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ORIGINAL ARTICLE

Correlates of virulence in a frog-killing fungal pathogen: evidence from a California amphibian decline

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The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has caused declines and extinctions in amphibians worldwide, and there is increasing evidence that some strains of this pathogen are more virulent than others. While a number of putative virulence factors have been identified, few studies link these factors to specific epizootic events. We documented a dramatic decline in juvenile frogs in a *Bd*-infected population of Cascades frogs (*Rana cascadae*) in the mountains of northern California and used a laboratory experiment to show that *Bd* isolated in the midst of this decline induced higher mortality than *Bd* isolated from a more stable population of the same species of frog. This highly virulent *Bd* isolate was more toxic to immune cells and attained higher density in liquid culture than comparable isolates. Genomic analyses revealed that this isolate is nested within the global panzootic lineage and exhibited unusual genomic patterns, including increased copy numbers of many chromosomal segments. This study integrates data from multiple sources to suggest specific phenotypic and genomic characteristics of the pathogen that may be linked to disease-related declines.

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Introduction

Disease impacts are traditionally viewed as an interaction between characteristics of host, pathogen and environment (the ‘epidemiologic triangle’). Many epizootic events are driven by the emergence of hyper-virulent strains of a pathogen (Lipsitch *et al.*, 2003; LaDeau *et al.*, 2007; Smith *et al.*, 2009). In these cases, pathogen characteristics overshadow the effects of hosts and environment. Understanding the genetic and physiological changes in a pathogen that contribute to hyper virulence requires an integrated research approach that links carefully designed virulence assays and detailed studies of putative virulence factors to specific epizootic events.

Batrachochytrium dendrobatidis (*Bd*) is an emerging fungal pathogen that has been implicated in

epidemics and extinctions of amphibians worldwide (Berger *et al.*, 1998; Lips *et al.*, 2006; Wake and Vredenburg, 2008). *Bd* causes the disease chytridiomycosis, and its effect on amphibians has been described as ‘the most spectacular loss of vertebrate biodiversity due to disease in recorded history’ (Skerratt *et al.*, 2007). There is considerable variation in the effect of *Bd* on amphibian populations, ranging from rapid extirpation of the host to long-term coexistence of host and pathogen (Kilpatrick *et al.*, 2010). The effect of *Bd* on amphibian hosts can be influenced by environmental factors, most notably temperature (for example, Kriger *et al.*, 2007; Richards-Zawacki, 2010) and host susceptibility (reviewed in Fisher *et al.*, 2009b). In addition, several laboratory experiments document variation in virulence between different *Bd* isolates (Berger *et al.*, 2005; Retallick and Miera, 2007; Fisher *et al.*, 2009a; Farrer *et al.*, 2011; Gahl *et al.*, 2012).

Recently there have been significant advances in our understanding of the mechanisms underlying *Bd* virulence (which we define as the host’s

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pathogen-induced loss of fitness). First, whole genome sequencing and expression studies have led to new insights into the phylogenetics and functional genomics of *Bd*. Specifically, genomic approaches identified distinct lineages within *Bd*, including a global panzootic lineage (GPL) that contains many of the most deadly *Bd* isolates (Farrer *et al.*, 2011; Rosenblum *et al.*, 2013); such analyses have also identified gene families that may have a role in *Bd* virulence (for example, Joneson *et al.*, 2011; Rosenblum *et al.*, 2012; Farrer *et al.*, 2013). Second, specific *Bd* traits have been linked to virulence, including zoospore production (Langhammer *et al.*, 2013), zoosporangium size (Fisher *et al.*, 2009a) and the ability to inhibit the growth of immune cells (Fites *et al.*, 2013). Here, we integrate field surveys of host and pathogen dynamics with a laboratory exposure experiment that provides a rigorous virulence assay, *in vitro* assays of putative virulence traits and genomic characterization of *Bd* isolates, thereby linking phenotypic and genomic virulence mechanisms to an amphibian decline in the field.

