

## Case Study

# Treatment of Chytridiomycosis in Laboratory Axolotls (*Ambystoma mexicanum*) and Rough-skinned Newts (*Taricha granulosa*)

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Chytridiomycosis is an infectious disease of amphibians caused by the fungal species *Batrachochytrium dendrobatidis* and *B. salamandrivorans* and has been implicated in the population decline of amphibian species worldwide. This case report describes a successful treatment protocol for chytridiomycosis in laboratory-maintained colonies of axolotls (*Ambystoma mexicanum*) and rough-skinned newts (*Taricha granulosa*). Over 12 mo, axolotls ( $n = 12$ ) in a laboratory-reared colony developed multifocal erythematous dermatitis, mainly on the distal limbs and tails. Wild-caught newts handled by the same lab personnel were housed in an adjacent room and occasionally presented with abdominal distension and lethargy. Differentials included poor water quality, pathogen infection, parasitic infestation, and trauma. Antibiotic treatment of animals according to results of bacterial culture and sensitivity, combined with bleach disinfection of aquaria, did not resolve clinical signs. Skin swabs from clinically affected axolotls submitted for a newly available commercial screen were positive for *B. dendrobatidis*. Additional PCR and sequencing analysis revealed chytrid-positive animals among group-housed newts in 2 clinically unaffected aquaria and suspected PCR-positives for 2 affected newt aquaria and an additional axolotl. Axolotls with skin lesions ( $n = 2$ ) and newts with abdominal distension and lethargy ( $n = 2$ ) underwent experimental treatment with itraconazole submersion (0.002% to 0.0025%; 5 min daily for 10 d). This pilot treatment was well tolerated and led to clinical resolution. Subsequent itraconazole treatment of the entire colony led to regrowth of extremities and restoration of normal coloration among axolotls. During treatment, the facility was decontaminated, and additional biosecurity measures were developed. PCR results after the pilot treatment and subsequent full-colony treatments (at 1 wk, 1 mo, and 6 mo after treatment) were negative for the presence of *B. dendrobatidis*. Because chytridiomycosis is a reportable animal disease in our state, colonies officially remained quarantined until negative PCR results were obtained at least 6 mo after treatment.

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*Batrachochytrium dendrobatidis* is a fungal pathogen of amphibians that originated in Asia, likely on the Korean peninsula,<sup>27</sup> and is now found on all continents that are home to amphibians.<sup>20</sup> Initial infection of an amphibian host is accomplished by motile, flagellated zoospores. These zoospores encyst within epidermal cells, germinate, and form intracellular sporangia, in which new zoospores develop, within the stratum corneum and stratum granulosum. Epidermal differentiation moves these sporangia to the skin surface, and mature zoospores are released into the external environment.<sup>34</sup> This intracellular proliferation of *B. dendrobatidis* within the host disrupts electrolyte balance and, in some cases, can lead to cardiac arrest and death.<sup>36</sup> Transmission of *B. dendrobatidis* between hosts occurs through a variety of methods, including close proximity to infected animals, which can shed considerable loads of infective zoospores into water systems; through direct contact, such as during mating;<sup>34</sup> and possibly through ingestion of infected prey.<sup>15</sup>

*B. dendrobatidis* infects all 3 orders of amphibians (anurans [that is, frogs and toads]; urodeles [salamanders]; and

caecilians)<sup>14</sup> and has been reported in more than 700 species of amphibians.<sup>20</sup> The spread of *B. dendrobatidis* has been linked to an overall global decline in amphibian populations, and this pathogen has contributed to population declines in almost 400 amphibian species that are currently listed as threatened.<sup>20</sup> The virulence of *B. dendrobatidis* infections varies among both host species and strains of the pathogen.<sup>2,20</sup> *B. dendrobatidis* has been shown to infect *Taricha granulosa* in the wild<sup>29</sup> and has been reported in captive-bred axolotls.<sup>11,12,23</sup> In addition, *B. dendrobatidis* can infect larval zebrafish,<sup>19</sup> creating potential concerns for spread between aquatic animal models when an outbreak is detected within a laboratory setting.

