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RESEARCH ARTICLE

A pathogenic skin fungus and sloughing exacerbate cutaneous water loss in amphibians

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ABSTRACT

Batrachochytrium dendrobatidis (*Bd*) is a pathogenic fungus that causes the cutaneous, infectious disease chytridiomycosis and has been implicated in population declines of numerous anuran species worldwide. Proximate cause of death by chytridiomycosis is asystolic cardiac arrest as a consequence of severe disruption to electrolyte balance. Animals heavily infected with *Bd* also experience a disruption to their skin sloughing regime, indicating that core functions of the skin, such as water retention, may be severely impacted. This study examined how skin sloughing, body size and *Bd* infection interact to influence water loss rates in five Australian frog species: *Litoria caerulea*, *Limnodynastes peronii*, *Lechriodus fletcheri*, *Limnodynastes tasmaniensis* and *Platyplectrum ornatum*. Rates of water loss more than doubled during sloughing in *L. caerulea*. During active periods across all species, water loss rates were on average 232% higher in *Bd* infected frogs than in uninfected frogs. This indicates that dehydration stress may be a significant factor contributing to the morbidity of severely *Bd* infected anurans, a symptom that is then exacerbated by an increased rate of sloughing. When taking size into account, smaller and/or juvenile anurans may be more at risk from dehydration due to *Bd* infection, as they lose a greater amount of water and slough more frequently than adults. This may in part explain the higher mortality rates typical for small and juvenile frogs infected with *Bd*. Understanding how *Bd* affects the core functions of the skin, including rates of water loss, can improve our predictions of disease outcome in amphibians.

KEY WORDS: *Batrachochytrium dendrobatidis*, Chytridiomycosis, Cutaneous water loss, Dehydration, Fungal pathogen, Skin shedding

INTRODUCTION

Amphibian skin not only provides the first line of defence against infection, but also serves as a semipermeable surface across which osmotic, ionic and respiratory exchanges can occur (Campbell et al., 2012; Mancini, 2004). The outermost layer of the skin, the stratum corneum, is composed of one to two thin layers of keratinised cells that allow an almost completely uninhibited flow of water from internal to external environments (Amey and Grigg, 1995; Liu and Hou, 2012). Consequently, many frogs are at risk of dehydration via evaporative water loss when they are away from aquatic or humid environments (Lillywhite, 2006). To counteract the risk of dehydration, some arboreal anuran species have developed substantial resistance to water loss across the skin (Lillywhite, 2006; Wygoda, 1984). These anurans often do not actively seek escape from hot, dry conditions but rather have evolved mechanisms to avoid desiccation and overheating (Amey and Grigg, 1995). The waterproofing mechanism in the majority of these species is a cutaneous layer of lipids that can either occur in the skin or be excreted from skin glands and wiped over the body (Amey and Grigg, 1995; Barbeau and Lillywhite, 2005).

All amphibians maintain the integrity of their skin by regularly shedding, or sloughing, the stratum corneum (Alibardi, 2003; Smith, 1975) and, with it, anything adherent to that tissue, such as microbial flora and fauna (Meyer et al., 2012). Sloughing occurs when the stratum corneum separates from the layer beneath (stratum granulosum) and becomes the 'slough'. When the stratum granulosum keratinises, it becomes the new stratum corneum and the slough is shed from the body (Alibardi, 2003; Smith, 1975). Sloughing can transiently alter electrolyte and osmotic movements across the skin in amphibians (Jørgensen, 1949; Wu et al., 2017). In *Bufo bufo*, *Rana temporaria* and *Rana esculenta*, large increases in both water permeability and transcutaneous sodium loss have been observed during sloughing (Jørgensen and Larsen, 1961). Although in *B. bufo* the physical separation of the stratum corneum and stratum granulosum begins approximately 3 h before the slough is removed from the body (Jørgensen and Larsen, 1961), changes in the rate of water uptake have been observed up to 12 h before a sloughing event occurs (Ewer, 1951; Jørgensen, 1949).

Sloughing not only plays an important role in amphibian skin function, but also in cutaneous immune function. Amphibians host a wide variety of microflora on their epidermis (Cramp et al., 2014; Culp et al., 2007) and recently it has been shown that sloughing can substantially reduce the microbial load on the skin, suggesting that it is an important process in regulating the abundance of cutaneous microbiota (Cramp et al., 2014; Meyer et al., 2012). One particularly important skin microbe, the chytrid fungus *Batrachochytrium dendrobatidis* Longcore, Pessier and D. K. Nichols 1999 (*Bd*), can establish itself in the skin of anurans and become pathogenic, resulting in the fatal disease chytridiomycosis. This disease has been implicated in the declines of up to 200 frog species worldwide (Skerratt et al., 2007). *Bd* propagates via sporangia that release zoospores and invade the superficial layers of the epidermis, predominantly the ventral surface and toes (Berger et al., 2005b). Frogs heavily infected with *Bd* slough more frequently (Ohmer et al., 2015) and this reduces the load of *Bd* on the skin (Ohmer et al., 2017); thus, this may be a defensive response to infection. However, given that sloughing also causes significant disruption to transcutaneous water and ion movements in healthy frogs (Wu et al., 2017), an increased rate of sloughing in infected frogs may exacerbate the ionic and osmotic disturbances associated with sloughing (Ohmer et al., 2015). The proximate cause of death from chytridiomycosis is asystolic cardiac arrest as a consequence of

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severe disruption to Na^+ and K^+ balance (Voyles et al., 2009). However, animals infected with *Bd* also display evidence of dehydration (Voyles et al., 2012), suggesting that water loss may be a significant factor contributing to the morbidity of anurans with chytridiomycosis.

Because heavy *Bd* loads also increase sloughing rates in infected animals (Ohmer et al., 2015), we hypothesised that *Bd* infection and increased sloughing frequency would synergistically interact to exacerbate sloughing-induced skin dysfunction in infected frogs, resulting in increased rates of water loss. Small frogs have a higher surface area to volume ratio and thus a higher rate of net water loss relative to body mass (Christian, 1978; Tracy and Christian, 2005; Withers et al., 1982). Body size also influences *Bd* infection outcome, with smaller frogs being more susceptible to fatal chytridiomycosis (Burrow et al., 2017; Carey et al., 2006; Langhammer et al., 2014; Ohmer et al., 2013). Consequently, we hypothesized that the magnitude of sloughing-induced increases in rates of water loss would be mass specific, with smaller frogs being substantially more affected than larger conspecifics. To test these hypotheses, we compared the effects of sloughing, body size and *Bd* infection on rates of water loss in five Australian frog species: *Litoria caerulea* (White 1790), *Limnodynastes peronii* Duméril and Bibron 1841, *Lechriodus fletcheri* Boulenger 1890, *Limnodynastes tasmaniensis* Günther 1858 and *Platyplectrum ornatum* Gray 1842. Understanding the physiology underpinning host–pathogen relationships is critical for understanding the patterns of susceptibility to disease both within and between frog species.

