A dilution effect in the emerging amphibian pathogen *Batrachochytrium dendrobatidis*

Catherine L. Searle[a,b,1], Lindsay M. Biga[a,b], Joseph W. Spatafora[c], and Andrew R. Blaustein[a]

[a]Department of Zoology, [b]Environmental Sciences Graduate Program, and [c]Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331

Global declines in biodiversity are altering disease dynamics in complex and multifaceted ways. Changes in biodiversity can have several outcomes on disease risk, including dilution and amplification effects, both of which can have a profound influence on the effects of disease in a community. The dilution effect occurs when biodiversity and disease risk are inversely related, whereas the amplification effect is a positive relationship between biodiversity and disease risk. We tested these effects with an emerging fungal pathogen of amphibians, *Batrachochytrium dendrobatidis* (Bd), which is responsible for catastrophic amphibian population declines and extinctions worldwide. Despite the rapid and continued spread of Bd, the influence of host diversity on Bd dynamics remains unknown. We experimentally manipulated host diversity and density in the presence of Bd and found a dilution effect where increased species richness reduced disease risk, even when accounting for changes in density. These results demonstrate the general importance of incorporating community structure into studies of disease dynamics and have implications for the effects of Bd in ecosystems that differ in biodiversity.

chytridiomycosis | emerging infectious disease | tadpole

The relationship between biodiversity and infectious disease is dynamic and often complex, especially in multihost pathogen systems. When multiple species are affected by the same pathogen, host species can vary in their ability to acquire the pathogen, defend themselves against the pathogen, and transmit the pathogen to others. Thus, changes in biodiversity that affect host species composition can dramatically alter disease dynamics within a community (1, 2).

Changes in biodiversity can have several outcomes on disease risk, including dilution and amplification effects (3). The dilution effect occurs when biodiversity and disease risk are inversely related. This pattern can occur through several pathways, including reduced encounter rates between susceptible and infectious individuals, reduced transmission rates, reduced host density, increased mortality of infected individuals, or accelerated recovery of infected individuals (3). The dilution effect has primarily been studied in vector-transmitted pathogens with frequency-dependent transmission (refs. 4–7 but see refs. 8 and 9).

The amplification effect, in contrast, occurs when there is a positive relationship between biodiversity and disease risk (3). This scenario occurs when low-diversity systems contain poorly competent hosts, and adding species to the community increases the number of highly competent hosts that easily acquire and transmit the pathogen. The amplification effect may occur, for example, when epidemics are driven by transmission from a reservoir species that is not present in low-diversity systems (10, 11).

Whether a dilution or amplification effect occurs can be context-dependent. For example, a comparative study in a grassland ecosystem showed the dilution effect to be more common than the amplification effect (12). Additionally, ecological models predict that dilution effects are more common than amplification effects in the intensely studied Lyme disease system, if species diversity is nonrandomly manipulated (13). However, other models have indicated that the dilution effect will primarily occur in pathogens with frequency-dependent transmission (14), which is common in vector-transmitted pathogens and not in pathogens that experience density-dependent transmission.

A dilution effect can occur if communities with higher biodiversity have a lower density of susceptible individuals. Fewer susceptible hosts lead to reduced pathogen transmission and a lower disease risk. In field studies, it is difficult to determine whether dilution effects occur from biodiversity changes or simply from changes in host density. Diversity per se does not always affect disease risk, but changes in host density as a result of altered diversity can create a dilution effect (12). Thus, it is essential to account for host density and biodiversity to understand the effects that each factor has on disease risk. Studies accounting for both are rare.

Neither the dilution nor the amplification effect has been well-studied with regard to amphibian disease ecology or amphibian population declines. However, such studies may elucidate some of the differences in amphibian disease dynamics in regions that differ in species diversity. For example, *Batrachochytrium dendrobatidis* (Bd) is an emerging amphibian pathogen responsible for numerous population declines around the world (15–17). Although there has been an extensive rise in the number of studies, publications, and citations regarding Bd since it was first described (18–21), very little is known about its biology in a community context (but see refs. 22 and 23). Bd is directly transmitted, and there is some evidence that Bd exhibits density-dependent transmission (24, 25), although transmission could potentially exhibit frequency dependence if a reservoir is present (26). Therefore, it may be possible for a dilution effect to occur in the Bd system. Understanding the relationships among Bd, host diversity, and host density is essential for a better understanding of Bd dynamics and transmission.

