In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic

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Ethics statement

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Abstract

The emerging infectious disease, amphibian chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (Bd), threatens hundreds of amphibian species globally. In the absence of field-based mitigation methods, the Amphibian Conservation Action Plan advocates captive assurance programmes to prevent extinction from this infectious disease. Unfortunately, with the cooperation of the entire global zoo community, the International Union for the Conservation of Nature Amphibian Ark estimates only 50 species could be saved. Clearly, if catastrophic losses are to be averted, alternative mitigation techniques need to be developed. There has been an absence of trialling laboratory proven interventions for chytridiomycosis in field settings, which must change in order to allow informed management decisions for highly threatened amphibian populations. We tested the in-situ treatment of individual mountain chicken frogs (*Leptodactylus fallax*) using the antifungal drug, itraconazole. Multi-state mark recapture analysis showed increased probability of survival and loss of Bd infection for treated frogs compared to untreated animals. There was evidence of a prophylactic effect of treatment as, during the treatment period, infection probability was lower for treated animals than untreated animals. Whilst long term, post-treatment increase in survival was not observed, a deterministic population model estimated antifungal treatment would extend time to extinction of the population from 49 to 124 weeks, an approximated 60% increase. In-situ treatment of individuals could, therefore, be a useful short-term measure to augment other conservation actions for amphibian species threatened by chytridiomycosis or to facilitate population survival during periods of high disease risk.

Keywords

In-situ treatment, Amphibian declines, *Batrachochytrium dendrobatidis*, Chytridiomycosis, Itraconazole, Antifungal

Abbreviations

Bd – *Batrachochytrium dendrobatidis*
CJS - Cormack-Jolly-Seber
CMR - capture-mark-recapture
DNA – deoxyribonucleic acid
GE – genome equivalent
IT – itraconazole treatment
NBC – non-bath control
PCR – polymerase chain reaction
PIT – passive Integrated Transponder
SWC – stream water control
1. Introduction

Emerging infectious diseases are a growing threat to both humans and biodiversity globally (Daszak et al. 2000; Morens and Fauci 2013). Three main strategies exist for the management of wildlife disease: prevention of introduction, mitigation of impact, and eradication (Wobeser 2002).

Globalisation, with its increased rate and volume of trade and travel, means preventing the introduction of novel diseases is increasingly difficult (Marano et al. 2007). Whilst neutralisation of threats has long been considered a pre-requisite for successful wildlife conservation (Caughley 1994), the emergence of threats which cannot be negated pose a difficult challenge to conservation managers. One example is amphibian chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which is implicated in the rapid decline or extinction of over 200 amphibian species globally (Skerrat et al. 2007), and has been described as “the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and it’s propensity to drive them to extinction” (Amphibian Conservation Summit 2005). This rapid global loss of amphibians is likely to have major implications for the environment (Whiles et al. 2006).

In the absence of in-situ mitigation for amphibian chytridiomycosis (Woodhams et al. 2011; Joseph et al. 2013), the Amphibian Conservation Action Plan advocates the creation of Bd-free captive populations for eventual release as a key conservation strategy (Gascon et al. 2007). Currently, conservation practitioners rely on such captive assurance programmes to prevent species extinctions (Mendelson et al. 2006), but this is only a short to medium term solution and Amphibian Ark estimates that only around 50 species can be saved in this way (Zippel et al. 2011). Even so, zoos are currently failing to prioritise species that are likely to require captive breeding programmes to prevent their extinction (Dawson et al. 2015). There is, therefore, an urgent need to change the research focus from the treatment of captive animals to in-situ mitigation (Scheele et al. 2014; Harding et al. 2015).