MATERIALS AND METHODS

Ethics statement

All experiments were conducted with the approval of The University of Queensland Animal Welfare Committee (certificate SBS/452/12/URG) and the Queensland Environmental Protection Agency (permit WISP12218412). This study was carried out in compliance with the standards of the Australian Code for the Care and Use of Animals for Scientific Purposes established under section 39 of the National Health and Medical Research Council Act (1992) and the Queensland Animal Care and Protection Act (2001).

Animal selection and maintenance

The primary study species was the green tree frog, *L. caerulea*, which is increasingly utilized as a model for chytridiomycosis research (Lillywhite, 2006; Ohmer et al., 2015). *Litoria caerulea*, in temperatures cycling over a naturalistic temperature range from 15 to 23°C, will slough approximately every 3–4 days (Ohmer et al., 2015). In an attempt to determine how chytridiomycosis influences water loss rates in other species, data from an additional four Australian frog species, *L. peronii*, *L. fletcheri*, *L. tasmaniensis* and *P. ornatum*, infected with *Bd* were also investigated. These additional species are common in south eastern Queensland, Australia, but have not yet demonstrated widespread evidence of declines as a result of *Bd* (Commonwealth of Australia, 2006).

Animal capture and husbandry

Limnodynastes peronii, *L. fletcheri*, *L. tasmaniensis* and *P. ornatum* were sourced as eggs from populations in southeastern Queensland and raised in captivity. Adult and juvenile *L. caerulea* were captured from non-protected areas (wet roads and vegetation) near Fernvale, Queensland, Australia, and transported to The University of Queensland. Animals were housed in either 5 or 10 l plastic containers (depending on frog size) lined with wet paper towels and half of a polyvinyl chloride pipe to provide shelter. Enclosures were cleaned and frogs fed vitamin dusted crickets on a weekly basis. Lighting and temperature cycles mimicked natural conditions, with a 12 h photoperiod from 03:00 to 15:00 h (chronologically shifted to facilitate monitoring of sloughing), a minimum temperature of 15°C at 03:00 h and a maximum temperature of 23°C at 15:00 h. Snout vent length (SVL) was measured using callipers (W77194 Callipers, Mitutoyo, West Heidelberg, Victoria, Australia) and body mass was measured using an electronic balance (EJ-303 Compact Precision Balance, A&D Weighing, Melbourne, Victoria, Australia). All animals were tested for *Bd* infection prior to experimentation (see below).

Monitoring sloughing frequency

Frog behaviour was monitored continuously using twelve 600TVL weatherproof infrared security cameras (model EN-CI20B-65H, Eonboom Electronics Limited) at a frame rate of 1.52 frames s^{-1}. Video was recorded on a 16 channel H.264 digital video recorder [DVR; model MDR688ZB (AU)-E, 600TVL]. Recordings were analysed to determine the timing and rate of sloughing and to predict future sloughs for each frog as detailed in Ohmer et al. (2015). Essentially, whenever sloughing occurred, the time and date were recorded and used to predict future sloughing times. Sloughing was easily recognised from a suite of characteristic behavioural actions, including limb movements and mouth gaping behaviour. For the purposes of this study, the total sloughing duration was measured from the first to the last mouth gape, and the time between successive sloughing events was termed the intermoult interval (IMI).

Rates of water loss

Water loss rates were measured within the same temperature-controlled room in which animals were housed. Following the methods of Amey and Grigg (1995), rates of water loss were determined by measuring body mass loss over time while the frog sat in a constant stream of air. The experimental setup is detailed in Fig. 1. Briefly, an air pump (HP40 Air Pump, Techno Takatsuki, Osaka, Japan) pushed air at an average rate of 466.7 ml min^{-1} through a plastic container in which a frog was positioned [containers were either 10.5×7×5 cm (SVL<45 cm) or 13.5×9.5×6 cm (SVL>45 cm)]. The container was positioned on top of a loading balance (EJ-303 Compact Precision Balance, A&D Weighing, Melbourne, Victoria, Australia) and changes in the mass of the frog were measured and automatically logged every 10 s (AD-1688 Weighing Data Logger, A&D Weighing). A Powerlab (ML866, ADInstruments, Bella Vista, New South Wales, Australia) was used to simultaneously collect air flow measurements (ML140 Spirometer, ADInstruments), changes in temperature (ML312 T-type Pod, ADInstruments) and video recordings (c170 Webcam; Logitech, Lausanne, Switzerland) during the experimental period. Humidity was monitored in 5-min increments with a humidity data logger (DS1923 Temperature/Humidity Logger, Maxim Integrated, CA, USA), and ranged between 34.7 and 81.4% [63.1±9.9% (mean±s.d.)]. Rate of net water loss (NWL; g h^{-1}) was determined using the equation:

$$\mathrm{NWL} = \Delta M/\Delta T, \quad (1)$$

where ΔM represents the change in body mass (g) over the total measurement period (ΔT, in h).

To examine how rates of water loss changed over the IMI in uninfected *L. caerulea*, water loss rates were measured every 24 h, for 30–65 min, throughout the entire sloughing cycle (3–4 days depending on the frog). To examine the effect of sloughing and *Bd*

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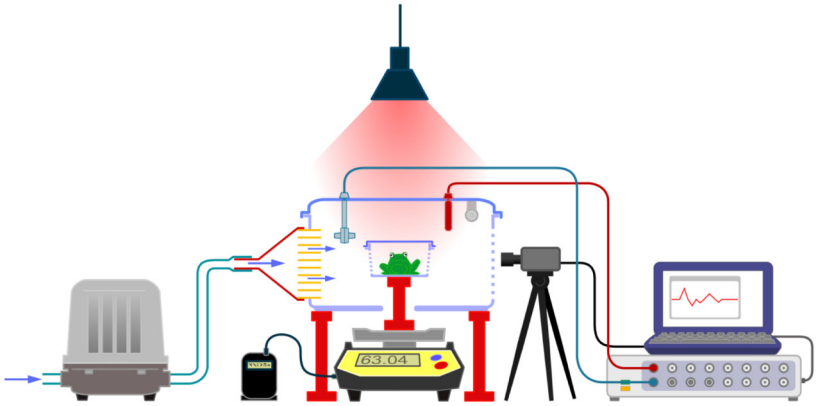

Fig. 1. The experimental setup used to measure water loss rates in five Australian frog species. Briefly, air was slowly pumped through a series of flow straighteners and into a container holding a frog, which was positioned on a logging balance. Mass changes were recorded every 10 s. Air speed, temperature and frog activity levels were captured and recorded using an ADInstruments Powerlab. Humidity was recorded at 5 min intervals using a separate humidity data logger. All measurements were made under red light to reduce disturbance of the animals. Figure drawn by H. Winwood-Smith.

infection on water loss rates in *L. caerulea*, rates of water loss were measured for 30–120 min immediately prior to sloughing, during sloughing and for a further 30–60 min after sloughing in both infected and uninfected frogs. Frogs were placed in the experimental setup close to when sloughing was predicted to occur based on previous video analyses. To compare water loss rates between *Bd* infected (*Bd*+) and uninfected (*Bd*–) frogs of all five species, frogs were placed in the experimental setup for 30–65 min at random points during their IMI (non-sloughing periods). All frogs were swabbed before and after water loss trials to monitor *Bd* infection load (see '*Bd* exposure and infection detection' section below). To control for any effect of swabbing, one half of the frog (randomly selected) was swabbed before the water loss trial and the other half was swabbed after the water loss trial (Ohmer et al., 2017).

During water loss trials, behaviour was monitored continuously using a video camera; measurement periods were divided into active and inactive periods, classified by whether or not frogs were active during each time period. If defecation occurred during experimentation, the session was aborted and repeated another day. Frogs were closely monitored to ensure that no individual lost more than 30% (for controls) or 10% (for infected frogs) of its original body mass during the experiment.

Bd culturing

Two Australian *Bd* isolates (*Waste point-Lverreauxii-2013-LB, RW, 2*, isolated by L. Berger, College of Public Health, Medical and Veterinary Sciences, James Cook University, and EPS4, isolated by E. P. Symonds, School of Veterinary Science, University of Queensland) were used for experimental infection. Cultures were kept at 4°C. Four to seven days before the exposure date the isolates were passaged onto 1% agar, 0.25% tryptone, 0.25% tryptone–soy plates and kept at 21°C. Zoospores were collected by flooding the plates with sterile distilled water for 30 min and gently agitating them periodically. Zoospore suspension was collected and concentration calculated using a hemocytometer as detailed by Boyle et al. (2004).

Bd exposure and infection detection

Throughout the course of the study frogs were exposed to *Bd* on four separate occasions (see Table S1 for details). During all exposures frogs were randomly allocated into a control or an exposure group. In order to increase the number of animals infected with *Bd*, we reallocated exposed animals that did not become infected to the control group and *vice versa*, and continued to monitor infection status of all frogs throughout experimentation (Table S1). No frog re-allocated to the control group developed *Bd* infection later in the experiment. In total, water loss rates of 14 *Bd*+ and 34 *Bd*– animals were measured (Table 1). Animals in the exposure group were exposed for 5 h to approximately 250,000–500,000 *Bd* zoospores in 40 ml of aged tap water in 300 ml plastic containers (Berger et al., 2005b; Ohmer et al., 2013). Control frogs were exposed, using the same methods, to aged tap water containing no zoospores. After exposure animals were placed back into their enclosures, sloughing rate was monitored continuously using video surveillance and clinical signs of chytridiomycosis (Berger et al., 2005a) were assessed twice daily. *Bd* load was assessed by swabbing the ventral surface of the frogs with a sterile cotton swab (MW100, Medical Wire, Corsham, Wiltshire, UK). Swabbing was completed before and after each water loss trial, and approximately every 14 days after *Bd* exposure, to monitor infection load in the exposed group and to ensure control animals remained uninfected. Swabbing involved firmly running a cotton swab three times over the frog's abdomen, sides, thighs, feet, webbing and toes (Kriger et al., 2006; Prunier et al., 2012; Retallick and Miera, 2007). Swabs were extracted in 50 µl Prepman Ultra (Applied Biosystems, Foster City, CA, USA) and analysed in triplicate with quantitative PCR following Boyle et al. (2004) and Hyatt et al. (2007). Quantitative PCR was conducted in a Mini Opticon real-time PCR detection system (Bio-Rad, CA, USA) with a 15 µl reaction volume per well (Ohmer et al., 2015). Before water loss trials began, each frog was lightly shaken dry. Water loss trials began for *Bd*+ frogs once infection loads exceeded 400 zoospore equivalents (ZE) or animals displayed symptoms of chytridiomycosis. Clinical signs of chytridiomycosis included, but were not limited to, discoloured skin, lack of appetite, sluggish behaviour, loss of righting reflex, abnormal posture, excessive sloughing of skin, and the development of ulcers on thighs and toes (Campbell et al., 2012; Hyatt et al., 2010; Ohmer et al., 2015).

No statistical difference in water loss rate was found between controls, *Bd*– animals, and previously exposed animals that did not become infected, so they were grouped together for analyses ($F_{2,79}$=0.93, P=0.40). If frogs began demonstrating severe clinical signs of chytridiomycosis, they were humanely euthanized.

Table 1. Final sample sizes for frogs of each study species infected with *Batrachochytrium dendrobatidis* (*Bd*+) and those that were uninfected (*Bd*–) at the time of water loss measurements

Frog species	*Bd*+	*Bd*–
Litoria caerulea	5	14
Limnodynastes peronei	2	4
Limnodynastes tasmaniensis	1	9
Lechriodus fletcheri	3	3
Platyplectrum ornatum	3	4

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Euthanasia was conducted by immersing the animal in a neutrally buffered solution of 0.3% tricaine methanesulfonate (MS-222).

Statistical analysis – *L. caerulea* only

All statistical analyses were conducted using the program R (https://www.r-project.org/). Water loss rates in *L. caerulea* were compared during inactive, active and sloughing periods in uninfected animals by using linear mixed effects models (function lme, package nlme, Pinheiro et al., 2013). 'Frog ID' was included as a random effect to take into account repeated measurements on the same individual, and 'number of previous *Bd* exposures' and 'SVL' were included as fixed effects.

Water loss rates during the IMI were examined in active and inactive uninfected *L. caerulea* utilizing mixed effects models (function lme, package nlme). 'Frog ID' was included as a random effect, and 'percent IMI', 'SVL' and the 'number of previous *Bd* exposures' were included as fixed effects. Percent IMI represents how far each individual frog was through its IMI at the time water loss was measured. Percent IMI was calculated by dividing how many hours post-sloughing the rate of water loss measurements were taken by the total number of hours in the IMI for that individual. This calculation took into account any variation in IMI across individuals.

The effect of *Bd* infection on water loss rates during sloughing was also tested utilising a mixed effects model (function lme, package nlme), with 'infection status' (uninfected or infected), 'SVL' and the 'number of previous *Bd* exposures' included as fixed effects and 'frog ID' included as a random effect.

In all models, the number of previous *Bd* exposures was insignificant, indicating that only current *Bd* infection was relevant to amphibian water loss rates.

Statistical analysis – all species

Rates of water loss in uninfected frogs were compared across all species during active and inactive periods with a linear mixed effect model (function lme, package nlme), with the fixed effects 'behaviour' (active or inactive), 'SVL' and the 'number of previous *Bd* exposures'. 'Frog ID' and 'species' were included as nested random effects. 'Frog ID' was used as a random effect to account for multiple measurements on the same animal, and 'species' was included to account for measurements being taken from five different species. A mixed effects model was also run to test the effect of both *Bd* infection and behaviour on water loss rates in all five species. Finally, to examine the effects of *Bd* load on rates of water loss, *Bd* load values were log+1 transformed due to the large scale of the data. Separate mixed effects models were run to examine the relationship between *Bd* load and water loss rates during activity and inactivity, including 'SVL' as a fixed effect and 'frog ID' nested within 'species' as a random effect.

'Wind speed', 'temperature' and 'humidity' were also analysed as model covariates; however, these variables were removed to improve model fit when no significance was found during active (temperature: $F_{1,20}$=1.27, P=0.27; air flow speed: $F_{1,20}$=1.69, P=0.21; humidity: $F_{1,20}$=1.33, P=0.26) and inactive (temperature: $F_{1,20}$=0.54, P=0.47; air flow speed: $F_{1,20}$=0.0013, P=0.97; humidity: $F_{1,20}$=0.0083, P=0.93) periods across all species. Models were fitted using maximum likelihood (ML).

RESULTS

The effect of sloughing and activity on water loss rates in *L. caerulea*

Litoria caerulea demonstrated a consistent sloughing rate with the average IMI lasting 73.9±17.7 h (N=19). The majority of all *L. caerulea* (N=19) sloughed between 15:30 h and 18:30 h, just after the lights went out, consistent with Ohmer et al. (2015). Sloughing behaviour lasted, on average, for 6.5±3.2 min and was as described by Ohmer et al. (2015) and Ohmer et al. (2017). Smaller frogs lost more water relative to body mass than larger frogs overall (linear mixed effects model, β=−0.43, P=0.01, 95% CI=−0.72, −0.13, Fig. 2A). Also, body size [SVL (mm)] had a significant positive relationship with the rate of water loss in sloughing frogs (β=0.013, P=0.046, 95% CI=0.0006, 0.0026) and in frogs active during IMIs (β=0.014, P=0.017, 95% CI=0.0021, 0.0027; Table S2; N=14). Water loss rates were significantly higher during sloughing when compared with inactive periods (Tukey *post hoc* comparison, β=0.41, P<0.0001, s.e.=0.066; see Table S3a for full model details, Fig. 2B; N=12), with sloughing frogs losing on average 105% more water per unit body mass than inactive frogs. Rates of water loss during inactivity before and after sloughing were combined for comparison as there was no significant difference between the two (Tukey *post hoc* comparison, β=−0.058, P=0.94, s.e.=0.077, N=12). Water loss rates were also high when frogs were active immediately after sloughing, but this was not significantly different from sloughing periods (Fig. 2B; β=0.10, P=0.69, s.e.=0.080; N=12). A substantial period of activity after sloughing was a common occurrence.

The effect of intermoult interval on water loss rates in *L. caerulea*

When investigating the change in water loss over the IMI, the rate of water loss increased significantly prior to sloughing in inactive *L. caerulea* (β=0.48, P=0.01, 95% CI=0.14, 0.82; Fig. 2C; N=7, Table S3b). However, this pattern was not seen in active *L. caerulea*, with rates of water loss varying largely and remaining consistently high over time (β=−0.13, P=0.71, 95% CI=−0.88, 0.60; N=7, Table S3b).

The effect of *Bd* infection and sloughing on water loss rates in *L. caerulea*

Sloughing in *Bd*+ *L. caerulea* occurred more frequently (average IMI: 57.01±7.19 h, N=4) and was more difficult to observe in the experimental setup, especially when frogs began displaying clinical signs of chytridiomycosis. Consequently, water loss rates during the process of sloughing were only collected from four infected *L. caerulea*. Behaviour during sloughing differed from uninfected frogs, with *Bd*+ individuals often failing to remove the entire slough and displaying drastically reduced limb movements. The sloughing duration of *Bd*+ frogs (N=4) was not significantly different from the sloughing duration of *Bd*– frogs (N=11; 6.5±4.3 min; β=-2.7×10^{5}, P=0.99, 95% CI=−0.059, 0.059). *Bd* infection had a significant effect on water loss rates during sloughing in *L. caerulea* (β=0.29, P=0.02, 95% CI=0.089, 0.29; Fig. 2D; Table S3c), with *Bd*+ frogs losing on average 121% more water than *Bd*− frogs.

The effects of *Bd* infection and activity on water loss rates in all frog species

When analysing all species combined, with 'frog ID' nested in 'species' as a random effect, the rate of water loss when frogs were inactive was significantly lower than the rate of water loss when frogs were active (β=−0.37, P<0.0001, 95% CI=−0.44, −0.30; N=29, Fig. 3; Table S4). All frogs were *Bd*-negative prior to the first experimental exposure to *Bd*. Control frogs remained healthy and *Bd*-negative throughout the experimental period. The majority of *Bd* exposed *P. ornatum*, *L. fletcheri*, *L. peronii* and *L. tasmaniensis* became infected with *Bd*. However, most *L. tasmaniensis* did not

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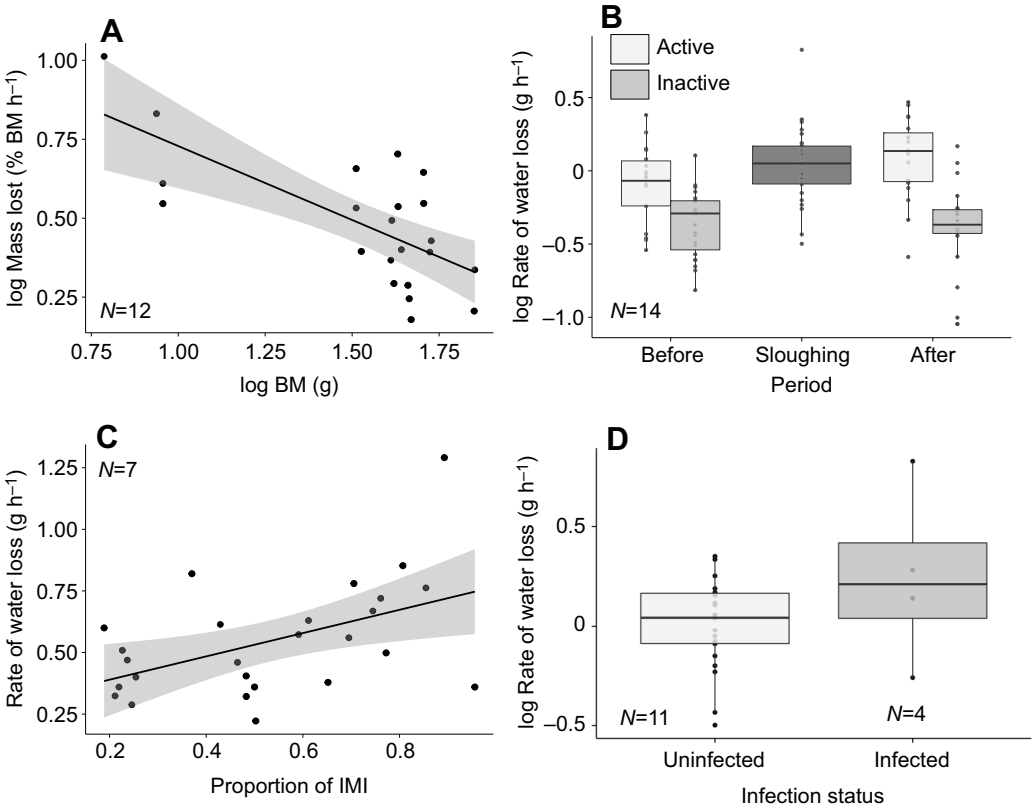

Fig. 2. Factors affecting the rate of water loss in Australian green tree frogs, *Litoria caerulea*. (A) The effect of body mass (BM) on rates of water loss in *L. caerulea*. Body mass had a significant effect on water loss rates, with smaller frogs losing a larger amount of water per unit time than larger frogs. (B) Activity levels had a significant effect on water loss rates in *L. caerulea*. Frogs lost substantially more water when active, relative to when they were still. The process of sloughing increased rates of water loss by 3-fold (relative to inactive periods). (C) Rates of water loss increased significantly as *L. caerulea* progressed through the intermoult interval (IMI). Frogs had the highest rate of water loss in the hours immediately preceding sloughing. (D) Rates of water loss were significantly higher in *Batrachochytrium dendrobatidis* (*Bd*) infected than uninfected *L. caerulea* during sloughing. The centre line is the 50th percentile, top and bottom of box represent 75th and 25th percentile, and whiskers extend to extreme data points (no more than 1.5 times the interquartile range).

display severe signs of chytridiomycosis and were able to maintain a low level of *Bd* on the skin or, in some instances, completely cure themselves of infection. Both *Bd* infection (β=0.19, P=0.001, 95% CI=0.08, 0.30) and activity (β=0.39, P<0.0001, 95% CI=0.33, 0.45; Table S5a) had a significant effect on water loss rates. During activity, infected frogs (N=13) lost on average 232% (2.76±2.12 g h^{-1}) more water per unit body mass than uninfected frogs (N=29; 1.13±0.45 g h^{-1}; Fig. 4), whereas, during inactivity, infected frogs (N=13) lost on average 17% (0.58±0.52 g h^{-1}) more water per unit body mass than uninfected frogs (N=29; 0.50±0.25 g h^{-1}; Fig. 4). As *Bd* load increased, the rate of water loss significantly increased across all frog species (N=22) during both active (β=0.38, P=0.021, 95% CI=0.10, 0.67) and inactive (β=0.10, P=0.013, 95% CI=0.031, 0.17; Table S5b) periods (Fig. 5; control animals were not included in these analyses).

DISCUSSION

Chytridiomycosis has been implicated in the decline and extinction of numerous frog species worldwide (Skerratt et al., 2007). Although cause of death by chytridiomycosis is suggested to result from restricted electrolyte uptake across the skin (Voyles et al., 2009), wild animals infected with *Bd* have also displayed evidence of dehydration (Voyles et al., 2012), suggesting that changes to water loss and/or uptake rates may also contribute to morbidity. This study showed that skin sloughing and infection with *Bd* substantially increased rates of water loss in five anuran species, with smaller frogs losing disproportionately more water per unit time than larger frogs. These results are consistent with the idea of dehydration stress in frogs with chytridiomycosis and provides a possible mechanism through which this may occur.

Behaviour and water loss rates

Across all species, frogs had a significantly higher rate of water loss when active compared with when they were inactive. Altering behaviour is one of the simplest ways frogs can manipulate their rate of water loss, with many species employing a suite of behavioural, physiological and morphological strategies (Shoemaker et al., 1992; Young et al., 2005). The type of habitat a frog utilises will also influence level of activity, meaning that different species (occupying different niches) will often have different activity patterns (Tracy et al., 2014). Differences in water loss rates among species may also reflect differences in body shape and levels of cutaneous resistance (Lillywhite, 2006; Young et al., 2005).

Sloughing *L. caerulea* lost twice the amount of water per unit time on average than inactive frogs. When amphibians slough, the process of manipulating the shed skin from the body into the mouth involves a suite of relatively vigorous limb and mouth movements. The pattern of sloughing behaviour is relatively consistent across anuran species (Ohmer, 2016), generally consisting of the fore and hind limbs being used to push loose skin from dorsal and lateral skin surfaces up towards the head where gaping mouth movements draw the loose skin into the mouth. Therefore, it is likely that the physical

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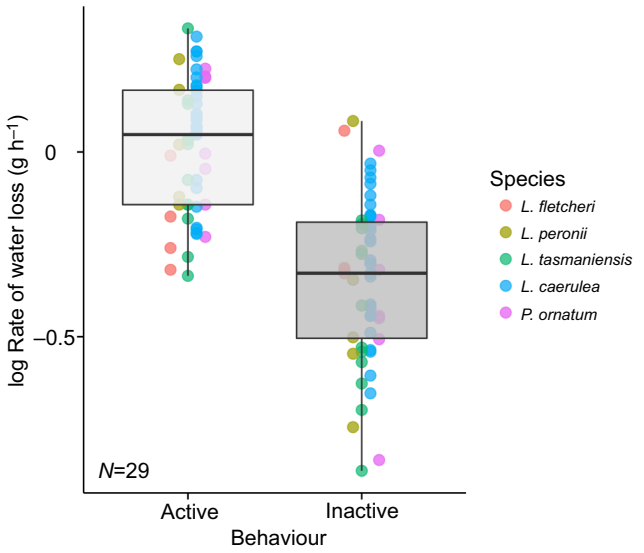

Fig. 3. Combined rates of water loss during activity and inactivity measured in five species of Australian frog: *Lechriodus fletcheri*, *Limnodynastes peronii*, *Limnodynastes tasmaniensis*, *Litoria caerulea* and *Platyplectrum ornatum*. Across all species, rates of water loss were comparable and significantly affected by activity level. The centre line is the 50th percentile, top and bottom of box represent 75th and 25th percentile, and whiskers extend to extreme data points (no more than 1.5 times the interquartile range).

movements that accompany sloughing have contributed to the increased rate of water loss during this period. In addition, immediately after sloughing occurs the new stratum corneum is not completely keratinised (Spearman, 1968) and may provide a less restrictive barrier to water movement. This, combined with the fact that most frogs became active immediately after sloughing, may explain why rates of water loss increased even further after sloughing had occurred (Fig. 2B). Of the species studied, the majority sloughed in the evening, which is also when these nocturnal species are typically most active (Gomez et al., 2006). Frogs, like most shedding species, are probably unable to actively repress sloughing behaviour, as sloughing is stimulated by a series of hormonal cues provided by the endocrine system (Barker Jørgensen, 1988; Herman, 1992; Jørgensen et al., 1965). Although the overall behavioural movements that accompany sloughing are relatively consistent across species (Ohmer et al., 2017), the duration of each sloughing event and the frequency of sloughing varies greatly amongst species (Ohmer, 2016), indicating that the cumulative effect of sloughing on water loss rates will most likely differ amongst species.

In *L. caerulea*, the rate of water loss changed throughout the IMI, gradually increasing until a sloughing event occurred. This indicates that permeability of the skin to water is altered even before sloughing takes place. Although rates of water loss during sloughing have not been measured before, previous work has shown that, in *B. bufo* and *Bufo regularis*, changes in the rate of water uptake across the skin occur up to 12 h before sloughing occurs (Ewer, 1951; Jørgensen, 1949). However, in *B. bufo* the separation of the stratum corneum from the stratum granulosum only begins 3 h before sloughing occurs (Jørgensen and Larsen, 1961), suggesting that changes in the permeability of the skin to water may precede the separation and loss of the stratum corneum. Whether the timing of the separation of the stratum corneum from the stratum granulosum influences the rate of water loss across the skin in *L. caerulea* remains unclear. Further research is needed to fully understand how permeability of the skin is affected by the morphological changes to the skin layers immediately preceding sloughing.

Body size had a significant effect on the rate of water loss in *L. caerulea*, with smaller frogs consistently losing a higher percentage of their original body mass. This is consistent with previous work showing that larger frogs have a smaller surface area to volume ratio than smaller frogs (Shoemaker et al., 1992; Tracy and Christian, 2005; Withers et al., 1982). This also means that smaller frogs are more susceptible to dehydration than their larger, adult counterparts.

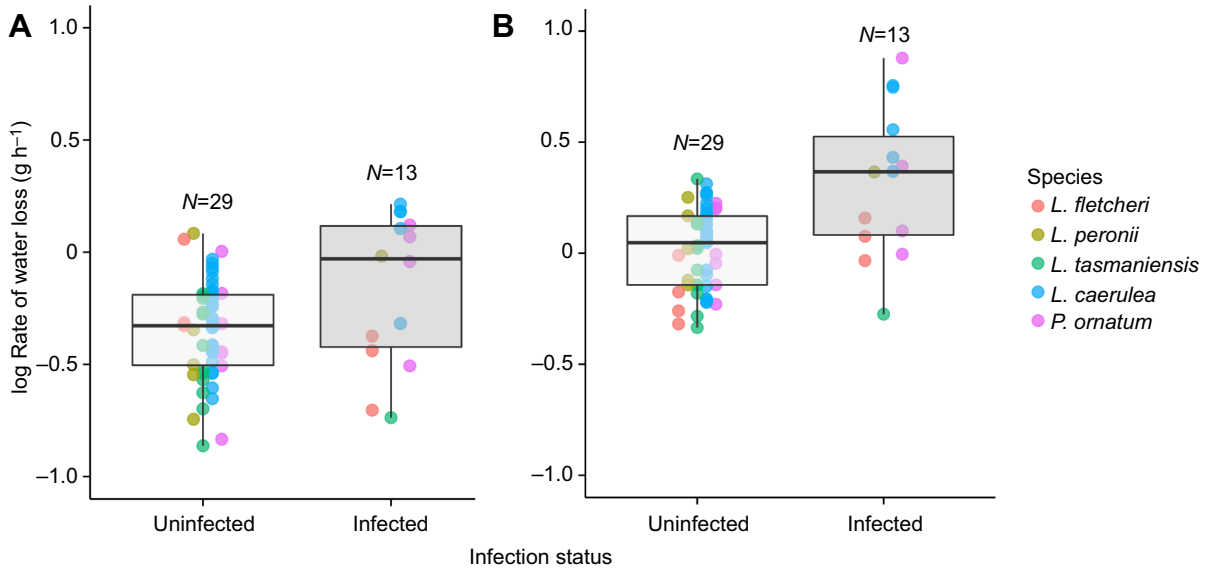

Fig. 4. The effect of infection with *Batrachochytrium dendrobatidis* (*Bd*) on rates of water loss while inactive and active in the Australian frogs *P. ornatum*, *L. fletcheri*, *L. peronii*, *L. tasmaniensis* and *L. caerulea*. *Bd* infection increased rates of water loss significantly, with active infected animals (B) losing up to 232% more water than uninfected animals (A). The centre line is the 50th percentile, top and bottom of box represent 75th and 25th percentile, and whiskers extend to extreme data points (no more than 1.5 times the interquartile range).

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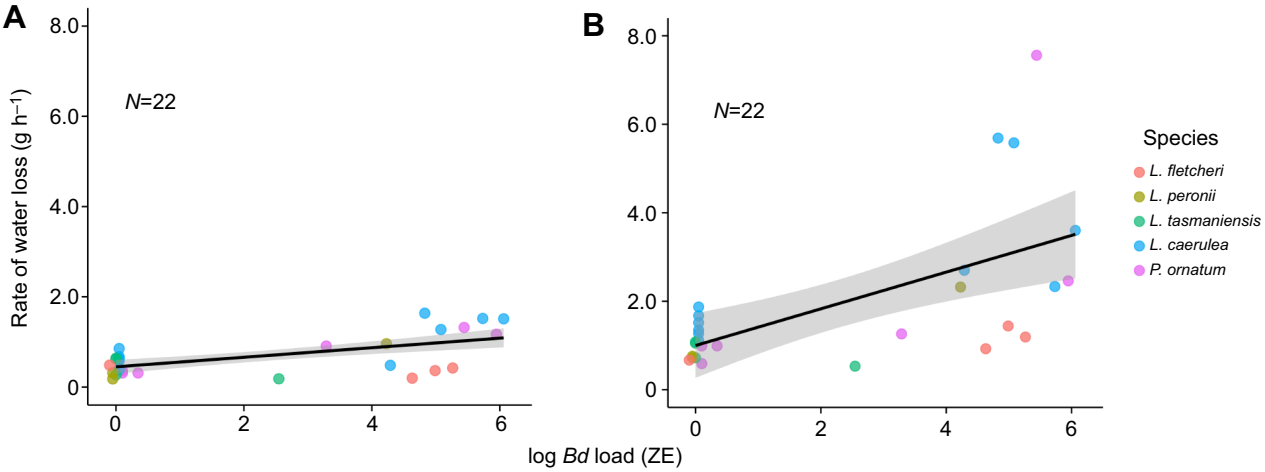

Fig. 5. The effect of *Bd* load on rates of water loss in frogs (all species). Rates of water loss increased significantly with *Bd* load both (A) while inactive and (B) during periods of activity.

Bd infection and water loss rates

In the majority of the frog species examined, *Bd* infection significantly increased rates of water loss. Although the stage within the IMI was found to have a significant effect on the rate of water loss, the effect of *Bd* infection was substantially higher, with infected *L. caerulea* losing approximately double the amount of water of uninfected frogs. However, to have a significant effect on rate of water loss, *Bd* zoospore load had to be high (on average $2.1\times10^5\pm3.4\times10^5$ ZE). This is consistent with Ohmer et al. (2015), who found that the effect of infection with *Bd* on IMI length in *L. caerulea* was *Bd* load dependent. In addition, Voyles et al. (2012) found that electrolyte and haematocrit levels only changed significantly in *Bd* infected *Rana muscosa* when zoospore levels were high (in this case, above 10,000 ZE). In all cases, *Bd* load needed to be relatively high before clinical signs of chytridiomycosis were observed. This suggests that low levels of *Bd* infection may not be sufficient to disturb skin function substantially and may explain why some species are able to resist developing chytridiomycosis if infection levels are kept low (Berger et al., 1998; Daszak et al., 2003; Lips et al., 2006). Consistent with this observation, Carver et al. (2010) found that *Bd* exposure had no significant effect on rate of water loss in *Litoria raniformis*. This is most likely because the effects of *Bd* on water loss rates were measured just 7 days after exposure to *Bd*, which is unlikely to have allowed sufficient time for infection loads to reach levels high enough to disturb skin function.

Bd infection load, sloughing rate and water loss rate

Heavy *Bd* infections substantially increased the amount of water lost during sloughing in *L. caerulea*. Although activity also increased the rates of water loss in animals with *Bd*, in general *Bd* infected frogs had considerably lower levels of overall activity than uninfected frogs, yet substantially higher water loss rates during sloughing. Animals infected with *Bd* may therefore regulate behavioural levels so as to limit excessive water loss rates. *Bd* infection also increased the rate of sloughing in *L. caerulea*, a result that has been seen previously (Ohmer et al., 2015). Therefore, *Bd* infection not only increases water loss rates during sloughing, but also increases the rate at which sloughing occurs in general, implying that increased sloughing rates could potentially contribute to morbidity in anurans with chytridiomycosis. This increase in the rate of sloughing may also exacerbate the effects of *Bd* infection, by continually disrupting osmotic and ionic homeostasis. Increases in haematocrit and plasma protein levels have also been observed in wild frogs with high *Bd* loads (Voyles et al., 2012). High haematocrit and protein levels are both indicative of dehydration (Billett, 1990; Lee, 2009; Voyles et al., 2012). Dehydration occurs when water loss rate exceeds water uptake rate, resulting in a net loss of water in the blood, and thereby elevating the concentration of red blood cells and plasma proteins in the blood. Moreover, water absorption rates are also reduced in *Bd* infected amphibians (Carver et al., 2010; Wardziak et al., 2013). Since sloughing cannot be actively controlled (Herman, 1992), occurs more frequently when animals are infected with *Bd* (Ohmer et al., 2015), increases rates of water loss (this study) and decreases the rate of water absorption (Carver et al., 2010; Wardziak et al., 2013), frogs infected with *Bd* are at a substantial risk of dehydration. Since smaller animals slough more frequently and lose more water relative to body mass, they are also more likely to develop an osmotic imbalance. This in turn may contribute to the explanation of why smaller frogs are more susceptible to *Bd*, and succumb quicker, than larger ones (Carey et al., 2006; Langhammer et al., 2014).

Conclusions

This study found that sloughing, activity levels and body size all significantly influenced rates of water loss in five species of Australian frog. These results were compounded by *Bd* infection, with rates of water loss increasing substantially in infected frogs. Given that frogs slough more frequently when infected with *Bd*, and *Bd* infected animals have a higher rate of water loss (this study) and a reduced water uptake capacity (Carver et al., 2010), it is likely that cutaneous osmotic dysfunction leads to dehydration and is a contributing factor in morbidity from chytridiomycosis. *Bd* has had a substantial effect on the health and abundance of numerous frog species worldwide, with many species experiencing severe declines and extinctions (Berger et al., 1998; Hyatt et al., 2010; Skerratt et al., 2007). Comprehending the host–pathogen relationship and the effects this pathogen has on basic skin functions is important for understanding variation in susceptibility and responses to *Bd* infection within and between amphibian species.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.E.B.O., R.L.C., C.E.F.; Methodology: C.J.M.R., M.E.B.O., R.L.C., C.E.F.; Validation: M.E.B.O.; Formal analysis: C.J.M.R., M.E.B.O.; Investigation: C.J.M.R., M.E.B.O., R.L.C.; Resources: R.L.C., C.E.F.; Data curation: C.J.M.R., M.E.B.O.; Writing - original draft: C.J.M.R.; Writing - review & editing: M.E.B.O., R.L.C., C.E.F.; Visualization: C.J.M.R., M.E.B.O.; Supervision: M.E.B.O., R.L.C., C.E.F.; Project administration: R.L.C., C.E.F.; Funding acquisition: C.E.F.

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Data availability
All data relevant to this manuscript are available from Figshare, under the DOI: 10.6084/m9.figshare.5202469.

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References

Alibardi, L. (2003). Adaptation to the land: The skin of reptiles in comparison to that of amphibians and endotherm amniotes. *J. Exp. Zool.* **298B**, 12-41.
Amey, A. P. and Grigg, G. C. (1995). Lipid-reduced evaporative water loss in two arboreal hylid frogs. *Comparative Biochemistry and Physiology A* **111**, 283-291.
Barbeau, T. R. and Lillywhite, H. B. (2005). Body wiping behaviors associated with cutaneous lipids in hylid tree frogs of Florida. *J. Exp. Biol.* **208**, 2147-2156.
Barker Jørgensen, C. (1988). Nature of moulting control in amphibians: effects of cortisol implants in toads *Bufo bufo*. *Gen. Comp. Endocrinol.* **71**, 29-35.
Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyati, A. D., McDonald, K. R. et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. USA* **95**, 9031-9036.
Berger, L., Hyatt, A. D., Speare, R. and Longcore, J. E. (2005a). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Dis. Aquat. Org.* **68**, 51-63.
Berger, L., Marantelli, G., Skerratt, L. F. and Speare, R. (2005b). Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Dis. Aquat. Org.* **68**, 47-50.
Billett, H. H. (1990). Hemoglobin and hematocrit. In *Clinical Methods: The History, Physical, and Laboratory Examinations* (ed. H. K. Walker, W. D. Hall and J. W. Hurst), pp. 718-719. Boston, USA: Butterworths.
Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Org.* **60**, 141-148.
Burrow, A. K., Rumschlag, S. L. and Boone, M. D. (2017). Host size influences the effects of four isolates of an amphibian chytrid fungus. *Ecol. Evol.* **7**, 9196-9202.
Campbell, C. R., Voyles, J., Cook, D. I. and Dinudom, A. (2012). Frog skin epithelium: Electrolyte transport and chytridiomycosis. *Int. J. Biochem. Cell Biol.* **44**, 431-434.
Carey, C., Bruzgul, J. E., Livo, L. J., Walling, M. L., Kuehl, K. A., Dixon, B. F., Pessier, A. P., Alford, R. A. and Rogers, K. B. (2006). Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth* **3**, 5-21.
Carver, S., Bell, B. D. and Waldman, B. (2010). Does chytridiomycosis disrupt amphibian skin function? *Copeia* **2010**, 487-495.
Christian, D. P. (1978). Effects of humidity and body size on evaporative water loss in three desert rodents. *Comparative Biochemistry and Physiology Part A: Physiology* **60**, 425-430.
Commonwealth of Australia (2006). *Threat Abatement Plan: Infection of Amphibians with Chytrid Fungus Resulting in Chytridiomycosis*. Canberra: Department of Environment and Heritage.
Cramp, R. L., McPhee, R. K., Meyer, E. A., Ohmer, M. E. and Franklin, C. E. (2014). First line of defence: the role of sloughing in the regulation of cutaneous microbes in frogs. *Conservation Physiology* **2**, cou012.
Culp, C. E., Falkinham, J. O., III and Belden, L. K. (2007). Identification of the natural bacterial microflora on the skin of Eastern newts, Bullfrog tadpoles and Redback salamanders. *Herpetologica* **63**, 66-71.
Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2003). Infectious disease and amphibian population declines. *Divers. Distrib.* **9**, 141-150.
Ewer, R. F. (1951). Water uptake and moulting in *Bufo regularis* reuss. *J. Exp. Biol.* **28**, 369-373.
Gomez, N. A., Acosta, M., Zaidan, F., III and Lillywhite, H. B. (2006). Wiping behavior, skin resistance, and the metabolic response to dehydration in the arboreal frog *Phyllomedusa hypochondrialis*. *Physiol. Biochem. Zool.* **79**, 1058-1068.
Herman, C. A. (1992). Endocrinology. In *Environmental Physiology of the Amphibians* (ed. M. E. Feder and W. W. Burggren), pp. 43-44. Chicago: The University of Chicago Press.
Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H. et al. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Org.* **73**, 175-192.
Hyatt, A. D., Speare, R., Cunningham, A. A. and Carey, C. (2010). Amphibian chytridiomycosis. *Dis. Aquat. Org.* **92**, 89-91.
Jørgensen, C. B. (1949). Permeability of the amphibian skin: II. Effect of moulting of the skin of anurans on the permeability to water and electrolytes. *Acta Physiol. Scand.* **18**, 171-180.
Jørgensen, C. B. and Larsen, L. O. (1961). Molting and its hormonal control in toads. *Gen. Comp. Endocrinol.* **1**, 145-153.
Jørgensen, C. B., Larsen, L. O. and Rosenkil, P. (1965). Hormonal dependency of molting in amphibians - effect of radiothyroidectomy in toad *Bufo Bufo* (L). *Gen. Comp. Endocrinol.* **5**, 248-251.
Kriger, K. M., Hines, H. B., Hyatt, A. D., Boyle, D. G. and Hero, J.-M. (2006). Techniques for detecting chytridiomycosis in wild frogs: Comparing histology with real-time Taqman PCR. *Dis. Aquat. Org.* **71**, 141-148.
Langhammer, P. F., Burrowes, P. A., Lips, K. R., Bryant, A. B. and Collins, J. P. (2014). Susceptibility to the amphibian chytrid fungus varies with ontogeny in the direct-developing frog, *Eleutherodactylus coqui*. *J. Wildl. Dis.* **50**, 438.
Lee, M. (2009). *Basic Skills in Interpreting Laboratory Data*. Bethesda, MD: ASHP.
Lillywhite, H. B. (2006). Water relations of tetrapod integument. *J. Exp. Biol.* **209**, 202-226.
Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proc. Natl. Acad. Sci. USA* **103**, 3165-3170.
Liu, J.-N. and Hou, P.-C. L. (2012). Cutaneous resistance to evaporative water loss in Taiwanese arboreal Rhacophorid frogs. *Zool. Stud.* **51**, 988-995.
Mancini, A. J. (2004). Skin. *Pediatrics* **113**, 1114-1119.
Meyer, E. A., Cramp, R. L., Bernal, M. H. and Franklin, C. E. (2012). Changes in cutaneous microbial abundance with sloughing: Possible implications for infection and disease in amphibians. *Dis. Aquat. Org.* **101**, 235-242.
Ohmer, M. E. (2016). Interactions between amphibian skin sloughing and a cutaneous fungal disease: infection progression, immune defence, and phylogenetic patterns. PhD Thesis, University of Queensland, Australia.
Ohmer, M. E., Herbert, S. M., Speare, R. and Bishop, P. J. (2013). Experimental exposure indicates the amphibian chytrid pathogen poses low risk to New Zealand's threatened endemic frogs. *Anim. Conserv.* **16**, 422-429.
Ohmer, M. E. B., Cramp, R. L., White, C. R. and Franklin, C. E. (2015). Skin sloughing rate increases with chytrid fungus infection load in a susceptible amphibian. *Funct. Ecol.* **29**, 674-682.
Ohmer, M. E. B., Cramp, R. L., Russo, C. J. M., White, C. R. and Franklin, C. E. (2017). Skin sloughing in susceptible and resistant amphibians regulates infection with a fungal pathogen. *Sci. Rep.* **7**, 3529.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and **R Development Core Team**. (2013). nlme: Linear and Nonlinear Mixed Effects Models: R package version 3.1-113.
Prunier, J., Kaufmann, B., Grolet, O., Picard, D., Pompanon, F. and Joly, P. (2012). Skin swabbing as a new efficient DNA sampling technique in amphibians, and 14 new microsatellite markers in the alpine newt (*Ichthyosaura alpestris*). *Molecular Ecology Resources* **12**, 524-531.
Retallick, R. W. R. and Miera, V. (2007). Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Dis. Aquat. Org.* **75**, 201-207.
Shoemaker, V. H., Hillman, S. S., Hillyard, S. D., Jackson, D. C., McClanahan, L. L., Withers, P. C. and Wygoda, M. L. (1992). Exchange of water, ions and respiratory gases in terrestrial amphibians. In *Environmental Physiology of the Amphibians* (ed. M. E. Feder and W. W. Burggren), pp. 125-150. Chicago: The University of Chicago Press.
Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B. and Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* **4**, 125-134.
Smith, P. G. (1975). Aldosterone-induced moulting in amphibian skin and its effect on electrical capacitance. *J. Membr. Biol.* **22**, 165.
Spearman, R. I. C. (1968). Epidermal keratinization in the salamander and a comparison with other amphibia. *J. Morphol.* **125**, 129-143.
Tracy, C. R. and Christian, K. A. (2005). Ecological significance of body size and skin resistance to terrestriality in frogs. *In 5th World Congress of Herpetology*. Stellenbosch, South Africa.

Tracy, C. R., Tixier, T., Le Nöene, C. and Christian, K. A. (2014). Field hydration state varies among tropical frog species with different habitat use. *Physiol. Biochem. Zool.* **87**, 197-202.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* **326**, 582-585.

Voyles, J., Vredenburg, V. T., Tunstall, T. S., Parker, J. M., Briggs, C. J. and Rosenblum, E. B. (2012). Pathophysiology in mountain yellow-legged frogs (*Rana muscosa*) during a chytridiomycosis outbreak. *Public Library of Science* **7**, e35374.

Wardziak, T., Luquet, E., Plenet, S., Léna, J.-P., Oxarango, L. and Joly, P. (2013). Impact of both desiccation and exposure to an emergent skin pathogen on transepidermal water exchange in the palmate newt *Lissotriton helveticus*. *Dis. Aquat. Org.* **104**, 215-224.

Withers, P. C., Hillman, S. S., Drewes, R. C. and Sokol, O. M. (1982). Water loss and nitrogen excretion in sharp-nosed reed frogs (*Hyperolius nasutus*: Anura, Hyperoliidae). *J. Exp. Biol.* **97**, 335-343.

Wu, N. C., Cramp, R. L. and Franklin, C. E. (2017). Living with a leaky skin: Upregulation of ion transport proteins during sloughing. *J. Exp. Biol.* **220**, 2026-2035.

Wygoda, M. L. (1984). Low cutaneous evaporative water loss in arboreal frogs. *Physiol. Zool.* **57**, 329-337.

Young, J. E., Christian, K. A., Donnellan, S., Tracy, C. R. and Parry, D. (2005). Comparative analysis of cutaneous evaporative water loss in frogs demonstrates correlation with ecological habits. *Physiol. Biochem. Zool.* **78**, 847-856.