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Microscopic Aquatic Predators Strongly Affect Infection Dynamics of a Globally Emerged Pathogen

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Summary

Research on emerging infectious wildlife diseases has placed particular emphasis on host-derived barriers to infection and disease. This focus neglects important extrinsic determinants of the host/pathogen dynamic, where all barriers to infection should be considered when ascertaining the determinants of infectivity and pathogenicity of wildlife pathogens [1–3]. Those pathogens with free-living stages, such as fungi causing catastrophic wildlife declines on a global scale [4], must confront lengthy exposure to environmental barriers before contact with an uninfected host [5–8]. Hostile environmental conditions therefore have the ability to decrease the density of infectious particles, reducing the force of infection and ameliorating the impact as well as the probability of establishing an infection [9]. Here we show that, in nature, the risk of infection and infectious burden of amphibians infected by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) have a significant, site-specific component, and that these correlate with the microfauna present at a site. Experimental infections show that aquatic microfauna can rapidly lower the abundance and density of infectious stages by consuming *Bd* zoospores, resulting in a significantly reduced probability of infection in anuran tadpoles. Our findings offer new perspectives for explaining the divergent impacts of *Bd* infection in amphibian assemblages and contribute to our understanding of ecosystem resilience to colonization by novel pathogens.

Results

We investigated the infection dynamics of one of the most devastating wildlife pathogens, *Batrachochytrium*

dendrobatidis (hereafter *Bd*) [10]. Although *Bd* is associated with species declines and mass mortalities of amphibians worldwide, prevalence varies significantly at local and regional scales [11–13]. Even a single highly susceptible host species, such as the European midwife toad *Alytes obstetricans*, can exhibit strong variation in the prevalence of infection across small geographic scales. Mortality in this species owing to chytridiomycosis correlates positively with altitude, which is due at least in part to the effects of environmental temperature [11, 13, 14]. However, this does not explain why sites with equivalent temperature regimes can still exhibit substantial variation in prevalence and mortality associated with infection [13], or why *Bd*-positive sites were found to be more similar to each other than would be expected based on chance [15]. We first used water sampled at amphibian breeding sites located in the Pyrenees with known histories of presence of *Bd* in the sentinel amphibian host species, *A. obstetricans*, to experimentally examine the effect of water and the aquatic microbial community on the probability of infection. The majority of these sites ($n = 23$) contain populations of *A. obstetricans* that exhibit low prevalence (<5%) or complete lack of infection (minimum sampling size = 30 individuals) and an absence of mortality in *A. obstetricans* across up to 6 years of field sampling, while a smaller number of populations ($n = 9$) have consistently exhibited high prevalence (usually $\geq 90\%$) over time (up to 10 years). Mass mortalities of recently metamorphosed *A. obstetricans* were observed over the same time span at those sites exhibiting 97% to 100% *Bd* prevalence (see Table S1 available online).

Dynamics of Motile and Immotile *Bd* Zoospores in Environmental Water

We investigated whether unfiltered environmental water affected the motility of *Bd* zoospores based on the prevalence of the source of the water in past years (experiment 1). We found that the number of motile zoospores varied significantly and in accordance with observed patterns of infection at the source of the water (generalized linear mixed model [GLMM]; $F_{1,244} = 14.18$; $p < 0.001$). Motile zoospores decreased as early as 2 hr after exposure to water from low-prevalence sites, while the number of motile zoospores in cultures that were exposed to water from high-prevalence sites only declined 33 hr after exposure (Figure 1). Counts of immotile zoospores did not differ significantly between low- and high-prevalence sites (GLMM; $F_{1,244} = 0.35$; $p = 0.554$; Figure S1). To determine whether loss of motility was associated with zoospore death (experiment 2), we assayed the viability of zoospores exposed for 24 hr to the two types of water using quantitative PCR. Zoospore survival was significantly greater in water from sites with high prevalence of infection (GLM; $F_{1,23} = 4.65$; $p = 0.042$). Water from high-prevalence sites also contained significantly reduced numbers of protozoans and microscopic metazoans compared to water from low-prevalence sites (generalized linear model [GLM]; $F_{1,23} = 8.14$; $p = 0.004$, Figure S2). Furthermore, the number of protozoans and microscopic metazoans was significantly and negatively correlated with the observed *Bd* prevalence in 2012 ($n = 25$; $r_s = -0.430$; $p = 0.033$) and was positively correlated with the reduction in the number of

