*B. luciae*, 100%; *A. lugubris,* 81.2%, and *A. lugubris* eggs, 75%) compared to other studies (58, 89, 92, 93). The swab culturing methodology used in this and other studies, however, is not perfect. For example, we did not test for other organisms (e.g., fungi) in the swab cultures, and it is possible that they could affect our bacterial culturing efforts. The high percentage of *B. luciae* bacterial isolates that inhibited the *Bd*-GPL culture is in accordance with our susceptibility trial data, in which *B. luciae* exposed to this *Bd* culture exhibited no mortality. This suggests that *Bd* inhibitory isolates on *B. luciae* skin may help this salamander species resist *Bd* induced mortality. The wild *Bd* was not available in culture, and thus we cannot draw direct conclusions about the bacterial inhibition on it. However, since previous studies examining populations of threatened amphibians have revealed a correlation between presence of *Bd* inhibitory symbiotic skin bacteria and resistance to *Bd* infections in the wild (87, 94, 95), we propose that some of the skin bacteria found living on *B. luciae* and *A. lugubris* may provide a mechanism for survival of *Bd*-infected individuals in the wild.

Our retrospective analysis shows *Bd* invasion, establishment, and emergence in two sympatric species of terrestrial salamanders. This is consistent with patterns in other hosts from previous retrospective studies of *Bd* in California (19, 21, 22, 25, 28, 30, 32). We suggest that other amphibian species in California, including the most diverse group [salamanders; (1)], may have been negatively affected by this pathogen in the past, but because the invasion and emergence occurred before the pathogen was described, this phenomenon may have been overlooked ["ghost of epizootics past"; (23)]. Population monitoring of salamanders that live cryptically is challenging, which makes it likely that any present or past effects of *Bd* on salamander populations may not have been measured. Although the two species we studied differ greatly in range size, abundance, and social behavior, the timing of emergence in both species is strikingly similar to each other and to other studies in western North America (19, 21, 30). However, our results are in

stark contrast to studies that used the exact same techniques to test for *Bd*-infection in museum specimens collected in other areas. For example, Talley et al. (60) found *Bd*-infected specimens consistently in ∼11% of specimens dating across over a century of collections (as far back as the 1890s) in specimens collected throughout Illinois, USA. In Brazil, Rodriguez et al. (96) found *Bd*-infected specimens consistently in ∼40% of specimens dating across all decades going back 100 years. For both of these studies, many *Bd*-infected specimens were discovered even in the oldest specimens, suggesting that the technique developed by Cheng et al. (50) is robust.

We found that heterospecific hosts may influence disease. For example, the presence of *A. lugubris* may influence disease prevalence and infection intensity of *B. luciae*. We found that *A. lugubris* have high prevalence of *Bd* in wild populations, which could possibly increase the chances of *Bd* transmission to other heterospecific hosts. Other species have been proposed as *Bd* reservoirs (53) or *Bd* super shedders (97), and *A. lugubris* shares some similar qualities. The arboreal salamander, *A. lugubris,* has an expansive geographic range that overlaps in distribution with 28 of California's 49 salamander species (1), has a relatively high proportion of infected individuals in nature, and is able to infect other host species (this study). This species is often found in close proximity to other hosts (terrestrial salamanders genera: *Aneides*, *Batrachoseps*, *Ensatina*), even under the same cover items (e.g., woody debris such as logs, bark). Several amphibian species sympatric with *A. lugubris* have been identified as *Bd*-positive (21, 22, 25, 30), but little is known about their susceptibility to *Bd* or whether their infection status might also be influenced by proximity to *A. lugubris* individuals. Reservoir species, and super shedders also pose a risk in that they could spread novel pathogens that may invade in the future (26). For example, *Batrachochytrium salamandrivorans* (*Bsal*), another recently discovered chytridiomycete pathogen, has caused mass die offs in wild salamander populations in Europe (98–101), but has not been found in North America (102). *Bsal*-infected animals are present in the international amphibian pet trade (103, 104), and this puts North American salamanders, including the terrestrial salamanders, at further risk of new epizootic disease (26, 105, 106). If we hope to use disease theory to help predict and mitigate disease risk in amphibians, we suggest that additional studies on chytridiomycosis in terrestrial salamanders are urgently needed.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://amphibiandisease.org/projects/?id=251, https://n2t.net/ark:/21547/Ajp2.

ETHICS STATEMENT

The animal study was reviewed and approved by San Francisco State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MC, VV, and AZ conceived and designed the experiments. MC and WS performed the experiments and conducted fieldwork. MC, HS, WS, TY, VV, and AZ analyzed the data. MC, WS, TY, HS, VV, and AZ wrote the manuscript. MK provided editorial advice. All authors contributed to the article and approved the submitted version.

FUNDING

This project was funded by NSF IOS – 1258133 AZ and VV, and through the Belmont Forum project: People, Pollution, and Pathogens (P^3); NSF 1633948 to VV. Funding also came from San Francisco State University Instructionally Related Activities Grants awarded to MC, and from the Whitman Internship Grant awarded to WS. Publication made possible in part by support from the Berkeley Research Impact Initiative (BRII) sponsored by the UC Berkeley Library.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.742288/full#supplementary-material

REFERENCES

1. AmphibiaWeb (2021). Available online at: https://amphibiaweb.org (accessed 1 May, 2021)
2. Wake DB, Koo MS. Amphibians. *Curr Biol.* (2018) 28:R1237–41. doi: 10.1016/j.cub.2018.09.028
3. Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci USA.* (2008) 105:11466–73. doi: 10.1073/pnas.0801921105
4. Barnosky AD, Matzke N, Tomiya S, Wogan GO, Swartz B, Quental TB, et al. Has the Earth's sixth mass extinction already arrived? *Nature.* (2011) 471:51–7. doi: 10.1038/nature09678
5. Alroy J. Current extinction rates of reptiles and amphibians. *Proc Natl Acad Sci USA.* (2015) 112:13003–8. doi: 10.1073/pnas.1508681112
6. Finlay JC, Vredenburg VT. Introduced trout sever trophic connections in watersheds: consequences for a declining amphibian. *Ecology.* (2007) 88:2187–98. doi: 10.1890/06-0344.1
7. Colón-Gaud C, Whiles MR, Kilham SS, Lips KR, Pringle CM, Connelly S, et al. Assessing ecological responses to catastrophic amphibian declines: patterns of macroinvertebrate production and food web structure in upland Panamanian streams. *Limnol Oceanography.* (2009) 54:331–43. doi: 10.4319/lo.2009.54.1.0331
8. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. *Science.* (2004) 306:1783–6. doi: 10.1126/science.1103538
9. Skerratt, LF, Berger L, Speare R, Cashins S, McDonald KR, et al. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecoealth.* (2007) 4:125–34. doi: 10.1007/s10393-007-0093-5

10. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci USA*. (1998) 95:9031–6. doi: 10.1073/pnas.95.15.9031
11. Fisher MC, Garner TW. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biol Rev*. (2007) 21:2–99 (2007). doi: 10.1016/j.fbr.2007.02.002
12. Fisher MC, Garner TWJ. Chytrid fungi and global amphibian declines. *Nat Rev Microbiol*. (2020) 18:332–43. doi: 10.1038/s41579-020-0335-x
13. Olson DH, Ronnenberg KL, Glidden CK, Christiansen KR, Blaustein AR. Global patterns of the fungal pathogen *Batrachochytrium dendrobatidis* support conservation urgency. *Front Vet Sci*. (2021) 8:685877. doi: 10.3389/fvets.2021.685877
14. Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. *et sp. nov., a Chytrid Pathogenic to Amphibians*. *Mycologia*. (1999) 91:219–27. doi: 10.1080/00275514.1999.12061011
15. 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 USA*. (2013) 110:15325–9. doi: 10.1073/pnas.1307356110
16. Rachowicz, LJ, Knapp RA, Morgan J. A. T., Stice MJ, Vredenburg VT, et al. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology*. (2006) 87:1671–83. doi: 10.1890/0012-9658(2006)87[1671:EIDAAP]2.0.CO;2
17. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc Natl Acad Sci USA*. (2010) 107:9689–94. doi: 10.1073/pnas.0914111107
18. Voyles J, Vredenburg VT, Tunstall TS, Parker JM, Briggs CJ, Rosenblum EB. Pathophysiology in mountain yellow-legged frogs (Rana muscosa) during a chytridiomycosis outbreak. *PLoS ONE*. (2012) 7:35374. doi: 10.1371/journal.pone.0035374
19. Vredenburg VT, McNally SVG, Sulaeman H, Butler HM, Yap T, Koo MS, et al. Pathogen invasion history elucidates contemporary host pathogen dynamics. *PLoS ONE*. (2019) 14:e0219981. doi: 10.1371/journal.pone.0219981
20. Briggs, CJ, Knapp RA, Vredenburg VT. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc Natl Acad Sci USA*. (2010) 107:9695–700. doi: 10.1073/pnas.0912886107
21. Sette, CM, Vredenburg VT, Zink AG. Reconstructing historical and contemporary disease dynamics: a case study using the California slender salamander. *Biol Conserv*. (2015) 192:20–9. doi: 10.1016/j.biocon.2015.08.039
22. Chaukulkar S, Sulaeman H, Zink AG, Vredenburg VT. Pathogen invasion and non-epizootic dynamics in Pacific newts in California over the last century. *PLoS ONE*. (2018) 13:e0197710. doi: 10.1371/journal.pone.0197710
23. James TY, Toledo LF, Rödder D, Silva Leite D, Belasen AM, Betancourt-Román CM, et al. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecol Evol*. (2015) 5:4079–97. doi: 10.1002/ece3.1672
24. Fisher MC, Garner TWJ, Walker SF. Global emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in space, time and host. *Ann Rev Microbiol*. (2009) 63:291–310. doi: 10.1146/annurev.micro.091208.073435
25. Sette CM, Vredenburg VT, Zink AG. Differences in fungal disease dynamics in co-occurring terrestrial and aquatic amphibians. *EcoHealth*. (2020) 17:302–14. doi: 10.1007/s10393-020-01501-z
26. Yap T, Koo M, Ambrose RF, Wake DB, Vredenburg VT. Averting a biodiversity crisis. *Science*. (2015) 349:481–2. doi: 10.1126/science.aab1052
27. Vredenburg VT, Koo MS, Wake DB. Declines of amphibians in California. *Threat Amphibians World*. (2008) 1:126. doi: 10.1016/B978-012226865-6/00578-X
28. De León ME, Vredenburg VT, Piovia-Scott J. Recent emergence of a chytrid fungal pathogen in California Cascades frogs (Rana cascadae). *EcoHealth*. (2017) 14:155–61. doi: 10.1007/s10393-016-1201-1
29. Hyde EJ, Simons TR. Sampling plethodontid salamanders: sources of variability. *J Wildlife Manage*. (2001) 65:624–32. doi: 10.2307/3803013
30. Yap TA, Gillespie L, Ellison S, Flechas SV, Koo MS, Martinez AE, et al. Invasion of the fungal pathogen *Batrachochytrium dendrobatidis* on California islands. *EcoHealth*. (2016) 13:145–50. doi: 10.1007/s10393-015-1071-y
31. Adams AJ, Kupferberg SJ, Wilber MQ, Pessier AP, Grefsrud M, Bobzien S, et al. Extreme drought, host density, sex, and bullfrogs influence fungal pathogen infection in a declining lotic amphibian. *Ecosphere*. (2017) 8:e01740. doi: 10.1002/ecs2.1740
32. Yap TA, Koo MS, Ambrose RF, Vredenburg VT. Introduced bullfrog facilitates pathogen invasion in the western United States. *PLoS ONE*. (2018) 13:e0188384. doi: 10.1371/journal.pone.0188384
33. Ostfeld RS, Glass GE, Keesing F. Spatial epidemiology: an emerging (or re-emerging) discipline. *Trends Ecol Evolut*. (2005) 20:328–336. doi: 10.1016/j.tree.2005.03.009
34. Schmid-Hempel P. Parasites and their social hosts. *Trends Parasitol*. (2017) 33:453–62. doi: 10.1016/j.pt.2017.01.003
35. White LA, Forester JD, Craft ME. Dynamic, spatial models of parasite transmission in wildlife: their structure, applications and remaining challenges. *J Anim Ecol*. (2017) 87:559–80. doi: 10.1111/1365-2656.12761
36. Bernardo-Cravo AP, Schmeller DS, Chatzinotas A, Vredenburg VT, Loyau A. Environmental factors and host microbiomes shape host–pathogen dynamics. *Trends Parasitol*. (2020) 36:616–33. doi: 10.1016/j.pt.2020.04.010
37. Morgan JAT, Vredenburg VT, Rachowicz LJ, Knapp RA, Stice MJ, Tunstall T, et al. Population genetics of the frog killing fungus *Batrachochytrium dendrobatidis*. *Proc Natl Acad Sci USA*. (2007) 104:13845–50. doi: 10.1073/pnas.0701838104
38. O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, et al. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*. (2018) 360:621–7. doi: 10.1126/science.aar1965
39. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D., Rodriguez D, et al. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proc Natl Acad Sci USA*. (2013) 110:9385–90. doi: 10.1073/pnas.1300130110
40. Jenkinson TS, Betancourt Román CM, Lambertini C, Valencia-Aguilar A, Rodriguez D, Nunes-de-Almeida CH, et al. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Mol Ecol*. (2016) 25:2978–96. doi: 10.1111/mec.13599
41. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc Natl Acad Sci USA*. (2011) 108:18732–6. doi: 10.1073/pnas.1111915108
42. Jockusch EL, Yanev KKP, Wake DB. Molecular phylogenetic analysis of slender salamanders, genus batrachoseps (Amphibia: Plethodontidae), from central coastal california with descriptions of four new species. *Herpetol Monographs*. (2001) 15:54–9. doi: 10.2307/1467038
43. Sodhi, NS, Bickford D, Diesmos AC, Lee TM, Koh LP, et al. Measuring the meltdown: drivers of global amphibian extinction and decline. *PLoS ONE*. (2008) 3:e1636. doi: 10.1371/journal.pone.0001636
44. Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, et al. The biodiversity of species and their rates of extinction, distribution, and protection. *Science*. (2014) 344:1246752. doi: 10.1126/science.1246752
45. Knapp RA, Fellers GM, Kleeman PM, Miller DA, Vredenburg VT, Rosenblum, et al. Large-scale recovery of an endangered amphibian despite ongoing exposure to multiple stressors. *Proc Natl Acad Sci USA*. (2016) 113:11889–94. doi: 10.1073/pnas.1600983113
46. Russell RE, Halstead BJ, Mosher BA, Muths E, Adams MJ, Grant E. H. C., et al. Effect of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on apparent survival of frogs and toads in the western USA. *Biol Conserv*. (2019) 236:296–304. doi: 10.1016/j.biocon.2019.05.017
47. Briggs, C, Vredenburg VT, Knapp RA, Rachowicz LJ. Investigating the population-level effects of chytridiomycosis, a fungal disease of amphibians. *Ecology*. (2005) 86:3149–59. doi: 10.1890/04-1428
48. 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) 60:141–8. doi: 10.3354/dao060141
49. Hyatt AD, Boyle DG, Olsen V, Berger L, Obendorf D, Dalton A, et al. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis Aquat Organ.* (2007) 73:175–92. doi: 10.3354/dao073175
50. Cheng TL, Rovito SM, Wake DB, Vredenburg VT. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc Natl Acad Sci USA.* (2011) 108:1–6. doi: 10.1073/pnas.1105538108
51. Richards-Hrdlicka KL. Extracting the amphibian chytrid fungus from formalin-fixed specimens. *Methods Evol.* (2012) 3:842–9. doi: 10.1111/j.2041-210X.2012.00228.x
52. Lynch JF, Wake DB. Aneides lugubris (Hallowell); arboreal salamander. *Catal American Amphibians Reptiles.* (1974) 1:159
53. Reeder NM, Pessier AP, Vredenburg VT. A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. *PLoS ONE.* (2012) 7:33567. doi: 10.1371/journal.pone.0033567
54. Kinney VC, Heemeyer JL, Pessier AP, Lannoo MJ. Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates areolatus*: affirmation of Vredenburg's "10,000 Zoospore Rule". *PLoS ONE.* (2011) 6:16708. doi: 10.1371/journal.pone.0016708
55. Berger L, Roberts AA, Voyles J, Longcore JE, Murray KA, Skerratt LF. History and recent progress on chytridiomycosis in amphibians. *Fungal Ecol.* (2016) 19:89–99. doi: 10.1016/j.funeco.2015.09.007
56. Boyle DG, Hyatt AD, Daszak P, Berger L, Longcore JE, Porter D, et al. Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. *Dis Aquat Organ.* (2003) 56:59–64 doi: 10.3354/dao056059
57. Bell SC, Alford RA, Garland S S, Padilla G, Thomas AD. Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis Aquat Organ.* (2013) 103:77–85. doi: 10.3354/dao02560
58. Holden WM, Hanlon SM, Woodhams DC, Chappell TM, Wells HL, Glisson SM, et al. Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (Rana sphenocephala) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol Conserv.* (2015) 187:91–102. doi: 10.1016/j.biocon.2015.04.007
59. Phillips, BL, Puschendorf R. Do pathogens become more virulent as they spread? Evidence from the amphibian declines in Central America. *Proc R Soc London B Biol Sci.* (2013) 280:20131290. doi: 10.1098/rspb.2013.1290
60. Talley BL, Muletz CR, Vredenburg VT, Fleischer RC, Lips KR. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biol Conserv.* (2015) 182:254–61. doi: 10.1016/j.biocon.2014.12.007
61. Ayer T, Chhatwal J, Alagoz O, Kahn CE, Woods RW, Burnside ES. Comparison of logistic regression and artificial neural network models in breast cancer risk estimation. *Radiographics.* (2010) 30:13–22 doi: 10.1148/rg.301095057
62. Therneau TM, Lumley T. *A Package for Survival Analysis in R*. (2015). Available online at: https://cran.r-project.org/package=survival
63. Rice WR. Analyzing tables of statistical tests. *Evolution*. (1989) 43:223–5. doi: 10.1111/j.1558-5646.1989.tb04220.x
64. Bradford DF. Mass mortality and extinction in a high-elevation population of Rana muscosa. *J Herpetol.* (1991) 25:174–7. doi: 10.2307/1564645
65. Sherman CK, Morton ML. Population declines of Yosemite toads in the eastern Sierra Nevada of California. *J Herpetol.* (1993) 27:186–98. doi: 10.2307/1564935
66. Vredenburg VT, Bingham R, Knapp R, Morgan JA, Moritz C, Wake DB. Concordant molecular and phenotypic data delineate new taxonomy and conservation priorities for the endangered mountain yellow-legged frog. *J Zool.* (2007) 271:361–74. doi: 10.1111/j.1469-7998.2006.00258.x
67. Huss M, Huntley L, Vredenburg VT, Johns J, Green S. Prevalence of *Batrachochytrium dendrobatidis* in 120 archived specimens of Lithobates catesbeianus (American bullfrog) collected in California, 1924–2007. *EcoHealth.* (2013) 10:339–43. doi: 10.1007/s10393-013-0895-6
68. Vredenburg VT, Felt SA, Morgan EC, McNally SVG, Wilson S, Green SL. Prevalence of *Batrachochytrium dendrobatidis* in Xenopus Collected in Africa (1871–2000) and in California (2001–2010). *PLoS ONE.* (2013) 8:e63791. doi: 10.1371/journal.pone.0063791
69. Mazzoni R. Emerging pathogen in wild amphibians and frogs (Rana catesbeiana) farmed for international trade. *Emerg Infect Dis.* (2003) 9:995–8. doi: 10.3201/eid0908.030030
70. Schloegel LM, Picco AM, Kilpatrick AM, Davies AJ, Hyatt AD, Daszak P. Magnitude of the US. trade in amphibians and presence of Batrachochytrium dendrobatidis and ranavirus infection in imported North American bullfrogs *(Rana catesbeiana)*. *Biol Conserv.* (2009) 142:1420–6. doi: 10.1016/j.biocon.2009.02.007
71. Ribeiro LP, Carvalho T, Becker GC, JenkinsonTS, Leite DdA, James TY, et al. Bullfrog farms release virulent zoospores of the frog-killing fungus into the natural environment. *Sci Rep.* (2019) 9:13422. doi: 10.1038/s41598-019-49674-0
72. Wells DK. *The Ecology and Behavior of Amphibians*. Chicago, IL: University of Chicago Press (2007). doi: 10.7208/chicago/9780226893334.001.0001
73. Ohmer MEB, Cramp RL, White CR, Franklin CE. Skin sloughing rate increases with chytrid fungus infection load in a susceptible amphibian. *Funct Ecol.* (2015) 29:674–82. doi: 10.1111/1365-2435.12370
74. Ezenwa VO, Archie EA, Craft ME, Hawley DM, Martin LB, Moore J, et al. Host behavior – parasite feedback: an essential link between animal behavior and disease ecology. *Proc Roy Soc B.* (2015) 283:20153078. doi: 10.1098/rspb.2015.3078
75. White LA, Forester JD, Craft ME. Using contact networks to explore mechanisms of parasite transmission in *wildlife*. *Biol Rev*. (2017) 92:389–409. doi: 10.1111/brv.12236
76. Kermack WO, McKendrick AG. A contribution to the mathematical theory of epidemics. *Proc R Soc A Math Phys Eng Sci.* (1927) 115:700–21. doi: 10.1098/rspa.1927.0118
77. Venesky MD, Kerby JL, Storfer A, Parris MJ. Can differences in host behavior drive patterns of disease prevalence in tadpoles? *PLoS ONE.* (2011) 6:e24991. doi: 10.1371/journal.pone.0024991
78. Lombardo MP. Access to mutualistic endosymbiotic microbes: an underappreciated benefit of group living. *Behav Ecol Sociobiol.* (2008) 62:479–97. doi: 10.1007/s00265-007-0428-9
79. Banning JL, Weddle AL, Wahl GW, Simon MA, Lauer A, Walters RL, et al. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. *Oecologia.* (2008) 156:423–9 doi: 10.1007/s00442-008-1002-5
80. Vredenburg VT, Briggs CJ, Harris RN. Host pathogen dynamics of amphibian chytridiomycosis: the role of the skin microbiome in health and disease. In: editors L Olson, E Choffnes, D Relman, L Pray Fungal, Diseases: *An Emerging Threat to Human, Animal, and Plant Health*. Washington, DC: National Academy Press. (2011). p. 342–55.
81. Gahl MK, Longcore JE, Houlahan JE. Varying responses of northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. *Conserv Biol.* (2012) 26:135–41. doi: 10.1111/j.1523-1739.2011.01801.x
82. Berger L, Marantelli G, Skerratt LF, Speare R. Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Dis Aquat Organ.* (2005) 68:47–50. doi: 10.3354/dao068047
83. Byrne AQ, Poorten TJ, Voyles J, Willis CKR, Rosenblum EB. Opening the file drawer: unexpected insights from a chytrid infection experiment. *PLoS ONE.* (2018) 13:e0196851. doi: 10.1371/journal.pone.0196851
84. Refsnider JM, Poorten TJ, Langhammer PF, Burrowes PA, Rosenblum BE. Genomic correlates of virulence attenuation in the deadly amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. *G3 Genes Genom Genet.* (2015) 5:2291–8. doi: 10.1534/g3.115.021808
85. Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, et al. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Natl Acad Sci USA.* (2006) 103:3165–70. doi: 10.1073/pnas.0506889103
86. Lam BA, Walke JB, Vredenburg VT, Harris RN. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog Rana muscosa. *Biol Conserv.* (2010) 143:529–31. doi: 10.1016/j.biocon.2009.11.015

87. Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, Flaherty DC, et al. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* (2009) 3:818–24 doi: 10.1038/ismej.2009.27
88. Walke JB, Becker MH, Loftus SC, House LL, Cormier G, Jensen RV, et al. Amphibian skin may select for rare environmental microbes. *ISME J.* (2014) 8:2207–17. doi: 10.1038/ismej.2014.77
89. Shaw, SD, Berger L, Bell S, Dodd S, James TY, et al. Baseline cutaneous bacteria of free-living New Zealand native frogs (Leiopelma archeyi and *Leiopelma hochstetteri*) and implications for their role in defense against the amphibian chytrid (*Batrachochytrium dendrobatidis*). *J Wildlife Dis.* (2014) 50:723–32. doi: 10.7589/2013-07-186
90. Prado-Irwin SR, Bird AK, Zink AG, Vredenburg VT. Intraspecific variation in the skin-associated microbiome of Ensatina, the terrestrial ring-species salamander. *Microb Ecol.* (2017) 74:745–56. doi: 10.1007/s00248-017-0986-y
91. Bird AK, Prado-Irwin SR, Vredenburg AG, Zink VT. Skin microbiomes of California terrestrial salamanders are influenced by habitat more than host phylogeny. *Front Microbiol.* (2018) 9:442. doi: 10.3389/fmicb.2018.00442
92. Flechas SV, Sarmiento C, Cárdenas ME, Medina EM, Restrepo S, Amézquita A. Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of Atelopus. *PLoS ONE.* (2012) 7:e44832. doi: 10.1371/journal.pone.0044832
93. Park ST, Collingwood AM, St-Hilaire S, Sheridan PP. Inhibition of *Batrachochytrium dendrobatidis* caused by bacteria isolated from the skin of boreal toads, anaxyrus (Bufo) boreas boreas, from Grand Teton National Park, Wyoming, USA. *Microbiol Insights.* (2014) 7:1–8. doi: 10.4137/M.B.I.S13639
94. Woodhams DC, Ardipradja K, Alford RA, Marantelli G, Reinert LK, Rollins-Smith LA. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Anim Conserv.* (2007) 10:409–17. doi: 10.1111/j.1469-1795.2007.00130.x
95. Becker MH, Walke JB, Cikanek S, Savage AE, Mattheus N, Santiago CN, et al. Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc R Soc B Biol Sci.* (2015) 282:20142881. doi: 10.1098/rspb.2014.2881
96. Rodriguez D, Becker CG, Pupin NC, Haddad CFB, Zamudio KR. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Mol Ecol.* (2014) 23:774–87. doi: 10.1111/mec.12615
97. DiRenzo GV, Langhammer PF, Zamudio KR, Lips KR. Fungal infection intensity and zoospore output of *Atelopus zeteki*, a potential acute chytrid supershedder. *PLoS ONE.* (2014) 9:e93356. doi: 10.1371/journal.pone.0093356
98. 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.* (2014) 346:630–1. doi: 10.1126/science.1258268
99. Cunningham AA, Beckmann K, Perkins M, Fitzpatrick L, Cromie R, Redbond J, et al. Emerging disease in UK amphibians. *Vet Rec.* (2015) 176:468. doi: 10.1136/vr.h2264
100. Spitzen-van der Sluijs A, Martel A, Asselberghs J, Bales EK, Beukema W, Bletz MC, et al. Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. *Emerg Infect Dis.* (2016) 22:1286. doi: 10.3201/eid2207.160109
101. 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. doi: 10.1038/nature22059
102. Waddle JH, Grear DA, Mosher BA, Grant E. H. C., Adams MJ, Backlin AR, et al. *Batrachochytrium salamandrivorans* (Bsal) not detected in an intensive survey of wild North American amphibians. *Sci Rep.* (2020) 10:13012. doi: 10.1038/s41598-020-69486-x
103. Laking A.E., Ngo HN, Pasmans F, Martel A, Nguyen TT. *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Sci Rep.* (2017) 7:44443. doi: 10.1038/srep44443
104. 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:e13845. doi: 10.1038/s41598-018-31800-z
105. Richgels KL, 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) 3:150616. doi: 10.1098/rsos.150616
106. Yap TA, Nguyen NT, Serr M, Shepack A, Vredenburg VT. *Batrachochytrium salamandrivorans* and the risk of a second amphibian pandemic. *EcoHealth.* (2017) 14:851–64. doi: 10.1007/s10393-017-1278-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 09 February 2022
doi: 10.3389/fvets.2022.755426

Ranavirus Amplification in Low-Diversity Amphibian Communities

Joe-Felix Bienentreu[1†], Danna M. Schock[2†], Amy L. Greer[3] and David Lesbarrères[1]*

[1] Department of Biology, Laurentian University, Sudbury, ON, Canada, [2] Sciences and Environmental Technology, Keyano College, Fort McMurray, AB, Canada, [3] Department of Population Medicine, University of Guelph, Guelph, ON, Canada

In an era where emerging infectious diseases are a serious threat to biodiversity, epidemiological patterns need to be identified, particularly the complex mechanisms driving the dynamics of multi-host pathogens in natural communities. Many amphibian species have faced unprecedented population declines associated with diseases. Yet, specific processes shaping host-pathogen relationships within and among communities for amphibian pathogens such as ranaviruses (RV) remain poorly understood. To address this gap, we conducted a comprehensive study of RV in low-diversity amphibian communities in north-western Canada to assess the effects of biotic factors (species identity, species richness, abundance) and abiotic factors (conductivity, pH) on the pathogen prevalence and viral loads. Across 2 years and 18 sites, with communities of up to three hosts (wood frog, *Rana sylvatica*; boreal chorus frog, *Pseudacris maculata*; Canadian toad, *Anaxyrus hemiophrys*), we observed that RV prevalence nearly doubled with each additional species in a community, suggesting an amplification effect in aquatic, as well as terrestrial life-history stages. Infection intensity among infected wood frogs and boreal chorus frogs also significantly increased with an increase in species richness. Interestingly, we did not observe any effects of host abundance or abiotic factors, highlighting the importance of including host identity and species richness when investigating multi-host pathogens. Ultimately, only such a comprehensive approach can improve our understanding of complex and often highly context-dependent host-pathogen interactions.

Keywords: amphibian decline, host richness, host identity, boreal forest, Canada, ranavirus, amplification

OPEN ACCESS

Edited by:
Rachel E. Marschang, Laboklin GmbH & Co. KG, Germany

Reviewed by:
Matthew J. Gray, The University of Tennessee, Knoxville, United States
Roberto Brenes, Carroll University, United States

****Correspondence:***
Joe-Felix Bienentreu
joe.bienentreu@gmail.com

†Present addresses:
Joe-Felix Bienentreu, Orchis Environmental, Sudbury, ON, Canada
Danna M. Schock, Palustris Environmental, Athabasca, AB, Canada

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *08 August 2021*
Accepted: *03 January 2022*
Published: *09 February 2022*

Citation:
Bienentreu JF, Schock DM, Greer AL and Lesbarrères D (2022) Ranavirus Amplification in Low-Diversity Amphibian Communities. Front. Vet. Sci. 9:755426. doi: 10.3389/fvets.2022.755426

INTRODUCTION

Host-pathogen interactions in natural communities are complex and context-dependent (1, 2). Pathogens can influence community processes at different trophic levels, including predator-prey interactions (3), inter-species competition (4), and social behavior. This includes, but is not limited to mate choice (5), avoidance of infected conspecifics (6), and higher activity rates in infected individuals (7). The identity and richness of suitable host species in a community can also influence infection prevalence and transmission of a pathogen, but effects are rarely straightforward (2, 8). The mode of transmission (e.g., frequency- vs. density-dependent), and individual traits such as life history and health of the host can also influence host-pathogen dynamics (2, 9–12). Overall, much remains to be explained regarding the transmission dynamics, and specific host-pathogen

interactions of infectious diseases in natural systems, and there is still limited understanding of how pathogens shape host communities, and vice-versa.

In general, pathogens can be classified as either generalist or specialist with respect to the number of host species they infect (13, 14). While specialist pathogens are often highly adapted to evade the immune system of a particular host, such specific adaptations can be detrimental for generalist multi-host pathogens due to functional trade-offs (15, 16). Yet, the majority of known pathogens infect more than one host species because of the advantages of facilitated invasion and transmission when infecting multiple host species in a community, rather than relying on a single route of transmission (15, 17). Pathogen transmission can be characterized as either density-dependent, where the relative contact rate between infected and susceptible individuals is a function of the population density, or frequency dependent, where the transmission rates are independent from the population density (18). If and how pathogen transmission is dependent on host density is fundamental to pathogen dynamics in natural systems (19) and determines whether a pathogen is capable of causing the decline of a host species (20–22). Additionally, the richness, diversity, and population dynamics of host species in an ecosystem can have a significant influence on pathogen presence, prevalence and transmission (2, 8, 23–25). When multi-host pathogens are involved, disease risk may escalate in low diversity ecosystems, but decrease in diverse ecosystems. This so called dilution effect was first investigated in vector-borne pathogen systems [e.g., *Borrelia burgdorferi*, (26), *West Nile virus,* (27)], but in recent years there has been an increasing number of studies in directly transmitted pathogen systems, in particular hantaviruses (8, 28–30). The opposite mechanism, called amplification effect, has also been observed whereby higher diversity amplifies the disease risk [e.g., *Laguna Negra virus*: (31); *Louping ill virus*: (32)]. Interestingly, it has been shown that dilution and amplification effects can actually occur simultaneously in the same system, where pathogen prevalence depends on the respective strength and interactions of the two effects (8). Thus, pathogen prevalence appears to be variously amplified or diminished by host richness, abundance, and the sequence in which host communities are assembled and disassembled (2, 25, 33, 34).

Pathogens are a natural part of ecosystems, but in the last few decades an increasing number of pathogen-induced mass die-offs and associated declines have been observed in vertebrate populations. On a global scale, amphibians have experienced the most severe disease-associated declines (35–37), most often linked to the chytrid fungi *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (38, 39), and viruses in the genus *Ranavirus* (40, 41). Infections with these pathogens can cause potentially lethal systemic diseases, including but not limited to metabolic dysfunctions and organ failure (42–45). Chytrid fungi and ranaviruses (RVs) are both capable of infecting multiple host species within a community (43, 46, 47), and their broad host ranges likely have significant influences on pathogen dynamics. Unfortunately, the effects of species identity and species richness are not commonly studied in wild amphibian host-pathogen systems [see (34, 48)]. For instance, RVs are globally distributed multi-host pathogens, infecting a variety of different host species across three classes of ectothermic vertebrates [amphibians, reptiles, and fish; (43, 49)] but studies investigating RV dynamics tend to focus on selected subsets or single amphibian hosts (and life-history stage), often within a community of several host species [reviewed in (50)]. Susceptibility and infection outcome vary greatly and typically depend on host identity as well as the life-history stage of the infected individual (11, 12). In amphibians, Ranids (true frogs) and Hylids (tree frogs) often show higher susceptibility, than other families, and aquatic life-history stages in particular (e.g., larvae, tadpoles, and metamorphs) often exhibit greater susceptibility to lethal infections when compared to post-metamorphic individuals (12, 51). To our knowledge, only two previous studies investigated community-level effects in an amphibian-RV system. First, a study on wild Californian amphibian communities showed an increase in host richness amplified RV prevalence (52). Second, Rosa et al. (53) reported spatially and temporally confined amplification effects of host community diversity on RV prevalence in Spain, but no general or repeated patterns over several years could be identified.

Here, we combined extensive sampling with habitat and community assessments to test whether host species identity or richness affect pathogen prevalence and viral loads. We focused on the multi-host pathogen RV in low-diversity amphibian communities in north-western Canada. The presence of only three amphibian species in the research area allowed us to conduct an assessment of a whole host-pathogen system under natural conditions. We also examined the influence of relative host abundance and wetland-specific pH and conductivity, which are factors that have been found to influence the dynamics of other amphibian host-pathogen systems (1, 54–56). Following previous studies (34, 48, 52, 57, 58), we expected to observe either a dilution or an amplification effect related to species identity or richness, with less influence from host abundance or environmental variables on epidemiological patterns in this system.

MATERIALS AND METHODS

Field Surveys and Sample Collection

Field-work was conducted in north-eastern Alberta and the southern Northwest Territories, Canada, predominately within the boundaries of Wood Buffalo National Park (**Figure 1**). The area is part of the Taiga Plains and Boreal Plains Ecoregions, featuring a distinctive landscape consisting of extensive areas of interconnected river systems, wetlands and lakes, surrounded by boreal forest (59–61); (see **Figures 2A–D**). The area is characterized by a sub-humid climate with low mean annual air temperatures (62), a high rate of environmental disturbances (e.g., wildfires and floods), and ecoregion specific characteristics such as (semi-) permafrost (63, 64). Field reconnaissance surveys began the first week of May in 2016 and 2017 when spring melt occurs in the area, and continued until the end of June. This timing maximized our ability to acoustically detect all amphibian species expected in the area as well as physically encounter multiple life stages of each species. To determine the

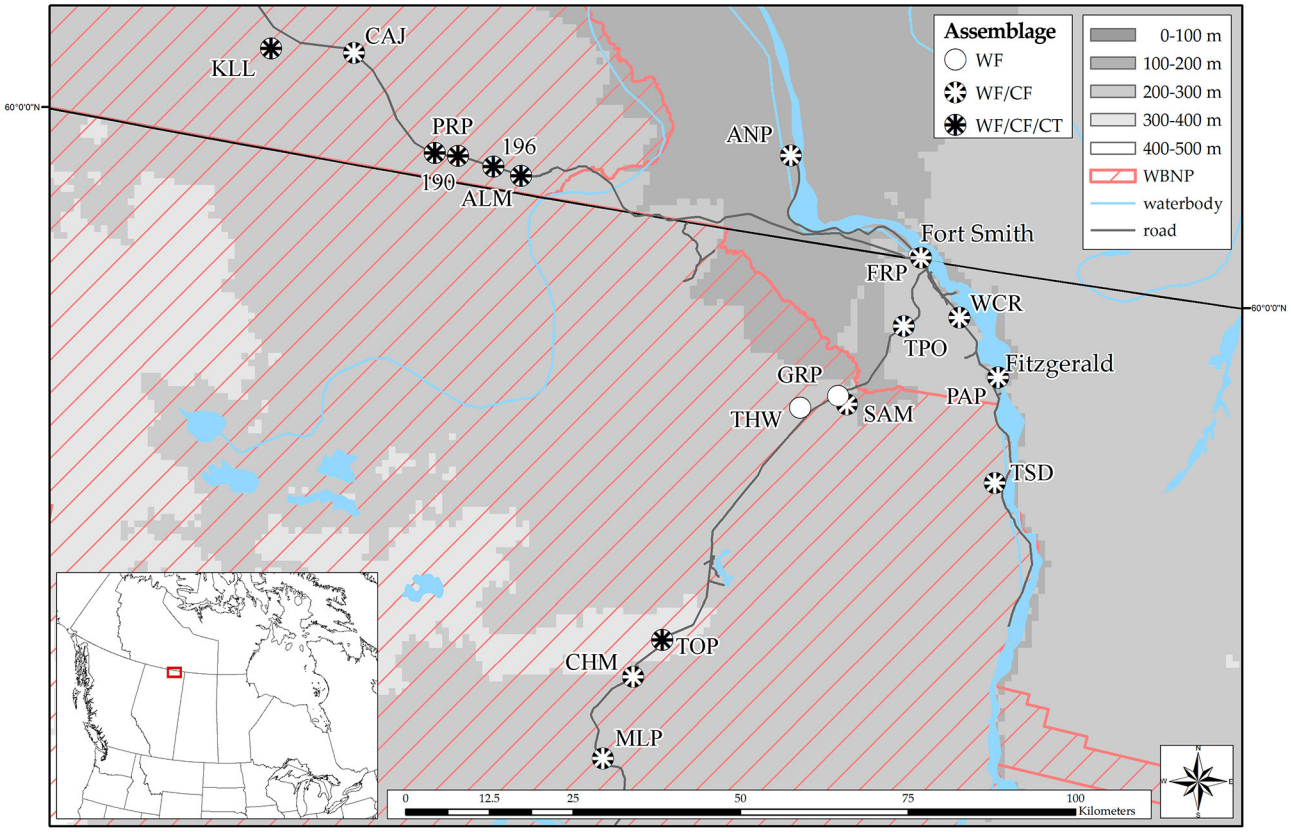


FIGURE 1 | Amphibian communities sampled in Alberta and the Northwest Territories in 2016 and 2017 (WF, wood frog; CF, boreal chorus frog; CT, Canadian toad; WBNP, Wood Buffalo National Park). Map was created using ArcMap10.5 (Esri, Redlands, CA, USA).

species present at each site and to estimate their abundance, we conducted a total of 221 visual, auditory, and dip netting surveys (75 in 2016; 146 in 2017) of suitable amphibian habitats such as floodplains, wetlands and meadows. Opportunistic surveys were conducted along the margins of the respective wetland, and down to an approximate water depth of 1 m. Three species of amphibians were encountered: wood frogs (*Rana sylvatica*, WF), boreal chorus frogs (*Pseudacris maculata*, CF), and Canadian toads (*Anaxyrus hemiophrys*, CT). A species was determined to be present at a site if the species was caught, or visually/auditorily identified even if we were unable to ultimately capture and collect tissue samples from the species at a site. Tadpoles of a given species were considered to be present when either visually confirmed, or when breeding behavior in adults was observed, and eggs were found. Throughout our extensive surveys we encountered three compositions: WF only, WF/CF, or all three species. Sites with CF or CT only, WF/CT, or CF/CT were never encountered.

