

EVALUATING TISSUE CONCENTRATIONS IN PANAMANIAN GOLDEN
FROGS (*Atelopus zeteki*) INFECTED WITH *Batrachochytrium dendrobatidis*
FOLLOWING A SINGLE 0.01% ITRACONAZOLE IMMERSION TREATMENT

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The undersigned, appointed by the dean of the Graduate school, have examined the thesis entitled

EVALUATING TISSUE CONCENTRATIONS IN PANAMANIAN GOLDEN FROGS (*Atelopus zeteki*) INFECTED WITH *Batrachochytrium dendrobatidis* FOLLOWING A SINGLE 0.01% ITRACONAZOLE IMMERSION TREATMENT

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ABSTRACT

Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd), continues to threaten amphibian biodiversity globally. The Panamanian golden frog (*Atelopus zeteki*), a species highly susceptible to Bd infection, remains extinct in the wild and dependent on ex situ conservation for survival. Itraconazole, a triazole antifungal, is widely used to treat Bd infections, yet the impact of Bd infection on itraconazole tissue absorption, distribution, and clearance in amphibians is still poorly understood. This study evaluated itraconazole tissue concentrations in Bd-infected (Bd⁺) and uninfected (Bd⁻) *A. zeteki* following a single 0.01% 10-minute immersion. Tissue samples were analyzed at 24, 48, 72, 144, 192, and 240 hours post-treatment, and itraconazole concentrations were measured in the skin, liver, kidney, and gastrointestinal tract using high-performance liquid chromatography (HPLC). Bd⁺ frogs exhibited consistently lower tissue concentrations and more rapid clearance compared to Bd⁻ frogs, likely due to Bd-induced physiological alterations. Tissue concentrations remained above the limit of quantification (LOQ; 0.1 ng/mg) at 240 hours in most tissues of Bd⁻ frogs but fell below the LOQ in most tissues of Bd⁺ frogs by 144–192 hours. These findings suggest that infection status influences itraconazole tissue absorption, distribution, and clearance, supporting the potential for reducing dosing frequency in Bd treatment protocols. Future treatment protocols should consider infection status, tissue-specific distribution, and optimized dosing intervals to improve efficacy and minimize adverse effects in amphibian chytridiomycosis management.

INTRODUCTION

Background

Amphibian populations have undergone significant global declines since the mid-20th century, with many species now facing extinction.^{14,29} Chytridiomycosis, an amphibian disease caused by the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal), has caused catastrophic declines in amphibian populations and is regarded as the largest recorded biodiversity loss associated with a pathogen.^{39,44,46} *B. dendrobatidis* has been detected worldwide and infects over 700 species from all three orders of amphibians (Anura, Caudata, and Gymnophiona), whereas Bsal has thus far only been reported in Asia and Europe, primarily affecting species within the order Caudata.^{8,32} It remains unclear whether Bd is a newly emerging pathogen or if its virulence has increased over time. Although the epizootic form was identified in the 1990s, analyses have detected *Batrachochytrium* spp. in amphibian specimens dating back to the 1800s.^{4,6} The effects of Bd infection vary widely among species, causing mass mortality in some while others remain asymptomatic.⁴¹ Research on treating captive populations has focused on developing antifungal protocols and management strategies to support conservation and reintroduction efforts.^{1,25,28} Currently, no effective or feasible strategies exist for mitigating Bd in wild populations due to the pathogen's persistence in native amphibian environments and the challenges of treating dispersed hosts in natural habitats.^{17,43}

Bd History, Ecology, and Anatomy

Although chytridiomycosis has contributed to amphibian declines since the 1970s, it was not identified as an infectious disease until the late 1990s. During the

1970s and 1980s, unexplained amphibian declines in Australia and the Americas were initially attributed to habitat loss and pollution, with no clear evidence of a pathogenic cause.^{4,32} Mass mortality events were observed in 1998 in wild amphibian populations in Panama and a captive population at the Smithsonian's National Zoological Park in Washington, D.C. The scale and rapid onset of these events prompted researchers to conduct histopathological analyses, ultimately leading to the discovery of a novel chytrid fungus, formally described as *Batrachochytrium dendrobatidis* in 1999.^{6,29} This fungus was the first chytrid species described to parasitize living vertebrates and cause pathogen-induced illness.⁶ Retrospective diagnoses of archival museum specimens revealed that Bd has been present on multiple continents for over 80 years, with the earliest confirmed detection in an Illinois amphibian specimen from 1888 using polymerase chain reaction (PCR) testing.⁴⁻⁵

B. dendrobatidis is a true fungus belonging to the division Chytridiomycota, order Rhizophydiales—a group characterized by aquatic lifestyles—and is an obligate parasite that requires a host to complete its life cycle. Its life cycle includes a motile zoospore stage that facilitates infection and a sessile zoosporangium stage in which the fungus reproduces and releases new zoospores.^{41,44} Flagellated zoospores spread primarily by passive movement in water currents and encyst on the surface of skin epithelial layers, where the flagellum is resorbed and a cell wall forms.² Once encysted, the zoospore develops a germination tube that penetrates the stratum corneum and forms an intracellular thallus, which matures from a sporangium into a zoosporangium that divides to produce additional flagellated zoospores.² In moist environments, these zoospores either exit via a discharge papilla through the skin cell

surface and disperse into the environment or penetrate deeper skin layers via the thallus.^{2,15}

B. dendrobatidis prefers cool, moist environments—including alpine, temperate, and tropical regions—where humidity and stable temperatures support its life cycle.⁴ Temperature plays a key role in influencing Bd virulence.^{32,41} The fungus thrives between 17°C and 25°C; cooler temperatures slow reproduction but prolong zoospore survival down to 4°C, overlapping with the optimal temperature range of most amphibians (15-25°C).⁴⁴ Conversely, higher temperatures (>25°C) reduce infection intensity, and prolonged exposure above 30°C is often lethal to Bd.^{4,43} Its flagellated zoospores rely on water for motility, allowing the pathogen to thrive in environments where amphibians breed and metamorphose, particularly in stagnant or slow-moving water bodies.⁶ The pathogen can persist in moist soil for weeks, providing a reservoir for reinfection and making environmental eradication particularly challenging.¹⁷ Growth of Bd is optimal at a pH range of 6-7, common to many natural freshwater systems.⁴⁴ Non-amphibian carrier hosts of Bd—including crayfish, fish, and waterfowl—may facilitate transmission as Bd adheres to the scales or feet of these species, enabling the fungus to disperse across aquatic habitats.^{35,44}