Our study focuses on the Cascades frog (*Rana cascadae*), a montane species that ranges from southern Canada to northern California. Laboratory experiments indicate that post-metamorphic *R. cascadae* are susceptible to *Bd* (Garcia *et al.*, 2006), and dramatic declines in California's Cascade Mountains are thought to be caused by *Bd* (Fellers *et al.*, 2008; Pope *et al.*, 2014). However, *Bd* is widespread in the range of *R. cascadae* (Adams *et al.*, 2010; Piovía-Scott *et al.*, 2011) and declines are not universal (Pearl *et al.*, 2009), suggesting that there is important variation in the effect of *Bd* on populations of this host amphibian. One study suggested that juvenile *R. cascadae* have higher *Bd* prevalence than other life stages (Piovía-Scott *et al.*, 2011), and many affected populations experience repeated recruitment failures in which very few individuals survive their first year after metamorphosis (Pope *et al.*, 2014), suggesting that juveniles are particularly vulnerable. Here, we document a dramatic decline in juvenile *R. cascadae* at a lake in northern California and show that this decline may have been linked to the emergence of a hypervirulent, highly immunotoxic strain of *Bd*.

Materials and methods

Host-pathogen dynamics in the field

We evaluated *R. cascadae* population size, *Bd* prevalence and *Bd* infection intensity at Section Line Lake in the Klamath Mountains and at Carter Meadow in the southern Cascade Mountains (Supplementary Figure S1). Between 2008 and 2013 we measured frog abundance using visual encounter surveys (Crump and Scott, 1994; Pope, 2008). Each site was visited multiple times over the course of each season (except for Section Line in

2012); we used the highest counts of adults and juveniles from a single survey as our abundance estimates for each year (Supplementary Table S1 shows count data from all surveys conducted during the study period). The count data presented in the results were collected under conditions conducive to observing frogs (mostly clear skies, warm air temperatures and patches of sunlight for basking).

We sampled frogs for *Bd* using sterile rayon swabs (Medical Wire and Equipment, Wiltshire, England). Each animal was swabbed five times along the ventral surface of the trunk, five times along the ventral surface of each thigh and five times across each foot. We evaluated the amount of *Bd* on each swab in terms of zoospore equivalents (ZE) using real-time quantitative PCR (Boyle *et al.*, 2004; Hyatt *et al.*, 2007; Retallick *et al.*, 2006; electronic Supplementary Information). *Bd* data was collected between 2008 and 2011. To avoid confounding effects of seasonality, we analyzed *Bd* data collected between late July and early September.

We tested for differences in *Bd* prevalence between the study sites using mixed logistic regression. *Bd* presence/absence on each frog swabbed (1 vs 0) was the response variable, study site, life stage and year (and the site-year interaction) were fixed predictors, and individual animal was used as a random effect to account for non-independence of observations from the same individual (see electronic Supplementary Material). In 2009–2011 we tested for differences between the two study sites in infection intensity for animals that tested positive for *Bd* (infection intensity data were not available from the 2008 surveys). We used analysis of variance (ANOVA) and applied the same fixed predictor variables as in the analysis of *Bd* prevalence. Few individuals were resampled in the load data set, so we used only one data point for each animal. Infection intensity data were log-transformed to meet ANOVA assumptions.

Laboratory exposure experiment

We used a laboratory experiment to test for differences in virulence between *Bd* isolated from Section Line Lake and Carter Meadow and to test for variation in the susceptibility of *R. cascadae*. In 2011, we isolated *Bd* from *R. cascadae* collected at Carter Meadow and Section Line Lake and collected *R. cascadae* eggs from five locations (Supplementary Table S3, Supplementary Figure S1). Frogs from each site were reared separately in a standardized environment. After metamorphosis frogs from each site were exposed to each of the two *Bd* isolates, or given sham 'control' exposures, in a fully crossed design. Frog survivorship and *Bd* infection intensity were then monitored for 14 weeks (see electronic Supplementary Material for details).

