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Occurrence of *Batrachochytrium dendrobatidis* in anurans of the Mediterranean region of Baja California, México

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ABSTRACT: Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and is regarded as one of the most significant threats to global amphibian populations. In México, *Bd* was first reported in 2003 and has now been documented in 13 states. We visited 33 localities and swabbed 199 wild-caught anurans from 7 species (5 native, 2 exotic) across the Mediterranean region of the state of Baja California. Using quantitative PCR, *Bd* was detected in 94 individuals (47.2% of samples) at 25 of the 33 survey localities for 5 native and 1 exotic frog species. The exotic *Xenopus laevis* was the only species that tested completely negative for *Bd*. We found that remoteness, distance to agricultural land, and elevation were the best positive predictors of *Bd* presence. These are the first *Bd*-positive results for the state of Baja California, and its presence should be regarded as an additional conservation threat to the region's native frog species.

KEY WORDS: Chytridiomycosis · Northwestern México · Amphibians · Diseases · Threats

INTRODUCTION

Chytridiomycosis, an infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), represents one of the most significant threats to global amphibian populations and is among the most lethal diseases affecting vertebrates (Skerratt et al. 2007). The impact of *Bd* on amphibian populations is highly variable and has been associated with several environmental and human-mediated factors, including temperature, precipitation, climate change, and habitat loss or alteration (Bosch et al. 2007, Andre et al. 2008, Raffel et al. 2015). As a result, some species and populations infected with *Bd* suffer massive declines, others appear to be more tolerant, and still others continue to be impacted even once *Bd* has reached an enzootic state (Daszak et al. 2004, Briggs et al. 2005, Fisher & Garner 2007, Catenazzi et al. 2017). *Bd*-tolerant species may act as biological reservoirs, and have been linked to the probable spread of the pathogen (Reeder et al. 2012, Huss et al. 2013).

In México, *Bd* was first reported in 2003, and has been detected since then in wild amphibian populations from the states of Baja California Sur, Chiapas, Guerrero, Hidalgo, Estado de México, Michoacán,

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Morelos, Oaxaca, Puebla, Sonora, Tamaulipas, Veracruz, and Distrito Federal (Mendoza-Almeralla et al. 2015). *Bd* outbreaks have been identified as a likely cause for amphibian declines in central and southern México (Lips et al. 2004, Cheng et al. 2011). In the 2 states that comprise the Baja California peninsula of México, a survey for *Bd* in the native treefrogs *Hyliola regilla* and *H. cadaverina* at 3 survey localities tested negative (Frías-Alvarez et al. 2008). In subsequent surveys, *H. regilla* in isolated oasis populations in Baja California Sur were positive for *Bd* (Luja et al. 2012). In adjacent California (USA), *Bd* has been detected in both native and exotic anuran species, and has been linked to severe declines in montane and foothill populations of ranid species (Rachowicz et al. 2006, Briggs et al. 2010, Adams et al. 2017a). Six anuran species native to the California Mediterranean region extend into Baja California: *Rana draytonii*, *H. regilla*, *H. cadaverina*, *Anaxyrus boreas*, *A. californicus*, and *Spea hammondii*. All of these species have tested positive for *Bd* in California (Lannoo et al. 2011, Piovia-Scott et al. 2011, Adams et al. 2017a, R. N. Fisher & A. Backlin unpubl. data), with massive die-offs observed for *A. boreas* (Colorado populations, Muths et al. 2003), and tolerance suggested for *R. draytonii* and *H. regilla* in California (Padgett-Flohr 2008, Reeder et al. 2012).

Although *Bd* models suggest that hosts in Mediterranean forest, woodlands, and scrub may have the lowest probabilities of *Bd* infection (Olson et al. 2013), amphibian declines have been documented in this region within California (Jennings & Hayes 1994, Adams et al. 2017a), suggesting that *Bd* may still be a cause for conservation concern (Vojar et al. 2017). Here, we report on the presence of *Bd* in native and exotic anurans across the Mediterranean region of Baja California, and discuss its importance as a potential threat to their populations.

