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Original Contribution

What Drives Chytrid Infections in Newt Populations? Associations with Substrate, Temperature, and Shade

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Abstract: The pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is considered responsible for the population declines and extinctions of hundreds of amphibian species worldwide. The panzootic was likely triggered by human-assisted spread, but once the pathogen becomes established in a given region, its distribution is probably determined by local drivers. To assess the relative importance of potential drivers of infection in red-spotted newts (*Notophthalmus viridescens*), we measured *Bd* levels in 16 populations throughout central Pennsylvania. Infected individuals were detected in all but four populations, indicating that *Bd* is widespread in this region. We quantified local factors hypothesized to influence *Bd,* and found that infection levels were best predicted by the proportion of the pond substrate consisting of leaf litter or vegetation, along with a significant effect of water temperature. *Bd* infection in amphibians is temperature-dependent, and one possible explanation of the apparent substrate effect is that tree cover and vegetation provide shade, reducing the availability of shallow, warm-water patches in which newts might reduce or clear *Bd* infections. Alternatively, leaf litter and emergent vegetation might increase *Bd* infection more directly, perhaps by providing substrates for environmental growth of the fungus. We also observed a curvilinear relationship between *Bd* load and snout-vent length (a proxy for age), hinting that newts might develop acquired resistance to *Bd* infection. Though correlational, these results add to a growing body of evidence suggesting that environmental temperature is an important driver of *Bd* infection dynamics.

Keywords: amphibian decline, parasitism, disease, age-intensity, acquired immunity, zero-inflated negative binomial

INTRODUCTION

Amphibian populations around the world are declining at an alarming rate, in part due to the spread of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*)

(Stuart et al., 2004; Lips et al., 2006; Skerratt et al., 2007). First discovered in 1998, this disease is thought to have emerged recently as a worldwide threat to amphibian populations (Lips et al., 2006; Kilpatrick et al., 2010). *Bd* has been recorded from many states in the USA and is considered an important threat to native amphibians, particularly in Western states (Muths et al., 2003; Lips et al., 2004; Rachowicz et al., 2006; Pearl et al., 2009). Determining what intrinsic and environmental factors influence this host–pathogen relationship will be important for

designing management efforts to mitigate *Bd*'s impact (Muths et al., 2008; Rohr and Raffel, 2010).

Although the primary cause of the *Bd* pandemic was likely human-assisted spatial spread of infected amphibians (Weldon et al., 2004; Lips et al., 2006; Fisher and Garner, 2007; Picco and Collins, 2008; Rohr et al., 2008), once the pathogen reaches an area, the extent and severity of the infection is probably determined by the availability of suitable hosts and local environmental variables (Rodder et al., 2008; Fisher et al., 2009). *Bd* is an aquatic pathogen that infects a wide range of amphibian hosts, attacking the skin of adults and the keratinized mouthparts of larvae (Longcore et al., 1999). Because *Bd* is directly transmitted through water or by host contact (Rachowicz and Vredenburg, 2004), host density might be an important factor (Rachowicz and Briggs, 2007; Hossack et al., 2009). *Bd* might also grow saprophytically on environmental keratin sources, making the type of substrate potentially important (Johnson and Speare, 2005; Mitchell et al., 2008). Climatic factors, like temperature and moisture, are also thought to be important drivers of *Bd* infection (Rohr and Raffel, 2010). Cooler temperatures generally favor the pathogen (Andre et al., 2008), and *Bd* outbreaks have been associated with cooler months (Berger et al., 2004) and higher altitudes (Kriger and Hero, 2008; Muths et al., 2008; Pearl et al., 2009). Amphibians can even clear the infection at temperatures above the optimum temperature for *Bd* growth (Woodhams et al., 2003).

To determine the distribution and abundance of *Bd* on adult red-spotted newts (*Notophthalmus viridescens*) in central Pennsylvania, we conducted a spatial survey of 16 newt populations. Newts are common aquatic amphibians in ponds throughout eastern North America, where *Bd* has been previously detected on this and other amphibian species (Drake et al., 2007; Padgett-Flohr et al., 2007; Campbell Grant et al., 2008; Rothermel et al., 2008). We quantified more than a dozen environmental variables that might influence *Bd* abundance, including variables related to the pond temperature profile, pond substrate, host density, water quality, and individual host characteristics. We then used model selection to determine which variables could explain among-population variation in infection levels. We predicted higher prevalence and abundance of *Bd* in ponds with higher densities of newts and potential alternate hosts, and in ponds with cooler water temperatures. We also used model selection to determine which characteristics of individual newts might affect *Bd* infection levels.

