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Age- and size-dependent resistance to chytridiomycosis in the invasive cane toad *Rhinella marina*

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ABSTRACT: In Australia, the cane toad *Rhinella marina* and chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) are examples of invasive species that have had dramatic impacts on native fauna. However, little is known about the interaction between *Bd* and cane toads. We aimed to explore the interaction of these 2 species in 3 parts. First, we collated data from the literature on *Bd* infection in wild cane toads. Second, we tested the susceptibility of recently metamorphosed cane toads to *Bd* infection. Finally, we modelled the distribution of the 2 species in Australia to identify where they overlap and, therefore, might interact. Through our data collation, we found that adult cane toads are infrequently infected and do not carry high infection burdens; however, our infection experiment showed that metamorphs are highly susceptible to infection and disease, but resistance appears to increase with increasing toad size. Niche modelling revealed overlapping distributions and the potential for cane toads to be affected by chytridiomycosis in the wild. While *Bd* can cause mortality in small juveniles in the laboratory, warm microhabitats used by wild toads likely prevent infection, and furthermore, high mortality of juveniles is unlikely to affect the adult populations because they are highly fecund. However, to demonstrate the impact of *Bd* on wild cane toad populations, targeted field studies are required to assess (1) the overall impact of chytridiomycosis on recruitment especially in cooler areas more favourable for *Bd* and (2) whether cane toad juveniles can amplify *Bd* exposure of native amphibian species in these areas.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · Wildlife disease · Marine toad · *Rhinella marina*

INTRODUCTION

Invasive species are among the top causes of biodiversity loss, but potential interactions among them can be difficult to predict. In Australia, little is known about interactions between 2 invasive species of particular conservation importance: the cane or marine toad *Rhinella marina* and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*).

The cane toad is a highly successful invasive species, having colonised >20 countries around the globe (Lowe et al. 2000, Lever 2001). One of the most notable invasions occurred in Australia, where the toad was deliberately introduced as a biological

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control for sugar cane pests in 1935 (Lever 2001). The toad population established, grew and expanded throughout much of eastern and northern Australia. The toad's distribution is predicted to keep expanding across the entire northern coast of Australia and towards the south (Kearney et al. 2008, Kolbe et al. 2010). The toad has already colonised areas outside the species' realized thermal niche in its native range in Central and South America (Kolbe et al. 2010). Cane toads are a conservation concern because they are highly toxic and can fatally poison native predators that attempt to consume them (Smith & Phillips 2006, Shine 2010). While it does not appear that post-metamorphic cane toads are affecting native amphibians by direct competition (Greenlees et al. 2007), there is direct competition among cane toad and native Australian frog tadpoles (Williamson 1999, Crossland et al. 2009). In addition, cane toads may act as a disease reservoir and thereby threaten native frog species. The only parasite thought to have been introduced to Australia with toads (the lung worm *Rhabdias pseudosphaerocephala*) has not been found in native frogs (Pizzatto et al. 2012). However, toads may have amplified native myxosporean pathogens and thereby increased native frogs' exposure to this and other pathogens (Hartigan et al. 2011).

One pathogen of particular conservation importance is *Bd*, the causal agent of amphibian chytridiomycosis. Chytridiomycosis is one of the most devastating wildlife diseases, infecting >600 amphibian species and suspected as the primary cause of decline of >200 species to date (Skerratt et al. 2007, Wake & Vredenburg 2008). In Australia, *Bd* is implicated in the decline of 34 frog species (Skerratt et al. 2016). *Bd* infects the outer layers of amphibian epidermis, causing electrolyte imbalances and ultimately cardiac arrest (Berger et al. 1998, Voyles et al. 2009). While chytridiomycosis has caused major declines in other species of toad, particularly *Alytes obstetricans, Bufo boreas* and species in the *Atelopus* genus (Bosch et al. 2001, Muths et al. 2003, La Marca et al. 2005), little is known about the impact of *Bd* infection on cane toads (Skerratt et al. 2007). Therefore, understanding *Bd* susceptibility will help guide future research on the impact of *Bd* on cane toads.

It has been suggested previously that cane toads might act as a reservoir host of *Bd* (Berger et al. 1999, Daszak et al. 2003, Johnson & Speare 2005, Fèvre et al. 2006, Kilburn et al. 2011). However, evidence for this claim was based on a single outbreak in captivity, where 14 of 63 wild-caught juvenile toads succumbed to infection (Berger et al. 1999). In that outbreak, infection was also detected in captive-bred tadpoles and resulted in high mortality after metamorphosis, while adult toads housed in the same room survived but were not tested for infection (Hyatt 1998, Berger et al. 1998, Berger 2001). This observation indicates that wild juvenile cane toads could be infected with *Bd*, but more empirical data are needed to assess the potential for cane toads to be reservoirs of chytridiomycosis that impacts native anurans. Thus, determining how toad ontogeny affects *Bd* susceptibility is important to understand the role this amphibian plays in *Bd* disease dynamics in native species. In addition to susceptibility, determining environmental niche overlap of both *Bd* and cane toad distribution is important to determine the potential for transmission of infection in metamorphic cane toads in the wild, which could affect the native co-occurring susceptible hosts as well as impact cane toads.

In this study, we examined the interaction between *Bd* and cane toads in 3 ways. First, we collated published and unpublished literature through a systematic literature review to identify studies in which cane toads have been analysed for *Bd* infection. Second, we conducted a laboratory exposure experiment on recent metamorphs and explored susceptibility based on body size. And third, given the potential role of cane toads in the spread of chytridiomycosis in Australia, we modelled host and pathogen distributions to identify areas where the 2 species overlap and, therefore, could interact in the wild. Distributional overlap results indicate where toads could amplify *Bd* dynamics and thereby harm native anurans or where *Bd* could impact toad populations.

