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EXPERIMENTAL EVIDENCE THAT THE BULLFROG (*RANA CATESBEIANA*) IS A POTENTIAL CARRIER OF CHYTRIDIOMYCOSIS, AN EMERGING FUNGAL DISEASE OF AMPHIBIANS

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To test the susceptibility of bullfrogs (*Rana catesbeiana*) to amphibian chytridiomycosis, groups of captive bred, recently metamorphosed bullfrogs were inoculated with zoospores of *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis, and assayed for clinical and pathological signs of infection. A novel technique for counting *B. dendrobatidis* zoospore inocula is described. Inoculation regimes varied from single exposures of 1-10 million zoospores per animal to inocula of 10 million zoospores per animal per day for a 31 day period. Twenty-five to fifty percent of each inoculated cohort was histologically positive for *B. dendrobatidis* on necropsy. However, lesions were focal, small with relatively little thickening of the keratinized epidermis and no clinical signs of chytridiomycosis were observed. Only one animal died during the experiment, due to cardiac puncture procedure. A fungal isolate used in these experiments was inoculated onto four metamorphosed poison dart frogs (*Dendrobates tinctorius*) to test whether *B. dendrobatidis* had become attenuated following repeated passage in culture. All four animals died within 30 days with severe chytridiomycosis, whereas two uninfected controls survived, demonstrating that the fungus had not become attenuated. These results provide the first experimental evidence that bullfrogs can be infected by *B. dendrobatidis*, but are relatively resistant to the disease chytridiomycosis, which is lethal to many other amphibian species. By demonstrating that *R. catesbeiana* is likely to be an efficient carrier of this pathogen, our experimental data add weight to the hypothesis that this host species is important in the spread of chytridiomycosis, particularly by commercial activities.

Key words: amphibian decline, *Batrachochytrium dendrobatidis*, chytrid fungus, frog

INTRODUCTION

Chytridiomycosis is an emerging fungal disease of amphibians that was first reported causing mass mortality associated with population declines in Central America and Australia (Berger *et al.*, 1998). The causative agent, a non-hyphal zoosporic fungus, *Batrachochytrium dendrobatidis*, was first isolated from captive Central American frogs (Pessier *et al.*, 1999) and Koch's postulates have since been fulfilled (Longcore, Pessier & Nichols, 1999). Chytridiomycosis has since been reported as the cause of mass mortalities and population declines in North America (e.g. Muths *et al.*, 2003) and Europe (Bosch, Martinez-Solano & Garcia-Paris, 2001), and as the cause of at least one, and possibly several, species extinctions (Daszak *et al.*, 2003; Cunningham *et al.*, in press). It is emerging in south-western Australia (Alpin & Kilpatrick, 2000) and New Zealand (Waldman *et al.* 2001) and has recently been reported from wild and museum specimens of amphibians from Ecuador (Ron & Merino, 2000), Venezuela (Bonaccorso *et al.*, 2003) and Africa (Lane *et al.*, 2003). Outbreaks of chytridiomycosis are often characterized by simultaneous die-offs of multiple amphibian species at affected sites (Berger *et al.*, 1998). Experimental infections and field data demonstrate that a range of frogs, toads and salamanders are susceptible to chytridiomycosis (Berger *et al.*, 1998; Speare *et al.*, 2001). However, the pattern of amphibian declines in the tropics is characterized by the presence of sympatric declining and non-declining species (Williams & Hero, 1998). It is unknown which, if any, amphibian species are resistant to infection, or to the disease chytridiomycosis.

Most emerging diseases of wildlife are driven by anthropogenic environmental changes such as altered land use, introduction of alien species, deforestation and others (Daszak, Cunningham & Hyatt, 2000; Daszak, Cunningham & Hyatt, 2001; Cunningham *et al.*, in press). Climate change, anthropogenic introduction of

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Batrachochytridum dendrobatidis and other factors have been hypothesized as drivers of chytridiomycosis emergence (Daszak *et al.*, 1999; Daszak *et al.*, 2003). The pattern of amphibian declines (Daszak *et al.*, 1999) and DNA sequence phylogeny of *B. dendrobatidis* isolates (Morehouse *et al.* 2003; Daszak, Cunningham & Hyatt, 2003) support the hypothesis that chytridriomycosis has been recently introduced into naïve populations in some regions. The presence of *B. dendrobatidis* in the national and international trades of amphibians for pets (Mutschmann *et al.*, 2000), amphibians destined for outdoor pond stocking (Groff *et al.*, 1991), laboratory animals (Reed *et al.*, 2000), zoo animals (Pessier *et al.*, 1999) and food (Mazzoni *et al.*, 2003) provides potential mechanisms for the anthropogenic introduction of this pathogen.

