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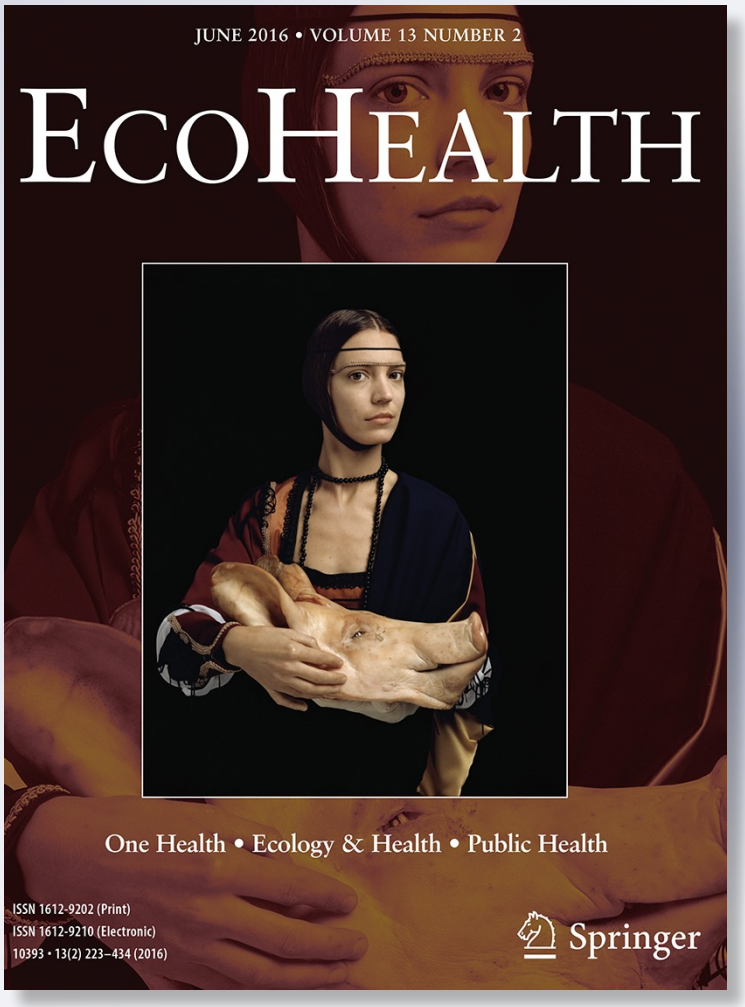

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Original Contribution

Leaf Litter Inhibits Growth of an Amphibian Fungal Pathogen

Aaron B. Stoler,[1,2] Keith A. Berven,[1] and Thomas R. Raffel[1]

[1]*Department of Biological Sciences, Oakland University, Rochester, MI*
[2]*Present Address: 1115 Center for Biotechnology and Integrative Sciences, Department of Biological Sciences, Rensselaer Polytechnic Institute, 110 8th St, Troy, NY 12180*

Abstract: Past studies have found a heterogeneous distribution of the amphibian chytrid fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). Recent studies have accounted for some of this heterogeneity through a positive association between canopy cover and Bd abundance, which is attributed to the cooling effect of canopy cover. We questioned whether leaf litter inputs that are also associated with canopy cover might also alter Bd growth. Leaf litter inputs exhibit tremendous interspecific chemical variation, and we hypothesized that Bd growth varies with leachate chemistry. We also hypothesized that Bd uses leaf litter as a growth substrate. To test these hypotheses, we conducted laboratory trials in which we exposed cultures of Bd to leachate of 12 temperate leaf litter species at varying dilutions. Using a subset of those 12 litter species, we also exposed Bd to pre-leached litter substrate. We found that exposure to litter leachate and substrate reduced Bd spore and sporangia densities, although there was substantial variation among treatments. In particular, Bd densities were inversely correlated with concentrations of phenolic acids. We conducted a field survey of phenolic concentrations in natural wetlands which verified that the leachate concentrations in our lab study are ecologically relevant. Our study reinforces prior indications that positive associations between canopy cover and Bd abundance are likely mediated by water temperature effects, but this phenomenon might be counteracted by changes in aquatic chemistry from leaf litter inputs.

Keywords: *Batrachochytrium dendrobatidis*, Resource subsidies, Amphibian pathogen, Temperate forests, Aquatic-terrestrial linkage

Introduction

The emergence of an infectious disease poses a challenge to the conservation of native populations, because it can be difficult to determine which factors cause the disease to spread or increase in virulence (Carey et al. 1999). The emergence of a pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) is an important example of this problem. Bd has been linked to numerous die-offs of amphibian populations, subsequently reducing the abundance of functionally important species (Lips et al. 2006; Whiles et al. 2006). The global distribution of Bd appears to be driven largely by climatic factors and by its recent spread through the international trade in amphibians and other aquatic organisms (Fisher and Garner 2007; Liu et al. 2013). However, there is substantial variation in Bd abundance at local scales (Pounds et al. 2006; Bosch et al. 2007; Laurance 2008; Bradley et al. 2015). Despite the

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Correspondence to: Aaron B. Stoler, e-mail: abstoler@gmail.com

continued presence of Bd as a threat to the global diversity of amphibians, reasons for this variation remain uncertain.

There is increasing recognition that the flux of material across open ecosystem boundaries can have substantial effects on community structure (Polis et al. 1997). Terrestrial leaf litter often constitutes a massive subsidy to wetlands inhabited by amphibians and Bd (Rubbo et al. 2008). This subsidy is a valuable source of energy and nutrients and serves as an important substrate for the growth of periphyton (i.e., algae, bacteria, and fungi) eaten by amphibian larvae (Skelly and Golon 2003). By increasing the resource base, litter input into wetlands can lead to increased growth and survival of amphibian larvae, especially when the litter species has high nutritional quality (Stephens et al. 2013; Stoler and Relyea 2016). Litter has also been proposed as a substrate for saprophytic growth of Bd. Indeed, ponds with higher levels of tree cover have been associated with increased Bd infection levels (Raffel et al. 2010; Becker et al. 2012), although this association has been attributed to the cooling effect of canopy. The direct implications of litter subsidies on Bd growth remain largely unknown.

In addition to providing a source of nutrients and substrate, leaf litter also alters aquatic chemistry in ways that might be detrimental to aquatic organisms. For example, leachate from red maple (*Acer rubrum*) litter can reduce tadpole resources by darkening the water column, decreasing light, and reducing algal growth (Stoler and Relyea 2016). Leachate can also contain phenolic acids (e.g., tannins) that inhibit growth of micro- and macro-consumers by inhibiting enzymatic function (Nicolai 1988; Stout 1989; Earl and Semlitsch 2015). Davidson et al. (2012) found that terrestrial tiger salamanders (*Ambystoma tigrinum*) experienced reduced pathogen loads when bathed in extracts from phenolic-rich litter species common to the southwest United States. This result suggests that litter leachate might inhibit Bd growth or sporulation, but it provides little mechanistic insight into how general litter chemistry alters Bd growth in wetlands.