We evaluated the effect of *Bd* treatment and frog collection site on frog survival using Cox's proportional hazard model. To test for effects of *Bd*

treatment and frog collection site on *Bd* prevalence and infection intensity in living frogs, we used mixed models. These *Bd* analyses were conducted using data from the first 2 weeks of the experiment, as there were sharp increases in mortality in some of the treatment groups after that point. Thus, the results provide insight into the rate of *Bd* prevalence and infection intensity increase prior to the onset of *Bd*-related mortality. *Bd* isolate, frog origin and week after exposure (week 1 or 2) were fixed predictors, and frog identity was included as a random factor to account for the non-independence of measurements taken from the same animal. Prevalence was analyzed using mixed logistic regression. Infection intensity in *Bd*-positive animals was analyzed using a linear mixed model. Number of ZEs was log-transformed to meet assumptions of normality.

Bd growth in vitro

We characterized the growth of three *Bd* isolates in liquid culture. In addition to the isolates from Section Line Lake and Carter Meadow, we tested an isolate collected from a bullfrog (*Lithobates catesbeiana*) at Finley Lake (in the foothills of the Cascade Mountains in northern California) in 2011. Cultures were started with 10 000 zoospores and grown in 96-well plates with 10 wells per isolate and 36 negative controls per plate. There were 30 replicate cultures of each isolate. Cultures were grown at room temperature (23 °C) and absorbance values were read daily at 490 nm using a PowerWaveXS Microplate reader (BioTek, Bath, UK). This experiment was run twice.

To characterize the growth of *Bd*, we fit logistic growth models to absorbance data. This model has three parameters: *r* or intrinsic growth rate; *K* or carrying capacity and *a* or initial size. A separate logistic model was fit for replicate cultures of each isolate on each plate, giving a total of 18 sets of parameter estimates (2 experiments × 3 plates × 3 *Bd* isolates). The first 3 days of each experiment were not used in model fitting, as minimal growth was observed during this period. Parameter estimates from logistic model fits were evaluated using a mixed-model ANOVA, with *Bd* isolate as a fixed effect and experiment and plate as random effects (plate was removed if it did not explain significant variation). Logistic models were fit using the *nls* function in R (R Development Core Team, 2012) and mixed models were fit using the *lme4* package (Bates *et al.*, 2014).

Immunotoxicity assay

We tested four *Bd* isolates for immunotoxicity. In addition to isolates from Section Line Lake, Carter Meadow and Finley Lake, we also assayed the type isolate JEL197, originally isolated by Longcore *et al.*, 1999 from *Dendrobates auratus*. Jurkat cells (from an immortalized line of human T lymphocytes) were

used as a surrogate for amphibian lymphocytes. The toxicity of *Bd* isolates against amphibian lymphocytes has been very thoroughly documented (Fites *et al.*, 2013). In the same paper, sensitivity of human and mouse lymphocytes was also documented. Thus, lymphocytes from multiple species are sensitive to the virulence factors released by *Bd*. The use of Jurkat cells provided a standardized target population and avoided possible genetic variability due to the use of outbred frog lymphocytes. To assess the extent to which *Bd* isolates inhibit the growth of Jurkat T cells, Jurkat cells were plated at 200 000 per well ($1 \times 10^6 \text{ ml}^{-1}$) and zoospores were added at a final concentration of 1×10^6 per well ($5 \times 10^6 \text{ ml}^{-1}$), with four to seven replicate wells per treatment in each assay. One control featured Jurkat cells only and the other control contained the DNA inhibitor etoposide (at $25 \mu\text{g ml}^{-1}$). After 2 days, the cultures were pulsed with $0.5 \mu\text{Ci } ^3\text{H-thymidine}$ ($5 \mu\text{Ci ml}^{-1}$, specific activity 2 Ci mmol^{-1} ; Perkin Elmer, Waltham, MA, USA) and harvested on the third day. Proliferation was quantified at counts per minute using a Wallac 1205 Betaplate Beta Liquid Scintillation Counter (Perkin Elmer).