In this paper, we report the results of a series of experimental infections of bullfrogs (*Rana catesbeiana*) with *B. dendrobatidis*. Our data suggest that *R. catesbeiana* is susceptible to infection by *B. dendrobatidis*, but resistant to the clinical effects of the disease chytridiomycosis, with no evidence of the severe lesions that are typical of chytridiomycosis, no evidence of behavioural changes associated with this disease and no mortality following infection. We discuss the results with reference to the ability of *R. catesbeiana* to act as a carrier of chytridiomycosis, and its potential involvement in the anthropogenic introduction of *B. dendrobatidis* to new regions.

MATERIALS AND METHODS

ANIMALS

Four experiments were conducted with recently metamorphosed, captive-bred bullfrogs (*Rana catesbeiana*), purchased from a commercial breeder (Rana Ranch Bullfrog Farm, Twin Falls, Idaho). All individuals were shipped less than three weeks after loss of final portions of tail bud. Frogs were purchased during July of 2000 (n=45), August of 2000 (n=20), November of 2000 (n=25) and March of 2001 (n=30). Because animals were captive-bred metamorphs, concerns about changes in immunological function at different seasons were considered irrelevant to the current study. To determine whether experimental groups were pathogen-free at the start of the experiment, five to ten frogs from each shipment were killed by soaking in a bath of 0.15% aqueous tricaine methane sulfonate (MS-222) buffered to pH 7.0 with sodium bicarbonate ($NaHCO_3$) for 10 minutes after cessation of respiratory activity, followed by pithing (Fowler, 1993). Three toes and webbing from each hind foot and approximately 3 cm^2 of skin from the ventral groin were fixed in neutral-buffered 10% formalin, embedded in paraffin, sectioned and stained with haemotoxylin and eosin and examined histologically for the presence of *Batrachochytrium dendrobatidis* zoosporangia (Pessier *et al.*, 1999). These representative samples from each experimental group were negative for *B. dendrobatidis*.

All experimental animals were captive bred and were acclimatized for one week prior to inoculation. Animals were individually housed in autoclaved plastic aquaria (29.8 cm length × 19.7 cm width × 20.3 cm depth) (Kritter-Keepers) within an environmental chamber with regulated thermal (20°C) and light conditions (fluorescent lighting for 14 hours per day) throughout the period of study. Deionized water (450 ml) was added to a bed of 200 ml of autoclaved gravel and a plastic shelter placed in the containers to provide a hiding place for the amphibians. Water was changed every three days. Frogs were fed commercially-bred crickets every two days. Tanks were covered with a mesh lids that prevented escape of crickets. All discarded fomites and water were sterilized with a 1% aqueous solution of sodium hypochlorite (diluted Chlorox) and containers were autoclaved prior to re-use. Used water was treated for 10 min with Chlorox to make a final 1% solution.

Six dendrobatid frogs of a species (*Dendrobates tinctorius*) known to be susceptible to chytridiomycosis (Nichols *et al.*, 2001) were purchased from a captive breeder. Four were inoculated with *B. dendrobatidis* to test if the pathogen had become attenuated by repeated passage in culture and two served as negative controls. The numbers used were small due to the difficulty in obtaining suitable animals, and for ethical (conservation) reasons.

PATHOGEN CULTURES AND ZOOSPORE COUNTS

Cultures of *B. dendrobatidis* were maintained on TGhL agar plates (Longcore *et al.*, 1999) at 17°C. Zoospores were harvested by flooding plates with a dilute salt (DS) solution used by mycologists to mimic pond water in pH and salt concentration (Fuller & Jaworski, 1987) and incubating at 15°C for 24 hrs. We determined zoospore concentration in the zoospore-rich supernatant as follows: First, we added 29 µl of the zoospore-rich supernatant to 1 µl of Lugols Iodine, which killed zoospores and stained them light brown. Using a Neueberg counting chamber and phase contrast microscopy, we counted flagellated zoospores and calculated the number of zoospores per ml of supernatant. Cells without flagella were not counted.