Methods

Field Survey

In May–June 2004, we conducted a survey of 16 red-spotted newt populations in and around Centre County, Pennsylvania (Table 1), as described previously by Raffel et al. (2006a). These wetlands represented a range of newt habitats, including small temporary ponds, permanent landlocked ponds of various sizes, and permanent ponds connected to stream systems. Dip net (30 × 60-cm aperture; 3-mm mesh) surveys were used to measure densities of newts and alternate hosts as described previously (Raffel et al., 2006a). Briefly, we took a meter-long sweep of the net every four steps in a gridded pattern along the shoreline, sampling out to 50-cm depth (Raffel et al., 2006a). We also recorded whether each sweep contained dead tree leaves and/or live emergent vegetation, and quantified the proportion of sweeps with leaves, the proportion of sweeps with vegetation, and the proportion of sweeps with either leaves or vegetation, the latter of which will be referred to hereafter as "substrate complexity."

Table 1. Variables Included as Potential Predictors in Models of *Bd* Infection in Red-spotted Newts

Individual-level analysis	
Age, sex, and breeding	Immune parameters
SV-length	Lymphocytes
SV-length$^{2}\star$	Neutrophils
Mass	Eosinophils
Sex	Basophils$\star$
Breeding status	
Pond-level analysis	
Pond characteristics	Densities of potential hosts
Temperature$\star\star$	Adult newts
Leaves$\star$	Amphibian larvae (total)
Vegetation	Water quality
Substrate complexity$\star$	Ca^{+2} concentration
Tree cover	Nitrate concentration
Individual-level variables	pH
Average SV-length	DO
(Average SV-length)^{2}	Seasonality
Average eosinophils	Date
Average basophils	

Bd, *Batrachochytrium dendrobatidis*; SV-length, snout-vent length; DO, dissolved oxygen
$\star$ $P < 0.05$ in univariate analyses
$\star\star$ $P < 0.05$, but only with removal of an outlier

We estimated tree cover around the sampled perimeter of each pond by accessing an aerial photo with Google Earth 5.2 (Google Inc., Mountain View, CA; accessed August 18, 2010). Aerial photos were taken by the USDA Farm Service Agency on June 6, 2005 (all ponds except for Mothersbaugh) and the U.S. Geological Survey on March 1, 2007 (Mothersbaugh). Commercially available Landsat infrared photographs taken in the spring and summer of 2004 were used to help identify pond margins (Image Trader, Nehalem, OR). Tree cover was quantified as the proportion of sampled pond perimeter that was obscured by trees in the aerial photos.

Water temperature, dissolved oxygen (DO), and pH were measured 10 cm below the water surface at a single point in the pond where the water was knee deep (approximately 50 cm), using a model 95 dissolved oxygen meter (YSI Inc., Yellow Springs, OH) and a model 313 pH/temperature meter (Corning Life Sciences, Acton, MA). We also collected a water sample for further analyses by the Penn State Institutes of the Environments Water Quality Laboratory (University Park, PA) (Ca^{+2}: standard methods 3500A-Ca AA spectrometric; NO_3–N: standard methods 4500F automated cadmium reduction).

Newt Collection and Measurements

Following each survey, 10 newts were collected from each pond and transported to the lab in 250-ml Nalgene® containers filled with pond water. Mass, sex, and snout-vent length were recorded for each newt, as well as breeding status, assessed based on the presence of dark keratinization on the legs (male) or cloaca (female). Within 3 hours of initial capture, they were anesthetized with a drop of 15% benzocaine ointment (Orajel®; Del Laboratories, Inc., Uniondale, NY) on the head, and killed by decapitation. Blood smears were made and processed as described by Raffel et al. (2006b) to obtain differential leukocyte counts (lymphocytes, neutrophils, eosinophils, and basophils, quantified as cells per 5000 erythrocytes), and specimens were stored in 70% ethanol (30% water). Erythrocyte numbers might also vary, obscuring the interpretation of leukocyte numbers relative to erythrocytes, but time constraints and small quantities of blood prohibited quantification of cells per unit volume.