A total of 18 unique sites were chosen for sampling (**Figure 1**; see **Appendix I** for full names and coordinates). Tissue sampling of terrestrial individuals (fully metamorphosed young of the year, juveniles, and adults) was conducted at 13 sites between May 15 and June 23, 2016. Tissue sampling of aquatic stages [tadpoles and metamorphs; Gosner stages 26 – 42 (65)] was conducted at 16 sites (11 identified in 2016 + 5 new in 2017) between June 14 and June 29, 2017. Due to logistical constraints, two of the sites sampled in 2016 were not sampled in 2017. To allow for comparisons of amphibian abundance among sites, we sampled each wetland using the same methodology, conducting opportunistic sampling for all species potentially present in the area. We quantified relative abundance as Catch Per Unit Effort (CPUE): the number of individuals encountered per unit of search time (here Ind./hour) according to (66). To allow for sufficient sample sizes for pathogen detection and statistical analysis, we selected sites with a minimum abundance of 10 terrestrials or 30 aquatic individuals per 1 h search time. If the required sample size could not be achieved at the first sampling, sites were sampled a second time within a 2 week time-frame of the initial sampling. To characterize abiotic characteristics of our study sites, temperature, water pH, and conductivity were recorded during each sampling event using an YSI 556 MPS (YSI Inc., Yellow Springs, United States), and were averaged if a site was sampled more than once. To more completely assess temperature profiles in our study wetlands, we deployed temperature data-loggers (Onset HOBO Fresh Water Data Logger U24-001) at three wetlands that differed in terms of size and other abiotic characteristics. The loggers were deployed 10 May−1 July, 2017, and were attached to a stationary

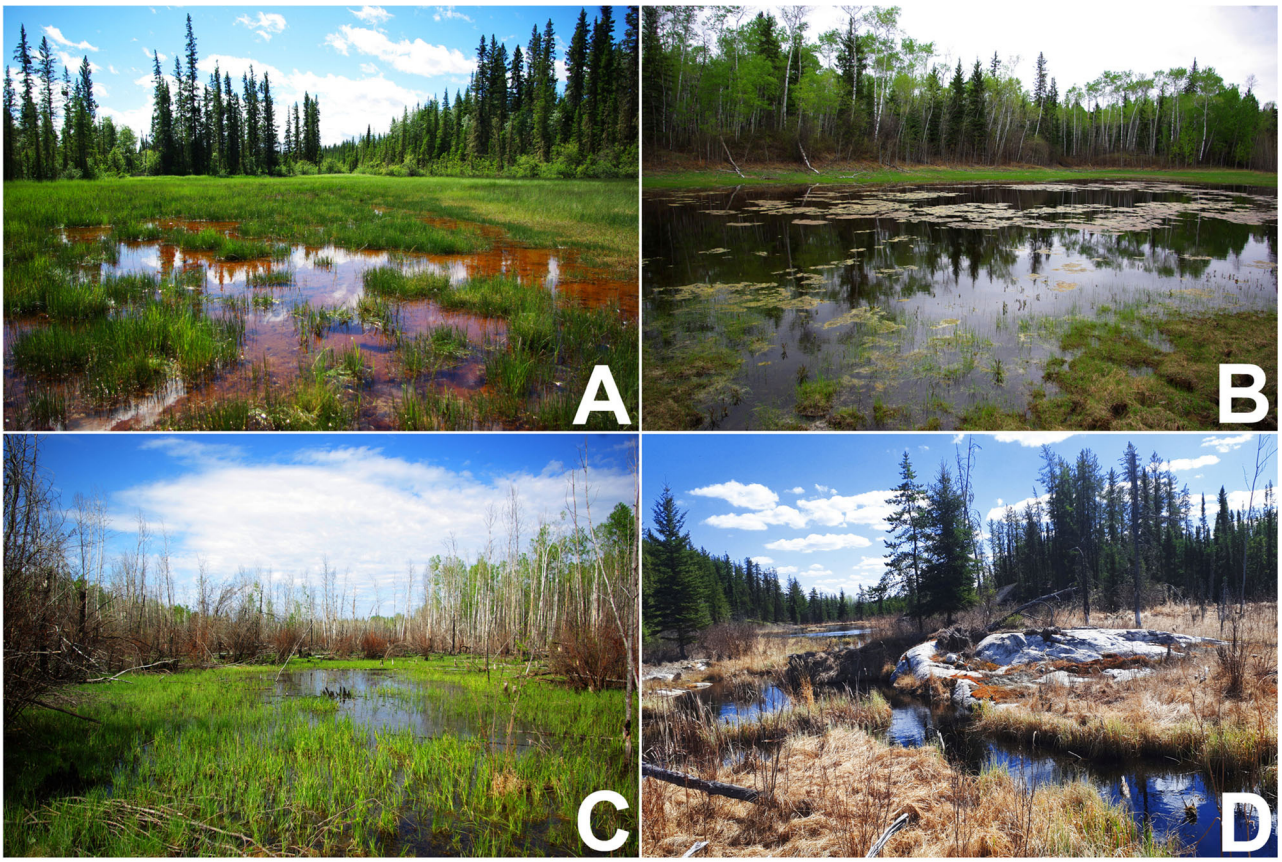


FIGURE 2 | Representative amphibian habitats sampled in Alberta and the Northwest Territories in 2016 and 2017: Shallow marsh **(A)**, water-filled sinkhole **(B)**, floodplain **(C)**, and interconnected ponds and streams **(D)**. All photos by JF Bienentreu.

pole approximately 50 cm below the water surface. Due to the landscape in the area, with vast areas of karst topography (i.e., sinkholes), it was not feasible to determine wetland depth.

Individual amphibians were caught by hand or by dip-netting and then held individually in new, single use plastic bags containing air and pond water (for aquatic stages), or wet vegetation (for terrestrial stages), until processed (<30 min). Care was taken to replenish air in the bags frequently and to protect the animals from direct sun and overheating. Aquatic individuals were measured (snout-vent-length; SVL) and staged (65) in their respective bag. Terrestrial individuals were weighed (g) and their SVL was measured. To test for RV infections, we non-lethally collected a ∼1 mm toe-clip (terrestrials) or tail-clip (aquatic stages) from each individual. To prevent cross-contamination and spread of pathogens among individuals during handling, a new pair of nitrile gloves and a new scalpel blade was used for processing each animal, and all surfaces and tools were regularly disinfected with 99% isopropyl. Tissue samples were stored individually in 1.5 ml microcentrifuge tubes (filled with 95% ethanol, Eppendorf AG, Hamburg, Germany). To prevent accidental transfer of pathogens among sites, all gear and equipment that came into physical contact with the processed individuals, including nets, boots and waders were disinfected for 5 min with a 10% bleach solution, rinsed with clean tap water, and allowed to dry before being used at the next site [see (67)]. We solely used non-lethal tissue samples for ethical reasons and because they are easy to collect and store under remote field conditions. Tail and toe clips have been shown to be a viable alternative to lethal sampling and are in a generally high agreement with results from corresponding organ samples, but often slightly underestimate infection prevalence (44, 68–71). There are no known long-lasting negative effects on the amphibians when a single phalanx or tail clip is collected aseptically (68–70), and non-lethal sampling significantly decreases the impact on biodiversity, particularly when rare or threatened species are studied (44, 69, 72).

PCR-Based Ranavirus Detection and Quantification

Ranavirus detection and quantification was performed using quantitative PCR (qPCR) following the cycling conditions described by Leung et al. (73), and quantification described by Hoverman et al. (11). DNA was extracted from toe and tail clips using Qiagen DNEasy® Blood and Tissue kits according

to manufacturer specifications (QIAGEN Inc., Valencia, CA, USA). A Synergy H1 Hybrid Multi-Mode Reader was used to quantify concentration of genomic DNA in each sample (BioTek, Winooski, VT, USA). Briefly, the qPCR mixture contained 10 μl TaqMan Universal PCR Master Mix 2X (Thermofisher Scientific, Waltham, MA, USA), 1 μl forward primer MCPRV_F-5GTCCTTTAACACGGCATACCT3 (10 μM), 1 μl reverse primer MCPRV_R-5ATCGCTGGTGTTGCCTATC3 (10 μM), and 0.05 μl TaqMan probe MCP_NFQ-5TTAT AGTAGCCTRTGCGCTTGGCC3 (100 μM). Subsequently, we added 250 ng of template DNA and DNA grade water to a final reaction volume of 20 μl. The qPCR was performed using an Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s, and 60 C for 30 s. Samples were run in duplicate in 96-well-plates. Each 96-well-plate included a no-template control (molecular grade water), and a serial dilution of a known quantity of cultured FV3 (1×10^6-10^1 copies/μl) to create a standard curve with precise fit ($R^2 > 0.95$). Individuals were considered positive if the target DNA was clearly amplified in both of the duplicates (i.e., surpassed the respective cycle threshold). If only one of the two wells amplified, a third reaction was conducted to either confirm or dismiss the previous positive result. Standard curves from each plate were used to calculate viral load following Yuan et al. (74) and reported as viral copies/250 ng of gDNA, as recommended by Gray et al. (69). Due to large standard deviations, viral loads are reported as log10 copies/250 ng gDNA (hereafter "logVL"). Primers used in our study target a consensus Major Capsid Protein (MCP) sequence of the major amphibian associated RV isolates: FV3: GenBank No AY548484, (75); TFV: AF389451, (76); CMTV: JQ231222, (77); EHNV, FJ433873, (78), and allow for a high analytical sensitivity when used in combination with a TaqMan probe (73). Pathogen identification and infection was confirmed through immunohistochemistry and *in-situ* hybridization on a subset of collected tissues (79).

Statistical Analyses

Statistical analyses were conducted using R 3.3.3 (80) in RStudio Version 1.1.383 (81). Sample sizes allowed for reasonable prevalence estimates of RV at each site [95% confident that prevalence was $< \sim 5\%$ if no animals tested positive at a given site; (82, 83)]. We conducted analyses on the community level including all three species. Analyses at the individual and population level were conducted for WF only because single species sites were found only for this species. Aquatic and terrestrial life stages were analyzed separately. We used Pearson's Chi-squared tests to compare prevalence (percentage of positive individuals in a community) among the three different community compositions and used *post-hoc* pairwise comparisons to determine significance. We conducted one-way analyses-of-variance (ANOVAs) to test for differences in mean viral load among communities. We used Tukey's HSD test to detect differences among means for ANOVAs where the overall null hypothesis was rejected. To analyze RV prevalence among populations and communities, we performed beta regressions using the package "betareg" (84) for beta-distributed, dependent percentage data. To assess the influence of predictor variables on viral loads at the individual, population and community level, we performed generalized linear mixed model regressions fitted with a Poisson family and log link, using the package "lme4" (85). Each level of analysis included site-specific conductivity, pH, and species richness (WF, WF/CF, WF/CF/CT), as well as total relative community abundance (number of individuals caught per person-hour) as fixed effects. Due to the wide range of measurements for conductivity, it was scaled into seven categories (low to high conductivity). Site was treated as a random effect in all mixed model analyses. We used the dredge function in the R package "MuMIn" to create a sub-set of best-supported models of all possible sub-models from ranavirus presence and infection global models (86). The best supported models were compared separately using Akaike's Information Criterion [AIC; (87, 88)]. Due to the overall small sample sizes, we used AIC corrected for small sample sizes (AICC) in all our analyses (87, 89). Model comparison and averaging were performed using R packages 'MuMIn' (86) and 'AICcmodavg' (88), and can be found in the **Supplementary Material** (see **Appendices II, III**).

RESULTS

Amphibian Community and Environmental Assessments

Three amphibian species were encountered in three types of community composition (**Figure 1**, **Tables 1**, **2**), and we sampled a total of 1,244 individuals across all species and sites (**Table 3**). Wood frogs occurred at all sites where amphibians were found and were the only species we observed by itself ($n = 2$ sites; **Table 4**). The most commonly encountered community consisted of WF and CF ($n = 10$ sites; **Table 4**). If the sites featured large, shallow areas and pools, and provided nearby suitable hibernacula sites (e.g., sand dunes and pits), we often found CT as well ($n = 6$ sites). Abundance varied greatly among the three amphibian species: in terrestrial phases (2016), an average of 12 WF were caught per hour (range 3–37 individuals/hour) across 13 sites, whereas an average of 4 CF were caught per hour (range 1–9 individuals/hour) across nine sites (**Table 1**). For terrestrial stages, CT were caught at three sites at an extremely low average rate (1 individual/hour) and were observed at only four sites overall (**Tables 1**, **4**). For aquatic stages (2017), an average of 40 WF were caught per hour across 16 sites (range 4–60 individuals/hour) whereas an average of 14 CF were caught per hour across 11 sites (range 2–41 individuals/hour; **Tables 2**, **4**). Aquatic stages of Canadian toads were caught at two sites at an average rate of 9 individuals per hour (range 8–10 individuals/hour). We observed CT breeding activities at four additional sites, but could not find any aquatic stages during sampling. Although abundance varied among species, CF abundance did not vary significantly across the communities in which they were found (WF/CF and WF/CF/CT) in either terrestrial or aquatic life stages. Wood frog abundance however was lower in WF/CF/CT communities as compared to the two less complex communities in both terrestrial and aquatic stages.

TABLE 1 | Field sites in Alberta and the Northwest Territories where terrestrial amphibian life stages were sampled for ranavirus in 2016.

Site	μS/cm	pH	sr	Species	Prevalence	log10 VL	hem	ind./hr
GRP	1,256 ± 250	7.41 ± 0.8	1	WF	2/30 (7%)	2.58 ± 0.11	0/30 (0%)	14
THW	215 ± 0	6.20 ± 0	1	WF	3/30 (10%)	2.50 ± 0.23	0/30 (0%)	17
ANP	1,168 ± 0	6.55 ± 0	2	WF	5/30 (17%)	2.57 ± 0.07	2/30 (6.7%)	23
				CF*	n/a	n/a	n/a	0
CAJ	960 ± 315	7.18 ± 0.03	2	WF	4/30 (13%)	2.90 ± 0.32	0/30 (0%)	12
				CF	1/30 (3%)	2.57 ± 0	0/30 (0%)	5
FRP	332 ± 0	7.34 ± 0	2	WF	0/30 (0%)	-	0/30 (0%)	37
				CF	0/30 (0%)	-	0/30 (0%)	6
MLP	290 ± 54	6.68 ± 0.75	2	WF	0/17 (0%)	-	0/17 (0%)	5
				CF	1/4 (25%)	2.49 ± 0	0/4 (0%)	1
PAP	493 ± 11	7.10 ± 0.08	2	WF	5/30 (17%)	2.82 ± 0.33	0/30 (0%)	11
				CF	2/20 (10%)	2.57 ± 0.04	0/20 (0%)	4
SAM	8,290 ± 0	6.87 ± 0	2	WF	0/3 (0%)	-	0/3 (0%)	5
				CF*	n/a	n/a	n/a	0
WCR	381 ± 45	7.46 ± 0.12	2	WF	5/30 (17%)	2.68 ± 0.34	0/30 (0%)	8
				CF	1/4 (25%)	2.65 ± 0	0/4 (0%)	1
ALM	822 ± 7	6.79 ± 0.08	3	WF	5/30 (17%)	3.03 ± 0.24	0/30 (0%)	8
				CF	0/8 (0%)	-	0/8 (0%)	1
				CT	0/14 (0%)	-	0/14 (0%)	1
PRP	724 ± 88	7.98 ± 0.06	3	WF	13/30 (43%)	2.99 ± 0.33	0/30 (0%)	3
				CF	1/20 (5%)	2.79 ± 0	1/20 (5%)	4
				CT	0/7 (0%)	-	0/7 (0%)	1
TOP	73 ± 10	6.4 ± 0.1	3	WF	3/30 (10%)	3.37 ± 0.04	3/30 (10%)	5
				CF	2/30 (7%)	3.02 ± 0.17	0/30 (0%)	9
				CT*	n/a	n/a	n/a	0
190	1,270 ± 108	7.58 ± 0.34	3	WF	3/19 (16%)	3.08 ± 0.20	2/19 (10.5%)	3
				CF	0/16 (0%)	-	0/16 (0%)	3
				CT	0/1 (0%)	-	0/1 (0%)	1

The data includes site-specific mean (+/− SD) conductivity (μS/cm), temperature (°C), and pH, species richness (sr), species-specific sample sizes, abundance, ranavirus prevalence (%), viral load (VL), and percentage of individuals with hemorrhages (hem) in wood frogs (WF), boreal chorus frogs (CF) and Canadian toads (CT). Abundance is presented as individuals caught per person-hour. Viral loads are presented as mean log10 viral copies/250 ng of gDNA ± SD. If a species was determined to be present at a site, but ultimately not captured and sampled, values are stated as n/a and species is marked with an asterisk.

Across all our study sites, the pH ranged from 6.18 to 7.98 in 2016 and 6.90 to 8.71 in 2017 (**Tables 1**, **2**). Conductivity ranged from 73 μS/cm to 8,290 μS/cm in 2016 and 70 μS/cm to 7,132 μS/cm across all sites (**Tables 1**, **2**). Although we collected water temperature at the time of tissue sampling, it was not included in our analyses. Single-point measurements of temperature under field conditions are highly biased by sampling time and environmental conditions. This observation was further supported by the marked daily variability in our temperature data collected at three selected wetlands, with the means not differing from one another (see **Appendix IV** for data-logger recordings). Individual measures such as snout-vent length, weight, and Gosner stage for aquatic individuals, did not have any statistical significance in preliminary model comparisons and were excluded from the final analyses.

Ranavirus Distribution and Prevalence

Ranavirus was detected in individuals at all sites sampled (2016, $n = 13$ and 2017, $n = 16$; **Tables 1**, **2**) and in all species (**Table 3**). The overall prevalence of RV across all species and sites was 16% in terrestrial individuals (2016) and 32% in aquatic stages (2017; **Table 4**). The mean infection prevalence in WF was 19% in terrestrial stages, and 37% in aquatic stages (**Table 3**). In CF, the mean infection prevalence was 9% in terrestrial stages, and 49% in aquatic stages (**Table 3**). We did not detect any infected terrestrial CT and the mean infection prevalence in CT aquatic stages was 16% (**Table 3**). It is notable that all RV-positive CT aquatic stages were detected at a single site during a RV die-off involving WF and CF [**Tables 2–4**; see Forzán et al. (79) for histological data on a subset of collected individuals]. Among all terrestrial individuals sampled, only 1% exhibited typical signs of ranavirosis such as hemorrhages at the toe-tips and ventral surfaces of the abdomen (44); see **Table 1** for population level data). By contrast, 29% of all aquatic individuals sampled exhibited typical signs of RV infection such as hemorrhages in developing hind legs, at the base of the tail, and on the abdomen (44); (98 WF, 63 CF, 1 CT; see **Table 2** for population level data), In addition to the aforementioned signs, aquatic individuals (*n*

TABLE 2 | Field sites in Alberta and the Northwest Territories where aquatic amphibian life stages were sampled for ranavirus in 2017.

Site	μS/cm	pH	sr	Species	Prevalence	log10 VL	hem	ind./hr
GRP	1382	8.71	1	WF	4/30 (13%)	2.67 ± 0.21	0/30 (0%)	60
ANP	822	7.35	2	WF	7/30 (23%)	3.44 ± 0.32	0/30 (0%)	90
				CF*	n/a	n/a	n/a	0
CAJ	819	7.36	2	WF	13/30 (43%)	2.57 ± 0.26	13/30 (43.3%)	30
				CF	9/30 (30%)	2.55 ± 0.15	8/30 (26.7%)	30
CHM	525	7.00	2	WF	7/30 (23%)	2.50 ± 0.19	5/30 (16.7%)	15
				CF*	n/a	n/a	n/a	0
FRP	447	6.90	2	WF	4/30 (13%)	2.45 ± 0.31	1/30 (3.3%)	60
				CF	2/8 (25%)	2.40 ± 0.08	0/8 (0%)	5
PAP	443	6.99	2	WF	2/30 (7%)	2.43 ± 0.22	9/30 (30%)	53e
				CF	3/30 (10%)	2.53 ± 0.21	9/30 (30%)	15
SAM	2,147	7.53	2	WF	7/30 (23%)	2.37 ± 0.10	4/30 (13.3%)	60
				CF*	n/a	n/a	n/a	0
TPO	429	7.89	2	WF	16/30 (53%)	2.55 ± 0.23	7/30 (23.3%)	60
				CF*	n/a	n/a	n/a	0
TSD	884	7.04	2	WF	7/30 (23%)	3.02 ± 0.51	9/30 (30%)	60
				CF	3/8 (38%)	2.95 ± 0.07	2/8 (25%)	8
WCR	327	7.53		WF	5/30 (17%)	3.34 ± 0.16	11/30 (36.7%)	20
				CF*	n/a	n/a	n/a	0
ALM	484	7.12	3	WF	5/30 (17%)	3.03 ± 0.24	0/30 (0%)	8
				CF	0/8 (0%)	-	0/8 (0%)	1
	e			CT*	0/14 (0%)	-	0/14 (0%)	0
KLL	7,132	7.70	3	WF	13/13 (100%)	4.69 ± 0.41	5/13 (38.5%)	4
				CF	30/30 (100%)	3.60 ± 0.48	10/30 (33.3%)	10
				CT	10/30 (33%)	3.32 ± 0.19	1/30 (3.3%)	10
PRP	1,072	8.46	3	WF	0/35 (0%)	-	2/35 (5.7%)	9
				CF	3/32 (9%)	2.56 ± 0.33	1/32 (3.1%)	8
				CT	0/31 (0%)	-	0/31 (0%)	8
TOP	70	7.36	3	WF	13/30 (43%)	3.94 ± 0.40	7/30 (23.3%)	15
				CF	4/5 (80%)	3.61 ± 0.63	2/5 (40%)	3
				CT*	n/a	n/a	n/a	0
190	1,212	7.90	3	WF	10/30 (33%)	3.93 ± 0.39	18/30 (60%)	60
				CF	2/3 (67%)	3.95 ± 0.37	3/3 (100%)	2
				CT*	n/a	n/a	n/a	0
196	244	7.97	3	WF	17/21 (81%)	4.92 ± 0.41	17/21 (81%)	21
				CF	39/41 (95%)	4.38 ± 0.62	22/41 (53.7%)	41
				CT*	n/a	n/a	n/a	0

The data includes site-specific conductivity, temperature, and pH, species richness (sr), species-specific sample sizes, abundance, ranavirus prevalence, viral load (VL), and percentage of individuals with hemorrhages (hem) in wood frog (WF), boreal chorus frog (CF) and Canadian toad (CT). Each site was sampled in a single day in 2017. Abundance is presented as individuals caught per person-hour. Viral loads are presented as mean log10 viral copies/250 ng of gDNA ± SD. If a species was determined to be present at a site, but ultimately not captured and sampled, values are stated as n/a and species is marked with an asterisk.

= 72) sampled during two die-off events in 2017 commonly exhibited edema. We found no effects of specific sampling dates on prevalence data for aquatic individuals ($Z = 1.61$, $p = 0.11$) or terrestrial individuals ($Z = 0.03$, $p = 0.98$), and we did not detect any temporal trends (**Appendices V–VII**).

Viral Loads

The mean viral loads among positive terrestrial WF and CF individuals were low (logVL < 2.83), and RV was not detected in terrestrial CT (**Tables 1**, **3**). Viral loads in aquatic stages varied greatly, with more than half of the individuals (61%) exhibiting higher mean viral loads in comparison to terrestrials (logVL < 3.65, **Tables 2**, **3**). Wood frog and CF aquatic stages sampled during mass die-off events ($n = 72$) exceeded the commonly observed viral loads (38 individuals with logVL > 3.65 and 34 individuals with logVL > 4.65).

Ecological Correlates of Ranavirus Prevalence and Viral Loads in Terrestrial Amphibian Life Stages (2016)

The percentage of RV positive individuals in a community increased with the number of species, but not significantly

TABLE 3 | Amphibian species sampled in 2016 and 2017, with respective life-stage, number of sampling sites, sampled individuals, ranavirus prevalence (% +/− SD), and viral loads.

Year	Species	Stage	Sites	*n*	Prevalence	log10 VL	hem
2016	WF	terrestrial	13	323	19 ± 11	2.83 ± 0.34	2
	CF	terrestrial	9	162	9 ± 6	2.71 ± 0.22	1
	CT	terrestrial	3	22	0	-	0
2017	WF	aquatic	16	459	37 ± 28	3.42 ± 1.00	31
	CF	aquatic	10	217	49 ± 32	3.73 ± 0.85	33
	CT	aquatic	2	61	16 ± 0	3.32 ± 0.19	2

Wood frog (WF), boreal chorus frog (CF), Canadian toad (CT), viral load (VL), percentage of individuals with hemorrhages (hem). Viral loads are presented as mean log10 viral copies/250 ng of gDNA ± SD.

