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Linking manipulative experiments to field data to test the dilution effect

Matthew D. Venesky[1,*†], Xuan Liu[2], Erin L. Sauer[1] and Jason R. Rohr[1]

[1]*Department of Integrative Biology, University of South Florida, Tampa, FL 33620, USA;* [2]*Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang, Beijing 100101, China*

Summary

1. The dilution effect, the hypothesis that biodiversity reduces disease risk, has received support in many systems. However, few dilution effect studies have linked mechanistic experiments to field patterns to establish both causality and ecological relevance.

2. We conducted a series of laboratory experiments and tested the dilution effect hypothesis in an amphibian-*Batrachochytrium dendrobatidis* (*Bd*) system and tested for consistency between our laboratory experiments and field patterns of amphibian species richness, host identity and *Bd* prevalence.

3. In our laboratory experiments, we show that tadpoles can filter feed *Bd* zoospores and that the degree of suspension feeding was positively associated with their dilution potential. The obligate suspension feeder, *Gastrophryne carolinensis*, generally diluted the risk of chytridiomycosis for tadpoles of *Bufo terrestris* and *Hyla cinerea*, whereas tadpoles of *B. terrestris* (an obligate benthos feeder) generally amplified infections for the other species. In addition, *G. carolinensis* reduced *Bd* abundance on *H. cinerea* more so in the presence than absence of *B. terrestris* and *B. terrestris* amplified *Bd* abundance on *H. cinerea* more so in the absence than presence of *G. carolinensis*. Also, when ignoring species identity, species richness was a significant negative predictor of *Bd* abundance.

4. In our analysis of field data, the presence of *Bufo* spp. and *Gastrophryne* spp. were significant positive and negative predictors of *Bd* prevalence, respectively, even after controlling for climate, vegetation, anthropogenic factors (human footprint), species richness and sampling effort. These patterns of dilution and amplification supported our laboratory findings, demonstrating that the results are likely ecologically relevant.

5. The results from our laboratory and field data support the dilution effect hypothesis and also suggest that dilution and amplification are predictable based on host traits. Our study is among the first to link manipulative experiments, in which a potential dilution mechanism is supported, with analyses of field data on species richness, host identity, spatial autocorrelation and disease prevalence.

Key-words: amphibian, chytridiomycosis, dilution effect, disease ecology, species richness, tadpole

Introduction

Infectious diseases of wildlife and humans are emerging at an unprecedented rate; thus, it is important to understand the ecological drivers of disease dynamics (Harvell *et al.* 1999; Daszak, Cunningham & Hyatt 2000; Dobson & Foufopoulos 2001). Many emerging pathogens infect multiple host species that vary in their resistance (ability to prevent or clear infections) and tolerance (ability to minimize the fitness consequences of infections) to pathogens (Raberg, Graham & Read 2009; Rohr, Raffel & Hall 2010). Hence, certain host species might amplify pathogen abundance, whereas others might reduce abundance. For example, the addition of tolerant host species to a community might increase pathogen prevalence and transmission (Roy & Kirchner 2000). In contrast, increased host

*Correspondence author. E-mail: mvenesky@gmail.com
†Current address: Department of Biology,Allegheny College,520 North Main Street, Meadville, PA 16335, USA

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diversity might reduce pathogen transmission if the relative abundance of competent hosts for the pathogen decreases as a function of increased biodiversity because transmission events are 'wasted' on low competent hosts (LoGiudice *et al*. 2003; Johnson *et al*. 2008).

This variation in host species susceptibility to pathogens, coupled with substantial spatiotemporal variation in host community composition and widespread losses of biodiversity, has generated considerable interest in the relationship between biodiversity and disease risk (e.g. Dobson *et al*. 2006; Keesing, Holt & Ostfeld 2006; Keesing *et al*. 2010). The 'dilution effect', which is the hypothesis that increased biodiversity (including host and non-host species) generally reduces disease risk, has garnered considerable support in a number of host–pathogen systems (reviewed in Keesing *et al*. 2010; but see Randolph & Dobson 2012 for a critique of the dilution effect). Several mechanisms have been proposed for how biodiversity can reduce disease risk, such as by reducing encounters between hosts and parasites, reducing transmission after encounters have occurred, increasing host recovery from infection, increasing mortality of infected hosts or decreasing the density of susceptible hosts (Keesing *et al*. 2010). However, to date, few dilution effect studies have linked mechanistic experiments to field patterns to establish both causality and ecological relevance (but see Johnson *et al*. 2013). These experimental links are important because they can transform dilution effect studies from being phenomenological to studies that can offer specific predictions regarding when and where pathogens might be problematic and how to manage emerging diseases.

