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TRANSMISSION OF *BATRACHOCHYTRIUM DENDROBATIDIS* TO WOOD FROGS (*LITHOBATES SYLVATICUS*) VIA A BULLFROG (*L. CATESBEIANUS*) VECTOR

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ABSTRACT: Chytridiomycosis, an emerging infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis*, threatens anuran populations worldwide. Effects of *B. dendrobatidis* on frog species are variable. Some species typically develop nonlethal infections and may function as carriers; others typically develop lethal infections that can lead to population declines. Nonlethal infections in the bullfrog (*Lithobates catesbeianus*) are well-documented. In contrast, recently metamorphosed wood frogs (*L. sylvaticus*) can die from chytridiomycosis. We conducted an ex-situ experiment between May and July 2010 to determine whether *B. dendrobatidis*-infected bullfrogs could transmit the fungus to wood frog tadpoles when the two species shared a body of water. We tested for *B. dendrobatidis* infections with quantitative polymerase chain reactions (qPCR) in a subsample of the wood frog tadpoles and in all metamorphosed wood frogs and compared risk of death of froglets exposed and unexposed to infected bullfrogs. We detected *B. dendrobatidis* sporadically in subsampled treatment tadpoles (nine of 90, 10%) and frequently in treatment froglets (112 of 113, 99.1%). Pooled risk of froglet death was higher ($P<0.001$) in treatment enclosures than in control enclosures. Our results indicate that, at the low infection loads bullfrogs tend to carry, swabbing for PCR analyses may underestimate prevalence of *B. dendrobatidis* in this species. We highlight bullfrog disease screening as a management challenge, especially in light of exotic bullfrog colonies on multiple continents and large-scale global trade in this species. We document the importance of quantifying lethal and sublethal effects of bullfrog vectors on *B. dendrobatidis*-susceptible species.

Key words: Amphibian, anuran, carrier, chytridiomycosis, vector.

INTRODUCTION

A major driver of global declines and extinction of anurans is spread of the chytrid fungus *Batrachochytrium dendrobatidis* (Longcore et al., 1999). This fungus colonizes keratin-containing amphibian tissue, causing the disease chytridiomycosis. In anurans, keratin is restricted to mouthparts of tadpoles, is gradually distributed to the skin during metamorphosis, and remains in the stratified epidermis postmetamorphosis (Marantelli et al., 2004). Although *B. dendrobatidis* seems capable of infecting all anurans and urodeles, effects of the fungus on species range from subclinical infection to morbidity with mortality (e.g., Lips, 1999; Hanselmann et al., 2004). The bullfrog (*Lithobates catesbeianus*) typically develops nonlethal infections and is considered a carrier of *B. dendrobatidis* (Daszak et al., 2004). The hypothesis that bullfrogs serve as vectors, transmitting *B. dendrobatidis* to susceptible species, has not been verified (Daszak et al., 2004; Hanselmann et al., 2004; Gahl et al., 2009). Understanding the role of asymptomatic carrier species in the epidemiology of *B. dendrobatidis* may further understanding of the dynamics of transmission of this disease in natural ecosystems.

Here, the wood frog (*L. sylvaticus*) serves as a model *B. dendrobatidis*-susceptible species. Recently metamorphosed wood frogs can die from chytridiomycosis under laboratory conditions (Gahl et al., 2012). In parts of the northeastern United States, wood frogs and bullfrogs may occupy the same ephemeral pools annually for early stages of development (wood frog) and for foraging (bullfrog). In coastal Maine, for example, bullfrogs foraged at

seasonal pools between mid-May and mid-July, a period corresponding with the presence of wood frog eggs, tadpoles, and juveniles (Gahl et al., 2009). *B. dendrobatidis* has been detected in regions and habitats where wood frogs and bullfrogs co-occur (e.g., Longcore et al., 2007). If young wood frogs are susceptible to *B. dendrobatidis* in the wild and bullfrogs serve as reservoir and vector, recruitment of wood frogs may be impeded by disease-induced mortality. We conducted an ex-situ experiment between May and July 2010 to determine whether *B. dendrobatidis*-infected bullfrogs could transmit the fungus to wood frog tadpoles when the two species shared a body of water. We tested for *B. dendrobatidis* infections with quantitative polymerase chain reactions (qPCR) in a subsample of the wood frog tadpoles and in all metamorphosed wood frogs and compared risk of death of froglets exposed and unexposed (control) to infected bullfrogs.

