MR. THOMAS J BURNS (Orcid ID : 0000-0003-0408-8014)

DR. BEN SCHEELE (Orcid ID : 0000-0001-7284-629X)

DR. LAURA BRANNELLY (Orcid ID : 0000-0003-2975-9494)

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Indirect terrestrial transmission of amphibian chytrid fungus from reservoir to susceptible host species leads to fatal chytridiomycosis

Thomas J. Burns[1]*, Ben C. Scheele[2], Laura A. Brannelly[3], Nick Clemann[4], Deon Gilbert[5], Don A. Driscoll[1]

[1]Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University Geelong, Burwood, 3125, Australia

[2]Fenner School of Environment and Society, Australian National University, Canberra, 2601 Australia

[3]Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Werribee, 3030, Australia

[4]Arthur Rylah Institute for Environmental Research, Department of Environment, Land, Water and Planning, PO Box 137, Heidelberg, 3084, Australia

[5]Wildlife Conservation and Science. Zoos Victoria, Parkville, 3052, Australia

*Address for correspondence: Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, (Burwood Campus), Geelong, Australia. Email: tomb-09@hotmail.co.uk, d.driscoll@deakin.edu.au

Abstract

The amphibian chytrid fungal pathogen (*Batrachochytrium dendrobatidis,* henceforth *Bd*) has had a devastating impact on biodiversity, causing the decline or extinction of over 500 amphibian species. Yet, our understanding of *Bd* transmission pathways remains incomplete, in particular for host species with weak aquatic associations, and between reservoir and susceptible host species. We examined *Bd* transmission from a potential reservoir host to a potentially susceptible critically endangered host; directly assessing the capacity of the former to transmit *Bd,* and the susceptibility of the latter as a *Bd* host. Using cohousing versus sequential use of the same enclosure by the two species, we distinguished the effects of direct versus indirect (environmental) transmission. Our study provides clear evidence that both direct and indirect terrestrial transmission from a reservoir to susceptible host species results in fatal chytridiomycosis. Transmission mode had no effect on overall morbidity or disease progression in the susceptible species. Our results demonstrate that reservoir and susceptible hosts do not need to be in the same place at the same time, or within an aquatic environment for transmission to occur. Our demonstration of indirect terrestrial transmission from a reservoir to susceptible host identifies mechanisms by which *Bd* may drive ongoing declines in populations where the pathogen is now endemic. Identifying these transmission pathways is important for understanding long-term extinction vulnerability of remnant populations of declining species challenged by disease.

Keywords: *Batrachochytrium dendrobatidis*, environmental transmission, itraconazole treatment, *Crinia signifera*, *Philoria frosti,* species decline, transmission pathways, chytrid

1. Introduction

Pathogens are a substantial and increasing threat to biodiversity (Daszak, Cunningham, & Hyatt, 2000; Fisher et al., 2012). The global spread of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has resulted in catastrophic amphibian declines across all continents in which amphibians occur, with the exception of Asia, the likely origin of the pathogen (O'Hanlon et al., 2018; Scheele et al., 2019b). *Batrachochytrium dendrobatidis* has had the greatest documented negative impact on biodiversity of any single pathogen, causing the decline or extinction of 500 amphibian species (Scheele et al., 2019b). In regions where *Bd* has spread and is now endemic, amphibian species that experienced initial declines exhibit a range of long-term responses, including population recovery, stabilisation and ongoing decline (Scheele et al., 2017b, 2019b; Whitfield et al., 2017). Although up to 90 species may have already become extinct due to *Bd,* preventing further extinctions is dependent on our ability to identify species experiencing ongoing declines, mechanisms driving declines, and implementing effective conservation interventions.

Community composition can influence disease dynamics and the risks to susceptible species within multi-host pathogen systems. *Batrachochytrium dendrobatidis* disease dynamics are complicated because impacts on amphibians vary with host species, population, environment and *Bd* lineage (Van Rooij et al., 2015; Lips, 2016). Among hosts, there exists a spectrum, from species that are highly susceptible to those that are tolerant or resistant to *Bd* (Van Rooij et al., 2015). Greater host diversity has been associated with a dilution of *Bd* risk in amphibian communities (Searle et al., 2011; Becker et al., 2014; Han et al., 2015), while the presence of *Bd* reservoir hosts may exacerbate disease impacts by maintaining high levels of a pathogen within a system even as the density of susceptible host species declines (Scheele et al., 2017a; Brannelly et al., 2018).

In the context of *Bd*, competent reservoir hosts are species that remain infected and shed infectious zoospores, but do not develop clinical symptoms of infection (Brannelly et al., 2018). The probability of spill-over from reservoir to susceptible host species is highly dependent upon the habitat overlap of reservoir and susceptible hosts, and fine-scale *Bd* transmission pathways, of which there is currently limited understanding (Garner, 2018). Although tadpoles can act as within-host *Bd* reservoirs (Briggs, Knapp, & Vredenburg, 2010), reservoir hosts are generally considered with reference to inter-specific transmission. While *Bd* reservoir host species have been suggested for several systems (Stockwell et al., 2016; Yap et al., 2018; Hudson et al., 2019), few studies have examined this phenomenon in detail (Reeder, Pessier, & Vredenburg, 2012; Scheele et al., 2017a; Brannelly et al., 2018). Consequently, there is a major knowledge gap surrounding the capacity for potential reservoir host species to transmit *Bd* infections to susceptible hosts, the transmission pathways through which this may occur, and how reservoir hosts may influence disease progression.

