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Original Contribution

Recent Emergence of a Chytrid Fungal Pathogen in California Cascades Frogs (*Rana cascadae*)

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Abstract: The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) has been associated with global amphibian declines, but it is often difficult to discern the relative importance of *Bd* as a causal agent in declines that have already occurred. Retrospective analyses of museum specimens have allowed researchers to associate the timing of *Bd* arrival with the timing of past amphibian declines. Cascades frogs (*Rana cascadae*) have experienced dramatic declines in northern California, but it is not clear whether the onset of these declines corresponds to the arrival of *Bd*. We used quantitative real-time PCR assays of samples collected from museum specimens to determine historical *Bd* prevalence in the northern California range of Cascades frogs. We detected *Bd* in 13 of 364 (3.5%) Cascades frog specimens collected between 1907 and 2003, with the first positive result from 1978. A Bayesian analysis suggested that *Bd* arrived in the region between 1973 and 1978, which corresponds well with the first observations of declines in the 1980s.

Keywords: chytridiomycosis, museum specimen, quantitative PCR, Klamath Mountains, Cascade Mountains

Introduction and Purpose

Amphibians are experiencing declines on a global scale (Wake and Vredenburg 2008). Whereas multiple factors have been associated with amphibian declines, it is often difficult to determine which of these factors had the greatest impact because historical data linking the timing of the decline with the presence or severity of particular stressors are unavailable. However, retrospective analyses are possible for some stressors. For instance, the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which has been associated with amphibian declines and extinctions worldwide (Berger et al. 1998; Lips et al. 2006; Skerratt et al. 2007; Lips et al. 2008; Vredenburg et al. 2010), can be detected on the skin of archived museum specimens (Cheng et al. 2011; Huss et al. 2013; Sette et al. 2015; Talley et al. 2015; Yap et al. 2016). The ability to link the timing of *Bd* arrival with the timing of amphibian declines can provide evidence implicating *Bd* as a causal factor in previously enigmatic declines.

The current study focuses on Cascades frogs (*Rana cascadae*) in northern California. These montane frogs experienced dramatic declines in the southern Cascade Range in the second half of the twentieth century, and only a handful of remnant populations remain in that region (Fellers and Drost 1993; Fellers et al. 2008; Pope et al. 2014). More populations remain in the adjacent Klamath Mountains (Piovia-Scott et al. 2011), but the absence of

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Table 1. *Batrachochytrium dendrobatidis* (*Bd*) Prevalence in Cascades Frog (*Rana cascadae*) Museum Specimens Collected in Northern California.

Decade	Number tested	Number positive	% positive	95% confidence intervals[a]	
				Lower	Upper
1900–1909	3	0	0	0.0000	0.71
1910–1919	2	0	0	0.0000	0.84
1920–1929	72	0	0	0.0000	0.05
1930–1939	18	0	0	0.0000	0.19
1940–1949	17	0	0	0.0000	0.20
1950–1959	73	0	0	0.0000	0.05
1960–1969	84	0	0	0.0000	0.04
1970–1979	60	1	1.7	0.0004	0.09
1980–1989	0	–	–	–	–
1990–1999	33	10	30.3	0.1559	0.49
2000–2009	2	2	100	0.16	1.00
Total	364	13	3.5	0.02	0.06

[a]Based on the binomial distribution.

historical survey data for the area makes it difficult to ascertain whether declines also occurred in the Klamath Mountains (Pope et al. 2014). Cascades frogs also have been shown to be susceptible to *Bd* in laboratory experiments (Garcia et al. 2006; Piovia-Scott et al. 2015), and the pathogen is linked to contemporary declines and population extirpations in both the Klamath Mountains and the southern Cascade Range (Pope et al. 2014; Piovia-Scott et al. 2015). However, other factors have also been linked to Cascades frog declines in northern California. Beginning in the late 1800s, trout were introduced into northern California montane lakes for recreational angling purposes. Cascades frogs are less likely to occupy lakes with introduced trout (Welsh et al. 2006), and trout removal leads to the recovery of Cascades frog populations (Pope 2008), indicating that these introduced predators may play a role in Cascades frog declines. However, the timing of trout introduction does not coincide with Cascades frog declines in the southern Cascade Range, and introduced trout are not as widely distributed in these mountains as other locations in California (Fellers et al. 2008). Pesticides also have been implicated in northern California Cascades frog declines (Davidson 2004). However, a recent survey of 73 contaminants in amphibian larvae and sediment failed to uncover a relationship between pesticides and Cascades frog declines (Davidson et al. 2012).