MATERIALS AND METHODS

Sample collection

During fieldwork conducted in spring 2013 and 2014, we swabbed amphibians in several arroyos in the Baja California Mediterranean region, from the US–México border south to Arroyo Santo Domingo, at elevations from sea level to 2070 m. We visited 33 localities and swabbed 199 individual anurans representing 5 genera and 7 species (see Table 1). For each site, we gathered data on elevation, distance to the nearest edge of an urban area (as a measure of remoteness), percentage of natural habitat within a 3 km buffer, and distance to the nearest edge of agricultural land, using Google Earth (http://earth.google.com; last accessed in April 2016). To collect *Bd* samples, we followed the technique described by Hyatt et al. (2007), using sterile rayon-tipped swabs (Medical Wire and Equipment). Each swab was preserved and stored in 95% ethanol and then dried to evaporate all residual ethanol prior to DNA extraction.

Molecular methods

DNA from swabs was extracted by adding 40 µl of PrepMan® Ultra (Applied Biosystems) and incubating at 100°C for 10 min. Tubes were then centrifuged at 13 000 × *g* for 3 min, and the supernatant was aspirated from swabs and placed in a new, sterile 1.5 ml tube. Samples were diluted 1:10 with sterile, DNase-free 0.25 × TE (Tris-EDTA). The qPCR analysis was performed on an Applied Biosystems StepOnePlus™ Real-Time PCR System, and PCR reactions and amplification parameters followed Boyle et al. (2004). Positive controls in quantities of 100, 10, 1, and 0.1 zoospores were run on each plate in triplicate, in addition to triplicate negative controls, and were used to create the standard curve. DNA from *Bd* used as standard DNA was developed by Mary Toothman, University of California, Santa Barbara, USA (isolate CJB7, from California, USA; ITS copy number = 60). Standards were quality-controlled for equivalent standard quantification to isolate AAHL 98 1810/3 prior to use in qPCR using a hemocytometer. To account for the dilution of the *Bd* extract and calculate the total amount of *Bd* DNA per swab, raw genomic output from qPCR results was multiplied by 80 to obtain a total number of zoospore equivalents (ZE) per swab. ZE can be used as an index of the intensity of infection or '*Bd* load' for individual frogs sampled.

Statistical analysis

To examine which factors best predict *Bd* load and prevalence in amphibians sampled, we used generalized linear mixed-effects models (GLMMs). With the GLMMs, we were able to account for non-independence of samples with the use of a variable ('survey event'), created to group frogs sampled within the same 2 wk time period and at the same site as a random effect. We excluded samples (n = 3) that were represented by 1 sample within a random effect level, and *Xenopus laevis* samples (n = 9) because they were all negative and therefore prevented model convergence; including these samples did not change our fundamental results. We included only *Bd*-positive samples (ZE > 0) in the *Bd* load analysis and log-transformed those ZE values. We hypothesized that *Bd* prevalence and load would vary with species, distance to agricultural land, elevation, percentage of natural area within a 3 km buffer, and remoteness (calculated as distance to the nearest town). We developed these hypotheses based on the literature, which suggests that *Bd* prevalence and load increase with elevation and vary by species and degree of habitat alteration (Becker & Zamudio 2011, Johnson et al. 2011, Saenz et al. 2015). We *z*-transformed all 4 continuous predictor variables so that effect sizes of different predictors were comparable. We ranked models according to Akaike's information criterion, corrected for small sample sizes (AIC_c) to determine the relative importance of predictor variables within each set of models (one set for *Bd* presence−absence and one for *Bd* load). We conducted all analyses in the R computing environment (version 3.3.2, R Development Core Team 2016).