In Australia, *Bd* is implicated in the decline or extinction of 43 species, six of which are experiencing ongoing declines and are at high risk of extinction (Skerratt et al., 2016; Scheele et al., 2017b). Adults of three of these six species primarily or entirely use terrestrial environments (the southern corroboree frog, *Pseudophryne corroboree*; northern corroboree frog, *Pseudophryne pengilleyi*; and the Baw Baw frog, *Philoria frosti*). Low adult aquatic association is not an ecology typically associated with amphibian declines linked to Bd (Lips, Reeve, & Witters, 2003; Bielby et al., 2008; Murray et al., 2011). However, some terrestrial species have been noted to experience *Bd* mediated population loss (Valenzuela-Sánchez et al., 2017) or reduced survivorship (Longo & Burrowes, 2010) in the absence of high *Bd* prevalence or a *Bd* epidemic. The abundant and widespread common eastern froglet, *Crinia signifera*, is implicated as a *Bd* reservoir host in the decline of the three aforementioned Australian species (Brannelly et al., 2018). In the region, *C. signifera* populations have high *Bd* prevalence, but have shown no signs of population decline or demographic impacts (Brannelly et al., 2018). In captivity, infected *C. signifera* remain *Bd* positive for a prolonged period

without developing clinical signs of chytridiomycosis (Scheele et al., 2017a; Brannelly et al., 2018). However, while it is clear that *C. signifera* is a competent reservoir host, transmission pathways that might result in spill-over into sympatric species have not been investigated, indicative of the broader knowledge gap surrounding *Bd* transmission pathways.

Our study species, the critically endangered Baw Baw frog *Philoria frosti*, is experiencing ongoing declines that commenced in the late 1980s/early 1990s (Hollis, 2004, 2011), coinciding with the spread of *Bd* through south-eastern Australia. Once abundant within their limited range, populations have declined by up to 98%, disappearing entirely from sub-alpine wetland areas occupied by populations of *C. signifera* (Hollis, 2004, 2011; Brannelly et al., 2018). Populations now remain only in lower elevation montane forest gullies, where *C. signifera* have not been recorded. Against the imminent threat of extinction in the wild, captive assurance populations and a captive breeding program were initiated in 2011 (Hunter et al., 2018). Given the temporal and geographical pattern of decline, *Bd* is the presumed primary threat to this species. However, their susceptibility to *Bd* and the likelihood of disease transmission from *C. signifera* remains unquantified.

In this study, we examined *Bd* transmission from a reservoir host (*C. signifera*) to a susceptible host (*P. frosti)*, assessing directly the capacity of the former to transmit *Bd,* and the susceptibility of the latter as a *Bd* host. We quantified both frog-to-frog (direct) and environmental (indirect) *Bd* transmission pathways. We also assessed the effectiveness of a common *Bd* treatment, itraconazole bathing, on *Bd* positive *P. frosti* displaying clinical signs of chytridiomycosis. It is important to determine the efficacy of *Bd* treatments as an emergency options in case of disease outbreaks in the captive breeding colony (Berger et al., 2010).

By examining different modes of transmission from a reservoir to a susceptible host species, we provide new insights into a poorly understood element of *Bd* disease dynamics. We demonstrate that species need not come into direct contact or have high aquatic associations for transmission to occur. Our work has application in the management of species threatened by *Bd*, particularly those experiencing ongoing declines in which transmission pathways and the role of other host species are unclear.

2. Methods

2.1 Husbandry

Seventy juvenile *P. frosti* (snout to vent length, SVL mean = 26.6mm, range = 16–33.8mm) from a captive population at Zoos Victoria's Melbourne Zoo, Parkville, Victoria were used in experiments. Animals were from two egg masses wild collected at the same oviposition site in the Mount Baw

Baw area in upland south-eastern Australia and raised in captivity under *Bd* sterile conditions for 34 months. These animals were surplus to the captive breeding program. Thirty-six adult *C. signifera* (SVL mean = 21.6mm, range = 13.4–28.2mm) were wild-captured from the Mount Baw Baw area, within the range of *P. frosti*.

Philoria frosti were housed individually for a minimum one-week acclimation period. *Crinia signifera* were cohoused in groups of four to six during a two-week acclimation period. We tested all animals for *Bd* (see 2.4 *Bd* testing) before the start of the experiment; all *P. frosti* were *Bd* negative and all *C. signifera Bd* positive.

Animals were housed in a 12:12 hour photoperiod, temperature and humidity controlled room (16.1±0.9 SD°C and 63.8±7.4 SD% relative humidity), within polypropylene enclosures (small enclosure, 19.5x12.5x11.5cm or large enclosure, 27.5x20.5x11.5cm) with a damp substrate (detailed in 2.2) and black plastic hide(s). We misted enclosures with reverse osmosis filtered water every one to three days, ensuring the substrate remained damp, but without standing water. Animals were visually monitored each day for signs of infection (detailed in Baitchman and Pessier, 2013; see Appendix S2 for detailed description of observations), fed 2-3 small (<10mm) gut loaded, calcium dusted crickets twice weekly and once a week inspected in hand as above, weighed and tested for *Bd*. Enclosures, substrate and hides were changed weekly. Enclosures were autoclaved and then washed prior to re-use.

2.2 Chytrid transmission experiments

We allocated 53 *P. frosti* into one of three experiments haphazardly: (1) shared terrestrial enclosure (*n*=12 *Bd* exposure, *n*=9 control), (2) shared aquatic enclosure (*n*=8 *Bd* exposure, *n*=8 control), and (3) indirect terrestrial transmission (*n*=8 *Bd* exposure, *n*=8 control). For a diagrammatic representation of the experimental design see Figure 1.

In the shared terrestrial enclosure experiment, *P. frosti* in the *Bd* exposure group were housed with *C. signifera* in mixed species pairs in small terrestrial enclosures with a paper towel substrate and hide, while the control *P. frosti* were held alone, but otherwise in the same conditions. In the shared aquatic enclosure experiment, *P. frosti* in the *Bd* exposure group were housed in mixed species pairs in large aquatic enclosures. These held 1cm of reverse osmosis filtered water and two terrestrial retreats (11cm diameter petri dish with a damp paper towel substrate), each covered by a hide. In this experiment a central divider, intended to allow the movement of water but not frogs, initially separated species. However, after several dividers failed within the first 24 hours, we removed these from all aquatic enclosures allowing the two species to share the entire enclosure for the remainder

of the experiment. *Philoria frosti* in the control group of the shared aquatic experiment were housed alone in small aquatic enclosures with a single terrestrial retreat and hide. Animals in the shared-enclosure experiments were housed this way throughout the experiment.

