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NOTE

Virulence variation among strains of the emerging infectious fungus *Batrachochytrium dendrobatidis* (*Bd*) in multiple amphibian host species

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ABSTRACT: Emerging infectious diseases have been documented in numerous plant and animal populations. The infectious disease amphibian chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is associated with global amphibian population declines. While much *Bd*-amphibian research has centered on response variation in hosts, a paucity of information exists on how variation in the pathogen, such as strain differences, affects infection dynamics. To examine how different *Bd* strains may differentially impact multiple hosts, we conducted laboratory experiments to measure 2 infection outcomes, viz. host survival and pathogen load, in 3 amphibian host species (Pacific treefrog, western toad, and Cascades frog) after exposure to 3 different *Bd* strains (an additional fourth *Bd* strain was tested in toads only). Our results confirm that the infection response differs among host species. Western toads experienced significant mortality, but Pacific treefrogs and Cascades frogs did not. Interestingly, our experiment also captured strain-dependent virulence variation but only in 1 host species, the western toad. Increased mortality was observed in 2 of the 4 *Bd* strains tested in this host species. Toads were also the only host species found to have variable pathogen load dependent on strain type; individuals exposed to the Panama strain harbored significantly higher loads compared to all other strains. These findings underscore the dynamic nature of *Bd* infection, showing that virulence can vary contingent on host and strain type. We highlight the importance of both host- and pathogen-dependent factors in determining overall infection virulence and show the need for *in vivo* testing to fully assess pathogenicity.

KEY WORDS: Virulence · Chytridiomycosis · Strain · Parasite · Amphibian disease

INTRODUCTION

Emerging infectious diseases (EIDs) are increasing worldwide, threatening biodiversity and public health (Fisher et al. 2012). The increase in disease emergence is often linked to human activities such as urbanization, land-use change, wildlife trade, pollution, and climate change (Farrer et al. 2011, Fisher et al. 2012, Tompkins et al. 2015). Pathogenic fungi are responsible for many well-documented EIDs such as white nose syndrome in bats and wheat stem rust (Fisher et al. 2012). One high-profile fungal pathogen, the chytrid *Batrachochytrium dendrobatidis* (*Bd*), which causes the disease amphibian chytridiomycosis, has been found in over 500 amphibian species and is listed as a major contributor to global amphibian population declines (Fisher et al. 2012, Tompkins et al. 2015). *Bd* infection susceptibility and virulence are dynamic and can depend on both host- and pathogen-associated factors (Fisher et al. 2009,

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Farrer et al. 2011, Gervasi et al. 2013, Bradley et al. 2015). Additionally, the active global trade in live amphibians raises the risk of contact between non-native host species and pathogen strains (Schloegel et al. 2009). By investigating how infection virulence varies among different host and pathogen types, we can uncover virulence variation in the amphibian–*Bd* system that could help us understand the consequences of *Bd* strain introduction due to the live amphibian trade.

The effects of chytridiomycosis can vary among host species and life stages (Blaustein et al. 2005, Garner et al. 2009, Gervasi et al. 2013) and can even vary between populations of the same species (Bradley et al. 2015). *Bd* infects keratinized tissues in amphibians, i.e. the mouthparts in larvae and the skin in adults. Although *Bd* infection is thought to have the most profound effects on amphibians during and post-metamorphosis, exposure during the aquatic larval stage can also result in high larval mortality (Blaustein et al. 2005, Fisher et al. 2009, Searle et al. 2011; but see Gervasi et al. 2013) and can influence post-metamorphic fitness (Garner et al. 2009). Other host traits such as behavior and skin microbiota may also affect host susceptibility and survival (Venesky et al. 2014, Becker et al. 2015).

