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Front cover photos: Background: Quarry wetland supporting a large population of the Growling Grass Frog, Boral property, Campbellfield, Victoria;

Top-left inset: Adult female Growling Grass Frog, Somerton, Victoria;

Bottom-right inset: Swabbing a juvenile Growling Grass Frog for chytrid fungus, Donnybrook, Victoria.

Photo credits: G.W. Heard.

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Summary

The Growling Grass Frog *Litoria raniformis* is officially listed as threatened at a national level and in every state in which it occurs. Declines in this species typify those seen in declining amphibians from around the world. Whilst a range of threatening processes have been proposed to explain these declines, it is apparent that the fungal pathogen-induced disease chytridiomycosis is a consistent causal factor. Despite considerable research on *L. raniformis* by a range of workers, the effects of chytridiomycosis on this frog have been largely neglected. Consequently, we examined the prevalence of chytrid fungus across a well-studied metapopulation of *L. raniformis*, examined factors that influence the probability of infection of individuals in this metapopulation, and determined whether infection load affects survival in these frogs. Prevalence of infection was substantially lower amongst juvenile frogs than adults. Our study sites occurred in three clusters aligned on a north – south axis; higher rates of infection were detected at our northernmost cluster, although prevalence of infection showed no clear geographic trend. The strongest predictor of infection status of individual frogs was water temperature: higher water temperatures corresponded to lower probabilities of infection. As a consequence, the probability of infection can be expected to be much higher at the start of active season than at its conclusion. Frogs occupying two spring-fed quarries also had lower rates of infection. We found a clear relationship between infection load and the probability of recapture, with heavily infected frogs being far more likely to be recaptured. We recommend continued application of hygiene protocols within and between sites in order to lessen the impact of chytridiomycosis on *L. raniformis* and sympatric amphibian species. Based on our finding that infection affects the probability of recapture, we also recommend that monitoring programs for *L. raniformis* incorporate sampling and diagnostic testing for chytrid fungus in order to account for the effects of infection when deriving estimates of population size, survival probabilities, etc. This work serves to reinforce the value of quarry wetlands as vital, high-quality habitat for *L. raniformis* across urban Melbourne. Populations of *L. raniformis* inhabiting these wetlands display lower rates of infection, which suggests that they may act as partial refugia from disease. The relationship between water temperature and probability of infection has implications for wetland management, because it emphasises the likely importance of high insolation levels for this species, and the probable detrimental effects of wetland shading by over-storey trees, including those planted as part of riparian revegetation projects. We conclude that maximising the probability of persistence of *L. raniformis* in the face of chytridiomycosis requires maximising local population sizes through appropriate wetland management, and maintaining opportunities for recolonisation in the event of local population extinctions.

1 Introduction

Changes to the distribution and abundance of the Growling Grass Frog *Litoria raniformis* in south-eastern Australia in recent decades exemplify the worrying trends seen in many amphibian species around the world. Once common and abundant in most suitable habitats throughout its range, population declines and losses have resulted in *L. raniformis* being listed as 'Vulnerable to Extinction' under the federal Environment & Biodiversity Conservation Act 1999, 'Endangered' in Victoria (DSE 2007), listed as a threatened taxon under the Victorian *Flora and Fauna Guarantee Act 1988* and variously threatened in the other Australian states where the species occurs (Clemann and Gillespie 2008).

Several processes have been suggested as drivers of the decline of *L. raniformis*, including loss and degradation of habitat, barriers to movement, drought, disease and increased predation due to exotic species (Clemann and Gillespie 2008). A reliance on water renders *L. raniformis* susceptible to the water-borne fungal pathogen *Batrachochytrium dendrobatidis*. This pathogen causes the disease chytridiomycosis, which has been implicated in rapid declines of amphibians in several parts of the world (Berger *et al.* 1998; Skerratt *et al.* 2007). Chytridiomycosis is listed as a threatening process under Commonwealth legislation, as a key threatening process in New South Wales, and as a potentially threatening process in Victoria.

Despite the plausible influence of chytridiomycosis on the changing status of *L. raniformis* (Pyke 2002; Hamer *et al.* 2010; Heard *et al.* in press-a), and a relatively large number of studies of various aspects of the biology and ecology of the species (detailed in Heard *et al.* 2010), there has not been any specific study of the impact of this disease on *L. raniformis*. The Victorian Department of Sustainability and Environment has commissioned this study to improve its understanding of the prevalence, impacts and management of chytrid fungal disease in *L. raniformis*. The study had three objectives:

1. To determine the prevalence of chytrid infection across a well-studied metapopulation of *L. raniformis*;
2. To identify variables that influence the probability that individual *L. raniformis* are infected with chytrid fungus in this metapopulation, and;
3. To use mark-recapture methods to assess whether infection by chytrid fungus at first capture affects subsequent survival of *L. raniformis* in this metapopulation.

By providing an improved understanding of the prevalence and impacts of chytridiomycosis on this well-studied metapopulation of *L. raniformis*, the importance of this threatening process for populations of *L. raniformis* can be broadly assessed, and current management recommendations updated in light of this information.

2 Methods

2.1 Mark-recapture

The study was conducted in the middle reaches of the Merri Creek catchment, on the northern fringe of Melbourne (Figure 1). The Merri Creek catchment is a component of Melbourne's northern basalt plain: an undulating, volcanic landscape that rises to a maximum elevation of around 200 m above sea level. Patterns of land-use in the catchment are rapidly changing from livestock-based agriculture and extractive industries, to residential and industrial developments.

Mark-recapture was undertaken at 12 sites (Figure 1; Appendix 1) across the study area during the 2004/2005 and 2005/2006 active seasons of *L. raniformis* (October–April). Sites were distributed in three clusters centred on the suburbs of Campbellfield, Somerton and Donnybrook. They were selected using previous knowledge of wetland occupancy by the frog, and by a desire to sample clusters of sites to document dispersal patterns (Heard *et al*. in press-b). Lotic (flowing water) sites (n = 5) included chains of pools along the Merri Creek, and two of its ephemeral tributaries (Curly Sedge Creek and Kalkallo Creek). Lentic (still water) sites (n = 7) included farm dams, flooded quarries and swamps.

Logistic and access constraints did not permit all sites to be censused in both seasons, and resulted in some variation in survey intensity within seasons. All 12 sites were monitored in season 1, dropping to five sites in season 2. Surveys were conducted on a two or three week rotation, with all sites surveyed in one rotation before commencing the next rotation. Proximate sites were grouped for this purpose, and were surveyed over the course of one night during each rotation. The chronology of group surveys was randomised within each rotation, as was the chronology of site surveys. Survey techniques are described by Heard *et al*. (2006).