To improve our understanding of how leaf litter subsidies influence Bd, we explored how the growth and sporulation of Bd fungus are altered by leachate and substrate of litter from 12 plant species (Table 1) that vary widely in chemistry. First, we hypothesized that litter leachate would generally reduce Bd growth and sporulation, but that this effect would vary with the chemistry of the litter. Second, we hypothesized that Bd would use leaf tissue as a growth substrate, leading to increased growth in the presence of litter fragments. Based on these competing (but not mutually exclusive) hypotheses, we predicted reduced Bd growth and sporulation rates when exposed to leachate from litter with higher phenolic levels and lower phosphorus content, but higher growth in the presence of pre-leached leaf tissue. We tested these predictions by conducting two laboratory studies in which we grew Bd in cultures with multiple concentrations of leachate from each plant species or in the presence of leached litter fragments. To verify the ecological relevance of the leachate concentrations in our study, we further conducted a field survey of pond water phenolic chemistry for 14 ponds in southeastern Michigan.

Methods

Leaf Litter Species

We used leachate of 12 leaf litter species, including 10 deciduous tree species and two emergent macrophyte species (Table 1). All species are found throughout the northeastern United States. Among the deciduous tree species, several are of conservation concern such as black oak and white oak, which are declining due to overbrowsing by white-tailed deer (Abrams 2003). Similarly, elm is disappearing as a result of Dutch elm disease, and green ash is rapidly being decimated by the spread of the invasive emerald ash borer (Moser et al. 2009). Red maple, sugar maple, and black cherry are frequently observed to gain dominance where other species are undergoing reductions (Abrams 2003). Other tree species including cottonwood, black willow, and sassafras are characteristic of moderately to highly disturbed areas. Regarding the emergent macrophyte species, both phragmites and reed canary grass are common invasive wetland macrophytes that frequently crowd out native wetland plants and reduce open water habitat (Lavergne and Molofsky 2004).

For all species except phragmites and reed canary grass, we obtained data on litter nutrient and phenolic content from previously published analyses by Ostrofsky (1993, 1997). We used percent P as a measure of litter nutrient content. We chose to use percent P instead of N because tryptone is a protein-rich medium that should contain sufficient N for Bd growth, but lacks a source of phosphorus. For phenolics, we report values for total phenolics as determined by the spectrophotometric Folin-Denis assay (Ostrofsky 1993). As in Ostrofsky (1993), phenolic values are reported in units of optical density. To obtain nutrient and phenolic values for phragmites and reed canary grass, we used values from Stephens et al. (2013). Percent P was similarly determined with an elemental analyzer. Phenolic

Table 1. List of Leaf Litter Species Used in the Experiment, Including Common Names, Codes, Taxonomic Families, and Species Names.

Leaf species	Code	Species	C:N	Phenolics (optical density)
Sugar maple	SM	*Acer saccharum*	60.9	1.337
Red maple	RM	*Acer rubrum*	52.5	1.681
Black willow	BW	*Salix nigra*	21.0	0.631
Green ash	ASH	*Fraxinus americana*	46.6	0.256
Black oak	BO	*Quercus velutina*	93.6	0.598
White oak	WO	*Quercus alba*	67.3	0.946
American elm	ELM	*Ulmus americana*	74.3	0.893
Black cherry	CHER	*Prunus serotine*	29.8	0.744
American cottonwood	CTWD	*Populus deltoides*	19.7	0.613
Sassafras	SASS	*Sassafras albidum*	47.9	0.298
Phragmites	PHRAG	*Phragmites australis*	27.2	0.323
Reed canary grass	CAN	*Phalaris arundinacea*	20.7	0.443

Values for C:N and phenolics for all species except phragmites and reed canary grass are from Ostrofsky (1993, 1997). Chemical values for phragmites and reed canary grass are from Stephens et al. (2013). See text for details on chemical determination.

content was determined via the Folin-Ciocalteu assay, on samples of water in an outdoor mesocosm after allowing leaves to soak for several months. Although this method differs from that of Ostrofsky (1993), we found a close correlation between values of phenolic content from litter species that were analyzed by both studies (Pearson $r = 0.882$, $P = 0.048$). We used this correlation to adjust the values from Stephens et al. (2013) for consistency in chemical reporting. All chemical values are reported in Table 1.

Effect of Litter Leachate

To test the effects of litter leachate on Bd sporulation and growth, we conducted a full-factorial design in which we crossed 12 species of litter leachates with three level of leachate dilution (i.e., 36 total treatments). To contrast Bd sporulation in the presence and absence of leaf litter leachate, we also included a no-leachate control. We replicated each treatment combination and the control four times for a total of 148 replicates. Individual replicates consisted of 20 mL test tubes filled with diluted or non-diluted litter leachate. Control treatments contained reverse-osmosis (R/O) water. Due to the time needed to count spores in each replicate, we conducted the experiment using multiple temporal blocks spread all replicates out of over 10 days. However, this does not constitute a true blocking effect, as it was impossible to include all treatment combinations in each daily block. Instead, treatments were randomly assigned to temporal "blocks," resulting in an even distribution of treatments.

To generate leachate for the experiment, we collected freshly senesced litter from multiple forests across southern Michigan during the autumn prior to the experiment. We collected ash leaf litter from a forest in eastern Wisconsin where the emerald ash borer had yet to invade. To avoid intraspecific chemical variation within litter species (Martin and Blossey 2013), we collected individual species of leaf litter from a single forest plot. After allowing litter to air-dry in a closed garage, we extracted leachate by soaking 2.8 g of litter from each species in 1.4 L of R/O water in plastic containers within the laboratory. We added R/O water as needed to maintain a constant volume of water. The mass of litter (i.e., 2 g litter L^{-1}) that we added to containers is well within the natural range of litter concentrations observed in a survey of wetlands in southeastern Michigan (Stoler, *unpublished data*), although it is approximately double the concentration typically employed in mesocosm studies (e.g., Stoler and Relyea 2011). After allowing litter to soak for 18 days, we filtered the leachate through 1.2 µm glass filters (Whatman Inc). We then aliquoted the leachate of each litter species into 12 acid-rinsed scintillation vials, and immediately placed them into a −20°C freezer. This procedure allowed us to thaw leachate as needed over the 10 time blocks of the study.

Each time block included 15 randomly chosen treatment replicates, except for the last time block in which there were only 13 replicates remaining to test. We first

Table 2. Results for the 2-Way and 1-Way Mixed-Model ANOVAs on Final Spore and Sporangia Densities.

