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EASTERN WATER DRAGONS (*PHYSIGNATHUS LESUEURII*) ARE NOT IMPORTANT ALTERNATE HOSTS OF THE FROG CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS*

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***Abstract.*—The chytridiomycete fungus *Batrachochytrium dendrobatidis*, causative pathogen of the disease amphibian chytridiomycosis, sometimes occurs on environmental substrates and can grow *in vitro* on reptile skin. This suggests that susceptible reptiles associated with water bodies may act as both reservoirs of infection and vectors for spread of the disease. We sampled the semi-aquatic Eastern Water Dragon, *Physignathus lesueurii*, associated with streams and known infected frog populations for *B. dendrobatidis*. None of the 15 juvenile dragons returned positive results for the pathogen by qPCR assay. This suggests *P. lesueurii* are not important alternate hosts for *B. dendrobatidis* and do not act as important reservoirs or vectors of *B. dendrobatidis*.**

Key Words.—alternate host; amphibians; *Batrachochytrium dendrobatidis*; chytridiomycosis; *Physignathus lesueurii*; reptiles; water dragons.

INTRODUCTION

The chytridiomycete fungus *Batrachochytrium dendrobatidis* (Longcore et al. 1999) is the causative pathogen of the disease amphibian chytridiomycosis, responsible for the declines of amphibians worldwide. The disease has emerged due to anthropogenic and natural spread of *B. dendrobatidis* into naive amphibian populations (Skerratt et al. 2007). Transmission of the disease among amphibians has been demonstrated experimentally to occur via release of zoospores of *B. dendrobatidis* from infected amphibians into the environment and subsequent infection of uninfected amphibians by these zoospores or reinfection of the same individual (Berger et al. 1998; Berger et al. 2004; Rachowicz and Vredenburg 2004; Carey et al. 2006; Rachowicz and Briggs 2007). Empirical support of this transmission method in the wild has been provided by detection of *B. dendrobatidis* DNA in environmental substrates such as pond water (Kirshtein et al. 2007; Walker et al. 2007) and on the surface of streamside rocks or boulders (Lips et al. 2006), with which stream-associated amphibians are likely to have contact.

To date, alternative hosts of *B. dendrobatidis* have not been demonstrated (Rowley et al. 2007); however, cultures of *B. dendrobatidis* can grow on snake skin (Piotrowski et al. 2004; Bryan Windmiller and Alison Robbins unpubl. data), suggesting that susceptible reptiles associated with water bodies may act as both reservoirs of infection and vectors for spread of the disease. The Eastern Water Dragon, *Physignathus lesueurii* (Gray 1831), is a semi-aquatic, arboreal lizard that perches on emergent rocks and tree branches overhanging creeks and rivers. When disturbed, *P. lesueurii* drops into the water to escape. Its diet includes frogs (Cogger 1994). The adults are fairly sedentary, moving an average of 76 m between captures in one study (Thompson 1993). We surveyed *P. lesueurii* along streams with permanent anuran populations known to be infected with *B. dendrobatidis* to determine the likelihood of this reptile acting as an important alternate host for the pathogen. We regarded that water dragons would be an important alternate host if infection prevalences in water dragons were similar to those of anurans in the same locations.

MATERIALS AND METHODS

We captured *P. lesueurii* after chance encounters during surveys of stream-associated anurans in Murray Upper National Park (18º11′S, 145º52′E, elevation 250 m) in the wet topics of Australia. Dragons were caught by gloved hand and the ventral surface and inguinal folds swabbed with an MW100 tubed dryswab (Medical Wire and Equipment Co [Bath] Ltd, Corsham, Wiltshire, England). We wore latex gloves, which were changed between animals to prevent transfer of pathogens. We labelled swabs and stored them at < 10°C in the field for up to five days, prior to transport to James Cook University and storage at -80°C until processing.

We used a real-time TaqMan® (Applied Biosystems, Scoresby, Victoria, Australia) PCR assay to detect *B. dendrobatidis*. The assay followed the protocol described by Boyle et al. (2004) with the following modifications. We performed the analysis on the Rotor-Gene™ 6000 (Corbett Research, Mortlake, New South Wales, Australia) using Gene-Disc 100 tubes. A 15 µL reaction volume was produced by loading 10 µL of PCR reaction mix and 5 µL of the diluted sample extract, standard, diluted negative control, or water into Gene-Disc tubes with a CAS-1200™

TABLE 1. Results of the PCR assay performed on swabs from *Physignathus lesueurii*. Replicate Ct values (n = 3) of the swabs did not vary significantly from the Ct value of the negative control (Mean ± Standard Deviation [SD] = 27.43 ± 0.71).