Capture by hand or net was attempted for all *L. raniformis* encountered. Those captured were retained overnight and marked in the laboratory the following day. The snout-vent length (SVL) and body mass of each frog were measured prior to marking. Frogs were permanently marked by subcutaneous injection of either a Passive Integrated Transponder (PIT) tag (11.5 × 2.12 mm; Trovan Ltd, East Yorkshire, United Kingdom), or a Visible Implant Alphanumeric (VIA) tag (3.5 × 1.5 mm; Northwest Marine Technology, Shaw Island, USA). Visible Implant Alphanumeric tags were used to mark all *L. raniformis* smaller than 50 mm SVL, given evidence that PIT tags may not be safe for marking such frogs (Christy 1996; Pyke 2005). Protocols for VIA tagging are described by Heard *et al*. (2008). Protocols for PIT tagging followed Christy (1996) and Pyke (2005). In order to identify frogs that lost tags, the toe-pad on the right middle finger of the left hand was clipped using standard techniques (Donnelly *et al*. 1994).

Frogs were released at their points of capture within 24 hours of being caught. Subsequent to the initial survey at each site, all captured frogs were scanned for the presence of a PIT tag (using a Trovan® LID570 Pocket Reader), inspected for the presence of a VIA tag (using the torch and viewing glasses supplied by the manufacturer), or inspected for the presence of toe-clips in the absence of either tag. Unmarked individuals were retained for marking. Recaptured frogs were released immediately following data collection, which replicated that undertaken at initial capture.

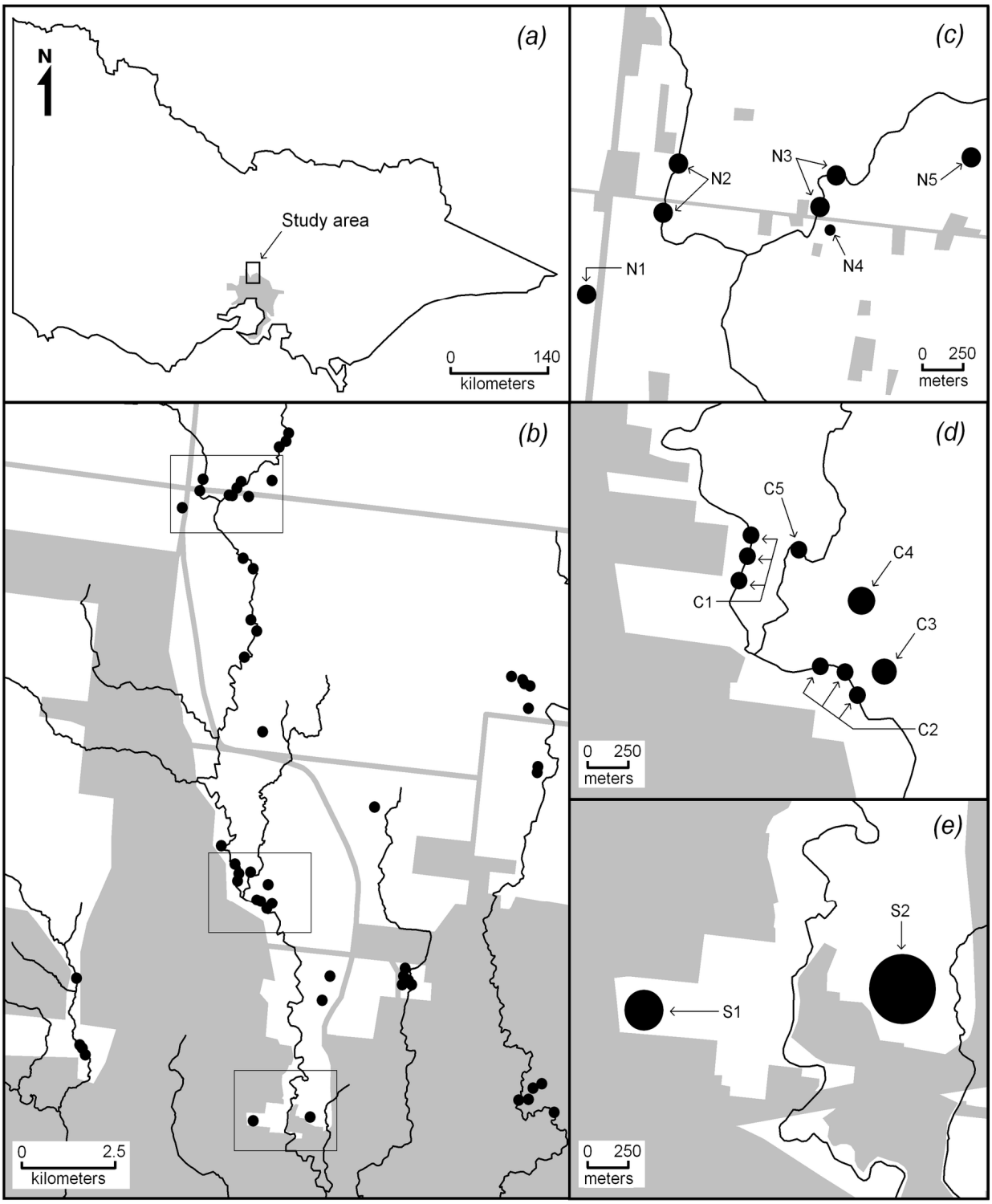

Figure 1. Map of the study area. Panel (a) shows the location of the study on the northern outskirts of the Melbourne metropolitan area (grey shading). Panel (b) shows the location of each of the cluster of wetlands where populations of *Litoria raniformis* were sampled for *Batrachochytrium dendrobatidis*. From north to south the clusters are centered on the suburbs of Donnybrook, Somerton and Campbellfield. Black dots represent wetlands known to support populations of *L. raniformis* in this area. The grey shaded area depicts the distribution of major urban infrastructure. Panels (c) - (e) display the distribution of sampling sites (black dots) in the Donnybrook, Somerton and Campbellfield clusters, respectively. Grey shading represents the distribution of buildings and roads. The black lines in panels (b) - (e) represent the Merri Creek, its tributaries and adjacent streams (Darebin Creek in the far-east and Yuroke Creek in the far-west).

2.2 Chytrid sampling and analysis

Swabbing the skin is the favoured technique for sampling chytrid zoospores from individual frogs; the technique is quick, inexpensive and causes little stress to the animal (Kriger *et al*. 2006). A haphazard sample of 521 *L. raniformis* was swabbed for chytrid on first capture during the mark-recapture study using medical grade, sterile swabs (Fine-tip MW100 swabs, Medical Wire and Equipment Pty Ltd, UK). Those not sampled were missed for logistical reasons - typically delays in obtaining swabs. Recaptured individuals were usually not re-swabbed.

At first capture individuals were swabbed the day following capture (prior to marking, as above). A standardised swabbing protocol was implemented in which the swab was rubbed gently over the frog's skin on the dorsal, lateral and ventral surfaces. We made sure to also rub the swab on the inner thighs and the palms of the front feet. Swabs were returned to the sterile housing, individually labelled with the frog's details (date, site of capture and tag code), and subsequently stored in a freezer at -18° C.