RESULTS

We detected *Bd* in 94 individuals (47.2% of samples) at 25 of the 33 survey localities (Table 1, Fig. 1). *Bd* was present in *Anaxyrus californicus*, *A. boreas*, *Hyliola cadaverina*, *H. regilla*, *Rana draytonii*, and *R. catesbeiana.* We did not observe any

Table 1. Sites where amphibians were sampled for *Batrachochytrium dendrobatidis* (*Bd*) presence. Site ID refers to numbers in Fig. 1. n: number of sampled individuals; Pr: *Bd* prevalence, defined as the fraction of infected individuals; mean *Bd* load: number of zoospores per infected individual; a.s.l.: above sea level; Ua: distance to nearest urban area; Al: distance to nearest agriculture land

Site ID	Locality	n	Pr (%)	Mean *Bd* load	Elevation (m a.s.l.)	Ua (km)	Al (km)
1	Abelardo L. Rodríguez Dam, Tijuana	1	0	0	118	0	22.0
2	El Carrizo Dam, Tijuana	1	100	925.21	272	1.0	8.0
3	Rancho las dos Cumbres, Tecate	2	0	0	620	1.0	0.1
4	Cañón El Alamo, Reserva Kumiai	6	17	9.26	488	9.0	8.4
5	El Gato, Cañón Las Parras, Ejido Guadalajara	1	100	3150.67	855	15.0	16.5
6	Rancho Talegas Arroyo las Calabazas	2	0	0	1187	21.0	23.3
7	Rancho San Faustino, Sierra Juárez	4	50	175.66	1270	25.0	25.0
8	Cañada El Morro, Rosarito	9	0	0	5	0	0.4
9	La Misión, Arroyo Guadalupe	10	20	6034.86	1	0	0.1
10	Cañada Salsipuedes	6	50	229.84	125	9.0	2.1
11	Rancho Madrigal, Ensenada	1	100	6.13	300	0	7.5
12	Cañada Miracielo, San Antonio de las Minas	5	20	1.57	358	6.6	6.8
13	Cañón Agua Caliente, Valle de Guadalupe	6	17	163.92	390	7.5	1.5
14	Arroyo El Barbon, Cañón Hondo	2	0	0	625	5.0	2.7
15	Rancho la Casa Verde, Arroyo Casa Verde	3	0	0	1370	20.0	20.1
16	Arroyo San Carlos, Ensenada	5	80	387.64	70	2.3	0.5
17	Rancho Agua Caliente, Arroyo Santo Tomas	1	0	0	495	19.0	7.8
18	Rancho Agua Caliente, Arroyo San Vicente	9	0	0	218	13.6	1.8
19	San Rafael, near junction of Arroyo La Palmita	20	30	1819.58	220	23.5	0.5
20	San Rafael (35 km E of Colonet)	2	50	1.93	340	29.8	11.0
21	San Rafael, 3 km NW of Mike's Sky Ranch	6	33	26.76	1036	30.0	24.5
22	Rancho Meling, Sierra San Pedro Mártir	9	89	265.75	620	40.0	15.5
23	Rancho El Molino, Sierra San Pedro Mártir	4	25	0.807	555	35.0	14.2
24	Santo Domingo, near Rancho La Canastilla	6	33	2.54	145	14.0	11.5
25	6 km downstream of Rancho Valladares	5	40	220.93	635	26.0	23.5
26	Rancho Valladares, Sierra San Pedro Mártir	11	82	165.30	720	30.0	27.0
27	Rancho El Potrero, Sierra San Pedro Mártir	7	57	8.98	890	37.0	35.0
28	Cañón La Jolla, Sierra San Pedro Mártir	3	100	1369.90	1560	43.8	30.9
29	Arroyo San Antonio Murillos	11	64	146.75	560	35.5	33.5
30	Rancho San Isidoro, Sierra San Pedro Mártir	4	100	2452.96	920	42.5	39.3
31	Arroyo La Víbora, Sierra San Pedro Mártir	5	60	2764.70	1880	46.6	44.5
32	Rancho Viejo, Sierra San Pedro Mártir	7	28	146.75	2050	51.0	65.0
33	La Grulla, Sierra San Pedro Mártir	25	92	379.28	2070	51.6	50.0