Additionally, infection dynamics may be driven by intrinsic pathogen-related factors (Salvaudon et al. 2005). Comparative genomics studies have identified a *Bd*-global panzootic lineage (GPL) containing North and Central American strains associated with high virulence and amphibian population declines (Farrer et al. 2011, Schloegel et al. 2012, Rosenblum et al. 2013). Like other emerging parasites, the global spread of *Bd* strains, particularly those in the *Bd*-GPL, has been linked to the commercial amphibian trade (Schloegel et al. 2009, 2012, Farrer et al. 2011). Amphibian trafficking activities increase the likelihood of contact between foreign hosts and strains via the release of live animals and/or housing water (Schloegel et al. 2009, 2012). Furthermore, *Bd* strain introduction may facilitate a sexual recombination event resulting in a hybrid. Schloegel et al. (2012) first detected a hybrid *Bd* strain on a wild Brazilian bullfrog; a multi-locus sequence typing analysis suggested it originated from a GPL and an endemic Brazilian strain. Even so, research exploring virulence differences among strains is still a nascent endeavor. Although virulence variation due to host factors has been documented in the amphibian–*Bd* system, most *Bd* studies investigating the effects of strain differences have only focused on responses within a single host species (Berger et al. 2005, Retallick & Miera 2007, Farrer et al. 2011, Piovia-Scott et al. 2015; but see Gahl et al. 2012). Due to the limited information on how infection virulence varies with different *Bd* strains and different host species, we sought to investigate the dynamic nature of this disease by measuring host survival and pathogen load in a comparative experiment using different North and Central American *Bd* strains and North American host species.

MATERIALS AND METHODS

Bd culture

All host species were tested with the following *Bd* strains: JEL425, isolated in Panama; JEL630, isolated in Oregon, USA; and JEL646, isolated in California, USA. All but one *Bd* strain (JEL425) in this study have been used in phylogenetic analyses placing them in the putatively virulent GPL (Schloegel et al. 2012, Rosenblum et al. 2013; Table 1). JEL425 was isolated in Panama 2004, a period when this region was experiencing amphibian population losses, but has never been used in a comparative genomics analysis to establish membership in the GPL clade. An additional GPL strain, JEL627 (Rosenblum et al. 2013), isolated in Maine, USA, was tested on western toads, a known sensitive species (Searle et al. 2011). These strains represent novel isolates from a range of geographic distances from the collection site of the 3 North American focal host species used in this study (Table 1). Strains will be referred to by their geo-

Table 1. *Batrachochytrium dendrobatidis* (*Bd*) strains used in this experiment including isolate identity, geographic origin, year isolated, and host species

Isolate	Geographic origin	Year isolated	Amphibian host
JEL425	El Cope, Coclé (Panama)	2004	*Bufo haematiticus*
JEL627	Bethel, Maine (USA)	2009	*Lithobates catesbeianus*
JEL630	Finley National Wildlife Refuge, Oregon (USA)	2009	*Lithobates catesbeianus*
JEL646	Point Reyes, California (USA)	2010	*Pseudacris regilla*

graphic origin (e.g. 'Oregon *Bd*' for JEL630). *Bd* strains were obtained from initial cryogenic stock from the lab of Dr. Joyce Longcore (University of Maine). All strains were cultured at ~20°C; Maine, California, and Panama isolates underwent 2 passages through 1% tryptone broth, while the Oregon isolate went through 5 passages before plating for use. Before the experiment, all strains were plated on 1% tryptone-agar plates for 1 to 2 wk.

Animal husbandry

Host species in this study are commonly found in the US Pacific Northwest: Pacific treefrog *Pseudacris regilla*, Cascades frog *Rana cascadae*, and western toad *Anaxyrus boreas*. Cascades frog and western toad population declines have been documented in the western USA and are both species of concern in this region (Muths et al. 2003, Piovia-Scott et al. 2015). Experimental evidence has demonstrated that Pacific treefrogs are able to tolerate relatively high *Bd* loads compared to sympatric species, making them a possible reservoir host (Reeder et al. 2012, Gervasi et al. 2013). To make sure study animals did not have prior *Bd* infections, newly laid eggs of each species were collected from the Cascades Range of Oregon and reared in the laboratory until they reached Gosner developmental stages 26 to 34 (first emergence of limb bud to initial differentiation of digits; Gosner 1960). Tadpoles were housed in 10 l glass aquaria at a density of 100 tadpoles tank^{-1} at ~15°C under a natural photoperiod. Tadpoles were fed every other day with a 3:1 ground mix of alfalfa pellets and fish flakes (Brine Shrimp Direct).

Experimental protocol

We examined the effects of *Bd* in the larval stage for all species. Once hatched, tadpoles were randomly assigned to different *Bd* strain treatment groups (Table 2). Individuals were housed in 1 l plastic cups filled with 600 ml of dechlorinated water and allowed to acclimate for 24 h before pathogen exposure. *Bd*-agar plates (7–8 plates per *Bd* strain group) were flooded with 10 ml of dechlorinated water, and actively swimming zoospores were quantified with a hemocytometer after 10 min. Each *Bd*-treated tadpole received 10 ml of *Bd* broth at a concentration of 1×10^4 zoospores ml^{-1} for a total of 1×10^5 zoospores (a dose used previously tested in the same larval host species by Gervasi et al. 2013). *Bd* broth was not filtered and thus contained both zoospore and sporangia structures. The process was repeated on sterile agar plates for the control group.