Strict protocols were applied to ensure that contamination of samples or cross-infection of individual frogs did not occur during the sampling process. In the field, standard protocols were followed to ensure that transmission of the disease between surveys sites did not occur (NPWS 2001). To ensure that the fungus was not passed between individuals during capture, all frogs were placed in separate plastic bags for retention or processing, and both the researcher's hands and capture net (when used) were sprayed thoroughly with 95% ethanol after each capture. In the laboratory, swabs were placed within their housing tube immediately after use, all handling equipment was discarded between individuals (gloves, holding bags etc.), and all marking equipment was sterilised by immersion in 95% ethanol and flaming for at least ten seconds.

Swabs were analysed using quantitative Polymerase Chain Reaction (qPCR) assays by Ecogene Ltd., New Zealand, following the approach of Hyatt *et al*. (2007). These assays amplify a genetically divergent segment of the fungus's ribosomal DNA, and not only provide a sensitive test for detection of the pathogen, but also an estimate the number of zoospores present in each sample based on the quantity of DNA (usually referred to as 'zoospore equivalents'; Hyatt *et al*. 2007). Ecogene applied a repeated sampling scheme to each swab, to account for possible per-test probabilities of detection that were less than one. We subsequently incorporated this source of error into our analysis of the prevalence of the disease, and its effects on survival and recapture rates of *L. raniformis* (see below).

2.3 Data analysis

All statistical analyses were undertaken within a Bayesian statistical framework using OpenBUGS version 3.1.2 (Thomas *et al*. 2006). Parameter estimates and their 95 % credible intervals (95 % CIs) were estimated using Markov Chain Monte Carlo (MCMC) sampling, as described by McCarthy (2007). Uninformative priors were used for all model parameters. Convergence was assessed by analysis of three replicate Markov chains with over-dispersed starting values using the Brooks-Gelman-Rubin statistic (Brooks and Gelman 1998), and by visual inspection of chain-histories. Convergence was achieved within 20,000 MCMC samples in all cases, and parameter estimates derived from a subsequent 10,000 samples.

We began by modelling relationships between the probability that an individual *L. raniformis* is infected with chytrid fungus and several intrinsic and extrinsic variables that we hypothesised may influence the prevalence and virulence of the fungus. Firstly, we considered the possibility that metamorphling *L. raniformis* may have a lower probability of infection, arising from the fact that

the keratinised tissue that chytrid fungus requires is only found in the mouthparts of tadpoles (Knapp and Morgan 2006). This could limit the pathogens' ability to infect tadpoles, producing carry-over reductions in the likelihood that newly metamorphosed individuals will display disease symptoms.

Experimental studies have shown that chytrid fungus is highly sensitive to environmental temperatures, with an optimum temperature for growth between approximately 16° C and 25° C, and an upper temperature tolerance of 29° C (Longcore *et al*. 1999; Piotrowski *et al*. 2004). Furthermore, the virulence of this fungus has been shown to be influenced by temperature, with infected frogs maintained at 27° C displaying higher survival rates than those maintained at 17° C and 23° C (Berger *et al*. 2004). We subsequently considered an effect of water temperature at the time of capture on the probability of infection, reasoning that higher temperatures may not only be less suitable for the fungus, but that the rate at which *L. raniformis* is able to overcome infection may also be influenced by water temperature.

There are also reasons to believe that particular water chemistry variables may influence the rates of infection. Piotrowski *et al*. (2004) report reduced growth rates of chytrid fungus at high pH (> 8). Recent work on *Litoria aurea* (a species closely related to *L. raniformis*) also suggests that the salinity tolerance of chytrid fungus is an important determinant of infection rates and virulence of the pathogen (M. Stockwell, University of Newcastle, pers. comm.). Amongst the set of wetlands sampled in this study, both pH and electrical conductivity (a common proxy for salinity; Smith *et al*. 2007) were considerably higher at two spring-fed quarries than all other sites (quarries: mean pH = 9.7, mean conductivity = 3845 mS cm^{-1}; remaining sites: mean pH = 8.2, mean conductivity = 909 mS cm^{-1}). For this reason, we explored the possibility that the probability of infection may have been lower at the spring-fed quarries we sampled relative to the other wetlands.

Following the recommendations of McClintock *et al*. (2010), we used a Bayesian formulation of a single-season occupancy model (MacKenzie *et al*. 2006) to model relationships between the probability of infection at first capture and the above variables. The approach was designed for occupancy studies in which the probability of detection during a single survey is assumed to be ≤ 1. Given a survey design that entails multiple visits to each site in which detection (1) or non-detection (0) is recorded, the approach of Mackenzie *et al*. (2006) allows the probability of occupancy (ψ) to be estimated while jointly estimating (and therefore accounting for) the probability of detection (p). Our sampling design was equivalent to a multi-visit occupancy survey, because the per-test probability of detecting chytrid for each swab was ≤ 1, and three replicate tests were conducted. Hence, applying the approach of Mackenzie *et al*. (2006) to these data simply requires re-interpretation of ψ and p: ψ becomes the probability of infection and p becomes the probability of detecting the pathogen during each of replicate test of the swab taken at first capture.

The approach of Mackenzie *et al*. (2006) provides considerable flexibility to model the effects of covariates on ψ. We adopted the standard approach here, using a linear equation and logistic link function to constrain the probability of infection of each frog (ψ_i) to be a function of age at first capture (age_i [0 = metamorphling, 1 = juvenile/adult]), water temperature at the time of first capture ($wtemp_i$ [in °C]), and a simple binary variable denoting whether or not each frog was captured in one of the two spring-fed quarries sampled during this study ($quarry_i$ [0 = not captured at a quarry, 1 = captured at a quarry]). Only simple linear, additive effects of these variables were considered in the model, as follows:

$$\log\left(\frac{\psi_i}{1-\psi_i}\right) = \alpha + \beta_1 \text{age}_i + \beta_2 \text{wtemp}_i + \beta_3 \text{quarry}_i \qquad \text{Equation 1}$$

where α is the intercept term and the β_1- β3 are regression coefficients for each covariate.

To explore the effect of chytridiomycosis on the survival rates of *L. raniformis*, we embedded the above model into a Bayesian Cormack-Joly-Seber (CJS) mark-recapture model, which we previously developed to estimate the survival rates of *L. raniformis* in the study area (Heard *et al*. in press-a). Similar to the occupancy approach described above, CJS models are built on the assumption that the detection (1) or non-detection (0) of an each individual during each survey of a mark-recapture study results from two processes: (1) the probability of survival between surveys (ϕ), and; (2) the probability of recapture during each survey (*r*). Hence, with multiple detection histories, CJS models allow ϕ to be estimated while jointly estimating (and accounting for) *r*. Our existing CJS model was constructed following McCarthy (2007), but with one important alteration to his approach. Given variation in the length of the interval between capture occasions (resulting from randomisation of survey chronology and the cessation of surveys over the winter inactive period), we rescaled ϕ in the model to be the daily probability of survival, rather than the probability of survival between surveys. This was achieved by raising ϕ for each interval to the number of days over which that interval extended.