MATERIALS AND METHODS

Experimental enclosures

Eight wood frog egg masses and nine juvenile bullfrogs (snout-vent lengths ranged from 4.1 to 4.7 cm at capture) were collected from the lower Penobscot River watershed, Maine, USA (44°58′N; 68°27′W), in April and May 2010. By collecting wood frogs at the egg phase, when keratinized tissue is lacking or inaccessible, we ensured that test animals were free of chytridiomycosis before the experiment (Marantelli et al., 2004). Before the experiment, wood frog eggs and newly hatched tadpoles were maintained communally in well water and bullfrogs were housed individually and separate from the wood frogs. We assigned animals to experimental enclosures and enclosures to experimental groups (treatment or control) with random numbers generated in Program R (R Development Core Team, 2010). Of nine enclosures, six were treatment enclosures (bullfrogs experimentally exposed to *B. dendrobatidis*; designated A, B, C, D, E, and F) and three were control enclosures (bullfrogs not experimentally exposed to *B. dendrobatidis*; designated G, H, and I).

At the onset of the experiment, each enclosure consisted of two plastic containers of different sizes (Sterilite, Townsend, Massachusetts, USA), the smaller of which (41.6 × 33.7 × 29.5 cm) was nested inside the larger container (84.1 × 51.4 × 37.5 cm). Each small container housed one bullfrog and contained a plastic ramp with an attached platform to provide a dry refuge. Each large container housed 35 wood frog tadpoles (except for one enclosure that housed 36 tadpoles). To allow exchange of water between the two containers of treatment enclosures, we drilled arrays of 0.3-cm holes into three vertical walls of the small containers. Arrays were 12 holes by 26 holes; holes were 1 cm apart. The purpose of control enclosures was to rule out husbandry problems in the event of frog mortality; we did not drill holes in control enclosures to prevent exposure of wood frogs to potentially naturally infected bullfrogs. We filled each enclosure with aged tap water to a depth of 20 cm. This required 55 liters of water in each enclosure, 40 liters of which filled the large container and 15 liters of which filled the small container.

Tadpoles were at Gosner stages 26 to 35 when the experiment began (Gosner, 1960). At metamorphosis (defined as onset of tail resorption), we transferred wood frogs to a third set of containers (43.2 × 28.3 × 16.5 cm; Sterilite) in which they remained separated by enclosure but no longer shared water with bullfrogs. These containers were tilted to create a pool of aged tap water on one side and a terrestrial area lined with moist paper towels on the other side.

Batrachochytrium dendrobatidis cultures and inoculations

We cultured *B. dendrobatidis* (strain JEL423) in 1% tryptone broth in 125-ml flasks. Two flasks functioned as long-term cultures from which pairs of fresh cultures were made bimonthly. For each passage, we transferred 1 ml of active culture to 75 ml of fresh broth. New cultures were incubated at 23 C for several days and then maintained at 4 C. To obtain zoospores for inoculations, 9-cm Petri plates (two per animal to be inoculated) containing 1% tryptone in 1% agar were each inoculated with 1 ml of cultured broth. Plates were dried in a laminar flow hood and incubated at 23 C for approximately 4 days before inoculation. On the day of inoculation, we confirmed with a compound microscope the presence of live zoospores on the plates and added up to 4 ml of sterile distilled water to each plate to form a zoospore suspension. We then combined the liquid contents of each plate in a sterile beaker and refrigerated the beaker with a cover at 4 C until immediately before inoculation (up to 1 hr later). We enumerated zoospores by

diluting a portion of suspension with an equal amount of Lugol solution and then counting zoospores in the diluted sample with a hemocytometer (e.g., Vazquez et al., 2009).

We inoculated bullfrogs on days zero, eight, 16, and 24 in individual 500-ml Ziploc containers (SC Johnson, Racine, Wisconsin, USA). To inoculate, we filled each container with enough aged tap water to cover the bottom (approximately 17 ml), placed each frog into a container, added 4 ml of zoospore suspension or 4 ml of aged tap water (control), and gently agitated each container. We inoculated with the following number of zoospores per frog: day zero, 6.6×10^6; day eight, 4.9×10^7; day 16, 1.9×10^7; day 24, 9.4×10^7. After inoculating, we examined a sample of the zoospore suspension to confirm continued presence of live zoospores. We left frogs in inoculant baths for 24 hr, rinsed them twice in aged tap water, and returned them to their respective enclosures.