A second member of the genus, *B. salamandrivorans*, was first described in 2013.<sup>21</sup> Like *B. dendrobatidis*, *B. salamandrivorans* appears to have originated in Asia<sup>27</sup> and infects epidermal tissue.<sup>21</sup> To date, *B. salamandrivorans* has been reported in salamanders and fire-bellied toads in the wild and has been shown to be capable of infecting anurans in the laboratory setting.<sup>26,32</sup> No reports of *B. salamandrivorans* infection in North America have been published. These 2 *Batrachochytrium* species are the only chytrid fungi known to infect vertebrates.<sup>2</sup>

The current case report describes the diagnosis and treatment of chytridiomycosis due to *B. dendrobatidis* in laboratory colonies of axolotls (*Ambystoma mexicanum*) and rough-skinned newts

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(*Taricha granulosa*; hereafter referred to as 'newts'). Axolotls are fully aquatic salamanders that have been bred in captivity for more than 150 y; axolotls are used mainly as model organisms for developmental biology and regeneration research.<sup>1</sup> Adult newts are semiaquatic and are model organisms for comparative neuroendocrinology research<sup>25</sup> as well as key contributors in classic long-term studies of predator-prey interactions.<sup>7</sup> Both amphibian species in the current report are used to study neurobiology and behavior of chemical communication and olfaction, as well as studies of skin peptides and toxins and their effects on the nervous system. The laboratory maintains populations of approximately 50 animals of each species; the axolotls are all captive-bred, and the newts are wild-caught. All animal use protocols were approved by the Michigan State University IACUC.

## Case Report

In late March 2017, about 12 animals in this laboratory colony of approximately 50 axolotls were reported for veterinary evaluation due to multifocal patches of erythematous dermatitis located mainly on the distal limbs, digits, and tails. Additional clinical signs included pale tissue originating at the tail tip and extending cranially along the lateral line, erythematous gills, generalized dulling of pigmentation, and sloughing, necrotic skin and ulcerations associated with the areas of dermatitis (Figure 1). Axolotls with lesions were housed in aquaria within the laboratory-maintained animal facility, with apparently unaffected animals cohoused in aquaria where those with lesions were found.

In general, the axolotls are purchased from the Ambystoma Genetic Stock Center (University of Kentucky, Lexington, KY), but transfers of adults from other researchers are accepted occasionally. The axolotls are maintained in groups of a maximum of 5 animals, in 3 rows of 30-gal aquaria, with animals segregated by sex and size (Figure 2). Each row constitutes a separate recirculating system. Within a row, the aquaria are refilled from an overhead reservoir; water from the surface of each aquarium drains through a mechanical filter to a sump, where the water is pumped across a biologic filter and passed through a UV sterilizer before being returned to the reservoir. Detritus (food particles, fecal material, and other debris) is siphoned from aquaria and additional water is removed to ensure that a minimum of 10% of the total water volume within each row is replaced daily. Each aquarium contains sections of PVC pipe (length, 6 to 8 in.) as enrichment devices to provide shelters (that is, 'hides'). Axolotls are maintained in 100% Holtfreter solution,<sup>1</sup> which contains 60 mM NaCl, 2.4 mM NaHCO<sub>3</sub>, 0.67 mM KCl, 0.81 mM MgSO<sub>4</sub>, and 0.68 mM CaCl<sub>2</sub> in reverse-osmosis-treated water (pH 7.5); adults each are fed 4 or 5 salmon pellets (Rangen, Buhl, ID) 3 times weekly. Axolotls with signs of illness may be removed from the aquarium system and housed in individual 4-L bowls filled with Holtfreter solution for periods of observation and treatment. The light cycle in the room is changed at the beginning of each month to match that of the animals' native habitat in Mexico City, and the temperature is 20 ± 1 °C year-round. The water chemistry in these tanks is tested weekly to verify that circulating ammonia levels are lower than 0.25 ppm.

Adult male newts were wild-caught from ponds and streams in Oregon and Idaho under permits from the Oregon Department of Fish and Wildlife and Idaho Department of Fish and Game. Newts are maintained in a separate room within the same animal housing facility as the axolotls. Approximately 50 newts are housed in groups of a maximum of 5 animals from the same capture location in 10-gal aquaria containing sponge filters as well as floating platforms that allow the animals to



**Figure 1.** (A) Clinically healthy axolotl with normal coloration. (B) Clinically affected axolotls with generalized dulling of coloration (left) and sloughing of tail tip (right) segregated into individual housing for pilot itraconazole submersion treatment.



**Figure 2.** Standard housing—1 of 3 rows of aquaria—for captive-bred axolotls.

crawl out of the water (Figure 3). Newts are maintained in a very low-salinity solution that mimics the composition of Replenish (Seachem, Madison, GA) and contains 302 µM CaCl<sub>2</sub>, 30.2 µM NaCl, 14.5 µM MgCl<sub>2</sub>, and 4.0 µM KCl in RO water





Figure 3. Standard aquarium housing for wild-caught newts.