TABLE 4 | Amphibian communities sampled in Alberta and the Northwest Territories in 2016 and 2017, with respective life-stage, number of sampling sites, sampled individuals (n), ranavirus prevalence (% +/− SD) and viral load.

Year	Composition	Stage	Sites	*n*	Prevalence	log10
2016	**WF**	terrestrial	2	60	9 ± 2	2.53 ± 0.18
	WF/CF	terrestrial	7	258	15 ± 4	2.70 ± 0.27
	WF/CF	terrestrial	7	170	16 ± 2	2.73 ± 0.29
	WF/**CF**	terrestrial	5	88	10 ± 7	2.57 ± 0.06
	WF/CF/CT	terrestrial	4	189	23 ± 16	3.07 ± 0.27
	WF/CF/CT	terrestrial	4	93	28 ± 16	3.11 ± 0.29
	WF/**CF**/CT	terrestrial	4	74	7 ± 1	2.94 ± 0.18
	WF/CF/**CT**	terrestrial	3	22	0	-
2017	**WF**	aquatic	1	30	13 ± 0	2.67 ± 0.21
	WF/CF	aquatic	9	346	28 ± 14	2.68 ± 0.40
	WF/CF	aquatic	9	270	29 ± 20	2.70 ± 0.43
	WF/**CF**	aquatic	4	76	26 ± 10	2.60 ± 0.22
	WF/CF/CT	aquatic	6	361	55 ± 27	4.03 ± 0.80
	WF/CF/CT	aquatic	6	159	53 ± 31	4.24 ± 0.83
	WF/**CF**/CT	aquatic	6	141	65 ± 33	3.94 ± 0.76
	WF/CF/**CT**	aquatic	2	61	17 ± 0	3.32 ± 0.19

Community level data are followed by a breakdown by species (if successfully sampled at respective site). Underlined species names indicate species considered in respective row: wood frog (WF), boreal chorus frog (CF), Canadian toad (CT). Viral loads (VL) are presented as mean log10 viral copies/250 ng of gDNA ± SD.

($\chi^2 = 2.19$, df = 2, $p = 0.335$; **Table 4**, **Figure 3A**). At sites where WF occurred alone, we observed a 9% mean infection prevalence, increasing to 15% when CF were also present in the community. When three species were present, the mean community prevalence increased to 23% per site on average with regression analyses showing a significant effect of species richness in explaining the observed pattern, but no effects of other variables (**Table 5**, **Appendix II**). We also observed a significant increase in mean community viral loads linked to species richness ($F_{2,7} = 6.82$, $p < 0.05$), while other variables had no explanatory power (**Tables 4**, **5**, **Figure 3B**).

At the population level (WF only), we observed a significant increase in infection prevalence from 9 (WF) to 16 (WF/CF), to 28% (WF/CF/CT; $\chi^2 = 8.5$, df = 2, $p < 0.05$; **Table 4**), but did not find any significant relationships of population prevalence and abiotic variables (**Table 5**). We also found a significant positive relationship of mean viral loads in terrestrial WF stages with the number of syntopic species in the community, at the individual and population levels (**Table 5**).

Ecological Correlates of Ranavirus Prevalence and Viral Loads in Aquatic Amphibian Life Stages (2017)

In the aquatic amphibian life stages we observed that at the community level, ranavirus prevalence nearly doubled with each additional species (**Table 4**, **Figure 3C**). Wood frog aquatic stages in sites with no other amphibian species exhibited a low RV infection prevalence of 13% while in communities consisting of WF and CF we found significantly higher infection prevalence across the two species (28%; **Table 4**). When CT were also present in the communities, the mean community infection prevalence increased significantly to 55% across the three species ($\chi 2 = 45.85$, df = 2, $p < 0.001$; **Table 4**). Regression analyses showed significant effect of species richness on RV prevalence at the community level, and no effects of other variables (**Table 6**, **Appendix III**). We also observed a significant increase in mean community viral loads ($F_{2,13} = 1.963$, $p < 0.05$) linked to species richness, while other variables had no explanatory power (**Tables 4**, **6**, **Figure 3D**).

At the population level (WF only), we observed an increase in prevalence from 13% (WF) to 29% (WF/CF), and to 53% (WF/CF/CT; $\chi^2 = 22.437$, df = 2, $p < 0.001$). Regression analyses showed a significant positive correlation of population prevalence in WF aquatic stages with the number of syntopic species in the community, with no significant effect of other variables (**Table 6**). The same patterns were observed for viral loads in WF aquatic stages at individual and population levels (**Table 6**). When data collected during mass die-off events were excluded from the statistical analysis, species richness remained the main predictor for prevalence and viral loads on community level and WF population level (see **Appendix VIII**).

DISCUSSION

There is still limited knowledge of how pathogens are shaping communities and vice-versa, and much more remains to be explained regarding transmission dynamics, relationships, and specific interactions in multi host-pathogen systems. Our data are consistent with amplifying effects of community richness, whereby an increase in the number of host species was positively correlated with pathogen prevalence and viral loads. We also observed strong inter- and intraspecific differences prevalence and viral loads linked to host identity and life-history stage. However, we did not find evidence for any effect of abundance or any abiotic factors on epidemiological patterns, contrary to other amphibian pathogen systems [e.g., Bd (56, 90, 91)].

Epidemiological patterns in multi-host pathogen systems can be directly dependent on the specific competence and number of host species involved (14, 92, 93), but studies on such relationships are sparse in the amphibian disease literature.

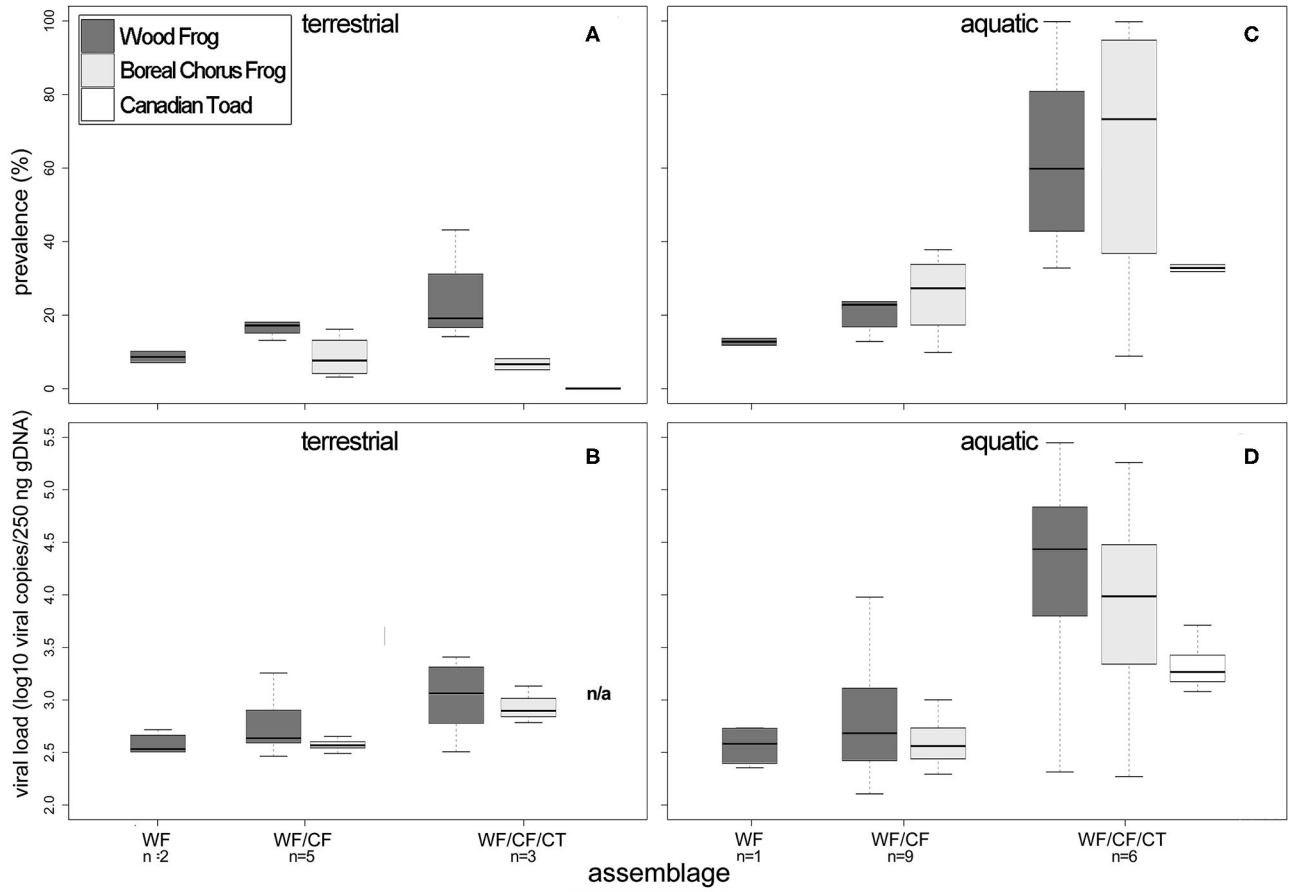


FIGURE 3 | Min/max, median, and inter-quartile ranges of ranavirus infection prevalence (%) and viral load (log10 copies/250 ng of gDNA) in terrestrial **(A,B)** and aquatic stage **(C,D)** amphibians in northern Alberta and the Northwest Territories in 2016 and 2017, arranged by community assemblage. The number of sampled communities is stated below the respective assemblage. WF, Wood frog; CF, boreal chorus frog; CT, Canadian toad.

In general, studies often investigate single hosts, or focus on surveillance rather than investigating the underlying host-pathogen relationships. Two major amphibian pathogens (Bd and RV) infect multiple host species within a community (43, 46, 47). Therefore it is critical to include host identity and species richness as factors when investigating the pattern of these pathogens in the wild. Changes in host species composition and their respective competence can influence the dynamics (e.g., transmission and persistence) of pathogens present in the system but may also lead to the introduction of new pathogens into naïve host communities (12, 58, 94, 95). In our system, we observed increases of pathogen prevalence and viral loads in concert with an increase in species richness in both aquatic and terrestrial life stages. Therefore, it is possible that host identity and richness were key drivers of host-pathogen interactions in our system. Similarly, a large-scale study of amphibian communities in California found that an increase amphibian host richness significantly raised site-level RV prevalence (52), and a study on amphibian ranaviruses in southern Europe reported localized amplification effects but did not observe generalizable spatial or temporal relationships (53). Comparable relationships of specific host identity and pathogen dynamics were observed in other amphibian-host-pathogen systems [Bd, (57, 96); *Ribeiroia ondatrae*, (58)].

Habitat characteristics are regularly considered in epidemiological studies involving amphibian pathogens. In particular, temperature, pH, and conductivity can have effects on pathogen dynamics under specific circumstances [reviewed by Bienentreu and Lesbarrères (50)]. We included pH and conductivity as abiotic factors in our analysis but did not find any significant effects. Our findings are consistent with other amphibian-RV studies where abiotic factors did not appear to be directly driving RV dynamics (52, 91, 97). Nonetheless, water characteristics such as pH and conductivity can have effects on amphibian overall body condition, which in turn may indirectly influence pathogen dynamics (50, 98–100). It is important to consider that the effects of abiotic factors are often observed under controlled lab conditions, but field studies regularly fail to find specific relationships. Under natural conditions, pathogen dynamics may be affected by unknown or uninvestigated habitat characteristics. As well, animals move throughout their habitat in search of preferred microhabitat

TABLE 5 | Generalized linear mixed model (GLMR) and beta regression (BETA) results for ranavirus prevalence and viral loads in terrestrial amphibian life-stages at the community and population level (wood frog only).

	Estimate	Std.Error	z value	Pr(>|z|)	Model type
IND_VL_WF					GLMR
(Intercept)	5.150	0.535	9.29	<0.001	
Richness	0.745	0.158	4.55	<0.001	
Conductivity	−0.067	0.107	0.60	0.549	
pH	−0.085	0.321	0.26	0.797	
Abundance	−0.008	0.026	0.30	0.764	
POP_PREV_WF					BETA
(Intercept)	−1.590	0.795	2.00	<0.05	
Abundance	−0.019	0.033	0.60	0.549	
Conductivity	0.066	0.139	0.48	0.635	
pH	0.046	0.222	0.21	0.834	
richness	0.022	0.161	0.14	0.890	
POP_VL_WF					
(Intercept)	6.180	0.800	6.88	<0.001	GLMR
Richness	1.280	0.316	3.09	<0.01	
pH	0.502	0.387	1.04	0.299	
Abundance	−0.042	0.036	0.93	0.354	
Conductivity	0.027	0.192	0.11	0.911	
COM _VL					
(Intercept)	5.250	0.370	12.12	<0.001	GLMR
Richness	0.554	0.137	3.35	<0.001	
Abundance	0.002	0.025	0.06	0.956	
Conductivity	−0.019	0.108	0.14	0.887	
pH	0.093	0.273	0.29	0.769	
COM _PREV					BETA
(Intercept)	−2.360	0.583	4.04	<0.001	
Richness	0.148	0.212	0.70	<0.05	
pH	0.054	0.171	0.32	0.751	
Conductivity	0.012	0.072	0.16	0.872	
Abundance	−0.001	0.013	0.06	0.953	

Individual (IND), population (POP), community (COM), viral load (VL), wood frog (WF). Please see ***Appendix II*** *for detailed output from statistical models.*

TABLE 6 | Generalized linear mixed model (GLMR) and beta regression (BETA) results for ranavirus prevalence and viral loads in aquatic amphibian life stages at the community and population level (wood frogs only).

	Estimate	Std.Error	z value	Pr(>|z|)	Model type
IND_VL_WF					GLMR
(Intercept)	4.97	2.010	2.47	<0.05	
Richness	4.43	1.060	4.15	<0.001	
pH	0.317	0.453	0.70	0.486	
Abundance	0.001	0.004	0.19	0.853	
Conductivity	−0.011	0.067	0.16	0.872	
POP_PREV_WF					BETA
(Intercept)	−1.950	1.900	1.03	0.304	
Richness	2.120	1.360	1.55	<0.05	
Conductivity	0.061	0.126	0.48	0.630	
pH	0.160	0.387	0.41	0.680	
Abundance	−0.002	0.006	0.26	0.797	
POP_VL_WF					
(Intercept)	7.930	1.500	4.84	<0.001	GLMR
Richness	4.050	1.050	3.44	<0.001	
Abundance	0.001	0.005	0.22	0.825	
pH	0.070	0.275	0.24	0.813	
Conductivity	−0.018	0.072	0.23	0.819	
COM _VL					
(Intercept)	0.886	3.220	0.26	0.797	GLMR
Richness	2.680	0.947	2.63	<0.01	
pH	0.350	0.685	0.49	0.625	
Abundance	0.001	0.009	0.10	0.921	
Conductivity	−0.019	0.131	0.13	0.898	
COM _PREV					BETA
(Intercept)	−2.740	1.260	2.17	<0.05	
Richness	1.020	0.369	2.78	<0.01	
Abundance	0.005	0.0108	0.50	0.619	
Conductivity	−0.017	0.141	0.12	0.906	
pH	−0.061	0.367	0.17	0.868	

Individual (IND), population (POP), community (COM), viral load (VL), wood frog (WF). Please see ***Appendix III*** *for detailed output from statistical models.*

conditions. Temperature has been shown to influence ranavirus dynamics, with an increase in temperature causing faster viral replication rates (40, 51). However, temperature in wetlands fluctuates throughout a single day, including at the three wetlands where we placed temperature loggers in 2017 (**Appendix IV**). Our data show that it is not uncommon for temperatures to fluctuate more than 12°C at a single location in a single day. Given that amphibians explore their environment throughout the day in pursuit of food and thermal optima, and to evade predators, the effect of temperature on RV infections is difficult to assess accurately, particularly in small bodies of water (101).

While amplification effects in other host-pathogen systems have been directly linked to the number of (susceptible) hosts in a specified area (102), ranavirus epidemics in amphibians are generally not following this pattern. A field study of RV epizootics in wood frog populations in the eastern United States found no effects of host density on the pathogen dynamics (97), and research conducted on amphibian communities in California similarly found no density effects at the site level (52). These findings are further supported by experimental RV exposure trials, where an increase in density lowered mortality in wood frog tadpoles (103). Although many of our wetlands are either part of bigger, inter-connected wetland and river systems, or semi-permanent bodies of water with drastic fluctuations in water-levels making their size undetermined, we quantified relative abundance as Catch-Per-Unit Effort (66). Using this effective time count method to gather relative abundance data for frogs (104), we did not find any significant effects of host abundance on pathogen prevalence or viral loads in our RV-amphibian system. Even when data collected at the sites where die-offs took place [see Forzán et al. (79)] were excluded from the analyses, species richness remained the most parsimonious explanatory variable for viral loads at

the individual, population, and community levels, regardless of the life-history stage. Therefore, it is plausible that die-off incidents in our study system are linked to the host identity and richness at a wetland rather than host density and/or abundance.

We found significant differences in inter- and intraspecific infection prevalence and viral loads, representing important indicators of the relative susceptibility of the host species (12). Among the three species present in our study system, WF and CF exhibited higher infection rates and viral loads than CT. Within species, post-metamorphic individuals exhibited considerably lower prevalence and viral loads than aquatic life-stages, and rarely showed typical signs of ranavirosis [e.g., lethargy, hemorrhages; see Miller et al. (44)]. Our findings are consistent with previous studies that demonstrated higher susceptibility to lethal RV infections in Ranids and Hylids than in Bufonids (51). Our findings are also consistent with other studies that demonstrated that larval amphibians are more likely to experience lethal RV infections than adults [(11, 12); see also (105) for multi-year data from our study region]. Notably, all positive CT tadpoles were sampled during a single die-off event of the other two species, but did not experience mass-mortality themselves [see (79)]. This could indicate spillover events from WF and/or CF into CT, only occurring when CT are exposed to a large number of infectious particles due to ongoing die-off events in other species.

Overall, our results also increase knowledge of RV distribution and dynamics in the Canadian north as the only other study in the region detected RV only in WF (94). A possible explanation for this discrepancy is the higher sensitivity of real-time qPCR methods used in our study relative to the conventional PCR methods used in the previous study (94). More than half of the positive individuals in our study exhibited low viral loads which could potentially lead to an increased number of false-negative samples with conventional PCR assays (70). The high percentage of sublethally infected individuals in our study area also supports the reservoir theory in amphibian-RV systems (51, 106), whereby low-susceptible host species or life stages can sustain sub-lethal infections and eventually infect individuals of other species in the long run. This possibility is supported by several experimental and field studies reporting sublethal RV infections (51, 103, 106–109) including an experimental study with WF collected in our study area wherein post-metamorphic individuals developed sub-lethal infections when exposed to environmentally relevant concentrations of RV virions via water bath (110). Wood frogs occur across our study region, and RV was found at all sampled wetlands and not restricted to a specific community of hosts. Therefore, it is possible that the WF in our system harbor low viral loads throughout hibernation and shed virions into the environment after returning to the wetlands in the spring, in turn infecting conspecifics and syntopic species in the community (106).

Due to their complexity, host-pathogen systems play a key role in many ecological communities. Our results underline the necessity for comprehensive habitat and community assessments in epidemiological studies involving multi-host pathogens. Ultimately, the combination of field studies with laboratory and/or mesocosm experiments will be critical to improve our understanding of the interactions between biodiversity and disease risk, providing further insights in managing wildlife pathogens that affect multiple host species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This research was approved by Laurentian University Animal Care committee (protocol #2013-04-01). Wildlife research and collection permits were received from the Government of the Northwest Territories (Wildlife Research Permit WL500433 and WL500510), the Government of Alberta (Research Permits #57578 and #57829, Collection Licence #57579 and #57830) and Parks Canada (WB-2016-21358 and WB-2017-23740).

AUTHOR CONTRIBUTIONS

JFB, DS, and DL: conceptualization, methodology, and writing–original draft. JFB: data collection, curation, and visualization. JFB and AG: data analysis and validation. JFB and DL: funding acquisition. JFB, DS, AG, and DL: writing–review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by NSERC to DL (RGPIN-2018-06877) and the Ontario Trillium Scholarship to JFB.