Amphibian Physiology and Impacts of Bd

Amphibians possess a uniquely specialized integumentary system that is more functionally diverse than other vertebrate taxa. Their skin is highly permeable and vascularized, playing critical roles in osmoregulation, respiratory gas exchange, thermoregulation, and toxin excretion.^{2,9} Electrolyte balance is regulated through the skin, with ions actively transported from the environment into the body via epithelial channels and ion pumps.^{2,10,15} Many species possess a specialized area in the ventral

pelvic region, commonly referred to as the drink patch or pelvic patch, which facilitates maximal uptake of water and oxygen from the environment.³⁴ The amphibian epidermis is notably thin, with the stratum corneum in most species consisting of a single layer of keratinized cells, which may contribute to their vulnerability to Bd infection.^{16,41}

Amphibians infected with Bd exhibit a range of behavioral and physical signs—including lethargy, anorexia, abnormal posture, loss of righting reflex, ataxia, tremors, and integumentary abnormalities.^{2,12} Infection of keratinized epidermal cells by Bd leads to progressive hyperkeratosis, which disrupts cutaneous homeostasis, including respiration and hydration.^{13,41} Infected individuals often exhibit excessive skin sloughing, epidermal thickening, and eventual loss of skin function, which impairs respiration, hydration, and behavior.²⁴ Disruption of sodium (Na^+), potassium (K^+), and magnesium (Mg^{2+}) homeostasis results in osmotic stress, impairing neuromuscular and cardiac stability.^{4,41} Mortality from Bd is associated with this disruption of cutaneous osmoregulation, as severe electrolyte imbalances ultimately lead to cardiac arrest.^{15,43}

B. dendrobatidis employs multiple mechanisms to suppress amphibian immune responses and facilitate infection. The fungus releases immunosuppressive compounds that inhibit lymphocyte activation and induce apoptosis of T and B lymphocytes, which weakens the adaptive immune system and compromises the host's ability to effectively respond to infection.^{13,18} Unlike many other pathogen-host systems, amphibians do not appear to develop robust acquired immunity against Bd, as reinfection studies show that prior exposure does not consistently improve

survival.^{14,42} This absence of protective acquired immunity is atypical for vertebrate host-pathogen systems and presents significant challenges to long-term resistance.

In addition to suppressing immune function, Bd infection disrupts the composition and functional capacity of the amphibian skin microbiome.³ Amphibian skin hosts a diverse microbial community, including bacteria that produce antifungal metabolites, which decline with Bd infection.²⁶ Disruptions to the microbiome may compromise natural skin defenses, allowing Bd to further colonize. Such microbiome shifts may persist after antifungal treatment, potentially impacting the amphibian's long-term health and increasing susceptibility to reinfection.^{3,26}

Panamanian Golden Frogs and Bd

The Panamanian golden frog (*Atelopus zeteki*), classified as critically endangered by the International Union for Conservation of Nature (IUCN), is an amphibian species severely affected by Bd, having experienced near-total population collapse.^{22,25} This species plays a critical role in its native ecosystem: as a predator, *A. zeteki* helps control insect populations, and as prey, it supports the food web, contributing to the biodiversity of its native cloud forest habitat.⁴⁶ *A. zeteki* holds deep cultural significance in Panama, historically depicted in Indigenous art and currently recognized as the national animal. This species is considered a symbol of good luck, with legendary associations in Panamanian folklore. Following the last sighting of a wild *A. zeteki* in 2009, the species now survives only in managed assurance colonies in Panama and the United States.^{16,22} Conservation efforts have focused on captive breeding and potential reintroduction, but Bd remains a major barrier to the reintroduction success of *A. zeteki* because of the species' high susceptibility to infection and lack of effective immune response.³² This species experiences nearly

100% mortality within weeks of experimental exposure to Bd.^{8,29,32} *A. zeteki* may survive Bd infections longer under warm and dry conditions, but in natural environments where optimal Bd conditions often prevail, infections have led to catastrophic population declines.^{4,31}

Experimental trials of Bd exposure and infection have shown that *A. zeteki* is an acute supershedder, releasing large quantities of Bd zoospores into the environment, thereby increasing infection risks for cohabitating amphibian species before succumbing to infection.^{13,32,44} Given the high transmission potential of infected *A. zeteki*, effective antifungal treatments are critical for both preventing disease spread in captive settings and enhancing survival prospects for wild reintroduction efforts.²⁸ *A. zeteki* lacks sufficient antifungal skin peptides, which in other species help inhibit Bd growth by producing antimicrobial compounds.^{7,9,42} Without these innate defenses, *A. zeteki* remains highly vulnerable to Bd colonization and infection. Bd's optimal growth conditions closely align with those of the montane cloud forests inhabited by *A. zeteki*, enabling the pathogen to persist in the environment and infect new hosts even in the absence of amphibians.¹⁷ The environmental stability of Bd presents a significant challenge to reintroduction initiatives. In addition to Bd infection, *A. zeteki* faces threats from habitat loss, which reduces suitable breeding sites; climate change, which may alter disease dynamics; and the illegal wildlife trade, which increases the risk of pathogen introduction and further population declines.^{22,31}

Treatment of Bd with Itraconazole

Effective treatment of Bd infections is essential for amphibian conservation, maintaining ecosystem stability, and preserving global biodiversity, as amphibians

play vital roles in food webs and nutrient cycling.⁴⁶ Currently, no feasible or effective methods exist for treating Bd infections in wild populations, primarily due to the difficulty of reaching all individuals and the pathogen's persistence in the environment.^{6,17,39} In captive settings, a variety of therapeutic approaches are used or are under investigation to manage Bd infections. These include antifungal drugs such as triazoles, terbinafine, chloramphenicol, and malachite green, as well as non-pharmaceutical interventions like heat therapy, probiotics, and microbiome manipulation.^{2,4,14,15,22}