We tested the effects of host density and species richness (taxonomic diversity) on disease risk from Bd. Bd infects keratinized cells of amphibians (larval mouthparts and postmetamorphic skin) and appears to cause higher mortality at the postmetamorphic stage (23, 27, 28). However, larval amphibians can suffer both lethal and sublethal effects of infection and may act as important reservoirs for Bd, maintaining persistence of Bd even in the absence of postmetamorphic hosts (24, 29).

We focused our study on Western toads (*Anaxyrus boreas*) because they are particularly susceptible to Bd (28, 30, 31) and have suffered population declines associated with Bd in portions of their range (32). In the Cascade Mountain Range of Oregon, *A. boreas* is sympatric with several other amphibian species that host Bd, including Cascades frogs (*Rana cascadae*) and Pacific tree frogs (*Pseudacris regilla*) (32–35). However, they can also be

found in habitats where other amphibians are rare or absent (33). *A. boreas* larvae aggregate at high densities either in single-species or mixed-species schools (33, 36, 37). Thus, this is an ideal system to study the interrelationships among biodiversity and density as well as to test for dilution and amplification effects.

Results

We first analyzed disease risk for our focal species (*A. boreas*), then expanded our analyses to include all individuals, with five measures of disease risk: toad infection severity (concentration of infection in *A. boreas*), toad infection prevalence (percentage of *A. boreas* infected), all-species infection severity (concentration of infection in all species), all-species infection prevalence (percentage of all species infected), and total infection [sum of Bd per replicate (in gross amount, not concentration)] (Table 1). We also analyzed gross infection [raw quantitative PCR (qPCR) output], which is closely related to infection severity (*SI Results*). Toad infection severity was reduced as species richness increased ($F_{1, 50} = 18.31$, $P < 0.01$; Fig. 1*A* and Table 1). Neither the number of *A. boreas* individuals nor the total number of individuals was a significant predictor of infection. The same results were found with gross infection of toads (*SI Results* and Fig. S1). Additionally, species richness decreased toad infection prevalence ($F_{1, 52} = 22.67$, $P < 0.01$; Fig. 1*B* and Table 1), but it was not affected by the number of *A. boreas* individuals or the total number of individuals.

When incorporating all species, infection severity again demonstrated a negative relationship with species richness ($F_{1, 50} = 9.86$, $P < 0.01$; Fig. 1*C* and Table 1), whereas neither the total number of individuals nor the number of *A. boreas* individuals was a significant predictor. The same results were found with gross infection (*SI Results* and Fig. S1). All-species infection prevalence was predicted by both species richness and the density of hosts (richness: $F_{1, 52} = 22.37$, $P < 0.01$; density: $F_{1, 52} = 4.43$, $P = 0.04$; Fig. 1*D* and Table 1), where more animals and more species led to a reduction in the percentage of individuals infected. However, species richness and total density were not significant predictors of total infection (richness: $F_{1, 40} = 0.03$, $P = 0.86$; density: $F_{1, 40} = 0.86$, $P = 0.36$). Total infection was best predicted by the number of *A. boreas*, where fewer toads led to lower total Bd ($F_{1, 40} = 7.81$, $P = 0.01$; Fig. 2 and Table 1).