ACKNOWLEDGMENTS

We respectfully acknowledge that field work was conducted on Treaty 8 Territory and lab work conducted at Laurentian University on Robinson-Huron-Treaty Territory. Considerable logistical support was provided by Parks Canada, Fort Smith, NWT.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.755426/full#supplementary-material

REFERENCES

1. Echaubard P, Leduc J, Pauli B, Chinchar VG, Robert J, Lesbarrères D. Environmental dependency of amphibian–ranavirus genotypic interactions: evolutionary perspectives on infectious diseases. *Evol Appl.* (2014) 7:723–33. doi: 10.1111/eva.12169
2. Keesing F, Belden LK, Daszak P, Dobson A, Harvell CD, Holt RD, et al. Impacts of biodiversity on the emergence and transmission of infectious diseases. *Nature.* (2010) 468:647–52. doi: 10.1038/nature09575
3. Han BA, Kerby JL, Searle CL, Storfer A, Blaustein AR. Host species composition influences infection severity among amphibians in the absence of spillover transmission. *Ecol Evol.* (2015) 5:1432–9. doi: 10.1002/ece3.1385
4. Kiesecker JM, Blaustein AR. Pathogen reverses competition between larval amphibians. *Ecology.* (1999) 80:2442–8. doi: 10.1890/0012-9658(1999)080[2442:PRCBLA]2.0.CO;2
5. Loehle C. Social barriers to pathogen transmission in wild animal populations. *Ecology.* (1995) 76:326–35. doi: 10.2307/1941192
6. Behringer DC, Butler MJ, Shields JD. Avoidance of disease by social lobsters. *Nature.* (2006) 41:421. doi: 10.1038/441421a
7. Han BA, Bradley PW, Blaustein AR. Ancient behaviors of larval amphibians in response to an emerging fungal pathogen, *Batrachochytrium dendrobatidis*. *Behav Ecol Sociobiol.* (2008) 63:241–50. doi: 10.1007/s00265-008-0655-8
8. Luis AD, Kuenzi AJ, Mills JN. Species diversity concurrently dilutes and amplifies transmission in a zoonotic host–pathogen system through competing mechanisms. *Proc Nat Acad Sci.* (2018) 115:7979–84. doi: 10.1073/pnas.1807106115
9. Echaubard P, Little K, Pauli B, Lesbarrères D. Context-dependent effects of ranaviral infection on northern leopard frog life history traits. *PLoS ONE.* (2010) 5:e13723. doi: 10.1371/journal.pone.0013723
10. Strauss AT, Bowling AM, Duffy MA, Cáceres CE, Hall SR. Linking host traits, interactions with competitors and disease: mechanistic foundations for disease dilution. *Funct Ecol.* (2018) 32:1271–9. doi: 10.1111/1365-2435.13066
11. Hoverman JT, Gray MJ, Haislip NA, Miller DL. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. *Ecohealth.* (2011) 8:301–19. doi: 10.1007/s10393-011-0717-7
12. Hoverman JT, Gray MJ, Miller DL. Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and a novel virus isolate. *Dis Aquat Organ.* (2010) 89:97–107. doi: 10.3354/dao02200
13. Malpica JM, Sacristán S, Fraile A, García-Arenal F. Association and host selectivity in multi-host pathogens. *PLoS ONE.* (2006) 1:e41. doi: 10.1371/journal.pone.0000041
14. Bowden SE, Drake JM. Ecology of multi-host pathogens of animals. Nature education knowledge. (2013) 4. Available online at: http://www.nature.com/scitable/knowledge/library/ecology-of-multi-host-pathogens-of-animals-105288915 (accessed March 15, 2021).
15. Woolhouse ME, Taylor LH, Haydon DT. Population biology of multihost pathogens. *Science.* (2001) 292:1109–12. doi: 10.1126/science.1059026
16. Elena SF, Agudelo-Romero P, Lalić J. The evolution of viruses in multi-host fitness landscapes. *Open Virol J.* (2009) 3:1–6. doi: 10.2174/1874357900903010001
17. Cleaveland S, Laurenson MK, Taylor LH. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philos Trans R Soc Lond B Biol Sci*. (2001) 356:991–9. doi: 10.1098/rstb.2001.0889
18. Begon M, Bennett M, Bowers RG, French NP, Hazel SM, Turner J, et al. clarification of transmission terms in host-microparasite models: numbers, densities and areas. *Epidemiol Infect.* (2002) 129:147–53. doi: 10.1017/S0950268802007148
19. Lloyd-Smith JO, Cross PC, Briggs CJ, Daugherty M, Getz WM, Latto J, et al. Should we expect population thresholds for wildlife disease?. *Trends Ecol Evol.* (2005) 20:511–9. doi: 10.1016/j.tree.2005.07.004
20. De Castro F, Bolker B. Mechanisms of disease-induced extinction. *Ecol Lett.* (2005) 8:117–26. doi: 10.1111/j.1461-0248.2004.00693.x
21. McCallum H. Disease and the dynamics of extinction. *Philos Trans R Soc.* (2012) 367:2828–39. doi: 10.1098/rstb.2012.0224
22. McCallum H. Models for managing wildlife disease. *Parasitology.* (2016) 143:805–20. doi: 10.1017/S0031182015000980
23. Becker CG, Rodriguez D, Toledo LF, Longo AV, Lambertini C, Corrêa DT, et al. Partitioning the net effect of host diversity on an emerging amphibian pathogen. *Proc R Soc B Biol Sci.* (2014) 281:20141796. doi: 10.1098/rspb.2014.1796
24. Han BA, Searle CL, Blaustein AR. Effects of an infectious fungus, *Batrachochytrium dendrobatidis*, on amphibian predator-prey interactions. *PLoS ONE.* (2011) 6:e16675. doi: 10.1371/journal.pone.0016675
25. Halliday FW, Rohr JR. Measuring the shape of the biodiversity-disease relationship across systems reveals new findings and key gaps. *Nat Commun.* (2019) 10:5032. doi: 10.1038/s41467-019-13049-w
26. Ostfeld RS, Keesing F. Straw men don't get lyme disease: response to wood and lafferty. *Trends Ecol Evol.* (2013) 28:502–3. doi: 10.1016/j.tree.2013.05.009
27. Swaddle JP, Calos SE. Increased avian diversity is associated with lower incidence of human West Nile infection: observation of the dilution effect. *PLoS ONE.* (2008) 3:e2488. doi: 10.1371/journal.pone.0002488
28. Clay CA, Lehmer EM, Jeor SS, Dearing MD. Sin nombre virus and rodent species diversity: a test of the dilution and amplification hypotheses. *PLoS ONE.* (2009) 4:e6467. doi: 10.1371/journal.pone.0006467
29. Dizney LJ, Ruedas LA. Increased host species diversity and decreased prevalence of *Sin Nombre virus*. *Emerg Infect Dis.* (2009) 15:1012. doi: 10.3201/eid1507.081083
30. Dearing MD, Clay C, Lehmer E, Dizney L. The roles of community diversity and contact rates on pathogen prevalence. *J Mammal.* (2015) 96:29–36. doi: 10.1093/jmammal/gyu025
31. Yahnke CJ, Meserve PL, Ksiazek TG, Mills JN. Patterns of infection with laguna negra virus in wild populations of *Calomys laucha* in the central paraguayan chaco. *Am J Trop Med Hyg.* (2001) 65:768–76. doi: 10.4269/ajtmh.2001.65.768
32. Gilbert L, Norman R, Laurenson KM, Reid HW, Hudson PJ. Disease persistence and apparent competition in a three-host community: an empirical and analytical study of large-scale, wild populations. *J Anim Ecol.* (2001) 1:1053–61. doi: 10.1046/j.0021-8790.2001.00558.x
33. Johnson PT, Preston DL, Hoverman JT, Richgels KL. Biodiversity decreases disease through predictable changes in host community competence. *Nature.* (2013) 494:230–3. doi: 10.1038/nature11883
34. Wilber MQ, Johnson PT, Briggs CJ. Disease hotspots or hot species? Infection dynamics in multi-host metacommunities controlled by species identity, not source location. *Ecol Lett*. (2020) 23:1201–11. doi: 10.1111/ele.13518
35. Muths E, Hero JM. Amphibian declines: promising directions in understanding the role of disease. *Anim Conserv.* (2010) 13:33–5. doi: 10.1111/j.1469-1795.2010.00410.x
36. Grant EH, Miller DA, Schmidt BR, Adams MJ, Amburgey SM, Chambert T, et al. Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *Sci Rep.* (2016) 6:1–9. doi: 10.1038/srep25625
37. Cohen JM, Civitello DJ, Venesky MD, McMahon TA, Rohr JR. An interaction between climate change and infectious disease drove widespread amphibian declines. *Glob Chang Biol.* (2019) 5:927–37. doi: 10.1111/gcb.14489
38. 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.* (2013) 110:15325–9. doi: 10.1073/pnas.1307356110
39. Lips KR. Overview of chytrid emergence and impacts on amphibians. *Philos Trans R Soc Lond B Biol Sci.* (2016) 371:20150465. doi: 10.1098/rstb.2015.0465
40. Brunner JL, Storfer A, Gray MJ, Hoverman JT. "Ranavirus ecology and evolution: from epidemiology to extinction". In: Gray MJ, Chinchar VG, editors. *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Cham: Springer Nature (2015). p. 71–104.
41. Chinchar VG, Hick P, Jancovich J, Subramaniam K, Waltzek T, Whittington R, et al. Create eight new species, remove three existing species in the family Iridoviridae. Available online at: https://talk.ictvonline.org/ictv/proposals/2018.007DAv1.Iridoviridae_8sp3sprem.zip (accessed March 5, 2020).
42. Voyles J, Rosenblum EB, Berger L. Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity. *Microbes Infect.* (2011) 13:25–32. doi: 10.1016/j.micinf.2010.09.015

43. Duffus AL, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, et al. "Distribution and host range of ranaviruses". In: Gray MJ, Chinchar VG, editors. *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Cham: Springer Nature (2015). p. 9–57.
44. Miller DL, Pessier AP, Hick P, Whittington RJ. "Comparative pathology of ranaviruses and diagnostic techniques". In: Gray MJ, Chinchar VG, editors. *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Cham: Springer Nature (2015). p. 171–208.
45. Grogan LF, Skerratt LF, Berger L, Cashins SD, Trengove RD, Gummer JP. Chytridiomycosis causes catastrophic organism-wide metabolic dysregulation including profound failure of cellular energy pathways. *Sci Rep*. (2018) 8:8188. doi: 10.1038/s41598-018-26427-z
46. Schock DM, Bollinger TK, Gregory Chinchar V, Jancovich JK, Collins JP. Experimental evidence that amphibian ranaviruses are multi-host pathogens. *Copeia*. (2008) 2008:133–43. doi: 10.1643/CP-06-134
47. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. Mapping the global emergence of Batrachochytrium dendrobatidis, the amphibian chytrid fungus. *PLoS ONE*. (2013) 8:e56802. doi: 10.1371/journal.pone.0056802
48. Scheele BC, Hunter DA, Brannelly LA, Skerratt LF, Driscoll DA. Reservoir-host amplification of disease impact in an endangered amphibian. *Conserv Biol*. (2017) 31:592–600. doi: 10.1111/cobi.12830
49. Brunner JL, Olson DH, Gray MJ, Miller DL, Duffus AL. Global patterns of ranavirus detections. *FACETS*. (2021) 6:912–24. doi: 10.1139/facets-2020-0013
50. Bienentreu JF, Lesbarrères D. Amphibian disease ecology: are we just scratching the surface? *Herpetologica*. (2020) 76:153–66. doi: 10.1655/0018-0831-76.2.153
51. Gray MJ, Miller DL, Hoverman JT. Ecology and pathology of amphibian ranaviruses. *Dis Aquat Organ*. (2009) 87:243–66. doi: 10.3354/dao02138
52. Tornabene BJ, Blaustein AR, Briggs CJ, Calhoun DM, Johnson PT, McDevitt-Galles T, et al. The influence of landscape and environmental factors on ranavirus epidemiology in a California amphibian assemblage. *Freshw Biol*. (2018) 63:639–51. doi: 10.1111/fwb.13100
53. Rosa GM, Sabino-Pinto J, Laurentino TG, Martel A, Pasmans F, Rebelo R, et al. Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. *Sci Rep*. (2017) 7:43260. doi: 10.1038/srep43260
54. Rachowicz LJ, Briggs CJ. Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the mountain yellow-legged frog Rana muscosa. *J Anim Ecol*. (2007) 76:711–21. doi: 10.1111/j.1365-2656.2007.01256.x
55. Briggs CJ, Knapp RA, Vredenburg VT. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc Nat Acad Sci*. (2010) 107:9695–700. doi: 10.1073/pnas.0912886107
56. Battaglin WA, Smalling KL, Anderson C, Calhoun D, Chestnut T, Muths E. Potential interactions among disease, pesticides, water quality and adjacent land cover in amphibian habitats in the United States. *Sci Total Environ*. (2016) 566:320–32. doi: 10.1016/j.scitotenv.2016.05.062
57. Venesky MD, Liu X, Sauer EL, Rohr JR. Linking manipulative experiments to field data to test the dilution effect. *J Anim Ecol*. (2014) 83:557–65. doi: 10.1111/1365-2656.12159
58. Johnson PT, Calhoun DM, Riepe T, McDevitt-Galles T, Koprivnikar J. Community disassembly and disease: realistic—but not randomized—biodiversity losses enhance parasite transmission. *Proc Royal Soc B*. (2019) 286:20190260. doi: 10.1098/rspb.2019.0260
59. Marshall IB, Smith CS, Selby CJ. A national framework for monitoring and reporting on environmental sustainability in Canada. *Environ Monit Assess*. (1996) 39:25–38. doi: 10.1007/BF00396133
60. Ecosystem Classification Group. *Ecological Regions of the Northwest Territories - Taiga Plains*. Yellowknife, NT: Department of Environment and Natural Resources, Government of the Northwest Territories (2009). 184 p.
61. Marshall IB, Schut PH. *A National Ecological Framework for Canada*. Ottawa, ON: Environment Canada and Agriculture and Agri-Food Canada (1999). p. 132.
62. Devito K, Creed I, Gan T, Mendoza C, Petrone R, Silins U, et al. framework for broad-scale classification of hydrologic response units on the boreal plain: is topography the last thing to consider? *Hydrol Process*. (2005) 19:1705–14. doi: 10.1002/hyp.5881
63. Federal, Provincial and Territorial Governments of Canada. C*anadian Biodiversity: Ecosystem Status and Trends 2010*. Ottawa, ON: Canadian Councils of Resource Ministers (2010). p. 142.
64. Garet J, Raulier F, Pothier D, Cumming SG. Forest age class structures as indicators of sustainability in boreal forest: are we measuring them correctly? *Ecol Indic*. (2012) 23:202–10. doi: 10.1016/j.ecolind.2012.03.032
65. Gosner KL. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. (1960) 16:183–90.
66. Heyer R, Donnelly MA, Foster M, McDiarmid R, editors. *Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians*. Washington, DC: Smithsonian Institution (2014). p. 320.
67. Olson DH, Haman KH, Gray MJ, Harris R, Thompson T, Iredale M, et al. Enhanced between-site biosecurity to minimize herpetofaunal disease-causing pathogen transmission. *Herpetol Rev*. (2021) 52:29–39.
68. St-Amour V, Lesbarrères D. Genetic evidence of *Ranavirus* in toe clips: an alternative to lethal sampling methods. *Conserv Genet*. (2007) 8:1247–50. doi: 10.1007/s10592-006-9242-6
69. Gray MJ, Brunner JL, Earl JE, Ariel E. "Design and analysis of ranavirus studies: surveillance and assessing risk". In: Gray MJ, Chinchar VG, editors. *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Springer Nature (2015). p. 209–40.
70. Greer AL, Collins JP. Sensitivity of a diagnostic test for amphibian *Ranavirus* varies with sampling protocol. *J Wildl Dis*. (2007) 43:525–32. doi: 10.7589/0090-3558-43.3.525
71. Gray MJ, Miller DL, Hoverman JT. Reliability of non-lethal surveillance methods for detecting ranavirus infection. *Dis Aquat Organ*. (2012) 99:1–6. doi: 10.3354/dao02436
72. Duffus AL, Pauli BD, Wozney K, Brunetti CR, Berrill M. *Frog virus 3*-like infections in aquatic amphibian communities. *J Wildl Dis*. (2008) 44:109–20. doi: 10.7589/0090-3558-44.1.109
73. Leung WT, Thomas-Walters L, Garner TW, Balloux F, Durrant C, Price SJ, et al. quantitative-PCR based method to estimate ranavirus viral load following normalisation by reference to an ultraconserved vertebrate target. *J Virol Methods*. (2017) 249:147–55. doi: 10.1016/j.jviromet.2017.08.016
74. Yuan JS, Reed A, Chen F, Stewart CN. Statistical analysis of real-time PCR data. *BMC Bioinformatics*. (2006) 7:85. doi: 10.1186/1471-2105-7-85
75. Tan WG, Barkman TJ, Chinchar VG, Essani K. Comparative genomic analyses of Frog virus 3, type species of the genus *Ranavirus* (family *Iridoviridae*). *Virology*. (2004) 323:70–84. doi: 10.1016/j.virol.2004.02.019
76. He JG, Lü L, Deng M, He HH, Weng SP, Wang XH, et al. Sequence analysis of the complete genome of an iridovirus isolated from the tiger frog. *Virology*. (2002) 292:185–97. doi: 10.1006/viro.2001.1245
77. Mavian C, López-Bueno A, Balseiro A, Casais R, Alcamí A, Alejo A. The genome sequence of the emerging common midwife toad virus identifies an evolutionary intermediate within ranaviruses. *J Virol*. (2012) 86:3617–25. doi: 10.1128/JVI.07108-11
78. Jancovich JK, Bremont M, Touchman JW, Jacobs BL. Evidence for multiple recent host species shifts among the *ranaviruses* (family *Iridoviridae*). *J Virol*. (2010) 84:2636–47. doi: 10.1128/JVI.01991-09
79. Forzán MJ, Bienentreu J, Schock DM, Lesbarrères D. Multi-tool diagnosis of an outbreak of *ranavirosis* in amphibian tadpoles in the Canadian boreal forest. *Dis Aquat Organ*. (2019) 135:33–41. doi: 10.3354/dao03369
80. R Core Team. R: A language and environment for statistical computing. *R Foundation for Statistical Computing. Vienna*. (2013). Available online at: https://www.r-project.org
81. R Studio Team. *RStudio: Integrated Development for R*. RStudio Inc, B*oston* MA (2016). Available online at: http://www.rstudio.com/
82. Amos KH. *Procedures for the Detection and Identification of Certain Fish Pathogens*. Fish Health Section, American Fisheries Society (1985). p. 114.
83. Thoesen JC. *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*. Fish Health Section, American Fisheries Society (1994).
84. Cribari-Neto F, Zeileis A. Beta regression in R. *J Stat Softw*. (2010) 34:1–24. doi: 10.18637/jss.v034.i02
85. Bates D, Maechler M, Bolker B, Walker S. *lme4: Linear Mixed-Effects Models Using 'Eigen' and S4. R Package Version 1.1–7*. (2014). Available online at: https://cran.r-project.org/web/packages/lme4/index.html (accessed January 10, 2022).

86. Barton K. *MuMIn: Multi-Model Inference, R Package Version 0.12*. (2010). Available online at: https://cran.r-project.org/web/packages/MuMIn/index.html (accessed January 10, 2022).
87. Anderson DR, Burnham KP. Avoiding pitfalls when using information-theoretic methods. *J Wildl Manag*. (2002) 66:912–8. doi: 10.2307/3803155
88. Mazerolle MJ. *AICcmodavg-Model Selection and Multimodel Inference Based on (q) aic (c), R Package*. (2017). Available online at: https://cran.r-project.org/web/packages/AICcmodavg/index.html (accessed January 10, 2022).
89. Burnham KP, Anderson DR. Multimodel inference: understanding AIC and BIC in model selection. *Sociol Methods Res*. (2004) 33:261–304. doi: 10.1177/0049124104268644
90. Strauss A, Smith KG. Why does amphibian chytrid (*Batrachochytrium dendrobatidis*) not occur everywhere? An exploratory study in Missouri ponds. *PLoS ONE*. (2013) 8:e76035. doi: 10.1371/journal.pone.0076035
91. Clulow S, Gould J, James H, Stockwell M, Clulow J, Mahony M. Elevated salinity blocks pathogen transmission and improves host survival from the global amphibian chytrid pandemic: implications for translocations. *J Appl Ecol*. (2018) 5:830–40. doi: 10.1111/1365-2664.13030
92. Holt RD, Dobson AP, Begon M, Bowers RG, Schauber EM. Parasite establishment in host communities. *Ecol Lett*. (2003) 6:837–42. doi: 10.1046/j.1461-0248.2003.00501.x
93. LoGiudice K, Duerr ST, Newhouse MJ, Schmidt KA, Killilea ME, Ostfeld RS. Impact of host community composition on Lyme disease risk. *Ecology*. (2008) 89:2841–9. doi: 10.1890/07-1047.1
94. Schock DM, Ruthig GR, Collins JP, Kutz SJ, Carrière S, Gau RJ, et al. Amphibian chytrid fungus and *ranaviruses* in the Northwest Territories, Canada. *Dis Aquat Organ*. (2010) 92:231–40. doi: 10.3354/dao02134
95. Young HS, Parker IM, Gilbert GS, Guerra AS, Nunn CL. Introduced species, disease ecology, and biodiversity–disease relationships. *Trends Ecol Evol*. (2017) 32:41–54. doi: 10.1016/j.tree.2016.09.008
96. Scheele BC, Hunter DA, Banks SC, Pierson JC, Skerratt LF, Webb R, et al. High adult mortality in disease-challenged frog populations increases vulnerability to drought. *J Anim Ecol*. (2016) 85:1453–60. doi: 10.1111/1365-2656.12569
97. Hall EM, Brunner JL, Hutzenbiler B, Crespi EJ. Salinity stress increases the severity of ranavirus epidemics in amphibian populations. *Proc Royal Soc B*. (2020) 287:20200062. doi: 10.1098/rspb.2020.0062
98. Sanzo D, Hecnar SJ. Effects of road de-icing salt (NaCl) on larval wood frogs (*Rana sylvatica*). *Environ Pollut*. (2006) 140:247–56. doi: 10.1016/j.envpol.2005.07.013
99. Hopkins GR, Brodie ED Jr. Occurrence of amphibians in saline habitats: a review and evolutionary perspective. *Herpetol Monogr*. (2015) 29:1–27. doi: 10.1655/HERPMONOGRAPHS-D-14-00006
100. Hall EM, Goldberg CS, Brunner JL, Crespi EJ. Seasonal dynamics and potential drivers of *ranavirus* epidemics in wood frog populations. *Oecologia*. (2018) 188:1253–62. doi: 10.1007/s00442-018-4274-4
101. Youker-Smith TE, Boersch-Supan PH, Whipps CM, Ryan SJ. Environmental drivers of *ranavirus* in free-living amphibians in constructed ponds. *Ecohealth*. (2018) 15:608–18. doi: 10.1007/s10393-018-1350-5
102. Dobson A. Population dynamics of pathogens with multiple host species. *Am Nat*. (2004) 164:S64–78. doi: 10.1086/424681
103. Reeve BC, Crespi EJ, Whipps CM, Brunner JL. Natural stressors and *ranavirus* susceptibility in larval wood frogs (*Rana sylvatica*). *Ecohealth*. (2013) 10:190–200. doi: 10.1007/s10393-013-0834-6
104. Bibby CJ, Jones M, Marsden S. *Bird Surveys*. London: Expedition Advisory Centre (1998).
105. Bienentreu JF. *Epidemiology of Ranaviruses in Amphibian Populations in the Boreal Forest of Northwestern Canada*. PhD dissertation, Laurentian University, Canada (2019).
106. Brunner JL, Schock DM, Davidson EW, Collins JP. Intraspecific reservoirs: complex life history and the persistence of a lethal *ranavirus*. *Ecology*. (2004) 5:560–6. doi: 10.1890/02-0374
107. Ariel E, Kielgast J, Svart HE, Larsen K, Tapiovaara H, Jensen BB, et al. *Ranavirus* in wild edible frogs *Pelophylax* kl. esculentus in Denmark. *Dis Aquat Organ*. (2009) 85:7–14. doi: 10.3354/dao02060
108. Crespi EJ, Rissler LJ, Mattheus NM, Engbrecht K, Duncan SI, Seaborn T, et al. Geophysiology of wood frogs: landscape patterns of prevalence of disease and circulating hormone concentrations across the eastern range. *Integr Comp Biol*. (2015) 55:602–17. doi: 10.1093/icb/icv096
109. Vilaça ST, Bienentreu JF, Brunetti CR, Lesbarrères D, Murray DL, Kyle CJ. *Frog virus 3* genomes reveal prevalent recombination between *Ranavirus* lineages and their origins in Canada. *J Virol*. (2019) 93:e00765–19. doi: 10.1128/JVI.00765-19
110. Bienentreu JF, Grayfer L, Schock DM, Guerreiro M, Mehes-Smith M, DeWitte-Orr SJ, et al. Sublethal effects of wild-type and a vIF-2α-knockout Frog virus 3 on postmetamorphic wood frogs (*Rana sylvatica*): potential for a stage-specific reservoir. *FACETS*. (2020) 5:738–57. doi: 10.1139/facets-2020-0001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 04 March 2022
doi: 10.3389/fvets.2022.756686