Bd Analysis

Two toes were sampled from each newt specimen (the longest on each hind foot) to improve the detection probability for *Bd*, because *Bd* is not always present on every toe of an infected individual (Boyle et al., 2004). The two toes were placed in the same vial before DNA extraction and PCR analysis. Seven specimens (three from LA and one each from CO, GB, IR, and MR; see Table 2 for site acronyms) were judged too degraded for analysis and omitted. We assessed *Bd* infection intensity using the real-time Taqman PCR assay described by Boyle et al. (2004) and modified by Hyatt et al. (2007), using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). We used a TaqMan® Exogenous Internal Positive Control (Exo IPC) Reagent (Applied Biosystems) to assess inhibition of the PCR reaction, as described by Hyatt et al. (2007). In this IPC system, a standardized concentration of a second DNA sequence is added to each reaction well, with its own set of primers and separate fluorescent probe. We found no significant inhibition of samples run at 1:100 dilution (instead of the standard 1:10), and therefore used this dilution for all analyses.

All samples with detectable *Bd* DNA amplification contained greater than one zoospore equivalent, the detection limit reported by Boyle et al. (2004), so we scored them all as positive samples for calculation of *Bd* prevalence. The prevalence data (Table 1) should be interpreted with caution, due to the potential for false-positive and false-negative samples when the *Bd* level in a sample is lower than 10 zoospore equivalents (Kriger et al., 2006). In this study, only 8 of 63 positive samples were below this threshold.

Statistics

We conducted analyses using R statistical software (R Development Core Team, 2008). To avoid problems due to potential biases in the prevalence data, and to make full use of the qPCR data, we conducted analyses directly on the measurements of *Bd* load (zoospore equivalents per sample). For the within-population analysis of *Bd* load on individual newts, we fit the *Bd* load data to a zero-inflated, negative binomial, generalized linear model (function "zeroinfl" in package "pscl") (Jackman, 2008; Zeileis et al., 2008). In a zero-inflated model, variation in the response is divided into two components: the zero-inflation component (binomial distribution with a logit link), which in this case models whether or not individuals become infected, and the count component (negative binomial distribution with a log link), which in this case models the infection intensity of infected individuals. In such a model, indi-

Table 2. Prevalence of *Bd* and Mean *Bd* Loads (Average Intensity or Log Intensity Including 0 s) on Newts in the 16 Ponds Sampled[a]

Pond	Prevalence	*Bd* load (mean ± SE)	Log *Bd* load (mean ± SE)
Beaver 1 (BE)	0.5 (5/10)	1068.6 ± 680.1	1.38 ± 0.5
Clearcut Pond (CC)	0.8 (8/10)	789.3 ± 368.1	1.96 ± 0.43
Catty Ninetails (CN)	0.4 (4/10)	53.8 ± 35.3	0.69 ± 0.32
Colyer Lake (CO)	0 (0/9)	0	0
Cranberry Lake (CR)	0 (0/10)	0	0
Deep Woods (DW)	0.7 (7/10)	32.7 ± 11.5	1.06 ± 0.26
False Beaver (FB)	0.8 (8/10)	1332.6 ± 872.9	1.90 ± 0.44
Greenbriar 1 (GB)	0.67 (6/9)	560.3 ± 371.5	1.64 ± 0.45
Irrigation Pond (IR)	0.22 (2/9)	38.2 ± 36.1	0.42 ± 0.3
Little Acre (LA)	0.57 (4/7)	8.1 ± 5.2	0.57 ± 0.24
Mothersbaugh (MB)	0 (0/10)	0	0
Mystery Newt (MN)	0.3 (0/10)	83.1 ± 71.7	0.59 ± 0.33
Muskrat Pond (MP)	0.67 (6/9)	132.4 ± 49.2	1.47 ± 0.38
Penn Roosevelt (PR)	0 (0/10)	0	0
Twin Pond (TP)	0.7 (7/10)	116.3 ± 36.6	1.49 ± 0.34
Turtle Shell (TS)	0.3 (3/10)	876.7 ± 862.3	0.76 ± 0.43

Bd, *Batrachochytrium dendrobatidis*
[a]Pond descriptions and GPS coordinates are available in Raffel et al. (2006a)

viduals with zero *Bd* load can arise from either the binomial process (infected vs. uninfected) or the negative binomial process (false negatives due to low zoospore numbers). For this analysis, we used a relatively simple model with a single parameter for the zero-inflation component, meaning that all the negative counts had the same probability of being true negatives. This model fit the data better than a regular negative binomial model (function "glm.nb" in package "MASS") (Venables and Ripley, 2002) or a more complex model with a separate zero-inflation parameter for each predictor (negative binomial: AIC = 1051.8; simple zero-inflated: AIC = 1032.8; complex zero-inflated: AIC = 1035.9). All subsequent models for the within-population analysis incorporated a blocking variable pond to control for among-population variation, and the four ponds without detectable *Bd* were excluded from this analysis. Pond was treated as a fixed effect because mixed-effects models have not yet been implemented for zero-inflated negative binomial models in R, to our knowledge. Snout-vent length was centered before calculating the quadratic term (SV-length^2). Because this quadratic effect of snout-vent length is marginal to the linear effect (SV-length), this linear term was included in all models containing the quadratic term. We conducted a univariate test for each predictor variable (Table 1) before obtaining a full model using forward stepwise selection. In each step, we added the