Dragon #	Replicate Ct Value	Replicate Ct SD	Notes
1	27.15	0.29	
2	28.21	0.75	
3	27.25	0.76	
4	28.26	0.24	
5	28.18	0.26	
6	27.18	0.07	Ct value of sample statistically compared with Ct value of negative control
8	27.49	0.52	
9	26.81	0.27	
10	26.93	0.61	
11	28.69	0.28	
13	27.08	0.39	
14	27.18	0.10	
15	27.49	0.87	
7	26.87	0.45	Initial software analysis failure, sample repeated in second PCR assay.
12	26.56	0.28	One replicate initially inhibited, PCR assay repeated at 1 in 100 dilution

pipetting robot (Corbett Robotics, Mortlake, New South Wales, Australia). We diluted the sample extract and negative control one in 10. We performed triplicate analyses for each sample, negative control and no-template-control, and quadruplicate analyses for each standard (100, 10, 1, 0.1 zoospore equivalents). The master mix included the addition of 400 ng/μL of bovine serum albumin (BSA) in order to reduce inhibition of PCR by substances such as humic acids and other inhibitors (Kreader 1996; Garland et al. 2009).

To test for false negatives due to inhibition of the PCR assay, we performed a repeat triplicate analysis incorporating the TaqMan® Exogenous internal positive control, IPC (0.6x Exo IPC Mix, 0.6x Exo IPC DNA; Applied Biosystems, Scoresby, Victoria, Australia) into the assay (Hyatt et al. 2007). We also made an extra one in 100 dilution of the extract for subsequent reanalysis when Ct differentials between the sample and the negative control were greater than three. Inhibition would be indicated by Ct values significantly higher than those obtained for the negative control, so a one-sample t-test ($\alpha = 0.05$) was performed to compare the sample Ct values. We calculated the exact confidence interval for the proportion of infected water dragons, with the assumption that the proportion infected could be any number in the range of zero to one, with all possibilities being equally likely.

RESULTS

We caught 15 juvenile *P. lesueurii* (snout vent length < 12 cm) at Murray Upper National Park in October 2006 (n = 8), December 2006 (n = 2), and October 2007 (n = 5). Captures were limited to opportunistic encounters during routine monitoring of stream-associated anurans. We did not encounter adult water dragons on the stream during frog surveys. None of the swabs of these dragons returned positive results for *B. dendrobatidis* by qPCR assay in any of the triplicate wells. These results were not false negatives, as the PCR assay was not inhibited. The Ct value for the swabs from *P. lesueurii* analysed by PCR assay did not vary significantly from the Ct value of the negative control ($t = 0.616$, df = 12, $P = 0.556$; Table 1). The qPCR assay for one swab was repeated in an additional run due to a software analysis failure. The assay for an additional swab was inhibited at the standard concentration, but inhibition was overcome when it was further diluted to one in 100. The Ct values for both of the above swabs were lower than that of the negative control (Table 1) and therefore inhibition did not occur. The exact 95% shortest confidence interval for this 0% estimate of prevalence of *B. dendrobatidis* in water dragons is 0–17%. The prevalence of infection of *Batrachochytrium dendrobatidis* in stream-associated anurans in 2006 and 2007 differed significantly ($\chi^2_c = 3.91$, df = 1, $P = 0.048$; Table 2) but not the intensity of infection ($t = 1.031$, df = 71, $P = 0.377$; Table 2).

DISCUSSION

Juvenile *P. lesueurii* were captured in periods of high prevalence of *B. dendrobatidis* among stream dwelling frogs (Table 2), indicating that the chytrid fungus was abundant in aquatic habitats. However, all *P. lesueurii* were negative for *B. dendrobatidis* by qPCR assay. Although the upper 95% confidence limit for the 0% prevalence estimate of *B. dendrobatidis* in water dragons was 17% this was much lower than the mean prevalence in frogs of 46% and the lower 95% confidence limit of 36% for frogs at similar times of the year. Therefore, we concluded that water dragons are not important alternate hosts and ceased sampling. However, we cannot rule out low infection prevalences in water dragons. It is unlikely that innate susceptibility to infection varies greatly in different populations of water dragons as

TABLE 2. The prevalence (%) and intensity (mean # zoospore equivalents, range) of infection of *Batrachochytrium dendrobatidis* (*Bd*) in stream-associated anurans in the Murray Upper National Park, Australia.

Month, Year	# Anurans Sampled	Prevalence of *Bd*	Intensity of Infection
Oct.-Dec. 2006	113	37%	343, 1–4232
Oct. 2007	57	54%	213, 1–23771

this does not occur within naive species of amphibians (McDonald et al. 2005). Determining if low prevalence of infection may occur in water dragons would require much larger sample sizes, which are difficult to obtain. A sample size of 298 would be required to be confident that the prevalence of *B. dendrobatidis* in water dragons was < 1%. Experimental infections to assess host susceptibility would be more cost efficient than pursuing large and expensive surveys. However, the cost versus benefits of experimental infection trials of water dragons indicates that it is not currently warranted given the other immediate priorities for research on *B. dendrobatidis*.