Water was changed once a week for the duration of the experiment (20 d). Animals were monitored daily for survival, and deceased individuals were preserved in 95% ethanol (EtOH). At the end of the experiment, all remaining animals were euthanized in MS-222 and preserved in 95% EtOH. For all individuals, ending measurements of mass and snout–vent length (SVL) were taken.

DNA extraction and qPCR

A subsample of 15 *Bd*-exposed animals and 3 control animals from each species underwent quantitative PCR (qPCR) analysis to determine *Bd* infection load. Whole mouthparts were excised and processed following Boyle et al. (2004) except that we used 60 µl of Prepman Ultra (Applied Biosystems) instead of 40 µl in all DNA extractions. Each sample was run in triplicate, and an average genome equivalent (GE) for each sample was calculated against *Bd* standards made from transgenic *Escherichia coli* cultures carrying plasmids of the *Bd* internal transcribed spacer region with standard titrations from 10^{-1} to 10^2 (USGS, https://water.usgs.gov/nrp/microbiology/resources/resources.html#Bd

Table 2. Experimental hosts and *Batrachochytrium dendrobatidis* (*Bd*) strain treatment groups with sample sizes; (–) no treatment

Host species	Strain treatments (sample size)				
	Control	California (JEL646)	Maine (JEL627)	Oregon (JEL630)	Panama (JEL425)
Pacific treefrog *Pseudacris regilla*	14	14	–	16	15
Western toad *Anaxyrus boreas*	30	29	30	30	30
Cascades frog *Rana cascadae*	15	23	–	24	24

_std). Quantitative PCR was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems) using primers and probes developed by Boyle et al. (2004). A no-template control containing nanopure water was included in each qPCR plate and always tested negative. An animal was considered positive if 2 of the 3 qPCR replicates tested positive. There was never an instance where only 1 replicate tested positive. GEs were averaged from all positive qPCR replicates for each *Bd*-exposed animal.

Data analysis

Cox proportional hazards models were employed to compare rates of survival among *Bd* treatments for each host species (Cox 1972). This test compares survival curves to analyze the probability of mortality from different variables (likelihood ratio test). The probability of mortality due to a factor is represented by a hazard ratio. Infection load data (in units of GE ind.$^{-1}$) were log-transformed to meet parametric assumptions (log-GE + 1) before conducting statistical tests (Table 3). We conducted analyses of variance (ANOVA) with each amphibian host species to test *Bd* strain and experimental days alive as main effects. After log transformation, 3 extreme outliers were detected (>3× the inter-quartile range) and removed from load analysis in Pacific treefrogs to meet data assumptions of normality (*Bd* treatment groups California, Oregon, and Panama each had 1 individual removed; Oregon and Panama outliers had no detectable *Bd* load). Significant results were followed by a Tukey's HSD test. All analyses were performed in R with the package 'survival' (R Core Team 2014).

RESULTS

Host survival

Western toad survival was not affected by exposure to Oregon and Maine *Bd* strains (Fig. 1A). However, western toad mortality increased significantly by a factor of 2.21 (95% CI: 1.02–4.79) and 2.51 (95% CI: 1.16–5.45) when exposed to the Panama and California *Bd* strains, respectively (Fig. 1A). No *Bd* strains affected survival of Pacific treefrog and Cascades frog tadpoles when compared to control groups ($p > 0.05$ in all cases, Fig. 1B,C).

Host pathogen load

We used ANOVAs followed by Tukey's HSD tests to determine infection load differences among treatments for each host species. *Bd* load was not predicted by number of days alive in any species. In Cascades frogs and Pacific treefrogs, strain type did not significantly affect pathogen load. However, *Bd* load did significantly differ among strain types in western toads ($F_{3,55} = 21.9$, $p < 0.001$). Toads exposed to Panama *Bd* had significantly higher loads compared to California, Maine, and Oregon *Bd* treatments (Tukey's HSD test $p \leq 0.001$ in all cases, Fig. 2A). *Bd* infection loads did not differ among the other strains. In Pacific treefrogs, California and Panama *Bd* treatment groups each had 1 animal that did not have detectable *Bd* infection following qPCR analysis. Weight and SVL were not associated with pathogen load. All control animals sampled for qPCR tested negative for *Bd* infection. Overall, Cascades frogs had lower loads compared to western toads and Pacific treefrogs (Fig. 2). Variation in *Bd* load due to