predictor that most improved the model according to likelihood ratio tests (function "lrtest" in package "lmtest,") (Zeileis and Hothorn, 2002), stopping when no additional predictors contributed significantly to the model ($P > 0.05$). To determine whether the results were robust to the selection procedure, we repeated this analysis using backward stepwise selection, starting with a maximum model including all nine predictors (Table 1). Significance of predictors was also assessed using likelihood-ratio tests.

Rather than test within- and among-population predictors together in a single nested analysis, which is not yet possible for zero-inflated negative binomial models, we used a separate model for the among-population analysis, using an average for each pond as the unit of replication and weighting by the number of newts sampled in each pond. *Bd* load and newt density (e.g., number of newts per meter-sweep) were log-transformed [$\log(N + 1)$] to improve normality before calculating pond averages. Mean log *Bd* load was approximately normally distributed, allowing us to use normal linear regression for the among-pond analysis. Proportional predictors (leaves, vegetation, substrate complexity, and tree cover) were arc-sine-square-root transformed to improve normality. Individual-level variables (e.g., mass and SV-length) were averaged for each pond and included in the among-population analysis if they were significant predictors in the within-population

analysis (Table 1). We again conducted a univariate test for each predictor variable before conducting best subsets model selection (function "regsubsets" in package "leaps"), using the exhaustive selection algorithm and selecting the top 10 models in order of adjusted R^2. To reduce the risk of over-fitting, we set a maximum number of four predictor variables, according to Crawley's (2007) rule of thumb not to add more than N/3 parameters to a regression model. Once a final regression model was selected, we used variance partitioning to assess relationships among potentially collinear predictors using the function "commonalityCo-efficients" in package "yhat" (Nimon et al., 2008; Nimon and Roberts, 2009).

Results

We found evidence of Bd infection in 12 of the 16 red-spotted newt populations, indicating widespread occurrence of this pathogen in central Pennsylvania wetlands (Table 2). None of the newts sampled from these ponds showed overt signs of chytridiomycosis, such as unusual skin sloughing or lethargy (Voyles et al., 2009). Indeed, most of the newts appeared visibly healthy aside from occasional infection by other more visible parasites (Raffel, 2006; Raffel et al., 2006a, 2008).

There were two significant univariate predictors of Bd load in the within-population analysis (Table 1): SV-length^2 (coefficient [coef.] = −0.073, X_1^2 = 8.0, P = 0.005) and basophils (coef. = −0.048, X_1^2 = 4.6, P = 0.032). Both of these predictors were also significant in the final model following stepwise selection, in addition to mass, which was only significant when controlling for the quadratic effect of SV-length (Table 3). Forward and backward selection both resulted in the same model, in which Bd load was higher in newts with fewer circulating basophils and with lower mass (Table 3). The quadratic effect of SV-length appears to have been driven by a rapid increase in Bd load with age for smaller newts (< 43 mm), followed by a gradual decrease in load for larger newts (> 43 mm; Fig. 1).

In the among-population analysis, two variables were significant univariate predictors of log Bd load: substrate complexity (the proportion of sweeps containing leaves and/or vegetation; coef. = 1.82, $F_{1,14}$ = 38.3, P < 0.001; Fig. 2a) and the proportion of sweeps containing dead leaves (coef. = 0.63, $F_{1,14}$ = 4.8, P = 0.047; Fig. 2b). Water temperature (measured where the water was 50-cm deep)

**Table 3.** Final Model for the Within-population Analysis of Bd Load on Individual Newts[a]

Predictor	β	X^2	df	P
Pond	NA	46.3	11	< 0.001
SV-length	0.500	6.3	1	0.012
SV-length^2	−0.066	7.1	1	0.008
Basophils	−0.051	5.2	1	0.022
Mass	−1.511	4.8	1	0.029