		F	*df*	*P*
(A) Effects of litter species and leachate concentration on spore densities				
2-Way ANOVA results				
Litter species		34.469	11,90	<0.001
Leachate concentration		23.324	2,90	<0.001
Inoculum spore density		2.321	1,8	0.167
Litter species × leachate concentration		4.221	22,91	<0.001
		Litter species		
1-Way ANOVA results				
2.0 g litter mL^{-1}	Litter species	20.835	11,25	<0.001
	Inoculum density	0.610	1,9	0.454
1.0 g litter mL^{-1}	Litter species	13.502	11,28	<0.001
	Inoculum density	0.788	1,8	0.402
0.5 g litter mL^{-1}	Litter species	9.286	11,24	<0.001
	Inoculum density	3.99	1,8	0.083
(B) Effects of litter species and leachate concentration on sporangia densities				
2-Way ANOVA results				
Litter species		10.076	12,94	<0.001
Leachate concentration		20.191	2,93	<0.001
Litter species × leachate concentration		2.547	22,94	<0.001
1-Way ANOVA results				
2.0 g litter mL^{-1}		5.116	11,26	<0.001
1.0 g litter mL^{-1}		9.273	11,28	<0.001
0.5 g litter mL^{-1}		3.209	11,27	0.007

The 2-way model included litter species, leachate concentration, and their interaction as fixed variables. The 1-way models focused on effects of litter species within levels of leachate concentration. All models included inoculum spore density as a covariate and temporal block as a random variable. Degrees of freedom (*df*) are reported as treatment *df* followed by error *df*.

allowed leachate to slowly thaw at room temperature. For non-diluted treatments (i.e., 2.0 g litter L^{-1}), we placed 14.25 mL of leachate into acid-rinsed, screw-topped borosilicate test tubes. For 1:2 diluted treatments (i.e., 1.0 g litter L^{-1}), we added 7.125 mL of leachate and an equal amount of R/O water. For 1:4 diluted treatments (i.e., 0.5 g litter L^{-1}), we added 3.56 mL of leachate and 10.69 mL of R/O water. For controls (i.e., 0.0 g litter L^{-1}), we added 14.25 mL of R/O water. To all treatments, we added 0.75 mL of a 20% tryptone solution in order to achieve a 1% solution. Combining leachate (or R/O water) with tryptone allowed us to provide Bd with a source of nitrogen for growth.

After autoclaving all replicates within a single block, we inoculated each test tube with 100 μL of Bd from a stock culture. We used Bd strain SRS 812, which was cultured from a bullfrog tadpole in 2006 at the Savannah River Site, SC, USA. Autoclaving does not alter the elemental composition of the leachate nor is there evidence to suggest that boiling phenolic acids alters their biological activity (Li and Shah 2013). In addition, preliminary analysis of pre- and post-autoclaved red maple leachate using a radial diffusion assay with bovine serum albumin (Graça et al. 2005) revealed no difference in protein-binding capacity. We cultured our stock in a 1% tryptone media under dark conditions within an incubator kept at 23°C. We set aside an aliquot of the stock solution at each time block to determine starting spore densities, which averaged 3.0 spores 10^3 mL^{-1} (±0.5 spores 10^3 mL^{-1}). Once inoculated, we immediately capped and vortexed the test tubes, and placed the tubes into the incubator. We repeated this procedure for each time block.

After allowing Bd to grow in replicates for 2 weeks, we enumerated spore and sporangia densities. We vortexed each replicate and placed 0.5 mL into a microcentrifuge tube with 0.1 mL of Trypan blue staining solution. We

Table 3. Regression Statistics for Correlations of Litter Chemistry with Spore (A) and Sporangia Densities (B) Grown in Cultures of Litter Leachate, and for Spore Densities (C) Grown in Cultures with Leached Litter Substrate.

	Chemical			Inoculum density			Marginal r^2	Conditional r^2
	F	*df*	*P*	*F*	*df*	*P*		
(A) Spore densities with litter leachate								
Phenolics	19.001	1,10	0.001	4.508	1,8	0.067	0.377	0.670
Phosphorus	4.467	1,10	0.061	4.418	1,8	0.070	0.214	0.691
(B) Sporangia densities with litter leachate								
Phenolics	17.043	1,10	0.002	1.578	1,8	0.244	0.147	0.486
Phosphorus	2.281	1,10	0.163	1.334	1,8	0.282	0.078	0.507
(C) Spore densities with litter substrate								
Phenolics	0.480	1,4	0.528				0.015	0.194
Phosphorus	1.679	1,4	0.267				0.040	0.218

All models include temporal block and leaf litter species as random effects. Models of spore and sporangia density in litter leachate include inoculum spore density as a covariate. Conditional and marginal r^2 values represent models with and without random effects, respectively.

counted spores with a hemocytometer, conducted duplicate measures for each replication, and averaged the two counts. Throughout the 10 time blocks, we noted contamination in seven replicates. We removed these replicates from the analysis, which resulted in the removal of no more than a single replicate from any treatment combination.

Effects of Litter Substrate

To test the effects of litter substrate on Bd growth and sporulation, we conducted another experiment in which we exposed Bd inoculum to cultures with leached litter. We used a subset of the litter species from the first experiment, including red maple, sugar maple, white oak, cottonwood, green ash, and phragmites. We also included a no-litter control which did not include any substrate. We replicated each treatment eight times, for a total of 56 total replicates. Individual replicates consisted of 20 mL test tubes filled with R/O water and tryptone to attain a 1% solution. The final solution volume in each test tube was 15 mL.

For each replicate (except no-litter controls), we generated two 3.75 mm × 0.7 mm strips of pre-leached leaf litter of standardized length and width (i.e., surface area = 2.63 mm^2). To cut the strips, we soaked leaves of each species in R/O water for 1 h. We removed the leaves from the water and used a stencil to cut strips from the leaves, and avoided all major veins. We then autoclaved the strips three times (1 h cycles at 121°C) to completely leach all soluble compounds from the litter. After the third autoclaving cycle, there was no visible leachate. After placing the strips into the test tubes with tryptone solution, we autoclaved and inoculated all replicates (including the no-litter controls) on the same day with the same procedure as in the first experiment. Inoculum spore densities were 3.0 spores 10^3 mL^{-1}.

After allowing Bd to incubate with litter strips for 2 weeks, we enumerated spore densities. Because it was possible that sporangia lived in or on the leaf litter, counts of sporangia in these cultures might not represent accurate densities. Hence, we did not enumerate sporangia densities in this experiment. We counted spores over 4 days. Our process for counting spores was identical to the first experiment.

Field Survey of Phenolic Concentrations

To determine the ecological relevance of the leachate concentrations that we used in our study, we conducted a field survey of pond water phenolic chemistry. We determined the phenolic levels in 14 semi-permanent, woodland ponds located in the north unit of Bald Mountain State Recreation Area (BMSRA) near Lake Orion, MI, USA (exact pond coordinates are found in Table 4). BMSRA is an 1877 hectare state park composed of a largely mature temperate deciduous forest with a diverse assemblage of tree species. We collected water samples (~50 mL) from three areas of each pond on 13 May and again on 10 June in 2015. We determined phenolic concentration of pond water samples using the Folin-Ciocalteu method (Clesceri and Eaton 1998) and measured in mg tannic acid equivalent L^{-1}. We

Table 4. Values of Phenolic Concentrations in the 14 Ponds Surveyed within the BMSRA on 13 May 2015 and 10 June 2015.

