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The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*

Trenton W. J. Garner[1,*], Matthew W. Perkins[1], Purnima Govindarajulu[2], Daniele Seglie[3], Susan Walker[1,4], Andrew A. Cunningham[1] and Matthew C. Fisher[4]

[1]*Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK*
[2]*Department of Biology, University of Victoria, PO Box 3020, Station CSC, Victoria, British Columbia V8W 3N5, Canada*
[3]*Department of Human and Animal Biology, University of Torino, Via Accademia Albertina 13, 10123 Torino, Italy*
[4]*Department of Infectious Disease Epidemiology, Imperial College, St Mary's Hospital, Norfolk Place, London W2 1PG, UK*
**Author for correspondence (trent.garner@ioz.ac.uk).*

***Batrachochytrium dendrobatidis* is the chytridiomycete fungus which has been implicated in global amphibian declines and numerous species extinctions. Here, we show that introduced North American bullfrogs (*Rana catesbeiana*) consistently carry this emerging pathogenic fungus. We detected infections by this fungus on introduced bullfrogs from seven of eight countries using both PCR and microscopic techniques. Only native bullfrogs from eastern Canada and introduced bullfrogs from Japan showed no sign of infection. The bullfrog is the most commonly farmed amphibian, and escapes and subsequent establishment of feral populations regularly occur. These factors taken together with our study suggest that the global threat of *B. dendrobatidis* disease transmission posed by bullfrogs is significant.**

Keywords: *Rana catesbeiana*; *Batrachochytrium dendrobatidis*; introduced species; emerging disease; epidemiology; amphibian

1. INTRODUCTION

When introduced species become established, they frequently cause radical alterations of local community dynamics and structure. Proposed mechanisms by which introduced species negatively impact endemics include the translocation of pathogens (Daszak *et al.* 2000; Hochachka & Dhondt 2000). Introduced species carry fewer parasites and benefit from this reduced parasitic load (Torchin *et al.* 2003), yet many of the parasites carried by introduced species fail to establish because they are incompatible with genetic, physiological and environmental factors of natives and their habitat (Agrawal & Lively 2002, 2003; Lively *et al.* 2004). However, a limited pathogen diversity vectored by introduced hosts may prove to have high fitness within a novel environment, and, as a consequence, have the capacity to become established. These pathogens then have the capability to incur substantial costs in endemics that have not had the benefit of coevolutionary challenges (Agrawal & Lively 2002, 2003).

Amphibian communities have a history of being susceptible to introduced species. Notably, the North American bullfrog (*Rana catesbeiana*) has been introduced globally, and is implicated in amphibian declines (Kats & Ferrer 2003; Daszak *et al.* 2004). It is known that bullfrogs are effective generalist predators and interspecific larval competitors, and it is thought that through these mechanisms bullfrogs negatively affect local communities (Kats & Ferrer 2003). However, bullfrogs coexist with many species of amphibian while preying on or competing with them (McAlpine & Dilworth 1989; Hirai 2004; Laufer 2004). This suggests that predation and competition may not be the only explanations for declines associated with bullfrog introductions.

Pathogens have been identified that have the capability to regulate amphibian populations, including the chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*, Stuart *et al.* 2004). This fungus is broadly and recently distributed, infects numerous amphibian species and is implicated in numerous species declines and extinctions (Morehouse *et al.* 2003; Stuart *et al.* 2004). *Batrachochytrium dendrobatidis* persistently infects bullfrogs, but infections are asymptomatic (Daszak *et al.* 2004). For this reason, bullfrogs have been proposed as likely vectors of *Bd* (Daszak *et al.* 2004; Hanselmann *et al.* 2004). Currently, no data are available for determining how general a threat *R. catesbeiana* pose as vectors of *Bd*. To determine this, we surveyed tissue samples from adult and larval bullfrogs introduced onto four continents for *Bd*. By using real-time PCR, histology and sequencing, we describe a global pattern of pathogen presence and variation in infection burden in introduced bullfrogs, and provide insight into the life history of *Bd*.

2. MATERIAL AND METHODS

We used toe clips and tadpole mouthparts of bullfrogs from two native populations from Ontario, Canada and 15 locations where bullfrogs have been introduced (table 1). All samples were extracted and amplified following the real-time PCR procedure of Boyle *et al.* (2004). We included amplification standards of 100, 10, 1 and 0.1 zoospore genome equivalents. All PCRs were replicated twice initially, and we considered successful amplification in both reactions as a provisional positive signal of *Bd* presence, pending assessment of infection burdens. No amplification in both replicates was taken as a true negative signal. Templates that amplified only once were subjected to one further amplification attempt, which, if successful, we took as provisional confirmation of infection.

