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Potential influence of plant chemicals on infectivity of *Batrachochytrium dendrobatidis*

Elizabeth W. Davidson*, Andrew Larsen, Crystal Meins Palmer

School of Life Sciences, Arizona State University, Tempe, Arizona 85287-4501, USA

ABSTRACT: We explored whether extracts of trees frequently found associated with amphibian habitats in Australia and Arizona, USA, may be inhibitory to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which has been associated with global amphibian declines. We used salamanders *Ambystoma tigrinum* as the model system. Salamanders acquired significantly lower loads of *Bd* when exposed on leaves and extracts from the river red gum *Eucalyptus camaldulensis*, and loads were also low in some animals exposed on extracts of 2 oak species, *Quercus emoryi* and *Q. turbinella*. Some previously infected salamanders had their pathogen loads reduced, and some were fully cured, by placing them in leaf extracts, although some animals also self cured when housed in water alone. A significant number of animals cured of *Bd* infections 6 mo earlier were found to be resistant to reinfection. These results suggest that plants associated with amphibian habitats should be taken into consideration when explaining the prevalence of *Bd* in these habitats and that some amphibians may acquire resistance to the fungus if previously cured.

KEY WORDS: *Batrachochytrium dendrobatidis* · *Ambystoma tigrinum* · *Eucalyptus* · *Quercus* · Oak · Inhibition

INTRODUCTION

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been associated with amphibian declines worldwide (Collins & Crump 2009, Lötters et al. 2009, Collins 2010, Kilpatrick et al. 2010). This fungus has been observed on amphibians in many habitats, but significant differences in prevalence have been reported among sites (e.g. McDonald & Alford 1999, Woodhams & Alford 2005, Peterson et al. 2007, Van Sluys & Hero 2009, Kriger & Hero 2008, Hossack et al. 2010, Murray et al. 2010, 2011, Skerratt et al. 2010). Many possible causes have been explored to explain this variation, including humidity, temperature, climate and seasonality (Bradley et al. 2002, Schlaepfer et al. 2007, Daskin et al. 2011, Murphy et al. 2011, Rohr et al. 2011, Rollins-Smith et al. 2011, Savage et al. 2011), skin peptides (Rollins-Smith et al. 2002, 2011, Rollins-Smith & Conlon 2005, Woodhams et al. 2006), chemicals (Lane & Burgin 2008, Threlfall et al. 2008, Boisvert & Davidson 2011), microbial flora (Harris et al. 2006, Brucker et al. 2008a,b, Becker & Harris 2010), host genetics (Savage & Zamudio 2011), species richness (Searle et al. 2011), environmental refuges (Puschendorf et al. 2011) and potential intermediate hosts (Daszak et al. 2004, Schloegel et al. 2010, Johnson et al. 2011, Kilburn et al. 2011, Gahl et al. 2012). However, one ecological factor that has not been widely explored is the potential influence of plants and plant chemicals in the habitats. Plant chemicals including phenols, tannins and alkaloids have been shown to influence the susceptibility of herbivorous insects to viral, fungal and bacterial pathogens (Cory & Hoover 2006). In this study we explore whether chemicals associated with tree species found around amphibian habitats could have an effect on the outcome of *Bd* infections.

*Email: e.davidson@asu.edu

In Australia, *Eucalyptus* spp. are common in the dryer and upland regions but are not common in the Queensland tropical rainforest, where the greatest loss of amphibians has occurred (Bowman 2000). In Arizona, USA, several oak (*Quercus*) species occur at intermediate and higher altitudes and near amphibian habitats (Elmore 1976). *Eucalyptus* and oak both regularly shed large quantities of dead leaves, seeds, and bark (Brooker 2002), which may fall into water and leach anti-fungal chemicals into it. *Eucalyptus* spp. produce oils, phenols, tannins, flavinoids and other chemicals with antimicrobial effects against bacteria and fungi (e.g. Deans 2002, Konoshima & Takasaki 2002, Ghalem & Mohamed 2008, Ben Marzoug et al. 2010, Gilles et al. 2010). The Australian tea tree *Melaleuca* spp. also produces antifungal compounds (Terzi et al. 2007). Oak leaves contain several antimicrobial chemicals such as phenolics (e.g. Güllüce et al. 2004, Karioti et al. 2011). We explored whether extracts from leaves of naturally occurring plant species may influence *Bd* growth and infection rates in tiger salamanders *Ambystoma tigrinum*.