Thus, while *Bd* has emerged as the leading hypothesis for historical Cascades frog declines in northern California, we do not know if the timing of *Bd* arrival in the region coincides with the timing of declines. In order to evaluate this hypothesis we examined museum specimens of Cascades frogs collected in northern California between 1907 and 2003 for *Bd*. We then used a Bayesian analysis to estimate the date of *Bd* arrival in the region and the post-arrival prevalence of *Bd* infection. Although recent sampling efforts show that *Bd* is now present throughout the northern California range of Cascades frogs (Piovia-Scott et al. 2011; Pope et al. 2014), this is the first retrospective survey to be conducted for this region.

Materials and Methods

We tested every historical Cascades frog specimen available from four California museum collections for *Bd*. Specimens were housed in collections at the California Academy of Sciences (CAS), Chico State University (CSU), UC Berkeley Museum of Vertebrate Zoology (MVZ), and the Los Angeles County Natural History Museum (LACM). Specimens were collected from Butte ($N = 41$), Plumas ($N = 110$), Trinity ($N = 27$), Shasta ($N = 49$), Siskiyou ($N = 90$), and Tehama ($N = 47$) counties from 1907 to 2003. Animals were stored in 70% ethanol. Specimens were fixed using formalin prior to being transferred to ethanol, as was the norm for amphibian specimen preparation during the period when most of the specimens were collected (Heyer et al. 1994). To see if we could establish an earlier date of arrival for *Bd* in the region, we also tested 42 specimens of three other anuran species (all from LACM) that occurred in the counties included in our study and were collected prior to the first *Bd*-positive Cascades frog. These specimens make up the entirety of the anuran specimens at the LACM that were collected from counties within our study region (Table 2). *Pseudacris regilla* (Pacific chorus frog; $N = 13$) and *Anaxyrus boreas* (western toad; $N = 12$) were collected from Shasta, Plumas, Trinity and Tehama counties between the years 1956 and 1972. These species' specimens were collected from mountainous areas (with the exception of one *A. boreas* collected at a low-elevation site), where these species often co-occur with Cascades frogs. *Rana boylii* (foothill yellow-legged frog; $N = 17$) inhabits low- to mid-elevation lotic habitats and is generally not sympatric with Cascades frog.

Museum specimens were tested for *Bd* following a protocol described in Cheng et al. (2011). Specimens were

Table 2. *Batrachochytrium dendrobatidis* (*Bd*) Prevalence in Cascades Frog (*R. cascadae*) Museum Specimens Collected from Six Northern California Counties.

County	Date range	Number tested	Number positive	% positive	95% conf. intervals		Years with positives
					Lower	Upper	
Butte	1923–1961	41	0	0.00	0.000	0.09	–
Plumas	1923–1975	110	0	0.00	0.000	0.03	–
Shasta	1923–2003	49	2	4.08	0.005	0.14	2003 (2)
Siskiyou	1914–1994	90	8	8.89	0.039	0.17	1990 (7), 1994 (1)
Tehama	1924–1961	47	0	0.00	0.000	0.08	–
Trinity	1907–1990	27	3	11.11	0.024	0.29	1978 (1), 1990 (2)

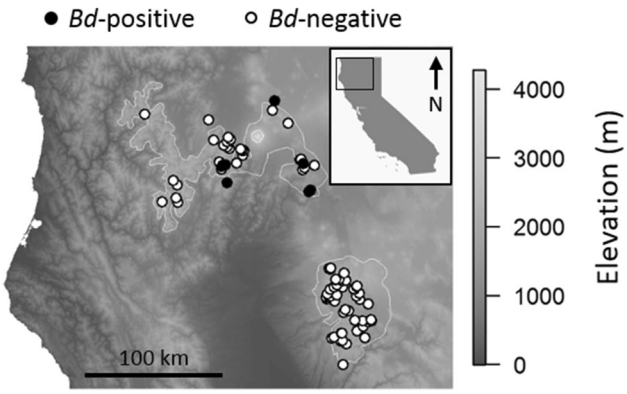

Figure 1. Map of the study region showing the collection sites for Cascades frog (*Rana cascadae*) museum specimens tested for *Batrachochytrium dendrobatidis* (*Bd*) in this study. The range of Cascades frogs in northern California (according to the California Wildlife Habitat Relationships information system; www.dfg.ca.gov/biogeodata/cwhr) is enclosed in a *thin white line*. Specimens from California State University Chico did not have latitude and longitude data associated with them; approximate locations were ascertained for all but four (not shown) of these specimens based on locality descriptions.

removed from preservation jars using clean forceps and rinsed thoroughly with diH2O and 70% ethanol to remove any free-floating cells that may contain *Bd* DNA from the environment or from other specimens. To prevent cross-contamination, forceps were cleaned thoroughly before handling a new specimen, and gloves were changed between specimens; Specimens were handled such that the areas being swabbed never came in contact with the forceps. Specimens were sampled for *Bd* by stroking a sterile swab (Medical Wire and Equipment) across the skin surface: 5 strokes along the ventral surface, 5 strokes along each inner thigh, and five strokes on each space of interdigital webbing. Swabs were stored in ethanol in 1.5 mL microcentrifuge vials and kept refrigerated until processing.