Bd, Batrachochytrium dendrobatidis; SV-length, snout-vent length
[a]The same model was obtained by forward stepwise selection and backward stepwise deletion. Bd load data were fit to a zero-inflated negative binomial generalized linear model with a single parameter for the zero-inflation component (Bd load $\sim \beta_{0,count} + \beta_1[\text{SV-length}] + \beta_2[\text{SV-length}]^2 + \beta_3[\text{Basophils}] + \beta_4[\text{Mass}] + \beta_5[\text{Pond}_1] + \cdots + \beta_{19}[\text{Pond}_{15}]|\beta_{0,zero-inflation})$. Raw coefficients ($\beta$) are presented for continuous predictors
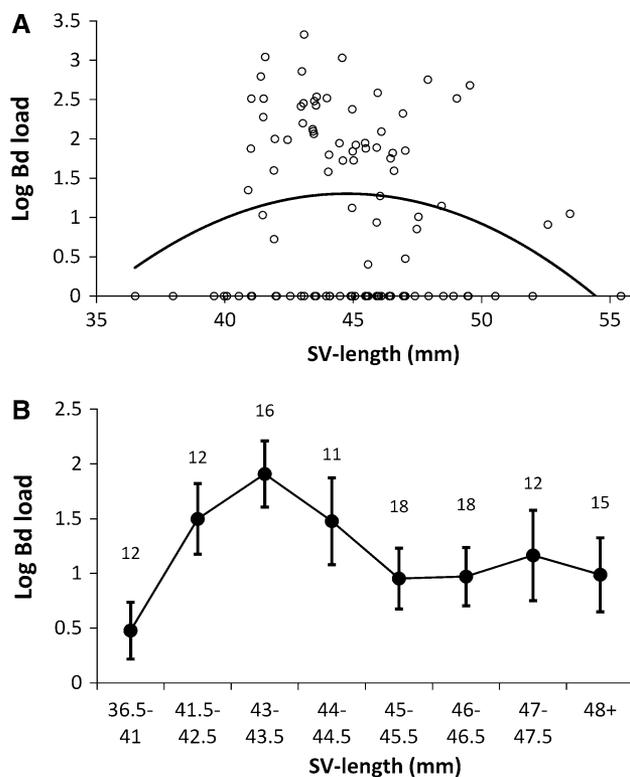
Figure 1. Age-intensity relationship for amphibian chytrid (Bd load, in zoospore equivalents per sample) on individual red-spotted newts, using snout-vent length (SV-length) as a proxy for age. (a) Continuous scatterplot of log Bd load versus SV-length (jittered), showing the quadratic fit to the full dataset (solid curve). (b) Categorical line plot of log Bd load versus SV-length, grouped so that size classes would have similar sample sizes (above each point). Error bars = SE.
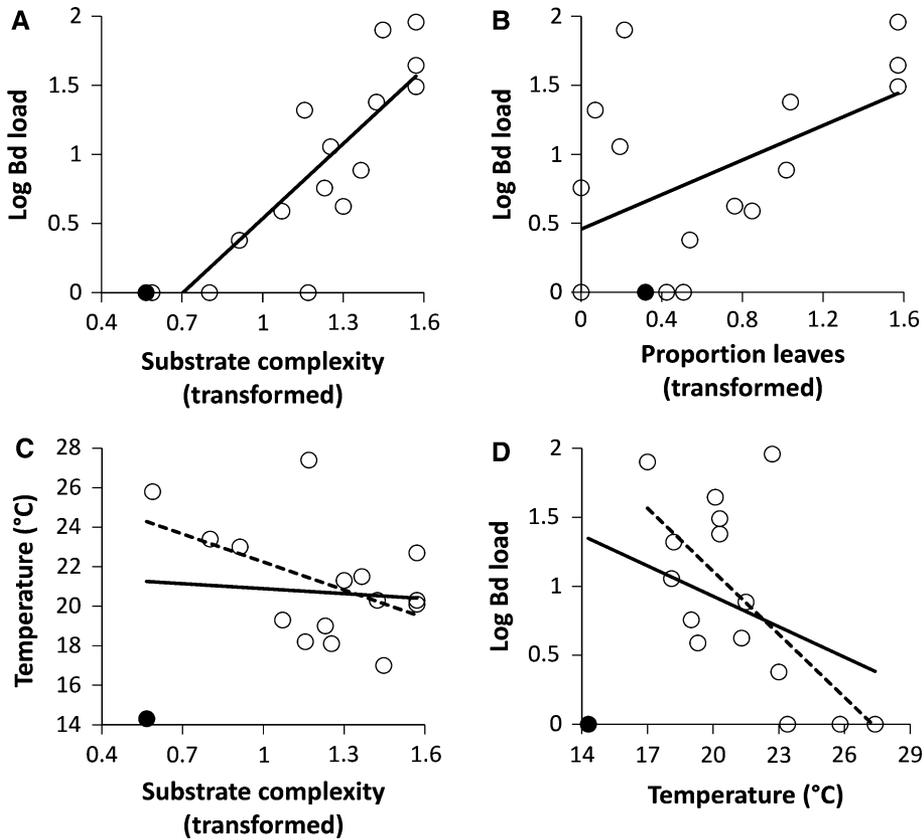
Figure 2. Patterns of among-population variation in *Bd* infection intensity (*Bd* load, in zoospore equivalents per sample) for 16 red-spotted newt populations, with respect to substrate variables and temperature. (a) Relationship between substrate complexity (the arcsine-square-root transformed proportion of sweeps containing dead leaves and/or emergent vegetation) and log *Bd* load. (b) Relationship between leaves (the arcsine-square-root transformed proportion of sweeps containing only dead leaves) and log *Bd* load. (c) Relationship between substrate complexity and water temperature. (d) Relationship between water temperature and log *Bd* load. Solid lines are best-fit lines for the full dataset, and dotted lines are best-fit lines after removal of an influential outlier, which is represented with a filled circle (pond PR; see Table 2).