Species did not significantly differ in infection severity ($F_{2, 192} = 1.12$, $P = 0.33$), although *A. boreas* and *P. regilla* had higher average Bd loads than *R. cascadae* did (Table S1). Gross infection differed by species ($F_{2, 187} = 11.58$, $P < 0.01$), where *R. cascadae* had lower average gross infection than the other two species (Table S1). All tested Bd-control (unexposed) individuals were negative for infection ($n = 10$). Infection severity, gross infection, and prevalence in *P. regilla* and *R. cascadae* did not differ among treatments, although average infection severity and gross in-

fection were lower in the presence of more species (*P. regilla* infection severity: $t(16) = 1.40$, $P = 0.18$; *P. regilla* gross infection: $t(16) = 1.78$, $P = 0.09$; *P. regilla* infection prevalence: $t(16) < 0.01$, $P = 0.99$; *R. cascadae* infection severity: $t(16) = 1.28$, $P = 0.22$; *R. cascadae* gross infection: $t(16) = 1.38$, $P = 0.19$; *R. cascadae* infection prevalence: $t(16) = 1.20$, $P = 0.25$; Fig. S2).

Mortality did not differ by species ($\chi^2 = 1.12$, df = 2, $P = 0.57$), Bd treatment ($\chi^2 = 0.16$, df = 1, $P = 0.69$), or density/richness treatment ($\chi^2 = 9.23$, df = 5, $P = 0.10$). Mass and length of *A. boreas* were not predicted by Bd treatment or species richness. However, *A. boreas* were heavier and longer when fewer total animals were present (mass: $F_{2, 224} = 16.70$, $P < 0.01$; length: $F_{2, 224} = 7.08$, $P < 0.01$) and when there were fewer *A. boreas* present (mass: $F_{1, 104} = 19.18$, $P < 0.01$; length: $F_{1, 104} = 15.10$, $P < 0.01$; Fig. S3). For *R. cascadae* and *P. regilla*, mass and length did not differ by treatment. Among-species comparisons revealed that both mass and length differed by species (mass: $F_{2, 387} = 136.97$, $P > 0.01$; length: $F_{2, 387} = 151.69$, $P > 0.01$). On average, *A. boreas* were the smallest of the three species, with *R. cascadae* being the largest and *P. regilla* as the intermediate (Table S1).

Discussion

Our results demonstrate a dilution effect in the Bd system. Increased species richness reduced disease risk in larval amphibians, even after controlling for changes in density. None of our measures indicated an amplification effect, and only one had a positive relationship with host density. Thus, this system follows trends predicted with the dilution effect.

Increased species richness caused a decrease in disease risk for four of the five measures tested. Specifically, both Bd prevalence and infection severity were reduced in treatments with greater species richness, whether we focused on *A. boreas* alone or all species together. Infection prevalence is an important measure of disease risk because it may indicate the likelihood of a susceptible host acquiring the pathogen. If fewer infected individuals are present in a system (as we found in treatments with high species richness), the encounter rate between susceptible and infected individuals is expected to be reduced. Infection severity, on the other hand, may be a good predictor of how strong an infection will become for an individual in a given system. Thus, because both measures of disease risk were negatively correlated with species richness, high-diversity systems may contain fewer infected individuals with reduced pathogen loads compared with systems with lower diversity.

The dilution effect has been found in other systems (4–9), and there are multiple mechanisms that could lead to this phenomenon. However, an inverse relationship between biodiversity and disease risk has not been previously reported regarding amphibians and Bd and has rarely been demonstrated in directly

Table 1. Summary information for the relationships among disease risk, focal species density (*A. boreas*), total species density, and species richness

Disease risk		Relationship with		
Measurement	Definition	All-species density	*A. boreas* density	Species richness
Toad infection prevalence	Percentage of *A. boreas* infected in each aquarium	None	None	Negative
All-species infection prevalence	Percentage of all individuals infected in each aquarium	Negative	None	Negative
Toad infection severity	Concentration of Bd in the mouthparts of *A. boreas*	None	None	Negative
All-species infection severity	Concentration of Bd in the mouthparts of all individuals	None	None	Negative
Total infection	Sum of Bd infection in each aquarium	None	Positive	None
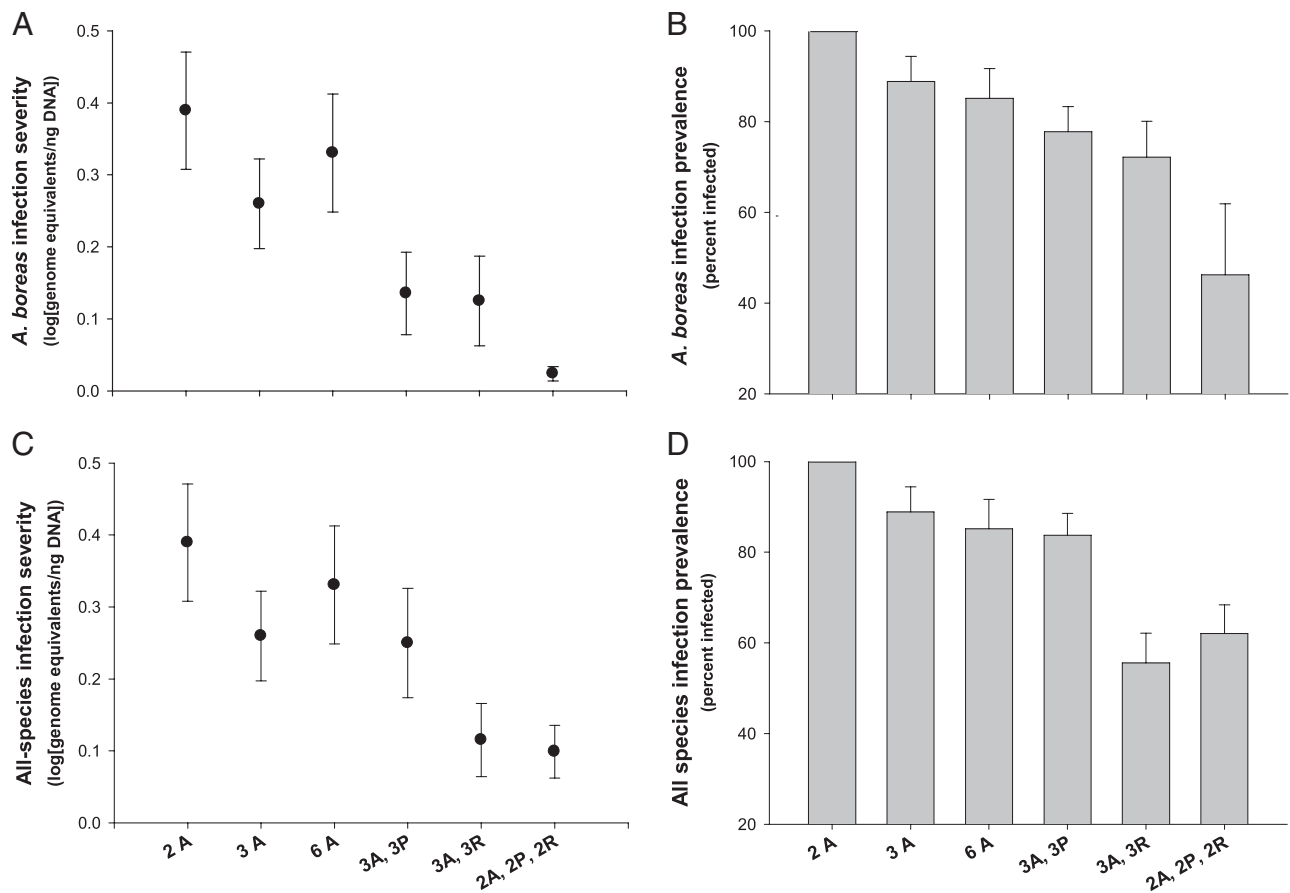
Fig. 1. Average infection severity and prevalence of Bd with varying host density and species richness. There were three *A. boreas*-only treatments with two, three, or six *A. boreas* individuals and three mixed-species combinations with six individuals and either two or three species. Treatments are labeled on the *x* axis representing the number of individuals of each species indicated as "A" for *A. boreas*, "P" for *P. regilla*, and "R" for *R. cascadae*. (*A* and *C*) Points represent the average Bd infection severity (log + 1-transformed; ±SE) for *A. boreas* (*A*) and for all species present (*C*). (*B* and *D*) Bars represent (+SE) the percentage *of A. boreas* testing positive for infection (*B*) and the percentage infection for all species (*D*). For the 2A treatment, all replicates showed 100% infection. Averages for all figures were calculated with all Bd-exposed aquaria, and each treatment was replicated nine times.

