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Successful clearance of chytrid fungal infection in threatened Chiricahua leopard frog (*Rana chiricahuensis*) larvae and frogs using an elevated temperature treatment protocol

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Chytridiomycosis, a skin disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al. 1999), is a major contributor to the decline of amphibian populations worldwide (Skerratt et al. 2007). *Bd* is responsible for the decline or extinction of over 200 amphibian species (Skeratt et al. 2007, Wake & Vredenburg 2008), with many already in decline due to other factors. *Bd* infections lead to mortality by disrupting important physiological processes such as osmoregulation in the amphibian's skin (Voyles et al. 2009). Infected individuals may display behavioral symptoms including the loss of righting reflex and lethargy as well as physical symptoms including cutaneous erythema and more frequent skin sloughing (Baitchman & Pessier 2013).

Treatment of *Bd* infections in amphibians has occurred *ex situ* with antifungal drugs, antibacterial drugs, and heat, with the various treatments differing in the duration of exposure required, impact on the amphibians, and success rate (Berger et al. 2010, Woodhams et al. 2012, Baitchman & Pessier 2013). Immersion in the antimicrobial drugs miconazole and itraconazole, but not trimethoprim-sulfadiazine, cured chytridiomycosis in dendrobatid frogs (Nichols et al. 2000). A combination of immersion in chloramphenicol, fluid therapy, and increased temperature cured terminal cases of chytridiomycosis in green tree frogs (Young et al. 2012). In vitro *Bd* has a low heat tolerance; exposure to temperatures of 32°C killed all zoosporangia within 96 h., with exposures to higher temperatures resulting in faster mortality (Johnson et al. 2003). However, a more recent study demonstrated that there are variations in temperature tolerance in different strains of *Bd* (Voyles et al. 2017), so there may be differences in success of heat treatments dependent on the species of amphibian and strain of *Bd*. In order to gain a better understanding of the conditions needed to successfully use heat treatments in amphibians, more studies encompassing a diversity of amphibians are necessary. To date, heat treatments have cured many species of amphibians with *Bd* infections (Woodhams et al. 2003, Retallick & Miera 2007, Chatfield & Richards-Zawacki 2011), and here we report on its first-known use to treat *Bd* in the North American Chiricahua leopard frog (*Rana chiricahuensis*).

Historically occupying aquatic habitats at high elevations in Arizona, New Mexico, and northern Mexico, the USA-Threatened/IUCN-Vulnerable Chiricahua leopard frog has experienced population declines due to loss of suitable habitat, introduction of invasive species, and *Bd* infection (Rosen et al. 1995, Bradley et al. 2002, U.S. Fish and Wildlife Service 2002, 2007). Since 1995, the Arizona Center for Nature Conservation/Phoenix Zoo (hereafter, Phoenix Zoo), in partnership with the U.S. Fish and Wildlife Service (USFWS) and Arizona Game and Fish Department, has head-started *R. chiricahuensis* to help with population recovery. To our knowledge, before this case, no *R. chiricahuensis* have been successfully treated for *Bd* using elevated temperature.

A group of 61 *R. chiricahuensis* larvae (Gosner stages 36–39; cf. Gosner 1960) was collected from central Arizona, USA, on 22 May 2015, hereafter day 0, by the USFWS and transported to the Phoenix Zoo's Arthur L. and Elaine V. Johnson Foundation Conservation Center (Johnson Center) quarantine facility. The larvae were housed in a

71.5 L tank filled with 56.8 L of aged tap water with an in-line heater to maintain temperature, a filtration system, and an air stone for aeration, with water and diet changed daily. One tank was used to house larvae, while a second tank included basking platforms and housed metamorphosed frogs. Johnson Center quarantine procedures were followed for the duration of the 60 day quarantine period.

Seven *R. chiricahuensis* individuals (six metamorphosed frogs and one larva) were sacrificed on day +3, in coordination and consultation with USFWS, and submitted as specimens for tissue extraction to San Diego Zoo Institute for Conservation Research Amphibian Disease Laboratory for *Bd* testing. Taqman real-time PCR was performed with modifications as previously described (Jones et al. 2012). Two specimens tested positive for *Bd* with total zoospore loads per sample of 11.657 and 12.325. Due to the positive *Bd* results, and since this group of head-started *R. chiricahuensis* originated from a single location and was co-housed in tanks, the population (54 individuals) remaining at the Johnson Center was presumed to be exposed to and positive for *Bd*.