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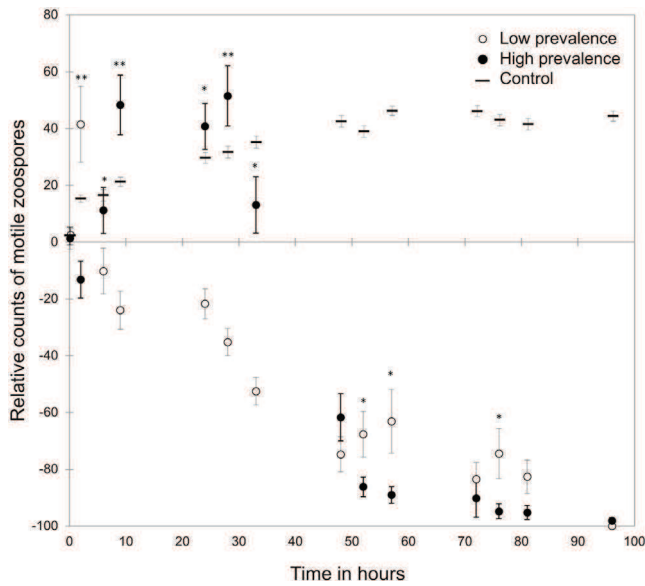


Figure 1. Dynamics of *Bd* Zoospores under Lab Conditions in Water of High- and Low-Prevalence Sites

Relative counts of viable zoospores (counts for each water sample were standardized relative to counts at $t = 0$) exposed to water from high-prevalence (●) versus low-prevalence sites (○). Controls are counts of viable zoospores in treatments where distilled water was added to the culture. Error bars indicate SEM across water samples in the same prevalence group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by post hoc pairwise test.

viable *Bd* zoospores ($n = 25$; $r_s = 0.682$; $p < 0.001$; Table S1). *Bd* prevalence in 2012 was positively correlated with the altitude of the site ($n = 32$; $r_s = 0.779$; $p < 0.001$), whereas water acidity and conductivity had no apparent effect on zoospore survival (Table S1). The best model explaining the reduction in viable zoospores in our experiment included both the number of protozoans and microscopic metazoans and altitude, but not pH ($F_{1,23} = 8.267$; $p = 0.002$, Akaike information criterion [AIC] 43.087), and *Bd* prevalence in 2012 was best explained by altitude ($F_{1,23} = 18.976$; $p < 0.001$, AIC 180.765; Table S1). Filtering out microorganisms with $0.45 \mu\text{m}$ cellulose acetate syringe filters (experiment 3) significantly reduced the ability of water from low-prevalence sites to cause mortality of *Bd* zoospores (GLMM; $F_{1,231} = 91.95$; $p < 0.001$; Figure S3). Our observations in the first set of experiments suggested that the process

through which *Bd* zoospore viability is affected is determined not by water quality but rather by the resident aquatic microfauna.

Challenge Experiments in Environmental Water

We directly tested the infectivity of zoospores in each different water type by exposing uninfected *A. obstetricans* tadpoles to zoospores in water from the two prevalence categories (<5% and >90% prevalence) and also zoospores in water from low-prevalence sites that had been heat treated (boiled for 15 min) to kill resident microfauna (experiment 4). Infection was significantly greater 8 days after exposure in tadpoles that had been exposed in either water from high-prevalence sites or heat-treated water from low-prevalence sites ($F_{2,93.1} = 17.14$; $p < 0.001$; Figure 2). Although prevalence in tadpoles exposed in water from low-prevalence sites increased 12 days postexposure, infections of these animals were significantly weaker than infections of animals exposed in water from high-prevalence sites (post hoc pairwise test, $p = 0.041$) and trended in the same direction as experiments using heat-treated low-prevalence water (Figure 2).

