1 **In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis**
2 **epidemic**
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23 **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

103 **1. Introduction**
104
105 Emerging infectious diseases are a growing threat to both humans and biodiversity globally (Daszak
106 et al. 2000; Morens and Fauci 2013). Three main strategies exist for the management of wildlife
107 disease: prevention of introduction, mitigation of impact, and eradication (Wobeser 2002).
108 Globalisation, with its increased rate and volume of trade and travel, means preventing the
109 introduction of novel diseases is increasingly difficult (Marano et al. 2007). Whilst neutralisation of
110 threats has long been considered a pre-requisite for successful wildlife conservation (Caughley
111 1994), the emergence of threats which cannot be negated pose a difficult challenge to conservation
112 managers. One example is amphibian chytridiomycosis, caused by the chytrid fungus
113 *Batrachochytrium dendrobatidis* (Bd), which is implicated in the rapid decline or extinction of over
114 200 amphibian species globally (Skerrat et al. 2007), and has been described as "the worst infectious
115 disease ever recorded among vertebrates in terms of the number of species impacted, and it's
116 propensity to drive them to extinction" (Amphibian Conservation Summit 2005). This rapid global
117 loss of amphibians is likely to have major implications for the environment (Whiles et al. 2006).
118
119 In the absence of in-situ mitigation for amphibian chytridiomycosis (Woodhams et al. 2011; Joseph
120 et al. 2013), the Amphibian Conservation Action Plan advocates the creation of Bd-free captive
121 populations for eventual release as a key conservation strategy (Gascon et al. 2007). Currently,
122 conservation practitioners rely on such captive assurance programmes to prevent species extinctions
123 (Mendelson et al. 2006), but this is only a short to medium term solution and Amphibian Ark
124 estimates that only around 50 species can be saved in this way (Zippel et al. 2011). Even so, zoos are
125 currently failing to prioritise species that are likely to require captive breeding programmes to
126 prevent their extinction (Dawson et al. 2015). There is, therefore, an urgent need to change the
127 research focus from the treatment of captive animals to in-situ mitigation (Scheele et al. 2014;
128 Harding et al. 2015).
129
130 A range of potential in-situ interventions to mitigate the impacts of chytridiomycosis have been
131 suggested, but so far these remain largely untested in the field (Berger & Skerrat 2012; Scheele et al.
132 2014). These include habitat manipulation to inhibit Bd (Scheele et al. 2014), reintroduction after
133 selection for resistance in captivity (Venesky et al. 2014), and in-situ use of antifungal treatments
134 (Berger and Skerrat, 2012). Some antifungal drugs, including itraconazole, are effective in the
135 treatment of Bd infection in captivity, but only following multiple daily applications (e.g. Forzan et al.
136 2008; Tamukai et al. 2011; Jones et al. 2012; Georoff et al. 2013; Brannelly et al. 2015). In addition to
137 being effective, the application of itraconazole is relatively easy, being via immersion in an aqueous
138 solution – albeit that repeated administration is required for successful treatment (Nichols &
139 Lamirande 2000). Whilst there have been some reported side-effects in certain species (Brannelly et
140 al. 2012; Brannelly 2014) and life stages (Garner et al. 2009; Woodhams et al. 2012), itraconazole is
141 considered to be the treatment of choice for amphibian chytridiomycosis (Holden et al. 2014).
142 Reducing the dose from 0.01% for 11 days to 0.0025% for 5 days has been shown to reduce side
143 effects while maintaining efficacy (Brannelly 2014). Bosch et al. (2015) described the eradication of
144 Bd from the wild Mallorcan midwife toad (*Alytes muletensis*) tadpoles by treating them with
145 itraconazole in captivity and returning them to the wild following chemical disinfection of their
146 breeding ponds and surrounding rocks. As other amphibians and vegetation were absent from the
147 disinfected sites, and as these were rock pools containing little organic matter (which rapidly
148 inactivates most disinfectants), this technique is unlikely to be transferable to many other species or
149 locations.
150
151 In-situ treatment regimens provide challenges in field settings due to, for example, large target
152 population sizes, low capture rates the potential of reinfection and the need for a continuous supply
153 of labour. As a result, previous studies have treated individuals with itraconazole in captivity prior to

154   re-release rather than treating them in-situ (Hardy et al. 2015). Environmental persistence of Bd
155   zoospores (Johnson & Speare 2003; 2005) and the possible presence of infected sympatric
156   amphibians (Daszak et al. 1999) mean animals treated in-situ would likely be exposed to Bd both
157   throughout and after the treatment period, increasing the likelihood of their extirpation (Retallick et
158   al. 2004; Mitchell et al. 2008). Antifungal treatment in a field setting, however, might enable treated
159   animals to persist by lowering their Bd infection load until the initial epidemic has passed (Briggs et
160   al. 2010; Vredenberg et al. 2010). There is some evidence that animals surviving the epidemic phase
161   persist by tolerating subsequent lower levels and frequencies of infection (Retallick et al. 2004;
162   Briggs et al. 2010). Also, repeated infection and clearance of Bd might allow the development of
163   resistance in some species (McMahon et al. 2014).
164
165   The Caribbean is a global hotspot of amphibian endemism, with 99% of the 197 species being
166   endemic (Fong et al. 2015), and it has the highest proportion (84%) of threatened amphibians within
167   a region (Stuart et al. 2008). One species, the mountain chicken frog (*Leptodactylus fallax*), has
168   suffered a precipitous decline due to chytridiomycosis (Magin 2003; Fa et al. 2010; Mountain
169   Chicken Recovery Programme 2014). *L. fallax* is classified as Critically Endangered on the IUCN Red
170   List of Threatened Species (Fa et al. 2010) and is restricted to only Dominica and Montserrat in the
171   Lesser Antilles. A 2005 survey found no evidence of Bd in amphibians on Montserrat (Garcia et al.
172   2007), but in January 2009 *L. fallax* mortality due to chytridiomycosis was first discovered on
173   Montserrat and this was rapidly followed by epidemic mortality across the island (Mountain Chicken
174   Recovery Programme 2014). The characteristically rapid rates of chytridiomycosis-driven declines
175   (Lips et al. 2006), such as those observed in *L. fallax*, limit the time available to react effectively.
176   Interventions that can reduce rates of decline can be valuable for providing extra time to implement
177   further conservation actions.
178
179   In this study we report the use of itraconazole treatment in a field setting in an attempt to mitigate
180   the impact of epidemic chytridiomycosis. We assess whether in-situ antifungal treatment is a
181   feasible and effective method for improving the survival of a critically endangered species
182   undergoing a precipitous decline due to epidemic chytridiomycosis. *L. fallax* is an ideal species to use
183   as a model for such in-situ treatment as it is a large territorial animal with predictable behaviours,
184   making it relatively easy to detect and individually identify. Also, the species has been studied for
185   over ten years on Montserrat, so there is a great deal of knowledge about its distribution,
186   abundance and behaviour and field sites were already established (Garcia et al. 2007; Martin et al.
187   2007). On Montserrat the presence of a sympatric amphibian fauna of species (*Eleutherodactylus
188   johnstonei* and *Rhinella marina*) able to carry Bd renders an in-situ treatment study realistic for
189   extrapolation to other species and regions where sympatric amphibians act as Bd reservoirs.
190   Effective treatment of chytridiomycosis in captive *L. fallax* using itraconazole has shown the drug to
191   be safe for this species (authors' unpublished observations). Finally, *L. fallax* has a voracious appetite
192   and requires large enclosures in captivity, therefore it is difficult and expensive to hold a large
193   enough captive population for a viable, long-term conservation breeding programme.
194
195   **2. Materials and methods**
196   **2.1. Study site**
197   Montserrat is a U.K. overseas territory in the Eastern Caribbean (16.45°N, 62.15°W). The centre of
198   the island comprises an active volcano which has been erupting regularly since 1995. As a
199   consequence *L. fallax* is restricted to a circa 17 km^2 mountainous area; the Centre Hills region which
200   is typified by montane rainforest and deep valleys (or ghauts – Fig. 1) (Young 2008).
201   The field site (Fairy Walk) is a forested relatively-shallow-sloped ghaut of approximately 1 km^2 on the
202   eastern flank of the Centre Hills at an approximate elevation of 250 m asl. Prior to 2009, Fairy Walk
203   was home to the highest known population density of *L. fallax* on Montserrat (Young, 2008) and, at