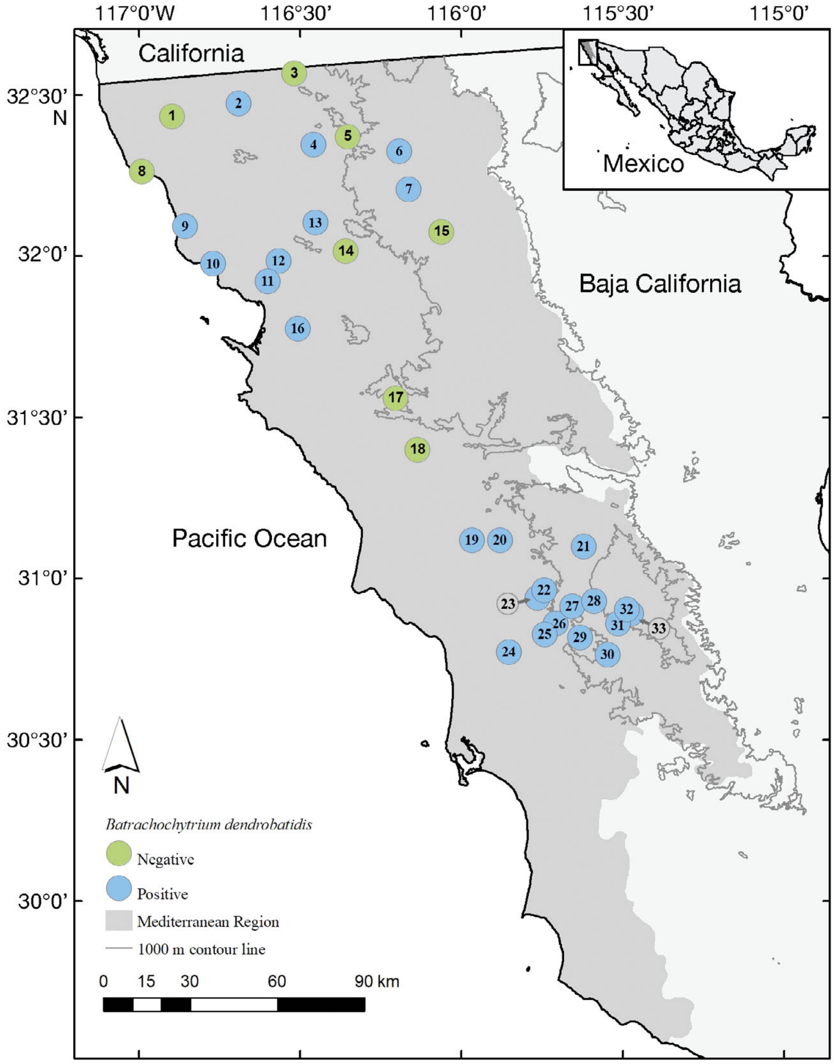

Fig. 1. Survey localities in the Mediterranean region of Baja California, México, and whether the amphibians sampled there tested positive or negative for *Batrachochytrium dendrobatidis* (inset: México). Numbers are site IDs (see Table 1)

Spea hammondii during our surveys; thus, information is still lacking for this species. Only the exotic *Xenopus laevis*, tested on 9 individuals from a single urban locality, was *Bd*-negative. *Bd* loads varied from zero ZE (i.e. no evidence of infection) to 12 067 ZE in an adult *H. regilla* from La Misión (Site 9). Across all sampling sites, *R. catesbeiana* exhibited the highest *Bd* prevalence (75%), followed by *R. draytonii* (68%) and *A. boreas* (40%) (Table 2). *Bd* loads were highest in *H. cadaverina* (mean ZE: 1634) and *H. regilla* (mean ZE: 1234), and lowest in *A. boreas* (mean ZE: 35). *Bd*-positive sites occurred at elevations ranging from sea level (i.e. Salsipuedes and La Misión) to 2070 m (i.e. La Grulla in the Sierra San Pedro Mártir). *Bd*-positive sites also ranged from immediately adjacent to urban areas up to 51.6 km away from the nearest town (Table 1). The best-fit models of *Bd* presence-absence indicated positive effects of remoteness, distance from agriculture, and elevation (Tables 3 & 4). None of the *Bd* load models were improved by predictors beyond the intercept-only model (Table 5). Despite differences, species was not a significant predictor of either *Bd* prevalence or load in the GLMMs (Tables 3 & 5).

