

# Clinical trials with itraconazole as a treatment for chytrid fungal infections in amphibians

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**ABSTRACT:** Due in large part to recent global declines and extinctions, amphibians are the most threatened vertebrate group. Captive assurance colonies may be the only lifeline for some rapidly disappearing species. Maintaining these colonies free of disease represents a challenge to effective amphibian conservation. The fungal disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is one of the major contributors to global amphibian declines and also poses a serious threat to captive assurance colonies. Many treatment options for *Bd* infection have not been experimentally tested and the commonly administered dosages of some drugs are known to have negative side effects, highlighting a need for clinical trials. The objective of this study was to clinically test the drug itraconazole as a method for curing *Bd* infection. We bathed *Bd*-positive juveniles of 2 anuran amphibian species, *Litoria caerulea* and *Incilius nebulifer*, in aqueous itraconazole, varying the concentration and duration of treatment, to find the combination that caused the fewest side effects while also reliably ridding animals of *Bd*. Our results suggest that a bath in 0.0025% itraconazole for 5 min d<sup>-1</sup> for 6 d reliably cures *Bd* infection and causes fewer side effects than the longer treatment times and higher concentrations of this drug that are commonly administered.

**KEY WORDS:** *Batrachochytrium dendrobatidis* · Chytridiomycosis · Fungicide · Itraconazole · White's green treefrog · *Litoria caerulea* · Gulf Coast toad · *Incilius nebulifer*

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## INTRODUCTION

Amphibians are currently experiencing the greatest decline in species richness and diversity of all vertebrate groups (Stuart et al. 2004, Hoffmann et al. 2010). Major contributors to these declines include habitat loss, overexploitation and disease. Chytridiomycosis, caused by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in the extinction or decline of up to 200 amphibian species (Skerratt et al. 2007). Because many species are declining at an alarmingly rapid rate, captive assurance colonies and breeding programs are important conservation measures and may be the only lifeline for some species (Gascon 2007, Garner et al. 2009a).

Establishing and maintaining disease-free colonies represents a significant challenge to amphibian management facilities. The current chemotherapeutic methods for treating *Bd* infections, although effective, can be harmful to the animals being treated, and no single treatment method has been successful across species and age classes of amphibians (Young et al. 2007, Berger et al. 2009, 2010).

### The amphibian chytrid fungus

*Bd* is widespread and has a broad host range. It has been detected on every continent where amphibians are found and at elevations spanning from sea level

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to more than 5000 m (Kriger & Hero 2009). Clinical signs of chytridiomycosis are non-specific and include irregular skin sloughing, lethargy, weight loss, ventral and hind-limb redness and loss of righting reflex. The motile zoospores of *Bd* infect the keratinized epithelium in the outer epidermal layers of post-metamorphic amphibians resulting in thickening of the epidermis (Stockwell et al. 2010). Infection seems to cause osmoregulatory dysfunction (Voyles et al. 2009a, 2012), though it is not clear whether this effect is caused directly by the fungus or perhaps by a toxin it produces (Voyles et al. 2007).

The spread of *Bd* may be facilitated by the increasingly global movement of amphibians for the pet trade, zoos, biological research and human consumption (Daszak et al. 2001). Because not all infected individuals are symptomatic, captive management facilities have unknowingly introduced *Bd*-infected amphibians into captive colonies (Pessier et al. 1999, Forzán et al. 2008, Miller et al. 2008). For example, the North American bullfrog *Rana catesbeiana* is a known carrier of *Bd* but does not tend to exhibit clinical signs of chytridiomycosis (Schloegel et al. 2010a). Bullfrogs are farmed and shipped all over the world

for human consumption (Garner et al. 2009a). Bullfrog breeding facilities are also perfect breeding grounds for *Bd* (Green & Dodd 2007) and infection rates as high as 78.5% have been reported (e.g. in Brazil, Schloegel et al. 2010a). While transportation of amphibians is important for cultural, conservation and economic reasons, until testing and a standardized method for the treatment of animals as they enter the amphibian trade is required, *Bd* infections will continue to pose a significant risk to captive amphibian colonies (Kriger & Hero 2009). *Bd* is now listed as a reportable infection to the World Organization for Animal Health (OIE). This requires that amphibians shipped internationally be either *Bd*-negative or treated for *Bd* prior to shipping (Schloegel et al. 2010b). With this regulation enforced an effective but safe treatment protocol is required.