Swabs were analyzed for *Bd* using quantitative real-time PCR (qPCR) following standard protocols (Boyle et al. 2004; Retallick et al. 2006; Hyatt et al. 2007). Briefly, vials with swabs were left open under a hood to allow ethanol to evaporate. Depending on the amount of ethanol soaking the swabs, swabs dried within one to 12 h. DNA was extracted from swabs using Prepman Ultra (Life Technologies). Extracted DNA was diluted by a factor of 10, and qPCR was run in singlicate (unless otherwise specified); negative controls and standards of known zoospore concentration were included in each assay. All positives showed distinct amplification curves and were assayed again in triplicate to ensure that they did not result from cross-contamination during the qPCR. To confirm that the earliest *Bd*-positive sample (from 1978) was not a false positive, we returned to the museum, rinsed and swabbed the putatively *Bd*-positive specimen and additional specimens housed in the same jar from the same year. Only the specimen that had previously tested positive tested positive again during this second round of testing. Swabs from CSU, CAS, and the MVZ were initially assayed at the University of California, Davis using an Applied BioSystems StepOnePlus real-time PCR system; initial assays of swabs from LACM and subsequent re-testing of *Bd*-positive swabs occurred at San Francisco State University using an Applied BioSystems 7300 real-time PCR system. Although different laboratories were used to conduct qPCR assays, the protocols used were essentially the same.

To characterize the temporal and spatial distribution of *Bd* in Cascades frogs, we calculated 95% confidence inter-

Table 3. Number of Non-Cascades Frog Museum Specimens Tested for *Bd*.

County	*Anaxyrus boreas*	*Pseudacris regilla*	*Rana boylii*
Lassen	3	0	0
Plumas	7	6	1
Trinity	1	3	7
Siskiyou	0	0	8
Tehama	1	1	0
Shasta	0	3	1
Total	12	13	17

The three native anurans, western toad (*Anaxyrus boreas*), pacific chorus frog (*Pseudacris regilla*), and foothill yellow-legged frog (*Rana boylii*) were collected from the same six northern California counties as the primary study animal, the Cascades frog. These samples were collected between 1952 and 1972; no samples tested positive for *Bd*.

vals for *Bd* prevalence in Cascades frog samples from each decade and from each county based on the binomial probability distribution. To estimate the arrival date and post-arrival prevalence of *Bd* in the northern California range of Cascades frogs, we used a Bayesian modeling approach where the process of *Bd* arrival is represented by a simple threshold model in which *Bd* switches from being absent to being present with some mean prevalence, and the number of infected frogs in each year in our data set is treated as a draw from a binomial distribution with a sample size equal to the number of frogs sampled in that year (Phillips and Puschendorf 2013). We used a uniform prior for arrival time that spanned our entire sampling period (1907–2003) and a flat beta prior ($\alpha = \beta = 1$) for the mean prevalence after *Bd* arrival. Due to the uneven distribution of sample dates within geographic regions, we did not conduct analyses that combined both geographic and temporal information. All statistical analyses were conducted in R (R Development Core Team 2015).

Results

Of the 364 total archived Cascades frog specimens tested, 13 tested positive for *Bd*, an overall *Bd* prevalence of 3.5% (Table 1). The 13 *Bd*-positive results were obtained from specimens from Trinity, Siskiyou and Shasta counties, a region that includes both the southern Cascade Range and Klamath Mountains. (Table 2; Figure 1). The earliest positive results were from a Trinity County specimen collected in 1978 (Table 2). Except for the *Bd*-positive specimen from 1978, all other positive specimens were collected between 1990 and 2003; we were not able to test any specimens collected in the 1980s (Table 1).

The Bayesian analysis gave 95% credible intervals of 1973–1978 for the date of *Bd* arrival in Cascades frog samples collected in the study region and 0.20–0.48 (mean: 0.33) for post-arrival *Bd* prevalence. For the 1920s, 1950s, and 1960s, we tested 72 or more specimens, which gives us a 95% confidence interval for *Bd* prevalence whose upper limit is 0.05 or less in each of these decades (Table 1). To see if we could establish an earlier date of *Bd* arrival, we sampled specimens of other anuran species collected prior to 1978 in our study region; none of these samples (12 *A. boreas*, 13 *P. regilla*, and 17 *R. boylii*) returned positive results (Table 3).