We first tested whether extracts of dead leaves, bark and seeds from various tree species could inhibit *Bd* growth in an *in vitro* bioassay. We then exposed tiger salamanders held in leaves and extracts or in water alone to *Bd*. *Ambystoma tigrinum* is highly susceptible to *Bd* but does not usually die from infection and may live for over a year with infection (Davidson et al. 2003). We observed salamander skin sloughs microscopically and used quantitative PCR (qPCR) analysis to determine the presence and quantity of *Bd* on the salamanders held in leaf versus control treatments. Because extracts of plants such as purple loosestrife *Lythrum salicaria* can have a strong negative effect on the development of tadpoles (Maerz et al. 2005), we monitored all salamanders for signs of distress or mortality during our study, whether exposed to *Bd* or not.

Because exposed salamanders were found to be significantly less susceptible to *Bd* while housed on certain leaf preparations, we placed heavily infected animals into leaf extracts to determine whether an infection could be reduced or cured.

MATERIALS AND METHODS

In vitro experiments

For initial exploration of leaf extract antifungal activity, dead leaves, seeds and bark were collected from 17 species of trees and shrubs native to Australia at the Boyce Thompson Arboretum, Superior, Arizona (with permission): *Casuarina crista, C. cunninghamiana, Melaleuca lanceolata, M. stypheloides, Acacia aneura, A. jennerae, Eucalyptus gilli, E. camaldulensis, E. polyanthemos, E. diptera, E. salubris, E. loxophleba, E. sideroxylon, E. salmonophloia, E. microtheca,* and *E. socialis*. For *in vitro* bioassays, ca. 15 g of leaves, bark and seeds were incubated with 225 ml distilled water in glass jars for 2 wk at 30°C. pH was taken using a Corning pH meter 430. Samples from each species were centrifuged and filter sterilized using a Pall Acrodisc (0.2 µm membrane). Sterile extracts were assayed in sterile 96-well plates against ca. 10^5 *Bd* zoospores ml^{-1} (*Pseudacris triseriata* strain, counted using a Bright-Line hemacytometer; Reichert Scientific), which had been cultured in tryptone-gelatin hydrolysate-lactose medium (TGhL; Longcore et al. 1999), at a final dilution of 25% plant extract to 75% TGhL with zoospores. Control wells contained either 25% extract and 75% TGhL but no zoospores, or 25% sterile distilled water and 75% TGhL with zoospores. For a second assay we diluted the most active extracts in sterile distilled water to 5% and 2.5% final concentrations in the zoospore/TGhL mixture. *Bd* densities at Day 0 and Day 2 were recorded using a Titertek Multiscan Plate Reader at 492 nm, and wells were also observed using an inverted microscope (Sheafor et al. 2008). Because plant extracts alone led to darkening of TGhL, which was detected by the Titertek monitor, readings for extracts with TGhL without zoospores present were subtracted from readings for extracts with zoospores.

Samples of oak (*Quercus*) extracts used in *in vivo* experiments were taken before salamanders were added to shoe boxes, filter sterilized and assayed using 96-well plates as described above.

