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NOTE

Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs

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ABSTRACT: The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been implicated in amphibian declines worldwide. *In vitro* laboratory studies and those done on wild populations indicate that *Bd* grows best at cool temperatures between 17 and 25°C. In the present study, we tested whether moderately elevating the ambient temperature to 30°C could be an effective treatment for frogs infected with *Bd*. We acquired 35 bullfrogs *Rana catesbeiana* from breeding facilities and 36 northern cricket frogs *Acris crepitans* from the wild and acclimated them to either 23 or 26°C for 1 mo. Following the acclimation period, frogs were tested for the presence of *Bd* using qPCR TaqMan assays. The 12 *R. catesbeiana* and 16 *A. crepitans* that tested positive for *Bd* were subjected to 30°C for 10 consecutive days before returning frogs to their starting temperatures. Post-treatment testing revealed that 27 of the 28 frogs that had tested positive were no longer infected with *Bd*; only a single *A. crepitans* remained infected following treatment. This result indicates that elevating ambient temperature to a moderate 30°C can be effective as a treatment for *Bd* infection in captive amphibians, and suggests that heat may be a superior alternative to antifungal drugs.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytrid · Amphibian disease

INTRODUCTION

The pathogenic amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been associated with amphibian declines and extinctions worldwide (Berger et al. 1998, Daszak et al. 2003, Lips et al. 2006, Skerratt et al. 2007). Most *Bd*-associated declines have occurred during cooler periods of the year and in high elevation areas, suggesting that *Bd* is most pathogenic in cool environments (Berger et al. 1998, 2004, Bradley et al. 2002, Retallick et al. 2004, Kriger & Hero 2007, Brem & Lips 2008, Murray et al. 2009). This has been corroborated in many *in vitro* studies, which indicate that optimal temperatures for *Bd* growth are between 17 and 25°C (Longcore et al. 1999, Johnson et al. 2003, Piotrowski et al. 2004, Woodhams et al. 2008).

Numerous drugs have been tested for the treatment of chytridiomycosis, the often fatal disease caused by *Bd*. For example, a combination of formalin and malachite green was seemingly successful in the treatment of *Xenopus tropicalis* diagnosed with late-stage chytridiomycosis (Parker et al. 2002). However, results from that experiment were obtained using histology rather than PCR testing, so a subclinical infection may have remained post-treatment. Furthermore, this treatment is not generally recommended because of human health concerns and intolerance by some amphibian species (see Pessier & Mendelson 2010). The drug chloramphenicol has also been used to treat chytridiomycosis, although its efficacy remains uncertain (Bishop et al. 2009). The prescription antifungal drug itraconazole has been used to treat many amphibian

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species (Nichols et al. 2000, Lamirande & Nichols 2001, Garner et al. 2009), but appears to be toxic to larvae and recent metamorphs of some species at standard doses (see Pessier & Mendelson 2010). Much of what is known about treatments involving drugs comes from anecdotal accounts rather than systematic clinical trials (see Berger et al. 2010 for a review).

Laboratory experiments suggest that heat is promising as a treatment for chytridiomycosis. In 2 *in vitro* studies, 100% of *Bd* zoospores died after 4 h at 37°C (Johnson et al. 2003), and 50% of cultures died after 8 d at 30°C (Piotrowski et al. 2004). *In vivo*, Woodhams et al. (2003) found that adult *Litoria chloris* can be cured of *Bd* infection by exposure to 37°C for 16 h, and Retallick & Miera (2007) cured 50% of infected adult *Pseudacris triseriata* by exposure to 32°C for 5 d.

Despite the potential of heat as a treatment for *Bd* infection, antifungal drugs remain the most common method of treatment in captive amphibians. However, the benefits of heat treatment over antifungal drugs are potentially great and include fewer negative side effects, lower cost, and greater accessibility to managers of captive amphibians. In the present study, we tested whether moderate temperature elevation could be an effective treatment for frogs infected with *Bd*.

