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SHORT NOTE

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Batrachochytrium dendrobatidis infection and treatment in the salamanders *Ambystoma andersoni*, *A. dumerilii* and *A. mexicanum*

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In order to better understand the impacts and treatment of infection with *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) it is important to document host species, the effect of infection and response to treatment protocols. Here we report asymptomatic Bd infection detected through duplex qPCR screening of three Mexican ambystomatid salamanders; *Ambystoma andersoni*, *Ambystoma dumerilii* and *Ambystoma mexicanum* at three zoo collections, and *A. andersoni* and *A. mexicanum* in a private collection. Bsal was tested for but not detected. We also report the effectiveness and side effects of five treatment protocols in these species. Using the antifungal agent itraconazole, *A. dumerilii* were cleared of infection without side-effects using the granulated preparation (Sporanox). Morbidity and mortality occurred when *A. dumerilii* and *A. andersoni* were treated using a liquid oral preparation of the itraconazole (Itrafungol); infection was successfully cleared in surviving specimens of the latter species. *Ambystoma mexicanum* was successfully cleared without any side-effects using Itrafungol. Mortality and morbidity were likely caused by toxic effects of some component on the liquid preparation of itraconazole, but aspects of water quality and husbandry cannot be ruled out.

Key words: Bd, axolotl, *Ambystoma*, chytridiomycosis, itraconazole

Batrachochytrium dendrobatidis (Bd; Longcore et al., 1999) is a major fungal pathogen known to infect all orders of amphibian (Gower et al., 2013) and is a driver of population declines (Bosch et al., 2001; Lips et al., 2006; Briggs et al., 2010; Scheele et al., 2017). The recently described congener *B. salamandrivorans* (Bsal; Martel et al., 2013) also causes disease and its spread is a cause for major concern (Martel et al., 2014; Richgels et al., 2016; Spitzen-van der Sluijs 2016; Laking et al., 2017). Bsal is thought to be less cosmopolitan than the globally distributed Bd (Fisher et al., 2009; Sabino-Pinto et al., 2016); however, surveys of captive and free-living populations are far from comprehensive.

In captivity, a variety of chemical treatments as well as heat therapy (27-37 ˚C) have been used to treat the chytrid infections caused by both agents (e.g. Berger et al., 2004; Bowerman et al., 2010; Chatfield and Richards-Zawacki 2011; Martel et al., 2011; Woodhams et al., 2003, 2012; Brannelly et al., 2012; Blooi et al., 2015a; b). However, urodelan amphibians tend to exhibit low tolerances to elevated temperatures (e.g. Liu et al., 2006; Bury, 2008), which often precludes heat treatment as a viable method to clear Bd infection. It is therefore imperative that the efficacy of other treatment options is tested.

Successful therapy using itraconazole has been documented in numerous amphibian species from all three orders (Forzan et al., 2008; Tamukai et al., 2011; Brannelly et al., 2012; Rendle et al., 2015), but different species respond differently to the drug. Itraconazole toxicosis has been reported in some amphibian species treated at 'routine' doses of the drug (Garner et al., 2009; Woodhams et al., 2012). It has therefore been recommended to record the effects of Bd on host animals and to develop and test protocols for treating this widespread pathogen in a variety of species of amphibian (Woodhams et al., 2012).

We present data following Bd diagnosis and subsequent treatments of 40 *Ambystoma dumerilii*, 26 *A. mexicanum* and 10 *A. andersoni* in captivity (see Supplementary Materials for details). Animals were maintained according to the protocols outlined in Table 1 at the Zoological Society of London (ZSL), Chester Zoo (CZ), Parc de Thoiry (PT) and a private breeder in the UK (PB). Salamanders were sampled initially and post treatment (see Table 1) for Bd/Bsal screening by swabbing dorsum,

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Table 1. Husbandry and swabbing protocols for *Ambystoma* salamanders reported in this study