In vivo experiments, *Eucalyptus camaldulensis*

Eucalyptus camaldulensis leaves (15 g) and bark (10 g) were incubated in 1 l aged tap water for 3 wk at 30°C in each of 20 individual plastic shoeboxes. After 3 wk, 1 laboratory-reared *Ambystoma tigrinum* metamorph salamander was added to each shoebox. Before being added to shoeboxes, each salamander (ca. 30 g) was swabbed on its back and abdomen using sterile toothpicks to confirm absence of *Bd* using qPCR as described by Hyman & Collins (2012). Ten salamanders housed in leaf extracts and 10 housed in water alone were treated twice, 2 wk

apart, with 1 ml zoospores (*Pseudacris triseriata* strain) in TGhL medium (ca. 10^6 ml^{-1}). The 20 remaining salamanders (10 in leaf extracts and 10 in water alone) were treated with 1 ml TGhL medium twice, 2 wk apart, to act as *Bd* exposure controls. Salamanders were fed crickets twice weekly. Two weeks after the second exposure, all salamanders were moved to clean water and 1 d later swabbed on the back and abdomen with sterile toothpicks. Samples were extracted from the toothpicks and *Bd* loads determined using qPCR (Boyle et al. 2004, Hyatt et al. 2007). Salamander sloughs were also observed microscopically for the presence of *Bd* (Davidson et al. 2003).

In vivo experiments, *Quercus* spp.

Dead leaves, bark and acorns were collected from scrub (shrub) oak *Quercus turbinella* and Emory oak *Q. emoryi* at sites on the Mogollon Rim, near Payson, Arizona. Approximately 20 g of leaves, bark and acorns were added to 1 l of aged tap water in each shoe box and incubated at 30°C for 2 wk.

Salamander metamorphs were swabbed for qPCR as above and placed into boxes containing leaf extracts or water alone (8 per treatment). Four animals from each group were inoculated twice, 1 wk apart, with 1 ml *Bd* zoospores (ca. 10^6 ml^{-1}) while the remaining 4 were inoculated with 1 ml TGhL (controls). All animals were moved to clean water 20 d after initial treatment and *Bd* loads determined as above.

Curing experiments

To determine whether exposure to leaf extracts could cure *Bd* infections, 15 *Bd*-inoculated salamanders exhibiting heavy *Bd* loads (mean ± SD load 2900 ± 6116 zoospore genome equivalents, zsp GE) were swabbed for the presence of *Bd*. Five of these animals were placed in shoeboxes containing *Eucalyptus camaldulensis* leaves and extract, 5 in boxes containing *Quercus emoryi* leaves and extract, and 5 in water alone. After 4 wk, all were transferred to clean water. One day after their transfer to water, sloughs were collected and each animal was swabbed for qPCR analysis of *Bd* infection intensity. *Bd* loads were determined as above. Non-parametric Mann-Whitney *U*-tests and Kruskal-Wallis tests were used to compare zoospore loads between treatments (JMP version 5.1.2, SAS).

RESULTS

In vitro experiments

Most *Eucalyptus* spp. extracts inhibited the growth of *Bd* (3 to 66% increase in density) as compared to extracts from other species, some of which enhanced growth (96 to 499% increase) and the water control (125% increase) (Table 1). The pH of extracts ranged from 6.5 to 6.7; dissolved oxygen content was not determined.

In the presence of *Eucalyptus* extracts, zoospores almost immediately lost motility on Day 0, and no sporangia or live zoospores were observed by Day 2 (Table 1). The most active extracts were from river red gum *Eucalyptus camaldulensis,* which inhibited *Bd* growth at concentrations as low as 2.5% extract (Table 2). Although *Casuarina cunninghami* extract appeared inhibitory in the first assay, it did not inhibit growth in the second assay. Further research then focused on *E. camaldulensis*.

Quercus turbinella and *Q. emoryi* extracts also inhibited motility and growth of *Bd* as compared to the water control (Table 3).