A range of potential in-situ interventions to mitigate the impacts of chytridiomycosis have been suggested, but so far these remain largely untested in the field (Berger & Skerrat 2012; Scheele et al. 2014). These include habitat manipulation to inhibit Bd (Scheele et al. 2014), reintroduction after selection for resistance in captivity (Venesky et al. 2014), and in-situ use of antifungal treatments (Berger and Skerrat, 2012). Some antifungal drugs, including itraconazole, are effective in the treatment of Bd infection in captivity, but only following multiple daily applications (e.g. Forzan et al. 2008; Tamukai et al. 2011; Jones et al. 2012; Georoff et al. 2013; Brannelly et al. 2015). In addition to being effective, the application of itraconazole is relatively easy, being via immersion in an aqueous solution – albeit that repeated administration is required for successful treatment (Nichols & Lamirande 2000). Whilst there have been some reported side-effects in certain species (Brannelly et al. 2012; Brannelly 2014) and life stages (Garner et al. 2009; Woodhams et al. 2012), itraconazole is considered to be the treatment of choice for amphibian chytridiomycosis (Holden et al. 2014). Reducing the dose from 0.01% for 11 days to 0.0025% for 5 days has been shown to reduce side effects while maintaining efficacy (Brannelly 2014). Bosch et al. (2015) described the eradication of Bd from the wild Mallorcan midwife toad (*Alytes muletensis*) tadpoles by treating them with itraconazole in captivity and returning them to the wild following chemical disinfection of their breeding ponds and surrounding rocks. As other amphibians and vegetation were absent from the disinfected sites, and as these were rock pools containing little organic matter (which rapidly inactivates most disinfectants), this technique is unlikely to be transferable to many other species or locations.

In-situ treatment regimens provide challenges in field settings due to, for example, large target population sizes, low capture rates the potential of reinfection and the need for a continuous supply of labour. As a result, previous studies have treated individuals with itraconazole in captivity prior to
re-release rather than treating them in-situ (Hardy et al. 2015). Environmental persistence of Bd zoospores (Johnson & Speare 2003; 2005) and the possible presence of infected sympatric amphibians (Daszak et al. 1999) mean animals treated in-situ would likely be exposed to Bd both throughout and after the treatment period, increasing the likelihood of their extirpation (Retallick et al. 2004; Mitchell et al. 2008). Antifungal treatment in a field setting, however, might enable treated animals to persist by lowering their Bd infection load until the initial epidemic has passed (Briggs et al. 2010; Vredenberg et al. 2010). There is some evidence that animals surviving the epidemic phase persist by tolerating subsequent lower levels and frequencies of infection (Retallick et al. 2004; Briggs et al. 2010). Also, repeated infection and clearance of Bd might allow the development of resistance in some species (McMahon et al. 2014).

The Caribbean is a global hotspot of amphibian endemicity, with 99% of the 197 species being endemic (Fong et al. 2015), and it has the highest proportion (84%) of threatened amphibians within a region (Stuart et al. 2008). One species, the mountain chicken frog (Leptodactylus fallax), has suffered a precipitous decline due to chytridiomycosis (Magin 2003; Fa et al. 2010; Mountain Chicken Recovery Programme 2014). L. fallax is classified as Critically Endangered on the IUCN Red List of Threatened Species (Fa et al. 2010) and is restricted to only Dominica and Montserrat in the Lesser Antilles. A 2005 survey found no evidence of Bd in amphibians on Montserrat (Garcia et al. 2007), but in January 2009 L. fallax mortality due to chytridiomycosis was first discovered on Montserrat and this was rapidly followed by epidemic mortality across the island (Mountain Chicken Recovery Programme 2014). The characteristically rapid rates of chytridiomycosis-driven declines (Lips et al. 2006), such as those observed in L. fallax, limit the time available to react effectively. Interventions that can reduce rates of decline can be valuable for providing extra time to implement further conservation actions.

In this study we report the use of itraconazole treatment in a field setting in an attempt to mitigate the impact of epidemic chytridiomycosis. We assess whether in-situ antifungal treatment is a feasible and effective method for improving the survival of a critically endangered species undergoing a precipitous decline due to epidemic chytridiomycosis. L. fallax is an ideal species to use as a model for such in-situ treatment as it is a large territorial animal with predictable behaviours, making it relatively easy to detect and individually identify. Also, the species has been studied for over ten years on Montserrat, so there is a great deal of knowledge about its distribution, abundance and behaviour and field sites were already established (Garcia et al. 2007; Martin et al. 2007). On Montserrat the presence of a sympatric amphibian fauna of species (Eleutherodactylus johnstonei and Rhinella marina) able to carry Bd renders an in-situ treatment study realistic for extrapolation to other species and regions where sympatric amphibians act as Bd reservoirs.

Effective treatment of chytridiomycosis in captive L. fallax using itraconazole has shown the drug to be safe for this species (authors’ unpublished observations). Finally, L. fallax has a voracious appetite and requires large enclosures in captivity, therefore it is difficult and expensive to hold a large enough captive population for a viable, long-term conservation breeding programme.