Effect of Different Microorganisms on *Bd* Infection Rate

To investigate whether specific microorganisms could be responsible for the observed patterns, we exposed *Bd* zoospores to 14 freshwater protozoans and microbial metazoans and again quantified zoospore viability (experiment 5). Two of these species (*Paramecium aurelia* and *Lecane stichaea*) were isolated from Pyrenean water samples, and 12 were sister species of microorganisms commonly found in Pyrenean lakes. The viability of *Bd* zoospores varied substantially when exposed to different microorganisms ($-0.04 \pm 0.19 \log_{10}$ genomic equivalent [GE] for the species associated with the smallest reduction in viable *Bd* zoospores [*Stentor coeruleus*] to $2.02 \pm 0.36 \log_{10}$ GE for the species associated with the greatest reduction in viable zoospores, the rotifer *Notommatidae* spp.; Figure 3). To determine the mechanism underlying this pattern, we observed the interactions between fluorescently stained zoospores and six microorganisms used in experiment 5: two that had the weakest impact on zoospore viability (*Dileptus anser* and *S. coeruleus*), two that had the greatest impact (*P. caudatum* and *Notommatidae* spp., experiment 6), and the two species isolated from Pyrenean sites (*P. aurelia* and *L. stichaea*). Our observations suggest that the process through which viability is affected is due at least in part to ingestion of zoospores (Figure S4). Interestingly,

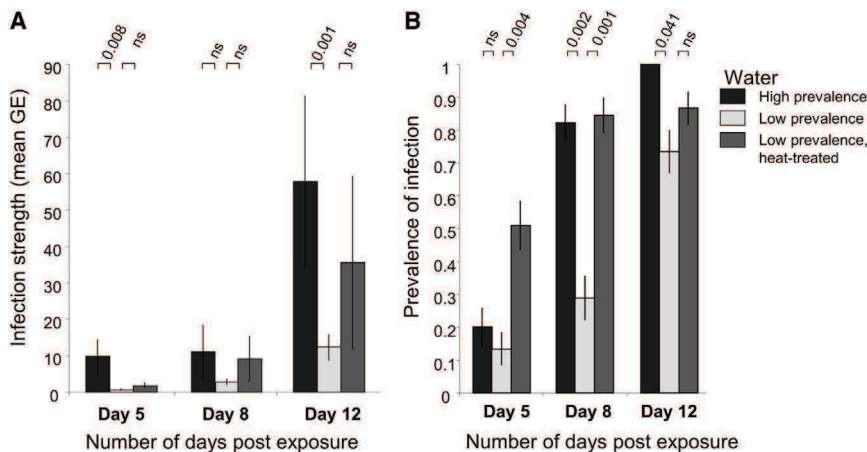


Figure 2. Infection Probability and Strength in Different Water Treatments

Impact of water source and heat treatment on infection strength (A) and infection probability at 5, 8, and 12 days postexposure in *A. obstetricans* tadpoles (B). Different bar shading indicates different water types and treatments. Strength of infection is the uncorrected qPCR estimate of the average number of zoospores (genomic equivalents, GE) for each water category. Pairwise p values from Tukey's post hoc tests are shown.

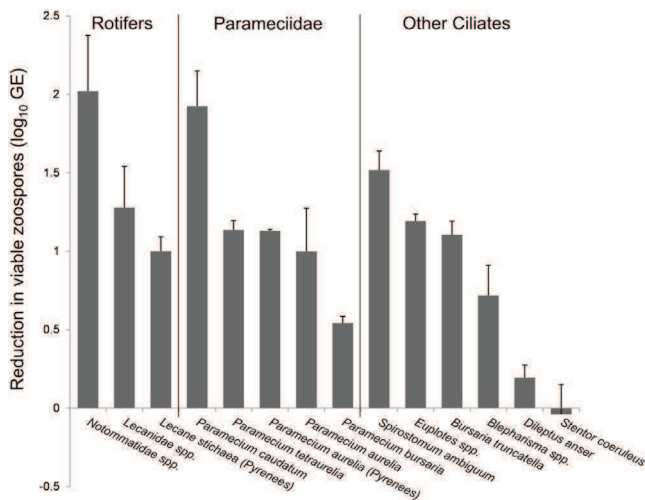


Figure 3. Reduction of Zoospore Viability Associated with the Presence of 14 Protozoan/Metazoan Species

Reduction of viability represented as log₁₀ GE of viable *Bd* zoospores after 24 hr + 1 hr SD. Error bars indicate SEM of three replicates per species. Two species were bred from samples stemming from our Pyrenean sites, the Lecanidae rotifer *Lecane stichaea* and the Parameciidae ciliate *Paramecium aurelia*.

despite substantial differences in body size (lorica size in rotifers; length along longest axis in ciliates), some ciliates (size range 40–750 μm) and rotifers (size range 20–180 μm) performed equally well in their ability to decrease the viability of *Bd* zoospores under laboratory conditions.