Table 3. Range, median, and mean of untransformed pathogen loads (genome equivalents) for different strains of *Batrachochytrium dendrobatidis* (*Bd*)

Host species	*Bd* strain	Range	Median	Mean
Pacific treefrog *Pseudacris regilla*	California (JEL646)	9.6–1273.3	417.3	493.9
	Oregon (JEL630)	0–3567.8	381.8	1170.5
	Panama (JEL425)	0–2471.5	1088.7	1032.0
Western toad *Anaxyrus boreas*	California (JEL646)	18.4–892.6	345.1	489.5
	Maine (JEL627)	55.3–984.0	168.7	268.3
	Oregon (JEL630)	26.7–612.1	200.7	227.3
	Panama (JEL425)	524.1–4338.3	1677.6	1760.2
Cascades frog *Rana cascadae*	California (JEL646)	3.8–406.6	22.1	54.4
	Oregon (JEL630)	1.4–78.8	25.3	28.4
	Panama (JEL425)	4–470.1	32.3	93.4

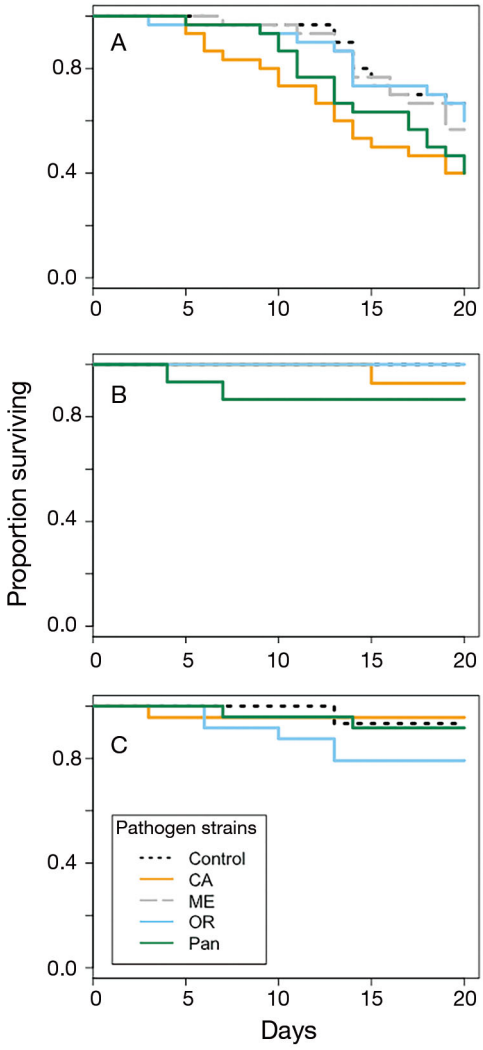

Fig. 1. Survival curves of *Batrachochytrium dendrobatidis* (*Bd*)-exposed animals and controls for each host species. (A) Western toad *Anaxyrus boreas* survival was significantly lowered in the Panama (JEL425) and California (JEL646) *Bd* treatment group compared to the control treatment group. (B) Pacific treefrog *Pseudacris regilla* survival was high and not affected by exposure to any *Bd* strain. The control survival curve is obscured behind the Oregon *Bd* survival curve; both treatments had 0 mortality. (C) Cascades frogs *Rana cascadae* did not show any significant survival differences among treatment groups. CA: California; ME: Maine; OR: Oregon; Pan: Panama

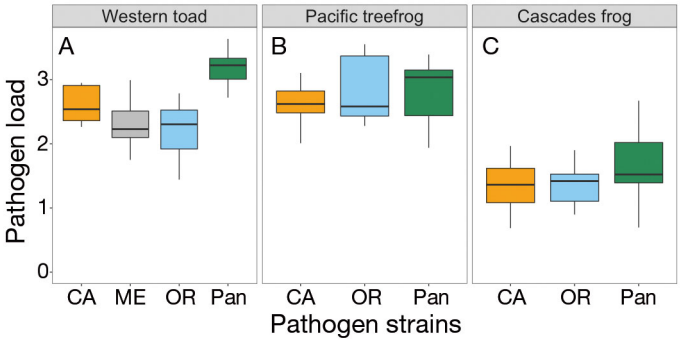

Fig. 2. Average pathogen load (log genome equivalents + 1) results. (A) Western toad *Anaxyrus boreas* pathogen loads were significantly higher in the *Batrachochytrium dendrobatidis* (*Bd*) Panama (JEL425) strain treatment group compared to the California (JEL646), Maine (JEL627), and Oregon (JEL630) treatment groups. (B,C) For Pacific treefrogs *Pseudacris regilla* and Cascades frogs *Rana cascadae*, there was no difference in average pathogen load among any *Bd* strain treatments. CA: California; ME: Maine; OR: Oregon; Pan: Panama. Thick horizontal lines: medians; boxes: interquartile range (IQR); whiskers: full data range within (±) 1.5 × IQR