was not a significant predictor in this initial univariate analysis ($F_{1,14} = 1.8$, $P = 0.198$). In the best subsets regression analysis, two variables were chosen in most of the top 10 models: substrate complexity and water temperature (Table 4). These variables contributed significantly to a model including both predictors (substrate complexity: coef. = 1.78, $F_{1,13} = 48.2$, $P < 0.001$; temperature: coef. = −0.06, $F_{1,13} = 5.5$, $P = 0.036$). No other predictor was significant when controlling for the effects of substrate complexity and temperature (all $P > 0.05$). Substrate complexity and water temperature were not significantly correlated using the full dataset ($r = −0.083$, $t_{14} = 0.31$, $P = 0.759$), but removal of an influential outlier (pond PR; Cook's distance = 1.39) revealed a nearly significant trend towards ponds with higher amounts of leaf litter and vegetation (i.e., substrate complexity) being cooler ($r = −0.474$, $t_{13} = 1.94$, $P = 0.074$; Fig. 2c). Revisiting the univariate analysis, we found that pond PR was also an influential outlier for the effect of water temperature on log *Bd* load (Cook's distance = 1.42; Fig. 2d). Removing this outlier revealed an apparent univariate effect of water temperature on log *Bd* load (coef. = −0.15, $F_{1,13} = 9.5$, $P = 0.009$; Fig. 2d). However, even with this

outlier removed, substrate complexity was still a better predictor of log *Bd* load than water temperature, and remained significant in a model controlling for temperature (shade: coef. = 1.62, $F_{1,12} = 22.1$, $P < 0.001$; temperature: coef. = −0.08, $F_{1,12} = 4.9$, $P = 0.047$). Tree cover was highly correlated with the proportion of sweeps containing leaves ($r = 0.815$, $t_{14} = 5.3$, $P < 0.001$), but was not a significant predictor of log *Bd* load ($F_{1,14} = 1.4$, $P = 0.264$).

Although it incorporated information about leaves and vegetation, the substrate complexity variable was not simply the sum of the proportion of sweeps with leaves and the proportion with vegetation, because some sweeps contained both substrate types. We therefore used variance partitioning to determine whether leaves or vegetation could account for more of the substrate complexity effect on log *Bd* load. Together, leaves and vegetation accounted for 78.0% of the variation in log *Bd* load due to substrate complexity, but substrate complexity accounted for more unique variation than either leaves or vegetation in a model containing all three variables (unique percent of explained variation: substrate complexity, 21.7%; leaves, 1.4%; vegetation, 1.0%). Leaves explained more of the variation due to substrate complexity than did vegetation (percent of

Table 4. Results of best subsets model selection for the pond-level analysis[a]