Table 1. *Batrachochytrium dendrobatidis* (*Bd*). Effect of Australian plant extracts on growth of *Bd* measured *in vitro* on Day 2 after exposure. Density change was determined between Day 0 and Day 2 and adjusted for density change in the negative control, i.e. extract and tryptone-gelatin hydrolysate-lactose medium (TGhL) with no zoospores present. Final dilution of extracts was 25% extract, 75% TGhL and zoospores. NG: no growth, dead zoospores; SPO: sporangia and live zoospores present

Species	Microscope observation	Density change (%)
Eucalyptus gylli	NG	3
E. camaldulensis	NG	8
E. polyanthemos	NG	10
E. diptera	NG	7
E. salubris	NG	2
E. loxophleba	NG	9
E. sideroxylon	NG	8
E. salmonopholia	NG	16
E. microtheca	NG	16
E. socialis	NG	38
E. yilgarniensis	NG	66
Casuarina cunninghami	SPO	5
C. crista	SPO	96
Melaleuca lanceolata	SPO	315
M. stypheloides	SPO	287
Acacia aneura	SPO	499
A. jennerae	SPO	396
Water control	SPO	125

Table 2. *Batrachochytrium dendrobatidis* (*Bd*). Effect of dilution of plant extracts on *Bd* growth determined *in vitro* on Day 2 after exposure. 75% tryptone-gelatin hydrolysate-lactose medium (TGhL) and zoospores was used for both extract dilutions, but the % sterile distilled water was 20% for the 5% extract and 22.5% for the 2.5% extract

Species	Density change (%)	
	5% extract	2.5% extract
Eucalyptus camaldulensis	0	0
E. sideroxylon	25	5
E. microtheca	12	8
Casuarina cunninghami	59	64
C. crista	55	56
Water control	38	38

In vivo experiments, *Eucalyptus camaldulensis*

Mean (±SE) zoospore load for salamanders exposed to *Bd* in water alone (n = 10) was 2259 ± 830 zsp GE, while the mean zoospore load for those exposed while housed on *Eucalyptus camaldulensis* (n = 10) was 170 zsp GE (±64) (Mann-Whitney *U*-test, df = 1, p = 0.011) (Fig. 1a). One animal exposed while housed on *E. camaldulensis* was completely negative for *Bd* by qPCR. Sloughing of skin by animals exposed to *Bd* in water and by animals exposed on *E. camaldulensis* was dramatically different. Animals exposed in water alone produced many large dark sloughs with obvious *Bd* colonies, while animals exposed while housed on *E. camaldulensis* produced few, small, lighter sloughs with few or no microscopically visible *Bd* colonies. No *Bd* was detected on control animals, and no animals housed on *E. camaldulensis* extracts died or showed distress during the experiment.

Quercus spp.

Mean zoospore load for those exposed in presence of *Quercus emoryi* extracts (n = 4) was 55.6 ± 87.2 zsp GE and for those exposed in presence of *Q. turbinella* extracts (n = 4) was 62 ± 78 zsp GE, while mean zoospore load for salamanders exposed to *Bd* in water alone (n = 4) was 16 237 ± 25 432 zsp GE (Fig. 1b). However zoospore loads were not significantly different between treatments due to the small sample size (Kruskal-Wallis, df = 2, p = 0.12). No *Bd* was detected on control animals, and no animals housed on *Quercus* spp. extracts died or showed distress.

Table 3. *Batrachochytrium dendrobatidis* (*Bd*). Effect of *Quercus* spp. extracts on *Bd* growth determined *in vitro* on Day 2 after exposure. Density change determined between Day 0 and Day 2. Final dilution of extracts: 25% extract, 75% tryptone-gelatin hydrolysate-lactose medium (TGhL) and zoospores

Species	Density change (%)
Quercus turbinella	30
Q. emoryi	21
Water control	123

Curing experiments

Four of 5 salamanders previously infected with *Bd* housed in *Eucalyptus camaldulensis* extracts had their zoospore loads greatly reduced after 4 wk (pre-treatment mean load 2900 ± 6116 zsp GE; post-treatment mean load 288 ± 644 zsp GE). Four of 5 housed in *Quercus emoryi* extracts also had their zoospore loads reduced (pre-treatment mean load 1256 ± 2284 zsp GE; post-treatment mean load 1132 ± 2532 zsp GE). Three of 5 salamanders housed on *E. camaldulensis* extract and 4 of 5 housed on *Q. emoryi* extract had their zoospore loads reduced to 0. Three of 5 animals housed in water alone were also fully