2. Materials and methods

2.1. Study site

Montserrat is a U.K. overseas territory in the Eastern Caribbean (16.45°N, 62.15°W). The centre of the island comprises an active volcano which has been erupting regularly since 1995. As a consequence L. fallax is restricted to a circa 17 km² mountainous area; the Centre Hills region which is typified by montane rainforest and deep valleys (or ghauts – Fig. 1) (Young 2008). The field site (Fairy Walk) is a forested relatively-shallow-sloped ghaut of approximately 1 km² on the eastern flank of the Centre Hills at an approximate elevation of 250 m asl. Prior to 2009, Fairy Walk was home to the highest known population density of L. fallax on Montserrat (Young, 2008) and, at
the commencement of this study, it contained the last remaining intact population following the emergence of chytridiomycosis on the island in 2009.

2.2. Study design

The field experiment took place between August 2009 and January 2010. We visited Fairy Walk three times a week for 24 weeks and surveyed a predefined 800 m transect along the stream (Fig. 1) at a slow walking pace in a team of five. On each occasion the team caught all *L. fallax* seen within 5 m of the transect and recovered any dead animals. We individually marked all captured frogs using a Passive Integrated Transponder (PIT) (11 mm x 2 mm, ID-100A Microtransponder, Trovan Ltd.), which we subcutaneously implanted in the dorsum where retention rates are maximal (Blomquist et al. 2008). We skin-swabbed each frog for Bd on every capture using a rayon-tipped swab (MW 100-100, Medical Wire and Equipment Co.) three times across each of the following sites: ventral abdomen, ventral thighs and calves, and plantar surfaces of both hind-feet. We assigned frogs to one of three groups during the study: itraconazole treatment (IT), stream water control (SWC), and non-bath control (NBC). On each capture, after skin-swabbing, we immersed each animal in the IT group for 5 minutes in a 0.01% aqueous solution of itraconazole (Sporanox, Janssen Pharmaceuticals, Inc.), prepared using stream water on site. We treated frogs in the SWC group similarly, but in stream water without itraconazole. We immersed each frog within a new, disposable food-grade plastic bag. We released frogs in the NBC group after swabbing with no further intervention.

During the first 2 weeks of the study, we randomly assigned animals to the IT and SWC groups at the time of first capture, with a 2:1 bias towards treatment. From week 3, we assigned all further captures to the NBC group. In order to examine any treatment-specific long term effect on survival or infection rate, we discontinued treatments after 15 weeks, but continued to capture and skin-swab re-sighted animals. We continued monitoring until week 24 when the study was prematurely ended by a major volcanic eruption.

2.3. Laboratory methods

We refrigerated skin-swabs until transport to the laboratory where DNA was extracted using methods adapted from Hyatt et al. (2007) (explained in Annex A). We diluted extracted DNA 1:10 in molecular grade water and examined it for the presence of Bd DNA using a Bd-specific TaqMan real-time PCR as described by Boyle et al. (2004) modified by the inclusion of bovine serum albumin to reduce PCR inhibition (Gerland et al. 2010). We tested samples in duplicate, incorporating two negative control wells containing laboratory grade distilled water and four positive controls (100, 10, 1, and 0.1 zoospore equivalents) in duplicate on each plate. A sample was considered positive if PCR amplification occurred in both duplicates. If duplicates generated conflicting results, the samples were re-run up to three times until matching results were obtained. If there was no consensus on the third occasion, the sample was considered negative.

Quantification of Bd DNA in each well was determined as Bd genome equivalents (GEs) by multiplying the real time PCR result by 120 (4 µl of 60 µl total elute used to make up the dilution (x15) and 5 µl of 40 µl 1:10 dilution used in qPCR (x8) [15 x 8=120]).

2.4. Bd infection intensity comparison

In order to test whether itraconazole treatment significantly reduced Bd infection intensity, we used a linear mixed effects model, with treatment group (control vs. IT) and time as fixed effects and frog ID as a random effect. Infection intensity was log transformed prior to analysis as values ranged over many orders of magnitude. Models were compared using AIC corrected for small sample size (AICc) and if no model was overwhelmingly supported (Akaike weight > 0.95), models with a ΔAICc<7 were considered for inference. Summed Akaike weight evidence ratios were used to assess variable importance (Burnham and Anderson, 2002).
2.5. Capture-mark-recapture analyses

We analysed our capture-mark-recapture (CMR) data using the software program Mark (White & Burnham 1999) in a multi-state CMR framework (Lebreton et al. 2009). Multi-state CMR models are an extension of Cormack-Jolly-Seber (CJS) which are used to model the probability of transition between states alongside estimating state dependent survival and recapture rates. These transitions were modelled as first order Markov processes in which the state at time t+1 is dependent only on the state at time t. For our study, we defined states as ‘uninfected’ (U), ‘infected’ (I), and ‘dead’ (D).