Predictors	Model rank									
	1	2	3	4	5	6	7	8	9	10
Substrate complexity[b]	1.06	0.93	0.89	0.83	0.95	0.80	0.81	0.92	0.87	0.85
Temperature		−0.30	−0.29		−0.30	−0.25	−0.30			−0.26
Calcium	−0.35	−0.28		−0.32		−0.17				
Date			0.20		0.24				0.27	0.18
Basophils	−0.37			−0.32				−0.30		
Nitrates						0.21	0.18			0.15
pH	0.31	0.28			0.18					
Tree cover								−0.49	−0.48	
SV-length^2			−0.18				−0.17			
Leaves								0.45	0.47	
Amphibian larvae				0.22						
R_{adj}^2	0.898	0.856	0.850	0.845	0.845	0.842	0.840	0.837	0.836	0.834
AIC$_C$	9.4	14.8	15.5	16.0	16.1	16.4	16.5	16.8	16.9	17.1
w_i	0.761	0.051	0.035	0.028	0.027	0.023	0.022	0.019	0.018	0.016

SV-length, snout–vent length

[a]Standardized coefficients are provided for the predictors included in each model. Models were ranked in order of adjusted R^2

[b]Proportion of meter-sweeps containing dead leaves and/or vegetation

explained variation in common with substrate complexity: leaves, 76.9%; vegetation, 43.0%).

DISCUSSION

Bd infection is widespread in central Pennsylvania ponds. We found *Bd* in most of the newt populations sampled, and all of the ponds with zero prevalence were within several miles of a pond with detectable *Bd* levels (Raffel et al., 2006a). Thus, it is possible that *Bd* exists at low levels in these populations but went undetected. *Bd*'s ubiquity in central Pennsylvania suggests that local conditions might drive *Bd* abundance in these populations. We also found no evidence for host density effects, suggesting that environmental drivers might be more important than intrinsic drivers of *Bd* infection dynamics in this system. The absence of overt signs of disease on infected newts, despite quite high infection intensity on some individuals (Fig. 1a), suggests that adult red-spotted newts are tolerant to *Bd* infection and might act as a reservoir species. Although we are unaware of recent amphibian declines in this region, the apparent ubiquity of *Bd* in Pennsylvania ponds is concerning, considering the impact this pathogen has had in other parts of the world (Kilpatrick et al., 2010).

Bd levels were higher in ponds with higher substrate complexity, i.e., a greater proportion of the substrate covered with dead leaves and emergent vegetation, and this variable was a better predictor of *Bd* load than any other quantified predictor, including direct measurements of water temperature. Both leaf litter and emergent vegetation appear to have contributed to this effect, but leaf litter explained a larger proportion of the variation. This correlation between substrate complexity and *Bd* in newts might indicate a direct effect of leaf litter and vegetation on *Bd* transmission, for example, by providing increased surface area on which *Bd* might grow in the environment. However, so far we lack empirical evidence that *Bd* grows in the environment on either leaf litter or vegetation, and substrate complexity is not the only pond characteristic indicated by leaf litter and emergent vegetation.

An alternative, and perhaps more likely, explanation of the substrate complexity effect is that leaf litter and dead leaves provide good indications of shade, which might be the true driver of higher *Bd* levels in newts. Tree cover, which was strongly correlated with leaf litter in this study, is known to substantially reduce the temperature of shallow water in wetlands (Poole and Berman, 2001). And although leaf litter accounted for more of the "substrate complexity" effect, emergent vegetation also lowers water temperature

and reduces the diurnal temperature range (Rose and Crumpton, 1996), which might affect *Bd*–host interactions (Rohr and Raffel, 2010). Shade around the periphery of the pond should substantially decrease the temperature of shallow water, and this in turn might increase susceptibility to infection in newts, which tend to concentrate in the shallow water near the shoreline (Petranka, 1998). We therefore propose that sunny patches in shallow water might provide thermal refugia where newts can avoid or perhaps clear *Bd* infection. This shade hypothesis is especially plausible if infected newts use behavioral fever to assist with pathogen clearance, as suggested by previous studies with other amphibian species (Kluger, 1991; Richards-Zawacki, 2010), though random movement of infected newts into warm shallow areas could also account for the observed pattern. Experiments will be necessary to determine whether newts use behavioral thermoregulation to clear themselves of *Bd* infection.

Three patterns in this study seemed at first to contradict the hypothesis that shade drives *Bd* infection in these populations, but further reflection or closer examination of the data showed them to be consistent with, if not in support of, this hypothesis. First, substrate complexity was not correlated with direct measurements of water temperature, which might be expected if substrate complexity indicates shade. However, the direct measurements of water temperature were taken at a single location in each pond, out where the water was a half-meter-deep, and might not be representative of shallow-water temperatures. This was especially true of Penn Roosevelt pond, where temperature was measured near the input point of a large mountain stream, leading to a much colder temperature at this location than in other sections of the shallow, unshaded periphery [T.R. Raffel, personal observations]. None of the other ponds had nearly such a large stream input, which might be why the correlation between water temperature and substrate complexity increased following removal of this datapoint (Fig. 2). Second, water temperature, the proposed proximate mechanism for the shade hypothesis, was a poorer univariate predictor of *Bd* load than substrate complexity, a potential indirect proxy for temperature in shallow water. However, this correlation also improved following removal of Penn Roosevelt, leading to a strong negative correlation between water temperature and log *Bd* load (Fig. 2). Third, the direct measure of tree cover from aerial photographs was a poorer predictor of *Bd* load than leaf litter, and neither was as good of a predictor as substrate complexity. However, leaf litter might be a better