In a CJS model, both ϕ and *r* can be constrained to be function of covariates using a linear equation and logistic link function (Nichols 2005). To assess the effect of infection on the probability of survival we included the (uncertain) infection status of each frog (z_i, as defined by the model of infection status described above) and the zoospore estimate for that frog ($zoospores_i$; log_e transformed) as a predictor of ϕ. Thus, ϕ_i was modelled as:

$$\log\left(\frac{\phi_i}{1-\phi_i}\right) = \alpha + \beta_1 \text{age}_i + \beta_2 z_i \text{zoospores}_i \qquad \text{Equation 2}$$

In addition to an effect of infection on the probability of survival, we considered it possible that infection status and pathogen load may influence the probability of recapture for *L. raniformis*, due to heavily infected individuals being less alert or agile, and hence easier to catch. The probability of recapture for each frog (r_i) was therefore modelled as:

$$\log\left(\frac{r_i}{1-r_i}\right) = \alpha + \beta_1 z_i \text{zoospores}_i \qquad \text{Equation 3}$$

3 Results

3.1 Prevalence and intensity of infection

Table 1 summarises the prevalence[1] of chytrid fungus infection amongst *Litoria raniformis* sampled during this study, and the intensity of infection (as indicated by the estimated number of zoospores per swab). Overall, the prevalence of infection was substantially lower amongst juvenile and metamorphling frogs than adults (12.94 vs. 35.04%). However, amongst infected individuals, the intensity of infection showed no clear difference between age groups, averaging ~ 114 zoospores (= 4.74 $\log_e$ zoospores) per swab for juveniles and ~154 zoospores (= 5.02 $\log_e$ zoospores) per swab for adults. Infection intensity ranged from one zoospore (= 0.32 $\log_e$ zoospores) to 216,444 zoospores (= 12.29 $\log_e$ zoospores).

There was some variation in the prevalence of the disease between the three clusters of sites. Prevalence amongst juveniles differed between sites in Campbellfield and Somerton compared to those in Donnybrook. Only one juvenile *L. raniformis* from the first two areas tested positive for chytrid fungus, whereas 21 juveniles tested positive at Donnybrook. This trend was not as apparent amongst adults, although the prevalence of infection was slightly lower in Campbellfield and Somerton (mean prevalence at Campbellfield and Somerton = 30.89%; mean prevalence in Donnybrook = 35.53%). At a site-level, the highest prevalence of infection amongst adults was observed at a farm dam adjacent to Donnybrook Road in Donnybrook (site 'N4') and the Merri Creek in the vicinity of Freight Drive, Somerton ('C2'). However, the sample size at both these locations was small, so this figure is somewhat unreliable. Amongst juveniles, the highest prevalence was recorded at a farm dam adjacent to the Hume Freeway in Donnybrook ('N1'). Again however, this result may be spurious – all juveniles at this site were swabbed in early spring, when the prevalence of the disease is relatively high due to low water temperatures at this time (see below). Sites 'S2', 'C1' and 'N1' displayed low prevalence of infection amongst the adult population of *L. raniformis*. The first of these is one of the spring-fed quarries in Campbellfield, the second is the Merri Creek in the vicinity of O'Hern's Road, Somerton.

The intensity of infection, as indicated by estimated number of zoospores, displayed no clear geographical trends. Estimates were relatively high at two sites in Donnybrook ('N4' and the Kalkallo Creek in the vicinity of Donnybrook Road [site 'N2']). Frogs swabbed at the Merri Creek in the vicinity of O'Hern's Road and Freight Drive, Somerton ('C1' and 'C2') also displayed high mean zoospore loads, as did those from a farm dam on Spring Street, Donnybrook ('N5'). The 45 *L. raniformis* swabbed at the farm dam adjacent to the Hume Freeway in Donnybrook (site 'N1') displayed the lowest mean zoospore load (~ 24 zoospores per swab).

[1] Note that 'prevalence' in this study represents a period prevalence rather point prevalence, as the data are pooled over two seasons. Period prevalence is typically higher than point prevalence, as the former does not consider additions and removals from the infected population (D. Ramsey, ARIER, pers. comm.).

Table 1. Summary of the prevalence and intensity of chytrid fungus infection amongst populations *Litoria raniformis* sampled in the study area between 2004 and 2006. Data are provided separately for juveniles (defined here as ≤ 50 mm snout-vent length) and adults (> 50 mm snout-vent length). Prevalence is the percentage of frogs sampled at each site that tested positive for the pathogen over the two year period. Numbers in parentheses are the number of frogs tested for the pathogen at each site. Dashes signify that no frogs in that age group were sampled at that site. The intensity of infection is represented by the estimated number of zoospores per swab. Dashes signify either that no frogs of that age group were sampled at that site, or that no frogs of that age group tested positive for the disease. Sites are grouped by suburb. See Appendix 1 for a description of the sites.

Site	Prevalence		Zoospore estimate (log_e transformed) for infected frogs					
	Juvenile	Adult	Juvenile			Adult		
			Mean	Minimum	Maximum	Mean	Minimum	Maximum
Donnybrook								
N1	69.23 (13)	21.87 (32)	5.49	2.28	9.47	3.18	1.93	4.56
N2	12.00 (75)	46.87 (32)	3.46	0.80	6.23	6.05	1.65	10.06
N3	13.63 (22)	43.75 (96)	6.89	3.81	10.23	4.55	0.54	12.29
N4	-	55.56 (9)	-	-	-	7.37	4.77	8.83
N5	-	45.16 (31)	-	-	-	5.97	3.49	9.86
Somerton								
C1	-	20.45 (44)	-	-	-	5.09	1.73	9.88
C2	0 (5)	58.33 (12)	-	-	-	5.85	0.33	8.97
C3	0 (19)	28 (25)	-	-	-	4.50	2.08	8.56
Campbellfield								
S1	0 (13)	28.95 (38)	-	-	-	4.26	0.52	8.94
S2	4.35 (23)	18.75 (32)	3.08	3.08	3.08	4.58	1.49	9.90
Overall	12.94 (170)	35.04 (351)	4.74	0.80	10.23	5.02	0.33	12.28

3.2 Correlates of infection

There were clear relationships between the probability of infection (ψ_i) and the variables identified *a priori* as possible drivers of the prevalence and virulence of chytridiomycosis for *L. raniformis*. Table 2 provides the estimates of the regression coefficients for each relationship. The relationships are depicted in Figure 2.

As suggested by the raw data, adult *L. raniformis* displayed a higher probability of infection than juveniles: the regression coefficient for this effect was positive (1.36), with a 95 % CI that did not overlap zero (Table 2). The strongest predictor of probability of infection was water temperature. Infection rates declined steeply across the range of water temperatures observed during this study (Figure 2). For an adult frog occupying a non-quarry wetland, ψ is predicted to fall from 0.74 at a water temperature of 13° C to 0.18 at a water temperature of 27° C. Frogs occupying the two spring-fed quarries also displayed a lower probability of infection. The difference is not large (Figure 2), but the 95 % CI of the regression coefficient for this effect did not overlap zero, meaning that it is statistically significant.