Itraconazole, a triazole antifungal, is widely used to treat Bd infections in amphibians due to its broad-spectrum efficacy and relative safety compared to other antifungal agents.^{2,6} By inhibiting cytochrome P450-dependent ergosterol synthesis, itraconazole compromises the integrity of the fungal cell membrane, weakening the fungus and halting fungal growth.^{10,38} Itraconazole is highly plasma protein-bound, with over 99% of the drug binding to albumin and other plasma proteins, which influences its distribution, bioavailability, and tissue penetration.³⁷ Standard amphibian protocols administer itraconazole via immersion baths—typically for 5-10 minutes at a 0.01% concentration for 5–11 consecutive days—and have shown efficacy in clearing Bd infections in many species.^{2,40} However, concerns about itraconazole toxicity have emerged, with reports of depigmentation, hepatotoxicity, lethargy, and weight loss in some amphibian species following treatment.^{20,27,33} Since the skin microbiome contributes to disease resistance, changes induced by Bd or antifungal treatment may affect long-term health and immune function.^{3,26} The pharmacokinetic profile of itraconazole in amphibians, including its distribution, metabolism, and excretion, is not fully understood. A study by Rifkin et al. (2017)

found that in healthy *A. zetekii* itraconazole accumulates in tissues, with significant concentrations detected in the skin, liver, and heart up to 36 hours post-treatment with a single 10-minute immersion dose of 0.01% itraconazole. This extended persistence raises concerns about toxicity risks with daily treatment protocols, indicating that dosing adjustments may be warranted to avoid potential adverse effects.¹¹ However, the study's limited scope—examining only a 36-hour window and a subset of tissues in uninfected frogs—highlights the need for more comprehensive research to better characterize itraconazole pharmacokinetics and optimize treatment protocols.

Research Context and Aims

Despite its widespread use, the long-term persistence and tissue-specific distribution of itraconazole in *A. zetekii* remain poorly understood, which limits the ability to optimize treatment protocols and minimize toxicity risks.^{10,36} Most existing studies have focused on short-term pharmacokinetics, often limited to the initial hours post-treatment, leaving uncertainty regarding optimal treatment intervals and potential differences in drug behavior between Bd-infected and uninfected individuals.³⁸

This study aimed to evaluate itraconazole tissue concentrations in Bd-infected and uninfected *A. zetekii* following a single 10-minute immersion in 0.01% itraconazole. Samples were collected at 24, 48, 72, 144, 192, and 240 hours post-treatment to assess drug persistence and investigate whether infection status influences tissue concentrations. It was hypothesized that itraconazole would persist in tissues for multiple days, supporting the feasibility of extended dosing intervals in Bd treatment protocols. Additionally, Bd infection was expected to alter drug absorption and distribution, resulting in differential tissue accumulation between infected and uninfected frogs.^{2,41} The findings from this study will enhance understanding of antifungal treatment dynamics in amphibians and offer practical

guidance for managing chytridiomycosis in conservation breeding programs and reintroduction efforts.

METHODS

Overview

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Smithsonian's National Zoological Park and Conservation Biology Institute (NZCBI), approval number SI-24044. Prior to inclusion into this study, a veterinarian performed an individual visual assessment of 60 adult *A. zeteki* from a captive-managed breeding population with no known prior exposure to Bd, and all individuals were confirmed to be healthy. The frogs were randomly assigned to either the control group ($n = 30$; Bd⁻) or the infected group ($n = 30$; Bd⁺). Throughout the study, veterinary and husbandry personnel at NZCBI conducted regular health evaluations and monitoring.

Housing and Husbandry

Frogs were housed individually in 15 x 8 x 10 cm transparent plastic enclosures with secured screen tops and hinged access lids. The enclosures were placed on metal racks fitted with 54W, 46" 5.0 UVB bulbs (Zoo Med Laboratories, San Luis Obispo, CA, USA) on a 12-hour light cycle. Control and infected groups were maintained in separate rooms to minimize the risk of cross-contamination. The enclosures contained a 1" layer of hydroponic clay pebbles (Halatool, USA) covered by mesh screening, topped with a substrate composed of sphagnum moss mixed with Successoil Select (BioD, USA). Halved polyvinyl chloride pipes measuring 5.5 x 5.5 x 3.5 cm served as hides. The water used for bowls and misting was a 1:1 ratio of

reverse osmosis (RO) and carbon-filtered water. Small petri dishes (40 x 8 mm) served as water bowls and were refilled twice daily. Humidity was maintained in each enclosure above 70% by misting as needed. Room temperature was maintained between 20–24°C. Frogs were fed pinhead crickets (*Acheta domesticus*) or fruit flies (*Drosophila hydei*) three times per week. Prior to inoculation with Bd, all frogs underwent a seven-day acclimation period in their assigned enclosures within the study area.

***B. dendrobatidis* Culture and Inoculation**

B. dendrobatidis was cultured using a 1% tryptone broth and agar plates. Plates were flooded with Bd culture stock one day before inoculation and incubated overnight at 4°C. The culture was then filtered using a 20 µm filter (MilliporeSigma, Burlington, MA, USA) and quantified. A concentration of 1.5×10^6 zoospores per mL was diluted in ultrapure water (Merck Millipore, Darmstadt, Germany) to achieve a final concentration of 3.33×10^4 zoospores per mL.

On day 0, following the acclimation period, frogs in the infected group were inoculated with the prepared zoospore suspension. Each frog was placed in a 2 oz Whirl-Pak (Nasco, Fort Atkinson, WI, USA) containing 3 mL of the Bd solution with an air pocket, resulting in a total exposure of 1×10^5 zoospores per frog via an immersion bath. Control frogs underwent the same procedure but were placed in sham immersion baths containing 3 mL of ultrapure water. Prior to immersion, frogs were thoroughly rinsed with RO water to remove any substrate from the skin. Frogs remained in the immersion baths for six hours before being returned to their enclosures. Frogs were monitored throughout immersion to ensure their pelvic

patches remained submerged and no adverse effects were observed during the immersion period.

***B. dendrobatidis* Confirmation via qPCR**

On day 14, all frogs were swabbed for quantitative polymerase chain reaction (qPCR) analysis to assess Bd infection load. Frogs were thoroughly rinsed with RO water prior to swabbing. Sterile rayon swabs with standard tips (MWE, Corsham, Wiltshire, England) were used to collect samples by swabbing 5 strokes each along the ventral pelvis, hind limbs, and feet for a total of 20 strokes. Swabs were placed in cryovials containing silica beads and kept on ice during transport. DNA extraction was performed using a DNeasy Powersoil HTP 96 Kit (Qiagen, Hilden, Germany) with a bead-beating step of 90 seconds on a Biospec 96 machine (BioSpec Products, Bartlesville, OK, USA). qPCR analysis was conducted using the Bio-Rad CFX96 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested in duplicate to confirm Bd infection. Any frogs testing negative on day 14 were swabbed on day 17 prior to treatment.

Itraconazole Treatment and Sample Collection

On day 17, all frogs underwent a 10-minute immersion treatment in 5 mL of 0.01% itraconazole using 2 oz Whirl-Paks as described above for the inoculation procedure. The immersion baths were prepared by diluting a 10 mg/mL itraconazole oral solution (Camber Pharmaceuticals, Piscataway, NJ, USA) with a 1:1 mixture of 0.9% saline (Abbott Laboratories, North Chicago, IL, USA) and carbon-filtered water.

Following treatment, frogs were euthanized at six predetermined time points (24, 48, 72, 144, 192, and 240 hours), with five control and five infected frogs euthanized at each interval. Euthanasia was performed via intracoelomic

administration of tricaine methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, MO, USA) prepared at a concentration of 5 g/L in a 1:1 mixture of 0.9% saline and carbon-filtered water and buffered with sodium bicarbonate to a neutral pH. A veterinarian confirmed death by the absence of response to stimuli and cessation of a visible heartbeat. Immediately post-mortem, tissues—including skin, liver, heart, lungs, kidneys, and gastrointestinal tract—were collected, placed in cryovials, and stored at -80°C prior to shipment for analysis of itraconazole concentration.

Itraconazole Concentration Analysis

For itraconazole concentration analysis, samples were weighed upon arrival, prior to homogenization in 1 mL of methanol using a TissueLyser II (Qiagen, Hilden, Germany) for two 2-minute cycles at 20 Hz. Homogenization was conducted in 2 mL microcentrifuge tubes containing Zr/Si beads (BioSpec, Cat. 11079125z, Bartlesville, OK, USA). After homogenization, samples were centrifuged at 17,000 g for one minute. The supernatant was collected and pellets were stored at -80°C. The homogenization process was repeated three times, and the combined supernatants were concentrated with a SpeedVac Concentrator (Thermo Fisher Scientific, Waltham, MA, USA). The concentrated samples were reconstituted in 300 µL of methanol containing 10 ppm caffeine (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard. Samples were then filtered through 0.22 µm PTFE membrane filters (MilliporeSigma, Burlington, MA, USA) into LC vials (Agilent Technologies, Santa Clara, CA, USA) before analysis.

The samples were analyzed using a Thermo TSQ-Quantis Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using a Waters BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) under the following conditions:

column temperature at 40°C, flow rate of 0.5 mL/min, solvent A composed of 2 mM ammonium acetate with 0.1% formic acid, and solvent B composed of 2 mM ammonium acetate with 0.1% formic acid in methanol. The liquid chromatography (LC) gradient program was as follows: 0–0.15 min at 2% solvent B, 0.15–0.3 min increasing from 2% to 99% solvent B, 0.3–1 min maintaining at 99% solvent B, 1–1.2 min decreasing from 99% to 2% solvent B, and 1.2–3 min maintaining at 2% solvent B.

Mass spectrometry conditions for itraconazole detection were as follows: electrospray ionization in positive mode (ESI+), retention time of 1.9 minutes, parent ion (m/z) of 705, daughter ion (m/z) of 392, and collision energy (CE) of 35 eV. The limit of quantification (LOQ) for itraconazole in tissues was 0.1 ng/mg.

Statistical Analysis

Descriptive statistics (mean and standard deviation) were calculated for tissue itraconazole concentrations at each time point for both groups (Bd⁻ or Bd⁺). Prior to analysis, outliers were identified and excluded based on visual inspection of the individual values and plotted data distributions. Values reported as zero were interpreted as below the LOQ and replaced with 0.05 ng/mg (LOQ/2) for statistical purposes. No inferential statistics were used; the primary objective was to describe itraconazole distribution and persistence over time.

RESULTS

Study Population and Bd Infection Confirmation

Frog body weights at the time of dissection ranged from 1.03 to 2.15 g. In the Bd⁻ group, the median weight was 1.30 g, with a first quartile (Q1) of 1.13 g, and a

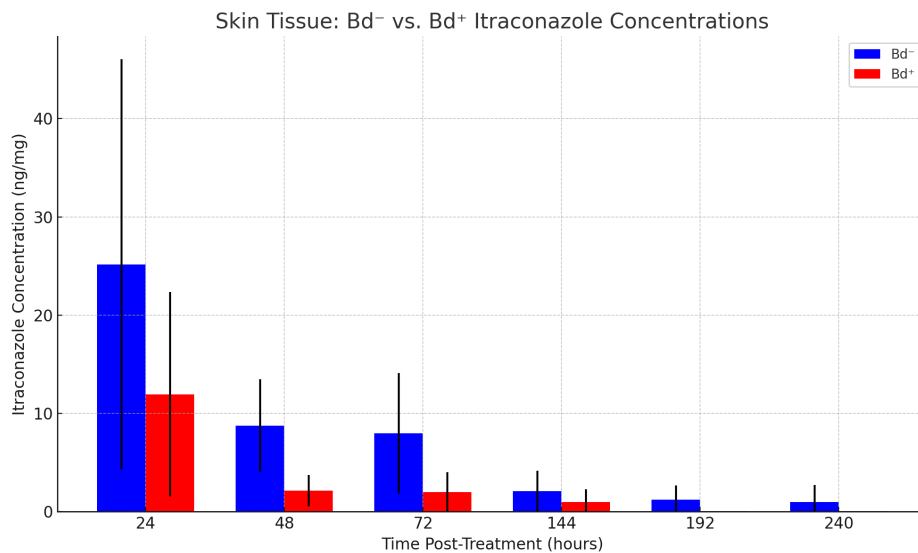
third quartile (Q3) of 1.50 g. In the Bd⁺ group, the median weight was 1.49 g (Q1: 1.32 g; Q3: 1.62 g). PCR testing confirmed Bd infection in 29 of 30 Bd⁺ frogs by day 14 post-inoculation; the remaining individual tested positive on day 17, immediately before itraconazole treatment.

Itraconazole Concentrations by Tissue

Skin

Skin peak concentrations occurred in both groups at 24 hours, with a higher mean value in the Bd⁻ group (25.17 ng/mg) than in Bd⁺ frogs (11.94 ng/mg). Concentrations for the Bd⁻ group were higher than those of the Bd⁺ group at each subsequent time point. In Bd⁺ frogs, concentrations fell below quantifiable limits (BQL) starting at 192 hours, whereas Bd⁻ frogs maintained detectable levels through 240 hours, with a mean of 0.96 ng/mg at that time point. Four outlier skin values were excluded at 72, 192, and 240 hours based on visual inspection.

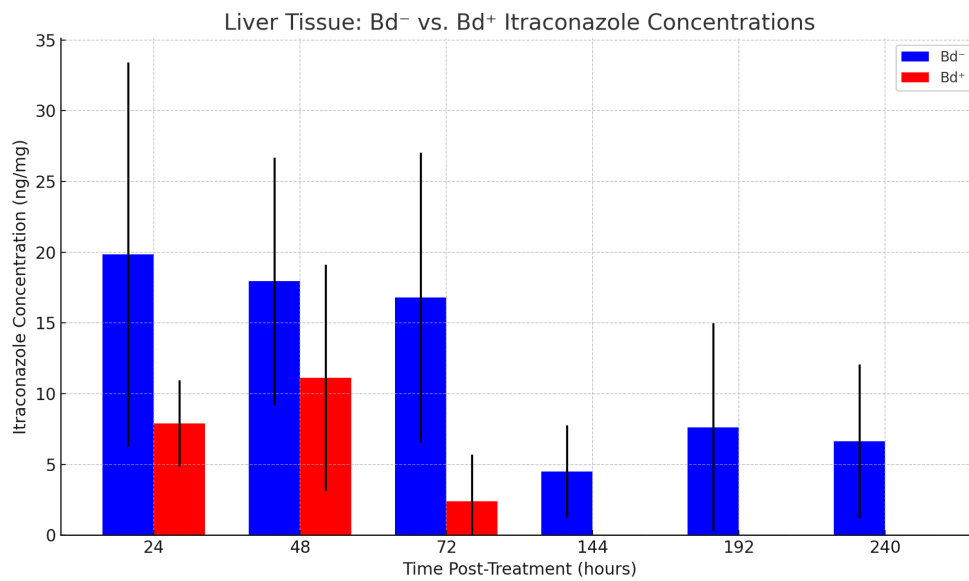
Figure 1: Mean itraconazole concentrations (\pm SD) in skin tissue samples



Liver

Liver peak concentrations occurred at 24 hours for the Bd⁻ group (19.86 ng/mg) and at 48 hours for the Bd⁺ group (11.11 ng/mg). Concentrations for the Bd⁻ group were higher than those of the Bd⁺ group at each time point. In Bd⁺ frogs, concentrations were BQL starting at 144 hours, whereas Bd⁻ frogs maintained detectable levels through 240 hours, with a mean of 6.63 ng/mg at that time point.

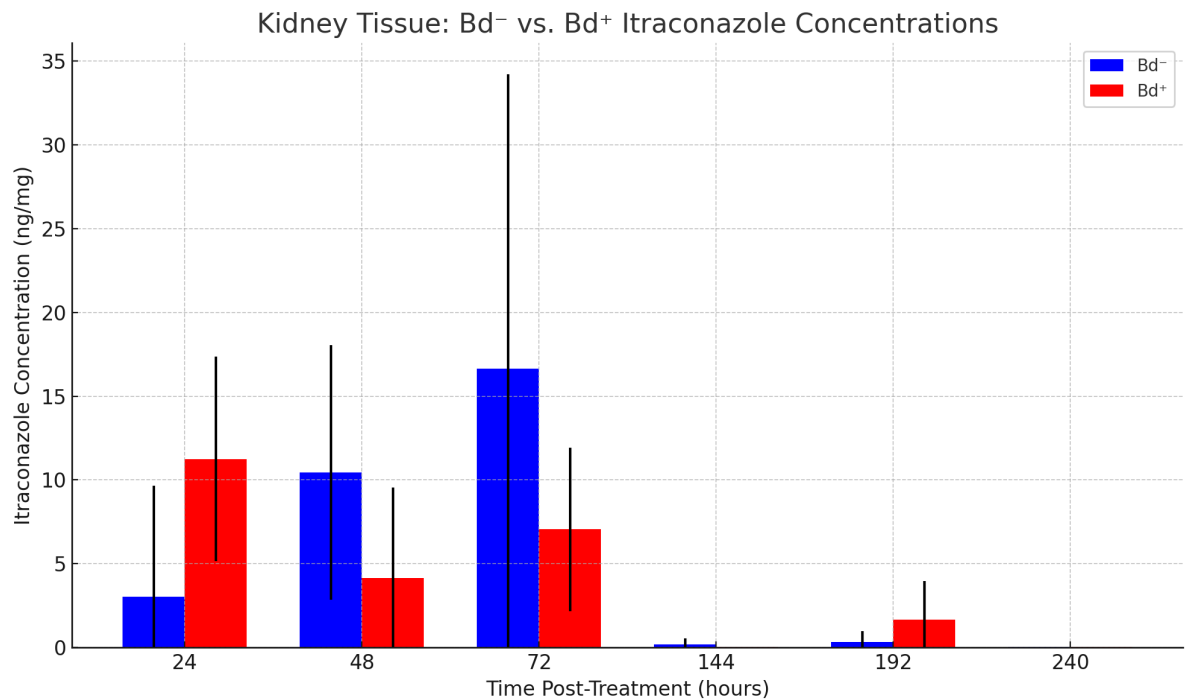
Figure 2: Mean itraconazole concentrations (\pm SD) in liver tissue samples



Kidney

Kidney peak concentration for the Bd⁻ group occurred at 72 hours (16.66 ng/mg) following a steady increase before and subsequent sharp decline to BQL at 240 hours. In Bd⁺ frogs, concentrations peaked at 24 hours at a lower value than the Bd⁻ peak (11.25 ng/mg). Bd⁺ frog values were BQL at 144 hours, rebounded at 192 hours (1.68 ng/mg), and returned to BQL at 240 hours.

Figure 3: Mean itraconazole concentrations (\pm SD) in kidney tissue samples



Gastrointestinal Tract

Gastrointestinal tract peak concentrations occurred in both groups at 24 hours, with a higher value in the Bd⁻ group (63.1 ng/mg) than in the Bd⁺ group (34.59 ng/mg). Concentrations for the Bd⁻ group were higher than those of the Bd⁺ group at each time point. Concentrations for this tissue remained above the LOQ at all time points in both groups.

Figure 4: Mean itraconazole concentrations (\pm SD) in GI tissue samples

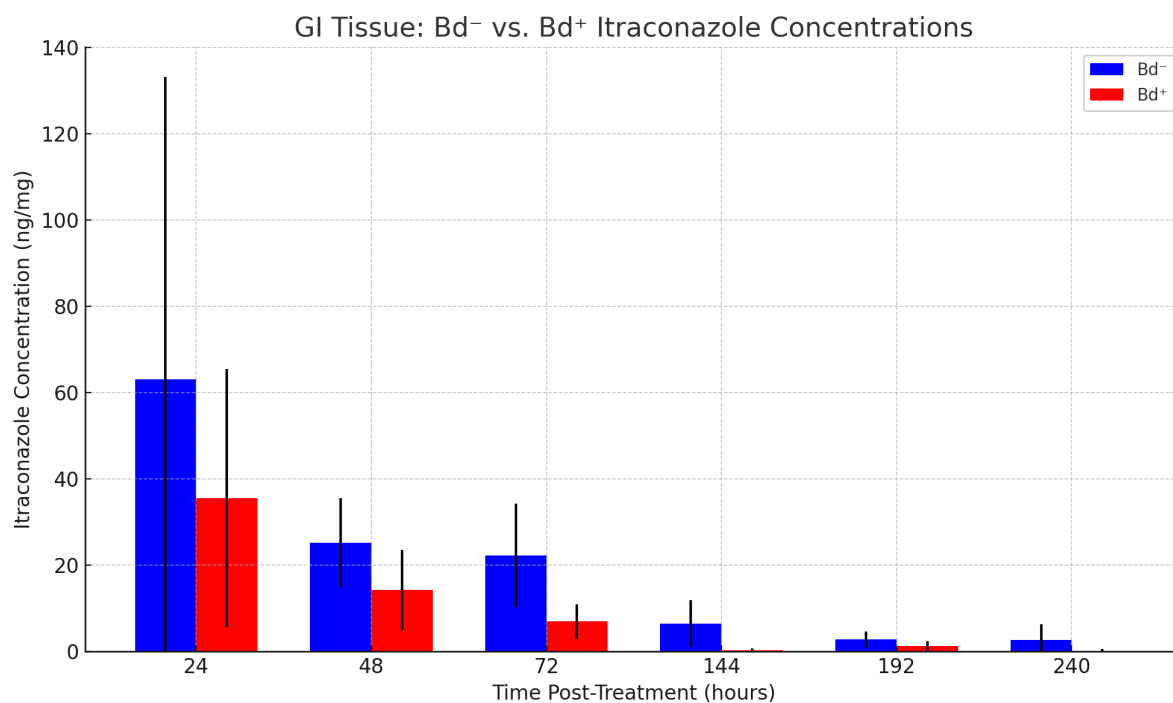


Figure 5: Mean itraconazole concentrations (\pm SD) in all tissue samples

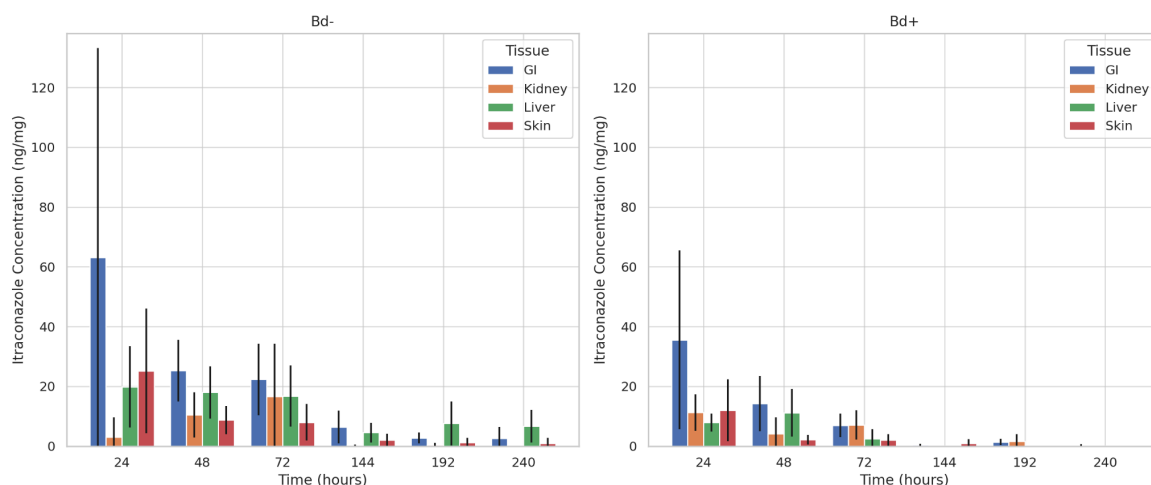


Table 1. Tissue concentrations of itraconazole over time in control (Bd^-) frogs

Time (hours)	Skin mean \pm SD (ng/mg)	Liver mean \pm SD (ng/mg)	Kidney mean \pm SD (ng/mg)	GI mean \pm SD (ng/mg)
24	25.17 \pm 20.87	19.86 \pm 13.57	3.02 \pm 6.63	63.1 \pm 70.05
48	8.74 \pm 4.71	17.94 \pm 8.76	10.44 \pm 7.59	25.23 \pm 10.34
72	7.97 \pm 6.14	16.79 \pm 10.25	16.66 \pm 17.55	22.29 \pm 12.04
144	2.07 \pm 2.11	4.49 \pm 3.26	0.2 \pm 0.33	6.4 \pm 5.46
192	1.21 \pm 1.46	7.62 \pm 7.34	0.34 \pm 0.65	2.75 \pm 1.82
240	0.96 \pm 1.73	6.63 \pm 5.43	0.05 \pm 0	2.61 \pm 3.7