Amphibian husbandry

To avoid cross-contamination, we wore new vinyl gloves when handling animals and used a set of husbandry equipment unique to each enclosure. The experiment lasted for 70 days. Laboratory conditions were 20 C ± 2 C with a 12-hr/12-hr light/dark cycle. We replaced half the water in enclosures with aged tap water weekly and adjusted enclosure water levels daily to achieve consistency. Solid waste was removed from enclosures as needed. Food for study animals varied throughout the experiment but diet was consistent among enclosures. We fed bullfrogs live mealworms or crickets every third day. Tadpoles received rabbit pellets or boiled and frozen kale every other day. Froglets received live flightless fruit flies, pinhead crickets, or mealworms daily. We refreshed water and paper towels in froglet containers weekly. We disinfected waste with bleach before disposing.

About every 12 hr for the first 50 days of the experiment, we gently lifted the small treatment enclosure containers (containing bullfrogs) above the corresponding large containers (containing wood frogs) until all water had drained into the large containers through the drilled holes. We then replaced the small containers inside the large containers. This ensured that water was regularly exchanged between the two containers of each enclosure.

We checked for mortality twice daily. We removed dead animals and immediately preserved them in 70% ethanol. We euthanized tadpoles that remained small with no development of limb buds on day 50 and surviving froglets on day 70. Froglets with clinical signs of chytridiomycosis (lethargy and excessive skin shedding) were immediately euthanized. Euthanasia was performed by placing animals in a solution of MS-222 (1g/l) buffered with an equal amount of sodium bicarbonate (Webb et al., 2005).

Assessment of *B. dendrobatidis* infection status

We used qPCR and wet mount cytology to assess status of *B. dendrobatidis* infection in study animals throughout the experiment (Boyle et al., 2004; Weldon and Du Preez, 2006). Analysis by qPCR was completed at the Vector Borne Disease Diagnostic Laboratory, School of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA. Samples for qPCR were processed with *B. dendrobatidis*-specific real-time Taqman (Applied Biosystems, Foster City, California, USA) PCR following Boyle et al. (2004) and Retallick et al. (2006). We removed ethanol with gentle heat prior to extraction. We ran appropriate negative and positive controls concurrently with samples; the limit of detection was 0.1 zoospore equivalents.

We documented infection status of bullfrogs with qPCR analysis of skin swabs. We swabbed each bullfrog on days zero, eight, 16, 24, 32, 40, and 50. Swabs from day zero were considered a record of natural infection status. On days when bullfrogs were both swabbed and inoculated (days zero, eight, 16, and 24), we swabbed immediately before inoculating. We swabbed by gently but firmly sweeping a sterile polyester-tipped applicator (Puritan Medical Products, Guilford, Maine, USA) several times on the ventral surface, limbs, and inter-digital membranes following Livo (2003) and Hyatt et al. (2007). We placed swabs in individually numbered plastic cryogenic vials, dried them in a laminar flow hood, and stored them frozen until shipment. Also, we collected samples of liquid from bullfrog inoculant baths after the third and fourth inoculations (days 17 and 25). We refrigerated samples for several hours to allow solid contents to settle and with a compound microscope examined aliquots of solid matter for infected skin sloughs.

We documented infection status of wood frogs using qPCR analysis of tissue samples. We harvested five tadpoles from each enclosure on days seven, 15, and 23 ($n=135$; five tadpoles removed from each of nine enclosures on 3 days) by blindly sweeping aquarium nets in enclosures. We immediately euthanized tadpoles, extracted mouthparts (up to Gosner stage 40) or one hind foot (past Gosner stage 40), and

TABLE 1. After sharing water with *Batrachochytrium dendrobatidis*-inoculated bullfrogs (*Lithobates catesbeianus*) in an ex-situ experiment, the proportion and total percentage of subsampled wood frog (*L. sylvaticus*) tadpoles that were positive for *B. dendrobatidis* by quantitative polymerase chain reactions.