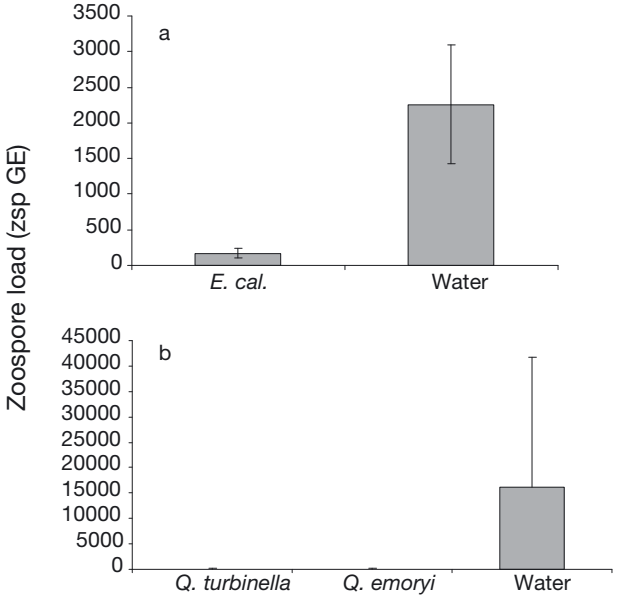

Fig. 1. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Ambystoma tigrinum.* Mean (±SE) zoospore loads of salamanders exposed to *Bd* when on (a) *Eucalyptus camaldulensis* (*E. cal.*) extracts (n = 10) or (b) *Quercus* spp. extracts (*Q. turbinella*, n = 4, and *Q. emoryi*, n = 4). Extracts from both genera were compared to those in water (n = 10 and n = 4, respectively). zsp GE: zoospore genome equivalents

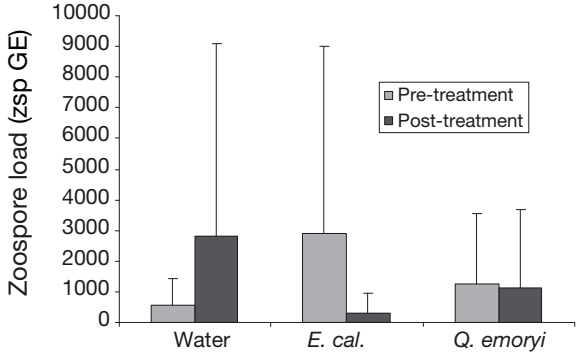

Fig. 2. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Ambystoma tigrinum.* Mean (±SE) zoospore loads of previously infected salamanders before and after being moved to water (n = 5), *Eucalyptus camaldulensis* (*E. cal.*) (n = 5) or *Quercus emoryi* (n = 5) leaves and extracts for 30 d. zsp GE: zoospore genomic equivalent

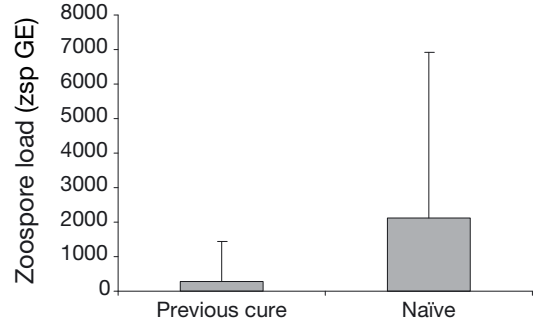

Fig. 3. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Ambystoma tigrinum.* Mean (±SE) zoospore loads in salamanders previously infected with *Bd* and cured by leaf extracts 6 mo earlier (n = 22) and in naïve animals (n = 8). zsp GE: zoospore genome equivalents

cured (pre-treatment mean 576 ± 863 zsp GE; post-treatment mean 2808 ± 6276 zsp GE) (Fig. 2). In each group, a single animal retained or increased its zoospore load, leading to the variation in the results; the number of animals losing infections was not significantly different between treatments.