INOCULATIONS

To maintain uniformity, inocula were prepared by adding DS to provide the desired number of zoospores for each frog. We pipetted a 5 ml inoculum over each frog individually while it sat in a small, sterile container. The containers were shaped such that the only position the frogs could maintain was to sit with their groins, abdomens and hindlegs submerged in the inoculum. After an initial inoculation period, each frog was returned to its individual aquarium. The inoculation period was varied from 1 hr to overnight (Table 1), so as to provide a range of intensity of exposures. To maintain an environment that resembled as natural a situation as possible, i.e. where *Batrachochytrium dendrobatidis* may persist as a saprobe in the water and on the substrate (Daszak *et*

al., 1999; Johnson & Speare, 2003), the inoculum that had been pipetted over each frog was added to the respective aquarium and mixed with the water already present.

HISTOLOGICAL ANALYSIS

From each frog necropsied, three toes and webbing from each hind foot and approximately 3 cm^2 of skin from the ventral groin were sampled and fixed in neutral-buffered 10% formalin, embedded in paraffin, sectioned and stained with haemotoxylin and eosin and examined histologically for the presence of *Batrachochytrium dendrobatidis* zoosporangia (Pessier *et al.*, 1999). Individuals were considered uninfected if 25 fields of view at low power (25×) were examined without finding *B. dendrobatidis* developmental stages. All carcass remains were autoclaved and discarded. All post mortem equipment was autoclaved after use.

RATIONALE FOR EXPERIMENTS 1-4

The aim of these experiments was to test whether bullfrogs could be infected in the laboratory with *B. dendrobatidis* and whether these infections would produce clinical signs of the disease chytridiomycosis and death (Berger *et al.*, 1998; Pessier et al 1999; Daszak *et al.*, 1999).

Experiment 1. This involved an initial high inoculum (20 million zoospores for one hour), mimicking an individual moving into a body of water during an epizootic, where infected individuals are hypothesized to produce large numbers of zoospores. This was followed by a repeat exposure around three weeks later, to boost infection rates. The temperature selected (21°C) was within the optimal range for maximum growth of *B. dendrobatidis* in culture (Piotrowski *et al.*, 2004). The isolates used were from North American ranid frogs (no. 216, *Rana muscosa*, California; no. 228, *R. yavapaiensis*, Arizona). The 33-day period of the experiment corresponded to the three week time course of chytridiomycosis observed previously (Berger *et al.*, 1998). Within this and other experimental groups, control frogs were housed individually within tanks that were placed between rows of inoculated frogs.

Experiment 2. Following the failure to produce severe infections or clinical disease in experiment 1, infections were conducted that followed the protocols of Nichols *et al.* (2001) by adopting a lower temperature of 15°C, using an isolate known to be lethal to a range of frogs in captivity (no. 198, *Dendrobates auratus*, captive National Zoo) and increasing the number of inoculations (5 million for 2 hours on days 1, 3, 5, 10, 15 & 20). The experiment was terminated on day 28.

Experiment 3. Following the failure of the Nichols *et al.* (2001) protocol to produce severe infections or clinical disease in bullfrogs, the protocol used in experiment 1 was repeated, using North American ranid frog isolates (no. 217, *Rana muscosa*, California; no. 270, *R. catesbeiana*, California; no. 260, *R. catesbeiana*, Quebec) but with inoculations of 5 million zoospores for 2 hours daily for 28 days at a temperature of 21°C.

Experiment 4. This was set up to test if the *B. dendrobatidis* isolates we were using had become attenuated during repeated passage in culture, i.e. in the absence of the amphibian host, or keratin. The isolate used in experiment 4 (no. 197, *Dendrobates auratus*, captive National Zoo) was collected during an outbreak of chytridiomycosis in multiple species of captive frogs, and was known to be lethal to dendrobatids and other species (Longcore *et al.*, 1999; Pessier *et al.*, 1999). It was one of the first two isolates of *B. dendrobatidis* collected and had been passaged approximately every 10-14 days for over two years, therefore was most likely to have become attenuated. Dendrobatid frogs of a known susceptible species were inoculated with 10 million zoospores for 2 hours daily for 31 days. The temperature selected (21°C) was within the optimal range for growth of *B. dendrobatidis* in culture.

