

This is the **Accepted Version** of a paper published in the  
journal FEMS Microbiology Letters:

Stockwell, Michelle P., Garnham, James I., Clulow, John,  
Bower, Deborah S., and Mahony, Michael J. (2016) *Low  
disease-causing threshold in a frog species susceptible to  
chytridiomycosis*. FEMS Microbiology Letters, 363 (12).  
pp. 1-6.

<http://dx.doi.org/10.1093/femsle/fnw111>

**Title**

Low disease causing threshold in a frog species susceptible to chytridiomycosis

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Low disease causing threshold to chytridiomycosis

**Keywords**

*Litoria aurea*, receiver operating characteristic, *Batrachochytrium dendrobatidis*, radio-tracking, susceptibility,

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## Abstract

A simple diagnosis of the presence or absence of an infection is an uninformative metric when individuals differ considerably in their tolerance to different infection loads or resistance to rates of disease progression. Models that incorporate the relationship between the progression of the infection with the potential alternate outcomes provide a far more powerful predictive tool than diagnosis alone. The global decline of amphibians has been amplified by *Batrachochytrium dendrobatidis*, a pathogen that can cause the fatal disease chytridiomycosis. We measured the infection load and observed signs of disease in *Litoria aurea*. Receiver operating characteristic curves were used to quantify the dissimilarity between the infection loads of *Litoria aurea* that showed signs associated with chytridiomycosis and those that did not. *Litoria aurea* had a 78% probability of developing chytridiomycosis past a threshold of 68 zoospore equivalents (ZE) per swab and chytridiomycosis occurred within a variable range of 0.5-490 ZE. Studies should incorporate a species-specific threshold as a predictor of chytridiomycosis, rather than a binary diagnosis. Measures of susceptibility to chytridiomycosis must account not only for the ability of *Batrachochytrium dendrobatidis* to increase its abundance on the skin of amphibians but also to determine how each species tolerates these infection loads.

## Introduction

Predicting the physiological impact of disease on infected individuals is a core component of health management but is complex because a single pathogen can have variable effects on its host (Linden 2006: 132-9). A simple diagnosis of the presence or absence of an infection is an uninformative metric when individuals differ considerably in their tolerance to different infection loads or resistance to rates of disease progression (Cecil, et al. 1996: 735-42).

Models that incorporate the relationship between the progression of the infection with the potential alternate outcomes provide a far more powerful predictive tool than mere diagnosis alone (Swets 1988: 1285-93). Moreover, they can provide a threshold level of infection beyond which a certain event (e.g. death) becomes highly probable without intervention.

Such thresholds can be used in decision-making to prioritise conservation efforts for populations or species most at risk and to direct, evaluate and optimise disease management.

One of the most significant diseases in wildlife biology is chytridiomycosis, caused by *Batrachochytrium dendrobatidis*, an invasive and globally distributed pathogenic fungus that is known to infect over 500 species ([www.bd-maps.net](http://www.bd-maps.net)) in all three amphibian Orders and has contributed to population declines, extirpations and extinctions world-wide (Berger, et al. 2016: 89-99, Berger, et al. 1998: 9031-6, Stuart, et al. 2004: 1783-6). Infection occurs primarily in the keratinised epidermis of developed individuals which can impair osmoregulatory function and cause cardiac arrest in susceptible species (Voyles, et al. 2009: 582-5). Infection also occurs in the keratinised mouthparts of larval amphibians but this does not result in chytridiomycosis (Berger, et al. 1998: 9031-6). The development of chytridiomycosis has been linked to infection with a higher abundance of *B. dendrobatidis* zoospores (Sih, et al. 2004: 274-6, Voyles, et al. 2009: 582-5, Vredenburg, et al. 2010: 9689-

94). However, individual infection loads can fluctuate widely without the onset of disease (Briggs, et al. 2010: 9695-700, Sapsford 2012) and can be affected by exposure to environmental inhibitors of fungal growth (e.g. warm temperatures (Stevenson, et al. 2014: 4053-64), salinity (Stockwell, et al. 2012: e36942, Stockwell, et al. 2015: 901-10) and phenolic acids (Stoler, et al. 2016: 1-13)), characteristics that reduce transmission potential (e.g. low densities (Briggs, et al. 2010: 9695-700) and aboreality (Rowley and Alford 2007: 1-9)) and immunological resistance (e.g. antimicrobial peptides (Woodhams, et al. 2007: 409-17) and cutaneous microflora (Harris, et al. 2006: 53-6)). This variability makes anticipating the outcome of infection difficult, even when individual infection loads and population prevalences are known. Currently, the most common determinant of population level susceptibility to chytridiomycosis is the observation of sick and dying individuals and the decline of infected populations (Berger, et al. 2016: 89-99).