We converted data from daily to weekly capture histories using weekly bins to generate weekly parameter estimates. Although grouping data in this way has been shown to produce biased parameter estimates of survival rate in a CJS model when survival rate is time-dependent (Barbour et al. 2013), fixed estimates of survival and transition rate were best supported by our data. Where we detected different states during a single weekly bin (n=32) we assigned frogs to whichever state we most commonly caught the individual in, unless one of those states was dead, which superseded other states. In the majority of cases (n=17) the different states recorded within a week reflected a transition between the state recorded in the previous week and the state in the following week, meaning there was no loss of transition in the weekly data. Where we caught the individual in two different states in the same week, we assigned the individual randomly to either state. As this might have hidden capture heterogeneity an ANOVA was used to test for a difference in the mean number of captures per week in each group.

We examined infection state (inf), treatment group (gr), sex and time dependence (time) in estimates of survival, recapture, and transition probabilities. Recovery rates of dead frogs were modelled as a function of treatment group, and sex. We also used models in which survival, recapture and transition rates were a function of group, but with two estimates for the IT group; one estimate during treatment with itraconazole (weeks 1-15), and one after this treatment had ended (week 16-24) (gr[split T]). This enabled us to test for any post-treatment effects. We tested for an effect of the immersion process by comparing models with one estimate for both control groups combined (gr[C]) and one where SWC and NBC were estimated separately (gr). No occasion-specific environmental variables were available. Juveniles were excluded from the analysis due to low sample size.

In order to reduce the potentially very large number of candidate models, we used a two-step process modified from Lebreton et al. (1992) to estimate parameters in the CMR analysis. In step one, we used the top model for survival and recapture probabilities from a preliminary Burnham dead recoveries analysis (Burnham 1993) to model dead recovery and transition rates. In step two, we used the best estimates of dead recovery and transition rates from step one to model survival and recapture probabilities. This led to the generation of a model set of 128 models.

2.6. Model selection and goodness of fit

We based model selection on AICc. To account for model selection uncertainty, robust estimates of the parameters were computed using weighted model averaging (Burnham & Anderson 2002).

We performed a preliminary diagnostic goodness of fit test for the multi-state models in program U-CARE (Choquet et al. 2009) which detected slight over-dispersion and so we altered the variance inflation factor to 1.15 and the adjusted QAICc was used for model selection.

Summed Akaike weight evidence ratios were used to examine the support for dependencies in the models. The strength of the support provided by the evidence ratios was extracted from Table 3 in Lucaks et al. (2007).
2.7. Population modelling

In order to predict how treatment with itraconazole would have affected the entire sampled population had it been applied across all frogs in this study, we produced a deterministic population model in a susceptible–infected–susceptible (SIS) framework using the transition and survival rate estimates from the CMR modelling. We excluded any recruitment to the adult population as no nests have been recorded on Montserrat since the onset of the chytridiomycosis epidemic. We defined population extinction as population size below 1.

We produced two versions of this model for a population of 228 frogs (the number of unique captures in this study). The first assumed that all frogs were treated at the same rate as the treated frogs in this study using the model averaged CMR transition and survival rate estimates for the IT group. We modelled the second population as untreated, using the model averaged CMR parameter estimates for the control groups. We initiated the simulation with one infected individual. The number of frogs in each state at each time step was calculated using the matrix below, following the notation in Lebreton et al. (2009) in which $\varphi(1,2)$ indicates the rate of transition between state 1 and state 2.

$$
\begin{pmatrix}
 n_I \\
 n_S \\
 n_D
\end{pmatrix}_{t+1}
=
\begin{pmatrix}
 \varphi(I, I) & \varphi(U, I) & 0 \\
 \varphi(I, U) & \varphi(U, U) & 0 \\
 \varphi(I, D) & \varphi(U, D) & 1
\end{pmatrix}
\begin{pmatrix}
 n_I \\
 n_S \\
 n_D
\end{pmatrix}_t
$$

where: $\varphi(I, I) = 1 - \varphi(I, U) - \varphi(I, D)$

and: $\varphi(U, U) = 1 - \varphi(U, I) - \varphi(U, D)$

In order to include model-averaged parameter uncertainty from the CMR models, we made two further models for each group, the shortest and longest times to extinction. To make the lowest time to extinction model we used the lower 95% CI estimate for the rate of loss of infection and the upper 95% CI estimates for infection and mortality rates. The opposite 95% CIs were used to make the longest time to extinction model. We present only the mean model graphically.