Here, we present the results of laboratory experiments and field surveys examining the dilution effect in an amphibian-chytridiomycosis system. Chytridiomycosis is an emerging disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) and is in particular need of management because it is implicated in the declines of hundreds of amphibian species world-wide (Rohr *et al*. 2008; Wake & Vredenburg 2008). Although the pathogen appears to cause greater mortality after than before metamorphosis, amphibian larvae are suspected to be important reservoirs for *Bd*, potentially maintaining and increasing *Bd* in the environment (Briggs, Knapp & Vredenburg 2010). Despite the extreme virulence of *Bd* and the potential value of having reliable predictions regarding where it might be problematic, the role of host diversity in the distribution, severity or emergence of this disease has rarely been considered (but see Searle *et al*. 2011). If *Bd* abundance or transmission is affected by biodiversity, then variation in biodiversity might help explain its spatial distribution and identify which amphibian communities might be buffered from the adverse effects of chytridiomycosis.

In our first experiment, we conducted feeding trials with tadpoles of Southern toads (*Bufo terrestris*), Green treefrogs (*Hyla cinerea*) and Eastern narrowmouth toads (*Gastrophryne carolinensis*) to test whether they can remove (e.g. filter) *Bd* zoospores from the water column. We then used a fully factorial laboratory experiment to examine both the effects of density and diversity on *Bd* abundance in replicated experimental aquaria. *Bd* infects keratinized cells of tadpole mouthparts, and tadpole species vary in the amount of keratin in their mouthparts and also in the degree to which they suspension feed (Altig & McDiarmid 1999). Thus, species differences in keratin and suspension feeding might reduce the density of *Bd* zoospores in the water column and thus host exposure to infection (Kagami *et al*. 2004; Hamilton, Richardson & Anholt 2012). We hypothesized that *G. carolinensis* would filter the most *Bd* zoospores from the water and would have the greatest potential to dilute *Bd* because tadpoles of this species are obligatory suspension-feeders and lack keratinized mouthparts (Wassersug & Rosenberg 1979). Tadpoles of *B. terrestris* use their keratinized mouthparts to scrape algae off surfaces (Venesky *et al*. 2011) and, along with other bufonids, are considered highly susceptible to *Bd*. Thus, we hypothesized that this species would consume few zoospores but would carry high *Bd* infections, thereby amplifying disease risk for the other species. *Hyla cinerea* tadpoles are mid-water feeders that have keratinized mouthparts and use a combination of algae-scraping and facultative suspension feeding (Altig & Kelly 1974). Hence, we hypothesized that this species would consume *Bd* zoospores but would also carry *Bd* infections and thus would have intermediate diluting potential relative to the other species.

We then examined *Bd* prevalence of 11 616 amphibians sampled in the U.S. to determine whether field patterns were consistent with our laboratory findings. We tested whether the presence of the focal genera used in our laboratory studies were positive or negative predictors of *Bd* prevalence, while controlling for sampling effort, climate, vegetation and the human footprint index because these variables can be significant predictors of *Bd* (Adams *et al*. 2010; Liu, Rohr & Li 2013; Olson *et al*. 2013). We predicted that *Bd* prevalence in the field would be diluted and amplified by the presence of *Gastrophryne* spp. and *Bufo* spp., respectively.

Materials and methods

ANIMAL COLLECTION AND HUSBANDRY

We collected early-staged *B. terrestris* and *G. carolinensis* tadpoles [Gosner (Gosner 1960) stages 26–30] and recently oviposited eggs of *H. cinerea* from three wetlands in Hillsborough County, FL, USA in July 2011. *Bd* has not been reported from these or other wetlands in Hillsborough County. Immediately after collection, the tadpoles and eggs were transported to the laboratory at The University of South Florida where they were separated by species and placed into three 37·85 L glass aquaria filled with approximately 25 L of artificial spring water. Prior to the start of the experiment, *B. terrestris* and *H. cinerea* tadpoles were fed a mixture of powered commercial algal-based food containing *Spirulina* and sea algae meal (Sera Micron®, Sera,

Germany) and spinach leaves *ad libitum* daily. *Gastrophryne carolinensis* tadpoles, which are obligatory suspension-feeders, were fed Sera Micron *ad libitum* daily. All tadpoles were maintained in the laboratory on a 12 L: 12 D photoperiod at 22 °C (±2 °C) until *H. cinerea* tadpoles developed to stage 26. At that point, we selected similarly staged (*c.* stage 29–32) tadpoles of each species to be used in our experiments.