(pH 6.5). Detritus is siphoned from aquaria daily and additional water removed to ensure that a minimum of 10% of the total water volume within each aquarium is replaced daily. In general, the newts are fed live blackworms (*Lumbriculus variegatus*) until satiety 3 times weekly but occasionally receive thawed bloodworms (chironomid larvae) from previously frozen stock instead. At the time of reporting of the axolotls with skin lesions, approximately 10 newts had been separated into 'quarantine' aquaria located apart from the main housing racks; signs of illness included bloated appearance, lethargy, erythematous ventral limb surfaces, and multifocal, raised white patches over the skin surface (Figure 4). The newts are maintained on a 14:10-h light:dark cycle at  $19 \pm 1^\circ\text{C}$  year-round. A room entry order is in place: handling of laboratory-reared axolotls occurs prior to wild-caught newts, and care of healthy animals is completed before handling those identified for possible illness.

Prior to the report of these cases, in the row of aquaria housing male axolotls, approximately 25% of the animals showed signs of multifocal erythematous dermatitis and the row was treated with tetracycline (1.5 mg/liter) for 10 d in spring 2017. In addition, quarantined newts were treated with Furan-2 (Aquarium Pharmaceuticals, Chalfont, PA) in their aquarium water (2.25 mg/liter) for 8 d, and Indian almond (*Terminalia catappa*) leaves were added to their aquaria around the same time period. Mild clinical improvement was seen during treatment of both species, but complete lesion resolution was not achieved. After veterinary assessment, initial differential diagnoses included complications with water quality, bacterial infection (*Aeromonas* spp., *Pseudomonas* spp., and other species associated with red-leg syndrome; *Mycobacterium* spp.), fungal infection (chytridiomycosis, saprolegniasis), parasitic infestation, and trauma.



Figure 4. Quarantine housing for newts with clinical abnormalities, including abdominal distension.

For mineral analysis and aerobic bacterial culture at the Michigan State University Veterinary Diagnostic Laboratory, water samples were collected from the main tap used for the axolotl aquarium system and 4 axolotl-housing aquaria, with care to select aquaria both with and without clinically affected animals and encompassing all 3 rows of aquaria. No abnormalities were noted on mineral analysis (Al, Sb, As, Ba, Cd, Cr, Cu, Fe, Pb, Mn, Hg, P, Se, Na, S, Tl, and Zn). Bacterial culture yielded no growth from the main tap sample, and *Pseudomonas* spp. (not identified as *P. aeruginosa*) was cultured from 2 aquaria housing clinically unaffected animals. *Pseudomonas* spp. and *Shewanella* spp. were each cultured from a single aquarium containing axolotls with dermatitis. For fungal and aerobic bacterial general culture, skin swabs were collected from the digital lesion of an affected adult male axolotl. This swab yielded no growth of fungus, whereas a few *Shewanella putrefaciens*, few *Pseudomonas aeruginosa*, and rare *Aerococcus viridans* were cultured.

Given the susceptibility results for *S. putrefaciens* and *P. aeruginosa* cultured from this initial axolotl, pilot treatment with enrofloxacin (10 mg/kg IM daily for 7 d; Baytril, Bayer, Shawnee Mission, KS) was started in one clinically affected adult male axolotl in May 2017. Two days after the start of this treatment, an adult female axolotl was found dead and submitted for full necropsy. This animal was described by lab personnel as lethargic with an erythematous area along the ventral surface of the right forelimb for a few days prior to death. Histologic evaluation of the skin noted heterophilic and lymphocytic dermatitis with epidermal hyperplasia and erosion. Evaluation of the gills was similar, noting heterophilic and lymphocytic bronchitis with epithelial hyperplasia. A final diagnosis of red-leg syndrome, due to the growth of enrofloxacin-susceptible *Aeromonas hydrophila* after bacterial culture of samples from skin, liver, and small intestine, was provided.

In an attempt to remove pathogenic organisms from the complex plumbing systems within each axolotl housing row, the axolotls were removed to individual bowls for 10 d in June 2017 and the aquaria were disinfected by adding bleach to create a 5% solution and allowing the bleach solution to circulate for 24 h. The bleach solution was then drained, and the aquaria and PVC hides were scrubbed and rinsed with tap water twice. Aquaria were then filled with RO water to circulate overnight, drained again, allowed to dry for 24 h, and then refilled with Holtfreter solution before the animals were returned to their home aquaria.