Endemic Lineages of *Batrachochytrium dendrobatidis* Are Associated With Reduced Chytridiomycosis-Induced Mortality in Amphibians: Evidence From a Meta-Analysis of Experimental Infection Studies

Anat M. Belasen[1,2,3], *Imani D. Russell*[4], *Kelly R. Zamudio*[1,3] *and Molly C. Bletz*[5]*

[1] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, United States, [2] Society for Conservation Biology, Washington, DC, United States, [3] Department of Integrative Biology, University of Texas at Austin, Austin, TX, United States, [4] Department of Ecology, Evolution, and Marine Biology, University of California-Santa Barbara, Santa Barbara, CA, United States, [5] Department of Biology, University of Massachusetts-Boston, Boston, MA, United States

OPEN ACCESS

Edited by:
Rachel E. Marschang,
Laboklin GmbH & Co. KG, Germany

Reviewed by:
Bruce Waldman,
Oklahoma State University,
United States
Koichi Goka,
National Institute for Environmental Studies (NIES), Japan
José Manuel Verdes,
Universidad de la República,
Uruguay, Uruguay

***Correspondence:**
Molly C. Bletz
molly.bletz@gmail.com

Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 10 August 2021
Accepted: 08 February 2022
Published: 04 March 2022

Citation:
Belasen AM, Russell ID, Zamudio KR and Bletz MC (2022) Endemic Lineages of Batrachochytrium dendrobatidis Are Associated With Reduced Chytridiomycosis-Induced Mortality in Amphibians: Evidence From a Meta-Analysis of Experimental Infection Studies. Front. Vet. Sci. 9:756686. doi: 10.3389/fvets.2022.756686

Emerging infectious wildlife diseases have caused devastating declines, particularly when pathogens have been introduced in naïve host populations. The outcome of disease emergence in any host population will be dictated by a series of factors including pathogen virulence, host susceptibility, and prior opportunity for coevolution between hosts and pathogens. Historical coevolution can lead to increased resistance in hosts and/or reduced virulence in endemic pathogens that allows stable persistence of host and pathogen populations. Adaptive coevolution may also occur on relatively short time scales following introduction of a novel pathogen. Here, we performed a meta-analysis of multi-strain *Batrachochytrium dendrobatidis* (Bd) infection experiments to test whether: (1) amphibian hosts exhibit lower mortality rates when infected with strains belonging to endemic Bd lineages relative to the Global Panzootic Lineage (Bd-GPL), hypothetically owing to long co-evolutionary histories between endemic Bd lineages and their amphibian hosts; and (2) amphibians exhibit lower mortality rates when infected with local Bd-GPL strains compared with non-local Bd-GPL strains, hypothetically owing to recent selection for tolerance or resistance to local Bd-GPL strains. We found that in a majority of cases, amphibians in endemic Bd treatments experienced reduced mortality relative to those in Bd-GPL treatments. Hosts presumed to have historically coexisted with endemic Bd did not show reduced mortality to Bd-GPL compared with hosts that have not historically coexisted with endemic Bd. Finally, we detected no overall difference in amphibian mortality between local and non-local Bd-GPL treatments. Taken together, our results suggest that long-term historical coexistence is associated with less disease-induced mortality potentially due to hypovirulence in endemic Bd lineages, and

that more recent coexistence between amphibians and Bd-GPL has not yet resulted in reduced host susceptibility or pathogen virulence. This corroborates previous findings that Bd-GPL introduced via the global amphibian trade has a high capacity for causing disease-induced mortality.

Keywords: *Batrachochytrium dendrobatidis* (Bd), meta-analysis, pathogen genotypes, experimental infection, amphibian, virulence, chytridiomycosis

INTRODUCTION

Emerging wildlife diseases pose significant threats to global biodiversity, but the mechanisms by which some hosts are able to survive recently emerged diseases are not yet fully understood (1). To aid in assessment of disease risk in host populations and species of concern, it is critical to understand the mechanisms that determine the outcome of infection. Hosts may be *susceptible* to infection and/or disease-induced mortality, or may survive infection due to *resistance* (i.e., the ability to prevent infection or reduce infection load) and/or *tolerance* (i.e., the ability to sustain infection without experiencing substantial negative impacts). Likewise, infection outcomes can also depend on variation in pathogen virulence, which can result from genotypic differences among pathogen strains or genotypes (2–4). While contemporary conditions (i.e., local abiotic and biotic environments) may significantly influence host susceptibility [e.g., (5)] and/or pathogen virulence [reviewed in Turner et al. (6)], prior interactions between host and pathogen populations may also contribute to significant variability in disease outcomes. For example, a pathogen introduced to naïve host populations may cause severe outbreaks (i.e., epizootic disease), while recently or historically co-occurring hosts and pathogens may show disease dynamics that are tempered with mild or no disease despite pathogen presence [i.e., enzootic disease or absent disease; (7, 8)]. When the co-evolutionary trajectory between host and pathogen takes the form of a classical oscillating arms race between host resistance and pathogen virulence, different host and pathogen populations may exhibit different levels of resistance and virulence at the same point in time (9). In these cases, current infection outcomes can vary according to host-pathogen co-evolutionary history, leading to a "geographic mosaic" of disease threat (10).

Amphibian chytridiomycosis caused by *Batrachochytrium dendrobatidis* (Bd) is one of the most devastating wildlife diseases documented in the scientific literature to date (11–13). Bd is believed to have originated in East Asia (14), and one hypervirulent lineage, the Global Panzootic Lineage (hereafter Bd-GPL) spread globally by the transcontinental amphibian trade (15). Bd-GPL has been implicated in die-offs around the world (12), and the prevailing hypothesis is that introductions of Bd-GPL were particularly damaging in naïve amphibian communities where many or all hosts lacked any effective form of Bd resistance (16, 17). In East Asia, Brazil, South Africa, and Switzerland, additional geographically restricted and early-diverged genotypic lineages of Bd have been identified that may have much longer coevolutionary histories with amphibians compared to Bd-GPL (14). These lineages named *Bd*-Asia, *Bd*-Brazil, *Bd*-Cape, and *Bd*-CH, are referred to as endemic Bd lineages. Studies have shown that at least some endemic Bd lineages were associated with low mortality rates in susceptible species (14) and that amphibian species that have coexisted historically with endemic Bd lineages appear to exhibit Bd resistance and/or tolerance [e.g. (18)]. These studies support the hypotheses that due to a long history of host-pathogen coevolution, endemic Bd lineages are broadly hypovirulent, and communities of amphibians in Bd-endemic areas could be protected from Bd outbreaks (16, 19).

However, as studies of Bd have increased, we have found surprising diversity in Bd lineages and their distributions, and also unpredicted variability in disease outcomes. Experimental results have varied widely according to focal host: for instance, initial experiments did not use hosts that had locally adapted to presumed hypovirulent genotypes [e.g. (14)], and later experiments showed host species-level variation in disease susceptibility even when infected by the same endemic Bd lineage (18). Retrospective studies of Bd infections in museum specimens revealed that Bd-GPL may have been present for longer than previously thought (i.e., predating amphibian trade) in areas such as southeastern Brazil (20) and the United States (21, 22), providing the opportunity for local adaptation to Bd-GPL. Furthermore, field studies have shown that multiple genotypes (endemic Bd and Bd-GPL) and multiple Bd-GPL strains can be present in sympatry in wild populations [e.g. (23)], with the potential to produce differing disease outcomes across amphibian communities. For example, local Bd-GPL strains, defined here as the only or predominant Bd-GPL strain in a host community, may protect hosts from more virulent non-local Bd-GPL strains found in surrounding communities (24). Given that in some cases Bd-GPL has been introduced through several independent events to disparate amphibian communities (25), we may expect variable levels of local, shorter-term co-adaptation between amphibian hosts and the Bd-GPL strains found in different host communities, climates, and geographies. Because Bd is globally distributed and is a highly generalist pathogen capable of infecting at least 500 amphibian species (12, 13), understanding how coevolution and potential co-adaptation of amphibians and Bd impact infection outcomes is critical to predicting and mitigating future chytridiomycosis declines.

Here, we perform meta-analyses of host mortality in multi-strain, controlled Bd infection experiments to determine whether opportunity for historical or recent selection alters disease outcomes in amphibian hosts that are susceptible to disease-induced mortality. Multiple factors may contribute to

experimental disease outcomes, including characteristics of the host source population; therefore we restricted our meta-analysis to controlled experiments and calculated pairwise effect sizes for Bd infection treatments involving hosts from the same source population or locality. This approach allows us to summarize the impact of Bd infection across experimental studies published to date using comparable quantitative effect sizes. We test two hypotheses: (1) the historical adaptation (HA) hypothesis posits that endemic Bd lineages show hypovirulence relative to Bd-GPL as a result of long coevolutionary histories with their amphibian hosts; and (2) the recent adaptation (RA) hypothesis posits that amphibians show lower susceptibility to local Bd-GPL strains relative to non-local Bd-GPL strains due to recent and rapid adaptation or co-adaptation of amphibian hosts and Bd. To test these hypotheses, we performed two meta-analyses to evaluate whether Bd genotype and/or locality impacts mortality due to standardized Bd infection. First, to test the HA hypothesis, we analyzed the effect of Bd genotypic lineage to determine whether endemic Bd strains result in lower mortality than Bd-GPL strains, regardless of host and Bd origin (i.e., collection locality). Second, to test the RA hypothesis, we analyzed the effect of recent geographic co-occurrence of host and pathogen to determine whether local Bd-GPL strains (collected from the same locality as the focal host) result in lower mortality relative to non-local Bd-GPL strains. Our results provide insight into the significance of host-pathogen adaptation at two temporal scales in chytridiomycosis outcomes, and suggest future directions for research that would aid conservation in the face of devastating wildlife disease.

METHODS

Data Compilation

To test the HA and RA hypotheses, we performed two meta-analyses of experimental Bd studies on susceptible amphibians, here defined as hosts that can experience chytridiomycosis-induced mortality. As a starting point for compiling experimental Bd studies, we used the systematic review of experimental studies performed by Blaustein et al. (26). This study utilized a Web of Science search supplemented with a Google Scholar search for studies between 1999 and 2017 using the search terms "*Batrachochytrium dendrobatidis* + amphibians + experiments (26)." We retained 14 studies identified by Blaustein et al. (26) that involved multi-strain infection experiments. We replicated this search to compile recent studies published between 2017 and 2021 using Web of Science (searched on April 21, 2021), supplemented with a Google Scholar search [October 7, 2021 and collated in Publish or Perish (27)], and included one unpublished study from the authorship team's collaborator (28).

Our supplemental search resulted in 3,177 publications total, of which four met our criteria of being multi-strain experiments that reported mortality in susceptible host species (i.e., species that exhibited mortality in at least one infection treatment; **Supplementary Figure 1**). Study units were excluded from analyses if: (i) mortality in control treatments significantly exceeded mortality in experimental infection treatments; (ii) Bd genotype (i.e., belonging to GPL or an endemic lineage) and/or collection locality information were not available; and/or (iii) hosts were fully resistant to chytridiomycosis (i.e., experienced zero mortality in all treatments). Study units were defined as a single study x species x treatment combination.

Between Blaustein et al. (26) and our supplemental search, we recovered 30 study units across 12 publications, covering 19 ecologically diverse species of amphibians and three amphibian life stages (**Figure 1** and **Supplementary Figure 1**). Studies used in the HA analysis ($n = 21$ study units, **Table 1**) included either wild-collected or captive-reared amphibians, and were required to include at least two genotypes of Bd (Bd-GPL plus one endemic genotype, i.e., Bd-Brazil, Bd-Asia, Bd-Cape, or Bd-CH) that were separately inoculated on test animals. If experimental Bd strains were not genotyped by the authors of the study, genotype information was sourced from papers that genotyped these strains using DNA sequencing and phylogenetic analyses (**Supplementary Table 1**). In one case, Bd genotype was inferred by the current authors to be Bd-GPL [isolate LC63 from a captive Australian frog (38)]. Studies used in the RA analysis ($n = 17$ study units, **Table 1**) were required to include wild-collected amphibians and at least two strains of Bd-GPL, at least one of which was collected locally (where focal hosts were collected) and at least one of which was collected non-locally.

Data Analysis and Visualization

Data extraction, analyses, and visualizations were performed in the R environment (39). For each study unit, we extracted the sample sizes for control and experimental infection treatments, and percent mortality for each genotype/strain of Bd. If mortality values were not directly present in publication text, we used the metaDigitise package in R (40) to extract numeric data from published survival curves. Mortality values were averaged in cases where there were multiple isolates from a given genotype x locality combination tested on the same amphibian host species in different treatments within a given study. We also compiled metadata for each study unit, including host adult ecology, host reproductive mode, host life stage at infection, and historical coexistence status with endemic Bd (i.e., whether the focal host species historically coexisted with the endemic Bd lineage used in the experiment).

In experiments using sensitive amphibian hosts, background mortality can be common. While we excluded studies that contained significantly higher mortality in controls relative to infection treatments, we included 24 study units with low levels of control mortality that did not significantly exceed treatment mortality. In these cases, the control mortality rate was subtracted from each treatment mortality rate to obtain a "background mortality-corrected" rate for each treatment. In cases where control mortality marginally exceeded treatment mortality ($n = 3$ studies in the HA analysis, $n = 1$ study in the RA analysis), the corrected treatment mortality value was rounded up to zero. In all of these studies, mortality was significantly higher in at least one infection treatment than in the control treatment, indicating Bd-induced mortality above potential background mortality due to captive conditions.

Unless otherwise stated, all calculations and analyses were performed in the R metafor package (41). To estimate the

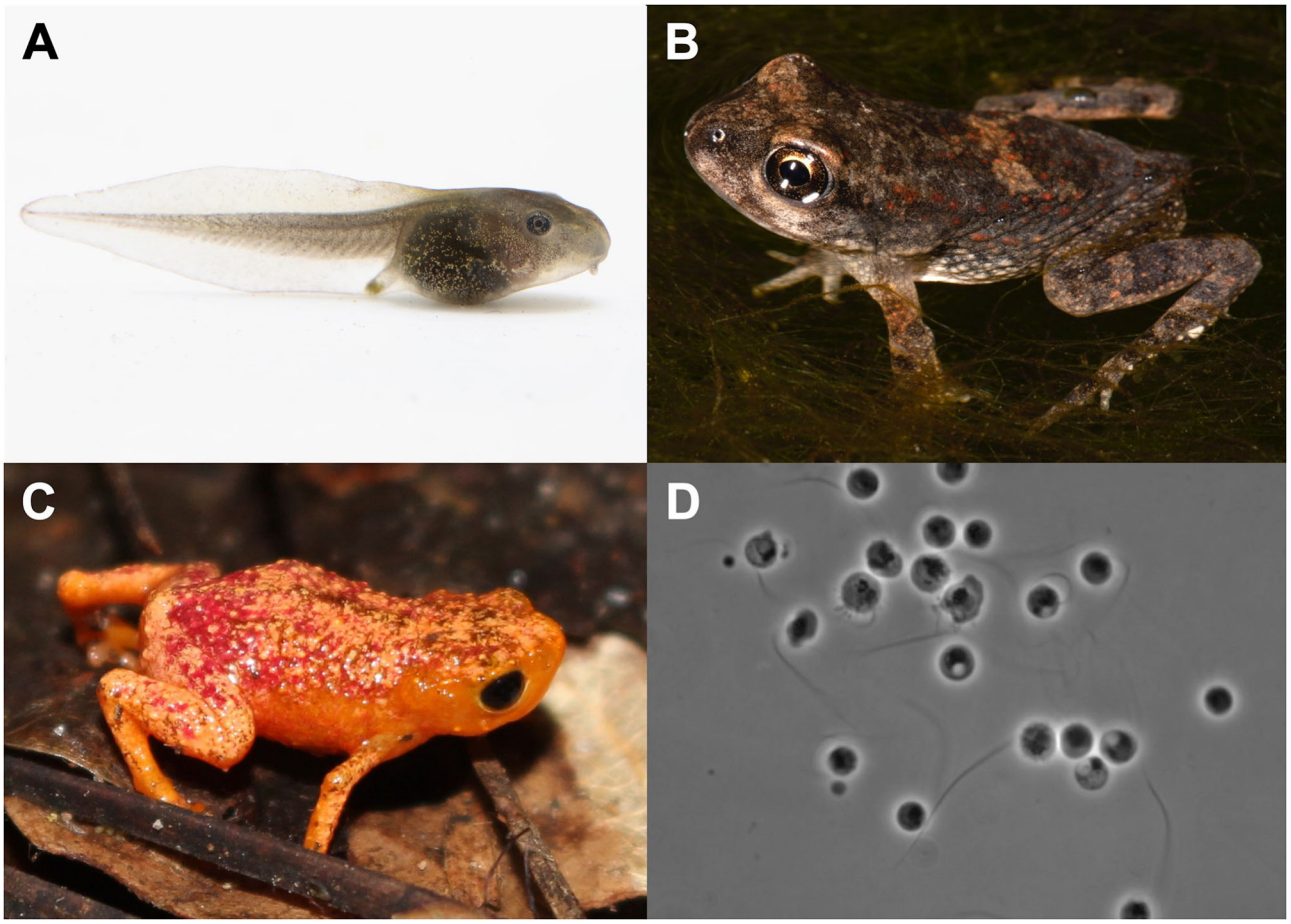


FIGURE 1 | Select study organisms included in the meta-analysis. **(A)** *Rana sylvatica* (wood frog) larva. This is one of three species for which data were available for larval experimental infections. Photo by Brian Gratwicke. **(B)** Bufonid metamorph. Recently metamorphosed amphibians are believed to be particularly susceptible to chytridiomycosis. Photo by Todd Pierson. **(C)** *Brachycephalus pitanga* (pumpkin toadlet) is a tiny terrestrial direct developer and is endemic to Brazil's Atlantic Forest. Photo by Anat Belasen. **(D)** *Batrachochytrium dendrobatidis* zoospores on 1%T visualized under a stereoscope. Photo by Joyce Longcore.

effect size of Bd genotype (HA analysis) or source locality (RA analysis) on mortality, log Risk Ratio was calculated for each study unit. Subsequently, linear mixed models were constructed to analyze effect sizes weighted by variance for each analysis. Models constructed for each analysis were fitted using a restricted maximum likelihood (REML) approach. A generalized, weighted least squares extension of Cochran's Q test (41) and I^2 (42) were calculated to test whether the heterogeneity among effect sizes was greater than expected. Outliers or influential cases were identified by calculating Cook's distances ($\geq$0.45), Hat values [$\geq$3*(1/k)], and dfbetas [$\geq$1; (41, 43)], and removed if at least two metrics identified the outlier.

To test whether variation among effect sizes was associated with any subgroup variables, three models were constructed for each analysis: (1) intercept-only (base) models; (2) multifactor (full) models that included all subgroup terms (HA only); and (3) single-factor models that consisted of separate models for each subgroup term. Subgroup variables included life stage at infection (both HA and RA) and historical coexistence (HA only). Host adult ecology and host reproductive mode were not included as subgroup moderators due to a lack of sufficient statistical power (little or no replication for some factor levels). In all models (including base models), study (publication) ID was included as a random term to account for non-independence among study units within publications (44). AIC was used to determine model ranking within each analysis. Average log Risk Ratios were exponentiated to calculate the Risk Ratio values for subgroup terms that were significant in single-factor models. Fligner-Killeen non-parametric tests for homogeneity of variance were used to evaluate variance among subgroup variables of interest. Results were visualized in metafor (41) and ggplot2 (45).

RESULTS

Historical Adaptation Analysis

In the HA analysis, Bd genotype was associated with a significantly positive log Risk Ratio according to the intercept-only model, indicating that Bd-GPL infections resulted in higher mortality relative to endemic lineages of Bd (RR = 1.79, $Z = 3.99$, $p < 0.0001$; **Figure 2**). The HA dataset showed a high degree of

TABLE 1 | Study units included in the two meta-analyses.