In our final experiment, we tested whether microorganisms with different impacts on zoospore viability determined the probability of infection in the predicted manner. We exposed tadpoles (*Discoglossus scovazzi*) to *Bd* zoospores in water containing one of the three presumed predatory microorganisms (*P. aurelia* isolated from the Pyrenees, *P. caudatum*, or the rotifer *Notommatidae* spp.; experiment 7). The presence of microorganisms significantly affected the probability of infection (GLMM; $F_{1,119} = 34.41$, $p < 0.0001$; Figure 4A; $F_{2,43} = 11.93$, $p = 0.003$; Figure 4C). None of the tadpoles exposed with *Notommatidae* spp. developed infections, 3 of 15 tadpoles were infected in the presence of *P. caudatum*, and 16 of 60 tadpoles exposed with the Pyrenean *P. aurelia* were infected (Figure 4). *P. aurelia* isolated from the Pyrenees also reduced significantly the strength of infection (GE) compared to control tadpoles (GLMM; $F_{1,66} = 18.82$, $p = 0.012$; Figure 4D), whereas this was not the case between control tadpoles and those housed with lab-reared *P. caudatum* treatments (GLMM; $F_{1,8} = 2.03$, $p = 0.757$; Figure 4B).

Discussion

The ability of microorganisms to forage on *Bd* zoospores has been postulated but never explicitly shown and then linked to both field conditions and experimentally derived patterns of infection [16, 17]. Here we demonstrate that ciliate and rotifer microorganisms are effective consumers of *Bd* zoospores in the Pyrenean mountain lakes, reducing the number of free-swimming, infectious zoospores. The presence of these microorganisms also reduces the probability of infection in two amphibian species that are highly susceptible to *Bd*. Due to the dose-dependent impact of infection by *Bd* on

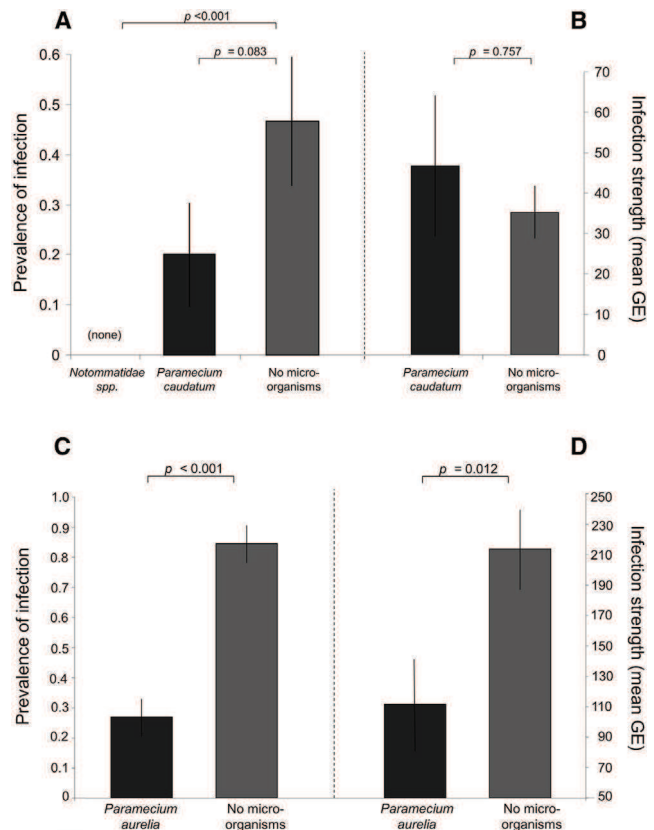


Figure 4. Challenge Experiment under the Influence of Microfauna

Number of infected tadpoles (A and C) and intensity of infection (B and D) of *Discoglossus scovazzi* tadpoles exposed repeatedly to *Bd* zoospores and cohoused with *Notommatidae* spp. (A), *Paramecium caudatum* (A and B), or *P. aurelia* from the Pyrenees (C and D) or housed in the absence of microorganisms.