Table 2: Tissue concentrations of itraconazole over time in infected (Bd^+) frogs

Time (hours)	Skin mean \pm SD (ng/mg)	Liver mean \pm SD (ng/mg)	Kidney mean \pm SD (ng/mg)	GI mean \pm SD (ng/mg)
24	11.94 \pm 10.39	7.88 \pm 3.05	11.25 \pm 6.1	34.59 \pm 29.91
48	2.14 \pm 1.6	11.11 \pm 7.99	4.13 \pm 5.42	14.23 \pm 9.24
72	1.97 \pm 2.06	2.4 \pm 3.3	7.05 \pm 4.88	6.97 \pm 3.95
144	0.98 \pm 1.28	0.05 \pm 0	0.05 \pm 0	0.33 \pm 0.41
192	0.05 \pm 0	0.05 \pm 0	1.68 \pm 2.27	1.35 \pm 1.05
240	0.05 \pm 0	0.05 \pm 0	0.05 \pm 0	0.23 \pm 0.41

DISCUSSION

Overview of Findings

This study investigated itraconazole tissue concentrations in *A. zeteki*, focusing on how infection with Bd may influence drug absorption, distribution, persistence, and clearance following a single 10-minute immersion bath at 0.01%. Itraconazole was detected in the skin, liver, kidney, and GI tissues, with concentrations declining over time and variations observed between control (Bd⁻) and infected (Bd⁺) tissues. Itraconazole concentrations were below the assay's LOQ (0.1 ng/mg) in multiple tissues and time points, highlighting the analytical sensitivity limits of the method used. In Bd⁻ frogs, measurable amounts of itraconazole persisted in most tissues throughout the 240-hour study period post-treatment, whereas in Bd⁺ frogs, drug concentrations declined more rapidly and often fell below the LOQ.

These findings align with previous research on itraconazole pharmacokinetics in amphibians and provide new insight into the influence of Bd infection on drug distribution and persistence. Consistent with Rifkin et al. (2017), rapid absorption and widespread tissue distribution of itraconazole in *A. zeteki* were observed, with peak concentrations occurring between 24 and 48 hours in most tissues. However, this study expands on those findings by demonstrating itraconazole persistence in tissues in both Bd⁻ and Bd⁺ frogs in an extended time frame. Notably, Bd⁺ frogs exhibited overall lower tissue concentrations and more rapid clearance from most tissues than their Bd⁻ counterparts, suggesting infection-induced changes in drug absorption or metabolism.

Tissue-Specific Considerations

Itraconazole is a highly lipophilic drug, and its tissue distribution is influenced by tissue composition, local pH, tissue permeability, metabolism, and excretion

pathways. The lipophilic nature of itraconazole facilitates absorption into lipid-rich tissues while limiting its penetration into tissues with higher water content or reduced lipid affinity. Due to its high affinity for keratinized tissues, itraconazole is particularly effective for treating fungal infections of the skin.¹⁰ The stratum corneum is lipid-rich, enabling itraconazole to penetrate and remain localized, due to its strong binding and long half-life.^{10,37–38} It is presumed that in amphibians, as in other vertebrates, the liver is the primary site for itraconazole metabolism via cytochrome P450 enzymes, leading to significant hepatic accumulation before clearance.³⁶ Itraconazole persistence in the GI tract may result from enterohepatic recycling, whereby drugs recirculate between the liver and intestines.^{2,12} Additionally, amphibians likely ingest shed skin containing itraconazole during natural behaviors, further contributing to drug accumulation in the GI tract.^{2,41} The kidneys serve a secondary role in itraconazole elimination, but some accumulation of metabolites or residual drug may occur via renal excretory pathways during systemic circulation and redistribution.³⁸ The high initial concentrations of itraconazole in the skin and liver following immersion treatment are consistent with previous studies, supporting the efficacy of transdermal administration.^{10,20,27,36}

Impact of Infection on Tissue Concentrations

The lower peak itraconazole concentrations observed in Bd⁺ frogs suggest that infection status may impact drug absorption, distribution, and clearance.

Bd-associated hyperkeratosis may impair transdermal absorption, as tissue thickening creates a physical barrier.^{2,13,41} Increased epidermal turnover associated with infection may lead to premature loss of itraconazole bound to keratinized tissues, reducing accumulation in other organs and contributing to lower, more variable tissue

concentrations.^{13,41} As itraconazole is administered transdermally, impaired skin absorption may also limit systemic distribution to other tissues.

Systemic infection with Bd may induce hepatic enzyme activity, causing accelerated metabolism and clearance of itraconazole.⁹ Bd-related immune and inflammatory responses may further alter hepatic and renal metabolism, potentially accelerating drug clearance and contributing to reduced tissue concentrations.^{12,18} Bd infection disrupts amphibian osmoregulation and electrolyte balance. Altered hydration status and ion transport may affect tissue permeability and drug distribution.^{15,41} Altered fluid distribution and plasma protein binding capacity in infected individuals may impact drug distribution and reduce tissue concentrations. Healthy frogs may exhibit more gradual systemic absorption of itraconazole over time than infected frogs. These findings support previous research suggesting that Bd infection alters skin permeability and may affect itraconazole bioavailability and tissue persistence.^{4,18,41}

Clinical Implications

The findings from this study have important practical and clinical implications for itraconazole use in treating amphibians infected with Bd. Understanding tissue-specific drug distribution and the influence of infection status with Bd can guide the development of optimized treatment protocols to enhance therapeutic efficacy and reduce the risk of toxicity in *A. zeteki*.^{6,25}

The observed persistence of itraconazole in tissues of both groups supports the feasibility of reduced dosing frequency or shorter treatment regimens compared to traditional protocols recommending daily immersion baths for 5-11 days.^{2,11,21} The

findings indicate that a single 10-minute 0.01% immersion dose may maintain inhibitory concentrations in critical tissues for several days, consistent with previous findings demonstrating extended itraconazole persistence above minimum inhibitory concentrations (MIC) for Bd in amphibian tissues.^{20–21,36} Reported MIC values for itraconazole range from 0.016-0.032 µg/mL, with the MIC for the Bd strain used in this study (JEL422) described as 0.018 µg/mL.⁴⁵ This can be converted to 0.018 ng/mg, assuming a tissue density of 1, which is below the LOQ for tissue concentration analysis.