	Host species	Study number	Life stage at Bd exposure	Exposure dose	Experiment duration	Endemic Bd used	Historical Bd coexistence	Citation
Historical Adaptation (HA) Analysis	*Alytes muletensis*	*1*	larva*†	Cumulative: 2.3 k zsp over 2w	160 days	Bd-Cape	Yes	Doddington et al. (29)
	Bombina orientalis	*6a*	adult	8 × 105 zsp	3 months	Bd-Asia1	Yes	Fu and Waldman (18)
	Brachycephalus ephippium	*7a*	adult	1.8 × 106 zsp	60 days	Bd-Brazil	Yes	Greenspan et al. (30)
	Brachycephalus pitanga	*9*	adult	3.9 × 106 zsp	15 days	Bd-Brazil	Yes	McDonald (28)
	Bufo bufo	*2a-b*	larva*†	Cumulative: 190 or 19k zsp every 4d over 2w (treatments averaged for analysis) Max total−5.7 × 10^4 zsp	80 days	Bd-Cape	No	Fisher et al. (2)
		3a-b	larva*	Cumulative: 3–17 k zsp every 4d × 8 reps; Max total−1.36 × 10^5 zsp	122 days	Bd-Cape	No	Farrer et al. (31)
		4a-c	larva*†	Cumulative: 7.5–37.5 k zsp every 4d × 8 reps; Max total = 3 × 10^5	42+ days	Bd-Asia1, Bd-Cape, Bd-CH	No	O'Hanlon et al. (14)
		4d-f	metamorph	Cumulative: 10–36 k zsp every 4 d × 5 reps; Max total = 8.5 × 10^4 zsp	22 days	Bd-Asia1, Bd-Cape, Bd-CH	No	O'Hanlon et al. (14)
	Dendropsophus minutus	*7b*	adult	1.8 × 106 zsp	60 days	Bd-Brazil	Yes	Greenspan et al. (30)
	Hymenochirus curtipes	*8*	adult	1.25 × 106 zsp	25 days	Bd-Brazil	No	Jenkinson et al. (32)
	Ischnocnema parva	*7c*	adult	1.8 × 106 zsp	60 days	Bd-Brazil	Yes	Greenspan et al. (30)
	Rana sylvatica	*5a*	larva*	106 zsp/tank at 0 and 17 d	70 days	Bd-Brazil	No	Becker et al. (4)
	Litoria caerulea	*6b*	adult	5 × 105 zsp	3 months	Bd-Asia1	No	Fu and Waldman (18)
Recent Adaptation (RA) Analysis	*Alytes obstetricans*	*10a-d*	metamorph	1 × 10^6 fo 24 h	13 weeks			Greener et al. (24)
	Anaxyrus americanus	*11a-c*	metamorph	3.33 × 105 zsp	73 days			Burrow et al. (33)
		12a	metamorph	10^{6-7} zsp + 10^{5-6} spg	40 days			Gahl et al. (34)
	Anaxyrus boreas	*13a-b*	larva†	105 zsp	20 days			Dang et al. (35)
	Bufo bufo	*2c*	larva*†	Cumulative: 190 or 19 k zsp every 4 d over 2 w (treatments averaged for analysis)	80 days			Fisher et al. (2)
		14	metamorph	4 ml of 4 × 10^6 zsp/ml inoculum for consecutive days	30 days			Meurling et al. (36)
	Physalaemus fernandezae	*15*	metamorph	6 × 10^4 for 5 h	14 days			Arellano et al. (37)
	Pseudacris regilla	*13c-d*	larva†	105 zsp	20 days			Dang et al. (35)
	Rana clamitans	*12b*	metamorph	10^{6-7} zsp + 10^{5-6} spg	40 days			Gahl et al. (34)
	Rana pipiens	*12c*	metamorph	10^{6-7} zsp + 10^{5-6} spg	40 days			Gahl et al. (34)
	Rana sylvatica	*12d*	larva*†	10^{6-7} zsp + 10^{5-6} spg	40 days			Gahl et al. (34)
		5b	larva*	106 zsp/tank	70 days			Becker et al. (4)
	Rana cascadae	*13e-f*	larva†	105 zsp	20 days			Dang et al. (35)
	Rana onca	*16a-b*	larva* & metamorph	3 × 106 zsp over 3d	18 weeks			Waddle et al. (38)

Studies in the top half of the table were included in the Historical Adaptation (HA) analysis, while those in the bottom half were included in the Recent Adaptation (RA) analysis. Study number corresponds with superscript notations in ***Figures 2***, ***4****. In studies where hosts were exposed to Bd as larvae, asterisks (*) denote that mortality values were reported post-metamorphosis, and crosses (†) denote that mortality values were reported pre-metamorphosis. Use of both symbols denotes mortality was recorded across both life stages. Abbreviations in the exposure dose column are as follows: zsp, zoospore; spg, zoosporangia; d, days; w, weeks; reps, repetitions. For HA analysis studies, the endemic Bd treatment column indicates the genotype of the endemic Bd lineage used in the experiment. Historical Bd coexistence is reported based on known or hypothesized historical coexistence of the focal host with the endemic Bd used in the experiment. Citation is provided with corresponding literature cited reference number in parentheses. See* ***Supplementary Table 1*** *for strain-specific genotyping information.*

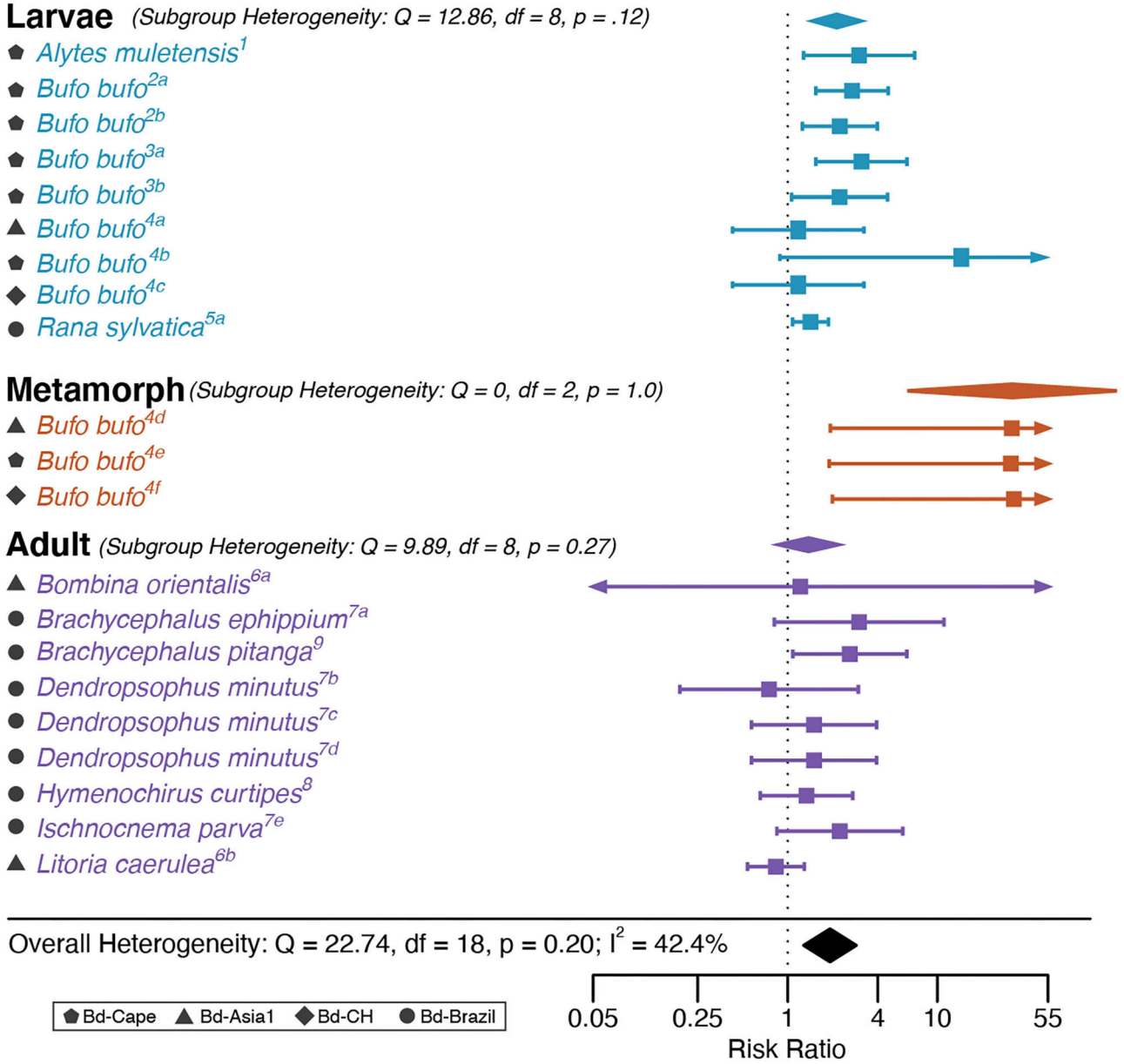


FIGURE 2 | Historical Adaptation analysis forest plot depicting Risk Ratio effect sizes for each life stage category. Squares indicate mean effect size for each study unit and error bars depict 95% Confidence Interval (CI). The meta-analytic mean effect size across study units (black diamond) was calculated without any subgroup moderator terms. Arrows on error bars indicate that the CI exceeds the provided x-axis scale. Study unit ID numbers correspond to **Table 1**. Heterogeneity test statistics (Q), degrees of freedom (df), and *p*-values for Cochran's Q test of heterogeneity are reported for each subgroup and the overall model.

heterogeneity ($I^2 = 42.38$ %; Q = 40.104, $p = 0.0048$). No outliers or influential cases were detected (Cook's D < 0.45; Hat < 0.14; dfbetas < 1).

The multifactor (full) HA model exhibited better fit than the intercept-only (base) model (AIC = 38.77 for the full model vs. 51.68 for the base model), and reduced heterogeneity in the dataset such that it was not significant (Q = 16.12, $p = 0.516$). The HA single-factor model that included host life stage was significant (QM = 14.24, $p = 0.0008$; **Figure 2**). Risk Ratio was higher in individuals infected as metamorphs compared with those infected as larvae or adults, and higher in individuals infected as larvae than as adults ($RR_{metamorph} = 6.9$, $RR_{larvae} = 1.99.1$, $RR_{adults} = 1.36$; **Figure 2**).

The single-factor model for historical coexistence was not statistically significant. However, data visualization showed evidence of greater variability in mortality due to infection with endemic Bd among hosts that had not historically coexisted with endemic Bd (**Figure 3** and **Supplementary Figure 2**). We further

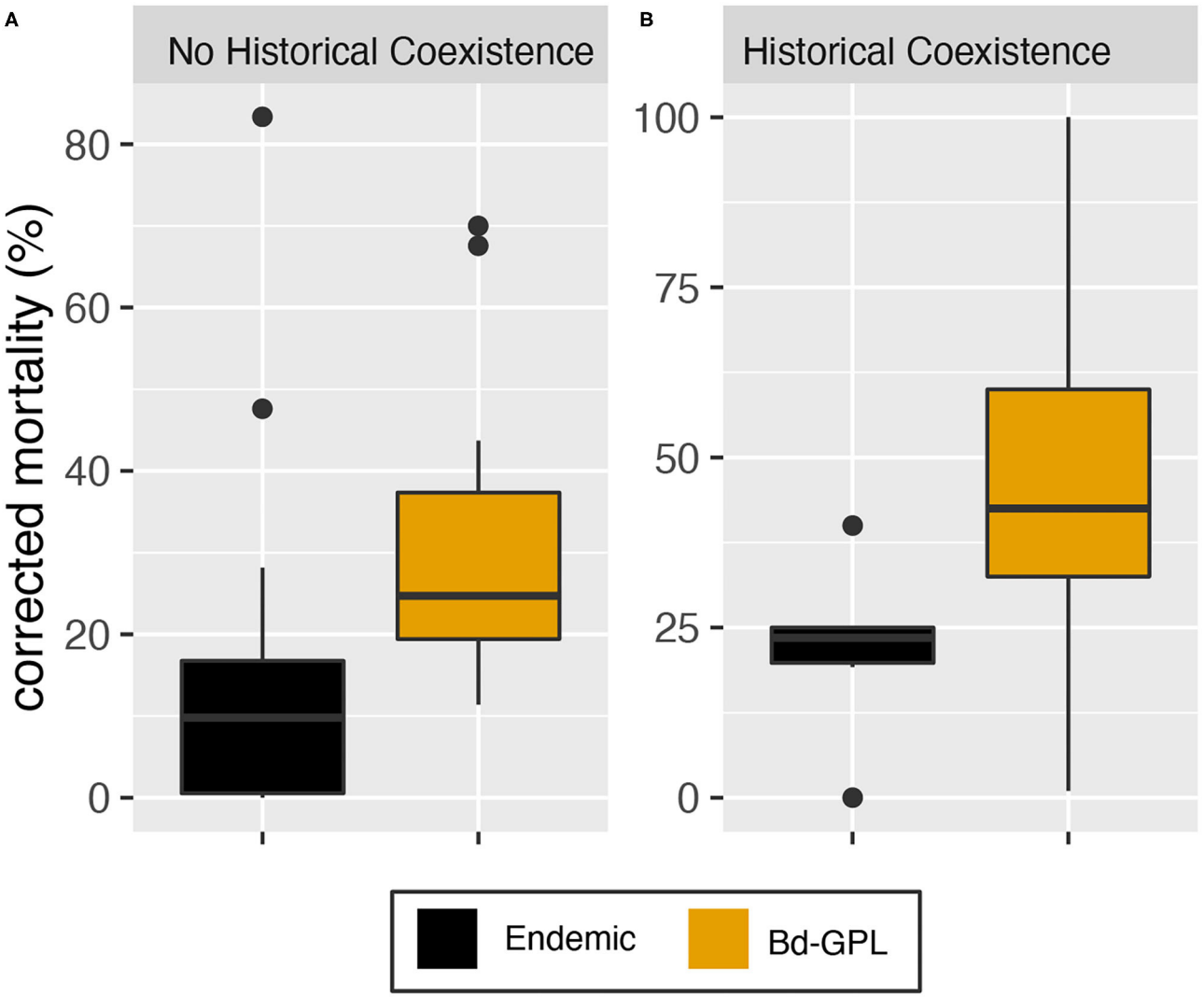


FIGURE 3 | Mortality outcomes pooled by Bd lineage. **(A)** Mortality in species that have not historically coexisted with the endemic Bd used in the experiment. **(B)** Mortality in species that have historically coexisted with the endemic Bd used in the experiment. Mortality values have been corrected for background (control treatment) mortality. Endemic Bd-associated mortality is depicted in black and Bd-GPL-associated mortality is depicted in ochre.

explored this relationship by testing for statistical differences in mortality variance in study units with historical coexistence vs. those without historical coexistence for endemic vs. panzootic (Bd-GPL) Bd genotypes. We found no statistically significant differences in variance for either Bd genotype group (Fligner-Killeen test: Bd-GPL–$X^2 = 1.57$, $p = 0.211$; endemic Bd–$X^2 = 0.46$, $p = 0.498$).

Recent Adaptation Analysis

In the RA analysis, the intercept-only model showed no significant effect of Bd-GPL collection locality on Risk Ratio (RR $= -0.996$, $Z = -0.024$, $p = 0.98$; **Figure 4**). The RA dataset showed a high degree of heterogeneity ($I^2 = 95.5\%$; Q = 66.54, $p = 0.001$). The moderator model including life stage at exposure did not exhibit better fit than the intercept-only model (AIC = 65.06 for the full model vs. 64.55 for the intercept-only model), and reduced but did not eliminate heterogeneity (Q = 64.27, $p < 0.001$). No outliers or influential cases were detected (Cook's D < 0.45; Hat < 0.18; dfbetas < 1).

DISCUSSION

Endemic Bd Lineages Are Associated With Reduced Chytridiomycosis Mortality

We performed meta-analyses of multi-strain Bd infection experiments to evaluate support for the hypotheses that the opportunity for historical or recent adaptation reduces chytridiomycosis-induced mortality. We found strong support for potential historical adaptation: we detected a significant effect of Bd genotype on Risk Ratio such that endemic Bd

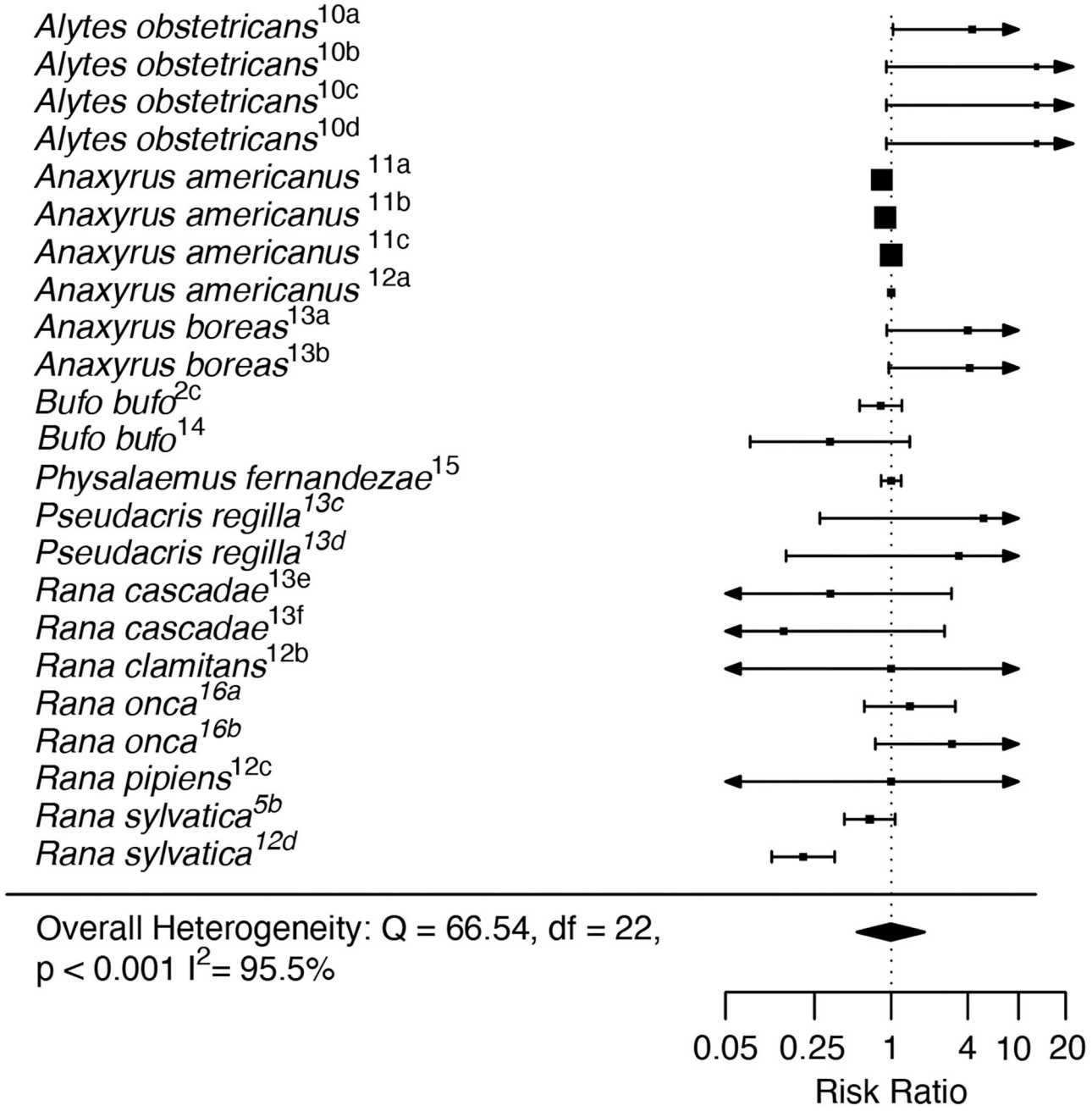


FIGURE 4 | Recent Adaptation analysis forest plot depicting Risk Ratio effect sizes across study units. Squares indicate mean effect size for each study unit, and error bars depict 95% Confidence Interval (CI). The meta-analytic mean effect across all study units (black diamond) was calculated without any moderator terms. Arrows on error bars indicate that the CI exceeds the provided x-axis scale. Study unit ID numbers correspond to **Table 1**. Heterogeneity test statistic (Q), degrees of freedom (df), and *p*-value is reported for Cochran's Q test of heterogeneity for the overall model.

lineages on average resulted in reduced mortality relative to Bd-GPL, regardless of whether hosts have historically coexisted with endemic Bd lineages. This apparent difference in virulence of Bd genotypes could have resulted through a number of mechanisms, including: historical coevolution between Bd and local amphibian hosts has led to a stable state of hypovirulence in endemic Bd lineages; genomic changes have resulted in hypervirulence in Bd-GPL; and/or, as a recently emerged lineage of Bd, Bd-GPL has escaped host defense mechanisms such that even hosts that have a long co-evolutionary history with endemic Bd are functionally naïve to Bd-GPL infections. These mechanisms are not mutually exclusive. For instance, hosts that

have historically coexisted with endemic Bd exhibited relatively consistent and generally low mortality rates when exposed to endemic Bd. This may indicate selection for reduced pathogen virulence and/or increased host resistance or tolerance in these systems. At the same time, ample phylogenetic and genomic evidence, the global spread of Bd-GPL, and its association with large-scale amphibian declines, support Bd-GPL as a recently emerged and widely introduced hypervirulent lineage (19, 31, 46). Independent of the mechanisms, our results confirm a strong difference in infection outcomes between endemic Bd lineages and Bd-GPL.