MATERIALS AND METHODS

Between January and June 2010, we acquired 35 bullfrogs *Rana catesbeiana* from commercial breeding facilities located in Louisiana and Georgia, USA, and collected 36 northern cricket frogs *Acris crepitans* from Joyce and Manchac Wildlife Management Areas in southeast Louisiana. Upon arrival in the laboratory, *R. catesbeiana* were housed individually in 36 × 20 × 18 cm containers filled with 3 l of filtered tap water, and *A. crepitans* were housed individually in 23 × 15 × 17 cm containers filled with approximately 0.3 l of filtered tap water. Water was changed and tanks cleaned and sterilized in a 10% bleach solution weekly for *A. crepitans* and twice weekly for *R. catesbeiana*. To provide a hide and a dry perching area, a modified inverted plastic bowl was provided to all *R. catesbeiana*. When handling frogs, we took precautions to avoid contamination by changing gloves between individuals. All frogs were fed 4 times each week exclusively on a diet of crickets (5 adult crickets for *R. catesbeiana* and five 6 mm crickets for *A. crepitans*), and were maintained at room temperature until the experiment began. Frogs were assigned at random to one of 2 temperature-controlled environmental chambers (Conviron) where they were acclimated to either 23°C (n = 18 *R. catesbeiana* and 18 *A. crepitans*) or 26°C (n = 17 *R. catesbeiana* and 18 *A. crepitans*) for 1 mo. Frogs were not tested for the presence of *Bd* prior to being assigned to the 23 or 26°C chambers.

At the end of the acclimation period, each frog was individually tested for *Bd*. This was done by swabbing each individual 30 times on the dorsal, lateral, and ventral surfaces using cotton-tipped swabs (Advantage Bundling SP). DNA was extracted from the swabs using Qiagen DNeasy Blood and Tissue DNA Extraction Kits. The presence of *Bd* DNA was detected using qPCR TaqMan assays following Boyle et al. (2004). All reactions were performed in triplicate, and samples were scored as positive if at least 1 replicate tested positive. An internal positive control VIC_{TM} dye (Applied Biosystems) was added to 1 replicate in order to detect the presence of PCR inhibitors which, if present, can cause false negative results (Hyatt et al. 2007).

After swabbing, frogs were returned to their original, individual tanks and environmental chambers. The temperature in each environmental chamber was then gradually increased to 30°C over a period of 2 d. All frogs were then held at an ambient temperature of 30°C for 10 d, after which they were re-acclimated to their starting temperatures (23 or 26°C). Frogs that had tested positive for *Bd* before the 30°C treatment period were tested again 6 d after the end of the treatment period. During the 10 d treatment period, bullfrog tanks were cleaned 3 times and cricket frog tanks were cleaned twice. During the 6 d between the treatment period and the post-treatment testing of frogs, bullfrog tanks were cleaned twice and cricket frog tanks were not cleaned.

As frogs had access to both dry substrate and water inside their tanks, we tested whether the temperature the frogs experienced while in the water differed from those experienced while on dry substrate. To do this, a Thermochron iButton (Embedded Data Systems) was placed in a small container of water (<0.5 l) and one was also left in the open in each of 2 environmental chambers for 3 d during the treatment period.

RESULTS

While there were no clinical signs of *Bd* infection observed at any point during the study, 12 out of 35 *Rana catesbieana* and 16 out of 36 *Acris crepitans* tested positive for *Bd* after the 1 mo acclimation period. Of the 28 swab samples scored as positive for *Bd*, 18 amplified *Bd* DNA in all 3 replicates, 3 amplified *Bd* DNA in 2 of 3 replicates, and 7 amplified *Bd* DNA in 1 of 3 replicates. Of the *R. catesbeiana* that tested positive, 10 frogs had been acclimated to 23°C and 2 had been acclimated to 26°C. Of the *A. crepitans* that tested positive, 7 frogs had been acclimated to 23°C and 9 had been acclimated to 26°C. No PCR inhibition was

detected. Since testing was not performed prior to the 30 d acclimation period at 23 or 26°C, we cannot determine whether any individuals caught or recovered from infection during this time, or whether this differed among acclimation temperatures.

After treatment at 30°C for 10 d, all 12 *Rana catesbeiana* and 15 out of 16 *Acris crepitans* tested negative, and only 1 frog (an *A. crepitans* originally acclimated at 23°C) tested positive for *Bd* infection. This individual's swab amplified *Bd* DNA in all 3 replicates.