Discussion

The arrival of *Bd* has been associated with precipitous amphibian declines in many regions, and this observation has been critical to establishing chytridiomycosis, the disease caused by *Bd*, as a causal factor in these declines (Lips et al. 2006; Vredenburg et al. 2010). In cases where *Bd* sampling was not conducted at the time amphibian declines began, museum specimens have provided valuable evidence linking the timing of the pathogen's arrival in the system to the timing of declines (Cheng et al. 2011). Our analyses suggest that *Bd* arrived in the study region between 1973 and 1978. Much of the power to identify 1973 as the earliest date of likely arrival derives from the abundance of Cascades frog specimens collected prior to 1978—324 samples collected in this period (89% of all Cascades frog samples in the study) tested negative. The mid-1970s arrival of *Bd* in the northern California range of Cascades frog is consistent with the timing of Cascades frog declines in the southern Cascade Range, which were first noticed in the 1980s (Fellers et al. 2008). Thus, our results are consistent with the hypothesis that the arrival of *Bd* is likely to have played an important role in causing the ongoing declines in Cascades frogs in the southern Cascade Range.

The mid-1970s arrival of *Bd* in the mountains of northern California also is consistent with previous studies that used museum specimens to determine the arrival and spread of *Bd* in central and northern California. Padgett-Flohr and Hopkins (2009) used histology to evaluate museum specimens from central California and found that the earliest *Bd*-positive specimen (an American bullfrog, *Lithobates catesbeianus*) was collected in the San Francisco

Bay Area in 1961. The authors hypothesized that *Bd* first arrived in that metropolitan area and subsequently spread out into the remaining areas of central California (Padgett-Flohr and Hopkins 2009). Using the same qPCR assay we used for museum specimens, Sette et al. (2015) reported the first *Bd*-positive result in native salamanders in central California in 1967 followed by an increase in *Bd* prevalence in the late 1970s, 1980s, and 1990s. Huss et al. (2013) also used the same qPCR assay and identified *Bd* on an introduced American bullfrog collected in 1928 in Sacramento County and Butte County in 1931 pushing the date of *Bd*'s arrival in California a few decades earlier. However, this animal in Butte County (a county included in our study) was collected ~95 km north of Sacramento in the Central Valley, where human activities have historically been concentrated, and it is possible that several decades may have elapsed before *Bd* reached the more remote mountainous areas of Butte County inhabited by Cascades frogs. It is also possible that these two early dates represent failed *Bd* invasions or local invasions with limited subsequent spread (Sette et al. 2015). Taken together, the historical observations of *Bd* derived from northern and central California museum specimens suggest a pattern of emergence and spread from centers of human activity in the Bay Area and Central Valley into more sparsely inhabited areas in the mountains. The early emergence of *Bd* in areas where human activity is concentrated is consistent with the hypothesis that human transport of certain amphibian species (e.g., American bullfrog, South African clawed frog [*Xenopus laevis*]) facilitated long-distance dispersal of *Bd*, including the introduction of the pathogen to previously unoccupied regions (Weldon et al. 2004; Rachowicz et al. 2005; Garner et al. 2006; Schloegel et al. 2009, 2012; Huss et al. 2013; Vredenburg et al. 2013; Yap et al. 2016).

Given the relatively small number of *Bd*-positive specimens identified in our study, we are not able provide much insight into patterns of local *Bd* invasions within the mountains of northern California. In particular, the paucity of specimens collected after 1990 and the absence of specimens collected in the 1980s hamper our ability to discern regional patterns. Nonetheless, it is important to point out that the earliest *Bd*-positive specimen we found was collected at Tamarack Lake, a site that is near a major transportation corridor (currently occupied by US Interstate 5) and is a popular destination for recreational angling and camping.

Conclusion

Our study suggests that *Bd* invaded the northern California range of the Cascades frog relatively recently (i.e., within the last 50 years), which is consistent with the hypothesis that this deadly pathogen played a role in the collapse of this species in parts of northern California. The pattern of relatively recent arrival and subsequent amphibian decline provides a valuable counterpoint to recent studies documenting longer-term endemism of *Bd* in other parts of North America (Talley et al. 2015). The relatively recent arrival of *Bd* in the mountains of northern California also raises the possibility that epizootic host-pathogen dynamics (i.e., disease outbreaks; Vredenburg et al. 2010) associated with *Bd* invasion could still be causing population extirpation in the Cascades frog. Cascades frog populations studied by Piovia-Scott et al. (2015) showed patterns consistent with both epizootic events and more stable enzootic host-pathogen dynamics, similar to those seen in studies of mountain yellow-legged frog (*Rana sierrae* and *R. muscosa*) populations in California's Sierra Nevada (Briggs et al. 2010; Vredenburg et al. 2010). Understanding the transition between these two phases is crucial for host persistence, as enzootic dynamics are associated with reduced risk of host extinction (Briggs et al. 2010). Thus, in order to provide a more comprehensive assessment of the threat posed by *Bd* to the regional persistence of Cascades frogs, future research should focus on determining what factors hasten the transition from epizootic to enzootic dynamics in this system.

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