Pond ID	Coordinates	May	June
1	42.78007 N; 83.20235 W	36.9 ± 2.9	50.3 ± 0.4
2	42.77984 N; 83.20343 W	35.4 ± 2.4	58.6 ± 0.8
3	42.77673 N; 83.20498 W	22.5 ± 0.2	25.3 ± 0.2
4	42.77696 N; 83.20706 W	6.7 ± 0.1	8.4 ± 0.1
5	42.77805 N; 83.20924 W	11.3 ± 0.9	19.1 ± 0.9
6	42.77891 N: 83.20948 W	8.1 ± 0.3	13.2 ± 0.5
7	42.77914 N; 83.21046 W	6.5 ± 0.8	26.4 ± 1.2
8	42.77908 N; 83.21573 W	37.8 ± 0.5	57.8 ± 1.9
9	42.77996 N; 83.21564 W	16.9 ± 0.1	19.2 ± 0.4
10	42.78175 N; 83.20984 W	13.7 ± 0.4	23.1 ± 2.7
11	42.78457 N; 83.20842 W	9.3 ± 0.3	17.1 ± 3.7
12	42.78453 N; 83.20551 W	6.2 ± 0.8	9.2 ± 2.2
13	42.78519 N; 83.20441 W	35.1 ± 0.9	32.8 ± 0.3
14	42.78484 N; 83.20074 W	12.0 ± 0.4	13.7 ± 0.5

Coordinates are in degree decimals; phenolic concentrations are reported as average mg tannic acid equivalent L^{-1} for three samples taken from each pond on each sample date.

then compared these values to phenolic concentrations measured in Stephens et al. (2013), which soaked leaf litter (1.5 g litter L^{-1}) in mesocosms, including many of the species used in our current study.

Statistics

To test our hypothesis that the addition of litter leachate reduces Bd spore and sporangia densities, we conducted mixed-model regression. We conducted separate analyses for spore and sporangia densities. Our models included leachate concentration (four levels, including the control) as a fixed effect, and both leaf litter species and time block as random effects variables. Because the densities of Bd inoculum differed across time blocks, we also included inoculum spore densities as a covariate. Preliminary analyses detected no interactions between treatments and inoculum spore density so we excluded all covariate interactions from the model.

To test our hypothesis that litter leachate has species-specific effects on Bd spore and sporangia densities, we conducted two-way mixed-model ANOVAs. Our models included fixed effects of litter species treatment, leachate concentration, their interaction, and a random effect of temporal block. Again, we included inoculum spore densities as a covariate. After finding a significant interaction of litter species and leachate concentration in both analyses, we conducted separate ANOVAs for each of the three leachate concentrations. Because our control treatment was not part of the full-factorial design, we excluded control responses from these analyses. However, we contrasted the control treatment with individual litter species at each level of leachate concentration by using Dunnett's tests with alpha correction (Benjamini and Hochberg 1995).

To test our hypothesis regarding the effect of litter substrate on Bd, we conducted a one-way mixed-model ANOVA with a model that included leaf litter species as a fixed effect and temporal block as a random effect. We only tested for effects on spore densities since we did not enumerate sporangia in the litter substrate experiment. We did not include inoculum spore densities as a covariate, because all cultures were started at the same inoculum density. As in the previous analysis, we conducted post hoc contrasts of individual litter species treatments with the control treatment using Dunnett's tests.

We explored the effects of litter nutrient and phenolic content on Bd when incubated with either litter leachate or substrate through mixed-model regression analysis. Models included fixed effects of either phenolics or P. For our analyses on the effects of litter leachate, we included inoculum spore density as a covariate. We included temporal block and leaf litter species as random effects in all analyses. Including litter species as a random effects variable ensured that the effects of nutrient and phenolic concentrations were assessed using species as the unit of replication ($N = 12$).

We conducted all analyses in program R (R Development Core Team 2014), using functions *lme* and *Anova* within *nlme* and *car* packages (Fox and Weisberg 2010; Pinheiro et al. 2013), respectively. We conducted all analyses using type II sums of squares. Prior to all analyses, we log-transformed spore numbers. We verified both normality and homoscedasticity of residuals following each analysis. In addition to the seven replicates removed due to contamination, we removed three replicates that were determined to have high leverage according to Cook's test. Further examination suggested that these points were outliers; removing these replicates greatly improved normality of the dataset and caused no qualitative changes in the results. Furthermore, all leaf litter species-leachate concentration combinations still possessed at least three replicates following outlier removal. For regression analyses, we determined both marginal (i.e., not including random effects) and conditional (i.e., including random effects) r^2 values as per the recommendation of Johnson (2014), using code available online (http://jonlefcheck.net/2013/03/13/r2-for-linear-mixed-effects-models/).

Results

Effects of Litter Leachate

We found a strong effect of leachate concentration on spore densities ($F_{3,47} = 9.644$, marginal $r^2 = 0.126$, conditional $r^2 = 0.752$, $P < 0.001$; Fig. 1a) and sporangia densities ($F_{3,55} = 10.196$, marginal $r^2 = 0.154$, conditional $r^2 = 0.558$, $P < 0.001$; Fig. 1b). Overall, both spore and sporangia densities were inversely correlated with leachate concentration.

Individual species of leaf litter had varied effects on spore and sporangia densities, and their effects differed by the level of leachate concentration (Table 2). One-way ANOVAs revealed strong effects of leaf litter species on spore and sporangia densities at all three leachate concentration levels (Table 2; Fig. 2). Mean comparisons of leachate treatments with the no-litter control revealed strong effects of individual litter species, and particularly red maple, sugar maple, white oak, green ash, and black willow. We found the strongest effects at the highest leachate concentration (i.e., 2.0 g litter L^{-1}); sugar maple, red maple, black willow, and green ash leachate reduced final spore densities by 77 to 97% relative to the no-leachate control ($P \leq 0.001$). Elm and white oak litter also reduced

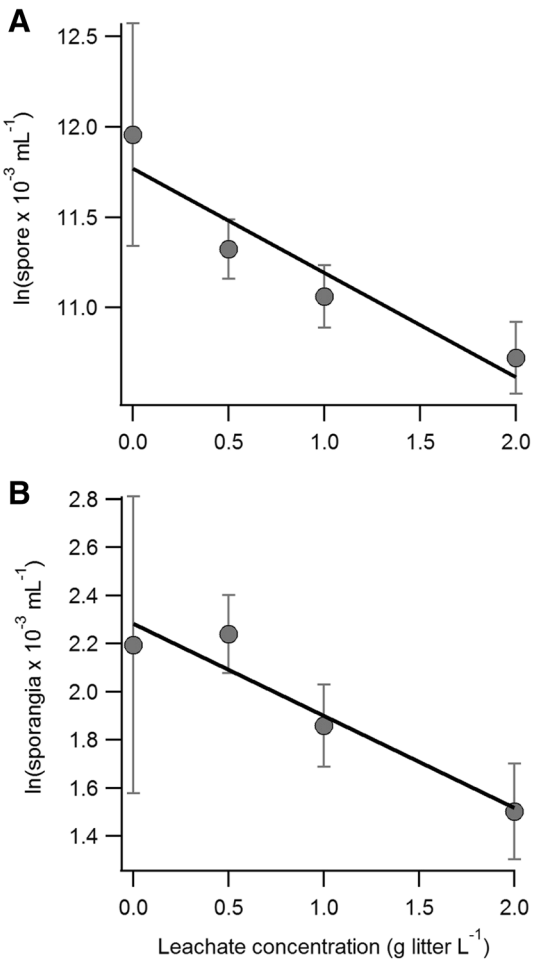

Fig. 1. Final zoospore (**a**) and sporangia (**b**) densities at each level of leachate concentration. *Points* represent pooled means for all litter species treatments. Values represent means of residuals added to least-squared estimated marginal means of ln-transformed values. *Bars* represent ±1 SE.