DISCUSSION

Our results indicate that *Bd* is widespread in most native anurans in the Mediterranean region of Baja California (*Spea hammondii* is still untested), and should be considered a potential threat to these anuran communities. We found *Bd* at sites from the coast to the mountains, in areas close to urban centers, and in remote, pristine habitats. Consistent with our expectations, *Bd* prevalence was positively associated with remoteness, distance to agricultural land, and elevation. High-elevation amphibian populations have been among the most affected by chytridiomycosis, as *Bd*-related die-offs have been documented in many montane regions (Stuart et al. 2004, Rachowicz et al. 2006, Bosch et al. 2007, Cheng et al. 2011). At our highest elevation site, and one of the most remote (also one of our most pristine) — La Grulla at 2070 m, *Bd* prevalence was 92%, and mean ZE was 195. Even though no massive die-offs have been observed at La Grulla or other locations, this could be the result of a lack of population monitoring. In addition, *Bd* die-offs can be abrupt events, so the probability of observing dead or dying animals is low in remote, infrequently visited regions (Piovia-Scott et al. 2011).

Our observation that *Bd* prevalence increases with distance to agricultural land and distance from the nearest town is consistent with previous work that suggests *Bd* is sensitive to temperature and moisture gradients introduced by more managed

Table 2. Amphibian species sampled for *Batrachochytrium dendrobatidis* (*Bd*) presence. n: sample size; Pr: *Bd* prevalence; ZE: zoospore equivalents (mean ± SD). See Fig. 1, Table 1 for site IDs

Species	n	Pr (%)	ZE (ind.$^{-1}$)	Site ID(s)
Anaxyrus boreas	5	40	35.9 ± 50.3	1, 7, 33
A. californicus	14	36	147.5 ± 321.6	15, 18, 19, 20, 24, 26
Hyliola cadaverina	49	39	1634.5 ± 2848.2	4, 6, 10, 14, 15, 18–21, 23–25, 28–31
H. regilla	46	35	1234.8 ± 3023.1	2–5, 7, 9, 10, 12, 13, 16–19, 21, 23, 24, 29, 32, 33
Rana draytonii	68	68	284.1 ± 1247.6	19, 22, 24–27, 29, 33
R. catesbeiana	8	75	259.8 ± 354.3	9, 11, 12, 16
Xenopus laevis	9	0	0	8

Table 3. Candidate mixed effects models used to determine the best predictors of *Batrachochytrium dendrobatidis* presence in sampled amphibians. AIC$_c$: Akaike's information criterion corrected for small sample sizes

Model rank	Model	No. of parameters	AIC$_c$	ΔAIC$_c$	Weight
1	Distance from agriculture	3	218.45	0.00	0.37
2	Remoteness	3	218.82	0.37	0.30
3	Elevation	3	220.04	1.59	0.17
4	Intercept only	2	220.73	2.28	0.12
5	Natural area	3	222.80	4.35	0.04
6	Species	7	226.32	7.87	0.01

Table 4. Parameter estimates for best-fit models (within 2 AIC$_c$; see Table 3) used to determine the best predictors of *Batrachochytrium dendrobatidis* presence/absence in amphibians. AIC$_c$: Akaike's information criterion corrected for small sample sizes. *$p < 0.05$

Model rank	Parameter	Estimate	SE	*Z*	p
1	(intercept)	–0.37	0.32	–1.15	0.25
	Distance from agriculture	0.70	0.33	2.14	0.03*
2	(intercept)	–0.32	0.33	–0.97	0.33
	Remoteness	0.63	0.32	1.90	0.05
3	(intercept)	–0.35	0.34	–1.04	0.30
	Elevation	0.54	0.33	1.64	0.10

Table 5. Candidate mixed effects models used to determine the best predictors of *Batrachochytrium dendrobatidis* load in sampled amphibians. AICc: Akaike's information criterion corrected for small sample sizes