Water temperature in the tanks was gradually raised from 26.1°C to 32.2°C from day +10 to day +16 with the elevated heat treatment for *Bd* starting on day +16. The water temperature was maintained at 31.7–32.2°C from day +16 to day +21, and the average ambient temperature on the basking platforms in the metamorphosed frog tank was 29.2 °C. The water temperature was lowered on day +22 and maintained at 29.4°C from day +22 to day +60, with the average ambient temperature on the basking platforms at 27.5°C.

The *R. chiricahuensis* larvae metamorphosed throughout the quarantine period and 16 individuals were lost to mortality, leaving 38 metamorphosed frogs remaining at day +60 (21 July 2015). No individuals were lost to mortality during the six-day elevated heat treatment period. Metamorphosed frogs (but not larvae) were swabbed (fine-tipped rayon swab with a plastic handle) and tested for *Bd* three times throughout the quarantine period (Pessier & Mendelson 2010). All individuals that had metamorphosed were swabbed (skin) for each testing day with multiple individuals (3–5 individuals) on a swab; 36 metamorphosed frogs on day +30, 38 metamorphosed frogs on day +46, and 38 metamorphosed frogs on day +51. All samples tested negative for *Bd* and there were no behavioral or morphological signs of chytridiomycosis in any of the surviving individuals. Five individuals tested individually as adults between days +278–285 were negative for *Bd*.

This study describes the first case of *Bd* infection in threatened *R. chiricahuensis* cured by an elevated temperature treatment. We demonstrate that *Bd* infections in *R. chiricahuensis* larvae and metamorphosed frogs can be treated by a noninvasive elevated temperature protocol (water temperature of 31.7–32.2°C for six days followed by 29.4°C for nine days). There were no short- or long-term adverse effects of heat treatment observed in the present case, as survivorship throughout the quarantine period was within the range for *R. chiricahuensis* reared at Phoenix Zoo. Previous treatment of chytridiomycosis in amphibians has focused on various antimicrobial and heat treatments in other species.

Other cases of successful heat treatment of *Bd* in frogs have also used temperatures at 30°C and above. A heat treatment of 30°C (ambient temperature) for ten days was effective in treating *Bd* in northern cricket frogs (*Acris crepitans*) and bullfrogs (*Rana catesbeiana*) (Chatfield & Richards-Zawacki 2011). Western chorus frogs (*Pseudacris triseriata*) infected with *Bd* were cured after incubation at 32°C for five days (Retallick & Miera 2007). A larger study on juvenile red-eyed tree frogs (*Litoria chloris*) found that *Bd*-infected frogs held at a constant 20°C or a naturally fluctuating heat treatment (13.5 to 23.2°C) did not survive the 94 day trial (Woodhams et al. 2003). However, ten of ten survived a 37°C thermal variation treatment, with all survivors successfully cured of *Bd* (Woodhams et al. 2003). A short heat treatment at 35°C for 24 h. did not clear *Bd* infection in northern leopard frogs (*Rana pipiens*), suggesting that a longer treatment is needed to clear *Bd* in that species (Woodhams et al. 2012).

Heat treatment of *Bd* infections in amphibians is an excellent alternative to antimicrobials because it is noninvasive, inexpensive, has no toxic effects, does not require a breach of quarantine procedures, and can treat both larval and metamorphosed life stages. However, some species may not tolerate the temperatures needed to clear the *Bd* and there may be differences in the thermal susceptibility of different *Bd* strains (Voyles et al. 2017). In the present case, even larvae undergoing metamorphosis, a potentially high-stress development period, were able to withstand high water temperatures with no detrimental effects. Because *Bd* occurs worldwide, the treatment of *Bd* will need to be feasible for all types of conservation efforts, and heat treatment provides a good option for a low cost, easily accessible treatment with minimal stress on the affected amphibians. As amphibian populations continue to decline, *ex situ* head-starting programs will become more important for population recovery; thus, treating diseases like *Bd* in conservation facilities will allow for propagation and reintroduction of healthy individuals and may be imperative for species survival.

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