204 the commencement of this study, it contained the last remaining intact population following the
205 emergence of chytridiomycosis on the island in 2009.
206
207 **2.2. Study design**
208 The field experiment took place between August 2009 and January 2010. We visited Fairy Walk
209 three times a week for 24 weeks and surveyed a predefined 800 m transect along the stream (Fig. 1)
210 at a slow walking pace in a team of five. On each occasion the team caught all *L. fallax* seen within 5
211 m of the transect and recovered any dead animals. We individually marked all captured frogs using a
212 Passive Integrated Transponder (PIT) (11 mm x 2 mm, ID-100A Microtransponder, Trovan Ltd.),
213 which we subcutaneously implanted in the dorsum where retention rates are maximal (Blomquist et
214 al. 2008). We skin-swabbed each frog for Bd on every capture using a rayon-tipped swab (MW 100-
215 100, Medical Wire and Equipment Co.) three times across each of the following sites: ventral
216 abdomen, ventral thighs and calves, and plantar surfaces of both hind-feet. We assigned frogs to one
217 of three groups during the study: itraconazole treatment (IT), stream water control (SWC), and non-
218 bath control (NBC). On each capture, after skin-swabbing, we immersed each animal in the IT group
219 for 5 minutes in a 0.01% aqueous solution of itraconazole (Sporanox, Janssen Pharmaceuticals, Inc.),
220 prepared using stream water on site. We treated frogs in the SWC group similarly, but in stream
221 water without itraconazole. We immersed each frog within a new, disposable food-grade plastic bag.
222 We released frogs in the NBC group after swabbing with no further intervention.
223
224 During the first 2 weeks of the study, we randomly assigned animals to the IT and SWC groups at the
225 time of first capture, with a 2:1 bias towards treatment. From week 3, we assigned all further
226 captures to the NBC group. In order to examine any treatment-specific long term effect on survival
227 or infection rate, we discontinued treatments after 15 weeks, but continued to capture and skin-
228 swab re-sighted animals. We continued monitoring until week 24 when the study was prematurely
229 ended by a major volcanic eruption.
230
231 **2.3. Laboratory methods**
232 We refrigerated skin-swabs until transport to the laboratory where DNA was extracted using
233 methods adapted from Hyatt et al. (2007) (explained in Annex A). We diluted extracted DNA 1:10 in
234 molecular grade water and examined it for the presence of Bd DNA using a Bd-specific TaqMan real-
235 time PCR as described by Boyle et al. (2004) modified by the inclusion of bovine serum albumin to
236 reduce PCR inhibition (Gerland et al. 2010). We tested samples in duplicate, incorporating two
237 negative control wells containing laboratory grade distilled water and four positive controls (100, 10,
238 1, and 0.1 zoospore equivalents) in duplicate on each plate. A sample was considered positive if PCR
239 amplification occurred in both duplicates. If duplicates generated conflicting results, the samples
240 were re-run up to three times until matching results were obtained. If there was no consensus on
241 the third occasion, the sample was considered negative.
242
243 Quantification of Bd DNA in each well was determined as Bd genome equivalents (GEs) by
244 multiplying the real time PCR result by 120 (4 µl of 60 µl total elute used to make up the dilution
245 (x15) and 5 µl of 40 µl 1:10 dilution used in qPCR (x8) [15 x 8=120]).
246
247 **2.4. Bd infection intensity comparison**
248 In order to test whether itraconazole treatment significantly reduced Bd infection intensity, we used
249 a linear mixed effects model, with treatment group (control vs. IT) and time as fixed effects and frog
250 ID as a random effect. Infection intensity was log transformed prior to analysis as values ranged over
251 many orders of magnitude. Models were compared using AIC corrected for small sample size (AICc)
252 and if no model was overwhelmingly supported (Akaike weight > 0.95), models with a ΔAICc<7 were
253 considered for inference. Summed Akaike weight evidence ratios were used to assess variable
254 importance (Burnham and Anderson, 2002).

255

256 **2.5. Capture-mark-recapture analyses**

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

263

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

276

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

287

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

294

295 **2.6. Model selection and goodness of fit**

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

298

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

302

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

306
307 **2.7. Population modelling**
308 In order to predict how treatment with itraconazole would have affected the entire sampled
309 population had it been applied across all frogs in this study, we produced a deterministic population
310 model in a susceptible–infected–susceptible (SIS) framework using the transition and survival rate
311 estimates from the CMR modelling. We excluded any recruitment to the adult population as no
312 nests have been recorded on Montserrat since the onset of the chytridiomycosis epidemic. We
313 defined population extinction as population size below 1.
314
315 We produced two versions of this model for a population of 228 frogs (the number of unique
316 captures in this study). The first assumed that all frogs were treated at the same rate as the treated
317 frogs in this study using the model averaged CMR transition and survival rate estimates for the IT
318 group. We modelled the second population as untreated, using the model averaged CMR parameter
319 estimates for the control groups. We initiated the simulation with one infected individual. The
320 number of frogs in each state at each time step was calculated using the matrix below, following the
321 notation in Lebreton et al. (2009) in which $\varphi(1,2)$ indicates the rate of transition between state 1
322 and state 2.
323
324