Species	***Ambystoma dumerilii***	***A. mexicanum***			***A. andersoni***
Collection	**ZSL London Zoo**	**Parc de Thoiry**	**Chester Zoo**	**Private collection, UK**	
Swab numbers	**Pre-treatment:** 3 individual swabs **Post-treatment (0, 30 and 180 days):** 11 individual swabs	**Pre-treatment:** 2 pooled swabs, 8 individuals each **Post-treatment (0 days):** 2 pooled swabs, eight individuals each **Post-treatment (40 days):** 5 pooled swabs, 3 individuals each; 1 individual swab	**Pre-treatment:** 13 individual swab **Post-treatment:** N/A	**Pre-treatment:** 1 pooled swab for 4 *A. mexicanum* **Post-treatment (30 days):** 1 pooled swab for 4 *A. mexicanum*	**Pre-treatment:** 1 pooled swab for 2 *A. andersoni* **Post-treatment (30 days):** 1 pooled swab for 2 *A. andersoni*
Bd/Bsal qPCR results	**Bd:** 3/3 +ve, **Bd infection load:** 6.48, 12.6, 2964.12 GE **Bsal:** 3/3 -ve	**Bd:** 1/2 two pooled swabs +ve. **Bsal:** 2/2 pooled swabs -ve.	**Bd:** 6/13 +ve, **Bd infection load:** 31, 41.64, 84.72, 97.44, 114.72, 704.76 GE **Bsal:** 13/13 -ve	**Bd:** +ve **Bsal:** -ve	**Bd:** +ve **Bsal:** -ve
Animal housing during treatment period	3-4 animals held in 100 x 30 x 30 cm aquaria Aquaria filtered using air-stream sponge filters.	5 animals in a 100 x 50 x 60 cm aquarium; 11 animals individually in 40 x 30 x 30 cm plastic boxes. Large aquarium filter with internal filter. Small boxes unfiltered; 100% water change performed daily.	4-5 animals held in 400L aquaria.	Large plastic boxes (varying capacity). No filtration. Daily 100% water changes and disinfection of enclosures.	
Water quality parameters during treatment period	**pH:** c. 8 **Ammonia (NH_3^+):** 0 - 0.03mg/L (with two brief instances of c. 0.5mg/L) **Nitrite (NO_2):** 0-0.04mg/L (with one instance of c. 0.5mg/L **Nitrate (NO_3):** <10 mg/l **Alkalinity:** 175-200mg/L **Temperature:** 15-17 ˚C	**pH:** 6.8 - 7.2 **Nitrite (NO_2):** 0mg/L **Nitrate (NO_3):** 50 - 75 mg/l **Conductivity:** 370 micro Siemens. **Temperature:** 18 ˚C	Water parameters not recorded.	**pH:** 7.9. **Temperature:** 16-20 ˚C.	

ventrum, cloaca, lips, tail base and plantar aspect of the feet using a sterile dry swab (see Table 1 for numbers of swabs collected and Supplementary Materials for swabbing methods). Duplex qPCR was used to test for the presence of Bd and Bsal DNA following protocols developed by Hyatt et al. (2007) and Blooi et al. (2013) (see Supplementary Materials). Itraconazole baths were used for treatment in all institutions using the protocols outlined in Table 2 and detailed in Supplementary Materials. Results of initial and post-treatment qPCR testing, including Genomic Equivalent (GE) values, are presented in Table 1; all species presented with at least some animals infected with Bd, but Bsal was not detected. All animals that survived treatment tested negative for both pathogens after treatment (Table 2). Identification of the lineage or strain of Bd infecting animals (Retallick & Miera, 2007) was beyond the scope of this work.

All surviving salamanders in all collections repeatedly tested negative for Bd post treatment (see Table 2). At ZSL and PT, there were no observed adverse side effects to treatment with Sporanox in *A. dumerilii*. Water quality was monitored at ZSL and PT, and remained good (see Table 1). At CZ, there was 100% mortality of animals shortly after exposure to the treatment regimen using Itrafungol. Water quality was not monitored at CZ. At CZ on day 7 of treatment with Itrafungol, six *A. dumerilii* were found dead. The remaining animals exhibited excessive mucus production, cloudy eyes, erratic movements and inappetence. At post mortem examination and subsequent histopathology, the salamanders were thin and presented acute dermatitis (sometimes ulcerative or necrotic) and branchitis. Some specimens showed hepatocyte vacuolation. Treatment was stopped but after two days, the condition of the remaining animals had continued to worsen and they were euthanased on welfare grounds. No CZ animals completed the treatment protocol.

At PB, *A. mexicanum* showed no clinical adverse effects of treatment with Itrafungol. In *A. andersoni*, 50% mortality was encountered when treated with Itrafungol.

Table 2. Protocols for and outcomes of itraconazole treatment in *Ambystoma* salamanders reported in this study.