### Treatment of chytridiomycosis

Protocols for treatment of *Bd* infections have been developed mainly through empirical treatment and from results of a few clinical trials (Table 1) (Berger et

Table 1. Alternative treatments for *Bd* infections in captive amphibians

Method	Advantages	Disadvantages	Protocol	Result	Reference
Itraconazole	(1) Short treatment time (2) Ease of application (3) Few side effects on humans	(1) Expensive (2) Harmful to some life stages and species	Baths in 0.0025–0.01% (adults) or 0.0005% (larvae) solution, 5 min d <sup>-1</sup> for 11 d	Effective treatment	Present study, Jones et al. (2012), Lamirande & Nichols (2002), Garner et al. (2009b)
Chloramphenicol	(1) Inexpensive (2) Well tolerated in adults and larvae (3) Ease of application	(1) Difficult for terrestrial species (2) Risk of aplastic anemia in humans	0.002% solution, constant exposure for 2–4 wk	Effective treatment	Page (1991), Bishop et al. (2009), Young et al. (2012)
Heat	(1) Few reported side effects	(1) High temperatures not tolerated by all species (2) Temperature control can be challenging	(1) 37°C for 16 h (2) 30°C for 10 d	(1) Effective treatment (2) 96% effective	Woodhams et al. (2003), Chatfield & Richards-Zawacki (2011)
Formalin and malachite green	(1) Short treatment time (2) Ease of application	(1) Not tolerated by some species (2) Carcinogenic to humans	Baths in 0.01% malachite green and 0.0025% formalin for 24 h, every other day for 4 treatments	Effective treatment but not recommended	Schreier et al. (1996), Sudova et al. (2007)
Fluconazole	(1) Short treatment time	Better for terrestrial organisms	0.0025% solution, sprayed 2 times d <sup>-1</sup> for 3–9 d	Not recommended	Essawy et al. (2005), Berger et al. (2009)
Benzalkonium chloride	(1) Short treatment time	Better for terrestrial organisms	0.0001% solutions, sprayed 2 times d <sup>-1</sup> for 3–9 d	Not recommended	Marple et al. (2004), Berger et al. (2009)

al. 2010, Pessier & Mendelson 2010). These treatment methods are used for captive animals but not wild amphibians. Because *Bd* is temperature sensitive, heat therapy is one potential treatment option. For amphibian species that can tolerate it, being held at an environmental temperature of 37°C for 16 h (Woodhams et al. 2003) or 30°C for 10 d (Chatfield & Richards-Zawacki 2011) has been successful. Chemotherapeutic treatments are another viable option for many species. Chloramphenicol, an antibiotic, has been used successfully to treat *Bd* infection in some species and can be used for both adult and larval anurans (Young et al. 2012). Treatment requires total bath immersion for an extended treatment time (2 to 4 wk), which may make it a more suitable treatment option for aquatic organisms than for terrestrial ones (Pessier & Mendelson 2010). However, chloramphenicol immersion has been used in terrestrial species as well (Bishop et al. 2009). Although chloramphenicol may be effective and appears to have few side effects for amphibians, its use in veterinary applications has been restricted in some countries because of the risk of chloramphenicol resistance in pathogens and because human exposure has been associated with a rare form of aplastic anemia (Page 1991). A *Bd* treatment involving formalin and malachite green, adapted from a parasite treatment method for fish, has been clinically tested in *Xenopus tropicalis* and may be an effective treatment for other aquatic species. However, its effectiveness in *X. tropicalis* was determined based on histological examination, which could have failed to detect persistent low-level *Bd* infections (Parker et al. 2002). Studies of non-chytrid pathogens in fish have demonstrated that the combination of formalin and malachite green is more effective at treating pathogens than using each chemical separately (Schreier et al. 1996, Sudova et al. 2007). However, both formalin and malachite green are carcinogens and, thus, this option poses potential health hazards for humans administering treatment (Sudova et al. 2007, Pessier & Mendelson 2010). Fluconazole, a triazole antifungal agent developed as a prophylactic antifungal for use after bone marrow transplantation, has also been proposed as a treatment for *Bd*, but there are concerns that it may cause alterations in amphibian blood cells comparable to carcinogenic chemicals (Essawy et al. 2005). Benzalkonium chloride is a quaternary ammonium compound that has been clinically used as an antimicrobial additive in both prescription and over-the-counter pharmaceutical products (Marple et al. 2004). It has been used in both fish and amphibian husbandry as a disinfectant (for

cleaning terraria and aquaria) and to sanitize skin prior to procedures such as toe clipping (Pessier & Mendelson 2010). Fluconazole and benzalkonium chloride treatments were shown to kill *Bd in vitro* (Berger et al. 2009). However, these treatment methods were not able to cure *Bd* infections in *Litoria caerulea* (Berger et al. 2009).

The most widely used chemotherapeutic treatment for chytridiomycosis in captive amphibians is itraconazole. Itraconazole is a broad spectrum antifungal (Grant & Clissold 1989, Yoon et al. 2005), and while it is naturally water insoluble, it is available in aqueous form (Sporanox<sup>®</sup>, Janssen Pharmaceutica). The benefits of itraconazole treatment include short treatment duration (11 d or fewer) and a low risk of adverse effects to human administrators. The disadvantages are that aqueous itraconazole is relatively expensive and that treatment is not tolerated well by all species and life stages of amphibians. The most common treatment (bath in 0.01 % aqueous solution) is fatal to larvae and recent metamorphs in species such as the Wyoming toad *Anaxyrus baxteri* (Pessier 2008, Pessier & Mendelson 2010). There also are sporadic reports of adult amphibians having adverse reactions to itraconazole treatment, especially in Ranidae (Pessier & Mendelson 2010). The mechanism for intolerance of itraconazole is unknown but may stem from the acidity of the solution, sensitivity to the chemicals used as a vehicle to get itraconazole into solution or a reaction to itraconazole itself. Skin irritation and osmotic dysfunction are potential side effects of treatment, but dilution in Amphibian Ringer's solution, which acts as an alkalizing agent, can reduce some of these effects. Other common side effects in species sensitive to itraconazole include treatment-associated anorexia and death (Pessier & Mendelson 2010).

The most commonly used protocol for treating *Bd* is to bathe infected amphibians in 0.01 % itraconazole in a solution of Amphibian Ringer's solution or Holtfreter's solution for 5 min d<sup>-1</sup> for 11 d (Nichols et al. 2000, Forzán et al. 2008, Pessier & Mendelson 2010). However, this concentration may be much higher than what is necessary to cure *Bd* infection as lower doses have also been successful in some cases (Garner et al. 2009b, Pessier & Mendelson 2010, Jones et al. 2012). Because of the potential for side effects, treatment in a 0.01 % itraconazole solution is not recommended for all amphibian species and life stages (Garner et al. 2009b, Pessier & Mendelson 2010). Clinical trials with this drug will facilitate the development of treatment protocols that are effective in ridding amphibians of *Bd* while also minimizing the potential for negative side effects. This will be especially important

in cases where the health of a captive animal is critical to the survival of the species. We conducted clinical trials using itraconazole to treat *Bd* infections in juveniles of 2 anuran species, White's green treefrog *Litoria caerulea* and the Gulf Coast toad *Incilius nebulifer*. We chose juveniles because they are often more susceptible to both chytridiomycosis and the side effects of itraconazole treatment (Lamirande & Nichols 2002, Kriger & Hero 2007, Pessier 2008, Pessier & Mendelson 2010). Our results suggest that to minimize negative side effects, careful consideration should be given to both the concentration and duration of itraconazole treatment, especially for sensitive life stages like new metamorphs and tadpoles.

## METHODS

### Study species

Our first treatment trial involved *Litoria caerulea*, an Australian species known to be susceptible to chytridiomycosis and which has experienced declines in the wild (Berger et al. 2009). *L. caerulea* has been treated clinically in previous studies using flucanazole, benzalkonium chloride (Berger et al. 2009), chloramphenicol (Young et al. 2012) and 0.01% itraconazole (Jones et al. 2012). *L. caerulea* is a hylid treefrog that is widely distributed in Australia. It is commonly found near streams on rocks or trees and can also inhabit urban or residential environments. It is also a common species in the pet trade. Our second trial involved *Incilius nebulifer*, a bufonid from the southeastern USA and northeastern Mexico. This toad occurs in a wide variety of habitats, including residential areas and the outskirts of cities (Mulcahy & Mendelson 2000). We chose *I. nebulifer* because we found it to be infected with *Bd* in Louisiana (4 of 21 animals sampled in June 2011 tested positive for *Bd*, authors' unpubl. data). Recently metamorphosed (juvenile) individuals of each species were used because this life stage is often more susceptible to *Bd* infection and chytridiomycosis than adults (Lamirande & Nichols 2002, Kriger & Hero 2007) and because juveniles also tend to exhibit more side effects with itraconazole treatment (Pessier 2008, Pessier & Mendelson 2010).

### Animal housing and management

For the *Litoria caerulea* trial, 50 *Bd*-negative juvenile *L. caerulea* (1 to 3 mo post-metamorphosis;

snout-to-vent length, 19.9 to 31.6 mm) were purchased from a commercial dealer. All animals were tested for *Bd* presence upon arrival (see 'Testing for *Bd*' section below) and were found to be *Bd*-negative. Animals were placed individually in 13 × 23 × 13 cm terraria containing filtered tap water (3–5 cm deep) and housed at 20°C in an environmental chamber where they were allowed to acclimate for no less than 1 wk before the start of the study. Animals were fed one-quarter inch (6.4 mm) crickets *Acheta domesticus* ad libitum daily.

For the *Incilius nebulifer* trial, 150 recently metamorphosed toads (snout-to-vent length, 12.8 to 29.0 mm) were captured from Tulane University's Herbert Center in Belle Chase, Louisiana (29° 53' 21.43" N, 89° 57' 7.77" W) and tested for *Bd* (all were negative). The toads were placed into communal enclosures (8 to 12 animals per 13 × 23 × 13 cm terrarium) with a damp paper towel substrate. These animals were also housed at 20°C in an environmental chamber and allowed to acclimate for no less than 1 wk before the start of the study. The *I. nebulifer* metamorphs were fed springtails (*Collembola* sp.) ad libitum daily.

For both trials, the amphibians were randomly assigned to a dosage level treatment group on the day itraconazole treatment began. In the *Litoria caerulea* treatment trial, animals were housed individually and each treatment group contained 9 frogs. On Day 6 of treatment in the *Incilius nebulifer* trial, each dosage level was split randomly into 2 equal sized groups (n = 15 toads per itraconazole treatment group, n = 17 toads in the *Bd*+ control group and n = 9 toads in the *Bd*- control group) to create the 6 d and 11 d treatment trial groups. Frogs were housed communally in the *I. nebulifer* treatment trial in order to more closely mimic the housing practices used in most amphibian captive management facilities. A treatment protocol was considered successful only if the whole treatment group was free of *Bd* infection at the end of the trial.

Enclosures were cleaned and disinfected (once a week for *Litoria caerulea* and twice a week for *Incilius nebulifer*) by removing the animals and rinsing each terrarium in a 10% bleach solution, followed by 2 rinses in a water bath. The terraria were dried completely for no less than 24 h before being used again. Because *Bd* is biohazardous to amphibians, each animal was handled with a clean pair of nitrile gloves, and all materials and equipment that came into contact with the amphibians or the contents of their enclosures (water or substrate) was disinfected using a 10% bleach solution. Household bleach was added

to all liquid waste (bringing it to 10% bleach) and all solid waste was autoclaved before disposal.

### Testing for *Bd*

We tested for *Bd* infection by using skin swabs and a quantitative real time PCR (qPCR) assay (Boyle et al. 2004). Swabbing is a non-invasive method of sampling for *Bd* on amphibian skin. The swabbing protocol was standardized by performing 40 strokes with a sterile swab (MW113, Medical Wire and Equipment) per animal, 5 on each of the dorsal surface, the ventral surface, each side and each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. Genomic DNA was extracted from the swabs using the Qiagen DNeasy Blood and Tissue Kit according to the instructions for animal tissue, with a final elution volume of 200  $\mu$ l. Extracted DNA was then analyzed using qPCR (Applied Biosystems 7500) following Boyle et al. (2004) with the following minor modifications: each sample was diluted 1:10 with doubly deionized water and 0.7  $\mu$ l of bovine serum albumin (BSA) (Applied Biosystems) was added to each reaction prior to amplification to prevent PCR inhibition (following Garland et al. 2010). For the *Litoria caerulea* experiment, we ran each sample in triplicate, with a positive and negative control, and a series of dilution standards (to estimate zoospore infection load). One of the 3 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. For the *Incilius nebulifer* experiment, we ran each sample in singlicate, where all samples contained an internal positive control.

### Experimental design

The *Litoria caerulea* experiment lasted 2 mo. The experiment began with a 1 wk acclimation period prior to inoculation. Treatment frogs were then inoculated with *Bd* zoospores (see below). After a 1 wk period of infection growth, each frog was treated for 11 d with itraconazole and monitored for 4 wk after treatment ended. Individuals were swabbed, weighed and measured 5 times throughout the experiment at the following stages: (1) immediately before the start of itraconazole treatment to ensure the presence of infection, and (2) 1 wk, (3) 2 wk, (4)

3 wk and (5) 4 wk after the end of treatment to assess whether the infection had been cleared. Swabbing 1 wk after treatment was suggested by Berger et al. (2010). We also swabbed at 2, 3 and 4 wk post-treatment to ensure that animals remained uninfected. Amphibians generally shed their skin fully every 14 d or less (Voyles 2009b); hence, at 2 to 4 wk after the completion of treatment the risk of dead *Bd* zoospores causing a false positive result was expected to be very low.

The *Incilius nebulifer* experiment was identical to the *Litoria caerulea* experiment except for the following modifications: toads were housed and treated communally (8 to 12 individuals per enclosure) for 11 d or 6 d and then were monitored individually for 4 wk after the end of treatment. Each toad was swabbed, weighed and measured 3 times throughout the experiment: (1) right before treatment (1 wk after inoculation), and (2) 2 wk and (3) 4 wk after the end of treatment.

### Zoospore harvesting and inoculation

Our *Bd* cultivation and inoculation procedure was adapted from previously published protocols (Berger et al. 2004). *Litoria caerulea* was inoculated with *Bd* strain JEL423 and *Incilius nebulifer* was inoculated with *Bd* strain JEL411 (both provided by J. Longcore). Both strains are from Guabal, Panama: JEL411 was originally isolated from *Atelopus varius*, while JEL423 was originally isolated from *Phyllomedusa lemur*. We chose to use 2 different strains of *Bd* in this study to ensure that our results were robust to differences in strains. *Bd* was grown in a tryptone broth containing penicillin–streptomycin for 7 d at 23°C. The broth culture was then transferred to agar and tryptone plates and incubated at 23°C for 7 d. Each plate was then flooded with 5 ml of filtered tap water for 5 min to harvest *Bd* zoospores. Each plate was flooded once and discarded. The number of zoospores in the flooded solution was estimated using a hemocytometer and the solution was diluted with filtered tap water to achieve the desired concentration of zoospores.

The inoculation protocol for *Litoria caerulea* and *Incilius nebulifer* differed slightly to accommodate for the fact that *I. nebulifer* is more terrestrial than *L. caerulea*. *L. caerulea* were inoculated by placing each frog in a 60 ml Solo® container containing 40 ml of inoculum (containing  $1 \times 10^7$  *Bd* zoospores) for 24 h. *I. nebulifer* were inoculated by placing pairs of toads in a 60 ml container and squirting 3 ml of ino-

culum (again containing  $1 \times 10^7$  zoospores) directly onto the ventral surface and limbs of each animal. Frogs and toads were then housed in the 60 ml containers for 24 h before returning them to their enclosures. After the inoculation procedure, the infection was allowed to develop for 1 wk before itraconazole treatment began.

### Treatment trials

Treatment trials for itraconazole were amended from Pessier & Mendelson (2010) by varying both solution concentration and treatment duration. The first experiment, using *Litoria caerulea*, investigated itraconazole concentration only. Infected frogs were bathed in a solution containing 1 of 4 concentrations of aqueous itraconazole (Sporanox® Oral Solution, Janssen Pharmaceutica; 0.01, 0.005, 0.0025 and 0.0005%,  $n = 9$  frogs per group) for 5 min  $d^{-1}$  for 11 d. Frogs were treated by placing them individually in 60 ml Solo® containers with 40 ml of treatment solution (itraconazole in Amphibian Ringer's solution) for 5 min and then returning them to disinfected terraria. There were 2 control groups for the *L. caerulea* experiment, one inoculated with *Bd* ( $n = 9$ ) and one sham inoculated (*Bd* negative) ( $n = 4$ ). The control groups were bathed in a sham treatment solution containing only Amphibian Ringer's solution. The highest treatment concentration, 0.01% itraconazole, was chosen because this is one most commonly used by managers of captive amphibians. The 0.005% itraconazole solution was chosen because it is thought to have been successful in treating *Bd* infections in *L. caerulea* (Pessier & Mendelson 2010, Jones et al. 2012). Our lowest concentration was 0.0005%, or one-twentieth the strength of the most commonly used protocol. This concentration has been successful in treating larvae (but caused depigmentation, Garner et al. 2009b) but has not been tested in metamorphosed amphibians.

Our second treatment experiment tested both itraconazole concentration and treatment duration for *Incilius nebulifer*. This experiment tested both 11 d of treatment and 6 d of treatment using itraconazole concentrations of 0.01, 0.005 and 0.0025% ( $n = 15$  toads per group). In both the 11 d treatment and the 6 d treatment there was a *Bd*-positive (inoculated with zoospores,  $n = 17$ ) and *Bd*-negative (sham inoculated,  $n = 9$ ) control group. During itraconazole treatment, toads were housed and treated communally by placing them in 200 ml of treatment solution in a 740 ml plastic container ( $4.5 \times 15 \times 15$  cm) for

5 min  $d^{-1}$  and then returning them to disinfected communal terraria.

Amphibians in all trials were monitored daily to check for clinical signs of chytridiomycosis and side effects of itraconazole treatment. If an animal was unable to right itself within 8 s after being placed on its back, we assumed that it would have died within 24 h and the animal was euthanized (Stockwell et al. 2010) with 0.2% MS-222 (Berger et al. 2004). All animals were euthanized upon completion of the experiment.

### Statistical analyses

A treatment protocol was deemed effective if all animals tested negative for *Bd* via qPCR upon final swabbing 4 wk after the completion of itraconazole treatment. Body condition (mass/snout-to-vent length) differences and zoospore loads were compared among groups using analyses of covariance (ANCOVA) and ANOVA, and survival differences were analyzed using a Cox regression. The assumption of proportional hazards for Cox regression was assessed by plotting  $\log[-\log(\text{survival})]$  versus  $\log$  of survival time for each trial. In all cases treatment group lines were parallel, indicating proportionality of hazards. All statistical analyses were done in SPSS v. 17.

## RESULTS

### Lowest effective dose

We determined the lowest effective itraconazole concentration for each experiment as the lowest dose in which all treated individuals tested *Bd* negative 4 wk after treatment. The most commonly used dosage of itraconazole for chytridiomycosis treatment, 0.01% for 11 d, was found to be effective in this study, but lower concentrations also provided effective treatment. The lowest effective concentration in the *Litoria caerulea* experiment (11 d treatment) was 0.0025% itraconazole. While treatment with 0.0005% itraconazole reduced zoospore load, the infection was not cured. The average ( $\pm$ SD) number of zoospore equivalents for this treatment 1 wk after inoculation was 475.07 ( $\pm$ 74.16), which decreased to 0.05 ( $\pm$ 0.02) zoospore equivalents 2 wk after treatment was completed. When tested 4 wk after the completion of treatment, the average zoospore load was 0.21 ( $\pm$ 0.12) zoospore equivalents. *Bd*+ control *L. caerulea* had a zoospore load of 2381.55 ( $\pm$ 727.43)

1 wk after inoculation and  $4686.67 (\pm 2798.00)$  at the end of the 6 wk experiment. The *Incilius nebulifer* experiment also supported 0.0025% as an effective dose, even when the treatment time was shortened to 6 d. One week after inoculation the zoospore load for *I. nebulifer* was  $1121.7 (\pm 17.34)$ , while after treatment the *Bd*+ control toads had a zoospore load of  $16\,765.12 (\pm 14\,643.08)$ .

### Survival and occurrence of side effects

Body condition (mass/snout-to-vent length) did not differ among any of the treatment groups on Day 1 of treatments (ANOVA: *Litoria caerulea*  $F_{6,43} = 0.65$ ,  $p = 0.69$ ; *Incilius nebulifer*  $F_{4,138} = 1.06$ ,  $p = 0.38$ ) or at the end of the experiment 4 wk after treatment concluded (ANOVA: *L. caerulea*  $F_{5,20} = 1.93$ ,  $p = 0.14$ ; *I. nebulifer* 11 d treatment  $F_{3,37} = 1.01$ ,  $p = 0.40$ ; *I. nebulifer* 6 d treatment  $F_{4,43} = 2.72$ ,  $p = 0.61$ ), although survival differed among treatment groups. For the *L. caerulea* experiment (Fig. 1a), treatment with itraconazole at all concentrations except 0.0025% resulted in reduced survival compared with sham-treated, *Bd*-positive frogs (Cox regression: 0.01% itraconazole  $\chi^2_1 = 4.61$ ,  $\exp(b) = 7.33$ ,  $p = 0.032$ ; 0.005% itraconazole  $\chi^2_1 = 5.547$ ,  $\exp(b) = 8.48$ ,  $p = 0.019$ ; 0.0005% itraconazole  $\chi^2_1 = 5.77$ ,  $\exp(b) = 8.75$ ,  $p = 0.016$ ). The effect of the 0.0025% itraconazole concentration on survival was marginally non-significant (Cox regression:  $\chi^2_1 = 3.36$ ,  $\exp(b) = 5.89$ ,  $p = 0.067$ ). For the *I. nebulifer* 11 d treatment trial (Fig. 1b), survival in the 0.0025% itraconazole treatment group did not differ from that of the *Bd*-positive sham-treated group (Cox regression:  $\chi^2_1 = 2.17$ ,  $\exp(b) = 20\,500$ ,  $p = 0.339$ ). However, survival in both 0.005% and 0.01% itraconazole treatments was significantly lower than in the *Bd*-positive and *Bd*-negative sham-treated groups (Cox regression: 0.01% itraconazole  $\chi^2_1 = 35.68$ ,  $\exp(b) = 307.54$ ,  $p < 0.01$ ; 0.005% itraconazole  $\chi^2_1 = 16.17$ ,  $\exp(b) = 9.40$ ,  $p < 0.01$ ). For the 6 d treatment trial using *I. nebulifer* (Fig. 1c) the 0.01% itraconazole treatment group had significantly reduced survival compared with the *Bd*-negative sham-treated group (Cox regression:  $\chi^2_1 = 7.261$ ,  $\exp(b) = 55.16$ ,  $p = 0.007$ ). However, survival did not differ between the *Bd*-negative sham-treated group and either of the lower concentration treatment groups (Cox regression: 0.005% itraconazole  $\chi^2_1 = 3.45$ ,  $\exp(b) = 47.59$ ,  $p = 0.63$ ; 0.0025% itraconazole  $\chi^2_1 = 3.50$ ,  $\exp(b) = 48.08$ ,  $p = 0.062$ ). The 0.0025% itraconazole treatment had the greatest percentage survival of all treatment groups in all 3 trials and had significantly greater sur-

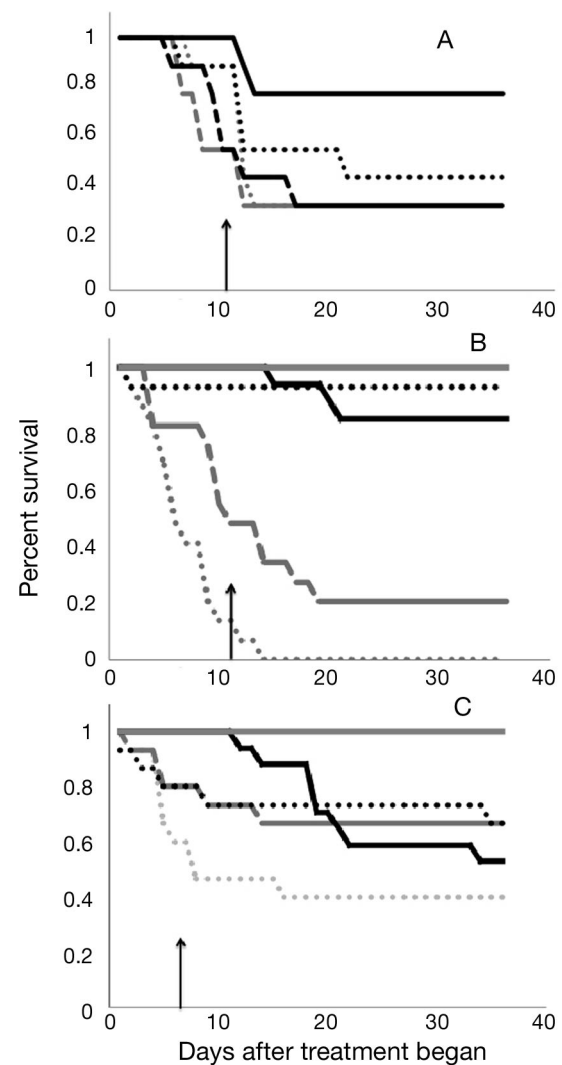


Fig. 1. *Litoria caerulea* and *Incilius nebulifer*. Differences in survival across itraconazole treatments. (A) *L. caerulea*: 11 d treatment ( $n = 9$  for each treatment group). (B) *I. nebulifer*: 11 d treatment ( $n = 15$  for each itraconazole concentration of 0.01, 0.005 and 0.0025%,  $n = 17$  for the *Bd*-positive control group and  $n = 9$  for the *Bd*-negative control group). (C) *I. nebulifer*: 6 d treatment ( $n = 15$  for each itraconazole concentration of 0.01, 0.005 and 0.0025%,  $n = 17$  for the *Bd*-positive control group and  $n = 9$  for the *Bd*-negative control group). Black arrows indicate the last day of treatment. Solid lines indicate survival of control groups (black = *Bd*-positive, gray = *Bd*-negative). Broken lines indicate survival of itraconazole treatment groups (gray dots = 0.01%, gray dashes = 0.005%, black dots = 0.0025%, black dashes = 0.0005% itraconazole)

vival than other treatment groups in both *I. nebulifer* treatment trials.

Aside from mortality, other negative effects of itraconazole treatment were observed but not quantified during our experiments; these included loss of appetite during treatment (as determined by the number uneaten of feeder insects remaining in the

tank), excessive skin sloughing, lethargy and skin discoloration. These effects were particularly prominent in the 0.01 and 0.005% itraconazole treatments for both *Litoria caerulea* and *Incilius nebulifer*, although they occurred in all treatment groups. Necropsy and histopathology were conducted on 2 *L. caerulea* individuals from each of the treatment groups that died during or shortly after the treatment period. Results of these analyses were inconclusive as to the cause of death, although no animals died of chytridiomycosis. Moderate autolysis that occurred between death and specimen preservation may have affected our ability to detect subtle differences between treatment groups.

## DISCUSSION

The goal of this study was to find an itraconazole treatment method that is effective at curing *Bd* infections in captive amphibians but that also minimizes the potential for treatment-associated mortality. The results of our clinical treatment trials on 2 amphibian species support the effectiveness of reduced concentrations of itraconazole (0.0025% as opposed to the most commonly used 0.01%) in ridding amphibians of *Bd*. Treatment in 0.0025% itraconazole was sufficient to cure *Bd* infection in 2 distantly related anuran species infected with different *Bd* strains. In *Incilius nebulifer*, reducing the treatment time from 11 to 6 d was also effective, even at this same low dose. Lower concentrations and shorter treatment times achieved the same desired effect (zero detectable *Bd*) with greater survival.

All 3 of our experiments demonstrate that treatment with itraconazole at the most commonly used concentration of 0.01% can be harmful to *Bd*-infected, newly metamorphed anurans. Treatment using the most commonly recommended duration (11 d) and concentration (0.01% itraconazole) caused 100% mortality in *Incilius nebulifer* metamorphs and 66% mortality in *Litoria caerulea* metamorphs, which was a higher mortality rate than that caused by *Bd* infection itself. Even half of this dose (0.005% itraconazole) resulted in high mortality in these anurans. Similar negative effects of itraconazole treatment have been observed in recent metamorphs with natural *Bd* infections (Pessier 2008). Treatment with 0.0025% itraconazole in both the *L. caerulea* (11 d) and *I. nebulifer* (6 and 11 d) trials resulted in survival that was not statistically different from survival in the sham-treated control groups. In the *I. nebulifer* study, the 6 d treatments all had higher survival

than the analogous 11 d treatment groups, and both the 0.005% itraconazole treatment group and the 0.0025% itraconazole treatment group showed no difference in survival compared with sham-treated control groups. All but the lowest (0.0005% itraconazole) treatment concentration were effective at curing *Bd* infection.

Of the protocols that effectively cured *Bd*, the one that resulted in the lowest mortality rate was a 6 d treatment using 0.0025% itraconazole. Our results suggest that shorter treatment durations and lower itraconazole concentrations are effective for recent metamorphs and should be considered to minimize side effects. However, caution should be exercised in generalizing our results to other species and age classes of amphibians.

The anurans used in this experiment were recent metamorphs, chosen because they are both more susceptible to chytridiomycosis than adults and because they are more negatively affected by itraconazole treatment than are adults (Pessier & Mendelson 2010). The high mortality in the 11 d treatment using both 0.01% itraconazole and 0.005% itraconazole doses in recent metamorphs may not be seen in adult animals. Stronger concentrations of itraconazole (0.01% and 0.005%) have been used effectively in adult *Litoria caerulea* and a variety of other amphibian species, apparently without the high mortality rates we observed in this study (Pessier & Mendelson 2010, Jones et al. 2012). However, there are some species, especially ranids, where adult individuals have anecdotally suffered negative side effects during itraconazole treatment (Pessier & Mendelson 2010, Woodhams et al. 2012). When treating a species or life stage for which there is no prior experience with itraconazole, it is recommended to test only a few animals at the lower dose before treating the entire group (Pessier & Mendelson 2010).

The cause of toxicity associated with itraconazole treatment is unknown and was not explored extensively in this study. Future studies could include more extensive postmortem examination and histopathology of animals that die during itraconazole treatment. Comparison of tissue changes between itraconazole treated and untreated animals may require euthanasia of subsets of animals at different time points during treatment to avoid problems with postmortem autolysis, which can occur rapidly and obscure subtle histologic lesions.

Finding a single *Bd* treatment regime that is effective for all species and age classes of amphibians may not be possible or even necessary (Woodhams et al.



2012). Nevertheless, this study demonstrates that changes to treatment concentration and duration can help to reduce the side effects associated with chemotherapeutic treatments. For species and life stages that are particularly affected by itraconazole, other treatment methods may provide a better solution. Heat therapy is one option that shows promise. Chatfield & Richards-Zawacki (2011) found that holding amphibians at 30°C for 10 d cleared *Bd* infection in 96 % of the animals. This temperature is likely to be well within the range of thermal tolerance for many species; however, more work needs to be done to develop a heat treatment protocol that is 100 % effective, especially for cool-adapted species. Heat therapy may be more difficult for managers of captive animals to put into practice because it requires close monitoring and control of thermal conditions for an extended period of time. In these situations, chemotherapeutic methods may be the best option. At present, itraconazole appears to be the most effective and widely used chemotherapeutic treatment for *Bd*. Chloramphenicol is another option, but it has not been extensively clinically tested, and because it requires constant immersion for 2 to 4 wk it is likely to be better tolerated by aquatic species. Based on results from the present study, we recommend that careful consideration be given to both species and life stages before designing an itraconazole treatment protocol for use in captive colonies.

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