Eleven assays were run, but not all treatments were included in each; each treatment was included in nine assays (Supplementary Table S6). For each assay, we found the mean counts per minute for each treatment and used these to calculate the degree of inhibition of Jurkat cell proliferation. We evaluated the difference between treatments in the degree of inhibition using mixed-model ANOVA. Treatment was used as a fixed effect and assay was a random blocking factor.

Genome sequencing

We extracted genomic DNA from cultures of the Section Line isolate and the Carter Meadow isolate that had undergone 10 laboratory passages. Prior to DNA extraction, we cultured the isolates for 5 days on two replicate 1% tryptone and 1% agar plates. We flooded the plates for zoospores and concentrated zoospores using a tabletop centrifuge. We extracted genomic DNA using a phenol–chloroform protocol (Zolan and Pukkila, 1986) modified for use in *Bd* (Joneson *et al.*, 2011). The Section Line and Carter Meadow genomes were sequenced using the Illumina MiSeq platform ($2 \times 250 \text{ bp}$ paired end reads) at the University of Idaho's Genomics Resources Core Facility. Sequence data have been contributed to the NCBI SRA (accession SRP049423).

Sequence alignment and single nucleotide polymorphism calling were performed as in the study by Rosenblum *et al.* (2013). We inferred phylogenetic relationships among isolates using a parsimony method, predicted genomic regions affected by past 'loss-of-heterozygosity' (LOH) events and estimated supercontig copy numbers using single nucleotide polymorphism allele frequencies (Rosenblum *et al.*,

2013; electronic Supplementary Material). We also assessed protease gene distribution across the 19 largest *Bd* contigs; we included three *Bd* protease families (metallo-, serine-type and aspartyl proteases) previously characterized in the study by Joneson *et al.* (2011).

To test for adaptive genomic evolution in the Section Line isolate, we used the sequence divergence test dN/dS and scanned for genes with an excess of nonsynonymous (dN) relative to synonymous (dS) substitutions in heterozygous single nucleotide polymorphisms. We used the Yang and Nielsen method (yn00) in PAML 4.6 (Yang, 2007) to calculate the pair-wise ratio of nonsynonymous substitution rate/synonymous substitution rate for each gene in the Section Line isolate compared with a basal isolate, UM142. Positive selection on a gene is indicated if the dN/dS ratio for that gene is >1 .

Ethics statement

This study was performed in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The protocol was approved by the University of California, Davis Institutional Animal Care and Use Committee (Protocol #16444).

Results

Field host/pathogen dynamics

At Section Line Lake, the abundance of juvenile frogs declined by $>99\%$ between 2009 and 2012; adult abundance increased steadily between 2008 and 2012, then declined sharply between 2012 and 2013 (Figure 1). The abundance of adults and juveniles at Carter Meadow was much lower than the maxima achieved at Section Line Lake, and there were no consistent declining trends (Figure 1). *Bd* prevalence was about five times higher at Section Line Lake (0.61: 98/161) than at Carter Meadow (0.12: 19/156) between 2008 and 2011 (Supplementary Table S2; $\chi^2 = 137.7$, $P < 0.0001$). For animals that tested positive for *Bd*, infection intensity was almost seven times higher at Section Line (mean: 3528 ZE) Lake than Carter Meadow (mean: 535 ZE) over the course of the study (Supplementary Table S2; $F_{1,96} = 91.8$, $P < 0.0001$). There were significant differences between years in both prevalence ($\chi^2 = 72.5$, $P < 0.0001$) and infection intensity ($F_{2,96} = 14.0$, $P < 0.0001$), and there was also a significant site \times year interaction for infection intensity ($F_{2,94} = 44.2$, $P < 0.0001$). Our analyses did not uncover statistical associations between life stage and *Bd* prevalence or infection intensity.