In the indirect terrestrial transmission experiment, we housed *P. frosti* in the *Bd* exposure group individually (for seven days) in small enclosures previously occupied by two *Bd* positive *C. signifera* (for five days). Enclosures held a living moss substrate and hide. *Philoria frosti* in the control group were housed individually (for seven days) in identical small enclosures that had been empty for five days. *Philoria frosti* from both *Bd* exposure and control groups were then transferred back to small individual enclosures with a paper towel substrate and hide for the remainder of the experiment.

The experimental endpoint for all *Bd* exposed animals in transmission experiments was morbidity, defined here as a delay or lack of righting response. When observed (alongside other clinical symptoms; see Appendix S2), animals were humanely killed by bathing in a neutral buffered 5g/L solution of MS-222 (Sigma-Aldrich, St. Louis, MO, USA). Control group animals were humanely killed at the end of the experiment; once no *Bd* exposed animals remained within their respective experiment.

2.3 Itraconazole treatment

We allocated a separate group of 17 *P. frosti* into *Bd* exposure (*n*=10) or control (*n*=7) itraconazole treatment groups haphazardly (Figure 1). *Bd* exposure group animals were inoculated with cultured chytrid zoospores (strain, MittaMitta-Lspenceri-2018-LB at P1). Zoospores were grown on TGHL agar plates for five days at 22°C. For harvest, plates were flooded with 2 mL of distilled deionised water and zoospores collected using a sterile pipette. Approximately $1.5x10^7$ cultured *Bd* zoospores, determined by counting using a haemocytometer, in 1.65mL of reverse osmosis filtered water, were poured across each animal's ventrum into a small inoculation enclosure (9x5.5x6cm) below containing 15mL of reverse osmosis filtered water. Animals were housed within inoculation enclosures for 14 hours, then transferred back to small individual enclosures with a paper towel substrate and hide for the remainder of the experiment. Control group animals were not inoculated, but were housed in identical small individual enclosures throughout the experiment.

Treatment to clear *Bd* infection in *P. frosti* commenced two weeks after we had inoculated *Bd* exposure group animals with cultured zoospores. *Bd* exposure and control group animals were bathed in a small zip-lock bag containing 20mL of itraconazole solution (Sporonox, Jansen-Cilag Pty Ltd, Macquarie Park, NSW, Australia), diluted to 0.05µg/mL in Amphibian Ringer's Solution, for five minutes each day for ten days (for descripton of similar protocols see: Brannelly et al., 2015, 2012;

Pessier and Mendelson, 2017). After each treatment bath, we placed animals into clean enclosures to prevent possible reinfection from shed zoospores (Pessier & Mendelson, 2017). After completion of the itraconazole treatment, we tested animals for *Bd* infection once weekly for four weeks.

2.4 *Bd* testing

Animals were tested for *Bd* by quantitative PCR (qPCR) screening of skin swabs. We swabbed animals before the commencement of experiments, weekly during experiments and prior to humane killing. Swabbing followed a standardised 35-stroke protocol; five rotating strokes across the venter, each flank, each thigh and each foot using a fine tipped rayon swab (MWE113, Medical Wire & Equipment, Wiltshire, England). Swabs were sent to a commercial laboratory (Cesar Pty. Ltd, Parkville, Victoria, Australia) for testing. DNA was extracted from swabs using a modified Chelex® extraction protocol (Walsh, Metzger, & Higuchi, 1991). The qPCR assay used was a modified Boyle et al., 2004 protocol. Samples were considered positive if all three reaction wells returned a positive result (see Appendix S1 for full details of extraction and qPCR protocols).

2.5 Statistical analyses

All statistical analyses were conducted with R software (R Core Team, 2018): 1) To compare survival curves of *Bd* exposed *P. frosti* across experiments we used log-rank tests. 2) To examine the effect of covariates on survival of *Bd* exposed *P. frosti* in transmission experiments we used Cox proportional hazard models. 3) To examine change in the *Bd* load of *P. frosti* and *C. signifera* during experiments we used linear mixed effects models. 4) To compare the *Bd* load of *P. frosti* across experiments at point of morbidity, and *C. signifera* across experiments immediately prior to use in transmission experiments, we used analysis of variance.

We used log-rank tests to compare survival curves and made post-hoc pairwise comparisons with a Bonferroni adjustment across shared aquatic enclosure, shared terrestrial enclosure and indirect terrestrial transmission experiments (package survival, Therneau, 2015). We used Cox proportional hazard models (package survival) to examine covariate effects on survival across *P. frosti* in transmission experiments (shared aquatic enclosure, shared terrestrial enclosure and indirect terrestrial transmission experiments). These models produce a hazard ratio (HR) indicating the effect size of the covariate, a HR > 1 indicates increased risk and HR < 1 indicates decreased risk of morbidity. As all *Bd* exposed animals reached the point of morbidity, and all control animals survived until the end of the trial, we modelled only *Bd* exposed *P. frosti*.

We analysed each transmission experiment separately, comparing three candidate Cox proportional hazard models for each. Models included time until morbidity as response variable (no censored

220 animals), modelled against snout-to-vent length (SVL) or *Bd* load of *C. signifera* used in transmission
221 (log (zoospore equivalents, ZSE+1)) as continuous explanatory variables, or a constant for the null
222 model (see Table 1 for all candidate models). As we used two *C. signifera* in each indirect terrestrial
223 transmission experimental enclosure, mean *Bd* load values for each pair were calculated and used
224 for this experiment. We compared and ranked candidate models using second order Akaike
225 Information Criterion (AICc). We assessed the proportional hazards assumption of models using the
226 cox.zph function (package survival).