MATERIALS AND METHODS

Collation of cane toad/*Bd* literature

First, we conducted a search for published and archived articles in the Google Scholar Database using keywords combining synonyms for both cane toad and *Bd*, such as 'cane toad', 'marina', 'marinus', 'marine toad' AND 'chytrid', 'Batrachochytrium', 'disease'. We then searched for published articles that cited the original case study that suggested cane toads might be a reservoir host, Berger et al. (1999) and Daszak et al. (1999), and the keywords 'cane toad' or 'disease reservoir' (or synonym). Then we searched the archives available both online and in print of all *Bd* surveys in *Herpetological Review* pub-

lished after 1999, because this journal is not searchable through Google Scholar. We read all *Bd* distribution surveys and looked for mentions of cane or marine toads. If species was not explicitly stated, but *Bufo* or *Rhinella* was mentioned, or more information was needed, we contacted the authors for more information. Finally, we contacted all individuals providing personal communication or unpublished data on *Bd* testing in cane toads in the published articles. Data collation and searches occurred in 2014 to 2016.

Host–pathogen interaction

Animal husbandry

Wild cane toads were caught from various locations around Townsville, Kirwan, and Alligator Creek, Queensland, Australia, with the largest distance between populations being 33 km. These animals were categorized as recent metamorphs because they were <30 mm in snout to vent length (SVL) (Cohen & Alford 1993). The size of the animals was not evenly distributed across all sizes but rather comprised 2 size classes: >18 mm (n = 29) and <16 mm (n = 59). We used size as a variable in our analyses but note that size covaries with other variables such as age and development (Cohen & Alford 1993). Thus, significant size effects in our analyses may actually reflect causal relationships with these size-correlated variables rather than with size itself. Because the animals used in this study were immature juveniles, sex could not be assessed. It was unlikely that the toads were exposed to *Bd* in the wild because Townsville and the surrounding region is a hot and dry climate (Murray et al. 2011a). However, we tested all collected toads for *Bd* prior to the experiment (see methods below) and confirmed that none were infected. A subset of the animals from this study were analysed after the completion of this infection experiment, post mortem, for immune parameters as reported by Brannelly et al. (2016c).

The animals were maintained in individual 10 × 6 × 6 cm terraria with damp paper towel substrate (Earthcare, ABC Tissue) that was changed twice each week and misted daily with reverse osmosis water. Enclosures were arranged on the countertop (not on shelving) so that each animal had the same exposure to the overhead fluorescent lighting on a 12 h light:12 h dark cycle. Animals had no opportunity to bask. The room temperature was maintained at 20 to 23°C because this is within the optimal temperature range for *Bd* growth (Piotrowski et al. 2004). Animals were fed 5 juvenile crickets *Acheta domestica* twice weekly. Crickets were dusted with amphibian vitamin powder supplement (Herptivite multivitamin, Rep-Cal) and gut loaded (Jurassiquench, JurassiPet). Animals were checked daily.

Inoculation

Animals were allowed to acclimate to their new environment for at least 7 d before the experiment. The toads were haphazardly divided, but evenly distributed across size classes, into 2 treatment groups: (1) exposure group (n = 54; 20 large, 34 small) and (2) control group (n = 34; 9 large, 25 small). The exposure group were inoculated with a New South Wales strain of *Bd* (AbercrombieR-L.booroolongensis-2009-LB1, Passage number 11). This isolate of *Bd* belongs to the Global Pandemic Lineage and is known to be virulent in susceptible species (Brannelly et al. 2015a, 2016a,b, 2017). *Bd* was harvested from tryptone, gelatin hydrolysate, lactose (TGhL) agar plates after incubation at 23°C for 5 d. Plates were flooded with 3 ml of aged spring water for 10 min to allow zoospores to be released from zoosporangia. Inoculum was poured off the plates, and zoospores were counted using a hemocytometer. The *Bd*-exposed animals were inoculated with 1×10^6 zoospores by applying 3 ml of inoculum onto the ventral side of the toad and were held inside a 40 ml inoculation container for 6 h, then transferred back into their terraria. This method of *Bd* exposure is an effective inoculation procedure for terrestrial anuran species (Brannelly et al. 2012a, 2016b). Control toads were mock-inoculated using the same procedure as above but with uninfected Petri plates.

Data collection

Once every week, we swabbed the animals for *Bd* (see 'Testing for *Bd*'), weighed them, and measured their SVL using dial callipers. Throughout the experiment, animals were euthanized by 0.5% buffered tricaine methansulfonate (MS-222, Sigma-Aldrich) bath if they displayed clinical signs of chytridiomycosis (reduced appetite, excessive and irregular skin slough, splayed legs) and their righting reflex was delayed (i.e. the animal was moribund). Animals were swabbed prior to being euthanized. We ended the experiment 93 d after inoculation, when all remaining control and exposed animals were swabbed and then euthanized. We used day of mor-

bidity/euthanasia for our survival analysis. We considered animals successfully infected if they tested *Bd*-positive at least twice during the experiment. We considered animals cleared if, following a successful infection, they returned *Bd*-negative results for at least 3 consecutive weeks (Berger et al. 2010).