Species	***Ambystoma dumerilii***	***A. mexicanum***	***A. andersoni***	
Collection	**ZSL London Zoo**	**Parc de Thoiry**	**Chester Zoo**	**Private collection, UK**
Therapeutic drug and preparation	Itraconazole (Sporanox; Janssen Pharamceutica N.V., Beerse B-2340, Belgium).	Itraconazole (Itrafungol; Elanco, Division Eli Lilly Canada Inc., 150 Research Lane, Suite 120, Guelph, ON, N1G 4T2, Canada)	Itraconazole (Itrafungol)	
Therapeutic itraconazole concentration, duration and temperature	0.01%. 15 minute baths daily for eleven days at c. 16 ˚C.	**Group 1 (n=8):** 0.01%. 7 minute baths daily for seven days. **Group 2 (n=8):** 0.005%. 15 minute baths daily for seven days Both versions at c. 18˚C.	0.01%. 5 minute baths daily for ten days, followed by 10 rest days and then a further ten days of 5 minute baths. Treatment course not completed due to mortality. Water temperature not recorded.	0.01% in buffered with one tsp NaHCl/5L tap water to maintain pH 7. 5 minutes per day, daily over six days. 16-20 ˚C.
Treatment protocol	Animals were moved to individual c. 1L containers of itraconazole solution. Filtered aquaria were not sterilised between treatments in order to preserve biological filtration.	Animals were to be bathed in 1 litre of solution in a clear plastic bag. Aquaria and filters sterilized with 1:500 F10 disinfectant after 5 and 10 days of treatment. Treatment was not completed.	Animals bathed in individual 1L containers. Enclosures sterilised between treatments.	
Mortality	0%	100% (animals either died from presumed toxicosis or were euthanased)	*A. mexicanum*: 0%	*A. andersoni:* 50%
Bd negative post treatment?	Y	Animals did not survive treatment	Y	Y

At PB, *A. mexicanum* showed no clinical adverse effects of the Itrafungol treatment. In *A. andersoni*, however, animals lost vigour during treatment and within one week of completion of treatment, five animals had died (50% mortality). Animals exhibited shrinking gill branches, loss of gill filaments both of which were noticeable in living and dead animals. Animals also showed reduced feeding behaviour. Ten days post treatment, surviving animals were removed from the established aquarium into which they had been placed and maintained in a 160L plastic box with 100% daily water changes. Following this intervention, mortality stopped and animals recovered to normal appearance and behaviour. No histological data are available from these animals.

Bd infection has not previously been reported from *A. dumerilii* or *A. andersoni*, but has been recorded in other neotenic Mexican Ambystoma (namely *A. altamirani*, *A. granulosum*, *A. mexicanum*, *A. rivulare*, *A. ordinarium* and *A. velasci*; Forzan et al., 2008; Frias-Alvarez et al., 2008; Galindo-Bustos et al., 2014; Spitzen-van der Sluijs et al., 2011). Animals of all three species in all four collections were apparently able to carry Bd infection without clinical disease. This has been reported in other ambystomatid salamanders (Spitzen-van der Sluijs et al., 2011; Davidson et al., 2003; Padgett-Flohr, 2008) and this may be linked to the production of skin peptides that inhibit the growth of Bd (Sheafor et al., 2008). Our data suggest that all three species investigated here may be able to act as reservoirs of Bd, at least within captive populations and, potentially, in nature.

In *A. dumerilii*, infection loads, in terms of GE per sample, were broadly similar overall between Chester Zoo and ZSL (Table 1), but variation within species was substantial. All but one swabbed animal (ZSL; 2964.12 GE) had very low loads. *Ambystoma mexicanum* in a Bd positive laboratory colony were reported to have loads of 0 - 1726.29 GE (Frias Alvarez et al., 2008). Although the highest infection load in *A. dumerilii* is approximately 40% higher than the maximum infection load reported in *A. mexicanum*, no measure of variation was given by Frias-Alverez et al. (2008) and so direct comparison with our data is not possible. The use of pooled swabbing at Parc de Thoiry for *A. dumerilii*, and for *A. andersoni* and *A. mexicanum* in the private collection precluded any estimation of infection intensity and so comparisons with the literature are not possible.

For unknown reasons, Bd was not detected on some *A. dumerilii* individuals within Bd positive groups; this can probably be regarded as representing the bottom end of the detected variation in infection loads between infected salamanders. This observation mirrors circumstances reported in colonies of *A. mexicanum* in both laboratory (Frias Alvarez et al., 2008) and zoo (Galindo-Bustos et al., 2014) settings. Labial swabs, alongside samples from other sites, were collected as some larval ambystomatid salamanders possess keratinized jaw sheaths that may act as infection foci for Bd (Venesky et al., 2010) as well as keratin elsewhere on the body (Bosch and Martinez-Solano, 2006), and so it is likely that swabbing was as efficient as possible for the collection of chytrid DNA. Although these results are likely to reflect real negatives, extremely low infection burdens below the detection threshold are also possible.