When attempting to infect salamanders for the curing experiment, 22 *Ambystoma tigrinum* metamorphs that had been previously infected with *Bd* more than 6 mo earlier and cured by leaf extracts were treated twice with high concentrations of zoospores (ca. 10^6 ml^{-1}). However the majority did not achieve high zoospore loads (mean 300 ±1135 zsp GE) while 8 naïve metamorphs of the same age treated at the same time acquired significantly higher levels (mean 2132 ± 4780 zsp GE; Kruskal-Wallis, df = 1, p = 0.0021) (Fig. 3).

DISCUSSION

Our results demonstrate that certain plant extracts in amphibian habitats may have an effect on the infectivity of *Bd.* These results are based on extracts of the leaves of only a few species of trees, but they suggest that certain plants may influence *Bd* growth and infectivity. More work needs to be done to assess what the natural levels of leaf extracts are and how these concentrations can influence *Bd* dynamics. In contrast to our results, leaf litter and vegetation were found to be related to higher *Bd* infection in Pennsylvania newts *Notophthalmus viridescens* which was also related to cooler water temperature (Raffel et al. 2010). This demonstrates that in natural conditions the effect of plants may be more indirect by providing shade or thermal refugia for *Bd* growth. Becker & Zamudio (2011) found that deforestation and habitat loss were negatively associated with the presence and intensity of *Bd* in Costa Rica and Australia and that disease was more common in undisturbed habitats. The tree species present in the studied habitats were not described in these studies, and as our *in vitro* studies demonstrated, activity of tree leaf extracts is highly species dependent, ranging from inhibition by some species to potential enhancement of growth by others. In addition to their presence in Australia, *Eucalyptus camaldulensis* and other species of *Eucalyptus* have been planted in Arizona and California, and species of *Quercus* are found throughout the USA, so chemicals from these trees may be found in many amphibian habitats. We do not yet know which of the many chemicals produced by *Eucalyptus* or *Quercus* (Deans 2002, Güllüce et al. 2004, Gilles et al. 2010, Karioti et al. 2011) are responsible for inhibition of *Bd* by the leaf extracts.

The ability of *Ambystoma tigrinum* previously cured of *Bd* infections to resist reinfection was unexpected. These animals had been free of infection for 6 mo or longer while housed in frequently changed water, and they regularly shed skin sloughs. Thus, it is unlikely that traces of plant extracts still remained in their skin. Not all animals appeared to acquire this resistance, but a large proportion of those we treated produced only low or no infection even after treatment with high levels of active zoospores. Resistance to *Bd* in previously cured *Leiopelma archeyi* frogs was reported by Shaw et al. (2010) and in toads *Anaxyrus* (*Bufo*) *boreas* by Murphy et al. (2011), suggesting that acquired resistance against this pathogen is possible, although an attempt to immunize *Rana muscosa* by injection of killed *Bd* culture was ineffective (Stice & Briggs 2010).

The ability of these extracts, in particular *Eucalyptus camaldulensis*, to reduce or eliminate infection in some *Bd*-infected animals is encouraging. Recently Woodhams et al. (2012) have shown that many current techniques for treatment of amphibians were unsuccessful in eliminating *Bd* from infected frogs and tadpoles, and some were toxic. Since no salamanders died while housed in *E. camaldulensis* or *Quercus* extracts, our results suggest that extracts of these trees should be explored further against more sensitive species, in particular tadpoles, to determine if they may be a useful method for curing *Bd*-infected amphibians.

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Editorial responsibility: Cynthia Carey, Boulder, Colorado, USA

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