spore densities by 48 to 51% relative to the control, although these comparisons were not quite significant ($P \leq 0.060$). Sugar maple, red maple, and black willow leachate reduced sporangia densities by 99% relative to the control ($P \leq 0.008$). Among treatments with 1.0 g litter L^{-1}, red maple, sugar maple, white oak, and black willow leachate reduced spore densities by 61 to 93% relative to the controls ($P \leq 0.003$). Similarly, red maple, sugar maple, and white oak leachate reduced sporangia densities by 52 to 99% relative to the control. However, green ash leachate actually promoted sporangia growth, leading to 147% higher densities than in the control. Among treatments with 0.5 g litter mL^{-1}, we found no significant differences between leachate and control sporangia densities at this level of leachate concentration ($P \geq 0.130$).

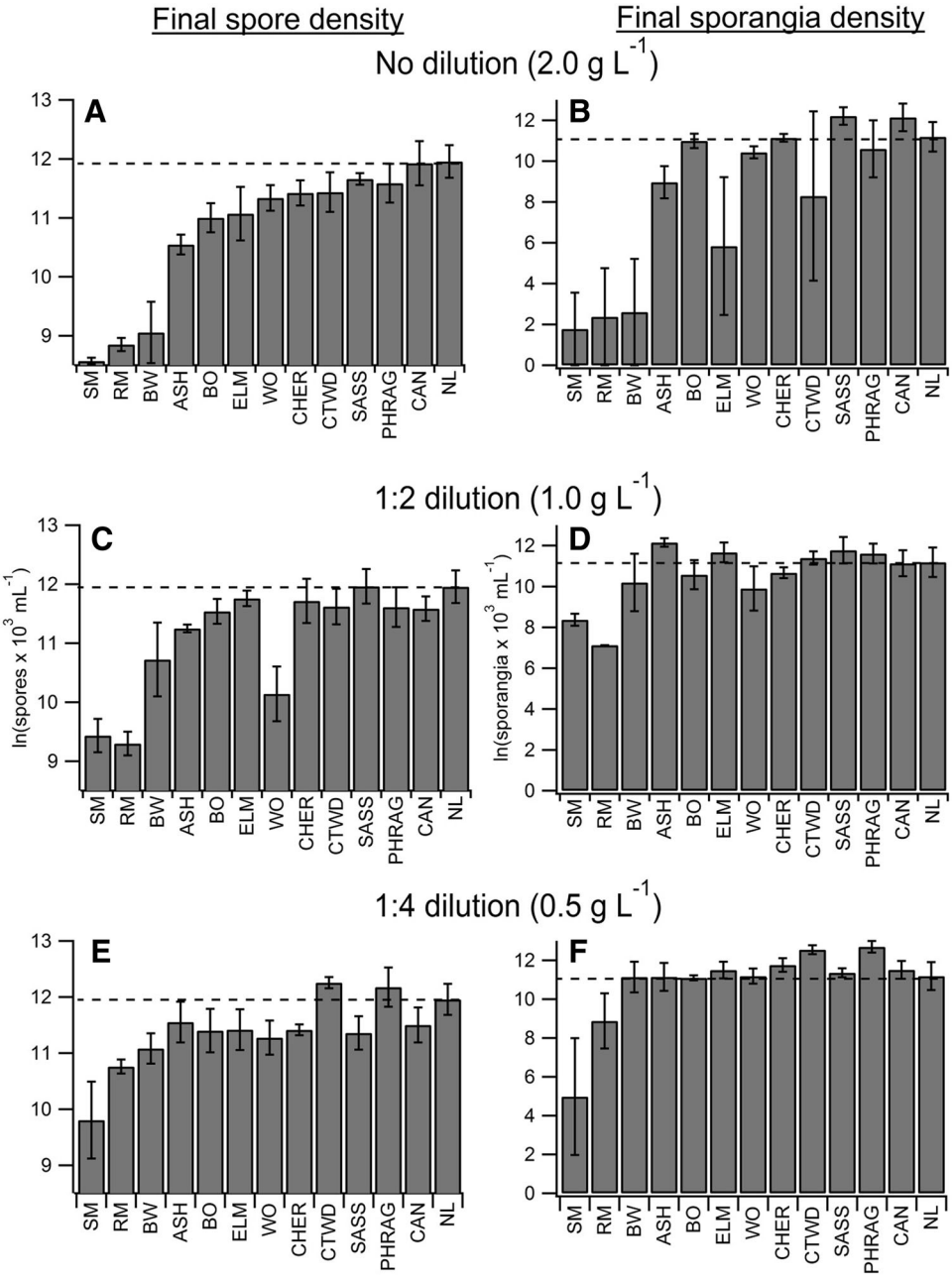

Fig. 2. Final spore and sporangia densities in all litter species treatments among 2.0 g litter mL^{-1} treatments (**a**, **b**), 1.0 g litter mL^{-1} treatments (**c**, **d**), and 0.5 g litter mL^{-1} treatments (**e**, **f**). The no-leachate response is shown as a *bar* and extended *dashed line* in each panel to aid in the interpretation of post hoc comparisons. Values represent means of residuals added to least-squared estimated marginal means of ln-transformed values. Treatment abbreviations are found in Table 1. *Bars* represent ±1 SE.

Effects of litter substrate

We found a strong negative effect of litter substrate on spore densities ($F_{6,48} = 5.345$, $P < 0.001$; Fig. 3). The presence of leached litter substrate, regardless of species, significantly reduced spore densities by 44 to 73% relative to the no-litter control ($P \leq 0.009$).

Effects of litter chemistry

We found a negative effect of litter phenolic content on final spore densities in leachate cultures, and a nearly significant negative effect of litter P content (Fig. 3a, b; Table 3A). We found negative effects of phenolic content sporangia densities in leachate cultures, but no effect of litter P content (Fig. 3c, d; Table 3B). There were no effects of either chemical attribute on spore densities in leached litter substrate cultures (Fig. 4e, f; Table 3C).