Genomic equivalents (GE) for all provisional positives were derived from standard curves. Our extractions were diluted 1/10 before PCR, so we corrected scores by a factor of 10 and considered a GE of 0.1 as the minimum acceptable value indicative of infection. All provisionally positive samples averaging less than 0.1 were definitively scored negative. We calculated prevalence as the proportion of individuals testing positive at 0.1 GE and calculated 95% confidence intervals (CI) for each prevalence rate using the two-tailed CI for proportions, or where prevalence was either 0 or 1, using the one-tailed CI, including either 0 or 1 in the interval.

We confirmed real-time PCR positives by examining histological sections of a large sub-sample ($n=31$) for evidence of *Bd* infection. We further confirmed real-time PCR results in over 25% of definitive positives using a nested PCR approach (Garner *et al.* 2005). Initial PCR amplification of the *ctsyn1* locus was completed following

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Table 1. Prevalence of chytrid infection of bullfrog (*Rana catesbeiana*) samples included in this study.

location	*N*	stage	% positive (95% CI)
Canada			
Beaver Lake, Vancouver Island, British Columbia	10	adult	50 (24–76)
Maltby Lake, Vancouver Island, British Columbia	10	adult	80 (44–96)
Wilbur Lake, Pembroke, Ontario	16	adult	0 (0–24)
Muskrat Lake, Pembroke, Ontario	16	adult	0 (0–24)
United States			
several locations near San Pedro River, Arizona	11	adult	45 (18–75)
Brazil			
210 km southwest Brasilia	6	larva	17 (1–64)
Goias State, Goiania	1	adult	0 (0–79)
Uruguay			
Canelones Department, Empalme Olmos, 35 km northeast Montevideo	1	juvenile	100 (21–100)
San Jose Department, Libertad, 46 km West Montevideo	1	adult	100 (21–100)
United Kingdom			
Kent, East Sussex border	14	adult	14 (3–44)
France			
Loir et Cher	16	adult	63 (36–84)
Bordeaux	4	adult	25 (1–78)
Arcachon	5	larva	100 (46–100)
Savoie	1	adult	0 (0–79)
Italy			
C. Monaco, Valfenera	3	larva	0 (0–69)
near Torino	33[a]	larva	0 (0–10)
	1	adult	100 (21–100)
Japan			
Nose ricefields near Osaka	17	larva	0 (0–23)
Mizorogaika pond near Kyoto	50	larva	0 (0–9)

[a] 32 of 33 larvae from this second sample from this location found dead.

Morehouse *et al.* (2003). Initial products were reamplified using primers developed from the *Bd ctsyn1* sequence (GenBank accession no. BH001045; primer sequences: ctsyn2F 5′-TTGACTCGC AAAAAGGTACG-3′, ctsyn2R 5′-TGAAATCCAGAGCAGTTT GC-3′). Nested amplifications contained 0.5 µl initial PCR product, 1.25 U recombinant *Taq* DNA polymerase, 0.5 M each primer, 3 mM $MgCl_2$, 2.5 µl 10× PCR buffer, 100 µM each dNTP and sterile water to 25 µl. We sequenced products using the published protocols and reagents for the BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems). Sequences were aligned using Mega3 (Kumar *et al.* 2004) and the published *ctsyn1* sequence.

3. RESULTS AND DISCUSSION

Over 20% of our samples tested positive for *Bd* infection (table 1). We detected no fungus in native bullfrogs from Ontario even though our sampling effort per pond was sufficient to detect 10% disease prevalence (DiGiacomo & Koepsell 1986). However, other research has shown that *Bd* has been present in bullfrogs from Eastern Canada since the 1960s (Ouellet *et al.* 2005), suggesting that there is spatial heterogeneity in infection within this region. Samples from Japan exhibited no sign of *Bd* infection. No reports of mass die-offs in Japan are published and amphibian declines are attributed to factors other than infectious disease (Ota 2000). Asia continues to stand out as an area rich in amphibian species but where *Bd* appears absent. Whether this truly indicates low prevalence or a lack of adequate screening for the pathogen remains to be seen, but it is notable that in one of our populations, sample sizes were large enough to have been able to detect a 10% prevalence of infection (DiGiacomo & Koepsell 1986). In general, New World populations in our study exhibited high prevalence (table 1) and extremely high burdens (table 2), the latter of which differed significantly among the two regions ($t_{0.05(2),20}=2.086$, $p=0.035$). We do not know why this is, but reduced burden does not mean Europe is relatively *Bd*-free. *Batrachochytrium dendrobatidis* is known to occur in six European countries and in over 20 species of European amphibian (Garner *et al.* 2005). Our study adds France to the list of European countries with amphibians that are known to be infected with *Bd*.