Testing for *Bd*

We tested animals for *Bd* infection by extracting the DNA from the skin swabs and quantifying the infection intensity using quantitative polymerase chain reaction (qPCR) (Hyatt et al. 2007). The standard protocol involves 45 strokes with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment, Corsham, Wiltshire, UK) per animal: 5 on the middle of the venter, 5 on each side of the venter, 5 on each thigh, and 5 for each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab, and to standardize the method so that each swab gathered is comparable to other samples within the experiment. DNA was extracted from the swabs using PrepMan® Ultra (ThermoFisher Scientific) and 2 min of high-velocity sample homogenization at maximum speed using 30 to 40 mg of 0.5 mm silica beads to break apart the fungal cell walls. We analysed the extract using qPCR following Boyle et al. (2004), in singlicate (Kriger et al. 2006, Brannelly et al. 2015b, 2016c) with a positive and negative control, and a series of dilution standards. We determined zoospore equivalents (ZE) of each whole swab using a series of 5 standards, which was then $\log_{10}$-transformed to quantify infection intensity. Swabs taken at morbidity were tested to confirm infection but were not used to quantify loads because intensity measures at late stages of infection can be artificially inflated due to degeneration of the epidermis and excessive epidermal shedding, which can result in greater amounts of DNA gathered on the swab for those animals in clinical stages of infection. Therefore, the samples taken at morbidity are unreliable for estimating the quantity of *Bd* in a way comparable to other animals in the study.

Histological preparation

We examined whole body sections histologically in a subset of animals (n = 25 *Bd* infected treatment group, n = 9 control group) to determine if morbidity in animals was caused by chytridiomycosis. Upon euthanasia, we dissected animals and fixed them in 4% phosphate buffered formaldehyde for 2 h and transferred the tissues to 80% ethanol prior to histological preparation. We used routine histological techniques to prepare the tissues for light microscopy following standard methods (Woods & Ellis 1994). Briefly, tissues were dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. Tissues with bone were decalcified in 10% formic acid for 24 h, rinsed in water and transferred to 70% ethanol prior to being embedded. All tissues were serially and longitudinally sectioned at 5 µm, affixed to glass slides and stained with haematoxylin followed by eosin counterstaining (H&E), and mounted with coverslips. We examined the dorsal, thigh and ventral skin from at least 3 randomly selected slides for each animal.

Analyses

Infection

We used a linear mixed effects model to determine if infection intensity of *Bd*-exposed animals varied over time. In the model, infection intensity $\log_{10}$(ZE) was the dependent variable, the week after inoculation was the fixed effect (i.e. 0, 1, 2, etc.), and individual was a random effect. Only *Bd*+ swab data points were included in this analysis. We analysed swab data collected every 2 wk to have more robust data under the assumption of independence.

Survival

We used a Cox regression to determine whether survival through the end of the experiment (93 d) was influenced by *Bd* exposure or body size, calculated as $\log_{10}$(SVL). We defined exposure status as exposed versus not exposed. For the Cox regression analysis, the dependent variable was time until morbidity (days), and the covariates (independent variables) were size, exposure, and an interaction between size and exposure. These analyses were performed in SPSS (v.21) (IBM).

Size and infection status

To evaluate how growth rate was affected by exposure, we used a mixed effects model where the growth rate, i.e. change in SVL each week =

($SVL_{Week\ i+1} - SVL_{Week\ i}$)/ $SVL_{Week\ i}$) was the dependent variable; week, *Bd* exposure and the interaction between week and exposure were fixed effects; and toad identity was included as a repeated random effect. We analysed weekly growth data every other week to have more robust data under the assumption of independence.

To determine if infection dynamics were affected by size in the *Bd*-exposed group, we used a linear regression models to determine if (1) the size of the animals, calculated as $\log_{10}$(SVL) and (2) body condition, calculated as $\log_{10}$(mass)/$\log_{10}$(SVL) at time of inoculation, affected infection intensity over time, as $\log_{10}$(ZE).

We used a binomial regression with logistic response to evaluate the effect of size on the animal's ability to clear infection, where infection status (*Bd*+ or *Bd*-cleared) was the dependent variable, and size (numeric variable, calculated as $\log_{10}$[SVL]) was the explanatory variable. All of the above analyses were performed in SPSS (v.21) (IBM). Effect size of animal size was determined using Cohen's *d* statistic in Microsoft Excel.

Distributional overlap

We generated a distribution model with Maxent to identify the areas where *Bd* might affect cane toads, particularly metamorphs (the most vulnerable post-metamorphic life stage as determined by our collation and laboratory infection described above), and areas where *Bd* presence might be amplified by metamorphosing cane toads (Phillips et al. 2006). Cane toad metamorphs typically stay within 3 m of water bodies until they grow to 25 mm SVL (Cohen & Alford 1993). Because of this close association with water at their most susceptible stage, we only included areas that were permanent or ephemeral water bodies to represent the maximum extent of niche overlap from the final distribution model where *Bd* and cane toads overlap.

Presence data for the distribution models was obtained from Murray et al. (2011b) for the *Bd* model and for the *R. marina* model from the Atlas of Living Australia (www.ala.org.au/). To control for the spatial autocorrelation and over-representation of certain frequently sampled areas in collections of presence records, we produced a series of thinned data sets with different distance thresholds (0.1 to 0.5°, roughly 10 to 50 km, in 10 km steps). The background points (50 000) for Maxent were selected randomly from the area assumed to be colonisable. We did not weight sampling by latitude because we used an equal area Australia-specific geographic reference system (GDA 94).