325
$$\begin{pmatrix} nI \\ nS \\ nD \end{pmatrix}_{t+1} = \begin{pmatrix} \varphi(I,I) & \varphi(U,I) & 0 \\ \varphi(I,U) & \varphi(U,U) & 0 \\ \varphi(I,D) & \varphi(U,D) & 1 \end{pmatrix} \begin{pmatrix} nI \\ nS \\ nD \end{pmatrix}_{t}$$

326
327 where: $\varphi(I,I)$ = 1 - $\varphi(I,U)$ - $\varphi(I,D)$
328 and: $\varphi(U,U)$ = 1 - $\varphi(U,I)$ - $\varphi(U,D)$
329
330 In order to include model-averaged parameter uncertainty from the CMR models, we made two
331 further models for each group, the shortest and longest times to extinction. To make the lowest time
332 to extinction model we used the lower 95% CI estimate for the rate of loss of infection and the upper
333 95% CI estimates for infection and mortality rates. The opposite 95% CIs were used to make the
334 longest time to extinction model. We present only the mean model graphically.
335
336 **3. Results**
337 In total we made 1735 captures of 228 frogs. We caught frogs assigned to the IT group (841 captures
338 of 80 frogs) more often in both absolute terms and relative to the group size than frogs from the
339 SWC group (326 captures of 42 frogs) and the NBC group (482 captures of 106 frogs). The sex ratio
340 was circa 1:1 in each treatment group. Frogs with clinical signs of chytridiomycosis were found
341 throughout the study and in all groups.
342
343 By the end of the study, 22% (n=50) of the frogs had been found dead (SWC=21% (n=9), NBC=18%
344 (n=19), IT=28% (n=22)). The proportion of animals known to be extant was greatest in the IT group
345 throughout the study, and this was especially evident towards the end of the study period (Fig. 2).
346
347 Across the study we captured, and therefore treated, frogs in the IT group an average of 0.98
348 (SE=0.06, min=0.16, max=2.50) times per week.
349
350 **3.1. Skin swab Bd data**
351 During the study 67% of the 1735 skin swabs taken tested positive for Bd (SWC=84% (n=317),
352 NBC=80% (n=463), IT=64% (n=819)). Until the itraconazole treatment ended at week 15, frogs in the
353 IT group were more likely to test negative for Bd than frogs in the control groups, after which the
354 likelihood of testing negative became the same across all groups (Fig. 2). We captured only 13 frogs

355 which never tested positive for Bd. Eleven of these were in the NBC group and were captured only
356 once (n=8) or twice (n=3). The remaining two were in the IT group and were captured 3 and 16
357 times. Bd infected animals in the IT group had a lower infection intensity during treatment than
358 animals in the control group (IT: naïve mean=5666 GE, SE=1879; Control: naïve mean=71 607 GE,
359 SE=24 218). The top linear mixed model for the treatment period contained a group-time interaction
360 and received overwhelming support (Akaike weight=0.9997). This provided evidence that although
361 the Bd infection intensity of infected animals was similar in the IT and control groups at the start of
362 the study (IT=168.81 GE, SE=1.63; Control=87.46 GE, SE=1.44), the rate at which the infection
363 intensity increased was much greater in the control group (on the log scale: IT=0.015 GE/week,
364 SE=0.03; Control=0.138 GE/week, SE=0.02; Annex B). In the post-treatment period, the infection
365 intensity of infected animals in the IT group increased (IT: naïve mean=47 002 GE, SE=19 169) and
366 there was very weak evidence (summed Akaike weight =0.4041, evidence ratio=0.7) of a difference
367 with the control group animals in the same period (Control: naïve mean=69 480 GE, SE=57 678)
368 suggesting the benefit of treatment were lost after treatment ended (see Annex B).
369
370 **3.2. Multi-state CMR models**
371 The top models (ΔQAICc<7) are listed in Table 1. As no model received overwhelming support (top
372 model Akaike weight 0.293), model averaging was used to generate robust parameter estimates to
373 account for model variation. Grouping captures into weekly bins may have hidden heterogeneity in
374 the capture rate between groups, but we found no evidence for a significant difference in the mean
375 number of captures per week between groups (ANOVA: SWC: mean=1.12, SE=0.08; NBC:
376 mean=0.97, SE=0.04; IT: mean= 0.98 SE=0.06; $F_{(2,225)}$=1.598, MSE=0.236, p=0.205).
377
378 All of the most parsimonious models (Akaike weight >0) contained a difference in survival between
379 the IT and control groups, and between Bd infected and uninfected animals. There was moderate
380 support for no difference in the SWC and NBC groups (summed Akaike weight=0.969; evidence
381 ratio=31.3) and so, only one estimate of survival for the two control groups is presented. Model
382 averaged parameter estimates showed that itraconazole treatment increased the weekly survival
383 rate of Bd infected animals by 11.6% compared to animals in the control groups (IT = 0.903, 95% CI =
384 0.860-0.934; Control = 0.809, 95% CI = 0.764-0.841; Fig. 3). All of the most parsimonious models,
385 however, included a second estimate for the IT group when treatment ended: the estimate
386 decreased to a value similar to the control groups (0.795, 95% CI = 0.709-0.864). Uninfected animals
387 had a higher weekly survival rate than Bd infected animals in both the IT (0.988, 95% CI = 0.972-
388 0.995, effect size = 9.4%) and control groups (0.974, 95% CI = 0.939-0.987, effect size = 20.3%; Fig.
389 3).
390
391 Each of the most parsimonious models contained a difference in recapture rate between Bd infected
392 and uninfected animals, and with time dependency. The top models also contained a difference in
393 the recapture rate of the IT and control groups, with limited support for a difference in the NBC and
394 SWC groups (summed Akaike weight = 0.877; evidence ratio = 7.1). There was very weak support for
395 an interaction between infection state and treatment group (summed Akaike weight = 0.095,
396 evidence ratio = 0.1). As time dependent recapture probability was best supported, mean estimates
397 averaged across each occasion are presented (Fig. 4 - full results). Model averaged parameter
398 estimates showed that Bd infection increased recapture probability by a mean of 99.1% in the IT
399 group (Uninfected(U) = 0.354, Infected(I) = 0.711), 120% in the SWC group (U = 0.310, I = 0.686), and
400 136% in the NBC group (U = 0.270, I = 0.637). Based on these estimates, the recapture rate of Bd
401 infected animals in the IT group was 3.6% greater than the SWC group and 11.6% higher than the
402 NBC group. The recapture rate of uninfected animals in the IT group was 14.1% greater than the
403 SWC group and 31.1% higher than in the NBC group. The recapture rate of Bd infected animals in the
404 SWC group was 7.2% higher than in the NBC group and 14.8% higher in uninfected animals.
405