3. Results

In total we made 1735 captures of 228 frogs. We caught frogs assigned to the IT group (841 captures of 80 frogs) more often in both absolute terms and relative to the group size than frogs from the SWC group (326 captures of 42 frogs) and the NBC group (482 captures of 106 frogs). The sex ratio was circa 1:1 in each treatment group. Frogs with clinical signs of chytridiomycosis were found throughout the study and in all groups.

By the end of the study, 22% (n=50) of the frogs had been found dead (SWC=21% (n=9), NBC=18% (n=19), IT=28% (n=22)). The proportion of animals known to be extant was greatest in the IT group throughout the study, and this was especially evident towards the end of the study period (Fig. 2).

Across the study we captured, and therefore treated, frogs in the IT group an average of 0.98 (SE=0.06, min=0.16, max=2.50) times per week.

3.1. Skin swab Bd data

During the study 67% of the 1735 skin swabs taken tested positive for Bd (SWC=84% (n=317), NBC=80% (n=463), IT=64% (n=819)). Until the itraconazole treatment ended at week 15, frogs in the IT group were more likely to test negative for Bd than frogs in the control groups, after which the likelihood of testing negative became the same across all groups (Fig. 2). We captured only 13 frogs
which never tested positive for Bd. Eleven of these were in the NBC group and were captured only once (n=8) or twice (n=3). The remaining two were in the IT group and were captured 3 and 16 times. Bd infected animals in the IT group had a lower infection intensity during treatment than animals in the control group (IT: naive mean=5666 GE, SE=1879; Control: naive mean=71 607 GE, SE=24 218). The top linear mixed model for the treatment period contained a group-time interaction and received overwhelming support (Akaike weight=0.9997). This provided evidence that although the Bd infection intensity of infected animals was similar in the IT and control groups at the start of the study (IT=168.81 GE, SE=1.63; Control=87.46 GE, SE=1.44), the rate at which the infection intensity increased was much greater in the control group (on the log scale: IT=0.015 GE/week, SE=0.03; Control=0.138 GE/week, SE=0.02; Annex B). In the post-treatment period, the infection intensity of infected animals in the IT group increased (IT: naive mean=47 002 GE, SE=19 169) and there was very weak evidence (summed Akaike weight =0.4041, evidence ratio=0.7) of a difference with the control group animals in the same period (Control: naive mean=69 480 GE, SE=57 678) suggesting the benefit of treatment were lost after treatment ended (see Annex B).

3.2. Multi-state CMR models

The top models (ΔQAICc<7) are listed in Table 1. As no model received overwhelming support (top model Akaike weight 0.293), model averaging was used to generate robust parameter estimates to account for model variation. Grouping captures into weekly bins may have hidden heterogeneity in the capture rate between groups, but we found no evidence for a significant difference in the mean number of captures per week between groups (ANOVA: SWC: mean=1.12, SE=0.08; NBC: mean=0.97, SE=0.04; IT: mean=0.98 SE=0.06; F(2,225)=1.598, MSE=0.236, p=0.205).

All of the most parsimonious models (Akaike weight >0) contained a difference in survival between the IT and control groups, and between Bd infected and uninfected animals. There was moderate support for no difference in the SWC and NBC groups (summed Akaike weight=0.969; evidence ratio=31.3) and so, only one estimate of survival for the two control groups is presented. Model averaged parameter estimates showed that itraconazole treatment increased the weekly survival rate of Bd infected animals by 11.6% compared to animals in the control groups (IT = 0.903, 95% CI = 0.860-0.934; Control = 0.809, 95% CI = 0.764-0.841; Fig. 3). All of the most parsimonious models, however, included a second estimate for the IT group when treatment ended: the estimate decreased to a value similar to the control groups (0.795, 95% CI = 0.709-0.864). Uninfected animals had a higher weekly survival rate than Bd infected animals in both the IT (0.988, 95% CI = 0.972-0.995, effect size = 9.4%) and control groups (0.974, 95% CI = 0.939-0.987, effect size = 20.3%; Fig. 3).