Table 2. Parameter estimates for the model relating the probability of infection of *Litoria raniformis* with chytrid fungus to frog age (juvenile or adult), water temperature (°C) and whether the frog occupied a spring-fed quarry or not. The regression coefficients for each effect (β) show the direction of the relationship and its magnitude (but note that the magnitude of the regression coefficients is not comparable across effects). The mean estimate of each regression coefficient is provided, along with the 95% credible interval of these estimates (95% CI).

Parameter	Mean	95% CI
α (intercept)	1.45	-0.40, 3.35
β_1 (age effect)	1.36	0.85, 1.91
β_2 (water temperature effect)	-0.19	-0.11, -0.26
β_3 (quarry effect)	-0.63	-0.08, -1.24

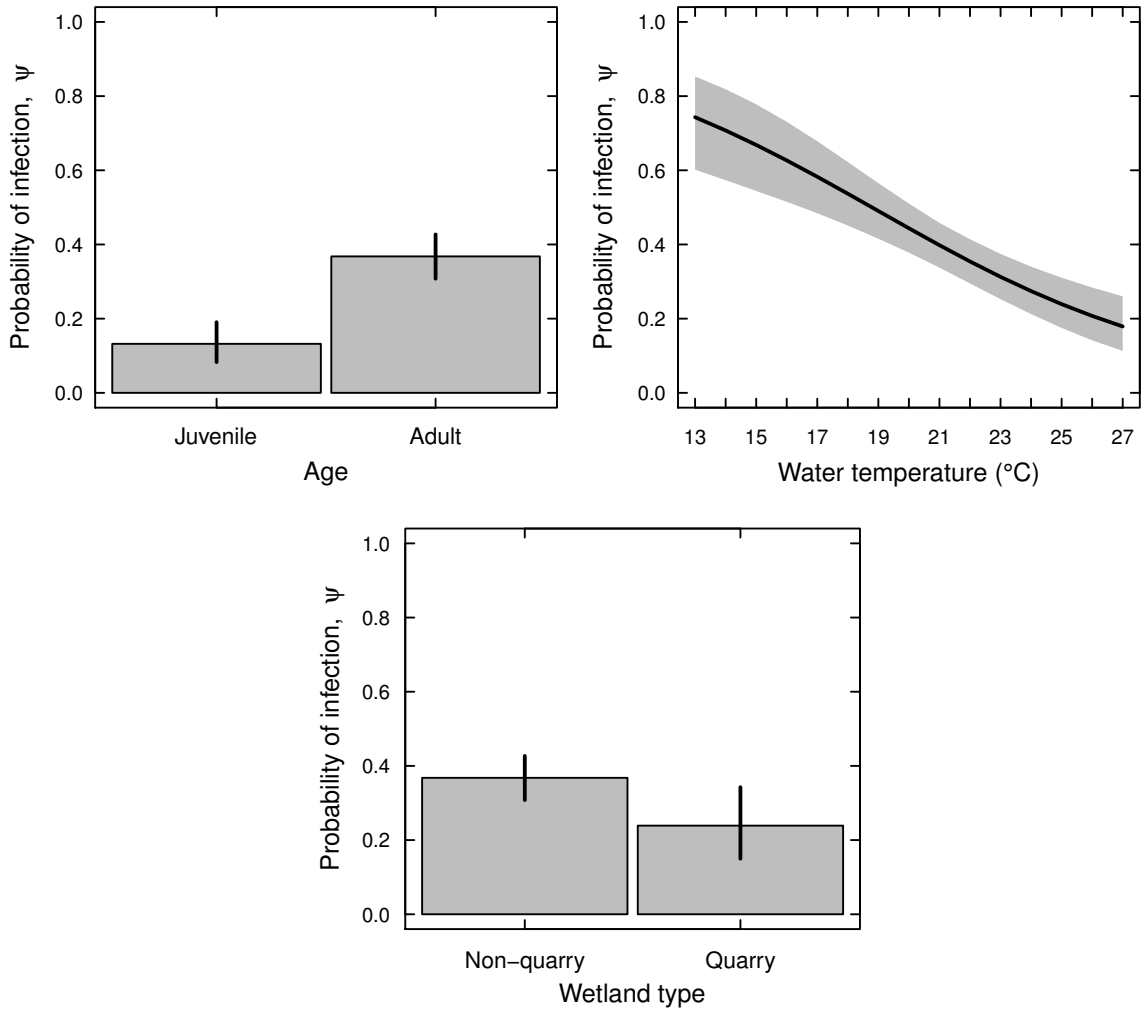

Figure 2. Relationships between the probability of infection of *Litoria raniformis* with chytrid fungus and frog age (top left-hand plot), water temperature (top right-hand plot) and wetland type (bottom plot). The relationship between the probability of infection and water temperature is for an adult frog in a non-quarry wetland. The relationships between the probability of infection and both frog age and wetland type are depicted with water temperature held at its mean. The bars show the mean estimates in the top-left and bottom plots, and the black lines the 95% CI of those estimates. The black line in the top-right plot shows the mean estimates and the grey shading the 95% CI of those estimates.

3.3 Consequences of infection

There was a weak effect of the intensity of infection on the daily probability of survival (ϕ) of *L. raniformis* during the mark-recapture study (Table 3; Figure 3). The regression coefficient for this effect was, as anticipated, negative (-0.03), but the 95 % CI of the coefficient overlapped zero (Table 3). Thus, while frogs with high zoospore counts displayed lower probabilities of survival overall, the effect was statistically equivocal.

Nevertheless, there was a clear relationship between infection load and the probability of recapture (r) for *L. raniformis* (Table 3; Figure 3). The regression coefficient for this effect was positive (0.1), and the 95 % CI of this coefficient did not overlap zero (Table 3). The probability of recapture is predicted to increase from 0.13 for a frog displaying ~ 3 zoospores per swab (= 1 $\log_e$ zoospores) to 0.32 for a frog displaying ~ 163,000 zoospores per swab (= 12 $\log_e$ zoospores) (Figure 3).

Table 3. Parameter estimates for the model relating the intensity of chytrid infection (estimated number of zoospores per swab) to the daily probability of survival (ϕ) and the probability of recapture (*r*) for *Litoria raniformis*. The regression coefficients for each effect (β) show the direction of the relationship and its magnitude (but note that the magnitude of the regression coefficients is not comparable across effects). The mean estimate of each regression coefficient is provided, along with the 95% credible interval of these estimates (95 % CI).