Enclosure	Day 7	Day 15	Day 23	Total (%)
A	0/5	0/5	0/5	0
B	0/5	1/5	1/5	13.3
C	1/5	1/5	0/5	13.3
D	0/5	0/5	0/5	0
E	0/5	0/5	0/5	0
F	2/5	1/5	2/5	33.3
Total (%)	10.0	10.0	10.0	10.0

placed samples in individually numbered plastic cryogenic vials containing 70% ethanol for shipment (Gosner, 1960). One hind foot of each preserved froglet was also extracted for qPCR after the experiment ended.

Statistical analysis

We assessed differences in wood frog froglet mortality between groups with Cox Proportional Hazards survival analysis (Cox, 1972). In this analysis, time until death is the response but individuals without a corresponding time until death (i.e., outlived the experiment or were lost from the study) are also included and are referred to as 'censored.' Censored individuals included tadpoles that survived until day 50 ($n=5$), tadpoles that were lost from the study because they were inadvertently killed ($n=1$), froglets that survived until day 70 ($n=63$), and froglets that were lost from the study because they were inadvertently killed ($n=3$) or drowned ($n=3$). Because infected frogs typically display clinical signs of chytridiomycosis within one or two days of death, froglets that were euthanized at onset of clinical signs were considered not censored (Berger et al., 2005). We performed all statistical analyses with Program R (Hothorn et al., 2008; R Development Core Team, 2010; Therneau and Lumley, 2009).

RESULTS

Wood frog tadpoles

Batrachochytrium dendrobatidis was not detected in control tadpoles but was sporadically detected in treatment tadpoles (zoospore equivalent mean ± SD=35±13). The number of positive detections in each of the three harvests was equal (three of 30, 10%) but the number of positive detections varied among enclosures (Table 1). We did not detect *B. dendrobatidis* in tadpoles from enclosures A, D, and E. For enclosures B and C, two of 15 (13.3%) tadpoles were PCR-positive. For enclosure F, five of 15 (33.3%) tadpoles were PCR-positive.

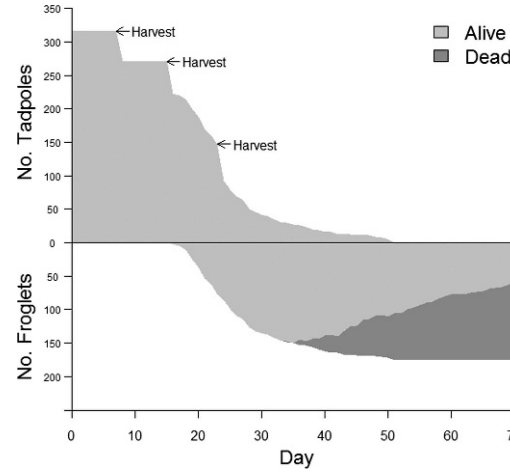

FIGURE 1. Outline of the experiment through time showing the initial number of wood frog (*Lithobates sylvaticus*) tadpoles ($n=316$; eight enclosures with 35 tadpoles each and one enclosure with 36 tadpoles), numbers of tadpoles removed from enclosures ($n=135$; five tadpoles removed from each of nine enclosures on three days), the pace of metamorphosis, and the fate of all froglets (alive or dead; $n=175$; 316–135 harvested tadpoles − six censored tadpoles). Wood frogs were exposed as tadpoles to water shared with *Batrachochytrium dendrobatidis*-inoculated bullfrogs (*L. catesbeianus*).

Wood frog froglets

Metamorphosis began on day 16, peaked between day 19 and day 28, and lasted until day 50 (Fig. 1). Of 175 (316–135 harvested tadpoles − six censored tadpoles) wood frogs that metamorphosed into froglets, 118 were from treatment enclosures and 57 were from control enclosures. Two treatment froglets displayed clinical signs of chytridiomycosis on days 42 and 63, respectively. Death of uncensored froglets was first observed on day 36 and continued steadily until day 70 (Fig. 1). We detected

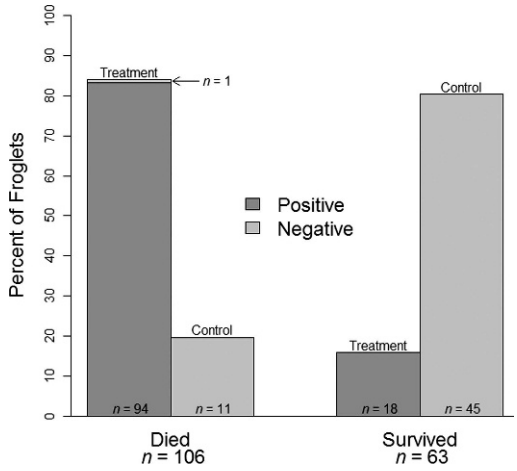

FIGURE 2. After sharing water with *Batrachochytrium dendrobatidis*-inoculated bullfrogs (*Lithobates catesbeianus*) as tadpoles in an ex-situ experiment, the number and percentages of treatment and control (bullfrogs not inoculated) wood frog (*L. sylvaticus*) froglets found positive or negative for *B. dendrobatidis* by quantitative polymerase chain reactions by mortality status (n=169; 175 − six lost to study). 'Died' refers to froglets that died and were not censored. 'Survived' refers to froglets that lived until day 70.

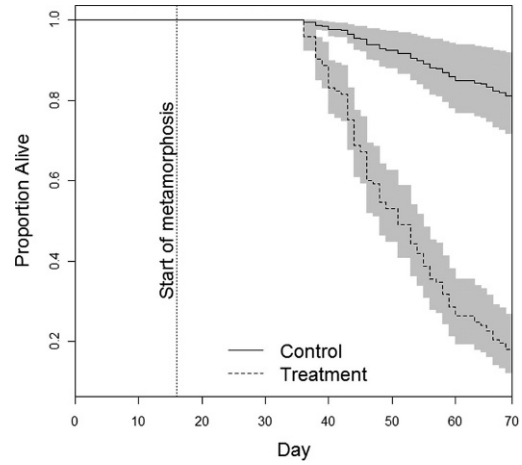

FIGURE 3. After sharing water with *Batrachochytrium dendrobatidis*-inoculated bullfrogs (*Lithobates catesbeianus*) as tadpoles in an ex-situ experiment, survival of wood frog (*L. sylvaticus*) froglets from pooled control enclosures (bullfrogs not inoculated; n=57) and pooled treatment enclosures (n=118). Confidence intervals (95%) are shaded.

no *B. dendrobatidis* in control froglets, indicating that wood frogs were not exposed to *B. dendrobatidis* before the experiment and providing evidence against occurrence of cross-contamination during the experiment. Of 56 control froglets (57 − one lost from study), 45 (80%) survived (Fig. 2). Of 113 treatment froglets (118 − five lost from study), 112 (99.1%) were PCR-positive for *B. dendrobatidis* (zoospore equivalent mean ± SD =998± 1,453) and 18 (15.9%) survived (Fig. 2). Pooled risk of froglet death was higher (P<0.001) in treatment enclosures than in control enclosures (Fig. 3). Risk of froglet death did not differ (P=0.072) among control enclosures. Risk of froglet death differed (P<0.001) among treatment enclosures, but survival curves generally followed the same trend (not shown).

Bullfrog observations

Four bullfrogs (C, D, F, and H) carried natural *B. dendrobatidis* infections for at least part of the study. Of 36 swabs collected from treatment bullfrogs between day eight and day 50, seven were PCR-positive for *B. dendrobatidis* (bullfrogs D and F were PCR-positive three and four times, respectively; zoospore equivalent mean ± SD =27±20). Three treatment bullfrogs (A, B, and E) did not produce any *B. dendrobatidis*-positive swabs. Among treatment enclosures, pooled risk of froglet death did not differ (P=0.081) between those housing bullfrogs on which *B. dendrobatidis* was detected (C, D, and F) and those housing bullfrogs on which *B. dendrobatidis* was not detected (A, B, and E). Skin sloughs containing zoosporangia, often in high densities, were found in inoculation chambers from all but one treatment bullfrog (E), which suggests that bullfrogs are efficient at ridding heavy infections and may have been carrying only light zoospore loads when returned to enclosures after inoculations. In enclosure E, froglet infection and mortality occurred although *B. dendrobatidis* was not detected in any bullfrog swabs, bullfrog sloughs, or wood frog tadpoles.