Each of the most parsimonious models contained a difference in recapture rate between Bd infected and uninfected animals, and with time dependency. The top models also contained a difference in the recapture rate of the IT and control groups, with limited support for a difference in the NBC and SWC groups (summed Akaike weight = 0.877; evidence ratio = 7.1). There was very weak support for an interaction between infection state and treatment group (summed Akaike weight = 0.095, evidence ratio = 0.1). As time dependent recapture probability was best supported, mean estimates averaged across each occasion are presented (Fig. 4 - full results). Model averaged parameter estimates showed that Bd infection increased recapture probability by a mean of 99.1% in the IT group (Uninfected(U) = 0.354, Infected(I) = 0.711), 120% in the SWC group (U = 0.310, I = 0.686), and 136% in the NBC group (U = 0.270, I = 0.637). Based on these estimates, the recapture rate of Bd infected animals in the IT group was 3.6% greater than the SWC group and 11.6% higher than the NBC group. The recapture rate of uninfected animals in the IT group was 14.1% greater than the SWC group and 31.1% higher than in the NBC group. The recapture rate of Bd infected animals in the SWC group was 7.2% higher than in the NBC group and 14.8% higher in uninfected animals.
All of the most parsimonious models contained a difference in state transition rates (infection and loss of infection) rates between the itraconazole treatment and control groups. There was very weak support for a difference in the transition rates of the two control groups (summed Akaike weight = 0.043; evidence ratio < 0.1), and in the different sexes (summed Akaike weight = 0.142; evidence ratio = 0.2). As a result one estimate for both control groups and sexes is presented. Itraconazole treatment reduced the weekly infection rate of uninfected animals by 19.3% compared to the control groups (IT = 0.208, 95% CI = 0.158-0.269: Control = 0.248, 95% CI = 0.185-0.330; Fig. 3). Itraconazole treatment also increased the weekly rate of loss of Bd infection of infected animals by 161% compared to the control groups (IT= 0.338, 95% CI = 0.254-0.433: Control = 0.129, 95% CI = 0.088-0.177). All top models included a second estimate for transition rate for the itraconazole treatment group when treatment ended, when infection rate increased to a similar level to the control groups (IT= 0.298, 95% CI = 0.194-0.430) and rate of loss of infection declined to levels similar to the control groups (IT = 0.083, 95% CI = 0.036-0.178; Fig. 3).

There was weak evidence for a treatment group difference in dead recovery rate (summed Akaike weight = 0.271; evidence ratio = 0.3). The model averaged parameter estimate was 0.241 (95% CI = 0.163-0.340) across all three groups.

3.3. Population models
The deterministic SIS models indicate that if the entire sampled population had been treated with itraconazole at the rate applied to frogs in the IT group, it would have survived an estimated 124 weeks (min = 79, max = 236) compared to 49 weeks (min = 33, max = 73) if no drug treatment had been given. Consequently, treatment would have increased time until extinction by an estimated 75 weeks (min = 6, max = 203) (Fig. 5). This represents an estimated weekly survival of 95.7% for the treated population compared to 89.4% for the untreated population.

4. Discussion
We used the emergence of amphibian chytridiomycosis in L. fallax on Montserrat as a model system to investigate the feasibility and impact of in-situ treatment of the disease using the antifungal drug, itraconazole. Our study shows that in-situ treatment of wild amphibians with itraconazole in the face of epidemic chytridiomycosis decreased the mortality rate of infected animals and increased their rate of loss of infection during the treatment period. Itraconazole treatment also reduced the infection rate of animals in the IT group during the treatment period, providing evidence of a short term prophylactic effect. On cessation of treatment, the benefits were lost and the rate of survival and loss of infection regressed and the infection rate increased to those of untreated individuals. It also suggests that, at least in L. fallax, repeated exposure to Bd and anti-fungal treatments does not facilitate resistance through the development of an immune response.

McMahon et al. (2014) reported that relatively small numbers of repeated exposures to Bd followed by clearances using heat treatment in captivity were sufficient to stimulate an immune response in Osteopilus septentrionalis resulting in a reduced mortality rate. Other studies have presented contradictory findings (Stice & Briggs 2010; Cashins et al. 2013; Fites et al. 2013), and it appears unlikely that this immuno-protective effect, if it does occur, can be stimulated in all species.

The decreased mortality rate conferred by itraconazole treatment in our study is encouraging considering each frog was treated on average just once a week. This is a substantially lower treatment rate than the once-daily treatment used in laboratory studies and recommended for captive animals (Pessier & Mendelson 2010).