Parameter	**Mean**	**95% CI**
Daily probability of survival (ϕ)		
α (intercept)	4.22	3.93, 4.61
β_1 (infection effect)	-0.03	-0.10, 0.04
Probability of recapture (*r*)		
α (intercept)	-1.90	-2.27, -1.59
β_1 (infection effect)	0.10	0.02, 0.19

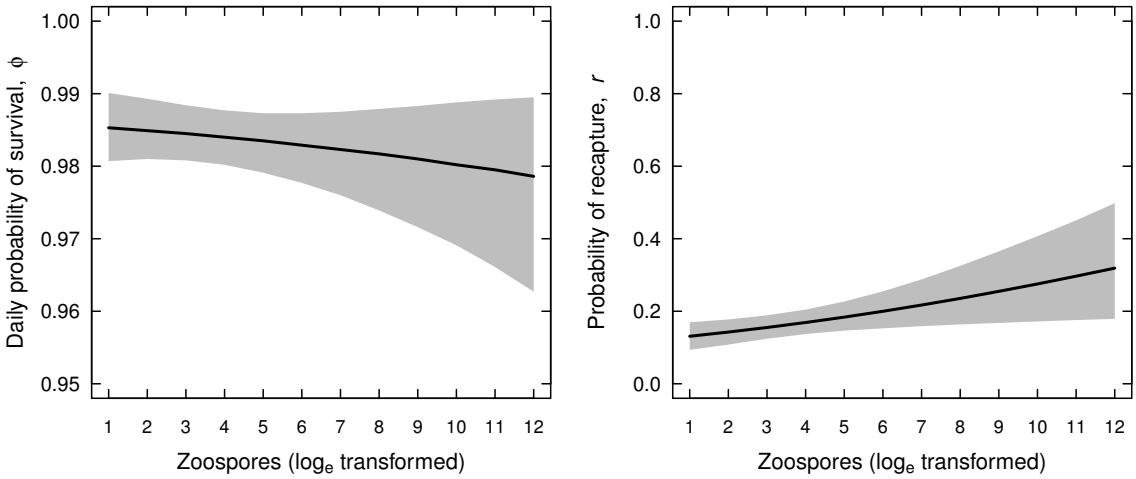

Figure 3. Relationships between the intensity of chytrid infection (estimated number of zoospores per swab) and both the daily probability of survival (ϕ) and the probability of recapture (*r*) for *Litoria raniformis*. The black lines show the mean estimates of these relationships and the grey shading the 95% CI of those estimates.

4 Discussion

The chytrid fungus (*Batrachochytrium dendrobatidis)* has been implicated in the decline of numerous Australian frog species (Berger *et al*. 1998), including the Growling Grass Frog (*Litoria raniformis)* from south-eastern Australia (Pyke 2002; Hamer *et al*. 2010). This species was formerly common and widespread across its range, but underwent significant range contractions and population declines during the latter half of the last century (Clemann and Gillespie 2008). This study represents the first assessment of the prevalence and dynamics of the chytrid fungus amongst populations of *L. raniformis* since that decline. It has identified important predictors of infection for *L. raniformis*, and provides insights into the effects of the disease on the survival rates of these frogs. In the following sections we compare the results of this study to similar research in Australia and elsewhere, and describe the implications of our results for the conservation of *L. raniformis*. The latter discussion focuses on the conservation of *L. raniformis* in Melbourne's urbanising landscapes, where this study took place.

4.1 Prevalence and intensity of infection

Chytrid fungus was ubiquitous amongst the populations of *L. raniformis* sampled during this study. Prevalence of the disease over the two year sampling period was 13% for juveniles and 35% for adults. These figures are higher than those reported for some other Australian frogs (e.g., Woodhams and Alford 2005), but similar to, or lower, than those reported for others[2]. For example, Retallick *et al*. (2004) reported a prevalence of chytrid infection of 18% for *Taudactylus eungellensis* and 28% for *Litoria wilcoxii* from an upland site in the tropics of central Queensland. Kriger and Hero (2006) reported an infection rate of 27% amongst *L. wilcoxii* at a lowland site in southeast Queensland. Stockwell *et al*. (2008) reported a prevalence of chytrid infection of 53% amongst juvenile *Litoria aurea* at a re-introduction site in coastal New South Wales. Clemann *et al*. (2009) report an overall prevalence of ~ 80% for populations of *Crinia signifera* and *Litoria verreauxii alpina* from the Australian Alps.

Our finding that chytrid infection was widespread amongst the sampled populations of *L. raniformis* is also congruent with results from other comparable systems in Australia. Retallick *et al*. (2004) detected the fungus at 66% of the sites they sampled, while Woodhams and Alford (2005) detected it at 100% of sites. Working on members of the *Litoria lesueurii* complex, Kriger *et al*. (2007) reported chytrid at 77% of sites they sampled along the eastern coast of Australia. Clemann *et al*. (2009) reported that chytrid was detected amongst the resident frog community at 75% of sites in the Australian Alps.

While there was some variation in the prevalence of chytrid fungus amongst the study sites, the intensity of infection was fairly consistent. Mean estimates were low, averaging 114 zoospores per swab for juveniles and 154 zoospores for adults (range = 1–216,444 zoospores per swab). Comparison of these results to other Australian frogs is limited by the relatively recent introduction of the qPCR approach used here to quantify pathogen loads (Hyatt *et al*. 2007). Kriger and Hero (2006) and Kriger *et al*. (2007) reported zoospores loads for *L. wilcoxii* and *L. lesueuri* that were similar to those reported here. For *L. wilcoxi*i, the maximum recorded load was in the order of ~ 100,000 zoospores per swab. For *L. lesueurii*, the mean zoospore count was 190 per swab, with a maximum of ~ 217,000 per swab. The study of Clemann *et al*. (2009) suggests high mean zoospores loads for *L. verreauxii alpina* (~ 17,000 zoospores), with a maximum in the order

[2] Note that comparison of the prevalence recorded during this study with that observed in others is complicated by the fact that the time period over which prevalence was measured varies amongst studies.

of 120,000 zoospores. The equivalent figures for *C. signifera* (which is appears to be relatively resistant to the pathogen) were 2,148 and ~ 35,000 zoospores per swab (Clemann *et al*. 2009).

4.2 Correlates of infection

Variation in prevalence of chytrid infection between different age groups of anurans has received some attention over recent years. The results are mixed. Retallick *et al*. (2004) reported no difference between adult and sub-adult *T. eungellenis* in central Queensland. Rachowicz *et al*. (2006) reported very high rates of chytridiomycosis-induced mortality (96%) amongst newly metamorphosed *Rana mucosa* at infected high-altitude lakes in the Sierra Nevada, California. Kriger *et al*. (2007) also reported a high prevalence of chytrid infection amongst juvenile *L. lesueurii* along the eastern coast of Australia, finding a strong negative relationship between body size and infection rate for this species. We found the opposite result for *L. raniformis*: juvenile frogs (defined here as 50 mm snout-vent length or less) displayed a much lower probability of infection than adults. We hypothesised that this effect could arise from the limited exposure of tadpoles to the pathogen, producing carry-over reductions in the likelihood of infection amongst newly metamorphosed individuals (which dominated our sample of juvenile frogs). However, such a result could also arise from the fact that the probability of infection for *L. raniformis* declines strongly with increasing water temperature, as newly metamorphosed frogs were almost exclusively captured between January and March. Nevertheless, our analyses did account for this interaction, because the model included effects of both age and water temperature on the probability of infection.