Laboratory experiment

To assess the virulence of *Bd* isolates collected from Section Line Lake and Carter Meadow, we conducted controlled laboratory exposures of 171 field-collected, captive-reared *R. cascadae* from five source

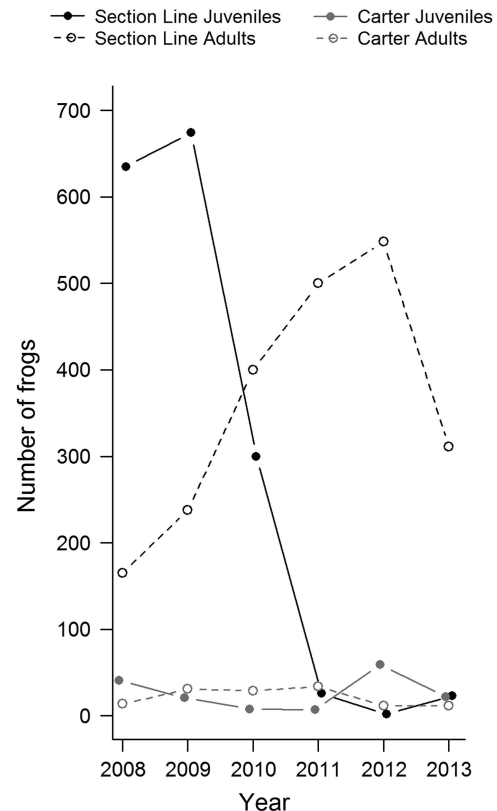


Figure 1 *Rana cascadae* population trends at Section Line Lake and Carter Meadow. The maximum number of adult and juvenile frogs observed during visual encounter surveys is shown.

populations (Supplementary Table S3). Over the course of the experiment 95% of the 56 frogs exposed to Section Line *Bd* died or had to be killed (with most mortality occurring 15–40 days after exposure), compared with 33% of the 57 frogs exposed to Carter Meadow *Bd* and 14% of the 58 control frogs (Figure 2; *Bd* strain effect: $\chi^2 = 121.34$, $P < 0.0001$); significant differences between all three treatments were confirmed with *post-hoc* Tukey tests.

Infection dynamics also differed between *Bd* isolates. In the first week after exposure, *Bd* prevalence was 80% higher in animals exposed to Section Line *Bd* than in animals exposed to Carter Meadow *Bd*; on week 2 *Bd* prevalence was $>150\%$ higher in the Section Line *Bd* treatment (Supplementary Figure S2; Supplementary Table S4; *Bd* strain \times week interaction: $\chi^2 = 13.65$, $P < 0.001$). For animals that tested positive for *Bd*, the average infection intensity on animals exposed to Section Line *Bd* increased from 218 to 60 916 ZE between weeks 1 and 2 after exposure, while the average infection intensity on animals exposed to Carter Meadow *Bd* was substantially lower—about 67 ZE in week 1 and 254 ZE in week 2 (Supplementary Figure S2; Supplementary Table S4; *Bd* strain \times week interaction: $\chi^2 = 36.04$, $P < 0.0001$).

We did not detect differences in survivorship between the five source populations (Supplementary Table S4). However, *Bd* prevalence and infection

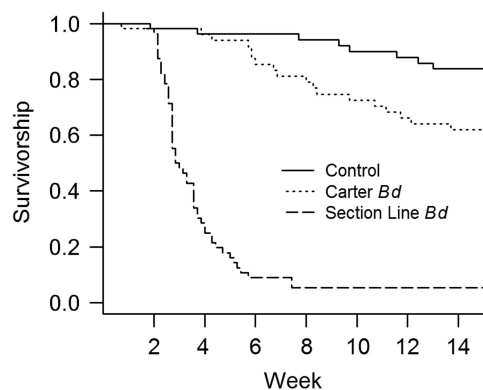


Figure 2 Survival of *Rana cascadae* metamorphs in a laboratory virulence assay. Frogs were exposed to *Bd* isolated from Carter Meadow ($n=57$), *Bd* isolated from Section Line Lake ($n=56$) and a control treatment containing no *Bd* zoospores ($n=58$) in the laboratory.

intensity differed between frog populations during the first 2 weeks of the experiment (Supplementary Table S4)—Tukey tests indicated that frogs from Old Cow Creek experienced higher prevalence than frogs from two of the four other collection sites, and higher infection intensity than all other populations (except for individuals exposed to *Bd* from Carter Meadow, for which there were no differences between populations). Frogs from Old Cow Creek differed from frogs from the other four source populations in that they were larger in size (length ANOVA: $F_{4,161}=42.41$; $P<0.0001$) and were exposed to a different preparation of the *Bd* inocula, because they matured about 1 month prior to frogs from the other sites (electronic Supplementary Material, Supplementary Table S3).