Each of the thinned data sets was used to fit a model with 2 different sets of environmental variables (Table S1 in the Supplement at www.int-res.com/articles/suppl/d131p107_supp.pdf); one using the first 4 components of a principle components analysis of the 18 Worldclim bioclimatic variables (Hijmans et al. 2005), 2 topographic variables (topographic wetness and stream power indices), and distance to ephemeral and perennial rivers, lakes, and reservoirs. We used a 1 km digital elevation model resampled to 5 km to match the resolution of the climatic data. The other set of variables included 3 of the bioclimatic variables, 2 hydrologic, and 1 topographical variable, which were specifically selected for *Bd* and the cane toad. We chose the most relevant variables for each species based on a niche views analysis. Niche views analysis consists of identifying variables with low colinearity and where the presence points are distributed close to the centre of a bivariate clouds of points within the area colonisable by the modelled species (Owens et al. 2013). Finally, for each thinned presence dataset and set of environmental variables, we generated a series of spatial patterns used for partitioning the data in training and testing sets (alternating longitude and latitude bands, and as per a chess board pattern). We used the presence records lying within the contiguous bands or squares for training and testing and vice versa. Each of the resulting models trained with the partitioned data were tested with a partial receiver operating characteristic (ROC) analysis, based on the area under curve (AUC) ratios of the random prediction and model predictions curves, using the partial ROC tool with various degrees of omission (predictive error allowed) (Barve 2008, Peterson et al. 2008). The authors' expert opinion was used to ground truth the predicted distribution models with known sites. The best-performing model was then re-fitted using the entire presence dataset with the thinning threshold that produced the best model. With these thorough training and testing methods and using Maxent default settings, we made sure our models (1) represent the distribution of both *Bd* and cane toads based on the occupied niche and (2) reliably estimate environmental suitability for both *Bd* and cane toads in areas beyond the presence data used to train the models.

The Maxent settings to fit the model included all default features and regularisation parameters, and

without clamping and extrapolation. The Maxent default settings result in highly irregular response curves (see the Supplement), which are typical of occupied niches. Fundamental niches, the native responses of species to environmental factors, tend to be smooth (Qiao et al. 2016). We chose not to adjust the default settings of Maxent because we wanted to characterise the invaded niches and represent the most likely areas already occupied by both species (*Bd* and *R. marina*). Given that all combined features describe the observed data well (with the loss of generality because we were not predicting beyond the most recent data points), we kept the most complex model achievable with Maxent (e.g. simpler models might only include linear of quadratic features for instance), which accurately predicted the data that was used for testing. Models were considered 'best' if they were able to predict independent data better than random. Niche models are blind to factors like mobility because the spatial resolution of the grid over which the model is projected is larger than the area covered by the typical home range of individuals (Soberón & Nakamura 2009).

RESULTS

Host–pathogen interaction

Collated cane toad survey data

Through the collation of the published literature, we found that prevalence of infection with *Bd* in adult cane toads tends to be low. From a total of 656 individuals sampled around the world, only 25 were found positive for *Bd* infection (Berger 2001, Sánchez et al. 2008, Lettoof et al. 2013, García-Roa et al. 2014, Mutschmann 2015), and only 5 of those positive animals were in the wild. In Central and South America, low intensity infections were detected at low prevalence in wild adult cane toads (Kosch et al. 2012, Drake et al. 2014) (Table 1). The only reports of *Bd* infection from wild cane toads in Australia involved 2 toads that had low infection intensity (Berger 2001, Lettoof et al. 2013). There is one report of an adult cane toad from the pet trade diagnosed with clinical chytridiomycosis (Mutschmann 2015, F. Mutschmann pers comm). We did not find reports of juvenile or tadpole cane toads infected with *Bd* from the wild

Table 1. Reports of *Batrachochytrium dendrobatidis* infection surveys of wild or captive cane toads *Rhinella marina* globally. The toads in these surveys were adult stage unless otherwise stated in the second column. Infection intensity (number of zoospore equivalents, ZE) is reported where available. Infections were not considered to be a cause of morbidity except for cases in Germany

Country	Wild or commercial	Number tested	Number positive	Method of detection	Source
Central America					
Mexico	Captive	3	0	qPCR	Galindo-Bustos et al. (2014)
Jamaica	Wild	14	0	qPCR	Holmes et al. (2012)
West Indies	Wild	100	0	Histology	Drake et al. (2014)
Nicaragua	Wild	5	1	qPCR, 1.27 ZE	García-Roa et al. (2014)
Costa Rica	Wild	21	0	qPCR	Goldberg et al. (2009)
Costa Rica	Wild	1	0	qPCR	Saenz et al. (2009)
Costa Rica	Wild	20	0	qPCR	Whitfield et al. (2013)
South America					
Ecuador	Wild	2	0	qPCR	McCracken et al. (2009)
Venezuela	Wild	20	2	qPCR, 11 ZE	Sánchez et al. (2008)
Peru	Wild	166	0	PCR	Kosch et al. (2012), T. Kosch (pers. comm.)
Australia					
New South Wales	Wild	44	1	qPCR, 2.94 ZE	Lettoof et al. (2013)
Queensland	Wild	4	1	Histology	Berger (2001)
	Captive (tadpoles)	8	3	Histology	Hyatt (1998), Berger et al. (1998), Berger (2001)
Cairns, Tully, and Normanton, Queensland	Wild	100	0	qPCR	Shine (2010), B. Phillips (pers. comm.)
Geelong, Victoria	Captive (juveniles)	63	14	Histology	Hyatt (1998), Berger et al. (1998)
Europe					
Germany	Captive	5	3	Histology	Mutschmann (2015), F. Mutschmann (pers. comm.)

other than the juvenile animals found positive for *Bd* from the Berger et al. (1999) and Hyatt (1998) studies, which were wild caught and held in captivity with adult toads for some time before *Bd* was detected.