All animals in this study tested negative for Bsal infection, although the animals were maintained within the optimal temperature range for this fungus (Martel et al., 2013; Blooi et al., 2015a). Negative results are important in delineating the overall presence of Bsal in captive populations. These results contribute to the current belief that Bsal infection is still relatively rare in captive urodelans (Sabino-Pinto et al., 2016).

Our results show that Bd infection can be eliminated using the established anti-fungal chemical itraconazole in neotenic *A. dumerilii*, *A. mexicanum* and *A. andersoni*. Treatment with itraconazole of confirmed Bd infection in neotenic *Ambystoma* has not been previously reported. Metamorphosed *A. tigrinum* were successfully cleared of Bd infection using a similar protocol to that described here (10 minute 0.01% itraconazole (Itrizole oral solution) baths every other day for seven treatments; Tamukai et al., 2011). The variations employed by Thoiry and the private keeper demonstrate that Bd infection can be treated, at least in this case, by using a lower itraconazole solution (0.005%) and shorter bath duration (5 minutes) than the 0.01% and 10-minute immersion time typically used for treating Bd infection (Jones et al., 2012). This is congruent with trials in the anurans *Litoria caerulea* and *Anaxyrus baxteri* (0.005% itraconazole baths in Sporanox form; Jones et al., 2012) and indicates that low dosage and short immersion time may be useful in a wide range of amphibian taxa, at least with low infection loads.

Different preparations of itraconazole appear to have different effects and efficacy in different *Ambystoma* species. The granule (Sporanox) preparation of itraconazole can apparently be used without deleterious side effects in *A. dumerilii* (this study) and *A. mexicanum* (Forzan et al., 2008), while liquid preparations are apparently safe for use in *A. mexicanum* (this study; Itrafungol) and in *A. tigrinum* (Itrizole Oral Solution 1%, Janssen Pharmaceutical K.K., Tokyo, Japan; Tamukai et al., 2011). However, our data suggest that use of the liquid preparation (Itrafungol) of itraconazole may be linked to rapid morbidity and mortality in *A. dumerilii* and *A. andersoni*. As water quality was not measured in collections using Itrafungol, and as other aspects of husbandry including disinfection of filter media co-varied with treatment regimen, it is possible that detrimental effects observed were caused by factors other than the drug. However, deleterious side-effects have also been recorded in anuran tadpoles treated with Itrafungol (Garner et al., 2009). We found no evidence in the literature suggesting either safe use or toxic effects of any non-itraconazole ingredient (see Supplementary Materials) of Itrafungol on amphibians. Although the active ingredient is the same in both compounds (itraconazole), there may also have been interactive effects between itraconazole and other compounds in the drug, for example through effects on bioavailability of the active compound. The Itrafungol solution was buffered at PB, but not at CZ. This may have an effect on its efficacy against Bd (e.g. pH affects Bd growth; Piotrowski et al., 2004) and any side-effects on the salamanders themselves, but this preparation led to morbidity and mortality in both collections. We recommend that the use of this preparation should probably be treated with caution in ambystomatid salamanders.

The deleterious side-effects reported here represent only impacts on health that can be detected in the short term. It is possible that other effects on health may be more subtle or require more longitudinal studies to detect. Furthermore, the treatment designs used here were based on previous reports of successful treatments and do not represent targeted or evidence-based approaches. The use of data from in vitro exposures of fungus to candidate treatment regimens could inform the selection of the lowest dose and shortest exposures possible to successfully eliminate the pathogen (e.g. Martel et al., 2011). Such an approach may avoid negative side-effects and reduce the chance of unforeseen negative outcomes of treatment attempts, although susceptibility of the fungus in vitro does not necessarily equate to successful therapy in vivo (Berger et al., 2010).

We also demonstrate that Bd infection can be successfully treated without sterilisation of biological filters. This is congruent with Rendle et al. (2015) and reinforces that a balance can be struck between effective therapy and the maintenance of appropriate environmental parameters. By disinfecting biological filters between itraconazole treatments, and unless other methods of dealing with nitrogenous waste (e.g. chemical filtration) are employed, the environment in which animals are kept may rapidly become toxic due to the accumulation of waste products. As Bd can, on the basis of these and other data, be eliminated without the disinfection of the environment, biological filters may be left intact during the treatment of aquatic amphibians for Bd. Bd can survive outside amphibian hosts (Johnson & Speare, 2003). We were unable to determine if infective colonies of Bd survived on the filter media post treatment, but repeated and long term negative Bd results suggest that such colonies were either absent or at least unable to re-infect salamanders.

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