transmitted pathogens with density-dependent transmission. Therefore, this study indicates that the dilution effect may be possible in systems with density-dependent transmission or that Bd can exhibit frequency-dependent transmission (25). Additionally, a dilution effect in Bd may be context-dependent. For example, a correlative study investigating the relationship between habitat loss and Bd infection found that Bd occurrence was higher in human-disturbed areas, where biodiversity was also lower (38). Controlled experimental studies are necessary to understand the mechanisms generating dilution effects.

Differential rates of transmission among species may be one mechanism that drives the dilution effect we found. For example, if low-diversity systems contain individuals that are particularly effective at transmitting pathogens, a dilution effect may occur. In our study, *A. boreas* may shed more infectious particles (zoospores) than the other species do, driving the pattern where aquaria with only *A. boreas* had higher infection prevalence and severity than aquaria with more species. However, few studies have attempted to quantify transmission rates of Bd among individuals, and none have done so in a comparative framework with multiple species (25, 29).

The observed dilution effect could also be caused by changes in behavior or physiology in response to density of conspecifics or the presence of other species. Larval amphibians alter their behavior in response to their biotic and abiotic environment (39, 40). Different densities of conspecifics or the presence of other

species may alter behaviors such as schooling or foraging, which could change their likelihood of encountering Bd or transmitting it to other individuals. Additionally, density and species richness may affect stress in *A. boreas*, altering their susceptibility to Bd. Stress hormones such as corticosterone affect amphibian immune function (41–43) and can be elevated in response to high larval density (44) and presumably in the presence of interspecific competition. Thus, our manipulations of larval density and species richness could have altered stress hormones, subsequently altering *A. boreas*' susceptibility to Bd.

If our findings had been driven by changes in host density, we would expect to see a positive relationship between density and disease risk. However, there was no clear pattern between these two factors. Infection prevalence was negatively correlated with host density but only when focusing on all species. Thus, treatments with more individuals had lower infection prevalence, which was also negatively correlated with species richness. In contrast, aquaria with more *A. boreas* had higher levels of total infection, which was not affected by species richness or total number of individuals.

Across species and treatments, there were no differences in mortality. It is possible that higher doses of Bd or a longer exposure period can cause lethal effects. Larval amphibians can suffer mortality from Bd exposure in some cases (30), and they can alter behavior and rates of growth and development in response to infection (22, 45, 46). Because tadpoles can act as
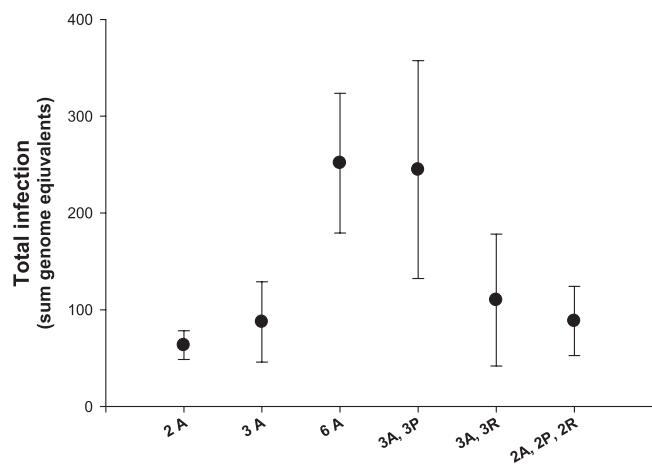
Fig. 2. Average total Bd infection by treatment. There were three *A. boreas*-only treatments with two, three, or six *A. boreas* individuals and three mixed-species combinations with six individuals and either two or three species. Treatments are labeled on the *x* axis representing the number of individuals of each species indicated as "A" for *A. boreas*, "P" for *P. regilla*, and "R" for *R. cascadae*. Points represent average total infection for Bd-exposed treatments (±SE). Only Bd-exposed treatments were used in calculating averages, and each treatment was replicated nine times.

important reservoirs for Bd (24, 29), it is essential to understand Bd dynamics in larvae so that we may better predict dynamics in communities.