Infection experiment

Infection. Of the 54 metamorphic toads exposed to *Bd*, 52 (96.3%) became infected. By the end of the 12 wk infection experiment, 10 of the 52 (19.2%) infected animals cleared the infection (they were *Bd*-negative for at least 3 consecutive weeks after they had become infected with *Bd*), and all of these *Bd*-cleared animals survived until the end of the experiment. Infection intensity increased over time in the exposed animals (mixed models: $F_{1,\ 174.763} = 243.48$, $p < 0.01$) (Fig. 1a). Prevalence was 63% (33 of 52 were positive) at 2 wk and increased to a peak of 90.6% (48 of 52 were positive) prevalence at 4 wk (Fig. 1b). A large proportion of the animals retained infection throughout the rest of the experiment (Fig. 1b). No control animals tested positive for *Bd* during the experiment.

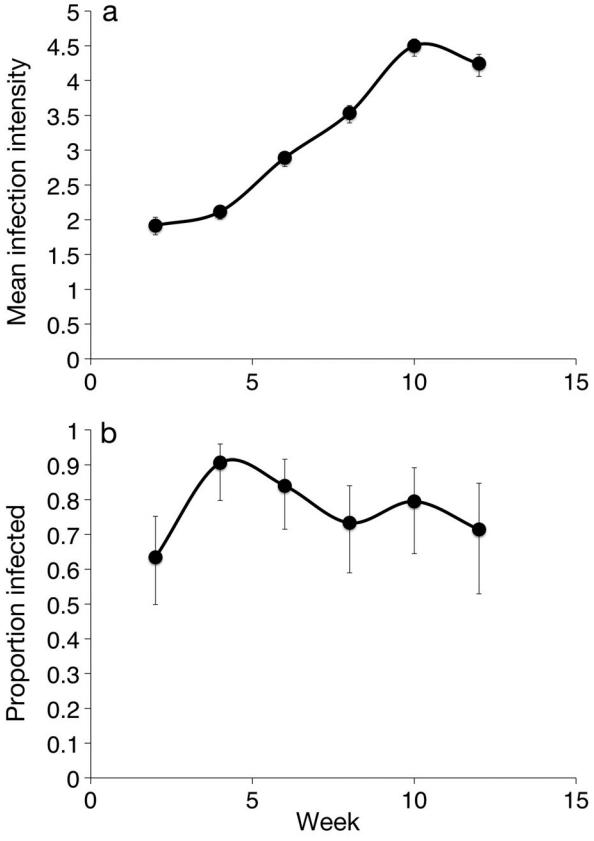

Fig. 1. Infection dynamics throughout the experiment. (a) Infection intensity of all successfully inoculated *Bd*+ (n = 52) individuals over time. Infection intensity is the $\log_{10}$(ZE). Error bars indicate standard error. (b) The proportion of all inoculated toads (n = 54) in the infection treatment group that returned positive qPCR swabs over the course of the experiment. Error bars indicate 95% confidence intervals

Survival. One aim of this study was to determine if the invasive cane toad was susceptible to *Bd* infection and could develop chytridiomycosis. The majority of metamorphic toads exposed to *Bd* became infected (96.3%), and these infected animals experienced a high rate of morbidity (79.2%) and many displayed signs of chytridiomycosis. Only 20.8% of the exposed animals survived to the end of the experiment, while 76.5% of the control animals survived (Fig. 2). Histological evidence indicated that exposed toads had extremely high infection intensities at morbidity (see results below).

Size played an important role in survival, where larger animals survived longer regardless of experimental treatment (Fig. 2; Cox regression: $e^B = 0.007$, $p < 0.01$). *Bd* exposure also significantly affected survival; exposed animals had lower survival than control animals (Cox regression: $e^B = 0.215$, $p < 0.01$). While there was no significant interaction between exposure and size (Cox regression: infection status × size class, $e^B = 0.0$, $p = 0.064$), there was a slight trend for an interactive effect of size and exposure with *Bd* causing greater mortality in smaller metamorphs (Fig. 2).

Size and infection status. Cane toad growth rates were positive each week but did not significantly differ between weeks or between *Bd*-exposed and non-exposed toads, and the interaction between week and

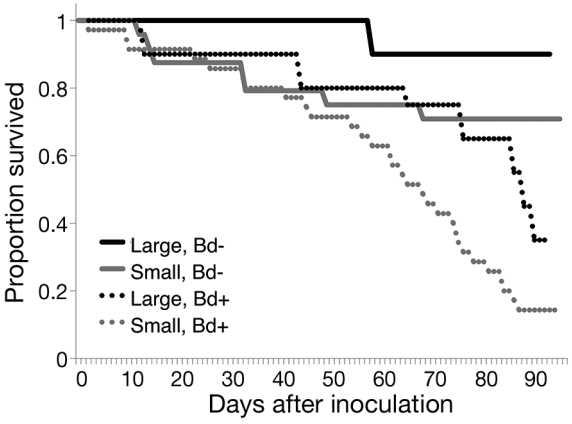

Fig. 2. Survival curves of exposed (*Bd*+) and unexposed (*Bd*–) toads, separated by size for clear visualization of the impact of size on survival (large = >16 mm; small = <16 mm at time of inoculation). Sample sizes for each group are: Large *Bd*–, n = 10; Large *Bd*+, n = 19; Small *Bd*–, n = 24; Small *Bd*+, n = 35. The lines indicate proportion of animals surviving plotted against days since inoculation/control treatments were administered. The experiment ended on Day 93 post inoculation