proxy for shade because it measures canopy cover in two dimensions and accounts for the density of tree foliage, and neither measure accounted for the potential shading effect of emergent vegetation. Unfortunately, no water temperature data were collected in the shallow margins of these ponds during the survey, so we cannot assess these relationships directly.

The most interesting pattern from the within-population analysis was the quadratic effect of snout-vent length on *Bd* load. Snout-vent length is a good proxy for age in adult red-spotted newts (Raffel et al., 2009), and parasite age-intensity relationships can provide useful information about parasite biology and ecology (Raffel et al., in press). Type III age-intensity relationships, in which parasite intensities decline with age in older hosts, sometimes indicate population-level effects of host-acquired immunity, in which repeated exposure to a parasite leads to increased resistance to infection (Raffel et al., 2009). The significant quadratic effect of snout-vent length on *Bd* load and the trend toward lower *Bd* load with length for newts more than 43-mm long (Fig. 2) are both suggestive of a Type III age-intensity relationship, similar to age-intensity patterns for two other newt parasite taxa (Raffel et al., 2009). Although this pattern is consistent with newts acquiring immunity following repeated exposure, other factors such as parasite-induced host mortality and increased innate resistance with age can also cause Type III age-intensity relationships (Raffel et al., in press).

Whether amphibians develop improved resistance to *Bd* following repeated exposure is an important but still unanswered question. A recent study found evidence for a robust acquired immune response to *Bd* infection in *Xenopus laevis* (Ramsey et al., 2010), but another study found no evidence of improved resistance to *Bd* infection in *Rana muscosa* following injection with dead *Bd* (Stice and Briggs, 2010). Injection of a dead pathogen is different from infection by a live pathogen, as indicated by the fact that live attenuated vaccines are generally more immunogenic than inactivated vaccines (Wareing and Tannock, 2001). Therefore, a direct test of whether amphibians develop acquired resistance to *Bd* will require repeated experimental exposure to live *Bd*. Although the apparent Type III age-intensity relationship in newts does not provide strong evidence for or against this hypothesis, it suggests that we should not rule out the potential for acquired resistance to influence *Bd* infection dynamics.

The apparent effects of increased mass and circulating basophils on *Bd* might indicate that newts with better body

condition or higher leukocyte levels are more resistant to infection. However, the direction of causation is uncertain. *Bd* infection might also reduce newt body mass, either via direct pathogenesis or by disrupting feeding behavior, or reduce circulating basophils by causing them to redistribute to the skin. Woodhams et al. (2007) found changes in levels of circulating leukocytes following experimental exposure of juvenile frogs to *Bd*, with some leukocytes decreasing following infection. However, the pattern for basophils was opposite that observed in this study. These problems illustrate the general difficulty with deriving conclusions from results of a correlative study, even when there are clear biological hypotheses for why variables might be causally related.

CONCLUSIONS

We found little evidence that environmental factors other than substrate complexity or temperature drive *Bd* infection dynamics in adult newts. Although a growing body of literature suggests that temperature is a key driver of *Bd* infection dynamics, to our knowledge, this is the first study to suggest that microhabitat temperature variation due to shade might be more important than mean wetland temperatures. Due to the correlative nature of this study and the lack of direct shallow-water temperature measurements, this hypothesis should be tested experimentally to determine whether shade is sufficient to drive increased infection by *Bd*. It will also be important to assess alternative hypotheses, such as leaf litter and vegetation directly enhancing *Bd* transmission, either by providing additional surfaces for *Bd* growth or via some other mechanism. If shade can indeed drive *Bd* infection in natural populations, however, one implication is that the availability of warm microhabitats should be considered when designing management strategies for controlling *Bd*. Although we discourage tree removal as an amphibian conservation strategy, particularly for red-spotted newts that depend on forest habitat for their terrestrial juvenile stage, these results suggest that cooler and more shaded sites might have higher infection levels and should be targeted for management efforts.

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