As *B. dendrobatidis* is capable of utilizing reptilian skin as a nutrient source (Piotrowski et al. 2004; Bryan Windmiller and Alison Robbins, unpubl. data), our results suggest that the preferred habitat of *P. lesueurii* along the vegetated edges of water bodies means it may occasionally contact contaminated substrates; however, its biology probably does not allow progression to infection. Lack of infection is probably because *B. dendrobatidis* is sensitive to desiccation (Johnson et al. 2003), and prevalence of *Bd*-infection among forest-dwelling frogs is far less than among stream-associated frogs (Kriger and Hero 2007). Adult *P. lesueurii* sometimes sleep with all but their nostrils submerged in water, but like most terrestrial reptiles, they are potentially a 'dry' lizard. During diurnal activity, they only enter water to escape a potential predator or briefly to cross water (Thompson 1993). Alternatively, live *P. lesueurii* skin simply may be unsuitable for *B. dendrobatidis* infection.

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ANDREA D. PHILLOTT was awarded her Ph.D. from Central Queensland University, Rockhampton, Australia, in 2003. Volunteer experience as an undergraduate student in a Bachelor of Applied Science (Biology; Honours) led to a strong attraction to marine and freshwater turtles, which expressed itself in her postgraduate studies of the fungal invasion of sea turtle nests. This in turn fostered an interest in wildlife diseases and her positions as Postdoctoral and Senior Research Fellows with the Amphibian Disease Ecology Group at James Cook University, Townsville, Australia. Dr. Phillott's current studies on the epidemiology of amphibian chytridiomycosis have awakened her to the wonders and intricacies of amphibian biology, although she still maintains an active research profile in sea turtle biology as an Honorary Research Fellow at Central Queensland University. Her current turtle project involves radio-tracking of Flatback Sea Turtle (*Natador depressus*) hatchlings from her sea kayak. Andrea does as much white water kayaking, rock climbing, trail running, and hiking as possible between field trips. (Photographed by Leah Denham)

STEPHEN GARLAND completed an undergraduate degree in Environmental Management, Fisheries Management and Aquaculture strand, Southern Cross University, Lismore, NSW, Australia, in 1995. He completed an Honours year in 1996 where he developed experience in molecular biology, assessing PCR-based markers for use in wildlife population genetics. In 1997 he commenced a Ph.D. developing PCR-based markers for rice breeding, working in the Centre for Plant Conservation Genetics at Southern Cross University. From 2000 to 2005, he worked as a research scientist with Queensland's Department of Primary Industries and Fisheries, where he established a molecular biology laboratory in Bowen, North Queensland, and developed and applied markers for the tomato, capsicum, and sweet corn breeding programs. After the closure of the Bowen Laboratory, Stephen worked as a research assistant at James Cook University's School of Medicine, Townsville, North Queensland. In this position, Stephen contributed to the development of research plans and performed experiments assessing the effect of short chain fatty acids on the expression of cytokines from preadipocytes and adipocytes. He developed theories on the inflammatory effect of short chain fatty acids produced by infecting bacteria. He currently operates a qPCR diagnostic service for causative agent of chytridiomycosis in amphibians at James Cook University and is undertaking research into marker development for the organism. (Photographed by Rebecca Webb)

LEE F. SKERRATT first became involved in wildlife health in 1991 when working on the toxic effects of mebendazole on macropods. He then completed a Bachelor of Animal Science Degree on the parasites and diseases of native animals, in particular the Common Wombat (*Vombatus urisinus*) in 1992, during the course of his veterinary degree. After working in mixed practice as a clinician, he worked on lead poisoning and Australian bat lyssavirus in flying foxes. Since completing his Ph.D. in 2001 at the University of Melbourne on sarcoptic mange in wombats, Dr. Skerratt has worked as a research associate at the University of Wisconsin and the National Wildlife Health Center (USA) in 2002/2003 on diseases of sea ducks. He was a senior lecturer in epidemiology and parasitology at James Cook University (JCU) from 2003–2007 conducting research on the amphibian chytrid fungus in frogs, avian influenza viruses in waterbirds, surra in Australian wildlife, and the health of endangered Proserpine Rock Wallabies (*Petrogale persephone*). He is currently a research fellow at JCU working on wildlife diseases. Specifically, Lee has demonstrated that the spread of the amphibian chytrid fungus has caused the global decline and extinction of frogs (Skerratt et al. 2007), which was recently acknowledged by the World Organisation for Animal Health when they made it a notifiable disease. He has shown that sarcoptic mange is an important disease in Common Wombats that may cause the extinction of small isolated wombat populations. He has identified pathogens that may be contributing to the decline of 10 of the 15 species of sea ducks in North America. He was the co-chair of the 11th International Wildlife Disease Association Conference: Wildlife Health in a Shrinking World, Ecology, Management and Conservation. (Photographed by Lee Berger)