This cleaning protocol did not prevent further cases of dermatitis in the axolotl colony, and enrofloxacin treatment was instituted in 4 additional animals over the next 6 mo. All enrofloxacin-treated axolotls were individually housed in bowls for the duration of administration. Moderate resolution, particularly of distal limb erythema and skin sloughing, was seen during the course of antibiotic administration; however, these clinical improvements were not sustainable, and lesions gradually returned over weeks to months once treatment was discontinued.

During this time, one adult female axolotl and one adult male newt were found dead and submitted for necropsy. The female axolotl had no overt clinical signs prior to death, whereas the newt had been relocated into a quarantine aquarium, due to a 2-wk history of abdominal distension, decreased feed intake, and weight loss. Gross assessment of the axolotl revealed a mild, focal area of hemorrhage over the gills. Histologic examination demonstrated mild chronic heterophilic bronchitis and mild chronic-active heterophilic and ulcerative dermatitis across multiple skin sections. Aerobic bacterial culture was positive for numerous *A. hydrophila* from samples of gill, skin, and liver, with a final diagnosis of septicemia due to *A. hydrophila*. The only abnormal finding on gross necropsy of the newt was hepatomegaly, whereas histology revealed systemic inflammation throughout the subcutaneous tissues, musculature, coelom, and viscera, including multifocal hepatic necrosis and severe multifocal lymphohistiocytic cellulitis across subcuticular sections. Similar to previous culture results, aerobic bacterial culture of the liver found rare occasions of *S. putrefaciens*, *P. aeruginosa*, and *Aeromonas* spp. suggesting bacterial septicemia. Susceptibility results for both cases continued to demonstrate enrofloxacin susceptibility among the cultured bacterial species.

In late 2017, IDEXX Bioresearch (now IDEXX BioAnalytics, Columbia, MO) debuted diagnostic testing for axolotls. According to instructions from IDEXX, in January 2018 skin swabs were collected from 2 adult male axolotls that had previously undergone enrofloxacin treatment and continued to show clinical signs and were submitted for testing using both the Axolotl Microbiology Panel and Axolotl Comprehensive PCR Panel. Cultures for *Aeromonas dhakensis*, *A. hydrophila*, *Flavobacterium columnare*, *P. aeruginosa*, *Salmonella enterica*, *Saprolegnia*, and *Serratia marcescens* were all negative, as were PCR tests for *Batrachochytrium salamandrivorans*, *Flavobacterium columnare*, *Mycobacterium marinum*, *Piscinoodinium pillulare*, *Ranavirus*, and *Salmonella*. The PCR test for *B. dendrobatidis* yielded positive results for both animals. Confirmatory testing was conducted by the Michigan State University Veterinary Diagnostic Laboratory using standard PCR procedures followed by DNA sequencing for confirmation; a real-time PCR assay for the detection of *B. dendrobatidis* in amphibian samples has been described previously.<sup>3</sup> Swabs from 2 adult male and 2 adult female axolotls, selected to include animals with and without clinical signs, were submitted. These axolotls all tested negative, although one female axolotl was considered 'suspect' for *B. dendrobatidis*, given that the PCR test produced a band of the correct size, but subsequent sequencing did not confirm *B. dendrobatidis*. In addition, swabs from 5 newts, encompassing 2 capture locations and each of 3 active quarantine aquaria, were submitted. Both samples from apparently healthy newts, caught in different locations, were positive on both PCR testing and sequencing analysis. Samples from the quarantine aquaria produced 2 suspected positive PCR results and 1 negative result.

The Michigan Department of Agriculture and Rural Development classifies *B. dendrobatidis* as a regulated disease, and

reporting of regulated diseases to the Department should occur within 24 h of diagnosis; the possible responses from this department include education and outreach, quarantine, and animal removal. The main goal of this reportable classification is to inhibit the spread of chytridiomycosis.<sup>24</sup> Therefore, the positive results were reported, and because of the considerable value of these captive salamander colonies to research efforts, a treatment protocol was discussed, and quarantine was instituted, in which treatment attempts were permitted to take place. Stipulations of the quarantine included confinement of all axolotls and newts to the premises of the housing facility, with permission for animals to be transferred for terminal procedures to laboratory space within the same building. Any newly acquired amphibians were to be quarantined and housed in separate aquaria, with appropriate biosecurity to prevent transmission of *B. dendrobatidis* between current colony animals and new arrivals. Finally, the quarantine was to remain in effect until both colonies tested negative on 3 consecutive PCR tests over a period of 6 mo, or until all animals with clinical signs or those testing positive were euthanized. If complete eradication of *B. dendrobatidis* from the facility was not possible, then colonies were to remain under strict quarantine in the locations specified earlier.