Field survey of phenolic concentrations

Among the 14 surveyed ponds, we found phenolic chemistry ranging from 6.2 to 37.8 mg L^{-1} (mean = 18.5 mg L^{-1}) during the mid-May sample and from 8.4 to 58.6 mg L^{-1}

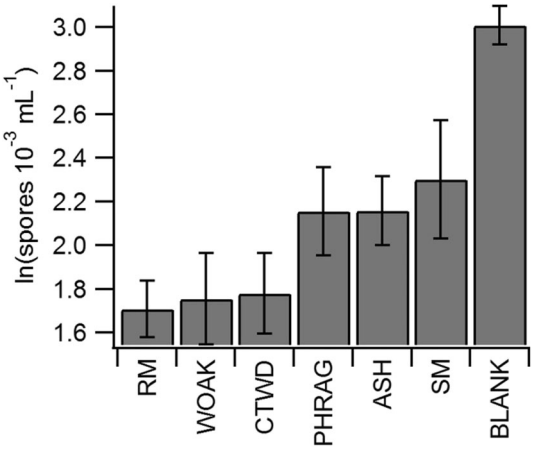

Fig. 3. Final spore densities in all litter species treatments for cultures growing with litter substrate. Values represent means of residuals added to least-squared estimated marginal means of ln-transformed means. Treatment abbreviations are found in Table 1; NL = no leachate. *Bars* represent ±1 SE.

(mean = 26.7 mg L^{-1}) during the mid-June sample (Table 4). These values are well within the range of aqueous phenolic concentrations measured by Stephens et al. (2013), which ranged from 3 mg L^{-1} in mesocosms with phragmites to 68 mg L^{-1} in mesocosms with red maple at a concentration of 1.5 g litter L^{-1}.

Discussion

Our study sought to determine whether the presence and identity of leaf litter leachate and substrate might influence the growth and sporulation of Bd fungus. Regardless of litter species, we found that sporangia and spore densities decreased with increasing concentrations of litter leachate (Fig. 1). However, we found that the magnitude of these negative effects varied dramatically among litter species, from minor effects on Bd growth by canary reed grass and phragmites to more than 1000-fold reduction in Bd growth with red or sugar maple (Fig. 2). We found similar effects of litter leachate on sporangia densities, although the leachate of at least one litter species (i.e., green ash) did promote sporangia growth at low leachate concentrations. Contrary to the hypothesis that litter promotes saprophytic growth of Bd, we found reduced spore densities in the presence of a pre-leached litter substrate. Hence, our laboratory study suggests that the presence of leaf litter likely presents a strong environmental filter reducing the presence of Bd in natural wetlands.

As hypothesized, both spore and sporangia densities were inversely correlated with the concentration of phenolics in litter. This result is consistent with prior studies that found reduced growth of micro- and macrofauna with increased phenolic acid concentrations in leaf litter (Nicolai 1988; Stout 1989). However, there were notable inconsistencies in this trend; for example, black willow leachate is relatively phenolic-poor but still reduced spore densities, whereas other phenolic-rich litter species in our study (e.g., elm, cherry) did not induce lower Bd densities. One possible explanation for this inconsistency is that individual litter species contain different types of phenolic acids that have varying effects on Bd. Indeed, phenolic acids are a very diverse set of molecules that have varying effects on aquatic consumers, although effects are generally negative (Earl and Semlitsch 2015). It is also possible that the effects of phenolics are counterbalanced by other components of litter chemistry. Indeed, prior studies have found neutral effects of phenolic concentrations on fungal abundance (McArthur and Richardson 2007; Tuchman et al. 2002), and the effects of litter chemistry on aquatic consumers are rarely predictable by single chemical elements (Stoler and Relyea 2016).

It is likely that multiple chemical components of the leaf litter acted in concert to produce the interspecific variation in Bd densities that we observed in our study. Processes of microbial colonization and decomposition are often regulated by a complex interplay between primary and secondary litter chemistry. For example, high litter nutrient content might reduce the negative effects of phenolic acids. However, we actually observed an inverse correlation between litter P content and Bd densities. Alternatively, high amounts of structural compounds (e.g., lignin, cellulose) might have reduced solubility of toxic compounds during our leaching process. For example, lignin degradation is a major source of phenolic compounds from litter, but this process is slow relative to the leachate of other litter phenolics (Kuiters and Sarink 1986). Bd densities might also be influenced by environmental factors associated with phenolic acids. For example, the presence of phenolic acids can reduce pH, and Bd growth is strongly reduced below pH 6 (Piotrowski et al. 2004). However, this pH effect cannot fully explain our results; although red maple has been associated with reduced pH, black willow litter does not have a similar effect (Stoler and Relyea 2016). Moreover, laboratory incubation of similar leaf litter species, but at higher concentrations than in the