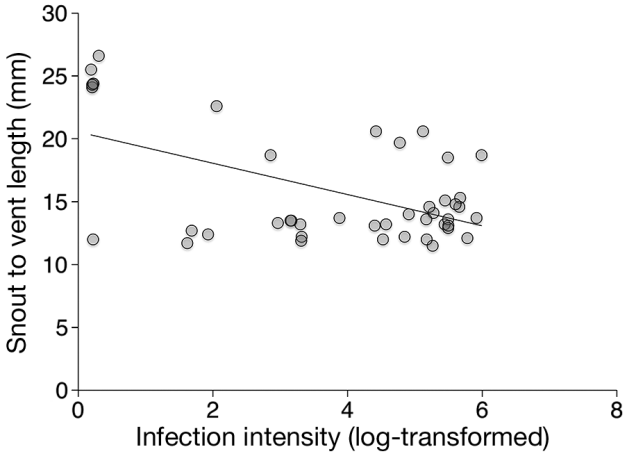

Fig. 3. *Bd* infection intensity compared to snout to vent length of exposed toads. Snout to vent length was measured in mm at the date of inoculation (*y*-axis) compared to log transformed zoospore equivalents (infection intensity) at date of morbidity or the end of the experiment (*x*-axis). Only animals infected on their last swab date (either the end of the experiment at Week 12, or at morbidity, n = 44) were included in this figure

exposure was not significant (mixed effects model: week, $F_{1,419} = 2.406$, p = 0.122; exposure, $F_{1,419} = 1.518$, p = 0.219; week × exposure, $F_{1,419} = 2.81$, p = 0.094).

Of the exposed toads, those that were larger (SVL) at the time of inoculation had lower infection intensities at the end of the experiment (or date of morbidity) (linear regression: Pearson's correlation = –0.495, p < 0.01, $t = -2.200$, p = 0.032) (Fig. 4). Body condition at the time of inoculation had no observable effect on infection intensity at the end of the experiment (linear regression: $t = 1.422$, p = 0.161).

Toad size at the start of the experiment also affected capacity to clear infection. Larger animals were more likely to clear infection (mean SVL for animals that cleared versus did not clear infection: 21.84 ± 7.39 mm [n = 10] versus 15.50 ± 4.21 mm [n = 44]; logistic regression: $e^B = 1.22$, p < 0.01).

Histological assessment. The histological analysis of ventral skin demonstrated extremely high infection intensity in exposed animals that died (Fig. 4), indicating the cause of morbidity was chytridiomycosis. Control animals that died during the experiment showed no signs of *Bd* infection, although the cause of death was unknown.

Distributional overlap

The predicted distribution overlap between *R. marina* and *Bd* in Australia comprises most of the eastern part of the country. Fig. 5 shows the distribution of ephemeral and perennial water bodies that could have *R. marina* infected with *Bd*. The most influential variable in the models was BIO 5 (maximum temperature of warmest month), contributing >50% towards predictions in the *Bd* model, with an overall negative effect on suitability. In the toad model BIO 12 (annual precipitation), with a contribution of nearly 60% (ESM 1), had a positive but asymptotic response peaking around 1000 mm. The best models performed better than random expectations based on the proportion of area predicted: AUC ratios were 1.37 with 25% omission (p < 0.05) and 1.41 (p < 0.05) with 25% omission, for *R. marina* and *Bd* respectively (models that perform no better than a random prediction will have an AUC ratio of 1).

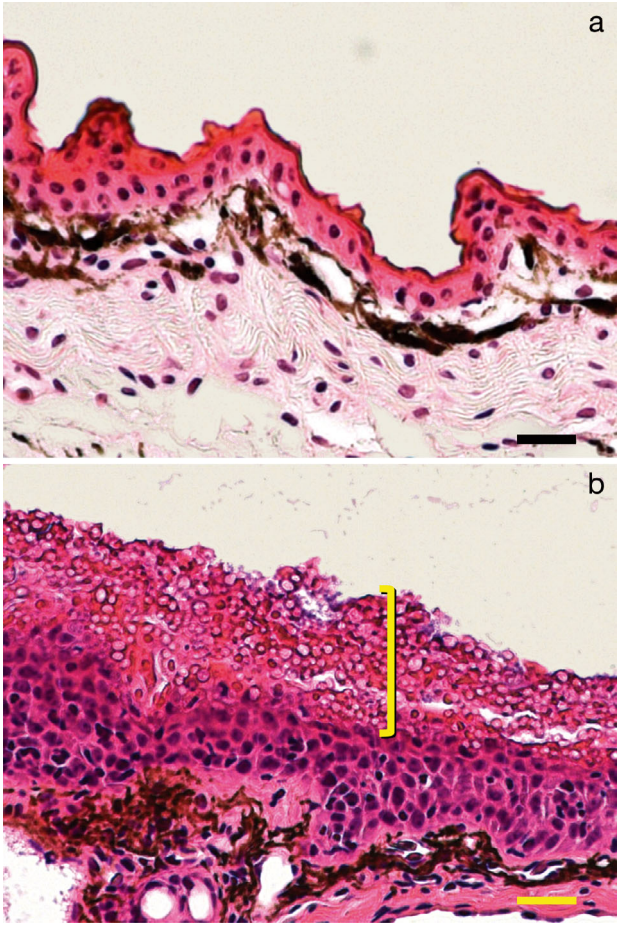

Fig. 4. Examples of both (a) healthy and (b) infected ventral skin of a juvenile toad. In (b), notice the severe epidermal hyperplasia (thick epidermal layers) and thick layer of *Bd* sporangia infecting the upper layers of the epidermis (yellow bracket). Infection of this magnitude was observed in all toads examined and suggests they are relatively tolerant of infections, persisting until infection becomes unusually heavy. Magnification is 200×; scale bars = 30 µm