Temperature is emerging as a key environmental constraint on chytridiomycosis in amphibians. Early laboratory studies suggests that the pathogen was sensitive to elevated temperatures (Longcore *et al*. 1999), and more recent studies have confirmed that the prevalence and virulence of chytrid fungus is influenced by environmental temperatures. Berger *et al*. (2004) found a clear increase in the rate of mortality due to chytridiomycosis in winter amongst various frog species from eastern Australia. This result accorded with their laboratory studies, in which the rate of infection and mortality from infection was significantly higher under 'cool' temperature treatments (17° C and 23° C). Retallick *et al*. (2004) and Woodhams and Alford (2005) reported the same winter increase in the prevalence of chytrid infection amongst several frogs along the eastern coast of Australia, while Kriger *et al*. (2007) documented a negative relationship between the probability of infection and water temperature for *L. lesueurii* in the same region. Internationally, higher rates of infection and susceptibility to infection with lower temperatures have been reported for *Alytes obstetricians, Atelopus zeteki, Lithobates areolatus* and *Lith. yavapaiensis* (Richards-Zawacki 2010; Geiger *et al*. 2011; Kinney *et al*. 2011; Savage *et al*. 2011). Our study confirms that the probability of infection for *L. raniformis* adheres to this trend, displaying a sharp decline with increasing water temperature. We can infer from this relationship that the probability of infection displays an annual cyclical trend, being highest in winter and lowest in late summer. This, and the probable interaction between temperature and the virulence of chytrid fungus, suggests that *L. raniformis* are most susceptible to the pathogen during the winter months. This patterns concurs with observations of sick and dying *L. raniformis* in winter around Melbourne (G. Heard pers. obs.), but not with previous analyses of the seasonal survival rates of the species in this area (Heard *et al*. in press-a). We discuss this issue further below.

The influence of water chemistry on the prevalence and virulence of chytrid fungus is also an area of current interest. In Australia, White (2006) suggested that increasing the salinity levels of ponds in which captive populations of *L. aurea* were housed raised their survival rates, apparently because the heightened salinity reduced the virulence of the fungus. More recent work indicates that *L. aurea* displays higher overwintering survival at more saline wetlands (M. Stockwell,

University of Newcastle, pers. comm.). Piotrowski *et al*. (2004) suggested that pH may also be an important constraint on chytrid development, with slower growth under alkaline conditions. Our finding of lower rates of infection of *L. raniformis* by chytrid fungus at quarry wetlands with relatively high salinity and pH is congruent with these findings. Nevertheless, as noted above, our data cannot identify the mechanism(s) underlying this result. It may be possible, for example, that other components of the water chemistry at these sites limit the fungus. Alternatively, these sites may display higher water temperature regimes than others, or offer enhanced opportunities to raise body temperature during basking (Richards-Zawacki 2010) because of high levels of incident solar radiation. Research on the mechanisms underlying the reduced rate of chytrid infection in quarry wetlands would be of considerable interest.

4.3 Consequences of infection

Amongst amphibians that are susceptible to chytridiomycosis, current knowledge suggests that the disease poses a significant threat to the viability of naïve populations, but that at least some populations that survive initial exposure may persist at reduced densities, despite cyclical outbreaks of the pathogen. For example, Retallick *et al*. (2004) did not find a strong effect of infection status on the probability of survival for *Taudactylus eungellensis* despite apparent severe population declines in this species, attributed to chytridiomycosis outbreaks during in the 1980s. Likewise, Woodhams and Alford (2005) reported widespread occurrence of the fungus amongst populations of frogs from central Queensland that had also undergone significant population declines. Pilliod *et al*. (2010) recently reported a clear relationship between survival probability of *Bufo boreas* in the field and chytrid infection status, but they also reported that population density was relatively stable during their study, suggesting that while chytrid is present in their study population, its influence on population trajectories may have stabilised. Kinney *et al*. (2011) report a similar result for *Lithobates areolatus*, although they did report local extinction of at least one population where a high rate of chytrid-related mortality was observed. Differential survival of geographically disjunct populations of the same taxon may also be affected by sympatric hosts of *B. dendrobatidis;* the presence of co-occuring amphibian species that are resistant to the effects of the fungus may increase the probability of local extinctions for non-resistant taxa (Clemann *et al.* 2009).

We did not find a clear relationship between the intensity of chytrid infection and the probability of survival for *L. raniformis* in the study area. The estimated effect of infection intensity on survival was negative, but the effect was not strong. This result might imply that populations of *L. raniformis* in the study area are now relatively robust to chytridiomycosis. However, there are several reasons to treat this conclusion with some caution. The first is that our analysis did not account for changes in infection status through time – it effectively assumed that frogs that were uninfected at first capture remained so, while those that were infected were unable to clear the pathogen. This is unlikely to be true (indeed, our repeat swabbing of some individuals showed some evidence of changes in individual's infection status through time), and so the lack of a clear relationship between infection status and survival could result from changes in infection status. The second problem is that our study was limited in duration, and was conducted during the warmer months of the year. As a consequence, we had little capacity to detect any winter die-offs resulting from chytrid infection, as discussed above. Nevertheless, as alluded to above, previous analyses of our mark-recapture data suggest that the survival rate of *L. raniformis* during the winter of this study was equivalent to that during the active season (Heard *et al*. in press-a), which runs counter to the expectations of winter die-offs resulting from chytridiomycosis. However, it is also true that the single autumn and winter period encompassed by this study was unusually warm, with minimum air temperatures that were above the long-term average in each month (BOM

2011). The final consideration is that our study may have lacked the required power to demonstrate a clear effect of infection intensity on survival rates, as few individuals displayed high infection loads. Only nine frogs displayed zoospores loads of > 10,000 per swab; the threshold above which chytridiomycosis is often considered fatal in a variety of amphibian species (Vredenburg *et al*. 2010; Kinney *et al*. 2011). Again, this may be due to the fact that we only conducted surveys during the warmer months of the year, and that this study occurred during an atypically warm period.

Interestingly, while we did not observe a clear effect of infection intensity on survival rates of *L. raniformis*, our analyses demonstrated a positive effect of infection on the probability of recapture during the mark-recapture study. Thus, individuals with more severe infections at first capture were more likely to be re-caught. This is a logical result, with more heavily infected individuals probably being less alert and/or agile than healthy frogs. Indeed, Waldman (2001) reported reduced likelihood of fleeing from capture amongst heavily infected *L. raniformis* in New Zealand.