The recent discovery of a disease-causing threshold (the *B. dendrobatidis* infection load that when exceeded results in a high probability of chytridiomycosis) in captive green and golden bell frogs *Litoria aurea* (Stockwell, et al. 2010: 62-71) may provide a predictive tool for chytridiomycosis-driven population decline. The disease-causing threshold for *L. aurea* in laboratory trials was 15 ZE when first exposed as tadpoles and 32 ZE when first exposed as juveniles (Stockwell, et al. 2010: 62-71). By comparison, infection load at death occurred at a wide range of infections loads, between 16-9439 ZE (Stockwell, et al. 2010: 62-71). These low infection thresholds and widely varying infection loads at death, suggest individuals of this species develop chytridiomycosis at a low infection load but infection load does not determine the timing of mortality (Stockwell, et al. 2010: 62-71). An overestimation of the disease-causing infection load would lead to false assumptions if it is considered a general proxy and used to identify susceptible or declining species. However, it is unknown whether

the lower thresholds of *L. aurea* in laboratory trials accurately reflect terminal stages of chytridiomycosis in adults of wild populations that may be exposed to different strains of *B. dendrobatidis* and environmental conditions that affect pathogen virulence or environmental stressors that may affect host susceptibility and ultimately, infection outcomes.

Our objective was to determine the disease-causing threshold in wild amphibian populations for a species in which this threshold has been previously estimated in the laboratory (Stockwell, et al. 2010: 62-71). Specifically we aimed to determine the sensitive and specific infection load threshold of *L. aurea* from two populations, above which individuals have a high probability of becoming diseased.

## **Methods**

### **Study site**

The study took place at two of the largest remaining populations of *L. aurea*. Sydney Olympic Park (32°51'S 151°44'E) is located 14 km west of Sydney's central business district (CBD) and contains 750 ha of remediated land, over half of which is dedicated to parklands including approximately 150 water bodies of varying size and hydrology. Sydney Olympic Park has a long-term average annual rainfall of 1132.9 cm (between 1905-2014) and mean minimum and maximum annual temperature of 12.3 and 22.2°C respectively (between 1909-2014) (BOM Station: 066131). The *L. aurea* population size has been estimated to be approximately 800 individuals (Pickett, et al. 2013: 156-62). Kooragang Island is situated 150 km north of Sydney, 5 km west of Newcastle's CBD and contains 2560 ha of reclaimed land. Newcastle has a long-term average annual rainfall of 1132.0 cm (between 1962-2014) and mean minimum and maximum annual temperature of 19.2 and 26.5°C respectively (between 1962-2014) (BOM Station: 061055). The *L. aurea* population has been estimated

to contain 1995 males (therefore, a total population estimate of 3990 assuming a 1:1 sex ratio) across 32 water bodies (Hamer, et al. 2007: 79-88).

### **Study method**

Sixteen adult *L. aurea* were radio-tracked on Kooragang Island between May and August 2007. An additional 24 adults were radio-tracked at Sydney Olympic Park between May and July in 2011. Each frog was fitted with an external single-stage transmitter (Titley Scientific, Australia) weighing less than 2.7 g (total weight less than 10% of the animal's body weight), attached by a silicone tube waist band (Richards, et al. 1994: 155-8) and released at the original point of capture. A REGAL 2000 telemetry receiver and a three element Yagi antenna (Titley Scientific, Australia) were used to track the location of each individual. Animals were tracked 2-3 times per week, monitored for skin abrasion caused by the waistband and signs of chytridiomycosis. Where skin abrasion was evident due to transmitter attachment, transmitters were immediately removed. All animals were swabbed for *B. dendrobatidis* infection twice on each hand, four repeat strokes along each of the ventral side and each inner and outer thigh, and two times on each foot (40 strokes) before being released back to their point of capture.

Where individuals displayed signs associated with chytridiomycosis including lethargy, unusual posture, a red ventral surface, skin shedding or lesions (Berger, et al. 2004: 434-40) their righting reflexes were tested by placing the animals on their back and watching their righting response. If they struggled or were unable to right themselves, the transmitters were removed, the animal was swabbed for *B. dendrobatidis*, and then transported to the University of Newcastle for heat treatment (Woodhams, et al. 2003: 65-7) before being released back to their points of capture. Without treatment, poor righting reflex is an

indication that death will occur within 48 hours (Berger, et al. 2005: 47-50). Animals that did not show signs of skin abrasion or disease were tracked for up to four months (the battery life of the transmitters). The transmitters were then removed, the animals swabbed and released back to their point of capture.