A notable exception to this overall pattern was Fu and Waldman (18), in which *L. caerulea* experienced high mortality from both Bd-Asia1 and Bd-GPL. The *L. caerulea* frogs used in this study were collected in New Guinea, which as of 2020 was entirely Bd-free (47). When compared with the other species in this study (*Hyla japonica, Bufo gargarizans,* and *Bombina orientalis*), which were collected from South Korea where Bd-Asia1 is historically endemic and widely distributed today (48), the pattern of disparity in outcomes due to differences in historical host adaptation opportunity is exemplified: Korean amphibians showed low mortality in both Bd-Asia1 and Bd-GPL treatments, suggesting that a long history with Bd-Asia1 may have led to evolution of broad Bd tolerance or resistance in these species. In contrast, entirely Bd-naïve *L. caerulea* frogs showed high chytridiomycosis mortality, even when infected with Bd-Asia1, which was previously reported to be hypovirulent (14). These findings suggest that Bd-Asia1 can be as virulent as Bd-GPL in naïve hosts, and that host defenses may be responsible for the apparent lack of epizootics due to Bd-Asia1 in Korean amphibian communities. Additional work is needed to assess the role of historical coexistence with endemic Bd in providing broad protection against lethal chytridiomycosis in other systems.

Individuals infected as metamorphs showed the highest Risk Ratios compared to all other life stages in the HA analysis. This may be due to the lag in the full development of the adaptive immune system following metamorphosis (49) and increased stress during this developmental transition (50, 51). Bd infects keratinized tissues in amphibians, which are restricted to mouthparts in larvae and become more widespread in post-metamorphic amphibian skin. In individuals infected as larvae, infection can shift through metamorphosis as more keratinized tissue becomes available on the body (52). Our results indicate that in the included studies, individuals infected at, rather than before, the metamorph stage, show the greatest increase in mortality due to Bd-GPL relative to endemic Bd. Metamorphosis is associated with immunosuppression, which functions to avoid a self-destructive autoimmune response as new adult-specific molecules develop but can also increase disease susceptibility in the vulnerable metamorph life stage (50). Full maturation of adult-like adaptive immunity takes a minimum of 2 months in amphibian species with fast development. Likewise, a full complement and mature levels of AMPs can be delayed until multiple months post-metamorphosis (53), although this is host species-dependent (49). Overall, our finding that individuals infected as larvae and metamorphs exhibit the highest Risk Ratios supports the idea that Bd-GPL poses a significant threat to these relatively poorly-surveyed life stages in the wild.

We note that the individuals that were infected as metamorphs in our dataset only represent a single study and species, *Bufo bufo* (14). However, this species was also exposed to comparable cumulative zoospore loads in two additional studies [57,000–136,000 zoospores over 2–4 weeks (2, 31); **Table 1**], and all three studies included the endemic lineage Bd-Cape. Comparing these three studies, *B. bufo* metamorphs show a higher Risk Ratio than larvae, indicating that metamorphs show the greatest difference in mortality between Bd-GPL and endemic Bd. Research on strain-specific and life stage-specific susceptibility in additional species is needed to determine whether these differences among larvae and metamorphs are common.

Recent Coexistence Is Insufficient to Reduce Lethal Chytridiomycosis

Our Recent Adaptation analysis comparing mortality due to infection by local vs. non-local Bd-GPL showed no overall effect on disease outcomes and no significant subgroup factors. We note that there were no available studies that were performed on adults, thus interpretation of our findings should potentially be limited to larval and metamorphic life stages. The relatively high variance in the results of the RA analysis may be explained by a number of factors that can influence disease outcomes. One important factor may be the host species from which Bd strains were isolated. Due to a lack of statistical power, we were unable to test the effect of host identity from which a Bd strain was isolated. However, we note one case study with disparities based on fine-scale origin of local Bd isolate. In Waddle et al. (38), the local Bd-GPL (LBP) was isolated from a *R. onca* frog collected from the same source population used for the experiment, while the non-local Bd-GPL (LC63) was isolated from a very sick captive White's tree frog (*Litoria caerulea*). It is noteworthy that the Bd-GPL strain isolated from a stable (non-epizootic) population of the focal host resulted in functionally zero mortality (no different from control), while the Bd-GPL strain isolated from a susceptible and sick host resulted in high mortality. In another treatment, *R. onca* were exposed to a relatively local Bd-GPL (SMR) that was instead isolated from *Pseudacris regilla* located ∼65 km from the nearest *R. onca* population. *P. regilla* is hypothesized to be Bd-tolerant and an important reservoir species in the western United States (54). Bd isolates from a stable population of a focal host, a susceptible species, or a reservoir species may have different consequences on the focal host as a result of selection for different virulence phenotypes. To aid in understanding the relationship between isolate origin and Bd virulence, it is important that future studies provide as much information as possible about the isolation history of Bd strains used in experiments.

In addition, local adaptation may be obscured by factors such as variation in Bd's phenotypic expression. Bd is known to exhibit phenotypic plasticity in response to temperature (55), and recent work has shown that Bd can be highly plastic over short time scales owing to functional variation in the pathogen's gene

expression (56). Although amphibians can exhibit relatively fast adaptive change in immune responses (57, 58), host evolution is still expected to lag behind that of a plastic pathogen. Nonetheless, in field studies, local recovery and persistence have been documented in post-epizootic amphibian communities (59) even where local Bd pathogenicity remains high (60). Our negative RA analysis results combined with findings from wild amphibian communities suggest that recovery following Bd epizootics may not necessarily be due to short-term adaptation to local Bd strains, but rather due to broad defense against or tolerance of Bd, or avoidance of infections altogether [e.g., see (61)].

Future Research Directions

Taken together, our results suggest that long periods of coexistence may be required for stable enzootic disease dynamics in the amphibian-chytridiomycosis system. We detected strong evidence across studies for hypovirulence in endemic Bd that may have arisen from historical coexistence with amphibian hosts. It remains unclear at what time scale adaptation of pathogen and/or host takes place, but our results do suggest that the relatively short time periods of coexistence with Bd-GPL in the geographic regions represented by the included studies (North America and Europe) have not yet been sufficient to protect many susceptible amphibian species from lethal chytridiomycosis. Further studies of the variation in disease outcomes resulting from endemic Bd lineages may provide a basis for identifying the mechanisms that reduce chytridiomycosis-induced mortality, and identifying cases where endemic Bd lineages retain virulence in naïve hosts. Similarly, host populations in which local adaptation may be beginning or in progress [e.g., in *Rana onca* (38)] may hold important insights into the potential for host adaptation to the highly successful and widely introduced Bd-GPL.

Our findings support the hypothesis that in many systems, endemic Bd lineages pose a lower threat to amphibian species than Bd-GPL. Recent studies have shown that previous infection with hypovirulent Bd-GPL strains can protect against severe chytridiomycosis due to hypervirulent Bd-GPL strains (24, 38). However, the question of whether historical coexistence with hypovirulent endemic Bd strains provide protection against Bd-GPL in the wild and/or in hosts that have not previously experienced Bd infection in their lifetimes remains to be adequately tested. Future research should focus on identifying the mechanisms that reduce lethal chytridiomycosis, which may include innate or adaptive immune mechanisms in the host, or reductions in virulence mechanisms in the pathogen. In addition, evaluating competitive dynamics between Bd-GPL and diverse endemic Bd strains may be critically important to determining the threat that Bd poses to local amphibian communities. To our knowledge, strain competition has only been assessed for Bd-GPL vs. Bd-Brazil to date (32). It remains unclear to what extent other endemic Bd lineages show similar competitive dynamics. Deepening our understanding of the relationships between co-evolutionary history, virulence, and disease outcomes will help predict and mitigate future hotspots of chytridiomycosis and aid in amphibian conservation efforts.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://github.com/m-bletz/Frontiers-meta-analysis-2021.

AUTHOR CONTRIBUTIONS

AB, MB, IR, and KZ conceived of the study. AB, IR, and MB compiled the data and created the figures. MB performed the analyses and visualized results. AB led manuscript writing. AB and MB co-led revisions. All authors contributed to writing and revising the manuscript.

FUNDING

This work was funded by a David H. Smith Postdoctoral Research Fellowship and a National Science Foundation Postdoctoral Research Fellowship in Biology that supported AB and NSF Career Grant to Douglas Woodhams that supported MB.

ACKNOWLEDGMENTS

The authors would like to thank A. Duffus and R. Marschang for inviting this contribution, C. McDonald for contributing unpublished data, and members of the Cannatella and Hillis labs for comments on the manuscript. MB would like to thank D. Woodhams for support during data analysis and manuscript preparation. The authors acknowledge that they performed this work as settlers on Indigenous Lands: AB and KZ worked on the Land of the Gayogo̱hó:nǫ' (Cayuga Nation), who are members of the Haudenosaunee Confederacy; IR worked on the Land of the Chumash Nation; and MB worked on traditional territories of Indigenous Peoples including the Pennacook and Massachuseuk Peoples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.756686/full#supplementary-material

REFERENCES

1. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, et al. Emerging fungal threats to animal, plant and ecosystem health. *Nature.* (2012) 484:186–94. doi: 10.1038/nature10947

2. Fisher MC, Bosch J, Yin Z, Stead DA, Walker J, Selway L, et al. Proteomic and phenotypic profiling of the amphibian pathogen Batrachochytrium dendrobatidis shows that genotype is linked to virulence. *Mol Ecol.* (2009) 18:415–29. doi: 10.1111/j.1365-294X.2008.04041.x

3. Lambertini C, Becker CG, Jenkinson TS, Rodriguez D, da Silva Leite D, James TY, et al. Local phenotypic variation in amphibian-killing fungus predicts infection dynamics. *Fungal Ecol.* (2016) 20:15–21. doi: 10.1016/j.funeco.2015.09.014
4. Becker CG, Greenspan SE, Tracy KE, Dash JA, Lambertini C, Jenkinson TS, et al. Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. *Fungal Ecol.* (2017) 26:45–50. doi: 10.1016/j.funeco.2016.11.007
5. Adams AJ, Kupferberg SJ, Wilber MQ, Pessier AP, Grefsrud M, Bobzien S, et al. Extreme drought, host density, sex, and bullfrogs influence fungal pathogen infection in a declining lotic amphibian. *Ecosphere.* (2017) 8:e01740. doi: 10.1002/ecs2.1740
6. Turner A, Wassens S, Heard G, Peters A. Temperature as a driver of the pathogenicity and virulence of amphibian chytrid fungus Batrachochytrium dendrobatidis: a systematic review. *J Wildl Dis.* (2021) 57:477–94. doi: 10.7589/JWD-D-20-00105
7. May RM, Anderson RM, Bodmer WF, Kingman JFC. Epidemiology and genetics in the coevolution of parasites and hosts. *Proc R Soc Lond B Biol Sci.* (1983) 219:281–313. doi: 10.1098/rspb.1983.0075
8. Retallick RWR, McCallum H, Speare R. Endemic infection of the Amphibian Chytrid Fungus in a frog community post-decline. *PLoS Biol.* (2004) 2:e351. doi: 10.1371/journal.pbio.0020351
9. Savage AE, Sredl MJ, Zamudio KR. Disease dynamics vary spatially and temporally in a North American amphibian. *Biol Conserv.* (2011) 144:1910–5. doi: 10.1016/j.biocon.2011.03.018
10. Thompson JN. Specific hypotheses on the geographic mosaic of coevolution. *Am Nat.* (1999) 153:S1–14. doi: 10.1086/303208
11. Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci USA.* (2008) 105:11466–73. doi: 10.1073/pnas.0801921105
12. 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.* (2019) 363:1459–63. doi: 10.1126/science.aav0379
13. Lambert MR, Womack MC, Byrne AQ, Hernández-Gómez O, Noss CF, Rothstein AP, et al. Comment on "Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity." *Science.* (2020) 367:eaay1838. doi: 10.1126/science.aay1838
14. O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, et al. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science.* (2018) 360:621–7. doi: 10.1126/science.aar1965
15. Schloegel LLM, Toledo LFL, Longcore JE, Greenspan SE, Vieira CA, Lee M, et al. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Mol Ecol.* (2012) 21:5162–77. doi: 10.1111/j.1365-294X.2012.05710.x
16. James TY, Toledo LF, Rödder D, da Silva Leite D, Belasen AM, Betancourt-Román CM, et al. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: Lessons from the first 15 years of amphibian chytridiomycosis research. *Ecol Evol.* (2015) 5:4079–97. doi: 10.1002/ece3.1672
17. Rachowicz LJ, Hero J-M, Alford RA, Taylor JW, Morgan JAT, Vredenburg VT, et al. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conserv Biol.* (2005) 19:1441–8. doi: 10.1111/j.1523-1739.2005.00255.x
18. Fu M, Waldman B. Ancestral chytrid pathogen remains hypervirulent following its long coevolution with amphibian hosts. *Proc R Soc B Biol Sci.* (2019) 286:20190833. doi: 10.1098/rspb.2019.0833
19. Farrer RA, Martel A, Verbrugghe E, Abouelleil A, Ducatelle R, Longcore JE, et al. Genomic innovations linked to infection strategies across emerging pathogenic chytrid fungi. *Nat Commun.* (2017) 8:14742. doi: 10.1038/ncomms14742
20. Rodriguez D, Becker CG, Pupin NC, Haddad CFB, Zamudio KR. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Mol Ecol.* (2014) 23:774–87. doi: 10.1111/mec.12615
21. Talley BL, Muletz CR, Vredenburg VT, Fleischer RC, Lips KR. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biol Conserv.* (2015) 182:254–61. doi: 10.1016/j.biocon.2014.12.007
22. Adams AJ, Pessier AP, Briggs CJ. Rapid extirpation of a North American frog coincides with an increase in fungal pathogen prevalence: historical analysis and implications for reintroduction. *Ecol Evol.* (2017) 7:10216–32. doi: 10.1002/ece3.3468
23. Jenkinson TS, Román CMB, Lambertini C, Valencia-Aguilar A, Rodriguez D, Nunes-de-Almeida CHL, et al. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Mol Ecol.* (2016) 25:2978–96. doi: 10.1111/mec.13599
24. Greener MS, Verbrugghe E, Kelly M, Blooi M, Beukema W, Canessa S, et al. Presence of low virulence chytrid fungi could protect European amphibians from more deadly strains. *Nat Commun.* (2020) 11:5393. doi: 10.1038/s41467-020-19241-7
25. Yap TA, Koo MS, Ambrose RF, Vredenburg VT. Introduced bullfrog facilitates pathogen invasion in the western United States. *PLoS ONE.* (2018) 13:e0188384. doi: 10.1371/journal.pone.0188384
26. Blaustein AR, Urbina J, Snyder PW, Reynolds E, Dang T, Hoverman JT, et al. Effects of emerging infectious diseases on amphibians: a review of experimental studies. *Diversity.* (2018) 10:81. doi: 10.3390/d10030081
27. Harzing A. *Publish or Perish.* (2007). Available online at: http://www.harzing.com/pop.htm
28. McDonald CA. *Too Little, Too Late: Amphibian Responses to Chytrid Fungus Across Time and Space* (2021).
29. Doddington BJ, Bosch J, Oliver JA, Grassly NC, Garcia G, Schmidt BR, et al. Context-dependent amphibian host population response to an invading pathogen. *Ecology.* (2013) 94:1795–804. doi: 10.1890/12-1270.1
30. Greenspan SE, Lambertini C, Carvalho T, James TY, Toledo LF, Haddad CFB, et al. Hybrids of amphibian chytrid show high virulence in native hosts. *Sci Rep.* (2018) 8:9600. doi: 10.1038/s41598-018-27828-w
31. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc Natl Acad Sci USA.* (2011) 108:18732–6. doi: 10.1073/pnas.1111915108
32. Jenkinson TS, Rodriguez D, Clemons RA, Michelotti LA, Zamudio KR, Toledo LF, et al. Globally invasive genotypes of the amphibian chytrid outcompete an enzootic lineage in coinfections. *Proc R Soc B.* (2018) 285:20181894. doi: 10.1098/rspb.2018.1894
33. Burrow AK, Rumschlag SL, Boone MD. Host size influences the effects of four isolates of an amphibian chytrid fungus. *Ecol Evol.* (2017) 7:9196–202. doi: 10.1002/ece3.3255
34. Gahl MK, Longcore JE, Houlahan JE. Varying responses of Northeastern North American Amphibians to the Chytrid Pathogen Batrachochytrium dendrobatidis. *Conserv Biol.* (2012) 26:135–41. doi: 10.1111/j.1523-1739.2011.01801.x
35. Dang TD, Searle CL, Blaustein AR. Virulence variation among strains of the emerging infectious fungus *Batrachochytrium dendrobatidis* (Bd) in multiple amphibian host species. *Dis Aquat Organ.* (2017) 124:233–9. doi: 10.3354/dao03125
36. Meurling S, Cortazar-Chinarro M, Siljestam M, Åhlen D, Ågren E, Höglund J, et al. Body size mediates latitudinal population differences in response to Bd infection in two amphibian species. (2021). 1–36. doi: 10.1101/2021.07.16.452656
37. Arellano ML, Natale GS, Grilli PG, Barrasso DA, Steciow MM, Lavilla EO. Host-pathogen relationships between the chytrid fungus Batrachochytrium dendrobatidis and tadpoles of five South American anuran species. *Herpetol J.* 27:33–39. Available online at: http://sedici.unlp.edu.ar/handle/10915/118298
38. Waddle AW, Levy JE, Rivera R, van Breukelen F, Nash M, Jaeger JR. Population-level resistance to Chytridiomycosis is life-stage dependent in an Imperiled Anuran. *EcoHealth.* (2019) 16:701–11. doi: 10.1007/s10393-019-01446-y
39. R Core Team. *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation of Statistical Computing (2021).
40. Pick JL, Nakagawa S, Noble DWA. Reproducible, flexible and high-throughput data extraction from primary literature: the METADIGITISE R package. *Methods Ecol Evol.* (2019) 10:426–31. doi: 10.1111/2041-210X.13118
41. Viechtbauer W. Conducting Meta-Analyses in R with the metafor Package. *J Stat Softw.* (2010) 36:1–48. doi: 10.18637/jss.v036.i03

42. Higgins JPT, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med.* (2002) 21:1539–58. doi: 10.1002/sim.1186
43. Harrer M, Cuijpers P, A FT, Ebert DD. *Doing Meta-Analysis With R: A Hands-On Guide*. 1st ed. Boca Raton, FL and London: Chapman & Hall/CRC Press (2021). doi: 10.1201/9781003107347
44. Noble DWA, Lagisz M, O'dea RE, Nakagawa S. Non-independence and sensitivity analyses in ecological and evolutionary meta-analyses. *Mol Ecol.* (2017) 26:2410–25. doi: 10.1111/mec.14031
45. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer (2009). doi: 10.1007/978-0-387-98141-3
46. James TY, Litvintseva AP, Vilgalys R, Morgan JA, Taylor JW, Fisher MC, et al. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathog.* (2009) 5:e1000458. doi: 10.1371/journal.ppat.1000458
47. Bower DS, Jennings CK, Webb RJ, Amepou Y, Schwarzkopf L, Berger L, et al. Disease surveillance of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Papua New Guinea. *Conserv Sci Pract.* (2020) 2:e256. doi: 10.1111/csp2.256
48. Bataille A, Fong JJ, Cha M, Wogan GOU, Baek HJ, Lee H, et al. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Mol Ecol.* (2013) 22:4196–209. doi: 10.1111/mec.12385
49. Rollins-Smith LA. Amphibian immunity–stress, disease, and climate change. *Dev Comp Immunol.* (2017) 66:111–9. doi: 10.1016/j.dci.2016.07.002
50. Rollins-Smith LA. Metamorphosis and the amphibian immune system. *Immunol Rev.* (1998) 166:221–30. doi: 10.1111/j.1600-065X.1998.tb01265.x
51. Robert J, Ohta Y. Comparative and developmental study of the immune system in Xenopus. *Dev Dyn Off Publ Am Assoc Anat.* (2009) 238:1249–70. doi: 10.1002/dvdy.21891
52. Marantelli G, Berger L, Speare R, Keegan L. Distribution of the amphibian chytrid Batrachochytrium dendrobatidis and keratin during tadpole development. *Pac Conserv Biol.* (2004) 10:173. doi: 10.1071/PC040173
53. Holden WM, Hanlon SM, Woodhams DC, Chappell TM, Wells HL, Glisson SM, et al. Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (Rana sphenocephala) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol Conserv.* (2015) 187:91–102. doi: 10.1016/j.biocon.2015.04.007
54. Reeder NMM, Pessier AP, Vredenburg VT. A reservoir species for the emerging amphibian pathogen Batrachochytrium dendrobatidis thrives in a landscape decimated by disease. *PLoS ONE.* (2012) 7:e33567. doi: 10.1371/journal.pone.0033567
55. Muletz-Wolz CR, Barnett SE, DiRenzo GV, Zamudio KR, Toledo LF, James TY, et al. Diverse genotypes of the amphibian-killing fungus produce distinct phenotypes through plastic responses to temperature. *J Evol Biol.* (2019) 32:287–98. doi: 10.1111/jeb.13413
56. McDonald CA, Ellison AR, Toledo LF, James TY, Zamudio KR. Gene expression varies within and between enzootic and epizootic lineages of Batrachochytrium dendrobatidis (Bd) in the Americas. *Fungal Biol.* (2020) 124:34–43. doi: 10.1016/j.funbio.2019.10.008
57. Savage AE, Zamudio KR. MHC genotypes associate with resistance to a frog-killing fungus. *Proc Natl Acad Sci USA.* (2011) 108:16705–10. doi: 10.1073/pnas.1106893108
58. Bataille A, Cashins SD, Grogan L, Skerratt LF, Hunter D, McFadden M, et al. Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. *Proc Biol Sci.* (2015) 282:20143127. doi: 10.1098/rspb.2014.3127
59. Scheele BC, Skerratt LF, Grogan LF, Hunter DA, Clemann N, McFadden M, et al. After the epidemic: ongoing declines, stabilizations and recoveries in amphibians afflicted by chytridiomycosis. *Biol Conserv.* (2017) 206:37–46. doi: 10.1016/j.biocon.2016.12.010
60. Voyles J, Woodhams DC, Saenz V, Byrne AQ, Perez R, Rios-Sotelo G, et al. Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. *Science.* (2018) 359:1517–9. doi: 10.1126/science.aao4806
61. Bell SC, Heard GW, Berger L, Skerratt LF. Connectivity over a disease risk gradient enables recovery of rainforest frogs. *Ecol Appl.* (2020) 30:e02152. doi: 10.1002/eap.2152

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership