Field and Laboratory Studies of the Susceptibility of the Green Treefrog (Hyla cinerea) to Batrachochytrium dendrobatidis Infection

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Abstract

Amphibians worldwide are experiencing devastating declines, some of which are due to the amphibian chytrid fungus (Batrachochytrium dendrobatidis, Bd). Populations in the southeastern United States, however, have not been noticeably affected by the pathogen. The green treefrog (Hyla cinerea) is abundant and widespread in the southeastern United States, but has not been documented to harbor Bd infection. This study examined the susceptibility of H. cinerea to two strains of Bd in the lab and the prevalence of infection in wild populations of this species in southeastern Louisiana. Although we were able to infect H. cinerea with Bd in the lab, we did not observe any clinical signs of chytridiomycosis. Furthermore, infection by Bd does not appear to negatively affect body condition or growth rate of post-metamorphic individuals. We found no evidence of infection in surveys of wild H. cinerea. Our results suggest that H. cinerea is not susceptible to chytridiomycosis post-metamorphosis and probably is not an important carrier of the fungal pathogen Bd in the southeastern United States, although susceptibility at the larval stage remains unknown.

Introduction

The amphibian chytrid fungus (Batrachochytrium dendrobatidis, Bd), and the disease it causes, chytridiomycosis, has been implicated as one of the main drivers of the global decline of amphibians [1,2]. However, unlike in many other regions, amphibian species in the southeastern United States have not experienced noticeable die-offs due to Bd [3]. Despite the presence of Bd throughout the region, declines due to chytridiomycosis have not been reported [3]. Several of the region’s anurans, such as Acris crepitans and Lithobates sevour, have experienced severe declines in parts of their ranges, but the role of Bd in those declines is uncertain [4-6].

The green treefrog, Hyla cinerea, was chosen for this study because it is one of the most abundant species across much of the southeastern United States [7,8]. While the species has never been reported to have Bd, field sampling has been sparse. In searching the literature, we only found mention of 24 individuals having been tested across three studies [3,9,10]. If this species can harbor Bd, then it has the potential to be an important carrier in southeastern United States amphibian communities because of its abundance and widespread distribution. If H. cinerea is a carrier species we would expect Bd to occur on this species in the wild, and individuals to maintain an infection under laboratory conditions with no clinical signs of chytridiomycosis.

The first objective of this study was to determine whether H. cinerea is capable of contracting a Bd infection. This was accomplished by inoculating post-metamorphic individuals under laboratory conditions that are optimal for Bd growth. Our second objective was to determine whether wild-caught H. cinerea are infected with Bd in areas where the pathogen is known to occur. Because Bd is known to inhabit cool environments [11] and prevalence varies seasonally [12,13], we sampled most heavily in the fall and spring, when environmental temperatures are suitable for Bd growth and H. cinerea are most active. If a Bd infection can be maintained in the lab, and if wild-caught individuals are found to harbor Bd, then H. cinerea may be an important carrier of Bd in the southeastern United States.

The third objective of this study was to test for differences in susceptibility and the course of infection between frogs infected with a native strain of Bd (from South Carolina) and those infected with a non-native strain (from Panama). There is some evidence suggesting that virulence varies among Bd isolates from different regions [14,15]. Many species have declined in Panama due to chytridiomycosis [16] and thus Panamanian strains are hypothesized to be highly virulent. Since no amphibian declines have been attributed to chytridiomycosis in the southeastern United States [3] it has been hypothesized that strains from this area are less virulent.

Materials and Methods

Ethics Statement

This study and its methods were approved by Tulane University’s Animal Use and Care Committee (protocol No. 0391). Permission to collect H. cinerea was granted by the Louisiana Department of Wildlife and Fisheries (permit Nos. WL-Research – 201043 and LNHP-10-030).
Field Data
A total of 782 amphibians from 15 sites in southeastern Louisiana were tested for Bd between November 2010 and October 2011 to provide Bd prevalence and seasonality data (Figs. 1 and 2, Table S1). Post-metamorphic Hyla cinerea (juveniles and adults, n = 258) were sampled from 9 of these sites (Table 1). Animals were captured using nitrile-gloved hands, swabbed (with swab MW113, Medical Wire and Equipment Co.), and then released where they were caught. The skin surface of each frog was swabbed 40 times: five times on each of the dorsum, venter and sides, as well as the underside of each foot. Clean gloves were used to handle each animal.

Testing for Bd
Genomic DNA was extracted from swab samples using the QiaGen DNeasy Blood and Tissue Kit according to the instructions for animal tissue, with a final elution volume of 200 μL. Extracted DNA was then analyzed using quantitative, real-time PCR (qPCR, Applied Biosystems 7500) following Boyle et al. [17] with the following minor modifications: each sample was diluted 1:10 with double deionized water and 0.7 μL of bovine serum albumin (BSA, Applied Biosystems) was added to each reaction well prior to amplification (following Garland et al. [18]). Positive and negative controls (extracted from swabs of known Bd-positive and Bd-negative captive Lithobates catesbeianus, respectively) and a series of dilution standards (provided by A. Hyatt, CSIRO, Melbourne, Australia) were included in each qPCR run. The laboratory animal samples were run in triplicate. One of the three replicates for each swab contained an internal positive control (VIC™ IPC, Applied Biosystems) to ensure that PCR inhibition was not affecting our results. Samples were scored as positive if Bd was detected in one or more of the triplicate wells. The field animal samples were run in singlicate, where all samples contained an internal positive control.

Laboratory Animals
Ten adult and ten juvenile Bd-negative H. cinerea were captured at Maurepas Swamp Wildlife Management Area in southeastern Louisiana in November 2010 and brought into the lab for the exposure experiment. The animals were tested and diagnosed as Bd negative by swabbing and qPCR analysis (see above) prior to beginning the experiment. Upon capture, adults were individually housed in 13×23×13 cm terraria. Initially, juveniles were individually housed in cylindrical terraria (11 cm diam.×14 cm tall), but by week 20 they were moved into the larger, rectangular terraria. In each enclosure, we placed 3 to 5 cm of filtered tap water. Animals were fed 1/4 in. crickets four times a week: adults were fed 16 crickets and juveniles were fed 8 crickets, until week 20 when all animals were considered adult and fed 16 crickets each. Animals were acclimated in individual terraria, inside an...
environmental chamber held at 20°C for at least one week prior to inoculation.

*Bd* is a biohazard and was treated as such. Materials that came in contact with fungal cultures or animals were sterilized. Terraria were sterilized once a week with a 10% bleach solution, rinsed in a water bath to remove any bleach residue, then dried completely before reuse. Nitrile gloves were changed between handling each animal in the lab and in the field to prevent cross contamination. Dry waste (e.g., gloves, swabs, paper towels) was autoclaved, and liquid waste was brought up to 10% bleach and then disposed of down the drain.

**Culturing *Bd* and Inoculation of Frogs**

Animals in the laboratory study were inoculated with one of two strains of *Bd*: JEL423 or SRS810 (provided by J. Longcore, University of Maine, Bangor, ME). JEL423 is a strain isolated from a lemur leaf frog (*Phyllomedusa lemur*) in Guabal, Panama and SRS810 was isolated from a bullfrog (*Lithobates catesbeianus*) from the Savannah River Site, near Aiken, South Carolina. To prepare the inoculate, *Bd* from each strain was cultured in a 10% tryptone broth for seven days, and then transferred to 10% agar +10% tryptone plates and allowed to grow at 23°C for five to nine days. Each plate was then flooded with 5 mL of 10% Holtfreter's solution for ten minutes to collect zoospores. The concentration of zoospores was estimated using a hemocytometer, and the inoculum was made by dilution with 10% Holtfreter’s solution.

### Table 1. qPCR assay results for sites in Southeastern Louisiana where *H. cinerea* were swabbed.

| Site                                      | Site number | Latitude | Longitude | Positive/Total | Months sampled                  |
|-------------------------------------------|-------------|----------|-----------|----------------|---------------------------------|
| Louisiana Immersive Technologies Enterprise Center | 1           | 30.221   | -92.043   | 0/24           | March–September (1–5 per mo.)  |
| National Wetlands Research Center         | 2           | 30.223   | -92.045   | 0/14           | March–May, September, October (1–6 per mo.) |
| Donner Canal                              | 3           | 29.693   | -90.966   | 0/5            | June                            |
| Nicholls State University Farm            | 4           | 29.767   | -90.784   | 0/11           | June                            |
| Dulac Marsh                               | 5           | 29.384   | -90.727   | 0/10           | July                            |
| Lower Dulac Marsh                         | 6           | 29.343   | -90.729   | 0/3            | July                            |
| Sandy Cove Site 50                        | 7           | 29.993   | -91.526   | 0/2            | March                           |
| Maurepas WMA                              | 8           | 30.108   | -90.435   | 0/178          | November (133), May (45)       |
| Herbert Center                            | 9           | 29.89    | -89.953   | 0/11           | June, August–November (3–4 per mo.) |

![Figure 2. Seasonal Bd prevalence estimates in Southeastern Louisiana.](image)

Monthly *Bd* prevalence for all *H. cinerea* (black diamonds) and other amphibian species (open gray circles) species tested across 15 field sites. Error bars are ±95% confidence intervals.

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Animals were inoculated by placing them individually in 100 mL containers containing 50 mL of inoculum for 24 hours. Each animal was inoculated with Bd zoospores three times over the course of six days, to ensure infection. Five adult and five juvenile H. cinerea were inoculated with JEL423 and an additional five adults and five juveniles were inoculated with SRS810. The number of zoospores used for each set of inoculations varied depending upon how many had been produced on the agar plates during the previous week.

One week after inoculation, the first data were collected. Animals were swabbed to test for the presence of Bd, weighed, and measured (snout-vent length, SVL). We examined body condition (mass/SVL) as an indicator of the overall health of individuals. Data was collected every 2–3 weeks for a total of 26 weeks. Frogs were monitored daily for clinical signs of chytridiomycosis, which include the presence of red legs, loss of appetite (as indicated by number of uneaten crickets), irregular skin sloughing, or loss of righting reflex. At the end of the 26-week experimental period, all animals were euthanized using a lethal dose of MS-222.

Because terraria were changed and sterilized weekly, and amphibians shed their skin approximately every two weeks [19], individuals who returned a positive qPCR result greater than 14 d after inoculation were considered to be carrying an active infection. While a positive qPCR result cannot assure the viability of Bd zoospores, many of the frogs in this study tested positive for Bd for six months, much longer than could reasonably be attributed to carryover from the inoculation process.

**Results**

**Field Study**

Despite the fact that Bd has been detected from other amphibian species in southeastern Louisiana (Fig. 2, Table S1), all H. cinerea we sampled (n = 258, Table 1) tested negative for Bd. Many of these individuals were sampled during the same site visits as other species that tested positive, suggesting that the absence of infection in H. cinerea comes despite the pathogen’s presence in the local amphibian community.

**Lab Study**

The zoospore concentrations used for inoculations varied with both strain and day. Animals inoculated with SRS810 received a dose of 2.7 × 10⁶ zoospores in the first inoculation, 1.6 × 10⁶ zoospores in the second (two days later) and 0.4 × 10⁶ zoospores in the third (another two days later). JEL423 animals were inoculated with 10.7 × 10⁶ zoospores in the first, 55.4 × 10⁶ zoospores in the second and 10.6 × 10⁶ zoospores in the third inoculation. These animals were inoculated on the same schedule as the SRS810 group. Although the inoculation doses varied between the two strains, the zoospore loads detected on the animals did not differ between the two treatment groups one week after the final inoculation (t-test: t₁₀₉ = 0.73, p = 0.47). All 20 lab animals contracted a Bd infection; however, some individuals cleared their infection over the course of the experiment. We considered an individual to have cleared its infection if its qPCR results were consistently Bd-negative after week 20. Over the course of the experiment there was no difference in body condition among frogs infected with different strains of Bd (JEL423 and SRS810, ANCOVA: F₁,₁₈ = 0.036, p = 0.856, see Fig. 3). Animals that retained an infection throughout the experiment also did not differ in body condition from animals that cleared their infection during the experiment (ANOVA: F₁,₁₈ = 1.611, p = 0.221, see Fig. 4).

The zoospore load was light for most of the animals throughout the experiment. While animals inoculated with JEL423 appeared to have higher zoospore loads near the end of the study, the pattern of variation did not differ among the two strains over the entire 26 weeks of the study (ANCOVA: F₁,₁₈ = 0.002, p = 0.965, see Fig. 5). Because they had very light infections, several animals tested negative for Bd, often times for several weeks, between positive swabs. Throughout the experiment, no animals showed clinical signs of chytridiomycosis.

**Discussion**

**Inoculation**

Previous inoculation studies involving Bd have used between 1 zoospore and 2 × 10³ zoospores [20]. Although the zoospore concentrations we used for inoculations varied among inoculation days and Bd strains, this did not appear to impact the infection load that resulted one week after the last inoculation. These results suggest that there may be no benefit to inoculating animals with 5.5 × 10⁶ zoospores (JEL423 at our highest dose) versus 2.6 × 10⁶ zoospores (SRS810 at our highest dose) in laboratory exposure experiments.

**Bd Infection in Hyla cinerea**

There was no difference in infection load or disease symptoms between animals exposed to the two strains of Bd. Our results suggest that H. cinerea is able to retain an infection under laboratory conditions, but we found no evidence of any negative effects of Bd on infected individuals. None of the animals developed clinical signs of chytridiomycosis during the 26 week study. Because our study used an established inoculation protocol [reviewed in 20] and a Bd strain (JEL423) that had produced disease symptoms in other species [21], these results suggest that H. cinerea can carry a Bd infection without developing disease symptoms. Using a similar protocol and JEL423 zoospores, Litoria caerulea individuals succumbed to a lethal chytridiomycosis infection, as diagnosed by the presence of clinical signs and high infection loads leading to death 3–4 months after inoculation [22, and unpublished data].

All animals captured in the field tested negative for Bd, despite the pathogen having been detected in other amphibian species from some of the same sites and sampling sessions (Fig. 2, Table S1). Other studies that tested for the presence of Bd across the southeastern United States have also failed to detect infected H. cinerea. One such study included nine H. cinerea captured from four locations across the southeast, all of which tested negative [10]. In another study, 14 individuals from across four states were tested, and again, none were infected with Bd [3]. In both cases, infections were found in other species at the same sites. These results, in addition to those presented here, suggest that H. cinerea shows weak, if any, susceptibility to Bd in the wild.

Considering both the laboratory and field data, we conclude that H. cinerea is not susceptible to chytridiomycosis and Bd infection, at least in post-metamorphic life stages. Although individuals were able to retain a low-level infection under laboratory conditions, they did not develop disease symptoms or show any sub-clinical signs of illness. This was true for both a southeastern United States strain (SRS810) and a putatively more virulent strain associated with die-offs in Panama [JEL423] [23]. Adult and juvenile H. cinerea were not found to be infected with Bd in the field, even at sites where other species are known to be infected. If the same pattern holds for H. cinerea tadpoles, this suggests that the species is neither an important carrier of Bd, nor likely to be susceptible to Bd-related declines.

This study focused only on post-metamorphic life stages and therefore we cannot rule out the possibility that H. cinerea larvae...
Some species of anuran larvae are known to carry *Bd* infections. While it is generally thought that tadpoles are not susceptible to chytridiomycosis, *Bd* infection can cause sublethal effects. For example, Garner et al. [24] found that tadpoles infected with *Bd* had a reduced mass at metamorphosis compared to uninfected tadpoles. This led them to suggest that investment of energy toward preventing *Bd* infection may overwhelm the early development process, reducing larval growth rates and even leading (directly or indirectly) to mortality at metamorphosis [24]. We do not know whether larval *H. cinerea* are susceptible to *Bd* infection; however, given that *H. cinerea* populations appear stable and abundant.
throughout their range, even if *Bd* infects the larval stage, it seems unlikely to be a major threat to *H. cinerea* populations.

While it is clear that amphibian species differ in their susceptibility to *Bd*, the reasons for this variation remain largely unclear. Differences in the immune system are one possible explanation for variation in susceptibility among species. This could include aspects of both the external immune system (e.g., antimicrobial bacterial colonies on the skin surface and/or antimicrobial skin peptides) and the internal immune system (e.g., lymphocytes and eosinophils) [25–28]. A particularly effective innate immune response in *H. cinerea* could explain the animals’ reduced susceptibility to *Bd* infections, but some other factor is then needed to explain why inoculated animals maintain an infection in the lab, while animals sampled in the field do not.

Climate and microhabitat may also influence susceptibility to *Bd*. It has been suggested that anurans that spend a large portion of their time in habitats that are ideal for *Bd* growth will be more likely to develop a *Bd* infection [29]. Post-metamorphic life stages of *H. cinerea* are largely arboreal and may not come into contact with infected water bodies as often as other species in the same habitat. Unlike in our exposure experiment, where frogs were maintained at near-optimal temperatures for *Bd* growth (20°C), wild *H. cinerea* may often be found at temperatures where the balance between immune function and zoospore growth is tipped in the frog’s favor. *Hyla cinerea* is known to bask in the sun [30], giving it one of the highest average body temperatures of arboreal frogs in the United States [30–32]. Basking is thought to improve digestion [33], but may also aid in eliminating chytrid infection since high temperatures are lethal to *Bd* [1,34–36]. We suggest that future research should aim to tease apart the factors contributing to *Bd* resistance by focusing on species like *H. cinerea*, which do not seem to be susceptible to infection.

**Supporting Information**

**Table S1** *Bd* prevalence in Southeastern Louisiana.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: LAB MWHC CLRZ. Performed the experiments: LAB MWHC. Analyzed the data: LAB CLRZ.

Contributed reagents/materials/analysis tools: CLRZ. Wrote the paper: LAB.

**References**

1. Berger L, Speare R, Hines HB, Marantelli G, Hyatt AD, et al. (2004) Effect of season and temperature on mortality in amphibians due to chytridomycosis. *Aust Vet J* 82: 434–439.

2. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, et al. (2004) Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1783–1786.

3. Rothermel BB, Walls SC, Mitchell JC, Dodd CK Jr, Irwin LK, et al. (2008) Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Dis Aquat Org* 82: 3–18.

4. Beasley VR, Each SA, Wiko B, Staehle C, Eisold J, et al. (2005) Risk factors and declines in northern cricket frogs (*Acris crepitans*). In Lannoo M editor. Amphibian declines: the conservation status of United States species. Berkeley: University of California Press. pp 75–96.

5. Lehtinen RM, Skinner AA (2006) The enigmatic decline of Blanchard’s cricket frog (*Acris crepitans blanchardi*): A test of the habitat acidification hypothesis. *Copeia*. pp 159–167.
6. Richter SC, Crother BI, Broughton RE (2009) Genetic consequences of population reduction and geographic isolation in the critically endangered frog, *Rana sylvatica*. Copeia. pp 799–806.

7. Ackleh AS, Carter J, Cole L, Nguyen T, Monte J, et al. (2010) Measuring and modeling the seasonal changes of an urban green treefrog (*Hyla cinerea*) population. Ecol Model 221: 281–289.

8. Zacharov M, Barichivich WJ, Dodd CK Jr. (2003) Using ground-placed PVC pipes to monitor hydrid treefrogs: Capture biases. Southeast Nat 2: 575–590.

9. Timpel EK, Graham SP, Gagliardo RW, Hill RL, Levy MG (2008) Occurrence of the fungal pathogen *Batrachochytrium dendrobatidis* in Georgia’s amphibian populations. Herpetol Rev 39: 447–449.

10. Lannoo MJ, Petersen C, Lovich RE, Nanjappa P, Phillips C, et al. (2011) Do frogs get their kicks on route 66? Continental US transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PLoS One 6: e22211.

11. Kriger KM, Hero J-M (2008) Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis*) infection in subtropical Australian frogs. Austral Ecol 33: 1022–1032.

12. Kriger KM, Hero J-M (2007) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. J Zool 271: 352–359.

13. Kinney VC, Heemeyer JL, Pessier AP, Lannoo MJ (2011) Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates arneoticus*: Affirmation of Vredenburg’s “10,000 zoospore rule”. PLoS One 6: e16708.

14. Berger L, Marantelli G, Skerratt LL, Speare R (2005) Virulence of the fungal pathogen *Batrachochytrium dendrobatidis* in amphibians. Herpetological Review 36: 345–346.

15. Gahl MK, Loncore JE, Houlahan JE (2011) Varying responses of Northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. Oikos 119: 823–826.

16. Berger L, Speare R (2005) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. Dis Aquat Org 68: 47–50.

17. Gahl MK, Loncore JE, Houlahan JE (2011) Varying responses of Northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. Conserv Biol. pp 1–7.

18. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, et al. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 95: 9031–9036.

19. Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60: 141–148.

20. Garland S, Baker A, Phillott AD, Skerratt LF (2010) BSA reduces inhibition in a TaqMan (R) assay for the detection of *Batrachochytrium dendrobatidis*. Dis Aquat Org 92: 113–116.

21. Voyles J (2009) Virulence and pathogenesis of chytridiomycosis: a lethal disease of amphibians. PhD Thesis. James Cook University.

22. Kilpatrick AM, Briggs CJ, Daszak P (2010) The ecology and impact of chytridiomycosis: An emerging disease of amphibians. Trends Ecol Evol 25: 109–118.

23. Becker MH, Harris RN (2010) Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS One 5: e10957.

24. Brannelly LA, Richards-Zawacki CL, Pessier AP. Clinical trials with iraconazole as a treatment for chytrid fungal infections in amphibians. In, review.

25. Gagliardo R, Crump P, Griffith E, Mendelson J, Ross H, et al. (2008) The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. Int Zoo Yearbook 42: 125–135.

26. Gagliardo R, Crump P, Griffith E, Mendelson J, Ross H, et al. (2008) The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. Int Zoo Yearbook 42: 125–135.

27. Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: An immunological perspective. Dev Comp Immunol 23: 459–472.

28. Conlon JM (2011) The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res 343: 201–212.

29. McCallum ML, Trauth SE (2007) Physiological trade-offs between immunity and reproduction in the northern cricket frog (*Acris crepitans*). Herpetologica 63: 269–274.

30. Rollins-Smith LA, Ramsey JP, Reinert LK, Woodhams DC (2011) Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. Integrative and Comparative Biology 51: E117–E117.

31. Woodhams DC, Allford RA (2005) Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. Conserv Biol 19: 1449–1459.

32. Wygodzka ML, Williams AA (1991) Body-temperature in free-ranging green tree frogs (*Hyla cinerea*) - a comparison with typical frogs. Herpetologica 47: 326–335.

33. McCallum ML, Trauth SE (2007) Physiological trade-offs between immunity and reproduction in the northern cricket frog (*Acris crepitans*). Herpetologica 63: 269–274.

34. Woodhams DC, Allford RA, Marantelli G (2003) Emerging disease of amphibians cured by elevated body temperature. Dis Aquat Org 55: 65–67.

35. Richards-Zawacki CL (2010) Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. P Roy Soc B-Biol Sci 277: 519–528.

36. Chatfield MWH, Richards-Zawacki CL (2011) Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. Dis Aquat Org 94: 235–238.