Swabs were stored at  $-4^{\circ}\text{C}$  within 8 hours of use. Taqman real-time PCR assays following standard procedures were used to detect and quantify *B. dendrobatidis* on swabs (Boyle, et al. 2004: 141-8), using a Rotor Gene 6000 DNA amplification system. Each swab was analysed in triplicate and the geometric mean of these replicates determined and multiplied by 10 to account for a dilution step in the PCR process (Vredenburg, et al. 2010: 9689-94). This geometric mean was used to indicate individual infection loads. Inhibition of template amplification was identified using TaqMan exogenous internal positive controls and when detected, samples were reanalysed with a further ten-fold dilution that was accounted for in the calculation of the geometric mean.

### **Data Analysis**

Receiver operating characteristic (ROC) curves were used to investigate whether radio-tracked *L. aurea* could be classified into two groups based on their infection loads; those that showed signs of terminal signs of chytridiomycosis and were considered deceased and those that did not show signs and were considered alive. The area under the ROC curve (AUC) was used as a measure of how dissimilar the infection loads were between these groups, where an AUC close to 1 represented a high level of dissimilarity. Where a high level of dissimilarity occurred, an infection load threshold was identified. ROC curves plot the proportion of true positives (i.e. the proportion of individuals correctly classified as having chytridiomycosis from their infection load; also called the sensitivity) against the proportion of false positives

(i.e. the proportion of individuals incorrectly classified as having chytridiomycosis based on infection load; also called 1-specificity, where specificity is the proportion of true negatives) as the discrimination threshold (i.e. the infection load threshold) changes (Zou, et al. 2007: 645-57). The point at which both sensitivity and specificity were at their highest was considered the infection load threshold that best predicted disease.

## Results

Of the 40 individuals radio-tracked, 11 had their transmitters removed due to abrasion at the attachment site, one was found dead as the transmitter antennae became tangled in wire, 19 were tracked until the end of the battery life and nine showed signs of chytridiomycosis and were treated. *Batrachochytrium dendrobatidis* was not detected on 17 individuals and none of those individuals showed signs associated with disease. Twenty three frogs were infected with *B. dendrobatidis*. In the nine individuals that displayed signs of disease, their infection loads ranged from 0.5-490 ZE, with a median of 145 ZE. In the remaining infected individuals that did not show signs of disease, their maximum infection loads ranged from 0.1-919 ZE, with a median of 15 ZE (Fig. 1).

There was a high level of dissimilarity between the infection loads of *L. aurea* that showed signs associated with chytridiomycosis and those that did not; the area under the ROC curve ( $\pm$  SE) equalled 0.86 ( $\pm$  0.06). The most sensitive and specific infection load threshold was 68 ZE per swab. Individuals with infection loads greater than this had a 78% chance of showing signs of chytridiomycosis, whereas individuals with infection loads less than this threshold had a 90% chance of not showing signs.

## Discussion

Our study demonstrated that individuals in wild *L. aurea* populations had a low infection load threshold for developing chytridiomycosis, as is consistent with previous laboratory studies (Stockwell, et al. 2010: 62-71). *Litoria aurea* had a 78% chance of developing chytridiomycosis past a threshold of 68 ZE per swab while chytridiomycosis occurred within a variable range of 0.5-490 ZE. Our results suggest that studies on *B. dendrobatidis* that consider infection presence or load as a predictor of chytridiomycosis will be improved by incorporating a species-specific threshold as a predictor of disease, rather than a binary diagnosis. For example, in multi-state transition models of survival (Nichols and Kendall 1995: 835-46), incorporating states of below and above the threshold may improve the predictive power of estimates. Where high or increasing (if monitored over time) proportions of a population have infection loads above the threshold, their use can highlight the need for ongoing monitoring, intervention in the form of rescuing the population's genetic potential by taking individuals (or their genetic material) into captivity, or disease management. Although there are no established methods for the management of *B. dendrobatidis* in a host population, there are many methods being trialled. These include reducing host density, treatment or bacterial bioaugmentation of hosts and their habitat, selective breeding for pathogen resistance, creation of climatic refugia and immunisation (Woodhams, et al. 2011: 8). For *L. aurea*, habitat creation trials that increase pond salinity for the control of *B. dendrobatidis* are underway (K.L. Klop-Toker unpubl data) and the use of the thresholds developed here will provide an important means of determining its effect on incidences of disease that would not have been previously possible due to low detectability of diseased and dead individuals.

By identifying populations or species with the highest proportion of individuals at risk of disease, infection load thresholds could also be used to direct and prioritise management

actions, provided methods are standardised prior to comparison. While previous research demonstrates intraspecific differences in susceptibility to chytridiomycosis (Langhammer, et al. 2014), it is unknown how a disease causing threshold may vary among populations. We found similar findings between the low infection threshold of juvenile *L. aurea* under a previous controlled laboratory experiment (32 ZE; Stockwell, et al. 2010: 62-71) and those of adults in wild populations (68 ZE, current study) when standardised methods were used. The range of infection loads at which wild *L. aurea* developed chytridiomycosis (0.5-490 ZE) were also comparable with individuals in the population that appeared healthy (0.1-919 ZE). During our study, two individuals developed chytridiomycosis at infection loads considerably below the predicted threshold. Previous studies have demonstrated that reducing immunocompetence of tolerant individuals and exposing them to *B. dendrobatidis* leads to the development of chytridiomycosis (Ramsey, et al. 2010: 3981-92). Therefore, it is logical to expect that individual differences in immunocompetence and factors that affect it such as access to warm temperatures, will determine the rate at which *B. dendrobatidis* causes damage (Ribas, et al. 2009: e8408). It is also possible that additive factors such as other pathogens, stress or predators (Sih, et al. 2004: 274-6) become more problematic during *B. dendrobatidis* infection, or the infection itself inhibits immune responses (Woodhams, et al. 2012: 1203-11); exploration of these interactions requires further examination.

Although we do not know how infection load thresholds vary between *L. aurea* populations, the individuals in the current study did appear to develop signs of chytridiomycosis at considerably lower infection loads than those reported for other species (Ratzlaff 2012, Voyles, et al. 2009: 582-5). However, this may also be also be explained by differences in *B. dendrobatidis* strain virulence (Berger, et al. 2005: 47-50, Retallick and Miera 2007: 201-7, Vredenburg, et al. 2010: 9689-94), variable sampling technique and swab strokes per

individual (n = 40, current study; n = 30 (Vredenburg, et al. 2010: 9689-94); n = 50 (Kinney, et al. 2011: e16708)) ITS1 copy number (which affects qPCR estimates of infection load Longo, et al. 2013: e59499), the use of different qPCR standards or the number of freeze-thaw cycles for standards and reagents, dilution factors applied to qPCR outcomes prior to data analysis (e.g. x 10 (Stockwell, et al. 2010: 62-71); x 80 (Vredenburg, et al. 2010: 9689-94)) or by differences in infection load at sampling versus those that would result from exponential growth in the final period of moribundity, where euthanasia is not induced (Carey, et al. 2006: 5-21). Therefore, such comparisons can only be made if methods from sample collection to data analysis are standardised. However, if future work confirmed that *L. aurea* is considerably less tolerant to infection than other species, then measures of susceptibility to chytridiomycosis may depend not only on the ability of *B. dendrobatidis* to increase in abundance on the skin of amphibians, but also how successfully an individual can actually tolerate different infection loads.

Previous research on factors that reduce susceptibility has largely focused on how individuals maintain low infection levels and thus examined the mechanisms that inhibit the transmission and growth of *B. dendrobatidis* through individual immunological, behavioural and habitat-use mechanisms (Harris, et al. 2006: 53-6, Heard, et al. 2013, Kriger and Hero 2007: 781-8, Stockwell, et al. 2014, Woodhams, et al. 2007: 409-17, Woodhams, et al. 2007: 390-8) and host population densities (Briggs, et al. 2010: 9695-700). Virulence and exposure dose of *B. dendrobatidis* can also affect the outcome of infection in hosts but it is unclear whether this occurs because of differences in infection loads or an alternative mechanism (Berger, et al. 2005: 47-50, Carey, et al. 2006: 5-21). Our study suggests that even low levels of infection can result in chytridiomycosis and therefore determining species specific disease-causing thresholds is important for assessing the impact of a pathogen; a critical component that

should be factored into future surveillance and research. For example, two individuals could have similar levels of protective skin peptides but if one host species has a lower disease-causing threshold, it is inherently far more susceptible than the other.