Itraconazole submersion is suggested as a potential treatment for chytridiomycosis in amphibians;<sup>16,38</sup> however, most studies investigating this treatment method have involved anurans. We chose to use a reduced concentration of itraconazole delivered over 10 d, in light of previously demonstrated efficacy in a frog species.<sup>4</sup> Prior to whole-colony treatment, pilot treatment of a small subset of animals was conducted to assess for potential efficacy and any unanticipated adverse effects.

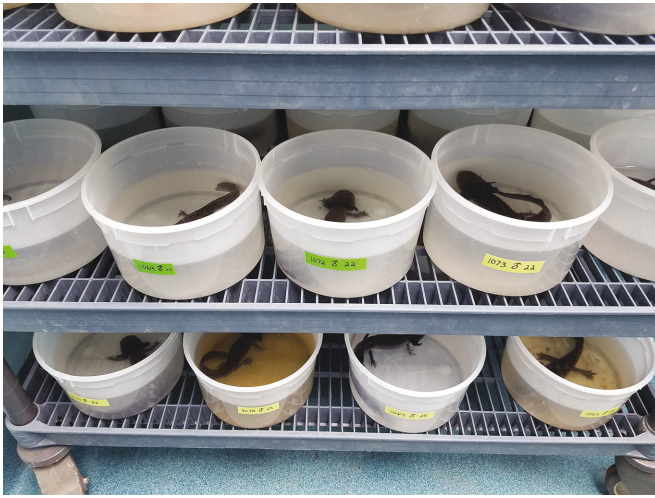
## Materials and Methods

All animals were rehoused and fasted during treatment. Axolotls were individually housed in white plastic bowls each containing approximately 2 L Holtfreter solution (Figure 5). Newts from each aquarium were housed according to groups in plastic shoeboxes lined with damp paper towels and provided with small plastic hides (Figure 6). After treatment but before return to their aquaria, axolotls were fed salmon pellets twice weekly, and newts were fed crickets 3 times weekly.

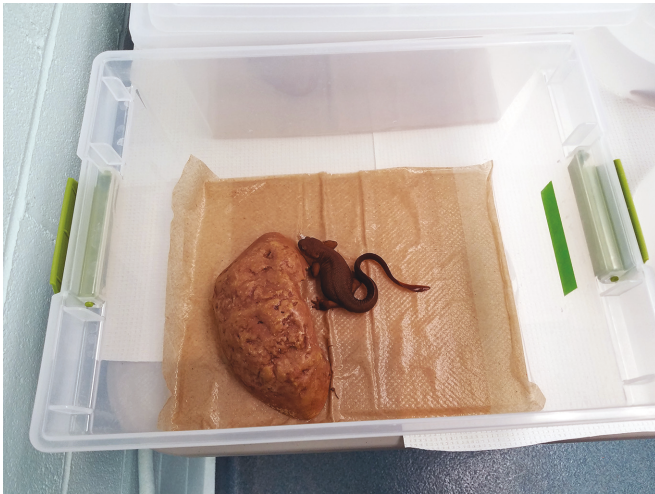
All animals were treated with a 0.002% to 0.0025% solution of itraconazole (Itrafungol, Elanco, Greenfield IN) for 5 min each day for 10 d. Specifically, itraconazole was diluted in Holtfreter solution (axolotls) or the minimal salt solution described earlier (newts) and shaken vigorously. The itraconazole solution was then poured into small plastic boxes with clip-on lids to a depth of 5 to 8 cm. Axolotls were treated individually; newts were treated in the same groups in which they were housed. After the animals were placed in a box and the lid sealed, the box was placed on a laboratory rotator at a gentle setting for the duration of the bath to ensure that the animals were thoroughly coated with the antifungal solution. After each submersion, animals were placed in clean, disinfected bowls (axolotls) or boxes (newts). Gloves were changed before handling each animal (axolotls) or group of animals (newts) both before and after treatment each day. Gloves, bench paper, and paper towels used in treatment were collected as hazardous waste and sent for incineration. Treatment steps were repeated at approximately 24-h intervals for 10 consecutive days.