406 All of the most parsimonious models contained a difference in state transition rates (infection and
407 loss of infection) rates between the itraconazole treatment and control groups. There was very weak
408 support for a difference in the transition rates of the two control groups (summed Akaike weight =
409 0.043; evidence ratio < 0.1), and in the different sexes (summed Akaike weight = 0.142; evidence
410 ratio = 0.2). As a result one estimate for both control groups and sexes is presented. Itraconazole
411 treatment reduced the weekly infection rate of uninfected animals by 19.3% compared to the
412 control groups (IT = 0.208, 95% CI = 0.158-0.269: Control = 0.248, 95% CI = 0.185-0.330; Fig. 3).
413 Itraconazole treatment also increased the weekly rate of loss of Bd infection of infected animals by
414 161% compared to the control groups (IT= 0.338, 95% CI = 0.254-0.433: Control = 0.129, 95% CI =
415 0.088-0.177). All top models included a second estimate for transition rate for the itraconazole
416 treatment group when treatment ended, when infection rate increased to a similar level to the
417 control groups (IT= 0.298, 95% CI = 0.194-0.430) and rate of loss of infection declined to levels
418 similar to the control groups (IT = 0.083, 95% CI = 0.036-0.178; Fig. 3).
419
420 There was weak evidence for a treatment group difference in dead recovery rate (summed Akaike
421 weight = 0.271; evidence ratio = 0.3). The model averaged parameter estimate was 0.241 (95% CI =
422 0.163-0.340) across all three groups.
423
424 **3.3. Population models**
425 The deterministic SIS models indicate that if the entire sampled population had been treated with
426 itraconazole at the rate applied to frogs in the IT group, it would have survived an estimated 124
427 weeks (min = 79, max = 236) compared to 49 weeks (min = 33, max = 73) if no drug treatment had
428 been given. Consequently, treatment would have increased time until extinction by an estimated 75
429 weeks (min = 6, max = 203) (Fig. 5). This represents an estimated weekly survival of 95.7% for the
430 treated population compared to 89.4% for the untreated population.
431
432 **4. Discussion**
433 We used the emergence of amphibian chytridiomycosis in *L. fallax* on Montserrat as a model system
434 to investigate the feasibility and impact of in-situ treatment of the disease using the antifungal drug,
435 itraconazole. Our study shows that in-situ treatment of wild amphibians with itraconazole in the face
436 of epidemic chytridiomycosis decreased the mortality rate of infected animals and increased their
437 rate of loss of infection during the treatment period. Itraconazole treatment also reduced the
438 infection rate of animals in the IT group during the treatment period, providing evidence of a short
439 term prophylactic effect. On cessation of treatment, the benefits were lost and the rate of survival
440 and loss of infection regressed and the infection rate increased to those of untreated individuals. It
441 also suggests that, at least in *L. fallax*, repeated exposure to Bd and anti-fungal treatments does not
442 facilitate resistance through the development of an immune response.
443
444 McMahon et al. (2014) reported that relatively small numbers of repeated exposures to Bd followed
445 by clearances using heat treatment in captivity were sufficient to stimulate an immune response in
446 *Osteopilus septentrionalis* resulting in a reduced mortality rate. Other studies have presented
447 contradictory findings (Stice & Briggs 2010; Cashins et al. 2013; Fites et al. 2013), and it appears
448 unlikely that this immuno-protective effect, if it does occur, can be stimulated in all species.
449
450 The decreased mortality rate conferred by itraconazole treatment in our study is encouraging
451 considering each frog was treated on average just once a week. This is a substantially lower
452 treatment rate than the once-daily treatment used in laboratory studies and recommended for
453 captive animals (Pessier & Mendelson 2010).
454
455 There was no difference in survival or infection state transition rates between the two control
456 groups, providing assurance that the physical action of handling and immersing frogs did not cause

457 stress sufficient to contribute to mortality or infection. This is important as there are limited
458 methods for the targeted delivery of antifungal compounds for *L. fallax* or for the application of this
459 technique to other amphibian species (Scheele et al. 2014). Hardy et al. (2015) recorded a prolonged
460 decrease in Bd prevalence and an increase in overwinter survival in *Rana cascadae* treated with
461 itraconazole in captivity prior to release into the wild. Although the pharmacokinetics of the drug
462 have not been studied in amphibians, these authors proposed that the itraconazole might have
463 persisted in the skin long enough for another mechanism of resistance to develop, but there is no
464 evidence for this (e.g. Cashins et al. 2013). In our study, itraconazole provided no prophylactic
465 protection from Bd infection beyond the treatment period.
466
467 During the post-treatment period, the infection rate in the IT group increased from that seen in the
468 treatment period to that seen in the control groups. The Bd infection intensity also increased in the
469 IT frogs from the levels found during the treatment period to those found in the control animals.
470 When Cashins et al. (2013) treated experimentally infected frogs (*Litoria booroolongensis*) with
471 itraconazole and then re-exposed them to Bd, they found higher infection prevalence and intensity
472 in frogs post-treatment than in frogs exposed only to Bd. These authors proposed an
473 immunosuppressant effect of itraconazole treatment although this is not a recognised side effect of
474 this drug in amphibians (Pessier & Mendelson 2010) or any other species (NOAH 2015). Itraconazole
475 at concentrations of up to 0.08 μg/ml has been shown not to inhibit the growth of multiple
476 symbiotic bacteria isolated from *Rana sphenocephala* skin (Holden et al. 2014). However, this is a
477 low concentration compared to the treatment used in our study and the study described by Cashins
478 et al. (2013) (0.1 mg/ml). At higher concentrations itraconazole solutions are lower in pH which
479 might result in skin irritation or osmotic dysfunction (Baitchman & Pessier 2013). Modifications such
480 as reducing the itraconazole concentration (Jones et al. 2012; Brannelly 2014) or using an alkalising
481 buffer (Brannelly et al. 2012), might help to reduce any such side effects. The similarity in infection
482 rate estimates in post-treatment and control group animals in this study suggests that any post-
483 treatment impact was not associated with changes to immune function or skin microflora and was
484 not ecologically important.
485
486 Using the mean parameter estimates from the CMR analysis, our population models predict a delay
487 of 75 weeks to population extinction for an itraconazole-treated population compared to an
488 untreated population; i.e. an approximated 60% increase in time to extinction. Whilst in-situ
489 itraconazole treatment at the intensity conducted in our study would not prevent population
490 extinction, it would prolong the period until extinction, thus allowing time to implement other
491 conservation measures, such as the establishment of an ex-situ conservation breeding population.
492 The prevalence of - and the risk of contracting - Bd infection have been repeatedly shown to vary
493 seasonally in response to environmental conditions (Kriger & Hero 2006; Longo et al. 2010). The
494 increased time until extinction predicted by our population model for populations treated in-situ
495 with itraconazole has the potential to maintain a susceptible population through seasonally high risk
496 periods.
497
498 In the current study, itraconazole treatment was applied for only 15 weeks, which was insufficient
499 time for the epidemic phase to come to an end and therefore high infection loads likely persisted in
500 untreated syntopic animals throughout this period. Should treatment have continued beyond the
501 epidemic phase, it is possible that a longer term benefit from itraconazole treatment, such as the
502 prevention of population extinction, could have occurred as exposure rates and inoculation doses
503 decreased and this would be worth investigating in other systems.
504
505 Previous studies have predicted the importance of Bd infection state in species detectability (Jenelle
506 et al. 2007), with reduced recapture probability of infected animals in populations where Bd is
507 endemic (Murray et al. 2009). Other studies have provided no evidence for a difference in recapture