Experiment 5. Following the production of lethal infections in experiment 4, 1-10 million of the same isolate (no. 197, *Dendrobates auratus*, captive National Zoo) was inoculated for 2 hrs daily onto bullfrogs for 29 days at 21°C to cross check whether this proven virulent strain, known to cause death in multiple frog species, would cause clinical chytridiomycosis or mortality in *R. catesbeiana*.

RESULTS

EXPERIMENTAL INFECTION OF BULLFROGS

Histological evidence for the presence of *B. dendrobatidis* was found in 25-50% of the bullfrogs inoculated in experiments 2, 3 and 5. We saw no significant differences in the degree of infection or percentage infected between cohorts kept at 15°C and 21°C (χ^2=0.274, P>0.5). Despite the presence of *B. denrobatidis* (i.e. infection by this parasite), the lesions were not consistent with the disease chytridiomycosis, which is characterized by extensive thickening of the keratinized epidermal cell layer (hyperkeratosis and hyperplasia). Experimentally infected frogs positive for *B. dendrobatidis* in experiments 2, 3 and 5 showed only infrequent lesions, which were focal, small (approximately 100-300 μm surface diameter on histological section) and consisted of developing and mature zoosporangia within areas of minimal thickening of the keratinized layer of the epidermis (up to a thickness of 3 cells maximum). No clinical signs of chytridiomycosis (inappetance, behavioural abnormalities) were observed in the exposed bullfrogs. Bullfrogs from the inoculated cohorts fed at the same rate as those in controls and gained weight during the experimental periods (data not shown). Only one infected bullfrog (animal no. 6 in experiment 1, on day 25) died during the experiments, shortly after a cardiac puncture procedure conducted as part of another experiment. One control animal was found infected in experiment 3. This animal had a low-level of infection with *B. dendrobatidis*.

TABLE 1. Experimental infection of bullfrogs (*Rana catesbeiana*) and dendrobatid frogs (*Dendrobates tinctorius*) with *Batrachochytrium dendrobatidis*, the agent of amphibian chytridiomycosis. *Clinical signs of inappetance and extensive sloughing of skin were observed prior to death.

Experiment number	Frog identification number	Isolate used	Number positive for *B. dendrobatidis* on necropsy
Expt. 1 *Rana catesbeiana*	1-5, 21-25 (controls)	-	0/10
	6-20	216	4/15
	26-40	228	0/15
Expt. 2 *R. catesbeiana*	1-5 (controls)	-	0/5
	6-20	198	4/15
Expt. 3 *R. catesbeiana*	1-3 (controls)	-	0/3
	4-7	270	2/4
	8-11	260	1/4
Expt. 4 *Dendrobates tinctorius*	1,2 (controls)	-	0/2
	3-6	197	4/4*
Expt. 5 *R. catesbeiana*	2,8,13 (controls)	-	1/3
	3-7, 9-12,14-22	197	4/11

EXPERIMENTAL INFECTION OF DENDROBATID FROGS

The inoculated frogs showed clinical signs of chytridiomycosis (inappetance, increased sloughing of their skin, listlessness). Histological evidence of intense *B. dendrobatidis* infection was found in all four inoculated *Dendrobates tinctorius*. No evidence of infection was found in the two controls. Lesions were numerous, extensive (70-100% of skin surface on histological sections) and revealed sporangia within areas of marked thickening of the keratinaceous layer of the epidermis (up to 10 cells thick). Two of the inoculated animals died (days 25 and 29) before the end of the experiment.

DISCUSSION

The experimental data presented here show that bullfrogs (*Rana catesbeiana*) are able to be infected by *B. dendrobatidis*, without progression to clinical chytridiomycosis or death. The distinction between infection by *B. dendrobatidis* and presence of the disease chytridiomycosis is based on the presence or absence of clinical and pathological signs of chytridiomycosis (Berger *et al.*, 1998; Pessier *et al.*, 1999; Nichols *et al.*, 2001). These include (1) The presence of a substantial area of hyperplasia and hyperkeratosis (between 5 and 10 keratinaceous cell layers thick) of the ventral skin containing substantial numbers of *B. dendrobatidis* developmental stages; (2) clinical signs such as inappetance, loss of righting reflex and increased epidermal sloughing; and (3) rapid progression to death. The absence of any of these signs of chytridiomycosis in the bullfrogs inoculated with zoospores in our experiments (including those that became infected) suggests that they are resistant to the disease, even though they can be infected by *B. dendrobatidis*. Bullfrogs showed no signs of chytridiomycosis, remained healthy, and did not die, even when inoculated with up to 10 million zoospores daily for over four weeks. This contrasts with experimental infections of highly susceptible species such as the Australian frog *Myxophyes fasciolatus*, individuals of which died 35 days after a single inoculation of 100 zoospores (Berger, pers. comm.). Our findings suggest that bullfrogs are relatively resistant to clinical chytridiomycosis, even though they are susceptible to *B. dendrobatidis* infection.