227 To examine the *Bd* load of both species during transmission experiments we used linear mixed
228 effects models (package lme4, Bates et al., 2015). For each species we ran a separate model for each
229 experiment, with experimental week as a continuous fixed effect and individual as a random effect.
230 For *C. signifera* we used data from week zero, the start point of transmission experiments, to week
231 three for the shared aquatic enclosure experiment (insufficient data in week four) and to week four
232 for the shared terrestrial enclosure and indirect terrestrial transmission experiments. For *P. frosti*
233 analysis was carried out from week one (post *Bd* exposure) to week four due to insufficient sample
234 size in the later weeks. We examined coefficient estimate 95% confidence intervals to infer how *Bd*
235 load changed and checked model assumptions by examining simulated residual plots (package
236 DHARMa, Hartig, 2019).

237 We used analysis of variance to assess variation in *Bd* load across transmission experiments for each
238 frog species. We analysed *C. signifera Bd* loads immediately prior to experiments to assess whether
239 reservoir host infection intensity varied across experiments. We analysed *Bd* exposed *P. frosti Bd*
240 loads at final screening prior to death to assess whether susceptible host infection intensity at point
241 of morbidity varied across experiments (package stats, R Core Team 2018). We included
242 experimental group as a categorical response variable and carried out post hoc pairwise
243 comparisons, with a Tukey adjustment (package emmeans, Lenth, 2019) across fixed effect levels.

244 **3. Results**

245 **3.1 Chytrid transmission experiment**

246 All *P. frosti* exposed to *Bd* zoospores through direct contact with *C. signifera* or indirect terrestrial
247 transmission became infected, developed clinical signs of chytridiomycosis and reached the point of
248 morbidity (see section 2.2, Figures 1 and 2). Kaplan-Meier survival curves varied across *Bd*
249 transmission experiments ($\chi2=15.9$, df=2, $p<0.001$); shared enclosure experiments were similar
250 (pairwise comparison, $p>0.05$), but differed significantly from the indirect terrestrial transmission
251 experiment ($p<0.001$ in each pairwise comparison; Figure 2). We observed no clinical signs of

chytridiomycosis in control group *P. frosti* and all were *Bd* negative at the end of the experiments. Several observations noted during daily monitoring were highly prevalent and occurred exclusively within *Bd* exposed groups, indicative of a strong association with chytridiomycosis in *P. frosti* (Appendix S2, Table S1 and Figure S1). These included a major change in posture, a change in gait, and skin that was swollen and/or red. Observations associated with chytridiomycosis tended to occur longer after *Bd* exposure in the indirect terrestrial transmission experiment (Figure S1).

3.1.1 Susceptible host; *Philoria frosti*

Greater *C. signifera Bd* loads were associated with greater risk to *P. frosti* (in this case faster morbidity) when the two species were in direct contact. The top ranked Cox proportional hazard models ($\Delta AIC_c \leq 2$) included *Bd* load of *C. signifera* used in transmission for all three experiments. However, for the indirect terrestrial transmission experiment, the null model showed the lowest AIC_c value (Table 1). For the shared aquatic and shared terrestrial experiments, the 95% confidence intervals (CI) of the coefficient estimates for *Bd* load of *C. signifera* in top-ranked models were greater than, and did not cross, one (hazard ratio of one indicates no effect; 1.02-29.54 and 1.83-72.71, respectively). For the indirect terrestrial transmission experiment, 95% CI for *C. signifera Bd* load crossed one (0.51-149.4), suggesting this was not an important predictor of time until morbidity for individuals infected indirectly.

The infection load of *Bd* exposed *P. frosti* increased with time post exposure (Figure 3). The 95% CI of the coefficient estimates for week post exposure for the shared terrestrial, shared aquatic and indirect terrestrial transmission experiments did not cross zero (1.44-1.69, 1.57-2.09, and 1.27-1.77, respectively), suggesting a strong effect. Mean infection load at point of morbidity was 5.84±0.35SD log (ZSE+1) and did not vary across experiments ($F_{2,25}$=0.24, p=0.783).

3.1.2 Reservoir host; *Crinia signifera*

We observed no clinical signs of chytridiomycosis in wild caught *C. signifera*. All survived to the completion of experiments, with the exception of two animals humanely killed early in the acclimation period that were displaying symptoms not consistent with chytridiomycosis (see Appendix S3 for description of symptoms).

During the acclimation period prior to transmission experiments, *Bd* prevalence increased from 60% upon entering the lab, to 100% one week later. Prevalence remained at this level for the remainder of the time animals were held, with the exception of one animal that was *Bd* negative for one week (Figure S2). The *Bd* loads of *C. signifera* used in *Bd* transmission experiments, measured immediately

prior to commencement, did not significantly vary across experiments ($F_{2,32}$=1.76, p=0.188; mean=2.16±0.62SD log (ZSE+1)).

During *Bd* transmission experiments, there was a slight increase in the *Bd* loads of *C. signifera* in the shared terrestrial and shared aquatic enclosure experiments (Figure S2). The 95% CI of the coefficient estimates for week post exposure in these experiments did not cross zero (0.19-0.34 and 0.06-0.46), suggesting a strong effect. This was not observed in the indirect terrestrial transmission experiment, where 95% CI crossed zero (-0.06-0.15).

3.2 Treatment of chytridiomycosis in *Philoria frosti*

At commencement of treatment (14 days after inoculation with cultured zoospores), all *P. frosti* in the *Bd* exposed experimental group had returned two *Bd* positive qPCR results, at one and two weeks post inoculation, and most were displaying clinical signs of chytridiomycosis (Table S1). Treatment with the antifungal itraconazole cleared *Bd* infection in all treated *P. frosti* (four sequential negative weekly *Bd* qPCR results, Figure 3).