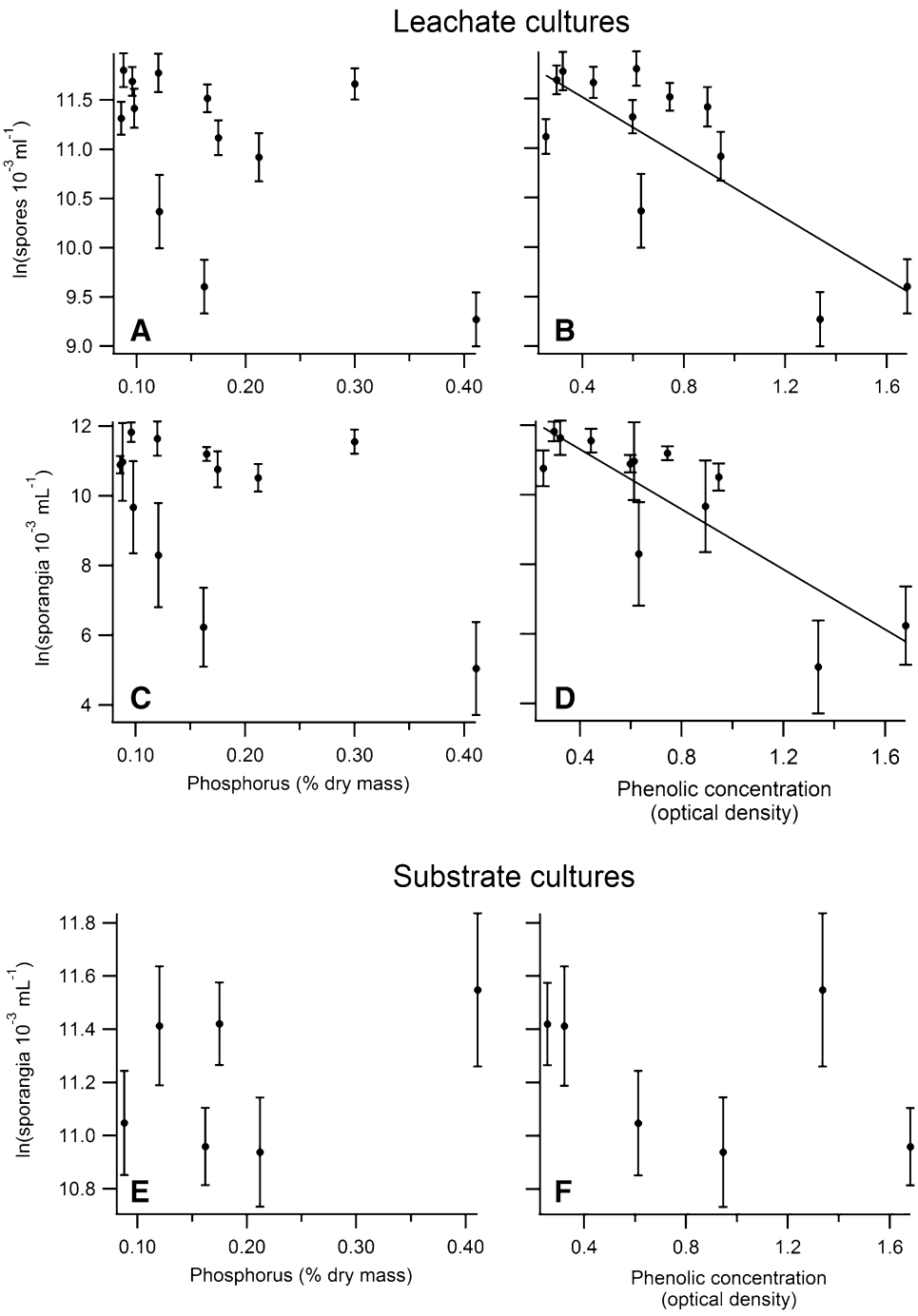

Fig. 4. Relationships between final spore and sporangia densities with litter C:N (**a**, **c**) and litter phenolic content (**b**, **d**) in leachate cultures, and relationships between final spore densities with litter C:N and leachate phenolic content (**e**, **f**) in leached substrate cultures. Only the correlations between spore and sporangia densities and phenolic content in leachate cultures are significant. Note that all densities are log-transformed. *Points* represent means of individual litter species. For leachate cultures, means are pooled across leachate levels. *Bars* represent ± 1 SE.

current study, revealed pH ranging from 7.0 to 8.5 (Stoler, *unpublished data*).

Contrary to our second hypothesis, we found no evidence that leaf litter substrate promotes saprophytic growth of Bd, as postulated by prior authors (Raffel et al. 2010). Spore densities were substantially lower in the presence of litter substrate from all litter species tested, relative to the no-litter control. Although the fragments were pre-leached and we observed no staining of the water after repeated autoclaving, negative effects were possibly caused by residual phenolic compounds remaining in litter fragments. However, it is more likely that multiple chemical and physical attributes of individual litter species contributed to the wide variation in Bd growth observed in our study. This is implied by the dramatic contrast in spore densities between red maple and sugar maple litter substrates, even though leachates from the two species resulted in similar effects on Bd growth. Regardless of the mechanism, our

results suggest that saprophytic growth of Bd on leaf litter is unlikely to offset negative effects of litter leachate on Bd growth.

Based on these combined results, we conclude that leaf litter should have net negative effects on Bd growth and abundance in wetlands. This result elucidates mechanisms underlying prior studies indicating that Bd abundance is positively correlated with canopy cover in natural wetlands (Raffel et al. 2010; Becker et al. 2012, 2015). This positive correlation was thought to be driven by the cooling effects of canopy cover, which reduced water temperatures to more optimal levels for Bd growth. The effect of temperature on Bd infection is well known (Raffel et al. 2013), and surveys of Bd abundance in temperate ecosystems indicate higher prevalence of the fungus during the spring and fall, when canopy cover combines with cooler temperatures (Lenker et al. 2014). Our results suggest that the positive correlation between Bd abundance and canopy cover is more likely mediated by shade effects on temperature than by direct positive effects of litter on Bd saprophytic growth. However, it is worth noting that the highest leachate concentrations used in our study might exceed concentrations naturally found during the spring, when amphibians are breeding. Although our surveys revealed a range of phenolic concentrations ranging from 6.2 to 58.6 mg L^{-1} during the spring and summer months, other surveys in northeastern temperate wetlands indicated concentrations that range from 0.4 to 11.0 mg L^{-1} (Freda and Dunson 1986; Maerz et al. 2005). Nevertheless, given that Bd was suppressed even at the lowest leachate dilution in our study, Bd growth rates in natural ponds with different levels of canopy cover likely depend on a balance between the positive thermal effects of canopy shading and the inhibitory effects of litter leachates.

Implications for Forest Change

Our results indicate that deforestation and changes in forest composition might influence the spread of Bd in complex ways. As wetlands are converted from forested to open-canopied systems through deforestation, the dominant litter types are necessarily converted from tree-dominated assemblages to emergent wetland plants. In our study, only species of deciduous litter had negative effects of Bd growth, whereas emergent wetland plants had little effect. Hence, our study suggests that reduced phenolics following deforestation ought to increase Bd infection. This is in contrast to field studies that found higher Bd prevalence or abundance in wetlands with greater canopy cover (Raffel et al. 2010; Becker and Zamudio 2011; Liu et al. 2013). The authors of these studies concluded that increased Bd infection in forested wetlands is likely driven by reduced temperatures in shaded wetlands. Our study lends support to this thermal shading hypothesis by excluding the alternative hypothesis that Bd benefits from saprophytic growth on litter in closed-canopy wetlands.

With regard to changes in forest diversity, our finding that several species of tree litter leachate inhibits Bd growth has potentially important implications. For example, our study suggests that the spread of red maple in many temperate forests might reduce Bd prevalence, due to high levels of phenolics relative to tree species being replaced (e.g., green ash and elm; Abrams 2003). Studies have shown that increased dominance of red maple is likely to reduce the growth and survival of amphibians due to its relatively low nutritional quality and high levels of phenolic compounds (Stephens et al. 2013; Stoler and Relyea 2016). However, our study suggests that the negative effects of maple litter on Bd growth might counteract this pattern in areas where Bd infection influences amphibian population dynamics. Indeed, it seems possible that some litter species might also reduce the sensitivity of larval anurans to Bd infection, and that leaf litter leachate might influence other amphibian pathogens (e.g., ranavirus). Further work, including coupled surveys of forest composition and Bd abundance, will be necessary to determine whether our laboratory findings translate to effects of leaf litter on infectious diseases in natural systems. If leaf litter does present a natural agent to reduce the spread and infectivity of Bd and other amphibian pathogens, our study suggests novel avenues of land management that could slow recent die-offs of amphibians. Indeed, selective harvesting of timber, managed seeding of forest landscapes, and control of emergent vegetation might induce changes to litter inputs that are beneficial for amphibian survival. Further field studies and controlled manipulations in natural environments will certainly help to inform future management plans.