The difference between air and water temperatures over 3 d during the treatment period was similar between the 2 environmental chambers. Mean ± SD air temperature over this period was 30.1 ± 0.4°C and mean water temperature was 28.5 ± 0.5°C.

DISCUSSION

We tested the hypothesis that elevating the air temperature to 30°C for 10 d would constitute an effective treatment for *Bd* infection in 2 species of captive North American frogs. Of 12 bullfrogs *Rana catesbeiana* and 16 northern cricket frogs *Acris crepitans* that tested positive for *Bd* prior to heat treatment, only a single cricket frog remained infected 4 d after the treatment period ended. This result indicates that elevating ambient temperature to a moderate 30°C can be effective as a treatment for *Bd* infection in captive amphibians.

Why did 1 frog remain positive after treatment while the remaining 27 frogs tested negative? One possibility is that this individual shed later or otherwise had dead zoospores present on its skin. Alternatively, *Bd* may have survived the elevated temperature, and the single positive sample may have reflected a very light infection. If the latter scenario were true, then some frogs which tested negative after the treatment may also have had light infections. Further experimentation involving repeated post-treatment testing is necessary to determine how long dead zoospores can remain on the skin or, alternatively, how long a light infection may be maintained after treatment. When using heat treatment in captive populations where it is important to assure that frogs test negative for infection (e.g. captive assurance colonies), we recommend conducting additional post-treatment tests.

Although air temperature in the environmental chambers was held constant at 30°C, frogs had access to water deep enough to cover their bodies throughout the study period. As the average water temperature was 28.5°C during the treatment period, a constant 30°C may not be necessary to cure amphibians of chytridiomycosis. Previous research has suggested that fluctuating ambient temperatures may slow the progression of the disease (Woodhams et al. 2003).

Quarantining frogs has become standard practice in zoos and captive assurance colonies (Daszak et al. 2001, Lynch 2001). General guidelines for the amount of time and the temperature at which individuals are held during quarantine, however, have not been established. Recent reviews recommend quarantining individuals at the 'maximum tolerable temperature' to rid them of *Bd* when first brought into captivity (e.g. Hadfield & Whitaker 2005). Determining the 'maximum tolerable temperature' for all incoming species may prove difficult or impossible given time, space, and funding constraints, and may expose the individuals to unnecessary levels of stress. The results presented here suggest that heat treatment can be effective at 30°C, making it suitable for species that may not tolerate higher temperatures (e.g. 32 to 37°C). Previous research suggests that 30°C lies within the range of temperatures experienced by many amphibians in the wild (Fitch 1956, Lillywhite 1970, Carey 1978) and is likely a more suitable temperature at which to house amphibians for an extended period of time. As a precaution, however, we recommend treating a subsample of individuals from species that are cold-adapted (i.e. restricted to high elevations or latitudes) prior to treating an entire captive population.

The results presented here support the idea that heat could be used as a general treatment for *Bd* infection, providing an alternative to antifungal drugs. Disadvantages to using such drugs are significant. For example, the most widely used drug in the treatment of *Bd* infection, itraconazole, presents problems for managers of captive amphibians for many reasons. First, it is only available by prescription and therefore may not be accessible to those working in remote areas or developing countries. Second, itraconazole can be prohibitively expensive for large numbers of individuals (approximately US$2.80 to treat a single individual under the currently recommended dosage of 0.01%; Pessier & Mendelson 2010). Third, the toxic side effects to larvae and recent metamorphs of many species (see Pessier & Mendelson 2010) prohibit use of this drug as a general treatment. Clinical trials are needed to determine whether there is a safe and effective dosage of itraconazole that can be used as a general treatment for *Bd* infection (Berger et al. 2010). Combining very low doses of itraconazole with short periods of slightly elevated temperatures may also prove effective.

In summary, heat treatment offers many advantages over antifungal drugs as it is inexpensive and has no toxic side effects. Other benefits of heat include its use in the treatment of animal enclosures, which may be contaminated with *Bd*, and its potential for use on fully aquatic species, which are often more difficult to treat (M. Jones pers. comm.). Lastly, heat treatment can be instituted concurrently with quarantine measures already taken.

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