4. Discussion

A recent global assessment found that 28% of species impacted by *Bd* have low or no aquatic association and that most of those are experiencing severe (62%) and ongoing declines (89%; values calculated from (Scheele et al., 2019b supplementary data). Reservoir hosts of *Bd* have been linked to the decline of susceptible species with low associations with aquatic habitat (Scheele et al., 2017a; Brannelly et al., 2018) and potential reservoir host species exist in several systems (Reeder et al., 2012; Stockwell et al., 2016; Yap et al., 2018; Hudson et al., 2019). We demonstrate direct and indirect terrestrial transmission of *Bd* from a reservoir to a susceptible host species, resulting in 100% morbidity of the susceptible host and no observed negative effects on the reservoir host. Our findings provide novel insights on *Bd* transmission pathways in the terrestrial environment, and between reservoir and susceptible hosts. A better understanding of terrestrial and reservoir host transmission pathways will be crucial for predicting long-term *Bd* risk and developing effective conservation actions for species with low aquatic associations experiencing ongoing declines.

Indirect *Bd* transmission has previously been discussed, for example when considering transmission in terrestrial systems (Kolby et al., 2015), and the role of waterways and non-amphibian vectors or hosts in *Bd* dispersal (Garmyn et al., 2012; McMahon et al., 2013; Sapsford, Alford, & Schwarzkopf, 2013; Ribeiro et al., 2019). Indirect transmission may also be an important pathway for some neotropical direct developing frogs (e.g. *Eleutherodactylids*), where greater *Bd* risk has been associated with younger age classes (Longo & Burrowes, 2010) and the cooler, drier season when

frogs congregate in moist refugia (Longo, Burrowes, & Joglar, 2010; Hudson et al., 2019). However, to our knowledge indirect *Bd* transmission has not previously been demonstrated in a terrestrial environment, and until recently, rarely quantified in aquatic systems (Carey et al., 2006; Courtois et al., 2017; Wilber et al., 2017). Notably, studies on zoospore temporal viability have been limited to sterile environments for *Bd* (Johnson & Speare, 2003, 2005), so it is impossible to predict how long zoospores may remain viable in terrestrial environments.

Our focal species, *P. frosti*, has experienced almost complete decline over 30 years (Hollis, 2004, 2011) and is critically endangered (Hero et al., 2004). Alongside the observed temporal and spatial pattern of decline (Hollis, 2004, 2011; Skerratt et al., 2016; Scheele et al., 2017b), our results provide additional support for *Bd* as the causal agent in the species' decline. Several lines of evidence indicate that low transmission rates and the presence of reservoir host populations are the likely driver of the species' ongoing decline towards extinction. 1) The rapid morbidity we document in *Bd*-infected *P. frosti* suggests maintenance of *Bd* within the *P. frosti* population alone is unlikely. 2) Initially, *P. frosti* were extirpated from sub-alpine habitat where *C. signifera* are abundant (Hollis, 2004, 2011). Yet, remnant populations continue to decline in montane habitat (Hollis, 2004, 2011), where no other amphibian species have been recorded calling, with the exception of colonisation by *C. signifera* and *Litoria ewingii* at some disturbed sites. 3) *P. frosti's* low reproductive output and long generation time (Hollis, 2004, 2011) precludes high recruitment compensating for *Bd* mediated mortality, as observed in other susceptible species persisting with endemic *Bd* (Scheele et al., 2015). 4) Research in other species has demonstrated that *Bd*-induced population extirpation can occur in the absence of an epidemic or high *Bd* prevalence (Valenzuela-Sánchez et al., 2017). In sum, low rates of *Bd* transmission from nearby *C. signifera* populations appear to be driving the ongoing decline of the last remaining *P. frosti* populations and will limit prospects for successful reintroduction.

Our demonstration of indirect terrestrial *Bd* transmission suggests it is important to consider not only species' breeding habitat (aquatic for many species), but also non-breeding terrestrial habitat when assessing pathogen exposure risk. Knowledge of this is often limited due a tendency of studies to focus on breeding sites where amphibians are generally more accessible and because of the difficulties in tracking small bodied species (Gourret et al., 2011; Rowley and Alford, 2007). However, many species occupy terrestrial habitat for much or all of their lifecycles, and co-occurrence or long-distance dispersal by reservoir hosts could expose susceptible terrestrial species to *Bd*. For example, a reservoir host occasionally passing through the range of remaining populations of a susceptible host, may cause a low level of *Bd*-related mortality that is nevertheless enough to drive slow declines. Determining long-term *Bd* risk across a landscape will be critical for informing

reintroduction strategies- the ultimate objective of many captive assurance programmes- for susceptible species in areas with endemic *Bd*.

Philoria frosti succumbs extremely rapidly to *Bd*; all animals exposed to *Bd* positive *C. signifera* or environments that they had used were moribund within 39 days, with earliest morbidity occurring at 21 days. Such rapid mortality and high susceptibility has been demonstrated for very few other species. For example, *Atelopus zeteki*, *Anaxyrus americanus* and *Pseudophryne corroboree* inoculated with cultured zoospores died within 18, 20 and 82 days of *Bd* exposure, respectively (Gahl, Longcore, & Houlahan, 2012; Brannelly et al., 2015a; Maguire et al., 2016). In line with previous work demonstrating that increased dose of *Bd* reduces time to death (Bustamante, Livo, & Carey, 2010), *Bd* loads of *P. frosti* directly inoculated with cultured zoospores appear, via qPCR skin swab testing, at least one week ahead of those exposed through transmission experiments (Figure 3). This suggests our observed times to morbidity for *P. frosti* may be conservative relative to experiments where animals are inoculated with thousands of cultured zoospores, as was the case in the aforementioned studies.