strain type was only apparent in western toads (i.e. we could not detect strain differences in other host species).

DISCUSSION

We found that variation in host mortality and infection load was driven by differences among amphibian hosts and pathogen strain type. By comparing disease outcomes among different combinations of host species and pathogen strains, we found *in vivo* virulence variation that may not have been detected if we had only tested a single host species or pathogen strain. We also tested amphibian hosts reared from eggs to ensure that infection virulence was not affected by prior host infection history. Exposure to all tested *Bd* strains did not significantly impact survival of Pacific treefrog and Cascades frog hosts, supporting previous work that found larvae of these species to be relatively robust to *Bd* infection (Blaustein et al. 2005, Reeder et al. 2012, Gervasi et al. 2013). Pacific treefrog adults have been previously named as an important reservoir host for *Bd* due to their ability to harbor high pathogen loads without incurring significant mortality (Reeder et al. 2012). Our study suggests this may be true in the larval stages as well. Cascades frogs had the lowest pathogen loads across all *Bd* strains, concurring with prior studies showing this species to be relatively resistant to *Bd* infection (Searle et al. 2011, Gervasi et al. 2013). One explanation for this consistent pattern is pathogen consumption by the host, although this has yet to be definitively tested in this species (Keesing et al. 2006, Venesky et al. 2014).

Although western toads and Pacific treefrogs carried similar pathogen loads, toads displayed decreased infection tolerance as evidenced by significantly increased mortality from 2 of the 4 strains (Fig. 1A). Since *Bd* infects larval mouthparts, impaired

feeding ability is expected to be the main mechanism that reduces survival, although other factors have been suggested, such as mortality due to an inadequate or costly immune response (Garner et al. 2009). While our results support research positing toads to be more vulnerable to *Bd*-induced mortality than other sympatric species (Carey et al. 2006, Searle et al. 2011), they also show that toad mortality can vary by *Bd* strain type. Toads exposed to *Bd* strains from Oregon, Maine, and California had similar infection loads but significant mortality was only seen in those exposed to the California strain. This difference in mortality may be due to underlying pathogen-related factors (e.g. strain-specific toxin production, local evolutionary history, growth rate). Our results emphasize the dynamic nature of infectious diseases, as virulence is an emergent property mediated here by the interactions between the host and pathogen.

Comparative intraspecific *Bd* research can also reveal the potential consequences of the global trade in amphibians for food, pets, bait, research, etc. The amphibian trade, especially in a highly *Bd*-tolerant host, the North American bullfrog *Lithobates catesbeianus*, is quickly facilitating the spread of *Bd* strains around the world (Schloegel et al. 2009, 2012). A 6 yr, 3 city survey of live amphibian markets found an overall *Bd* infection prevalence of 62% in live frogs sold for human consumption. Amphibians sampled for this survey (Schloegel et al. 2009) came from wild and captive populations in Asia and South America and did not include species involved in the pet trade. Another genomics study carried out by Schloegel et al. (2012) found Brazilian *Bd* strains present in a live bullfrog from a US market as well as in wild invasive bullfrogs in Japan. These studies provide evidence suggesting that global trafficking of amphibians can enable the global spread of novel *Bd* strains. Although our study was a controlled laboratory experiment, the data indicate functional virulence variation among geographically disparate *Bd* strains and suggest that realistic scenarios resulting in exotic *Bd* strain introduction, such as via the amphibian trade, may hold adverse consequences to susceptible, naïve amphibian hosts. Comprehensive *Bd* strain research can be used to inform amphibian trade policy and regulations by identifying and monitoring geographic regions and host populations in danger of exposure to or currently with high-virulence *Bd* strains in circulation. Additional experimental research exploring genomic *Bd* strain diversity and disease pathology is needed to better understand the underlying mechanisms mediating virulence in *Bd* infections.

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