Bd phenotype

The Section Line *Bd* isolate had a lower intrinsic growth rate (r), but a higher carrying capacity (K) in liquid medium than two other northern California *Bd* isolates, Carter Meadow and Finley Lake (Supplementary Table S5, Supplementary Figure S3). Immunotoxicity assays showed that the Section Line *Bd* isolate inhibited T-lymphocyte (Jurkat cell) growth ~22% more than the two other northern California *Bd* isolates; it was also more inhibitory than the type isolate for *Bd* (JEL197), which has been in continuous culture in the Rollins-Smith lab since about 1999 (treatment effect: $F_{4,31.9}=15.23$, $P<0.0001$; Supplementary Figure S4). In fact, the Section Line *Bd* isolate decreased Jurkat cell proliferation as much as the cytotoxic DNA synthesis inhibitor etoposide (used as a control).

Genomic analyses

Our genomics results are based on 13 696 single nucleotide polymorphisms across 17 supercontigs. We obtained a depth of coverage for aligned reads of $26\times$ in Section line and $3\times$ in Carter Meadow. We

included both isolates in all subsequent analyses, but focus more directly on the Section line isolate because this isolate has the most interesting virulence phenotype and the most robust genomic coverage. Using a phylogeny that included 29 isolates from a previous study (Rosenblum *et al.*, 2013), we found that the Section Line and Carter Meadow isolates nested (with high bootstrap support) within the most recently derived and widely distributed clade—the GPL (Supplementary Figure S5). Although the isolates were found in a subclade that includes some other California isolates, there was little geographic signature within the GPL, and close relatives were collected from many localities in North and South America.

The most compelling genomic patterns observed were chromosomal copy number variation and LOH in the Section Line isolate. Chromosomal copy number for the Section Line and Carter Meadow isolates varied from disomic to pentasomic and disomic to tetrasomic, respectively. Most notably, the Section line isolate showed an increase in copy number for 7 of 17 supercontigs when compared with the mean copy numbers of 29 isolates from a previous study (Rosenblum *et al.*, 2013; Supplementary Figure S6). Specifically, supercontigs 2, 3, 7, 8, 11, 12 and 14 showed higher copy numbers in the Section Line isolate than the average of other isolates studied to date. The genomic patterns of LOH for Section Line and Carter Meadow were generally within the range of variation observed in other isolates. However, Section Line contained three chromosomes (7, 8 and 15) that appeared to be affected by LOH events spanning the complete length of the chromosome. Only one of 1336 genes evaluated showed a dN/dS ratio >1 and it was a gene coding for an uncharacterized predicted protein.

Discussion

Bd has caused declines and extinctions in amphibians worldwide, but remarkably little is known about why this pathogen has catastrophic effects on some populations of susceptible hosts and not on others. We documented a dramatic decline in a montane amphibian population and used a laboratory experiment to show that *Bd* isolated in the midst of this decline was markedly more virulent than *Bd* isolated in the same year from a population of the same amphibian host that did not experience a dramatic decline during the same period. We then assessed phenotypic and genomic correlates of virulence for both strains and found that the highly virulent isolate exhibited greater immunotoxicity than other *Bd* isolates from northern California and attained higher optical densities in liquid culture. Both isolates were nested solidly within the GPL, and there was some evidence that the more virulent isolate experienced more rapid genomic evolution

than other GPL isolates. These findings suggest that a particularly virulent strain of *Bd* was responsible (at least in part) for a dramatic host decline, and highlight phenotypic and genomic characteristics of the pathogen that may be linked to this event.