Reducing the dosing frequency and treatment duration may decrease cumulative drug exposure, potentially minimizing itraconazole-related adverse effects such as hepatotoxicity, depigmentation, microbiome disruption, and stress from handling.^{12,26–27} While histopathology was not performed, no gross evidence of itraconazole toxicity—such as depigmentation or gross morphological abnormalities—was observed during sample collection. Achieving effective antifungal therapy while minimizing adverse effects requires further refinement of treatment protocols, including individualized dosing strategies based on infection status, frog size, and tissue-specific drug distribution.^{4,12}

Limitations and Future Directions

The small sample size at each time point may have increased variability in tissue concentration data and reduced statistical power. As *A. zeteki* are functionally extinct in the wild and maintained only in limited ex situ populations, increasing sample sizes for future research may be impractical or unfeasible. Manual removal of outliers may have introduced minor bias, though this approach was necessary to ensure an accurate representation of the data.

The diminutive size of the frogs posed an additional limitation, making anatomical dissection challenging—particularly for organs such as the kidney. This may have led to variability in sampling or resulted in partial or mixed tissue samples in some cases, potentially affecting the precision of itraconazole concentration measurements. One kidney sample (Bd⁺ group at 24-hour time point) could not be recovered. Tissues were weighed after freezing at -80°C for shipment, thawing, rinsing, and drying rather than at the time of dissection.

Physiological factors could have influenced drug metabolism, distribution, and absorption. Natural individual variations, including differences in body weights and metabolic rates, could influence drug absorption and clearance patterns. Frogs with higher Bd loads may have experienced more severe hydration imbalances and epidermal damage, potentially altering itraconazole absorption, distribution, and clearance.^{2,10,20,41}

The LOQ for tissue concentration analysis in this study may have limited the detection and interpretation of low-level drug concentrations, with itraconazole potentially persisting in tissues beyond the measured timeframes. As the LOQ exceeded the MIC for itraconazole, tissue concentrations may have remained at a therapeutically effective level beyond the final time point. Future studies would benefit from using more sensitive analytical equipment with a lower LOQ, allowing more accurate detection of low concentrations in tissues and enhancing the ability to determine the actual duration of drug levels above the MIC. As technology advances, future improvements in assay sensitivity may allow for even lower detection limits. Interpreting MICs for fungal pathogens presents additional challenges due to fungal growth variability and differences between in vitro and in vivo efficacy. In

amphibians infected with Bd, factors such as their unique skin structure, environmental conditions, and Bd life cycle may also affect drug efficacy.

The inability to directly assess extraction efficiency and percent recovery of itraconazole from tissue samples added another limitation to this study. Because *A. zetekii* is critically endangered, no surplus tissues could be collected or used for assay validation beyond those obtained from the study animals themselves. This restriction, while necessary for conservation reasons, may introduce some uncertainty regarding absolute tissue concentrations; however, all samples were processed consistently using established HPLC protocols.

Another limitation was the use of the LOQ/2 substitution method to estimate values BQL when calculating descriptive statistics. This approach, while appropriate for the dataset, introduces a degree of estimation and may not accurately reflect the true distribution of tissue concentrations, particularly when multiple values fall below the LOQ. While this method retains all data points and avoids bias from exclusion or censoring, it may underestimate variability and skew mean values, especially at later time points where BQL values were more frequent. Future research should consider advanced statistical methods for censored data, such as maximum likelihood estimation or Kaplan-Meier techniques, to improve analytical robustness.

Future studies should also refine antifungal protocols through population-based pharmacokinetic modeling to better predict itraconazole distribution and clearance patterns. Although overt itraconazole toxicity was not observed in this study, previous research has documented adverse effects—such as depigmentation, hepatotoxicity, and weight loss—in various amphibians receiving prolonged itraconazole treatment.²⁰ Given these risks, future work should prioritize treatment regimens that balance efficacy while minimizing adverse effects.

Key Findings

This study provides novel insights into itraconazole tissue concentrations in *A. zeteki*, highlighting potential Bd-induced alterations in drug absorption, distribution, and clearance, as well as prolonged persistence of itraconazole in tissues. These findings emphasize the need to optimize treatment protocols for clearing Bd infections and to expand research on antifungal pharmacokinetics in amphibians. Based on tissue concentration data from Bd⁺ frogs, itraconazole 0.01% immersion baths administered every 72 hours may be therapeutically effective when treating Bd infections in *A. zeteki*. Optimizing antifungal protocols could improve Bd treatment success in captive populations of many amphibian species and enhance the success of reintroduction efforts.²³

CONCLUSION

As chytridiomycosis continues to drive amphibian declines worldwide, understanding antifungal treatment dynamics in vulnerable species is critical to conservation success. This study advances the understanding of itraconazole tissue concentrations in the critically endangered Panamanian golden frog (*A. zeteki*), a species highly susceptible to Bd infection. Comparison of tissue concentrations between Bd-infected and uninfected frogs after a single 10-minute exposure to 0.01% itraconazole revealed distinct patterns of drug distribution and persistence that appear closely tied to infection status. Itraconazole remained above the LOQ in most tissues of uninfected frogs throughout the 240-hour observation period, while infected frogs exhibited lower peak concentrations and faster drug clearance, likely reflecting Bd-associated physiological disruptions.

These findings have direct implications for conservation breeding programs, where daily antifungal treatments can increase physiological stress, toxicity risk, and logistical challenges. The observed persistence of itraconazole above minimum inhibitory concentrations for several days after a single 0.01% immersion dose in both Bd-infected and uninfected frogs suggests that reduced-frequency protocols may remain effective while improving both animal welfare and treatment outcomes. Such strategies are particularly valuable in managing endangered populations, where the health and survival of each individual are critical. Future research should build upon these findings by evaluating the clinical efficacy of reduced-frequency and shorter-duration itraconazole treatment protocols. More sensitive analytical techniques and outcome-based assessments will help refine protocols to balance efficacy, safety, and practicality. As reintroduction efforts for *A. zeteki* and other amphibian species progress, optimizing antifungal protocols will be essential to support long-term survival and population resilience.

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