Crinia signifera is a competent *Bd* reservoir host and has been implicated in the decline of several sympatric species in south eastern Australia (Scheele et al., 2017a; Brannelly et al., 2018). Previous work has demonstrated that *C. signifera* can maintain high infection loads while displaying no clinical signs of chytridiomycosis under laboratory conditions and show no indication of decline or demographic responses to infection in the wild (Scheele et al., 2017a; Brannelly et al., 2018). We extend this work by demonstrating that *C. signifera* can directly and indirectly transmit *Bd* to *P. frosti*, while showing no signs of disease. Further, we found that the time to morbidity in *P. frosti* decreased with increasing *Bd* load of *C. signifera*. Therefore, identifying factors that may drive spatial and temporal variation in infection status and load of reservoir host species or communities is likely to be important for predicting risk to susceptible populations in systems where *Bd* is endemic.

Despite *Bd* having been identified as a threat to amphibians over two decades ago (Berger et al., 1998; Longcore, Pessier, & Nichols, 1999), there are limited options for mitigating the impacts of *Bd* in the wild (Scheele et al., 2014, 2019a; Garner et al., 2016). As a result, the number of ex situ captive assurance colonies established to prevent extinction has grown to include more than 70 species (Harding, Griffiths, & Pavajeau, 2016). The protection of these colonies, including those established for *P. frosti* (Hunter et al., 2018), from the risk of *Bd* infection has become crucial to the preservation of a substantial portion of amphibian diversity. We found that treatment with low doses of the antifungal itraconazole (among others, Brannelly et al., 2012, 2015b; Pessier & Mendelson, 2017) was highly effective in treating *P. frosti* displaying clinical signs of chytridiomycosis

(Figure 3). While empirical testing is not equivalent to the clinical trials necessary to fully assess a treatment (Berger et al., 2010; Brannelly et al., 2012), we suggest that low doses of itraconazole be considered for use in emergency treatment of captive *P. frosti* populations.

Our work demonstrates transmission pathways through which spill-over from reservoir to susceptible hosts could occur. However, further experiments examining *Bd* transmission are required to elucidate the role of these mechanisms in *Bd* dynamics. Experiments should aim to determine how long *Bd* zoospores shed into environments can transmit infection. Previous studies have examined these questions in part. For example, *Bd* zoospores have been demonstrated to survive periods in sterile aquatic (seven weeks) and sterile terrestrial (three months) environments (Johnson & Speare, 2003, 2005). Similarly, the related pathogen species *Batrachochytrium salamandrivorans* has been shown to be viable after 31 days in filtered environmental water, 48 hours in forest soil, and further to be transmissible between hosts via the terrestrial environment (Stegen et al., 2017). However, there remains a paucity of published information on *Bd* zoospore viability in non-sterile systems or possible environmental reservoirs. Our study design- using wild infected reservoir hosts and semi-natural terrestrial enclosures to test transmission- provides a framework upon which experiments to answer such questions for *Bd* could be built.

Our study demonstrates indirect terrestrial *Bd* transmission from a reservoir to susceptible host species, resulting in fatal chytridiomycosis. To our knowledge, this provides the first empirical evidence of this transmission mode for *Bd* and demonstrates the potential ease of transmission from reservoir to susceptible hosts. Our findings have implications for understanding *Bd* disease dynamics; in particular transmission pathways for species with low aquatic associations, the potential for pathogen spill over from reservoir host species, and mechanisms driving ongoing *Bd* declines.

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Tables and figures

Table 1. Candidate Cox proportional hazard models of time until morbidity of *Philoria frosti* exposed to *Batrachochytrium dendrobatidis* (*Bd*), through sharing enclosures with (direct aquatic and direct terrestrial transmission) or use of environments contaminated by (indirect terrestrial transmission) *Bd* positive *Crinia* signifera (*Cs*). Models displayed in order of decreasing second order Akaike Information Criterion (AIC_c) values. SVL, snout-to-vent length; df, degrees of freedom; and ΔAICc, delta AICc.

Transmission experiment	Model	df	AICc	ΔAIC_c	Akaike Weight
Direct aquatic	~*Cs Bd* load	1	18.9	0	0.712
	~1	0	21.2	2.3	0.225
	~SVL	1	23.8	4.87	0.062
Direct terrestrial	~*Cs Bd* load	1	34.4	0	0.871
	~SVL	1	39.3	4.91	0.075
	~1	0	40	5.57	0.054
Indirect terrestrial	~1	0	21.2	0	0.457
	~*Cs Bd* load	1	21.4	0.16	0.421
	~SVL	1	23.8	2.64	0.122