Our study focused on *A. boreas*, which has suffered population declines throughout much of its range, in some cases correlated with the presence of Bd (32, 47, 48). The results of our study indicate that *A. boreas* may experience the lowest risk from Bd in high-diversity systems. Thus, in developing a conservation plan for *A. boreas* and other species suffering Bd-related declines, it is essential to consider the presence of other amphibian species in predicting the prevalence and severity of Bd infection. Additionally, other susceptible amphibian species may exhibit similar patterns with Bd infection and species richness. Thus, loss of amphibian biodiversity from other factors such as habitat loss and pollution may produce communities that are more at risk of Bd-associated declines.

Materials and Methods

Collection and Rearing. To ensure that animals were not initially infected with Bd, we collected all species as eggs from sites in the Cascade Mountains, Oregon. *A. boreas* and *P. regilla* eggs were collected from Little Three Creeks Lake (Deschutes County, elevation 2093 m), and *R. cascadae* eggs were collected from Parrish Pond (Linn County, elevation 1130 m). Eggs were collected from at least six egg masses for each species and brought to the laboratory for rearing in 38-L aquaria filled with dechlorinated water. Temperature in the laboratory was maintained at 13.5–15.0 °C. Upon hatching, animals were fed a 3:1 ratio (by volume) of rabbit chow to fish food (TetraMin).

Experimental Procedure. Our experimental unit was a rectangular plastic aquarium (8.5 × 20 × 30 cm) filled with 2.5 L of dechlorinated water. We manipulated both the density of *A. boreas* and the number of species present in each aquarium. Our density treatments were aquaria with two, three, or six *A. boreas*. Species richness was manipulated by keeping density constant at six individuals and combining *A. boreas* with either *P. regilla* (three *A. boreas* and three *P. regilla*), *R. cascadae* (three *A. boreas* and three *R. cascadae*), or both (two *A. boreas*, two *P. regilla*, and two *R. cascadae*). Additionally, each aquarium was randomly assigned to be exposed to either Bd or control (unexposed). Therefore, we had 12 total treatments (6 density/richness treatments each either exposed to Bd or unexposed) that were each replicated nine times.

Animals were placed into their experimental aquarium and given 1 h to acclimate before application of their Bd treatment. We cultured Bd (JEL

strain 274, originally isolated from *A. boreas* in Colorado, stored at −80 °C, then grown in a tryptone solution for 19 mo) on 1% tryptone agar Petri plates that had been kept at ~14 °C for 15 d. We harvested Bd by flooding plates with distilled water and waiting for 30 min for zoospores to release into the water. We then pooled inoculum from 20 plates and quantified zoospores with a hemocytometer. This inoculum was then diluted 1:10, and 20 mL was added to each aquarium. After 5 days, before any mortality had occurred, the animals were exposed again in the same manner. Combined, these two inoculations added 2.47×10^4 zoospores to each Bd-exposed aquarium. Control animals were inoculated under an identical regime but using sterile agar plates to create the inoculum.