Model rank	Model	No. of parameters	AIC$_c$	ΔAIC$_c$	Weight
1	Distance to agriculture	4	455.12	0.00	0.40
2	Intercept only	3	456.15	1.04	0.24
3	Elevation	4	457.18	2.06	0.14
4	Remoteness	4	457.42	2.31	0.13
5	Natural area	4	457.88	2.76	0.10
6	Species	8	463.83	8.71	0.01

environments (Murrieta-Galindo et al. 2014, Saenz et al. 2015). Habitat loss has been inversely associated with *Bd* prevalence and load in the tropics, likely a result of microclimatic conditions caused by habitat alteration that inhibit *Bd* growth and persistence (Becker & Zamudio 2011). Similar results have been reported for anuran populations in Veracruz, México with higher *Bd* prevalence in native forest than agricultural fields (Murrieta-Galindo et al. 2014). In addition, infection may be associated with amphibian species richness, which is likely to be lower in more disturbed habitats (Saenz et al. 2015). Some pesticides may suppress amphibian immune defenses and may facilitate disease outbreaks (Davidson et al. 2007). Amphibian declines in California have been related to agricultural chemicals (Davidson et al. 2002), and pesticides or fertilizers may also affect *Bd* zoospores (Johnson et al. 2003, McMahon et al. 2013b), although the causal role of pesticides on *Bd* susceptibility remains largely unknown. Only 8 of 33 localities in this study were *Bd*-negative, although 5 of these sites contained small sample sizes ($n < 5$) and should not be considered conclusively *Bd*-negative. Two *Bd*-negative sites, Arroyo Santo Tomas ($n = 1$) and Arroyo San Vicente ($n = 9$), contain hot springs, and their higher water temperatures may inhibit *Bd* growth and infection (Forrest & Schlaepfer 2011). Both *Anaxyrus* species showed the lowest ZE values, potentially related to the primarily terrestrial life history strategies of these 2 species, which may reduce the risk of infection.

Rana catesbeiana and *Xenopus laevis* are known to be tolerant carriers of *Bd* and therefore have the potential to vector *Bd* to susceptible native amphibians (Greenspan et al. 2012, Vredenburg et al. 2013). In the present study, *R. catesbeiana* individuals were *Bd*-positive at 4 of 5 sites (Table 2), while *X. laevis* individuals from our single collection locality were *Bd*-negative. Since *R. catesbeiana* and *X. laevis* were only found at 6 of our 33 sites, it appears unlikely these species are the primary vector spreading *Bd* in Baja California, even though *R. catesbeiana* may be acting as a *Bd* reservoir host in the areas where it is sympatric with native amphibians (Adams et al. 2017b). Recent work has suggested that non-amphibian vectors, including crayfish, birds, and even rainwater, may have played an important role in the emergence and spread of *Bd* (McMahon et al. 2013a, Kolby et al. 2015).

Other *Bd* carriers may include native amphibians, particularly *Hyliola regilla*, a mobile, common species characterized by large upland foraging sites, multiple breeding ponds, and extensive movement between ponds and streams (Schaub & Larsen 1978). A retrospective analysis of museum specimens has shown that *Bd* occurrences in central California are associated with the presence of infected *H. regilla* (Padgett-Flohr & Hopkins 2009), and this species may act as a *Bd* reservoir, given its high *Bd* tolerance (Reeder et al. 2012). In the present study, mean ZE for both *Hyliola* species were the highest of any species sampled (*H. cadaverina*: 1634 mean ZE; *H. regilla*: 1234 mean ZE; Table 2). Also, the highest ZE value on an individual in this study was from *H. regilla* (12 000 ZE at La Misión), which is within the range of values considered lethal in *R. muscosa*, *R. sierrae*, and *Lithobates areolatus* (>10 000 ZE; Vredenburg et al. 2010, Kinney et al. 2011). Further work is needed to determine how both hylid species might be playing a role in carrying and transmitting *Bd* to other amphibians in the region.

We suggest that our initial surveys be followed with regular monitoring to determine the fate of these sensitive amphibian populations and to understand and manage for the dynamics and effects of *Bd* infection in the Mediterranean region of Baja California. In addition, the genetic identity of *Bd* in the region is currently unknown, and low prevalence might also be related to the presence of a less virulent strain than the global panzootic lineage of *Bd*, which has been responsible for declines and extinctions elsewhere in the world (Farrer et al. 2011). Until this is clarified, we suggest that any fieldwork conducted in the region include adequate decontamination and hygiene protocols to avoid *Bd* transmission between aquatic sites.

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