508   rates of infected vs uninfected animals (Phillot et al. 2013), therefore this effect is likely species- and
509   infection-load- specific. In our study, we found infection state to be an important predictor of
510   detectability, but with higher recapture rates for infected animals. A possible reason for this
511   difference from previous studies is that *L. fallax* is a large bodied and highly territorial species
512   (Martin et al. 2007), thus sick animals will be more easily detected than cryptic species such as tree
513   frogs. Our field observations showed that *L. fallax* frogs with clinical chytridiomycosis were lethargic,
514   active during the day, aggregated in ponds, and displayed decreased capture avoidance (authors'
515   unpublished observations). It is possible that the increased recapture probability of infected animals
516   may have increased the efficacy of itraconazole treatment, by increasing the likelihood of capture
517   and, hence, treatment of infected animals. This is unlikely to be the case for all amphibian species.
518
519   We found that animals in the IT group had higher recapture probabilities than those in the control
520   groups. At first, this seems to contradict our finding that infected animals were more likely to be
521   recaptured than uninfected animals (with a higher proportion of the IT group being uninfected than
522   the control groups). This result, however, appears to be due to a higher recapture probability of
523   uninfected animals in the IT group compared to the control groups (Fig. 4). Itraconazole treatment
524   has been reported to cause lethargy of some amphibians under laboratory conditions (Brannelly et
525   al. 2012), but in these cases the drug doses were higher as they were administered daily compared
526   to on average weekly in this study. Importantly, the apparent behavioural differences of animals in
527   the IT and control groups did not impact survival sufficiently to negate from the increased survival
528   resulting from itraconazole treatment.
529
530   The NBC group also had lower recapture probabilities than either the IT or the SWC group. This could
531   be because animals were assigned to the NBC group after the other groups and the first animals
532   caught and assigned to the IT and SWC groups might have been more territorial and, hence, more-
533   easily detected, and recaptured.
534
535   There has been little research into the potential for the development of antifungal resistance by Bd.
536   Such resistance has been widely reported in human fungal pathogens, including to triazoles, the
537   group of fungicides which includes itraconazole (e.g. Kanafani & Perfect 2008). It is possible,
538   therefore, that in-situ treatment with itraconazole could enhance the development of resistance to
539   this drug in Bd, especially if, as is the case in the field, treatment protocols cannot be conducted
540   rigorously and treatment regimens are suboptimal with Bd survival within the treated population.
541
542   **5. Conclusions**
543   Our study has shown that in-situ treatment of individual animals by immersion in an aqueous
544   solution of itraconazole is an effective tool for reducing the chytridiomycosis-induced mortality rate
545   in *L. fallax* in the short term. This treatment, however, is highly labour intensive and limited to
546   amphibian species for which recapture rates are relatively high.
547
548   A lack of capacity for captive assurance colonies for the large number of amphibian species at risk of
549   decline should Bd reach naïve amphibian hotspots (Bielby et al. 2008) means alternative responses
550   to the mitigation of Bd in-situ, such as anti-fungal treatment, are urgently required. The concurrent
551   in-situ treatment of multiple endemic and sympatric species, such as those in Madagascar (where
552   there is now evidence for Bd presence (Bletz et al. 2015)) and Sri-Lanka, could provide a more cost-
553   effective treatment regimen and justify the high effort required.
554
555   Further work is urgently required to test the efficacy of new and existing treatments for
556   chytridiomycosis in field settings. Field-trials such as ours should be replicated on species with
557   different life histories and in systems where Bd infection is endemic. Modifications to the treatment
558   protocol to include parallel electrolyte treatment (Baitchman & Pessier 2013; Brannelly et al. 2015),

559 and alterations in the concentration of itraconazole or the addition of pH buffers, should also be
560 considered. New delivery methods for antifungals, and the use of longer-acting drugs if they become
561 available, should be investigated to enable larger numbers of animals to be treated with lower effort
562 over longer time periods.
563
570
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862 **Tables**

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864 **Table 1. Multi-state mark recapture model selection table** showing the top models (ΔQAICc<2), the
865 next best models (ΔQAICc<7) and the general model (bottom row).

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867 Abbreviations: no group or time variation (.), infection state (inf), all group difference (gr), difference between
868 treatment and control groups (gr[C]), with two estimates for the treatment group: one during and one post-
869 treatment gr[splitT], time, sex, difference in AIC between selected model and top model (ΔQAICc), the QAICc
870 weight (W), the number of parameters (K). The AIC score is corrected for small sample size (AICc) and an
871 adjusted variance-inflation factor to account for slight-overdispersion (QAICc).

Survival	Recapture	Dead recovery	Transition	QAICc	ΔQAICc	W	K	QDeviance
inf+gr[C+splitT]	inf+gr+time	.	inf*gr[C+split T]	5057.429	0.000	0.296	37	4978.787
inf+gr[C+splitT]	inf+gr+time	gr	inf*gr[C+split T]	5057.937	0.508	0.230	38	4981.433
inf+gr[C+splitT]	inf+gr+time	.	inf*gr[C+split T]+sex	5059.732	2.274	0.095	38	4981.061
inf+gr[C+splitT]+sex	inf+gr+time	.	inf*gr[C+split T]	5059.857	2.429	0.088	38	4981.216
inf+gr[C+splitT]	inf*gr+time	.	inf*gr[C+split T]	5060.888	3.459	0.052	39	4980.104
inf+gr[C+splitT]	Inf+gr+time	.	inf*gr[split T]	5061.289	3.861	0.043	39	4980.506
inf+gr[C+splitT]	inf+gr[C]+time	.	inf*gr[C+split T]	5061.697	4.268	0.035	36	4985.193
inf+gr[C+splitT]	inf+gr[C]+time	gr	inf*gr[C+split T]	5062.263	4.834	0.026	37	4987.893
inf+gr[C+splitT]	inf*gr+time	.	inf*gr[C+split T]+sex	5062.526	5.097	0.023	40	4979.598
inf+gr[C+splitT]+sex	inf*gr+time	.	inf*gr[C+split T]	5062.776	5.347	0.020	40	4979.847
inf+gr[C+splitT]	inf+gr[C]+time	.	inf*gr[C+split T]	5063.183	5.754	0.017	37	4986.679
inf+gr[splitT]	inf+gr+time	gr	inf*gr[C+split T]	5063.433	6.004	0.015	42	4976.202
inf+gr[splitT]	inf+gr+time	.	inf*gr[C+split T]+sex	5063.483	6.055	0.014	41	4978.406
inf+gr[C+splitT]	inf+gr[C]+time	.	inf*gr[C+split T]+sex	5064.112	6.683	0.010	37	4987.608
inf*time	inf*gr+time	gr+time	inf*gr+time	5186.711	110.834	0.0000	124	5645.1061