Histological sections of positive samples from Canada (Vancouver Island), South America, France, the UK and Italy contained typical signs of *Bd* infection: hyperkeratosis, along with empty and zoospore-bearing zoosporangia embedded in the stratum corneum and stratum granulosum of bullfrog digits (figure 1). Sequences were generated from bullfrogs sampled in British Columbia, Canada ($n=3$ Beaver Lake Ponds, $n=5$ Maltby Lake) and France ($n=3$ Loir et Cher). We scored 238 bases of the *ctsyn1* locus and detected the single homo/heterozygote polymorphism published by Morehouse *et al.* (2003) located at position 117 of our sequences. Beaver Lake frogs included both homozygotes (2A, 1G), Maltby Lake samples included all three genotypes (1A, 3G, 1A/G) and Loir et Cher samples included both homozygotes (1A, 2G). Research by Morehouse *et al.* (2003) previously described heterozygosity at the *ctsyn1* locus in 32 out of 35 *Bd* isolates, including three bullfrog-derived isolates. The authors concluded that excess heterozygosity at the *ctsyn1* and another locus was evidence for rare, ancestral recombination,

Table 2. Genomic equivalents (GE) for all positive bullfrog samples in this study. (GE is corrected for a 1/10 dilution factor.)

location	sample	GE
Beaver Lake	BP2	5683.2
	BP4	5052.7
	BP5	2272.2
	BP6	4828.7
	BP10	3326.1
Maltby Lake	MB1	62.5
	MB3	561.1
	MB4	0.7
	MB5	12487.1
	MB6	211222.4
	MB7	7631.8
	MB8	69390.6
	MB10	663.3
San Pedro R.	44202	1740.3
	44578	52.6
	44600	176.2
	44601	210.1
	44604	169.5
Goias State	FORB2b	129000.0
Empalme	OHE16	32806.1
Libertad	OHE15	70567.8
Kent	C3/509/04	9.9
	C7/505/04	7.8
Loir et Cher	FB3	0.1
	FB4	22.4
	FB5	3.0
	FB6	0.6
	FB7	0.2
	FB8	2.6
	FB9	28.7
	FB10	5.5
	FB11	2.0
	FB13	0.5
Bordeaux	B4	1.1
Arcachon	A4	43.0
	A7	54.0
	A10	59.0
	A17	430.5
Valfenera	RC04	9.0

and that the predominant mode of reproduction in this species is clonal (Morehouse *et al.* 2003). The life history of *Bd* certainly suggests that recombination should be rare, as no sexual structures have yet been identified, and fixed heterozygosity is accepted evidence for an absence of typical segregation at meiosis. However, our results provide no evidence that heterozygosity is fixed, as only one of our 11 samples was heterozygous, and we observed nearly equal numbers of each homozygote.

The effects of introduced predators can be disastrous for native amphibian species (Kats & Ferrer 2003), and certain conditions could favour bullfrog predation leading to native amphibian population decline (Govindarajulu 2004). Nevertheless, evidence for bullfrogs causing declines through predation is equivocal. At many locations, native amphibians are rare or absent from the diet of introduced bullfrogs, including locations where natives are in decline (Kupferberg 1997; Hirai 2004; Laufer 2004; Govindarajulu *et al.* 2005; but see Wu *et al.* 2005). Bullfrogs are generalist