In addition to treatment of the animals, the facility in which the animals were housed was decontaminated. *B. dendrobatidis* thrives at cool temperatures and can be killed by heat, desiccation, ethanol, bleach, and Virkon S (Lanxess, Pittsburgh, PA).<sup>17,32,35</sup> After axolotls were moved to individual bowls, the





**Figure 5.** Individual housing of axolotls during facility disinfection and itraconazole treatment. After each daily itraconazole submersion, axolotls were transferred to clean, disinfected bowls containing fresh Holtfreter solution.



**Figure 6.** A newt with severe abdominal distension in terrestrial housing for itraconazole treatment. After each daily treatment, newts were transferred to clean, disinfected terrestrial habitats. This animal was singly housed for pilot treatment; during full colony treatment, newts were socially housed according to their previous aquarium grouping.

aquarium system was disinfected by using bleach as described earlier. All mechanical and biologic filtration devices and all nets used to catch animals from aquaria were air-dried and then discarded and replaced. After newts were moved to boxes, their aquaria and lids were drained, scrubbed, rinsed, liberally sprayed with 70% ethanol, and left to dry, as was the aquarium that housed the feeder blackworms. All sponge filters, tubing, air stones, air pumps, and floating platforms were air-dried and then discarded and replaced. The barrels in which water solutions are made and stored were disinfected with bleach, rinsed with tap water and then reverse-osmosis-treated water, and left to dry.

To assess the efficacy of the itraconazole submersion protocol, skin swabs were collected from treated animals at 7 d, 28 d, and 6 mo after the last day of treatment and submitted for *B. dendrobatidis* PCR analysis. Sterile, synthetic culture swabs (BBL CultureSwab, Becton Dickinson, Sparks, MD) were repeatedly passed over the dorsum, lateral sides, ventrum, and any areas with apparent skin lesions (at least 30 passes over the skin) of each tested animal. Animals enrolled in the pilot treatment

group were swabbed individually. For testing after full-colony treatment, individual swabs collected from axolotls and newts were pooled, because a recent study shows that pooling cutaneous swabs (maximum, 4) does not reduce the efficacy of detecting chytrid spores.<sup>31</sup> Swabs were pooled by housing room row (axolotls) or capture location (newts) in additive-free, red-top blood collection tubes (Monoject, Covidien, Mansfield, MA); 1 mL of sterile 0.9% NaCl (Hospira, Lake Forest, IL) was added to each tube to maintain swab moisture, as recommended by the Michigan State University bacteriology laboratory. All PCR samples were transported in insulated coolers to the testing laboratory immediately after collection.

## Results

We first carried out a pilot itraconazole treatment regimen with 2 adult axolotls displaying skin lesions and 2 newts with abdominal distension and lethargy. These animals included one adult male axolotl and 2 newts that tested positive for *B. dendrobatidis* on PCR analysis and one adult female axolotl with a potentially positive PCR result. Follow-up testing at 7 and 28 d after the last day of treatment produced negative results for all animals in the pilot group.

When the treatment was repeated for the entire colony, we again tested animals at 7 d, 28 d, and 6 mo after the end of treatment. Specifically, 7 d after the end of treatment, 3 or 4 environmental locations in and around the sumps for each row of aquaria in the axolotl facility were swabbed and pooled for each row; swabs from 4 adult male axolotls, 4 adult female axolotls, and 4 juvenile axolotls were pooled; and swabs from each newt in 3 aquaria that originated in 3 separate locales were pooled as well. In total, 3 pooled axolotl (4 axolotls per sample), 3 pooled newt (3 to 5 newts per sample), and 3 pooled environmental (3 or 4 locations per sample) samples were submitted at 7 d after treatment. All tests were negative for *B. dendrobatidis*. Follow-up samples of the same animals collected at 28 d and 6 mo after treatment were also negative. Furthermore, samples submitted from 2 adult male axolotls and one adult newt for the IDEXX Comprehensive PCR Panel at 6 wk after full-colony treatment were negative for *B. dendrobatidis*.

Axolotls did not display any overt signs of distress (such as escape behaviors or changes in swimming ability) during itraconazole submersion periods, nor did they show any novel clinical signs over the course of treatment. In contrast, the newts frequently tried to climb out of the treatment solution, and some animals in the full colony treatment group presented with lethargy and closed eyes beginning on the 3rd to 5th day of treatment and continuing through the remaining treatment period. During the month after treatment, 5 to 7 newts developed multiple spots of dark coloration on their skin that resolved within 2 wk without intervention. In addition, 2 of 46 axolotls died during the 10 d of treatment, whereas all 48 newts that were treated survived until the end of the testing period. Before the 10 d of treatment was complete, all axolotls showed at least some resolution of clinical signs, with regrowth of tissue on the digits and tail and restoration of normal coloration. Full-colony testing results from 7 d, 28 d, and 6 mo posttreatment were provided to the Michigan Department of Agriculture and Rural Development; after their review of these results, both colonies were officially released from quarantine, with all movement restrictions lifted.