4.4 Management implications

4.4.1 Implications for survey and monitoring

Existing protocols for the prevention of disease transmission during research on amphibians emphasise the need to minimise the chance of spreading chytrid fungus between wetlands or study areas. For this reason, it has been standard practice in research and monitoring of *L. raniformis* to disinfect footwear and equipment between wetlands (Heard *et al*. 2010). Our finding that chytrid fungus was essentially ubiquitous in the study area may provide a basis for questioning the need to maintain between-site disinfection protocols. However, we caution against discontinuation of between-site disinfection strategies for four reasons. Firstly, our study provides only a two-year snap-shot of the distribution of chytrid fungus. It may be that the distribution of the fungus is highly changeable through time, in which case survey protocols that do not include between-wetland disinfection strategies run the risk of re-introducing the fungus to populations of *L. raniformis* (and other species) that have become chytrid-free. Secondly, our study was conducted over a small component of the geographic distribution of *L. raniformis*: it is quite possible that the distribution of chytrid in this landscape is unrepresentative of that elsewhere in south-eastern Australia. Thirdly, there are various strains of chytrid fungus, and these strains vary in their virulence (Berger *et al*. 2005); although the fungus in any particular area may be a uniform strain, it is best to remain cautious in this regard. Finally, while the disinfection requirements for some types of surveys can be onerous (e.g., disinfecting nets used to sample tadpoles), disinfection of equipment and footwear is easily completed and inexpensive in most circumstances.

Our finding that higher infection loads increase the probability of recapture of *L. raniformis* has implications for monitoring programs for these frogs, and for future studies that seek to document rates of chytrid infection amongst populations of *L. raniformis*. Mark-recapture studies may be used to measure and monitor population sizes, recruitment rates and survival rates of *L. raniformis* (Heard *et al*. 2010). The results of the current study suggest that mark-recapture studies should consider the influence of chytrid infection on recapture rates, because this influence could bias mark-recapture estimates of the demographic parameters such as survival and abundance. We recommend swabbing each frog for chytrid on each capture occasion, so that infection status can be included as a covariate on the probability of recapture. Doing so will also provide the information required to understand the dynamics of the disease for *L. raniformis*, and its effects on the population dynamics of this species.

The relationship between chytrid infection intensity and recapture rate for *L. raniformis* also has ramifications for studies that seek to determine the prevalence of the disease through space or

time. If this relationship is not considered, the resulting estimates of prevalence may be biased high. Marking individuals represents the only way of accounting for this effect, but researchers should consider the ethical, logistical and financial burden that individual marking entails. Batch marking (applying a non-unique mark) could represent a compromise.

4.4.2 Implications for wetland management

Quarry wetlands are recognised as vital, high-quality habitat for *L. raniformis* across urban Melbourne, given the large size of these wetlands, their long hydroperiods, high water quality and diverse aquatic vegetation community, and because they generally lack exotic predatory fish (Robertson *et al*. 2002; Heard *et al*. 2004; Heard and Scroggie 2009). We have argued previously that these wetlands should be priorities for protection, because they likely represent key wetlands for metapopulation persistence (Robertson *et al*. 2002; Heard *et al*. 2004; Heard and Scroggie 2009). However, the finding that populations of *L. raniformis* inhabiting these wetlands display lower rates of chytrid infection suggests that they may be even more important for metapopulation persistence than originally recognised, because they may act as refuges from the fungus. It is therefore doubly concerning that populations of *L. raniformis* occupying quarry wetlands around Melbourne are highly threatened, due to the frequency that these quarries are used for landfill. Indeed, the two quarries included in this study are being land-filled, and will probably be destroyed in the next few years. We urge much greater emphasis on the protection of these wetlands across Melbourne.

It is clear from this study that water temperature is a key driver of chytrid prevalence for *L. raniformis*, with much higher probabilities of infection at lower water temperatures. This result is of some significance for wetland management for *L. raniformis*, because it further emphasises the detrimental effect of shading on wetland quality for this species. We have already documented a strong negative relationship between aquatic vegetation cover and the probability of extinction for *L. raniformis* in this study area (Heard *et al*. 2010). While this relationship has previously been attributed to the microhabitat requirements of *L. raniformis,* the relationship may also be indicative of the relationship between water temperature and chytrid infection rates for this species. The diversity and density of aquatic vegetation at wetlands around Melbourne is heavily influenced by insolation and water temperatures, with cool, shady sites (typically those overgrown with exotic riparian trees) invariably displaying limited aquatic vegetation. We encourage removal of exotic riparian vegetation to increase solar radiation and water temperatures, both because it represents a means of increasing aquatic vegetation cover (Heard *et al*. 2010), and because it may limit the prevalence of chytrid fungus. For the same reasons, we caution that some riparian revegetation schemes may ultimately prove detrimental to populations of *L. raniformis* if those schemes lead to excessive wetland shading and concomitant reductions in water temperature.

More generally, we highlight the fact that while our analysis has not indicated a clear effect of chytrid infection on survival rates of *L. raniformis*, the disease may represent an ongoing, cyclical stress for populations of this species, or one that periodically drives populations to local extinction (given outbreaks under appropriate environmental conditions). Both scenarios have implications for wetland management. The former suggests that the management of individual wetlands should strive to enhance local population sizes, because larger populations should be more robust to cyclical die-offs (Savage *et al*. 2011). We suggest enhancement of wetland area, hydroperiod and aquatic vegetation cover as a means of achieving this goal, following the approaches described by Heard *et al*. (2010). Maintaining opportunities for recolonisation in the event of local population extinction will also be crucial. As we have outlined previously (Heard *et al*. 2010), maintaining or developing dense networks of populations through the protection, enhancement and creation of wetlands, and maintenance of terrestrial dispersal corridors between them, are the key

requirements in this regard, because the probability of colonisation for *L. raniformis* is tightly controlled by the density of surrounding populations, and the ability of migrants from those populations to disperse to vacant wetlands.

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Appendix 1. Sites at which the mark-recapture study and swabbing of *Litoria raniformis* for chytrid fungus was undertaken.

Site	**Description**	**Easting (GDA94)**	**Northing (GDA94)**	**Total number *L. raniformis* swabbed on first capture**
Donnybrook				
N1	Dam adjacent to the Hume Freeway	318368	5842686	45
N2	Kalkallo Creek at Donnybrook Road	318829	5843122	107
N3	Merri Creek at Donnybrook Road	319825	5843192	118
N4	Dam on Donnybrook Road	319807	5843041	9
N5	Dam on Spring Street	320748	5843374	31
Somerton				
C1	Merri Creek at O'Hern's Road	319822	5833203	44
C2	Merri Creek in the vicinity of Freight Drive	320570	5832473	17
C3	Quarry at the former Epping Tip (now City of Whittlesea land)	320723	5832645	44
C4	O'Hern's Swamp	320580	5833068	0
C5	Curley Sedge Creek at Craigieburn Grasslands	320187	5833393	0
Campbellfield				
S1	Former Austral Bricks quarry at Bolinda Road	320194	5827075	51
S2	Boral Quarry on Transport Drive	321858	5827145	55

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