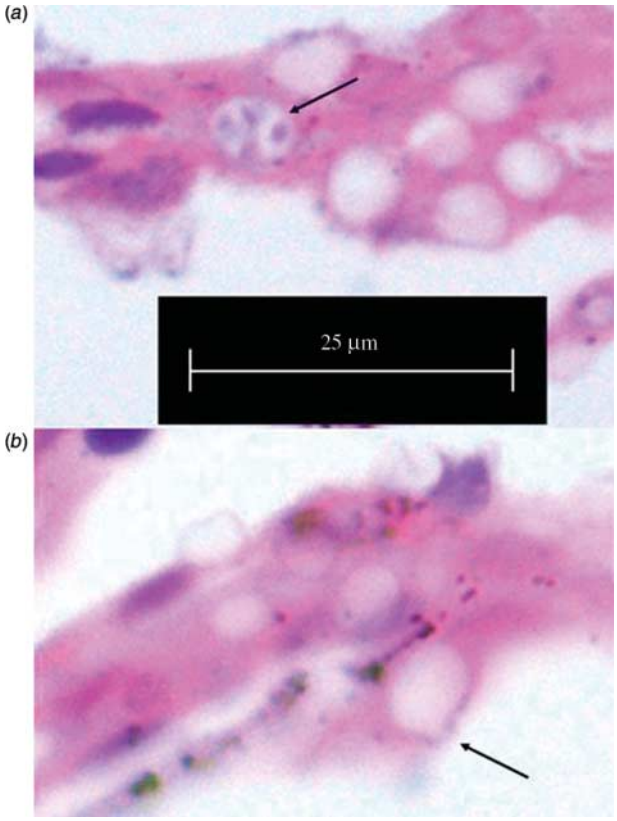

Figure 1. Histological evidence of *Batrachochytrium dendrobatidis* infection in a South American bullfrog. Multiple empty intracellular zoosporangia (sub-oval white structures) can be seen within the superficial, keratinized, layer of the epidermis. (*a*) A sporangium containing zoospores (arrow); (*b*) an empty zoosporangium with its discharge papilla clearly visible (arrow).

predators and frequency-dependent prey selection limits effects on any one species (Schmidt 2004), but experiments testing bullfrog predation capability have used amphibians without providing alternative prey (Kiesecker & Blaustein 1997; Pearl *et al.* 2004; Maret *et al.* 2006). Similarly, results from experiments examining bullfrog larval competitive abilities have revealed responses that are difficult to extrapolate to population dynamics of native species. These latter experiments are also confounded with disease presence; in every case, larvae were field collected and possibly infected with *Bd* and other pathogens (e.g. Kiesecker & Blaustein 1997; Kupferberg 1997; Lawler *et al.* 1998; Kiesecker *et al.* 2001). Results have shown native tadpoles responding in a similar fashion as tadpoles used in experimental investigations where *Bd* was included explicitly as a treatment (Parris & Baud 2004; Parris & Beaudoin 2004).

Bullfrog introductions have occurred throughout western North America (Kats & Ferrer 2003). While the relationship between bullfrog introductions and the spread of *Bd* has not been clearly established, studies of this area suggest *Bd* may be playing a powerful role in species declines. The Boreal toad (*Bufo boreas*) has tested positive for infection in the wild (Annis *et al.* 2004), suffers increased mortality after experimental exposure to *Bd* (Blaustein *et al.* 2005) and a recent analysis shows that *Bd* is the most likely agent of toad declines in the region (Scherer *et al.* 2005). In contrast,

Pacific tree frogs (*Pseudacris regilla*) do not seem to be affected by bullfrog introduction (Kupferberg 1997; Govindarajulu 2004) and experimental *Bd* exposure had no effect on *P. regilla* (Blaustein *et al.* 2005). We have detected infection in adult and apparently healthy adult *P. regilla* (T. W. J. Garner & P. Govindaraju 2005, unpublished data).

It is likely that disease, predation and competition all influence native species after bullfrog introductions. Amphibian declines are frequently due to a complexity of causative agents (Adams 1999; Blaustein & Kiesecker 2002; Relyea 2003; Pearman & Garner 2005), and though *Bd* alone can be responsible for rapid declines (Bosch *et al.* 2001), predation and competition would exacerbate the process; experiments have shown significant interactions between predators and *Bd* exposure (Parris & Beaudoin 2004). Although introduced bullfrogs can be removed as a conservation measure, *Bd* likely has a capacity to persist across a variety of environmental conditions (Piotrowski *et al.* 2004). Predation and interspecific competition are therefore manageable, but removing *Bd* from the environment has yet to be accomplished. Conservation plans for amphibian populations invaded by bullfrogs involve eliminating bullfrogs and the reintroduction of native species. These projects will likely yield unsatisfactory results until amphibian pathogens are also treated as invasive.

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