There was no difference in survival or infection state transition rates between the two control groups, providing assurance that the physical action of handling and immersing frogs did not cause
stress sufficient to contribute to mortality or infection. This is important as there are limited methods for the targeted delivery of antifungal compounds for \textit{L. fallax} or for the application of this technique to other amphibian species (Scheele et al. 2014). Hardy et al. (2015) recorded a prolonged decrease in Bd prevalence and an increase in overwinter survival in \textit{Rana cascadae} treated with itraconazole in captivity prior to release into the wild. Although the pharmacokinetics of the drug have not been studied in amphibians, these authors proposed that the itraconazole might have persisted in the skin long enough for another mechanism of resistance to develop, but there is no evidence for this (e.g. Cashins et al. 2013). In our study, itraconazole provided no prophylactic protection from Bd infection beyond the treatment period.

During the post-treatment period, the infection rate in the IT group increased from that seen in the treatment period to that seen in the control groups. The Bd infection intensity also increased in the IT frogs from the levels found during the treatment period to those found in the control animals. When Cashins et al. (2013) treated experimentally infected frogs (\textit{Litoria booroolongensis}) with itraconazole and then re-exposed them to Bd, they found higher infection prevalence and intensity in frogs post-treatment than in frogs exposed only to Bd. These authors proposed an immunosuppressant effect of itraconazole treatment although this is not a recognised side effect of this drug in amphibians (Pessier & Mendelson 2010) or any other species (NOAH 2015). Itraconazole at concentrations of up to 0.08 µg/ml has been shown not to inhibit the growth of multiple symbiotic bacteria isolated from \textit{Rana sphenocephala} skin (Holden et al. 2014). However, this is a low concentration compared to the treatment used in our study and the study described by Cashins et al. (2013) (0.1 mg/ml). At higher concentrations itraconazole solutions are lower in pH which might result in skin irritation or osmotic dysfunction (Baitchman & Pessier 2013). Modifications such as reducing the itraconazole concentration (Jones et al. 2012; Brannelly 2014) or using an alkalising buffer (Brannelly et al. 2012), might help to reduce any such side effects. The similarity in infection rate estimates in post-treatment and control group animals in this study suggests that any post-treatment impact was not associated with changes to immune function or skin microflora and was not ecologically important.

Using the mean parameter estimates from the CMR analysis, our population models predict a delay of 75 weeks to population extinction for an itraconazole-treated population compared to an untreated population; i.e. an approximated 60% increase in time to extinction. Whilst in-situ itraconazole treatment at the intensity conducted in our study would not prevent population extinction, it would prolong the period until extinction, thus allowing time to implement other conservation measures, such as the establishment of an ex-situ conservation breeding population. The prevalence of - and the risk of contracting - Bd infection have been repeatedly shown to vary seasonally in response to environmental conditions (Kriger & Hero 2006; Longo et al. 2010). The increased time until extinction predicted by our population model for populations treated in-situ with itraconazole has the potential to maintain a susceptible population through seasonally high risk periods.

In the current study, itraconazole treatment was applied for only 15 weeks, which was insufficient time for the epidemic phase to come to an end and therefore high infection loads likely persisted in untreated syntopic animals throughout this period. Should treatment have continued beyond the epidemic phase, it is possible that a longer term benefit from itraconazole treatment, such as the prevention of population extinction, could have occurred as exposure rates and inoculation doses decreased and this would be worth investigating in other systems.

Previous studies have predicted the importance of Bd infection state in species detectability (Jenelle et al. 2007), with reduced recapture probability of infected animals in populations where Bd is endemic (Murray et al. 2009). Other studies have provided no evidence for a difference in recapture
rates of infected vs uninfected animals (Phillot et al. 2013), therefore this effect is likely species- and infection-load- specific. In our study, we found infection state to be an important predictor of detectability, but with higher recapture rates for infected animals. A possible reason for this difference from previous studies is that \textit{L. fallax} is a large bodied and highly territorial species (Martin et al. 2007), thus sick animals will be more easily detected than cryptic species such as tree frogs. Our field observations showed that \textit{L. fallax} frogs with clinical chytridiomycosis were lethargic, active during the day, aggregated in ponds, and displayed decreased capture avoidance (authors’ unpublished observations). It is possible that the increased recapture probability of infected animals may have increased the efficacy of itraconazole treatment, by increasing the likelihood of capture and, hence, treatment of infected animals. This is unlikely to be the case for all amphibian species.