872 **Figures**

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874 **Figure 1**

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877 **Figure 1. Map of Montserrat and Fairy Walk study site**. The ghauts (steep sided valleys) of
878 Montserrat with the study transect in Fairy Walk ghaut highlighted, downstream of the Fairy Walk
879 spring on the East of Montserrat.
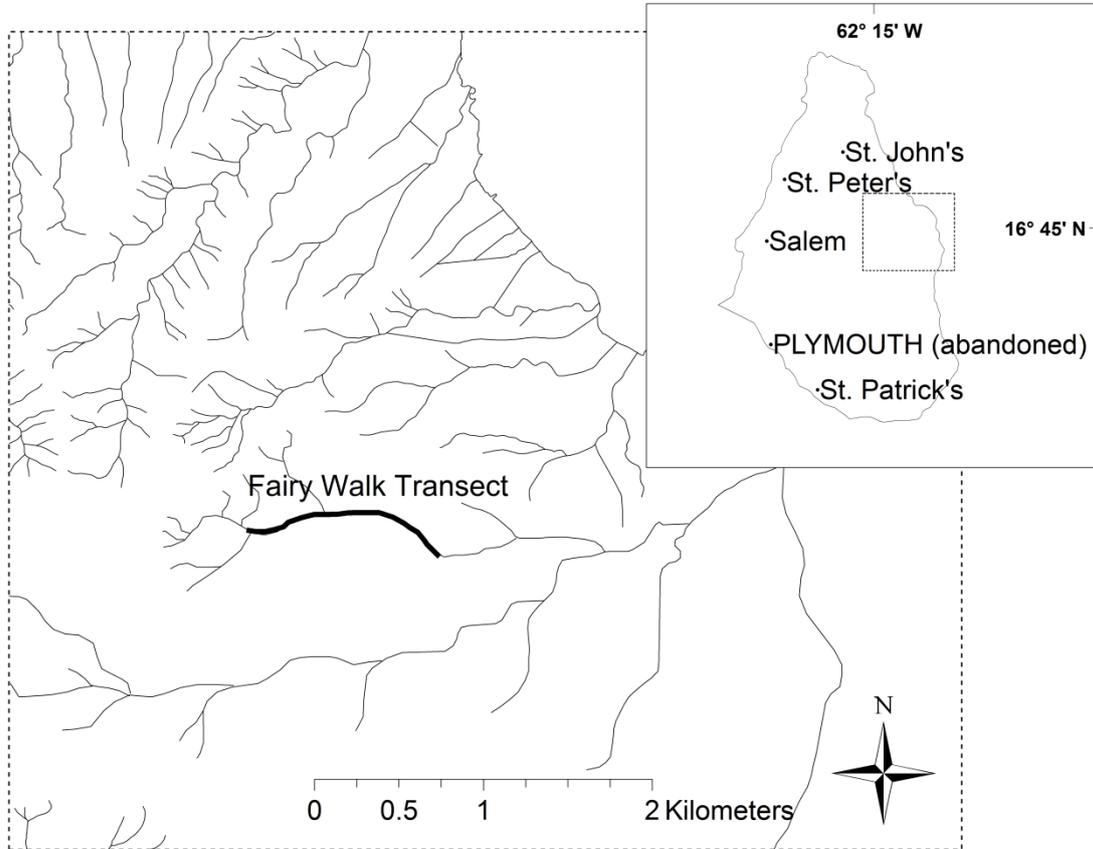
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898   **Figure 2**
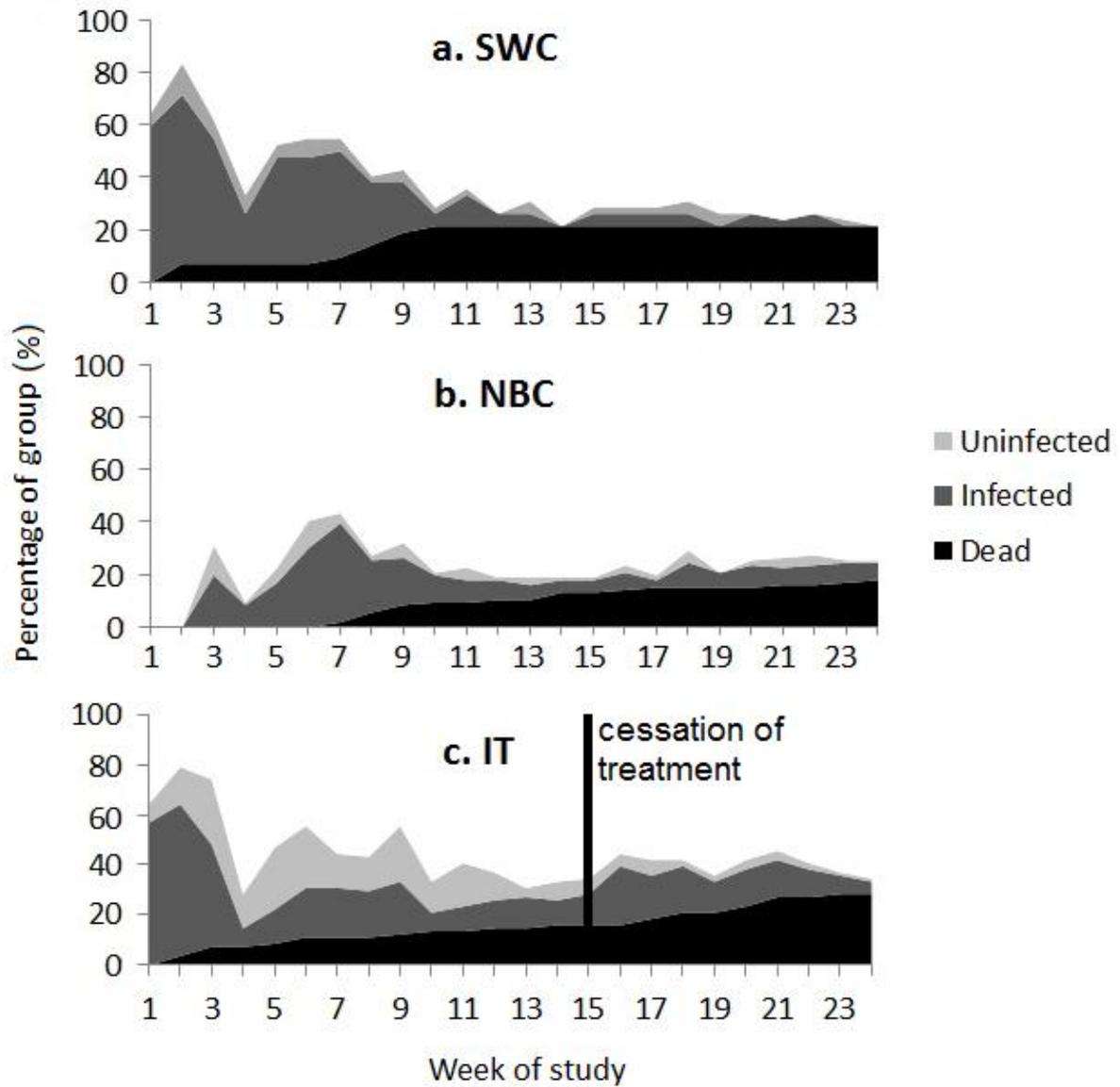
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900   **Figure 2. Weekly states of captured *L. fallax*** by proportion of total number in the (a) stream water
901   control group, (b) non-bath control group and (c) itraconazole treatment group. Higher levels of
902   uninfected individuals are visible throughout the study in the itraconazole treatment group and a
903   larger number of known extant individuals persist in that group at the end of the study.