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References

Abrams MD (2003) Where has all the white oak gone? *Bioscience* 53:927–939

Becker CG, Zamudio KR (2011) Tropical amphibian populations experience higher disease risk in natural habitats. *Proceedings of the National Academy of Science* 108:9893–9898

Becker CG, Rodriguez D, Longo AV, Talaba AL, Zamudio KR (2012) Disease risk in temperate amphibian populations is higher at closed-canopy sites. *PLoS ONE* 7:e48205

Becker CG, Rodriguez D, Longo AV, Toledo LF, Lambertini C, Leite DS, Haddad CFB, Zamudio KR (2015) Deforestation, host community structure, and amphibian disease risk. *Basic and Applied Ecology*. doi:10.1016/j.baae.2015.08.004

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological)* 57:289–300

Bosch J, Carrascal LM, Durán L, Walker S, Fisher MC (2007) Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society B* 274:253–260

Bradley PW, Gervasi SS, Hua J, Cothran RD, Relyea RA, Olson DH, Blaustein AR (2015) Differences in sensitivity to the fungal pathogen *Batrachochytrium dendrobatidis* among amphibian populations. *Conservation Biology* 29:1347–1356

Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. *Developmental and Comparative Immunology* 23:459–472

Clesceri LS, Eaton AD (1998) *Standard Methods for Examination of Water and Wastewater*, *20th ed.*, Washington DC: American Public Health Association

Davidson EW, Larsen A, Palmer CM (2012) Potential influence of plant chemicals on infectivity of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 101:87–93

Earl JE, Semlitsch RD (2015) Effect of tannin source and concentration from tree leaves on two species of tadpoles. *Environmental Toxicology and Chemistry* 34:120–126

Fisher MC, Garner TWJ (2007) The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduce amphibian species. *Fungal Biology Reviews* 21:2–9

Fox J, Weisberg S (2010) *An R companion to applied regression*, *2nd ed.*, Thousand Oaks: Sage Publishing

Freda J, Dunson WA (1986) Effects of low pH and other chemical variables on the local distribution of amphibians. *Copeia* 2:454–466

Graça MAS, Bärlocher F, Gessner MO (2005) *Methods to Study Litter Decomposition: A Practical Guide*, Dordrecht: Springer

Johnson PCD (2014) Extension of Nakagawa & Schielzeth's R^2_{GLMM} to random slopes models. *Methods in Ecology and Evolution* 5:944–946

Kuiters AT, Sarink HM (1986) Leaching of phenolic compounds from leaf and needle litter of several deciduous and coniferous trees. *Soil Biology and Biochemistry* 5:475–480

Laurance WF (2008) Global warming and amphibian extinctions in eastern Australia. *Austral Ecology* 33:1–9

Lavergne S, Molofsky J (2004) Reed canary grass (*Phalaris arundinacea*) as a biological model in the study of plant invasions. *Critical Reviews in Plant Sciences* 23:415–429

Lenker MA, Savage AE, Becker CG, Rodriguez D, Zamudio KR (2014) *Batrachochytrium dendrobatidis* infection dynamics vary seasonally in upstate New York, USA. *Diseases of Aquatic Organisms* 111:51–60

Li S, Shah NP (2013) Effects of various heat treatments on phenolic profiles and antioxidant activities of *Pleurotus eryngii* extracts. *Journal of Food Science* 78:C1122–C1129

Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Pessier AP, Collins JP (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academic of Sciences* 103:3165–3170

Liu X, Rohr JR, Li Y (2013) Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. *Proceedings of the Royal Society B* 280:20122506

Maerz JC, Brown CJ, Chapin CT, Blossey B (2005) Can secondary compounds of an invasive plant affect larval amphibians? *Functional Ecology* 19:970–975

Martin LJ, Blossey B (2013) Intraspecific variation overrides origin effects in impacts of litter-derived secondary compounds on larval amphibians. *Oecologia* 173:449–459

McArthur MD, Richardson JS (2007) Microbial utilization of dissolved organic carbon leached from riparian litterfall. *Canadian Journal of Fisheries and Aquatic Science* 59:1668–1676

Moser WK, Barnard EL, Billings RF, Crocker SJ, Dix ME, Gray AN, Ice GG, Kim M, Reid R, Rodman SU, McWilliams WH (2009) Impacts of nonnative invasive species on US forests and recommendations for policy and management. *Journal of Forestry* 107:320–327

Nicolai V (1988) Phenolic and mineral content of leaves influences decomposition in European forest ecosystems. *Oecologia* 75:575–579

Ostrofsky ML (1993) Effect of tannins on leaf processing and conditioning rates in aquatic ecosystems: an empirical approach. *Canadian Journal of Fisheries and Aquatic Sciences* 50:1176–1180

Ostrofsky ML (1997) Relationship between chemical characteristics of autumn-shed leaves and aquatic processing rates. *Journal of the North American Benthological Society* 16:750–759

Pinheiro J, Bates D, DebRoy S, Sarkar D (2013) nlme: linear and nonlinear mixed effect models. R package version 3.1-103. R Foundation for Statistical Computing, Vienna

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96:9–15

Polis GA, Anderson WB, Holt RD (1997) Toward an integration of landscape and food web ecology: the dynamics of spatially subsidized food webs. *Annual Review of Ecology and Systematics* 28:289–316

Pounds JA, Bustamante MR, Coloma LA, Consuegra JA, Fogden MPL, Foster PN, La Marca E, Masters KL, Merino-Viteri A, Puschendorf R, Ron SR, Sánchez-Azofeifa GA, Still CJ, Young BE (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* 439:161–167

R Development Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Version 3.1.2.

Raffel TR, Michel PJ, Sites EW, Rohr JR (2010) What drives chytrid infections in newt populations? Associations with substrate, temperature, and shade *EcoHealth* 7:526–536

Raffel TR, Romansic JM, Halstead NT, McMahon TA, Venesky MD, Rohr JR (2013) Disease and thermal acclimation in a more variable and unpredictable climate. *Nature Climate Change* 3:146–151

Rubbo MJ, Belden LK, Kiesecker JM (2008) Differential responses of aquatic consumers to variations in leaf-litter inputs. *Hydrobiologia* 605:37–44

Skelly DK, Golon J (2003) Assimilation of natural benthic substrates by two species of tadpoles. *Hydrobiologia* 59:37–42

Stephens JP, Berven KA, Tiegs SD (2013) Anthropogenic changes to leaf litter input affect the fitness of a larval amphibian. *Freshwater Biology* 58:1631–1646

Stoler AB, Relyea RA (2011) Living in the litter: the influence of tree litter on wetland communities. *Oikos* 120:862–872

Stoler AB, Relyea RA (2016) Leaf litter species identity alters the structure of pond communities. *Oikos* 120(6):862–872

Stout J (1989) Effects of condensed tannins on leaf processing in mid-latitude and tropical streams: a theoretic approach. *Canadian Journal of Fisheries and Aquatic Sciences* 46:1097–1106

Tuchman NC, Wetzel RG, Rier ST, Wahtera KA, Teer JA (2002) Elevated atmospheric CO_2 lowers leaf litter nutritional quality for stream ecosystem food webs. *Global Change Biology* 8:163–170

Whiles MR, Lips KR, Pringle CM, Kilham SS, Bixby RJ, Brenes R, Connelly S, Colon-Gaud JC, Hunte-Brown M, Huryn AD, Montgomery C, Peterson S (2006) The effects of amphibian population declines on the structure and function of neotropical stream ecosystems. *Frontiers in Ecology and the Environment* 4:27–34