host life-history traits, decreasing their infection burden will reduce the impact of *Bd* on larval development and probably postmetamorphic survival [18]. *Bd* infection may result in host mortality only when a threshold density of sporangia (infection intensity) is reached [18], implying that control may be achieved by limiting the number of *Bd* zoospores. Our study raises hope that the rate and intensity of *Bd* infection in amphibian populations can be manipulated by natural means, and that appropriate methods of natural augmentation of predatory microorganisms will significantly decrease the adverse effects of chytridiomycosis on amphibians and ecosystems.

The results of our experiments show that both the prevalence and intensity of infection in larvae of *A. obstetricans* are site dependent and correlate with the presence of indigenous predatory microorganisms in the water; we experimentally confirmed this latter pattern in a second susceptible species (*D. scovazzi*). We were able to show that rotifers and ciliates reduce the number of *Bd* zoospores in the environment and that this reduction might occur in several different ways, such as concomitant predation, predation of free-living stages, or passive consumption and filtration [19]. Predatory microorganisms such as the ciliates *P. caudatum* and *P. aurelia* and the rotifers *Notommatidae* spp. and *L. stichaea* appear to have a higher foraging efficacy for *Bd* zoospores, ingesting *Bd* zoospores more efficiently than

planktonic species with different foraging strategies, for example *D. anser* and *S. coeruleus*. Indeed, it is likely that only a few typical freshwater plankton species may be unable to ingest *Bd* zoospores. Microorganisms able to prey on *Bd* zoospores are expected to be numerous, as aquatic environments are rich with species of chytrid that utilize zoospores as a dispersal unit and therefore represent a rich source of potential nutrition [20]. This observation has been confirmed by laboratory-based studies showing that planktonic *Daphnia* species can consume *Bd* [16, 17]. Therefore, we suggest that many indigenous plankton species are preadapted to preying on *Bd* zoospores, as these are similar in size and form to endemic zoosporic aquatic fungi that likely form a key nutritional component of the microfaunal plankton diet [21].

Environmental factors have been identified that covary with the prevalence of infection and chytridiomycosis [11, 13]. Most prominently, lower temperature regimes at higher altitudes are associated with higher *Bd* infection probability [13]. Temperature may act to modify prevalence through direct and indirect pathways, either by directly influencing host immunity and pathogen growth rates or by indirectly influencing the activity of microorganisms across infected sites. This goes some way toward explaining why both prevalence of infection and mortality are more common in the Pyrenees when environmental temperatures are very low, and when laboratory estimates of *Bd* growth rates and zoospore production indicate that infection should be rare [13, 22]. Environmental factors may also explain the composition, density, and dynamics of the planktonic communities across seasons [23], with sites of high *Bd* prevalence being an enemy-free space for *Bd*, allowing the fungus to infect suitable host species rapidly and with a high intensity. Additional support for the generality of our findings comes from recent studies showing that *Bd*-positive ponds are more similar to each other than would be expected based on chance [15] and that the “dilution effect” hypothesis may apply to the amphibian-*Bd* system [24], suggesting links between pond ecology and local-scale epidemiological dynamics. More detailed ecological studies are now needed to better link abiotic variables to the composition, density, and dynamics of the planktonic communities and the outcome of the host/pathogen dynamic.

We here show the importance of predation in controlling infections in larvae of two amphibian species and provided direct evidence that zoospore ingestion is the mechanism through which infection is modified [19]. Development of methods that facilitate natural augmentation of predatory microorganisms as a form of *Bd* biocontrol may hold promise as a field mitigation tool that lacks the downsides associated with introducing nonnative biocontrol agents, such as the use of antifungal chemicals or release of nonnative skin bacteria into the environment, or the reliance of unpredictable environmental temperature to “cure” infections [25, 26]. However, before biocontrol can be safely attempted, additional study is required.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.11.032>.

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