## Discussion

This case report describes the successful treatment of *B. dendrobatidis* in laboratory colonies of *A. mexicanum* and *T. granulosa*

by using an itraconazole submersion protocol. Itraconazole acts as an antifungal through selective binding to cytochrome P450 isoenzymes, which are involved in many physiologic functions<sup>8</sup> in *B. dendrobatidis*. Binding of itraconazole to these enzymes is irreversible, ultimately resulting in structural degeneration of the fungi through enzymatic dysfunction and increases in membrane permeability.

Treatment of chytridiomycosis in amphibians with itraconazole submersion has been documented previously.<sup>10,29</sup> These studies have mainly been conducted in anurans, and the concentration of itraconazole used most often is 0.01%. A treatment period of 11 d is often recommended, presumably in light of prior administration guidelines for mammals. However, decreased concentrations and durations have demonstrated efficacy in some species of frogs.<sup>4,5</sup> In our current study, a treatment period of 10 d was adequate for resolution of chytridiomycosis as evaluated by PCR analysis at as long as 6 mo after treatment. However, this 10-d protocol was not completely innocuous for the newts, given that lethargy and closed eyes were observed in a subset of newts beginning on days 3 to 5 of treatment and continuing until treatment concluded. These signs resolved fully after completion of the treatment protocol. Further investigation is needed to determine whether shorter treatment durations at 0.002% to 0.0025% itraconazole would reduce adverse effects among *T. granulosa* yet remain effective for the treatment of *B. dendrobatidis* in laboratory settings.

Agent pharmacokinetics should also be considered when selecting treatment protocols for mycoses. Itraconazole is metabolized by the liver and has been associated with increases in liver enzymes and rare instances of hepatotoxicity in mammals. Treatment of Majorcan midwife toad tadpoles (*Alytes muletensis*) with itraconazole, although effective in eliminating *B. dendrobatidis*, also resulted in whole-body depigmentation. A possible hepatic effect of itraconazole was suggested, given that melanin is produced in many amphibian cells, including Kupffer cells within the liver.<sup>13</sup> In our case study, a contrasting effect was seen with itraconazole treatment: axolotls demonstrated a return to normal coloration as compared with the generalized dulling and focal loss of pigmentation observed prior to treatment. In addition, some newts developed patches of dark coloration in the month after treatment. These changes in coloration among newts resolved without intervention, but may represent a delayed, self-limiting effect of itraconazole on melanin production. Therefore, selection of itraconazole for use in amphibians should involve considerations of life stage and animal model; specifically, itraconazole may not be the antifungal of choice for models with hepatic impairment or otherwise evaluating hepatic function or melanin production.

High mortality rates have occasionally been reported after itraconazole treatment of amphibians. These rates seem to vary with species, life stage, the extent of initial infection, and treatment dosage and duration. For example, one study<sup>39</sup> tested a variety of treatments for *B. dendrobatidis* infection in 5 species of frogs at different life stages, including larvae, metamorphs, and adults. Use of a 0.01% itraconazole solution led to elevated mortality that appeared to be due to drug toxicity rather than the effects of the infection in adult common midwife toads (*Alytes obstetricans*), striped marsh frogs (*Limnodynastes peronii*), and mountain yellow-legged frogs (*Rana muscosa*). In addition, case reports of itraconazole treatment of 3 species of ambystomid salamander (*A. andersoni*, *A. dumerilii*, and *A. mexicanum*) involved bathing the animals with 0.005% to 0.01% itraconazole across several treatment durations.<sup>23</sup> Surprisingly, mortality for axolotls (*A. mexicanum*) varied widely, with no loss of animals

treated at one location and 100% mortality at another. The mortality rate for the axolotls in our laboratory (2 of 46 animals; 4.3%) was much higher than would be expected during a similar colony time period outside of disease treatment but was low compared with that described previously.<sup>23</sup> This difference may be related to the use of a relatively low (0.002% to 0.0025%) concentration of itraconazole in the protocol we described here.