Alternative explanations of the data can be ruled out. Firstly, repeated passage in culture of a range of pathogens (viruses, bacteria and others) often leads to attenuation, i.e. loss of virulence when inoculated onto susceptible hosts (Ford *et al.*, 2002). Our finding (in experiment 4) that an isolate of *B. dendrobatidis* produced clinical and pathological signs of severe chytridiomycosis and death in dendrobatid frogs, but only very mild, focal lesions, no clinical signs of chytridiomycosis and no mortality in bullfrogs, demonstrates that our cultures of *B. dendrobatidis* had not become attenuated despite repeated passage outside the host. It is important to note that this isolate originated from dendrobatid frogs during an outbreak that killed multiple frog species, therefore is proven to be lethal to a range of amphibians. Secondly, some pathogens exist as a series of strains that are species-specific, or specific to a geographical region. It is conceivable that the isolates of *B. dendrobatidis* are lethal only in amphibians from which they are isolated, therefore non-bullfrog isolates would not kill or cause disease in bullfrogs. However, in the current experiments, we used fungal isolates that are known to be lethal to a range of frogs (e.g. isolate nos. 197 and 198) and others isolated spe-

cifically from North American ranid frogs, including bullfrogs. None of these caused chytridiomycosis or death of bullfrogs. Furthermore, molecular data suggest that isolates of *B. dendrobatidis* collected from a range of sites and hosts (including some of those used in this study) are a single clonal strain (Morehouse *et al.*, 2003) and there are no significant morphological or physiological differences between isolates in culture (Longcore *et al.*, 1999; Piotrowski *et al.*, 2004). Finally, experimental infection of Australian frogs using isolates collected from other species and geographical sites produced lethal infections (Berger *et al.*, 1998; Berger pers. comm.).

The finding of an infected control frog in experiment 4, and other infected animals from the same supplier, suggests that it is possible frogs within the supply facility may have been contaminated with *B. dendrobatidis*. It could be hypothesized that the animals we inoculated with zoospores may have been partially immune to the pathogen, explaining the lack of disease or mortality. We doubt that this is a valid hypothesis, because the animals used in our studies were purchased less than three weeks (i.e. less than the clinical course of chytridiomycosis) after resorption of the tail bud. Only post-metamorphic amphibians have cutaneous chytridiomycosis, therefore if animals were infected at the beginning of the post-metamorphic period at the breeders, they should have been at a late stage of progression to chytridiomycosis by the time of arrival, if the species were susceptible. Instead, in each experiment, the animals we examined prior to the experiment were negative for *B. dendrobatidis* and no signs of chytridiomycosis were found in those used for experiments. The infection of this control animal may, therefore more likely be due to experimental error or inadvertent transmission of *B. dendrobatidis* between enclosures. It is unknown whether tadpoles infected at the suppliers would develop immunity to chytridiomycosis, or be able to carry immunity through metamorphosis.

In the current paper, we describe for the first time a simple, reliable protocol for counting *B. dendrobatidis* zoospores in inocula. Because chytrid pathogens are normally transmitted to new hosts via flagellated zoospores, this technique provides an accurate way to assess the number of zoospores (and hence the degree of challenge) in experimental inocula, while excluding non-motile (non-infectious) zoospores. We also describe a series of detailed techniques for inoculating, handling and housing experimental animals for future laboratory studies of chytridiomycosis.