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915 **Figure 3**
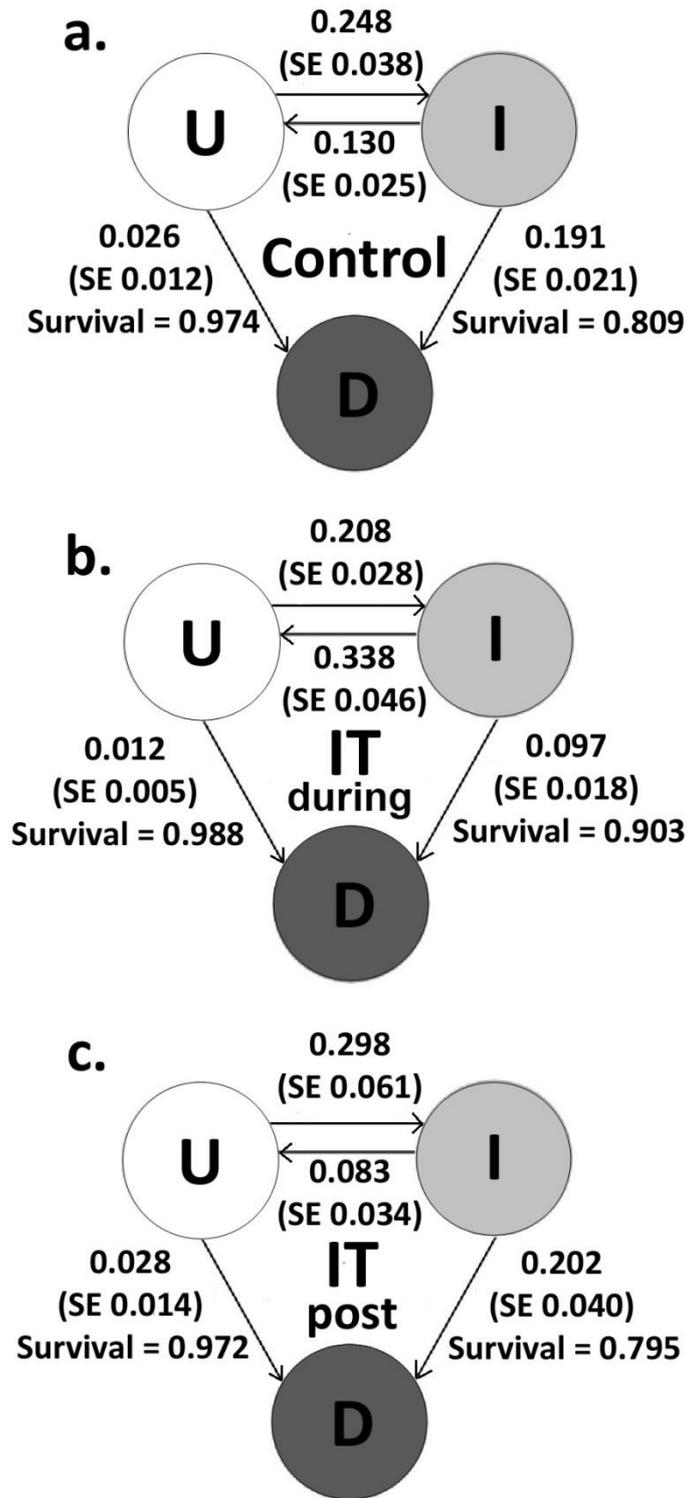
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917 **Figure 3. Model averaged weekly multi-state mark recapture parameter estimates** with
918 unconditional standard errors. Estimates are shown for control groups and itraconazole treatment
919 group (IT) during and after treatment. Abbreviations: uninfected state (U), infected state (I) and
920 dead (D).
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922 **Figure 4**
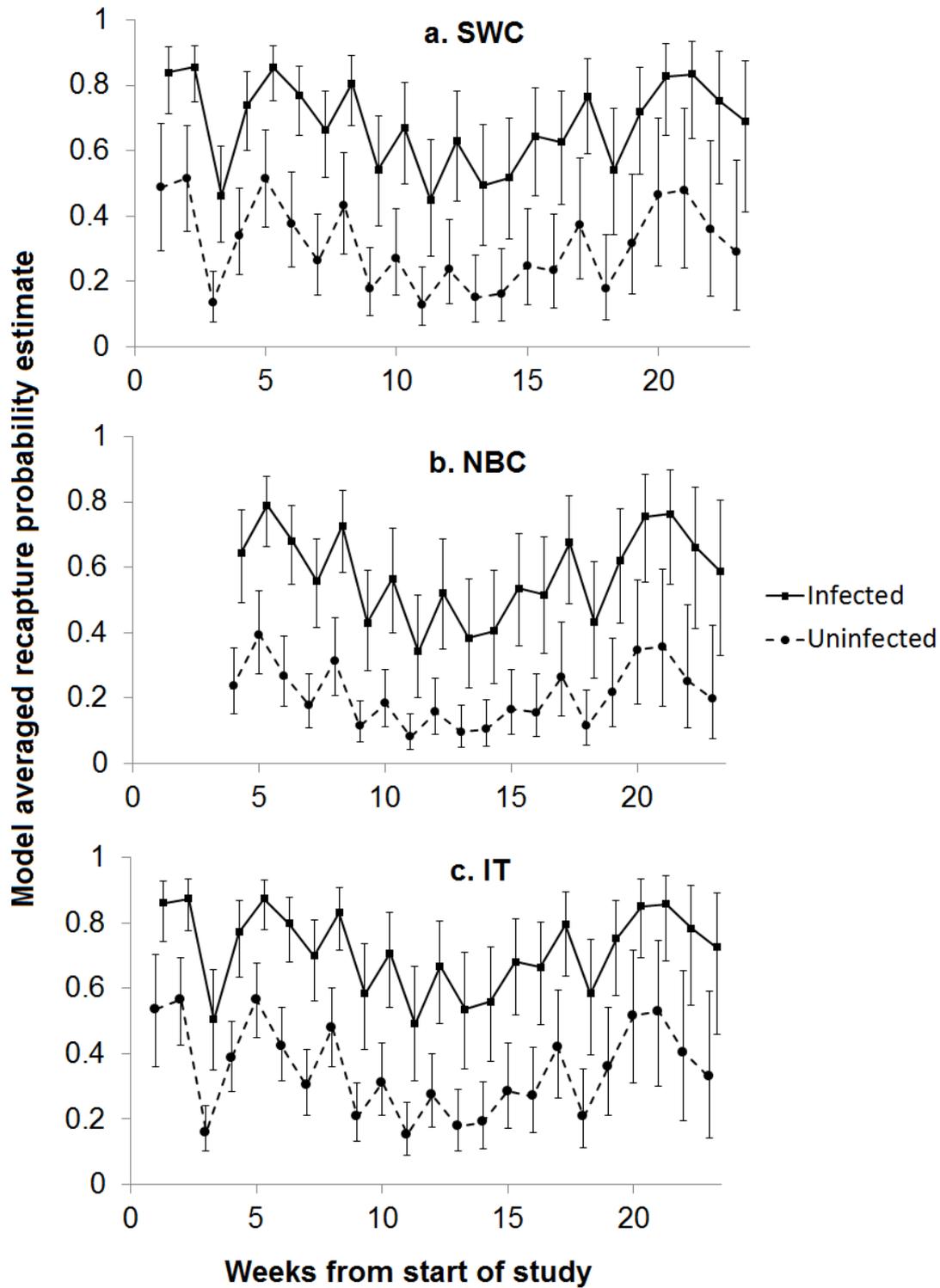
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924 **Figure 4. Model averaged estimates of recapture probability** from the multi-state capture-mark-
925 recapture model for infected and uninfected *L. fallax.* Estimates shown for the (a) stream water
926 control group, (b) non-bath control and (c) itraconazole treatment group. No animals were allocated
927 to the NBC group for the first two weeks of the study and so no estimates are shown.