DISCUSSION

Improved ability to quantify the influence of vectors on *B. dendrobatidis* infection rates could substantially enhance accuracy of predictive models for amphibian mortality events (see Gahl and Calhoun, 2008). Our finding that bullfrogs function as vectors of *B. dendrobatidis* supports the hypotheses of a number of authors (Daszak et al., 2004; Hanselmann et al., 2004; Gahl et al., 2009). *Batrachochytrium dendrobatidis*-inoculated bullfrogs, most likely carrying the low zoospore loads characteristic of this species, gave rise to a cascade of infection in experimental enclosures that led to wood frog infection at aquatic and terrestrial life stages. Our experimental design does not rule out a fitness cost to treatment wood frogs in response to bullfrog chemical cues (Petranka et al., 1987) but it is probable that mortality of treatment froglets was also attributable to *B. dendrobatidis* originating from bullfrogs. This is an important step in quantifying the lethal and sublethal effects of bullfrog vectors on *B. dendrobatidis*-susceptible species. A useful avenue for future research would be to determine the effects of bullfrog vectors on other species and under field conditions.

Treatment wood frog froglets were PCR-positive for *B. dendrobatidis* almost without exception, while subsampled tadpoles from the same enclosures rarely were PCR-positive. Several hypotheses could explain this observation: 1) PCR analysis of tadpole mouthparts yielded false negatives (Type II error). We believe this is unlikely because lethal sampling of tadpoles is deemed a sensitive method of detection (Retallick et al., 2006). 2) The number of tadpole infections remained low until a period of heightened susceptibility during metamorphosis that lasted into subadulthood. Our data do not support this because the number of *B. dendrobatidis*-positive tadpoles in each of the three harvests was equal even though the first harvest occurred more than 1 week before metamorphosis began (day seven), the second harvest occurred 1 day before metamorphosis began (day 15), and the third harvest occurred during the peak in metamorphosis (day 23). 3) *B. dendrobatidis* spread was infrequent at all tadpole stages and during metamorphosis but conspecific transmission was common among froglets. We believe this hypothesis is most probable given the smaller size of and smaller amount of water in froglet enclosures, the frequent contact we observed among froglets, and the larger amount of keratinized tissue in froglets compared to tadpoles. Similarly, Lamirande and Nichols (2002) found that newly metamorphosed poison dart frogs were particularly vulnerable to *B. dendrobatidis* infection. Because it is improbable that postemergent wood frog froglets occur at such high densities in the field, it could be that risk of infection via bullfrog vectors is low except under conditions that lead to crowding (e.g., drought) or other variables that increase disease susceptibility. Risk of infection at aquatic stages may be heightened in the field in comparison to experimental conditions, however, when vernal pool water levels fall and tadpoles are subsequently concentrated. Because of their small size and cryptic behavior and coloration, young wood frogs are rarely seen in the wild. Thus, if *B. dendrobatidis* infection tends to progress particularly rapidly in young wood frogs under certain environmental conditions, it is possible that froglet die-offs would occur unnoticed. Terrestrial stages of amphibians are critical for maintaining population viability (Patrick et al., 2008). We urge amphibian ecologists and wildlife managers to focus future research on late aquatic and early terrestrial life stages of this species to increase the chances of detecting mortality events.

In addition to the information we gained about the transmissibility of *B. dendrobatidis* from bullfrogs to wood frogs, we found that bullfrogs rapidly shed heavily infected skin and sometimes

carried infections that were undetectable by thorough swabbing. Future research could investigate the role of molting dynamics in *B. dendrobatidis* resistance. In one enclosure (E), froglet infection occurred even though *B. dendrobatidis* was not detected in any bullfrog swabs, bullfrog sloughs, or wood frog tadpoles. This suggests that, even with multiple screening methods, *B. dendrobatidis* surveys in natural water bodies implemented over short time periods may underestimate the extent of *B. dendrobatidis* in amphibian vectors. This is particularly critical from a conservation perspective given that the bullfrog has been introduced far beyond its native range (Kats and Ferrer, 2003; Pearl et al., 2007) primarily because of its high value in the food trade (Mazzoni et al., 2003). *Batrachochytrium dendrobatidis* has been found in introduced bullfrogs on multiple continents (Hanselmann et al., 2004; Cunningham et al., 2005; Garner et al., 2006; Schlaepfer et al., 2007), and Goka et al. (2009) hypothesize that introduced bullfrogs have transmitted *B. dendrobatidis* to native amphibian species in Japan. Other amphibian species, including the northern leopard frog (*L. pipiens*), are also considered potential vectors of *B. dendrobatidis* (Woodhams et al., 2008). Considering the challenges implicit in screening bullfrogs for disease, we discourage translocation of bullfrogs and emphasize the limitations of standard screening methods for this species.

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