We monitored aquaria daily for mortality, and dead animals were immediately removed and preserved individually in 95% ethanol. Animals were fed ad libitum, and water was changed every 2 wk. At 35 d after initiation of the experiment, which is long enough for multiple life cycles of Bd (20), we killed all animals in MS-222 and preserved them in 95% ethanol. We measured both mass (to the nearest milligram) and length (snout-vent length; to the nearest 0.1 mm) for all individuals. We then extracted whole mouthparts from all Bd-exposed animals and 10 randomly selected control animals for infection analysis with qPCR. Our qPCR methods followed those of Boyle et al. (49) except that we used 60 μL of PrepMan Ultra (Applied Biosystems) instead of 40 mL in DNA extractions. Extractions were diluted 1:10 and processed in an ABI PRISM 7500 (Applied Biosystems). We compared samples to known quantities of Bd (100, 10, 1, and 0.1 genome equivalents). Each sample was analyzed in triplicate, and the average number of genome equivalents Bd per animal was calculated. We refer to the raw qPCR output (average genome equivalents per sample) as "gross infection," which is a relative value of the total amount of Bd in each animal's mouthpart. A sample was considered positive if at least two of the three replicates were positive. Additionally, to account for potential differences in size among species and treatments, each sample was analyzed three times in a Nano-Drop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific) to determine the concentration of DNA in each sample. This information allowed us to standardize infection severity regardless of host size by calculating infection severity as genome equivalents (determined by qPCR) per nanogram of mouthpart DNA (determined on the NanoDrop spectrophotometer). "Infection severity" is therefore the concentration of Bd in the mouthparts of each animal. Similar methods are used to standardize pathogen loads in ranaviruses (50–52).

Statistical Analysis. All statistical analyses were performed in R statistical software environment (version 2.9.0). None of our Bd-control animals were infected, so all infection statistics were performed with data from Bd-exposed aquaria only. Our first analyses focused on the effects of density and species richness on our focal species, *A. boreas*, by using generalized linear models (GLMs). Our model analyzed log-transformed toad infection severity or gross infection (with and without length and weight as cofactors) with the following predictors: number of *A. boreas*, number of species, and total number of individuals. Individuals were nested by aquarium. Our findings were the same regardless of whether we included Bd-exposed *A. boreas* that tested negative for infection (i.e., with an infection severity of zero) or if we excluded these individuals from the analyses. We report statistics and figures that include all Bd-exposed animals. We also performed a GLM on the percentage of *A. boreas* individuals infected in each container (toad infection prevalence) by using the same predictors.

In addition to infection in our focal species, *A. boreas*, we also determined infection patterns across all species: all-species infection severity, all-species gross infection, all-species infection prevalence, and the total amount of Bd in all individuals within an aquarium (referred to as "total infection"; Table 1). We performed GLMs on log-transformed all-species infection severity, including all Bd-exposed individuals and again using only those that tested positive for infection. Our results were the same whether we included Bd-negative individuals in the analyses, so we report statistics and figures including all Bd-exposed animals. Gross infection was analyzed both with and without length and weight as cofactors. To determine total infection, we used the sum of the Bd quantity of all individuals in a replicate (gross amount, not in the form of Bd concentration). If a replicate contained an individual that died and was not recovered (presumably because of cannibalism), this replicate was not included in the analysis of total infection. For these analyses, we performed GLMs with predictors of number of *A. boreas* individuals, number of species present, and total number of individuals. Individuals were nested by aquarium for analyses of all-species infection severity and gross infection. Additionally, to determine the effects of treatment on *R. cascadae* and *P. regilla* infection, we analyzed log-transformed infection severity (averaged by aquarium), log-transformed gross

infection (averaged by aquarium), and prevalence for *R. cascadae* and *P. regilla* between treatments by using a two-tailed *t* test for each species.

We tested the effect of Bd treatment, density/richness treatment, and species identity on mortality by using logistic regression nested by aquarium. Mass, length, log-transformed infection severity, and log-transformed gross infection were compared among species by using a GLM with individuals nested by aquarium. To compare mass and length of *A. boreas* among treatments, we performed a GLM with predictors for number of *A. boreas* individuals, number of species, number of individuals, and Bd treatment (ex-

posed or unexposed). For *R. cascadae* and *P. regilla*, we tested the effects of Bd treatment and number of species present (two or three) on average mass and length by using a two-way ANOVA.

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