The findings reported here support a simple population model of chytridiomyocsis in which individual hosts, host populations and species vary in susceptibility to *B. dendrobatidis* (Daszak *et al.*, 1999; Daszak *et al.*, 2003). In this model, only a small proportion of biologically and ecologically predisposed populations undergo local extinctions and declines due to chytridiomycosis, whereas the majority of other species and populations remain relatively unaffected. Our data suggest that *B. dendrobatidis* would cause little, if any, mortality in populations of bullfrogs, and that the majority of individuals would be able to grow and breed normally. Our data are supported by reports of similar low intensity infections in farmed (Mazzoni *et al.*, 2003), feral (Hanselmann *et al.*, 2004) and wild-caught (Daszak *et al.*, 2003) bullfrogs. No evidence in any of these reports indicated that infection by *B. dendrobatidis* in bullfrogs caused the abnormal behavioural syndromes associated with chytridiomycosis or skin lesions consistent with those found in frogs that had died of chytridiomycosis (Berger *et al.*, 1998).

Our data have conservation significance for two reasons. Firstly, they suggest bullfrogs may be an efficient reservoir or alternative host of this emerging pathogen because they are able to harbour the fungus, but do not suffer clinical signs of infection. Although it is so far unknown whether the light *B. dendrobatidis* infections in bullfrogs are sufficient for transmission of the agent from one individual to another, the presence of fully formed sporangia in many of the positive animals suggests this is likely. Other authors have recently highlighted the role of such reservoir or alternative hosts in "apparent" or "parasite-mediated" competition (Holt and Lawton, 1994; McCallum & Dobson, 1995; Hudson & Greenman, 1998). Parasite-mediated competition occurs when two sympatric host species share a common parasite and the least susceptible host uses the differential impact of the parasite as a competitive edge. In some cases, parasite-mediated competition allows the less susceptible host to drive the more susceptible host to extinction. In areas where susceptible endemic amphibians occur sympatrically with introduced, *Batrachochytrium*-positive *R. catesbeiana*, apparent competition mediated by chytridiomycosis may be an outcome.

Secondly, the ability of *R. catesbeiana* to become infected by *B. dendrobatidis* – but not to suffer clinical signs of infection – suggests that introduced individuals could act as efficient carriers of this pathogen. *Batrachochytrium dendrobatidis* has been reported from bullfrogs that are part of an increasingly centralized and expanding trade in live animals for food within South American countries and between South America and the USA. (Mazzoni *et al.*, 2003). In Venezuela, bullfrogs introduced as a farmed food source have escaped, expanded their population locally, and support a high prevalence of *B. dendrobatidis* infection (Hanselmann *et al.*, 2004). Our findings of infected, but otherwise healthy, *R. catesbeiana* suggest that the trade in ranid frogs for laboratory or educational uses is yet another source for the introduction of chytridiomycosis. Bullfrogs have been introduced into Europe, Asia and the western USA. (Kupferberg, 1997). The western USA populations are descended from individuals introduced as a food source in the late 19th and early 20th centuries and have successfully colonized large areas, competing with endemic species and perturbing community structure (Kupferberg, 1997; Kiesecker &

Blaustein, 1997). The potential of *R. catesbeiana* to act as an efficient carrier of chytridiomycosis adds to its capacity as an invasive species threat to amphibian populations (Kats & Ferrer, 2003).

Previous authors have commented on the likely role of introduced species in the spread or emergence of chytridiomycosis (Berger *et al.*, 1998; Daszak *et al.*, 1999; Cunningham *et al.*, 2003). Molecular evidence also suggests anthropogenic introduction is involved in the spread or emergence of chytridiomycosis (Morehouse *et al.*, 2003; Daszak *et al.*, 2003). Amphibian chytridiomycosis is a key example of an emerging infectious disease of wildlife which, as a group, is responsible for a series of significant population declines and extinctions of wildlife species (Daszak *et al.*, 2000, 2001; Dobson & Foufopoulos, 2001). The most commonly cited driver of these diseases is the anthropogenic introduction of pathogens or hosts to new regions, or 'pathogen pollution' (Cunningham *et al.*, 2003). Our current paper provides evidence that *R. catesbeiana* is a potentially efficient carrier of chytridiomycosis and adds weight to its hypothesized role in pathogen pollution-mediated spread of chytridiomycosis. We urge that strong measures be taken to combat this threat to amphibian biodiversity. These should include quarantine regulations (Cunningham *et al.*, 2001; Johnson & Speare, 2003) and efforts to curtail the legal and illegal trade in amphibian species.

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