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

STUDY ANIMALS

All *Ambystoma dumerilli* were eight month old, captive bred specimens originating from a common, third party institution and recently imported and kept either at the Zoological Society of London (ZSL) (n=11), Chester Zoo (CZ) (n=13) or Parc de Thoiry (PT) (n=16). *Ambystoma mexicanum* and *A. andersoni* were long term, resident adult (ages unknown) populations as part of a private collection (PB), housed in permanent quarantine for several years since acquisition.

SWABBING PROTOCOL

Salamanders were sampled for Bd/Bsal screening by swabbing dorsum, ventrum, cloaca, lips, tail base and plantar aspect of the feet using a sterile dry swab (Dryswab MW100, Medical Wire & Equipment, Corsham, Wiltshire, SN13 9RT, UK). Swabs were sealed in their accompanying sleeves and kept at room temperature and immediately processed (ZSL) or posted at ambient temperature, to a diagnostic lab (CZ,PT, PB).

Bd/Bsal assays

Bd/Bsal assays from *A. dumerilii* at ZSL and CZ, and *A. mexicanum* at PB were carried out at ZSL.

The samples from the *A. dumerilii* held at PT were analysed at the University of Ghent, while samples from *A. andersoni* and *A. mexicanum* held at PB were processed by Pinmoore Animal Laboratory Services (PALS; Tarporley CW6 0EG, UK).

Bd/Bsal assays were performed using the same methods for all species. DNA was extracted from swabs following the method described by Hyatt et al. (2007) in a 1:10 dilution from the supernatant. A Bd/Bsal assay was then performed using duplex quantitative real-time polymerase chain reaction (qPCR), using Bd/Bsal primers specific to the ribosomal ITS-1/5.8S region, following Blooi et al. (2013). Bovine serum albumin (BSA) was included in reactions to reduce amplification inhibition (Garland et al. 2010). In combination with samples, positive controls of known Bd/Bsal concentration (100, 10, 1 and 0.1 zoospore genomic equivalents: GE) and negative controls were included in the assays. Samples were run in duplicate on PCR plates and repeated until both wells gave the same (positive or negative) result. Should two matching results not be obtained, samples would be re-assayed with the same diluted solution (1:10 from the supernatant), then with a new 1:10 dilution made up independently from the supernatant and finally with a 1:5 dilution, again made up independently from the supernatant. If no clear result was obtained at this point, this sample would be discarded as potentially contaminated.

Genomic Equivalents (GE) detected per sample, a measure of infection load, was calculated per swab for non-pooled CZ and ZSL samples. To correct for the dilution factors used here, each GE result per PCR was multiplied by 120 (Hudson et al., 2016).

ITRACONAZOLE BATHS

Iitraconazole baths were used to treat salamanders. These were made using itraconazole in two different preparations.

Sporanox capsules (Janssen Pharmaceutica N.V., Beerse B-2340, Belgium) were crushed and suspended in water, following Rendle et al. (2015) at two different concentrations of 0.005% and 0.01 %. Sporanox, uses itraconazole-coated beads of starch and sucrose along with hypromellose, polyethylene glycol (PEG) 20,000, titanium dioxide, FD&C Blue No. 1, FD&C Blue No. 2, D&C Red No. 22 and D&C Red No. 28. as inactive ingredients. Itrafungol liquid preparation (Elanco, Division Eli Lilly Canada Inc., 150 Research Lane, Suite 120, Guelph, ON, N1G 4T2, Canada) was diluted in water to a concentration of 0.01%). Itrafungol uses a suspension of itraconazole mixed with, propylene glycol (E1520), sorbitol 70% non-crystallizing solution, hydroxypropyl-b-cyclodextrin, concentrated hydrochloric acid, sodium hydroxide, and purified water, as well as sodium saccharin and caramel (E150) cherry flavour to improve palatability for mammals.

All baths were prepared at the same temperature as the holding aquarium using fresh water from the same source as that used to house animals. The salamanders at ZSL and PT were treated with unbuffered Sporanox baths with pH of approximately 7.8 at ZSL. At ZSL all salamanders received 11 days of daily 15 minute baths in 0.01% itraconazole, following Rendle et al. (2015). PT salamanders were split into two treatment groups, each composed of four individual salamanders from the group that yielded a Bd-positive pooled swab and four from the Bd-negative pooled swab group. Each group was bathed daily for seven days with one group (n=8) receiving 7 minute baths in a 0.01% itraconazole solution, while the other group (n=8) received 15 minute baths in 0.005%

solution (Jones et al., 2012). After each treatment the animals were returned to the original tank. Aquaria and filters were not sterilised between treatment baths.

The CZ animals received 0.01% itraconazole baths using Itrafungol. At CZ the animals were to receive 5 minute baths, daily for 10 days; the animals were to be placed in a clean tank after treatment on days one, 5 and 10, at which time the old tanks and filters were to be cleaned and disinfected with a 1:500 solution of F10 (Health and Hygiene, Unit 7 Windmill Road, Loughborough, LE11 1RA), and rinsed in itraconazole preparation to receive the animals on the next cycle.

At PB five minute 0.01% Itrafungol baths were performed daily for 6 days. Baths were made up by mixing Itrafungol to aerated tap water (low hardness, pH c. 6.5) and then buffered to a pH of c. 7 using approximately 7g of sodium bicarbonate per 5 litres of tap water, The animals were returned to the original tank after treatment.

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