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Itraconazole as a Successful Treatment to Manage *Batrachochytrium dendrobatidis* Outbreaks in *ex-situ* Frog Colonies in Argentina: Dose and Schedule for *Pleuroderma somuncurense*

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The El Rincon Stream Frog, *Pleurodema somuncurense*, is a Critically Endangered species that inhabits permanent hot springs and the warm headwaters of the Valcheta Stream, a watercourse located on the northern edge of the Somuncura Plateau (Patagonia, Argentina) (IUCN 2021). In 2015, an *ex-situ* assurance colony of this frog was established at the La Plata Museum (Buenos Aires, Argentina) as part of a conservation program to recover wild populations of this species. Since 2016, several reproduction events of this frog have occurred in the *ex-situ* facility (Velasco 2018). The high productivity associated with these reproductive events resulted in hundreds of froglets in good health, allowing a reintroduction program to be initiated aimed at reestablishing extinct populations of this species in the wild. Since then, eight successful reintroductions have been made in four restored habitats (Martínez Aguirre et al. 2019; FPK, pers. observ.). It is important to mention that during all the years of success of the *ex-situ* colony, only two massive mortality events occurred, both related to failures in the heating and cooling system. The first mortality event occurred in 2017 when a significant increase in temperature caused the sudden death of 15 adult frogs. The second case is described in this work.

In September 2020, the lockdown associated with the COVID-19 pandemic prevented the froglet translocation from the *ex-situ* facility to the field, resulting in overcrowding. During overcrowding, a failure in the heating and cooling system caused a significant decrease of the temperature in the *ex-situ* facilities from 23 to 14°C. After that, a massive frog death event (ca. 40 individuals) occurred within the facilities. Dead individuals were fixed in formalin, and necropsy was performed, evidencing an unusual heavy infection of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Most of the dead individuals were deposited in the herpetological collection of La Plata Museum. This fungus is the causative agent of the disease *Bd*-chytridiomycosis, which has caused declines and extinctions in a wide range of amphibian host species (e.g., Kilpatrick et al. 2010; Vredenburg et al. 2010). This high *Bd* infection was confirmed by observing shed skin samples under the light microscope at 400× (Fig. 1A). After *Bd* diagnosis, we immediately conducted a clearing treatment with Itraconazole solution. Many experiments have demonstrated the efficacy and the safety of Itraconazole in *Bd*-clearing treatment, varying doses from 0.0005% to the most commonly use 0.01%, and duration (4–28 d) (Garner et al. 2009; Brannelly et al. 2012; Georoff et al. 2013; Hardy et al. 2015; Knapp et al. 2022). Therefore, with this information and the fact that there was no information on the sensitivity of this species to antifungals, we conducted a clearing treatment following diagnosis using initially a low concentration (0.003%). Herein, we report our procedure to inform pre-emptive biosecurity measures to be considered in other rescue programs with captive headstarting facilities (see also Pessier and Mendelson 2017).

The purpose of our treatments was to mount a rapid response action to control a disease outbreak in a Critically Endangered species being bred in captivity for release to the wild, as such, we did not conduct traditional experiments with negative controls. We carried out two different treatments separated in time by 15 d. Both treatments consisted in common steps: 1) we kept frogs in group for one day in a plastic container (25 × 30 cm) with an absorbent towel at the bottom and 1 cm of Amphibian Ringer solution (Wright and Whitaker 2001), to keep them moist and to allow them to maintain osmotic equilibrium; 2) we moved the frogs individually to a container with Itraconazole solution (Itraconazole powder diluted in Amphibian Ringer solution), exposing them for 5 min; 3) we washed the individuals in a different container with Amphibian Ringer solution for another 5 min. After that, we returned all the individuals to the first step, meaning we returned the frogs to the container with an absorbent towel and Amphibian Ringer solution for another day. We repeated these steps for consecutive days, varying the time (see below). After each step, we sterilized the used containers (Fig. 2).

The first treatment included seven adults and a juvenile obtained from a common aquarium of 375,000 cm^3, with four other individuals. We exposed the frogs individually to three Itraconazole concentrations that increased over time for a total of 11 d. Initially the frogs were exposed to a 0.003% Itraconazole solution (Itrac 100-Cassara). This concentration was chosen based on a previous safe and effective protocol that used a very similar concentration of 0.0025% (Brannely et al. 2012). Originally our intention was to maintain the exposure for 10 days (from Pessier and Mendelson 2017), but the sudden death of three frogs made us decide to increase the Itraconazole concentration after day four. We exposed the remaining five frogs to 0.006% Itraconazole solution for three more days, and three additional individuals died. Because of these new deaths,

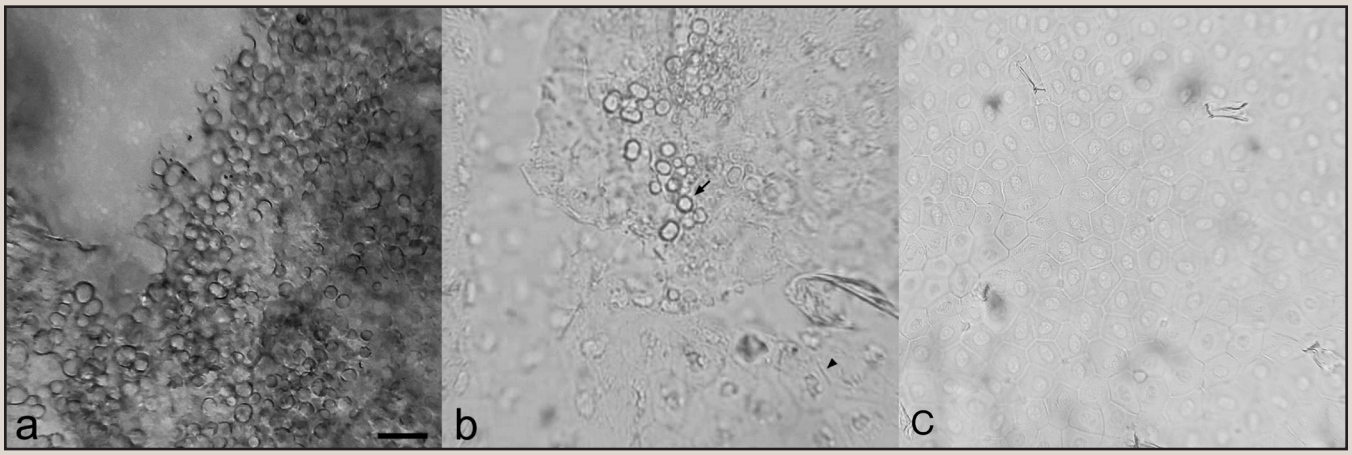

Fig. 1. Unstained wet shedding skin of El Rincon Stream Frogs, *Pleurodema somuncurense* (400×) with: A) an unusual high infection load with the total skin surface area covered by *Batrachochytrium dendrobatidis* sporangia; B) stable low-intensity infection with clusters of sporangia (arrows) on the epidermal skin surface; C) no *Bd* evidence in the skin surface, arrow shows epithelial cells. Scale bars = 30 µm.

we increased the concentration one more time, to 0.01% and exposed the two remaining frogs (one adult and one juvenile) for another four days. These two last frogs survived the treatment and skin samples showed no *Bd* evidence.

This initial trial informed a second treatment. Three days later we performed a second treatment, applying steps one to three for 10 consecutive days (Fig. 2). We exposed two groups of four and five individuals respectively, obtained from two different aquaria to a 0.01% Itraconazole concentration for 10 d. Each group was treated independently. None of these frogs died during or after treatment. Shed skin samples were recorded with no evidence of *Bd* on days four and seven, respectively (Fig. 1c).

The response to Itraconazole can vary among different species, depending on the concentration and duration of exposure. The lack of data about the sensitivity of the El Rincon Stream Frog to antifungal treatments made us experiment with different concentrations and times of exposure to respond to an emergency, with some of them resulting in ineffective doses. Based on this, we suggest conducting experiments to find the lowest effective dose before any emergency as a way to reduce risks. Our trials support use of a bath treatment with a 0.01% Itraconazole solution, five minutes a day, for at least eight days as being effective against *Bd* infection in the El Rincon Stream Frog, with no *Bd* infection in treated individuals one week after the start of the treatment. However, we note that our low sample size of tested individuals and use of microscope skin sample examinations are limitations of our study.

The same concentration of 0.01% caused the mortality of juvenile frogs of other species both in six and 11 days of treatment (Brannelly et al. 2012), whereas 10 d in our experiments resulted in test-animal survival and no known subsequent mortality related to treatment. The lower concentrations (0.003% and 0.006%) initially used in our study did not appear effective for clearing *Bd* infection quickly enough to prevent frog death, or the infection was too high in our frogs that mortality was unavoidable. It is possible that a low concentration would be effective with a less intense infection, as Brannelly et al. (2012) demonstrated with an even lower concentration of 0.025% over 11 d of exposure. However, we had an urgent situation with an endangered species die-off in captivity, and we were unaware of its antifungal sensitivity. For this reason, we followed a cautionary approach to find a minimum effective dose to reduce any risk

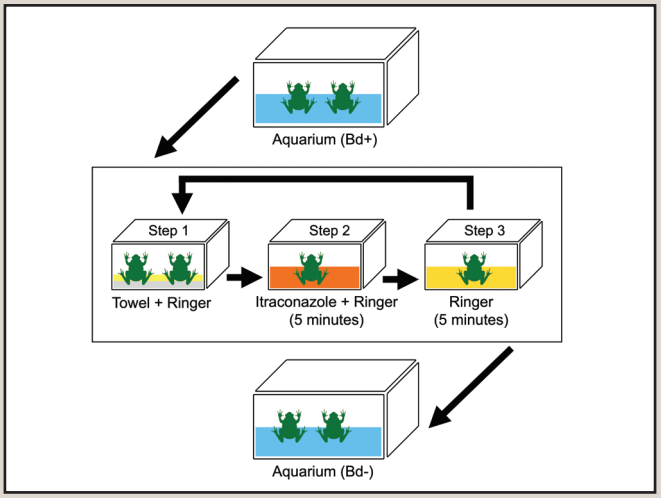

Fig. 2. Treatment steps to clear *Batrachochytrium dendrobatidis* infections from captive El Rincon Stream Frogs, *Pleurodema somuncurense*, in Argentina.

related to increased sensitivity to Itraconazole in this species. Given our approach and our findings, we provide support for a safe Itraconazole concentration and an adequate exposure time for *P. somuncurense*.

Although *Bd* occurrences had been known from both field populations and *ex-situ* colonies of El Rincon Stream Frog (Arellano et al. 2017; MLA, pers. obs.), no mortality cases related to this fungal pathogen have been recorded previously. Arellano et al. (2017) documented a low infection intensity in the field (1.2–29.3 zoospore equivalents), and laboratory studies of shed skin samples also showed a slight infection (10–15 sporangia/mm^2; MLA pers. obs.). The low and stable infection intensity in the field (18–26°C) and the laboratory (21–23°C) supports the ability of *P. somuncurense* to sustain a sublethal *Bd* infection within these temperature ranges (Fig. 1b). Stable temperatures also were associated with low infection loads and mortality in other amphibian species (Sonn et al. 2017; Campbell et al. 2019).

The sudden mortality event reported here led us to formulate the following hypothesis. In six years, the *ex-situ* colony had only two unusual mortality events. The first event was associated with

a significant increase in temperature range due to a failure of the heating and cooling system. During this event, temperatures increased to 32°C, far exceeding the temperature range of this species, which inhabits hot springs between 18°C and 26°C. The second event, the scenario described here, could be due to a combination of factors. Once again, the failure of the heating and cooling system caused a sudden temperature drop (14°C) recorded for two days. This temperature is similar to those that caused high infectious loads and host mortality in other species (Bustamante et al. 2010; Murphy et al. 2011; Soon et al. 2017; Robak and Zawacki 2018; Campbell et al. 2019). Another consequence of low temperatures and/or sudden temperature changes is its negative effect on the frogs' immune system (Raffel et al. 2006; Rollins-Smith and Woodhams 2012; Robak et al. 2019). In the case of El Rincon Stream Frog, a combination of stress of overcrowding due to the pandemic lockdown and the sudden decrease in temperatures could have caused both an increase in *Bd* loads and decreased immune system activity that led to mass mortality.

While our study has shown that a 0.01% Itraconazole bath safely cleared frogs in at least eight days, further research is needed to determine protocols for other life cycle stages of this species. This technique combined with egg mass and tadpoles management (see Arellano et al. 2018), appear to offer protocols that can be standardized for keeping the *ex-situ* colony of this species safe from mortality due to *Bd* infection. Overall, we recommend conducting formal experiments with different *Bd* doses and Itraconazole concentrations that includes negative controls for *P. somuncurense* and other species in ex-situ settings so that standardized protocols may be developed. In terms of management of *ex-situ* colonies for endangered amphibians, we suggest conducting cautionary annual treatments with minimum effective doses (below the dose suggested for treatment), even in the absence of *Bd*-chytridiomycosis.

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