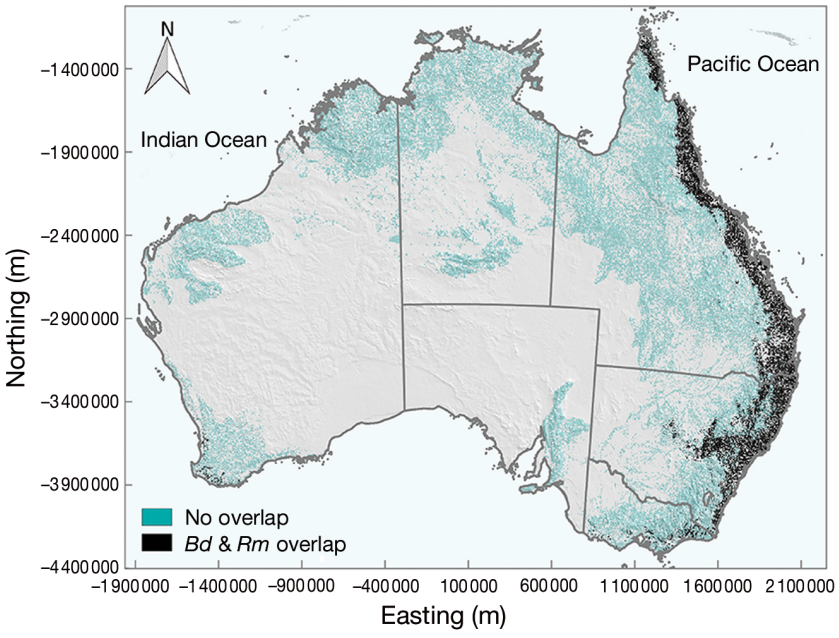

Fig. 5. Modelled potential overlap between *Rhinella marina* (*Rm*) and *Bd*. The black areas represent overlapping distributions. Green areas are where either *Rm* or *Bd* could be present but do not overlap. We have removed all areas that do not contain water bodies to show specifically where *Rm* metamorphs could overlap with *Bd*

DISCUSSION

In this study, we aimed to assess the impact of *Bd* infection on cane toads through literature collation, clinical experimentation and modelling distributional overlap. Through our collation of the literature, it appears that cane toad adults are not susceptible to infection. Our inoculation experiment demonstrated that recent metamorphs, by contrast, are susceptible to *Bd* infection and indicated that resistance increases with size. We found that the distribution of *Bd* in Australia largely overlaps the distribution of toads, but we suggest that it is unlikely to affect cane toads for several reasons. First, adults appear to be resistant. Second, breeding sites are usually in warm microhabitats (Hagman & Shine 2006), which are likely free from *Bd*. Finally, cane toads are highly fecund animals, and recent metamorphs have high mortality rates irrespective of *Bd* infection. Therefore, even high mortality of juveniles due to *Bd* may not impact adult populations (Cohen & Alford 1993, Alford 1994). Furthermore, toads are unlikely to be important in spread of *Bd* across the landscape because although metamorphs are susceptible and can have high infection, they stay near to the natal site until they are larger (Freeland & Kerin 1991, Cohen & Alford 1993). Adults are the mobile stage but are generally resistant. However, to determine if wild cane toad populations respond to *Bd* like our study predicts, future field studies need to include juvenile and tadpole life stages to determine if these animals are infected with and impacted by *Bd* in the wild, especially in cooler areas more favourable to *Bd*.

Cane toad susceptibility

Our collation of past field surveys revealed that *Bd* infection is rare in adult toads in natural habitats, consistent with the laboratory finding that adult toads generally clear experimental infection quickly (Poorten & Rosenblum 2016). Wild juvenile or tadpole cane toads were missing from these surveys, suggesting these groups have not been surveyed for *Bd* infection. Our infection experiment results indicate that toad metamorphs can readily die from chytridiomycosis. Susceptibility to *Bd* infection is known to vary with ontogeny in other anurans. Some species are affected as tadpoles and early metamorphs but not as adults (Pearl et al. 2007, Langhammer et al. 2014, Abu Bakar et al. 2016). It has been hypothesized that recent metamorphs are more susceptible to *Bd* infection because they have weaker or less-developed immune systems compared to adults (Rollins-Smith 1998, Gantress et al. 2003). While the vast majority of *Bd*-exposed cane toad metamorphs succumbed to infection in our inoculation experiment, a small proportion (19.2%) of animals cleared infection, and those animals that cleared infection tended to be larger. Future research could test if infection dynamics in wild juvenile toads follow the same pattern.

However, we predict infection is rare in cane toad metamorphs because the peak of breeding is in summer (Freeland & Kerin 1991, Cohen & Alford 1993) when the temperature is unsuitably warm for *Bd* (Berger et al. 2004, Brannelly et al. 2012b, McMahon et al. 2013, Phillott et al. 2013). Further, cane toads prefer to spawn in ponds with warm shallow edges (30–32°C) (Hagman & Shine 2006), and tadpoles often swarm along these warm edges, which are above the optimum range for growth of *Bd* (19–25°C) (Piotrowski et al. 2004, Stevenson et al. 2013). In addition, tadpoles that develop quickly and meta-

morphose out of the water body typically have lower *Bd* prevalence, probably due to less opportunity for exposure (Skerratt et al. 2010). Therefore, as cane toad tadpoles can reach metamorphosis quickly (within 3 wk) (Tyler 1975, Lever 2001), they are likely to have low *Bd* prevalence as tadpoles.