We found that animals in the IT group had higher recapture probabilities than those in the control groups. At first, this seems to contradict our finding that infected animals were more likely to be recaptured than uninfected animals (with a higher proportion of the IT group being uninfected than the control groups). This result, however, appears to be due to a higher recapture probability of uninfected animals in the IT group compared to the control groups (Fig. 4). Itraconazole treatment has been reported to cause lethargy of some amphibians under laboratory conditions (Brannelly et al. 2012), but in these cases the drug doses were higher as they were administered daily compared to on average weekly in this study. Importantly, the apparent behavioural differences of animals in the IT and control groups did not impact survival sufficiently to negate from the increased survival resulting from itraconazole treatment.

The NBC group also had lower recapture probabilities than either the IT or the SWC group. This could be because animals were assigned to the NBC group after the other groups and the first animals caught and assigned to the IT and SWC groups might have been more territorial and, hence, more-easily detected, and recaptured.

There has been little research into the potential for the development of antifungal resistance by Bd. Such resistance has been widely reported in human fungal pathogens, including to triazoles, the group of fungicides which includes itraconazole (e.g. Kanafani & Perfect 2008). It is possible, therefore, that in-situ treatment with itraconazole could enhance the development of resistance to this drug in Bd, especially if, as is the case in the field, treatment protocols cannot be conducted rigorously and treatment regimens are suboptimal with Bd survival within the treated population.

5. Conclusions

Our study has shown that in-situ treatment of individual animals by immersion in an aqueous solution of itraconazole is an effective tool for reducing the chytridiomycosis-induced mortality rate in \textit{L. fallax} in the short term. This treatment, however, is highly labour intensive and limited to amphibian species for which recapture rates are relatively high.

A lack of capacity for captive assurance colonies for the large number of amphibian species at risk of decline should Bd reach naïve amphibian hotspots (Bielby et al. 2008) means alternative responses to the mitigation of Bd in-situ, such as anti-fungal treatment, are urgently required. The concurrent in-situ treatment of multiple endemic and sympatric species, such as those in Madagascar (where there is now evidence for Bd presence (Bletz et al. 2015)) and Sri-Lanka, could provide a more cost-effective treatment regimen and justify the high effort required.

Further work is urgently required to test the efficacy of new and existing treatments for chytridiomycosis in field settings. Field-trials such as ours should be replicated on species with different life histories and in systems where Bd infection is endemic. Modifications to the treatment protocol to include parallel electrolyte treatment (Baitchman & Pessier 2013; Brannelly et al. 2015),
and alterations in the concentration of itraconazole or the addition of pH buffers, should also be considered. New delivery methods for antifungals, and the use of longer-acting drugs if they become available, should be investigated to enable larger numbers of animals to be treated with lower effort over longer time periods.

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### Table 1. Multi-state mark recapture model selection table

Showing the top models (ΔQAICc<2), the next best models (ΔQAICc<7) and the general model (bottom row).

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<tr>
<th>Survival</th>
<th>Recapture</th>
<th>Dead recovery</th>
<th>Transition</th>
<th>QAICc</th>
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Figure 1. Map of Montserrat and Fairy Walk study site. The ghauts (steep sided valleys) of Montserrat with the study transect in Fairy Walk ghaut highlighted, downstream of the Fairy Walk spring on the East of Montserrat.
Figure 2. Weekly states of captured *L. fallax* by proportion of total number in the (a) stream water control group, (b) non-bath control group and (c) itraconazole treatment group. Higher levels of uninfected individuals are visible throughout the study in the itraconazole treatment group and a larger number of known extant individuals persist in that group at the end of the study.
Figure 3. Model averaged weekly multi-state mark recapture parameter estimates with unconditional standard errors. Estimates are shown for control groups and itraconazole treatment group (IT) during and after treatment. Abbreviations: uninfected state (U), infected state (I) and dead (D).
Figure 4. Model averaged estimates of recapture probability from the multi-state capture-mark-recapture model for infected and uninfected *L. fallax*. Estimates shown for the (a) stream water control group, (b) non-bath control and (c) itraconazole treatment group. No animals were allocated to the NBC group for the first two weeks of the study and so no estimates are shown.
Figure 5. Deterministic SIS models (with mortality) of the total individuals in each disease state (and total live) using model averaged parameter estimates generated by the multi-state mark-recapture modelling for the (a) control group (untreated population) and (b) itraconazole treatment group (itraconazole treated population).