Factors that affect the variation in the infection load at which individuals succumb to disease have not been previously determined for amphibians with *B. dendrobatidis*. Chytridiomycosis causes epidermal hyperplasia and hyperkeratosis (thickening of the *stratum corneum*) in amphibian hosts, which impairs osmoregulatory function, through inhibition of electrolyte transport on the epidermis of infected individuals (Voyles, et al. 2009: 582-5). If *L. aurea* do develop chytridiomycosis with comparatively lower zoospore counts than some other species, this highlights the possibility that physiological differences in the structure or function of the skin of *L. aurea* may leave them more vulnerable to epidermal damage. Cree (1988: 119-25) demonstrated that the osmotic water flow through rate in the pelvic skin in *L. aurea* was higher than the 22 other species tested and related this to the functional requirement for a species to uptake water efficiently. Therefore, it is possible that the rate of osmotic water flow through amphibian skin affects the tolerance to damage and thus affects species disease-causing thresholds. It would be useful to compare levels of epidermal damage during low level infections between *L. aurea* and other susceptible species that succumb to disease at higher zoospore counts to determine whether the skin of *L. aurea* is comparatively more damaged, or alternatively whether *L. aurea* are less tolerable of low levels of damage and how this tolerance interacts with the health status of individuals.

The apparent susceptibility of *L. aurea* to chytridiomycosis at low infection loads also highlights the possibility that many other frog species may also succumb to chytridiomycosis at similarly low loads. This requires further research because assumptions that species with

low loads are not at risk of succumbing to chytridiomycosis would be false, leading to poor conservation outcomes. The generation of disease-causing thresholds can be done for any system where infection loads can be monitored in a sufficient sample of host individuals over time. ROC curves then provide a useful tool to diagnostically determine the threshold at which diseases are likely to occur and are used widely in the human health field (Søreide, et al. 2011: 27-34, Zweig and Campbell 1993: 561-77). Although the range of infection loads at which individuals succumbed to chytridiomycosis was variable in our study, by using analyses that could incorporate sensitivity and specificity, the estimated threshold incorporated a level of confidence that would otherwise be unclear. Using ROC analysis provided a standard method to compare with other studies that aim to identify measures of susceptibility through disease causing thresholds to chytridiomycosis. It provided a tool to relate infection loads of *L. aurea* to probable outcomes and thus a defensible decision making process to direct management, or to incorporate into models. Determining the disease causing thresholds of other populations and species can therefore help to predict, understand and prevent decline events in susceptible hosts.

## **Funding**

This study was supported by Port Waratah Coal Service, the Kooragang Wetland Rehabilitation Project and an ARC Linkage Grant LP0989459 (Industry partners: Australian Research Council, Sydney Olympic Park Authority, Strathfield council, South Australian Museum, NSW Roads and Traffic Authority, NSW Department of Environment and Climate Change).

## **Acknowledgements**

Katherine Oxenham, Jenny O'Meara, Kerry Darcovich and Peggy Svoboda are gratefully acknowledged for site access and constructive input into our research and manuscript. We also thank the many volunteers that assisted with field work. Approvals were granted by the University of Newcastle Animal care and ethics committee (A-2008-165 and 949 0506) and NSW National Parks scientific licences (SL100190 and SL10382).

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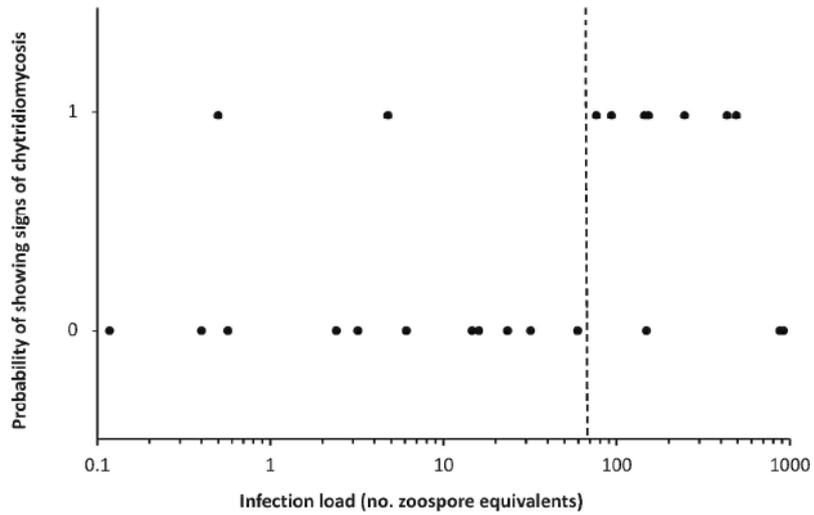


Fig.1. Relationship between the probability of showing signs of chytridiomycosis and infection load, measured as the number of zoospore equivalents of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* detected on *Litoria aurea* radiotracked on Kooragang Island and Sydney Olympic Park over the non-breeding season. The dotted line shows the most sensitive and specific infection load threshold of 68 zoospore equivalents.