Effect of *Bd* on cane toad populations

While we found high *Bd*-induced mortality in recently metamorphosed cane toads in the lab, high juvenile mortality might not impact cane toad populations in the wild because they are highly fecund animals. Severe population declines due to chytridiomycosis have typically occurred in frogs that lay small clutches, and the effects of *Bd* on frogs can be partially counteracted by high recruitment (Hero et al. 2005, Scheele et al. 2015, Grogan et al. 2016, Brannelly et al. 2016a). Cane toads are a highly fecund, r-selected species and produce a large number (up to 35 000 eggs at a time; Hearnden 1991) of low-quality offspring. They have high natural mortality at the recent metamorph stage, ranging from 53 to 90% mortality within the first year post-metamorphosis (Alford et al. 1995a). Our laboratory experiment supported findings that cane toad metamorphs generally experience high mortality. Of the uninfected control toads, 23.5% died during the 93 d experiment, with most of the mortality occurring in smaller animals. The most important variable predicting mortality in this experiment was size, not *Bd* exposure. This is similar to previous work with young captive toads (Berger 2001), where mortalities were thought to be due to imperfect husbandry or natural attrition as per the wild (Alford et al. 1995a).

In the wild, cane toad tadpoles and recent metamorphs often occur at very high densities and experience density-dependent mortality (Cohen & Alford 1993, Alford 1994). Lower population density for metamorphs has been shown to increase overall recruitment because animals developing in low-density environments have improved body condition and growth rate (Cohen & Alford 1993, Ward-Fear et al. 2010, Cabrera-Guzmán et al. 2013). For this reason, it has been argued that population control tactics for cane toads should focus on reducing adult toads, rather than tadpoles or recent metamorphs (Alford et al. 1995a,b, Hyatt 1998, Lampo & Leo 1998). Thus, the impact of chytridiomycosis on cane toad metamorphs in the wild, if *Bd* infection occurs, may have little effect on cane toad populations.

Cane toad and *Bd* niche overlap

Our models suggest that the potential distribution of *Bd* and cane toads in Australia is larger than their current realized distributions. To date, cane toads have not spread south of Sydney (Phillips et al. 2008), and *Bd* is not as far north as Cape York (Murray et al. 2010). In the more southern temperate forests, the temperatures are cool and outside the natural thermal limits of the cane toad (Newell 2011, Wijethunga et al. 2016), which might explain why these animals have not spread further south (Tingley et al. 2014, Trumbo et al. 2016). However, cane toads have already expanded outside the thermal niche of their native habitat in Australia, and they are predicted to keep expanding southward (Kearney et al. 2008, Kolbe et al. 2010). In these more southern edges of their current distribution, cane toad population densities are often much lower, which could be due to the lack of suitable breeding water bodies for breeding (Newell 2011) or temperatures too cold for development (Wijethunga et al. 2016). However, it is worth considering whether the lower population densities in the cooler regions of Australia may also, in part, be due to *Bd* limiting toad population growth and spread, because *Bd* prefers cooler temperatures and cane toads develop more slowly, which could leave them more vulnerable to infection. These cooler areas of the toad's distribution are suitable for *Bd* and for the cane toad (Figs. S5 & S6). The differences between the species' predicted distributions in the model and their realised distributions may mean these species can still spread to those areas in the future. Alternatively, the models may over-predict the species' distributions by failing to account for other factors that limit these species, such as chytridiomycosis in the case of cane toads, or they may include presence records in areas where the cane toads can disperse but not breed (White & Shine 2009).

Role of the cane toad in the spread and amplification of *Bd*

Spread of disease across landscapes may be more likely via adults and large juvenile toads because these ontogenetic life stages are not restricted to water bodies and can travel large distances (Freeland & Kerin 1991, Cohen & Alford 1993). However, adult and juvenile cane toads are less probable as vectors for *Bd* because they have low susceptibility and prefer hot and dry microhabitats unfavourable to *Bd* infection (Freeland & Kerin 1991, Cohen & Alford 1993). In contrast, our results show that recently

metamorphosed cane toads (<25 mm) are susceptible, but they often stay within 3 m of the water's edge due to high risk of desiccation (Freeland & Kerin 1991, Cohen & Alford 1993). Therefore, they are less likely to facilitate spread of disease into new areas. However, the high burdens we observed on juvenile toads in the laboratory indicate that suitable environmental conditions enabling occurrence of abundant and diseased metamorphosing toads could result in amplification of *Bd* within a water body.

CONCLUSIONS

Through our data collation, laboratory infection experiment and niche model, as well as considering the ecology of the cane toad, we suggest that *Bd* does not greatly affect cane toads and that cane toads are not an important carrier of *Bd* in Australia. Reviewing available information shows that adult toads have low susceptibility to *Bd* and are rarely found infected and, therefore, do not appear to be important as *Bd* reservoirs or in driving the spread of this pathogen. Although recent cane toad metamorphs were highly susceptible to *Bd* in the laboratory, infection in early life stages is likely to be uncommon over much of the toad's distribution. This is because the cane toad's prime breeding habitat is warmer, lowland, ephemeral pools (i.e. unsuitable for *Bd*) and because cane toad tadpoles and metamorphs develop rapidly and thereby minimise their exposure. Additionally, cane toad metamorphs rarely move >3 m from the water's edge of their natal site (Freeland & Kerin 1991, Cohen & Alford 1993), thereby reducing the potential for cane toad metamorphs to spread disease. The cane toad has colonised regions of Australia that experience thermal regimes outside those within its native range, but their expansion south into cooler habitat is much slower than their expansion across tropical northern Australia (Newell 2011). This slower southern progression may be due to the toad's thermal preferences/limits, but it is possible that *Bd* contributes to their lower abundance in cooler habitats. Initial further work could test *Bd* prevalence in wild tadpole, metamorph and adult cane toads that occur in these cooler forests where the disease has a greater potential to affect this invasive anuran.

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