Phenotypic characteristics that have been linked to virulence in *Bd* include immunotoxicity (Fites *et al.*, 2013) and traits associated with growth, such as zoospore production (Langhammer *et al.*, 2013) and zoosporangium size (Fisher *et al.*, 2009a). With regard to immunotoxicity, the Section Line *Bd* isolate inhibited the growth of human immune cells more than other northern California *Bd* isolates; Fites *et al.*, (2013) showed that *Bd* has similar toxicity to amphibian, mouse and human lymphocytes. This lends support to the hypothesis that the deleterious effect of *Bd* on host organisms is linked to the pathogen's ability to inhibit critical immune functions, suggesting that enhanced immunotoxicity may drive amphibian declines. The Section Line *Bd* isolate attained a higher optical density but had a lower exponential growth rate in culture than other northern California *Bd* isolates. This finding suggests that the ability to maintain growth at high conspecific density may be an important aspect of *Bd* virulence. This is the first time that optical density at carrying capacity has been linked to virulence; more work needs to be done to determine if this is a general pattern. The results of our *in vitro* growth experiment were not consistent with the results of our laboratory exposure experiment, as *Bd* loads increased much more rapidly on animals exposed to the Section Line isolate than those exposed to *Bd* from Carter Meadow (Supplementary Figure S2). Thus, it seems that maximum growth rate in culture may not correspond to the rate at which *Bd* density increases on susceptible hosts (as in Raffel *et al.*, 2013), and that care should be taken when using phenotypic characteristics of *Bd* laboratory culture to infer effects on particular host species.

There were also genomic differences between Section Line *Bd* and other *Bd* isolates. Our phylogenetic analysis placed the Section Line isolate within the GPL, which is thought to be responsible for most of the worldwide amphibian declines and extinctions. However, the Section Line isolate was distinguished from other sequenced isolates in the GPL by high rates of polysomy in particular regions of the genome and elevated LOH. Copy number variation and LOH have been proposed as mechanisms for rapid evolution in *Bd* (Farrer *et al.*, 2013; Rosenblum *et al.*, 2013). Thus, it is possible that these genomic processes have altered copy number or allele frequencies for genes that relate to virulence. Chromosomal segments that should be targets for future study include 7 and 8, which show unusual copy number variation and LOH patterns in the Section Line isolate, and 12, which is found at a high copy number in the Section Line isolate and contains a high density of protease gene copies, that

are hypothesized to have a role in *Bd* pathogenesis (Rosenblum *et al.*, 2013). Although our genomic analyses highlighted important genomic processes and broad-scale regions of the genome, we did not find evidence for selection on particular genes-of-interest. Methods to scan for selection in single genomes generally have low power, so additional work is required to understand how the genomic patterns revealed here may relate to specific virulence properties of *Bd*.

At Section Line Lake, there was a >99% reduction in juvenile abundance between 2009 and 2012. In 2010 juveniles still numbered in hundreds; in July 2011 we observed only 3 juveniles and 16 carcasses (observations of frog carcasses have been extremely rare in thousands of hours of visual survey we performed in this system, so this number of carcasses is quite unusual); only 2 juveniles were seen in our 2012 survey. Adults began to decline 3 years after juveniles, which is consistent with the 3–5 years of age at maturity observed for this species in this region (Pope *et al.*, 2014). These observations support our contention that high levels of mortality led to the disappearance of most juvenile frogs from this population. High overwinter mortality in juveniles is consistent with declines associated with *Bd*, and we did not observe evidence of other causes of recruitment failure or juvenile mortality such as desiccation or elevated levels of predation. Furthermore, *Bd* prevalence and load were relatively high at Section Line Lake during the study period.