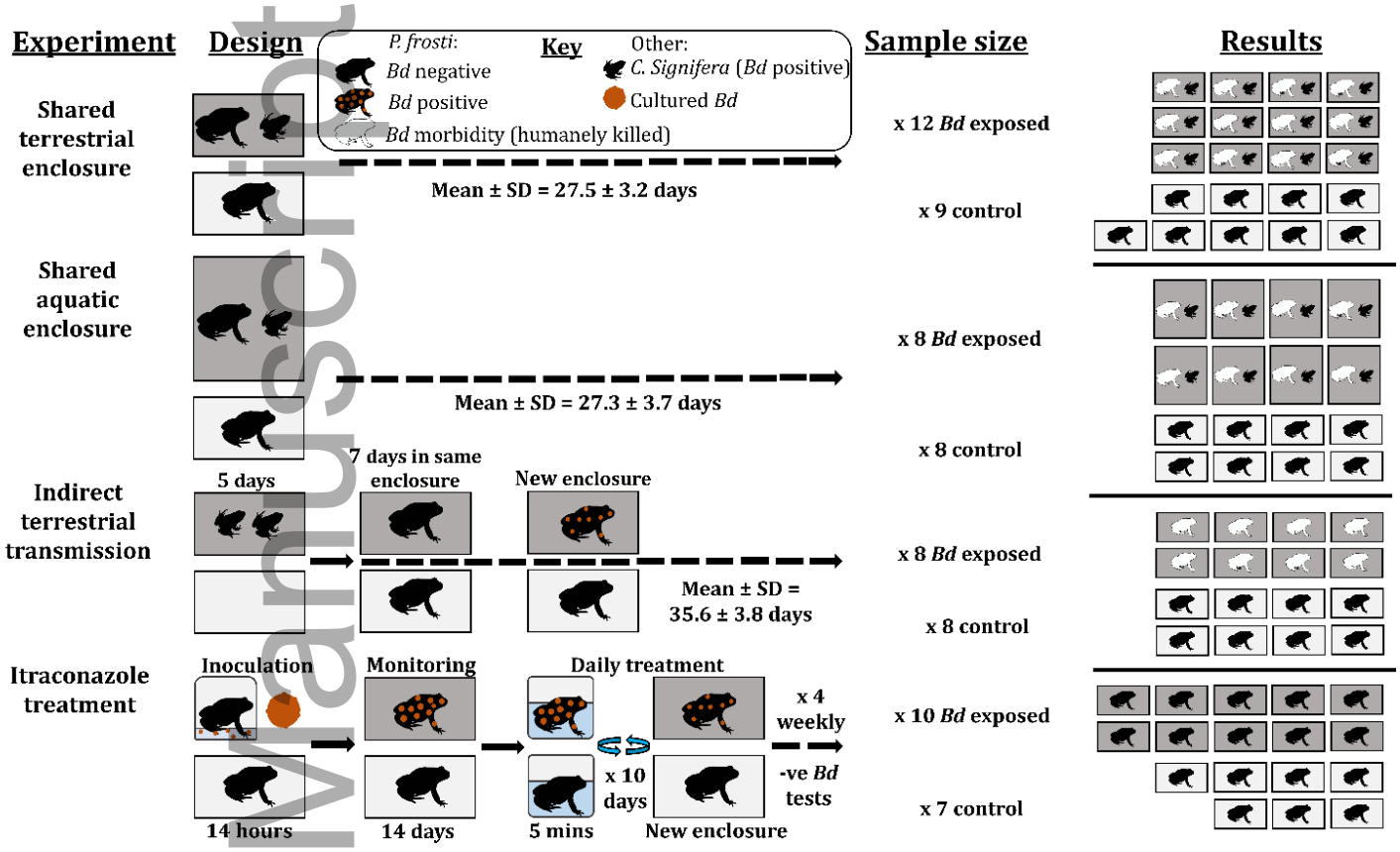

Figure 1. Experimental design and results summary for *Batrachochytrium dendrobatidis* (*Bd*) transmission and treatment experiments. *Philoria frosti* were housed with *Crinia signifera* (shared aquatic or terrestrial enclosure experiments) or in enclosures with a living moss substrate previously occupied by *C. signifera* (indirect terrestrial transmission experiment). *Philoria frosti* in the treatment experiment were inoculated with cultured *Bd* zoospores before treatment with low dosage itraconazole baths (blue liquid). Dashed arrow line represents time between first *Bd* exposure (or final bath in treatment experiment) and the experimental endpoint. Frog silhouettes by M. Mark.