Bd ZOOSPORE FILTERING TRIALS

We assigned the following treatments to 100-mL plastic containers filled with 60 mL of autoclaved DI water: one *B. terrestris* tadpole, one *H. cinerea* tadpole, one *G. carolinensis* tadpole or no tadpole. Each treatment was replicated six times for a total of 24 experimental containers. To account for time to collect data, we split the experiment into two temporal blocks (that differed only in their starting time), each containing 12 containers. At the start of the experiment, the tadpoles had developed to Gosner stages 29–32 (*B. terrestris*: 31·2 ± 0·75; *H. cinerea*: 31·7 ± 0·56; *G. carolinensis*: 31·5 ± 0·62; mean ± SE). After a 24-h acclimation period, we inoculated each container with 1·0 mL of tryptone broth containing approximately 1×10^6 *Bd* zoospores of strain SRS 812. *Bd* SRS 812 was originally cultured from a bullfrog (*Rana catesbeiana*) tadpole at the Savanna River Site, SC, USA in August 2006 and has since been maintained in culture at 4 °C. After 4 h, we removed the tadpole and pipetted 150 μL of water from the surface, middle and bottom of each container (total of 450 μL/container) and counted the total number of zoospores on a hemocytometer. We used this value as an estimate of the zoospores not removed (i.e. not consumed) from the water by a tadpole. We hypothesized that tadpoles of *G. carolinensis* would consume *Bd* zoospores because they are obligatory suspension feeders and that *B. terrestris* would not consume *Bd* zoospores because they feed by scraping algal-covered surfaces. We recognize that we cannot discriminate between reductions in ambient zoospores that occur because of successful infection of a tadpole and zoospores that are removed via consumption by a tadpole. We used a generalized linear model with a Poisson error distribution to test for effects of species present (*B. terrestris*, *H. cinerea*, *G. carolinensis* or no tadpole) and temporal block on the number of zoospores recovered from the water. We tested for all main effects and two-way interactions using log-likelihood ratio tests. Analyses for the zoospore feeding experiment were conducted in Statistica (v. 6·1) (Statsoft, Inc. Tulsa, OK, USA).

In a corollary experiment, we exposed tadpoles of *G. carolinensis* and *B. terrestris* ($N = 6$ per species) to an infectious dose of *Bd* as described above or to tryptone broth alone ($N = 6$ per species). We removed each tadpole after four hours, euthanatized them with an overdose of MS222 and dissected each intestine using sterile equipment. We then swabbed inside of each intestine and tested for the presence of *Bd* using quantitative PCR (qPCR) analysis (see Supplemental Methods for further details, Supporting information). We hypothesized that we would only detect *Bd* DNA in the intestines of the tadpole species that filter feed *Bd* zoospores (i.e. *G. carolinensis*).

DILUTION EFFECT EXPERIMENT

The *Bd* zoospore feeding experiment was designed to test for a potential mechanism for *Bd* dilution. In the present experiment, we tested whether differences in *Bd* consumption could dilute risk of chytridiomycosis. We conducted a fully factorial laboratory experiment to isolate the main and interactive effects of tadpole density and diversity on *Bd* abundance. We manipulated the total density of tadpoles by placing either six or 12 tadpoles in rectangular plastic containers (33 × 17·75 × 11·4 cm) filled with 3·0 L of artificial spring water. Within each density treatment, we then manipulated species diversity such that each host species was raised in either monospecific or heterospecific (i.e. two and three-species) communities (Table S1, Supporting information). The resulting treatment combinations were replicated four times totalling 56 experimental units.

On 20 July 2011, we randomized the position of the containers on three laboratory shelves, added the tadpoles to their assigned containers and allowed them to acclimate for 24 h. On 21 July 2011 (Day 0), we inoculated each container with 6·0 mL of tryptone broth containing approximately $4{\cdot}25 \times 10^5$ *Bd* zoospores (SRS 812). On Day 9, we inoculated each container a second time with 3·0 mL of tryptone broth containing approximately 1×10^5 *Bd* zoospores. We monitored the containers twice daily for mortality and preserved dead tadpoles individually in 100% ethanol. Throughout the experiment, we changed 50% of the volume of water in each aquarium every 4 days by pouring out 1·5 L through an aquarium net and replacing it with an equal volume of artificial spring water. Immediately after each water change, we fed the tadpoles a mixture of Sera Micron® and spinach leaves *ad libitum*. On day 21, we euthanized all the tadpoles with MS-222 (Webb *et al.* 2005) and preserved them in 100% ethanol. We then measured the mass (to the nearest milligram) and the Gosner stage of every tadpole, dissected their mouthparts and stored mouthparts at −20 °C for qPCR analysis (see Supplemental Methods for further details, Supporting information).

During the course of the experiment, we dipped all of our equipment (e.g. aquarium nets, forceps and iridectomy scissors) sequentially in 10% bleach, 1% Novaqua® (Kordon, LLC. San Francisco, CA, USA) (which neutralizes bleach), and deionized water whenever handling different species within a replicate, or before switching to another replicate, to prevent any accidental transfer of *Bd* zoospores or DNA.

To test for effects of factors on *Bd* abundance, we used mixed-effects models with a zero-inflated negative binomial distribution, nested individual tadpoles within tanks (a random effect) and tested for significance using log-likelihood ratio tests (using the 'glmmADMB' function in the 'glmmADMB' package in R). Zero-inflated negative binomial distribution assumes that the response variable is a function of a binomial process (uninfected vs. infected) and a count process (negative binomial distributed infection intensity) and had the lowest Akaike Information Criterion (AIC_c) value of six error distributions tested.

In our first generalized mixed-effects model, we analysed the effects of density and diversity on the total amount of *Bd* in each tank (ignoring species identity). In this model, we included the total density of tadpoles and the number of species present as predictors. We then tested for differences in species' resistance, measured as *Bd* abundance (zoospore genome equivalents) of each species when raised in the absence of heterospecifics. We included density and mass of each focal species as predictors in our analyses. We used a sequential Bonferroni alpha adjustment to compare levels of species richness (1 vs. 2, 1 vs. 3 and 2 vs. 3). Lastly, we analysed the effects of the presence of heterospecific tadpoles on *Bd* abundance for each focal species (*B. terrestris*, *H. cinerea*, and *G. carolinensis*).