It is possible that factors other than elevated pathogen virulence contributed to a *Bd*-related juvenile decline at Section Line Lake. For example, environmental factors may have exacerbated the effects of *Bd* on *R. cascadae* at this particular site—at 2182 m, Section Line Lake is near the upper end of the elevation range occupied by *R. cascadae* in California, and *Bd* prevalence can increase with elevation for *R. cascadae* (Piovía-Scott *et al.*, 2011). In contrast, Carter Meadow is lower in elevation (1865 m) and features a network of streams and pools, which may be less favorable for the maintenance of *Bd* infections (Pope *et al.*, unpublished data). In addition, the density of frogs at Section Line Lake was quite high (1.2 frogs per meter of shoreline in 2008), having increased dramatically following the removal of introduced trout from this lake in 2003 (Pope, 2008, Pope *et al.*, unpublished data), and theoretical models of interactions between *Bd* and amphibian hosts indicate that high host density can facilitate the buildup of high *Bd* loads and increase host mortality (Briggs *et al.*, 2010). However, a site 18 km from Section Line Lake at a similar elevation has sustained high frog densities in the presence of *Bd* without experiencing dramatic declines (Piovía-Scott *et al.*, unpublished data), suggesting that high elevation and high frog density do not always lead to epizootic events. Our laboratory experiment suggested that frogs from different source populations differ in the level of

Bd infection they develop, which may indicate that populations differ in their ability to resist infection. However, we found no evidence that frogs from Section Line Lake were more susceptible to *Bd* than frogs from other sites, suggesting that the presence of particularly susceptible host genotypes was not one of the principal drivers of juvenile decline. Finally, it is possible that the hyper-virulent isolate used in our laboratory experiment was not the dominant strain present at Section Line Lake during the decline. However, a second isolate from Section Line Lake collected in 2013 also showed exceptional virulence compared with several other northern California isolates (Piovio-Scott *et al.*, unpublished data), suggesting that the isolate described in the current paper may not represent a rare virulence phenotype. Future research should focus on quantifying the relative importance of pathogen virulence, abiotic environmental factors and host density as drivers of *Bd*-related amphibian declines and on evaluating within-population variation in *Bd* virulence.

Why does the Section Line *Bd* isolate exhibit high virulence? Exponential host population growth and high host density have been linked to the *in situ* evolution of increased virulence (Bull, 1994; Ewald, 1994; Lenski and May, 1994), and these conditions were present at Section Line Lake shortly before the isolate was collected. The low virulence of *Bd* from the smaller and more stable Carter Meadow population is consistent with the expectation that reduced virulence can emerge in low density populations (Bull, 1994; Ewald, 1994; Ebert and Bull, 2008). Unfortunately, the rapid course of *Bd*-related mortality in some montane amphibians may mean that many populations go extinct before reduced virulence can evolve (Vredenburg *et al.*, 2010). Alternatively, *Bd* virulence at a site may be driven largely by dispersal and gene flow (that is, the characteristics of propagules that colonize the site), rather than within-site selective forces. For example, the virulence of *Bd* may increase as the pathogen spreads across a previously unoccupied landscape (Phillips and Puschendorf, 2013). However, recent surveys of the study region did not uncover any evidence of an invasion front (Piovio-Scott *et al.*, 2011; Pope *et al.*, 2014), and the absence of detailed information on the introduction and spread of *Bd* in northern California makes it difficult to ascertain whether high virulence of the Section Line isolate is likely to be a product of invasion history in this region. More regional-scale population genetic studies (for example, Rodriguez *et al.*, 2014) are needed to outline the processes driving variation in *Bd* virulence across landscapes.

In summary, this study expands our understanding of virulence in a devastating pathogen by providing clear links between putative virulence factors (for example, immunotoxicity), rigorous virulence assays and a specific host decline. Interestingly, the high-virulence isolate was collected in

a region where the host amphibian is widespread (Piovio-Scott *et al.*, 2011), while the low-virulence isolate came from a remnant population in a region where dramatic host declines have occurred (Fellers *et al.*, 2008). This inconsistency between site-specific host–pathogen interactions and regional patterns of host decline highlights the importance of local processes in determining host–pathogen dynamics and the continued potential for disease outbreaks in populations that appear to be persisting with a pathogen.

Conflict of Interest

The authors declare no conflict of interest.

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