For the duration of itraconazole treatment and housing facility disinfection, axolotls were individually housed in bowls and newts were group-housed in moist, terrestrial habitats different from that of their standard aquaria housing. Overall, the axolotls appeared to tolerate the bowl environment well, although bowl housing reduced the amount of space available per animal, limited the provision of enrichment, and led to daily spikes in ammonia levels (some as high as 1 ppm) prior to water changes. In contrast, newts housed terrestrially displayed unanticipated effects, including decreased activity and poor appetite, across the majority of animals. Terrestrial housing was selected to mimic prior anuran protocols,<sup>5</sup> and these effects were noted throughout terrestrial housing regardless of the time point during antifungal treatment. These signs resolved with the relocation of newts back to aquatic housing. Therefore, maintaining aquatically housed newts with standard husbandry during itraconazole treatment may be preferred over relocation to terrestrial housing, because aquatic housing did not alter treatment efficacy in axolotls.

Several biosecurity measures were instituted during the animal treatment period. For the quarantine period, signs were placed on the doors to the housing rooms, noting that animals within either tested positive or were potentially exposed to *B. dendrobatidis*, and disposable PPE used for animal handling were collected by our institutional environmental health and safety department as hazardous waste. Other measures instituted during treatment have remained in effect as standard lab practices. Entry to these facilities is limited to laboratory personnel and veterinary staff, and refuse collection is coordinated between laboratory and janitorial staff such that janitorial staff do not need to enter animal areas.

African clawed frogs (*Xenopus* spp.) are another commonly used aquatic animal model that is susceptible to *B. dendrobatidis* infection, with the earliest reported case of chytridiomycosis found in an *X. laevis* frog.<sup>37</sup> Clinical signs seen in *Xenopus* spp. are similar to those we described here, including hyperpigmentation, cutaneous lesions, excessive skin shedding, lethargy, and weight loss.<sup>9,28</sup> *Xenopus* spp. are not currently housed at our institution, but personnel working with the axolotl and newt colonies were made aware of potential susceptibility among other aquatic vertebrates on campus. Laboratories using aquatic species within the same building were informed of the state-mandated quarantine, and personnel from the salamander lab did not enter any other laboratories that worked with aquatic animals until the infection was cleared.

The source of *B. dendrobatidis* entry into the facility could not be identified. Potential sources of contamination include animals entering the facility from outside laboratories or the field, food sources, or transmission on clothing or equipment brought into the housing space by laboratory personnel. All animals brought into the facility have been apparently healthy on arrival. However, chytridiomycosis infection can be inapparent in a variety of aquatic species, with clinically unaffected species suggested as potential reservoir hosts.<sup>6,20</sup> Various food sources, such as crayfish, have been implicated as a source of chytrid infection for salamanders;<sup>22</sup> food source evaluation is, therefore, a prudent inclusion in outbreak investigations. In our case, live



blackworms are purchased from a large commercial supplier, with no reports of pathogen transmission concerns noted by the supplier or colleagues at this or other institutions that purchased blackworms from this source. Furthermore, PCR testing of the housing for these blackworms was negative for *B. dendrobatidis*. Transmission on nonliving objects (fomites) has also been suggested, given that worldwide geographic movement of *B. dendrobatidis* has been speculated to be due to movement of both infected amphibians as well as infected water or moist soil.<sup>17</sup> Furthermore, *B. dendrobatidis* can survive for as long as 7 wk after introduction into water sources.<sup>18</sup>

For ongoing practices at our institution, new water taps were installed to reduce the possibility of cross-contamination between axolotls and newts. Retraining was provided to personnel regarding the use of disposable, extended-cuff nitrile gloves and hand washing with Bacdown hand soap (Decon Labs, King of Prussia, PA) during daily animal care. All personnel now have dedicated, washable, slip-resistant clogs for wear in the animal facility, and they change gloves between cleaning rows of aquaria. New animals entering the facility are subject to a quarantine period, including testing and, as warranted, treatment for *B. dendrobatidis* before release from quarantine and prior to introduction to existing housing rooms. Animals in quarantine are handled after all other husbandry tasks are completed, and personnel do not reenter colony housing rooms after working in quarantine. Our current study has presented an effective protocol for the treatment of chytridiomycosis in axolotls and rough-skinned newts; ultimately, adherence to quarantine protocols—developed with consideration for the bacterial, viral, fungal, and parasitic pathogen susceptibilities of the species of interest and possible exposures prior to arrival—is essential to the prevention of disease transmission in aquatic animal models in the laboratory setting.

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