RELATIONSHIP BETWEEN HOST IDENTITY AND *Bd* PREVALENCE IN THE FIELD

To test for consistencies between our laboratory experiments and field patterns of host identity and chytridiomycosis risk within the U.S., we used published records of *Bd* infection prevalence from the Global *Bd*-Mapping Project (www.spatial-epidemiology.net/Bd/) and unpublished data on *Bd* prevalence from our laboratory and calculated the average *Bd* prevalence in each of the 881 grid cells (2·5 arc-minutes grid) across the U.S. Amphibian species richness at each grid cell was acquired by overlaying GIS layers of amphibian range maps from the IUCN Global Amphibian Assessment (Stuart *et al.* 2004). For each grid cell, we also obtained 19 climate variables from the WorldClim data base (1950–2000) (Hijmans *et al.* 2005), the normalized difference vegetation index (NDVI; from 1982–2000, excluding 1994; http://edit.csic.es/Soil-Vegetation-LandCover.html), and an estimate of human influence on the landscape, expressed as the human footprint index (Sanderson *et al.* 2002; data available at http://www.ciesin.columbia.edu/wild_areas/). NDVI has been widely used in ecological studies by epidemiologists as a surrogate measure of vegetation type and ground moisture, which may affect the survival and prevalence of many pathogens (Rogers *et al.* 2002; Pettorelli 2006). We conducted a factor analysis on the 19 climate variables and used the scores from the first three principal component axes (hereafter PC1, PC2 and PC3), which accounted for 81·7% variation. All of the predictors and *Bd* prevalence were rescaled to the same resolution as amphibian species richness (2·5 arc minutes) using a bilinear function, which is considered more realistic than the simpler nearest-neighbour method (Phillips, Anderson & Schapire 2006).

There were not enough data to evaluate our species composition hypotheses at the level of species, so we conducted our analyses on the three genera, assuming that there is less variation within than among genera (Olson *et al.* 2013). We first trimmed our U.S. data base into three subsets by overlaying GIS layers of *Bufo*, *Hyla* and *Gastrophryne* range maps from the IUCN Global Amphibian Assessment (Stuart *et al.* 2004) onto our data base. This resulted in 796, 343 and 167 grid cells within the range of *Bufo*, *Hyla* and *Gastrophryne*, respectively. We used the software package Spatial Analysis in Macroecology (Rangel, Diniz & Bini 2010) to control for spatial autocorrelation while testing for relationships between the presence of each focal genus (predictor variable) and *Bd* prevalence of the amphibian community at each grid cell (response variable; i.e. logistic regression) while statistically controlling for the effects of climate, species richness, vegetation, sampling effort and human footprint. In this analysis, we did not test whether *Bd* prevalence was lower within the geographic range of one genus compared with another genus (e.g. whether *Bd* prevalence was lower within the range of *Gastrophryne* compared with the range of *Hyla*). Instead, within the geographic range of each host genus *Bufo*, *Hyla* and *Gastrophryne*), we tested whether the presence of amphibians of each host genus was a positive or negative predictor of *Bd* prevalence of the entire amphibian community (e.g. whether *Bd* prevalence of the amphibian community was higher or lower when *Gastrophryne* were present at a site within their geographic range). We hypothesized that the presence of *Bufo* and *Gastrophryne* would be positive and negative predictors of *Bd* prevalence within their respective geographic ranges.

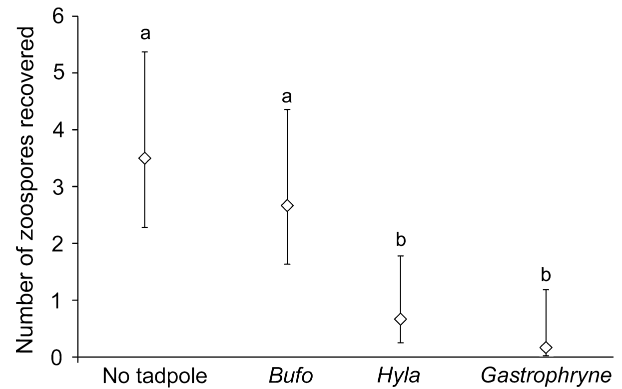

Fig. 1. Average number of *Batrachochytrium dendrobatidis* zoospores recovered from 450 μL of water in each replicate. A single tadpole of each species (*Bufo terrestris*, *Hyla cinerea* or *Gastrophryne carolinensis*) was allocated to each container, which was filled with 60 mL of autoclaved DI water and inoculated with $1{\cdot}0 \times 10^6$ zoospores. The treatment group without a tadpole served as the control. Values are represented as means (±95% CI). Data points with different letters are significantly different from one another ($P < 0{\cdot}05$).

Results

Bd ZOOSPORE FILTERING TRIALS

There was a main effect of species on the number of zoospores recovered from the containers (log-likelihood ratio test: $\chi^2 = 30{\cdot}17$, $P < 0{\cdot}0001$; Fig. 1). Compared with containers without a tadpole, significantly fewer zoospores were recovered in containers with *H. cinerea* or *G. carolinensis* (log-likelihood ratio test: $\chi^2 = 12{\cdot}67$, $P = 0{\cdot}0003$; $\chi^2 = 22{\cdot}36$, $P < 0{\cdot}0001$, respectively). The number of zoospores in containers with *B. terrestris* did not differ from containers without a tadpole (log-likelihood ratio test: $\chi^2 = 0{\cdot}68$, $P = 0{\cdot}410$). Neither temporal block nor the block-by-species interaction ($P > 0{\cdot}259$) was significant. In addition, the intestines of five of the six tadpoles of *G. carolinensis* exposed to *Bd* were positive for *Bd,* whereas 0 of the six tadpoles of *B. terrestris* had *Bd* DNA in their intestines. None of the control tadpoles tested positive for *Bd*.

DILUTION EFFECT EXPERIMENT

Mortality in this experiment was negligible (~ 2%) and occurred evenly across density treatments (i.e. in four and five replications in low- and high-density treatments, respectively). No more than one individual died within a single replicate.

We first analysed single- and mixed-species treatments with overall *Bd* abundance per tank as the response variable. These analyses revealed a significant negative relationship between number of host species and *Bd* abundance (log-likelihood ratio test: $\chi^2 = 3{\cdot}840$, $P = 0{\cdot}050$; Fig. 2), but neither density (log-likelihood ratio test: $\chi^2 = 0{\cdot}220$, $P = 0{\cdot}639$) nor the interaction between density and

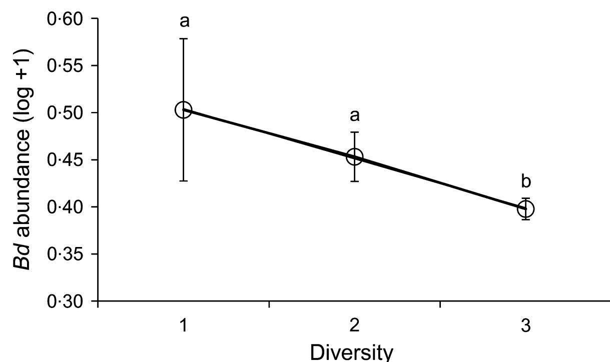

Fig. 2. The relationship between *Bd* abundance and species diversity (ignoring species identity) in the laboratory dilution effect experiment ($\chi^2 = 3{\cdot}840$, $P = 0{\cdot}050$). Values are tank means (±SE). Data points with different letters are significantly different from one another ($P < 0{\cdot}05$).

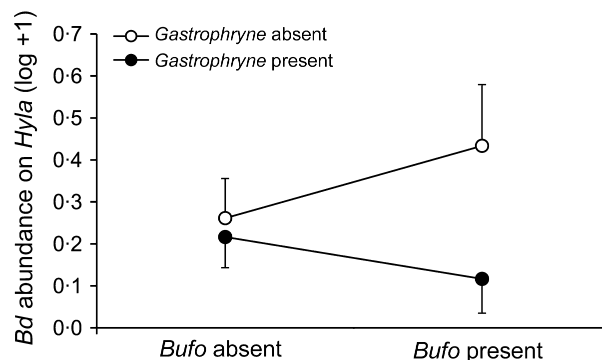

Fig. 3. The interactive effects of *Gastrophryne carolinensis* and *Bufo terrestris* tadpoles on *Bd* abundance of *Hyla cinerea* tadpoles ($\chi^2 = 6{\cdot}874$, $P = 0{\cdot}0087$). Values are tank means (±SE).

diversity (log-likelihood ratio test: $\chi^2 = 0{\cdot}700$, $P = 0{\cdot}403$) was significant. Further analyses revealed that increases in species richness from either one or two species to three species significantly reduced *Bd* abundance (log-likelihood ratio test: $\chi^2 = 4{\cdot}970$, $P = 0{\cdot}026$; $\chi^2 = 8{\cdot}356$, $P = 0{\cdot}003$; respectively). We then examined *Bd* abundance within single-species treatments and found an overall difference among species in their *Bd* abundance (log-likelihood ratio test: $\chi^2 = 8{\cdot}896$, $P = 0{\cdot}011$), which was independent of the density at which the tadpoles were raised (log-likelihood ratio test: $\chi^2 = 0{\cdot}716$, $P = 0{\cdot}398$). *Bufo terrestris* had the highest *Bd* abundance followed by *H. cinerea* and then *G. carolinensis* (Fig. S1, Supporting information).

Several predictions arise from our findings thus far if we assume that the filtering rate is independent of heterospecifics. First, given that *G. carolinensis* had the highest filtering rate and *B. terrestris* produced the most zoospores, we would expect *G. carolinensis* to have the greatest diluting effect on *B. terrestris* and *B. terrestris* to have the greatest amplifying effect on *G. carolinensis*. Secondly, we would expect the diluting effect of *G. carolinensis* on *H. cinerea* to be greater in the presence of *B. terrestris* because *G. carolinensis* would be filtering more zoospores when *B. terrestris* is present than when it is absent (because it produces the most zoospores). Based on similar logic, we would expect the amplifying effect of *B. terrestris* on *H. cinerea* to be greatest in the absence of *G. carolinensis*. Given that density was never significant in any of the analyses above, we tested these species composition hypotheses excluding the effect of density.

As predicted, *G. carolinensis* strongly reduced *Bd* abundance on *B. terrestris* (log-likelihood ratio test: $\chi^2 = 12{\cdot}962$, $P < 0{\cdot}0004$, coefficient: $-1{\cdot}216$) and *B. terrestris* strongly increased the *Bd* abundance on *G. carolinensis* (log-likelihood ratio test: $\chi^2 = 4{\cdot}342$, $P = 0{\cdot}0372$, coefficient: 0·232). *H. cinerea* also reduced *Bd* abundance on *G. carolinensis* (coefficient: 0·202, $\chi^2 = 4{\cdot}232$, $P = 0{\cdot}0397$) but had no other significant main effects or interactions (log-likelihood ratio test: $\chi^2 = 2{\cdot}286$, $P > 0{\cdot}1304$). Also as predicted, *G. carolinensis* and *B. terrestris* interacted to affect *Bd* abundance on *H. cinerea* (log-likelihood ratio test: $\chi^2 = 6{\cdot}874$, $P = 0{\cdot}0087$; Fig. 3). *Gastrophryne carolinensis* reduced *Bd* abundance on *H. cinerea* more so in the presence than absence of *B. terrestris* and *B. terrestris* amplified *Bd* abundance of *H. cinerea* more so in the absence than presence of *G. carolinensis* (Fig. 3). When examining the net effects of each species on the tested heterospecifics, *B. terrestris* generally amplified risk of chytridiomycosis, *H. cinerea* weakly diluted risk, and *G. carolinensis* was generally the strongest diluter (Fig. 4a).

RELATIONSHIP BETWEEN DIVERSITY AND *Bd* PREVALENCE IN THE FIELD

Even after accounting for climactic, vegetation and anthropogenic (human footprint) factors known to influence the prevalence of *Bd* (Adams *et al.* 2010; Liu, Rohr & Li 2013; Olson *et al.* 2013), the presence of *Bufo* spp. was a significant positive predictor of *Bd* prevalence, whereas the presence of *Gastrophryne* spp. was a significant negative predictor of *Bd* prevalence of all amphibians in a sampled community (Table 1; Fig. 4b). The presence of *Hyla* spp. was not a significant predictor of *Bd* prevalence. These results were consistent with the results of our laboratory experiments.

Discussion

The central principle of the dilution effect is that increased biodiversity will reduce disease risk because the relative abundance of competent hosts decreases as a function of increasing species richness (Ostfeld & Keesing 2000). In our experiments, we found that *Bd* abundance at the tank level (i.e. considering all species) decreased as a function of increasing tadpole diversity. We did not find any support that density could explain our results, despite other studies showing density effects for different host–parasite systems (e.g. Raffel *et al.* 2010).

Increased biodiversity can reduce disease risk through a variety of mechanisms, most notably by reducing encounters

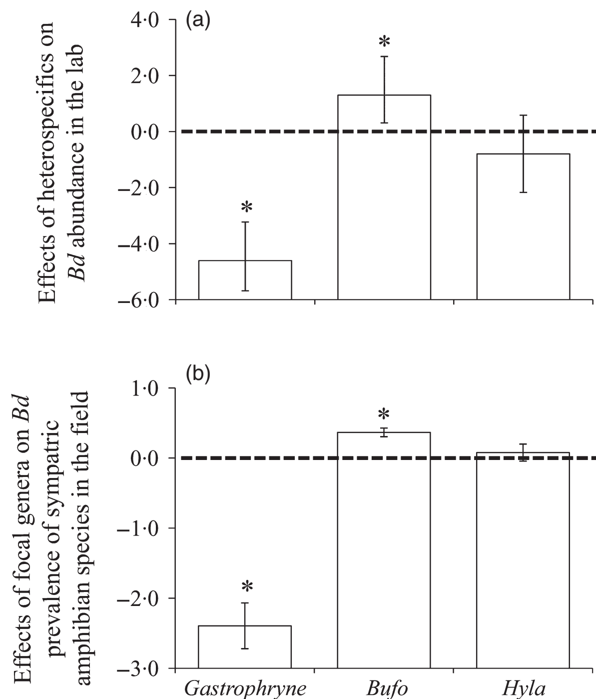

Fig. 4. (a) The net effects of each species on heterospecific *Bd* abundance in the laboratory dilution effect experiment (coefficient ± SE from a zero-inflated negative binomial model on *Bd* abundance of heterospecifics with species, density, focal genus and a density-by-focal genus effects and tank as a random factor). (b) The net effects of each genus on *Bd* prevalence in the field (coefficient ± SE from a logistic regression model on *Bd* prevalence of all amphibians screened for *Bd* in localities containing the focal genera controlling for spatial autocorrelation). For both panels, coefficients with asterisks are significantly different from zero.

between hosts and parasites or reducing transmission events after encounters have occurred (reviewed in Keesing, Holt & Ostfeld 2006; Johnson & Thieltges 2010). Understanding which mechanisms lead to a dilution effect and whether different host–pathogen systems share a common mechanism can help improve our ability to predict when the dilution effect will/will not occur. The results from our *Bd* zoospore removal experiments provide a mechanistic link between our dilution effect experiment and field patterns with *Gastrophryne* spp., indicating that the filtering capacities of *G. carolinensis* tadpoles might effectively remove *Bd* from aquatic environments and reduce the risk of chytridiomycosis ('encounter reduction' sensu; Keesing, Holt & Ostfeld 2006). The fact that *Bd* DNA was found in the intestine of the obligatory suspension-feeding tadpole *G. carolinensis* but not in the algal-scraping tadpole *B. terrestris* further supports this mechanism. This result is also consistent with a recent study, which found that suspension-feeding cladocerans (*Daphnia* spp.) can consume *Bd* zoospores (Buck, Truong & Blaustein 2011; Hamilton, Richardson & Anholt 2012). Indeed, it is common for tadpoles of some suspension-feeding species to filter algal cells between 2·7–5·1 μm in

Table 1. The univariate effects of the eight variables on *Batrachochytrium dendrobatidis* prevalence within the geographic range of *Bufo* spp., *Hyla* spp. and *Gastrophryne* spp. Principle component axes 1–3 ('PC1–3′') represent the most important climactic variables and 'NDVI' is a measure of the vegetation index

Variable	Coefficient	SE	P
Geographic range of *Bufo* spp.			
Bufo spp. present	0·367	0·062	<0·001
Number of amphibians sampled	−0·008	0·001	<0·001
Amphibian species richness	0·001	0·004	0·789
PC1	0·223	0·024	<0·001
PC2	0·119	0·016	<0·001
PC3	−0·098	0·016	<0·001
NDVI	0·004	0·002	0·095
Human footprint	−0·002	0·002	0·365
Geographic range of *Hyla* spp.			
Hyla spp. present	0·078	0·122	0·524
Number of amphibians sampled	−0·007	0·001	<0·001
Amphibian species richness	0·029	0·006	<0·001
PC1	0·236	0·037	<0·001
PC2	0·153	0·059	0·009
PC3	−0·225	0·030	<0·001
NDVI	−0·016	0·004	<0·001
Human footprint	0·015	0·003	<0·001
Geographic range of *Gastrophryne* spp.			
Gastrophryne spp. present	−2·394	0·327	<0·001
Number of amphibians sampled	0·001	0·001	0·804
Amphibian species richness	0·048	0·011	<0·001
PC1	0·578	0·081	<0·001
PC2	−1·145	0·142	<0·001
PC3	−0·226	0·086	0·008
NDVI	−0·047	0·012	<0·001
Human footprint	0·002	0·005	0·666

diameter (Seale & Wassersug 1979), within the range of *Bd* zoospores (which range 3–5 μm in diameter; Longcore, Pessier & Nichols 1999). There is a possibility that our estimate of zoospore filtering also includes *Bd* encystment. However, if our measure of zoospore recovery was a proxy for differences in *Bd* encystment rate rather than filtering rate, we would have expected to recover the fewest zoospores in containers with *B. terrestris* tadpoles because they are least resistant to infection (Fig. S1, Supporting information). Because we observed the opposite pattern, we are confident that our results reflect differences in filtering capacities of these tadpole species. Interestingly, in a recently published study on the dilution effect in an amphibian-chytridiomycosis system, Searle and colleagues found that the addition of *Rana cascadae* tadpoles dilutes *Bd* risk for *Anaxyrus boreas* tadpoles (Searle *et al.* 2011). Here, the diluting species (*R. cascadae*) are not obligatory suspension-feeders and have keratinized mouthparts and thus differ fundamentally from the diluting species in our study, suggesting that there are multiple mechanisms for pathogen dilution in the amphibian-*Bd* system.

Our laboratory and field results reinforced the importance of species identity to pathogen dilution and amplification (e.g. LoGiudice *et al.* 2003; Ostfeld & LoGiudice 2003). At the tank level, *Bd* abundance was reduced only in treatments with *G. carolinensis* tadpoles, whereas

abundance was generally amplified in treatments with *B. terrestris* tadpoles (Fig. S1, Supporting information). These results are supported by patterns of *Bd* prevalence in the field, where the presence of *Bufo* spp. and *Gastrophryne* spp. were positive and negative predictors of *Bd* prevalence, respectively, even after controlling for climatic, vegetation and anthropogenic (human footprint) factors known to affect the prevalence of *Bd* (e.g. Liu, Rohr & Li 2013). Collectively, our findings build upon and complement a body of research suggesting that species identity is an important metric that influences the outcome of pathogen dilution. For example, in the Lyme disease system, larval ticks (vectors for bacterium *Borrelia burdorferi*) that attempted to feed on opossums were less likely to survive than ticks that fed on mice (Keesing *et al.* 2009), suggesting that these host species have a diluting and amplifying potential, respectively. Indeed, in model simulations, the removal of diluting or amplifying host species resulted in a relative increase or decrease in infected larval ticks, respectively (Keesing *et al.* 2009).

Our study also identifies context-dependent relationships among heterospecific tadpoles that lead to either *Bd* dilution or amplification. For example, the magnitude of the diluting effects of *G. carolinensis* and the amplifying effects of *B. terrestris* on *H. cinerea* predictably depended on the presence of the other species (Fig. 3). This presumably is because *G. carolinensis* filters more zoospores from the water in the presence of *B. terrestris*, because densities of zoospores are higher in the presence of this amplifier, whereas *B. terrestris* has a greater amplifying effect in the absence of the diluter. It is also possible that the presence of *G. carolinensis* altered the behaviour of *H. cinerea*, thereby affecting its probability of *Bd* exposure (i.e. ‘apparent competition’; reviewed in Raffel, Martin & Rohr 2008). These results suggest that the magnitude of the effect of a diluter or amplifier can depend on the presence of other amplifiers or diluters in the ecosystem and highlights that species composition, not just species identity, can be important in influencing the magnitude of dilution or amplification (LoGiudice *et al.* 2003, 2008). Future studies would be necessary to discriminate among behavioural, physiological or other potential hypotheses for our results.

One unexpected result from our experiment was that *Bd* prevalence in *G. carolinensis* was ~12%. This was unexpected because *Bd* is strongly associated with keratinized tissues (although it can grow *in vitro* on media without keratin; Voyles, Rosenblum & Berger 2011), and tadpoles of *Gastrophryne* lack keratinized mouthparts (Altig & McDiarmid 1999). One explanation is that *Bd* zoospores were associated with the non-keratinized epidermis of the heads of *G. carolinensis* tadpoles. A more likely alternative is that our qPCR detected *Bd* zoospores located in the branchial food traps or gill filters in the tadpole’s mouth (Wassersug & Rosenberg 1979). Because *G. carolinensis* did not have discrete keratinized mouthparts, as did the other species that we studied, we likely dissected some of the branchial food traps/gill filters when we processed tadpoles of this species. We ended our experiment 12 days after our final *Bd* inoculation, and *Bd* zoospores can survive and remain motile in tap water for up to 3 weeks (Johnson & Speare 2003); thus, it is possible that *G. carolinensis* tadpoles were still filtering *Bd* zoospores from the water column, resulting in positive qPCR samples. This explanation is consistent with our findings that *G. carolinensis* consumed *Bd* zoospores. Even though we are unable to ascertain whether *G. carolinensis* were actually infected with *Bd*, our statistical analyses demonstrated that this species had a consistent diluting effect on the other species. Thus, if our infection data on *G. carolinensis* are false positives, their diluting potential would actually be stronger than our analyses suggest.

In conclusion, our study is among the first to link manipulative experiments, in which a potential dilution mechanism is supported, with analyses of field data on species richness, host identity, spatial autocorrelation and disease prevalence. We demonstrated that tadpoles appear to suspension feed *Bd* zoospores and that the degree of suspension feeding seems to be positively associated with their dilution potential. Furthermore, field data from across the U.S. revealed dilution and amplification patterns that supported our laboratory findings, suggesting that our manipulative experiment demonstrating causality was ecologically relevant. Our results have important implications for how we understand the relationship between biodiversity and disease risk and emphasize the need to understand host traits that influence their dilution or amplification potential. Specifically, identifying host species that amplify pathogen abundance, or those that are diluters, should be a priority for wildlife disease management.

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Data accessibility

The data used in this paper can be found at: http://dx.doi.org/10.6084/m9.figshare.810480 (Venesky *et al.* 2014).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Methods S1. A detailed description of our qPCR extractions and analyses.

Fig. S1. Average *Batrachochytrium dendrobatidis* (Bd) prevalence and infection intensity with varying host diversity. Species combinations are labeled on the *x*-axis and represent the number of individuals of each species ('B' for *Bufo terrestris*, 'H' for *Hyla cinerea* and G for *Gastrophryne carolinensis*). Values are averaged across low- and high-density treatments and represented as tank means (+SE) because neither density ($\chi^2 = 0{\cdot}220$, $P = 0{\cdot}639$) nor the interaction between density and diversity ($\chi^2 = 0{\cdot}700$, $P = 0{\cdot}403$) significantly influenced *Bd* prevalence or infection intensity.

Table S1. Fully factorial design to test the effects of tadpole diversity (*Bufo terrestris*, *Gastrophryne carolinensis* and *Hyla cinerea*) and density (six or 12 tadpoles) on *Batrachochytrium dendrobatidis* (*Bd*) abundance.