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930   **Figure 5**

a. Untreated population

b. Itraconazole treated population
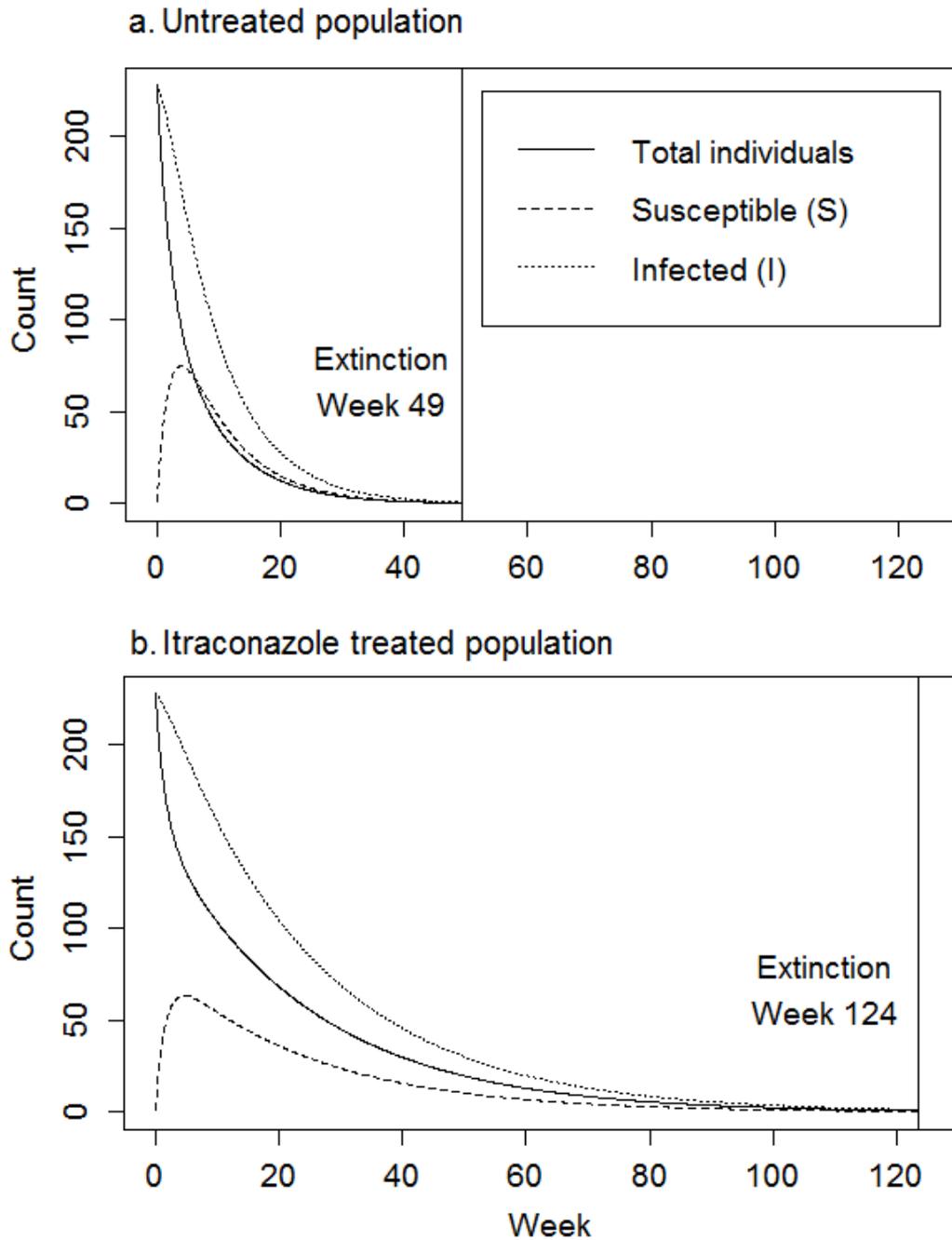
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932   **Figure 5. Deterministic SIS models** (with mortality) of the total individuals in each disease state (and
933   total live) using model averaged parameter estimates generated by the multi-state mark-recapture
934   modelling for the (a) control group (untreated population) and (b) itraconazole treatment group
935   (Itraconazole treated population).