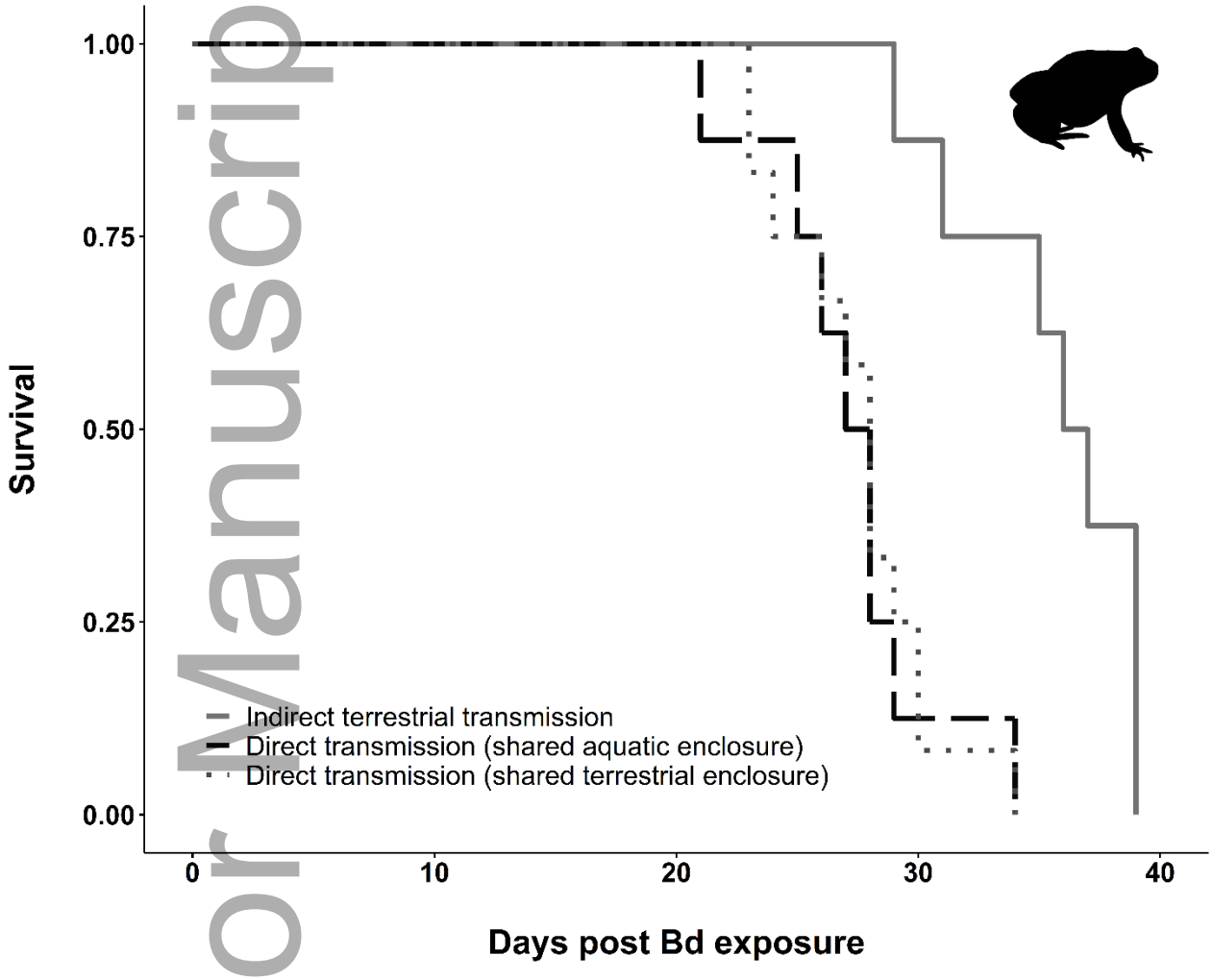

Figure 2. Kaplan-Meier survival curves for Philoria frosti exposed to Batrachochytrium dendrobatidis (Bd) under controlled laboratory conditions. Philoria frosti were housed with wild caught Crinia signifera (shared aquatic or terrestrial enclosure experiments) or in enclosures previously occupied by C. signifera (indirect terrestrial transmission experiment). Frog silhouette by M. Mark.

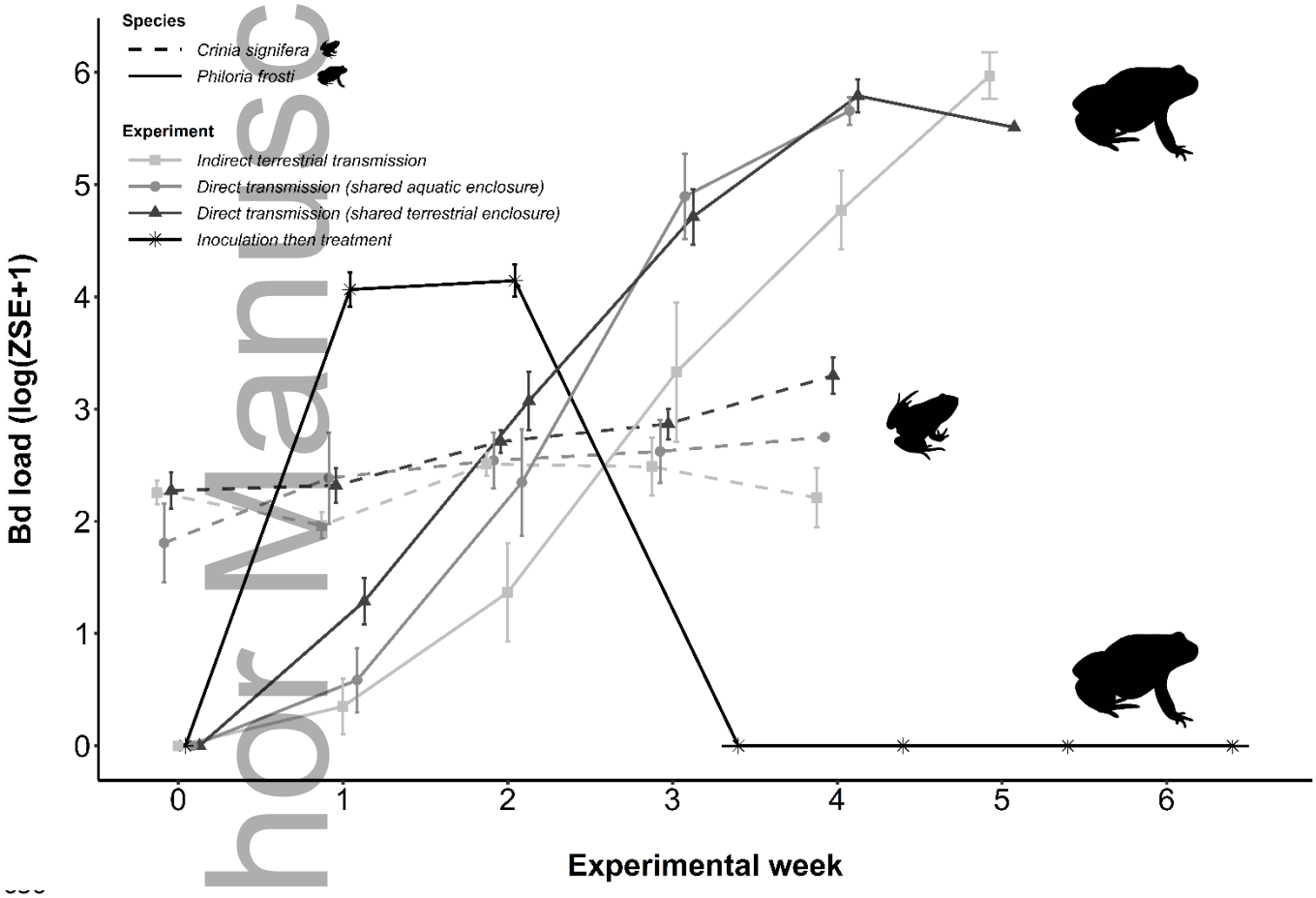

Figure 3. Batrachochytrium dendrobatidis (Bd) infection loads of Philoria frosti and Crinia signifera during experiments examining Bd transmission, susceptibility and treatment in P. frosti and the capacity of C. signifera to act as a Bd reservoir host. We display data only for Bd exposed Philoria frosti; that were housed with wild caught C. signifera (shared enclosure experiments), within enclosures previously occupied by C. signifera (indirect terrestrial transmission experiment) or inoculated with cultured Bd zoospores before treatment by itraconazole bathing 14 days later. We commenced experiments over a three-week period, as such experimental week is relative to the start of each experiment. Error bars show standard error of the mean. ZSE = Bd zoospore equivalents, determined by qPCR assay of skin swabs. Frog silhouettes by M. Mark.