Title: Predation by zooplankton on *Batrachochytrium dendrobatidis*: Biological control of the deadly amphibian chytrid fungus?

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Abstract

Batrachochytrium dendrobatidis (hereafter *Batrachochytrium*), a fungal pathogen of amphibians, causes the disease chytridiomycosis which is responsible for unprecedented population declines and extinctions globally. Host defenses against chytridiomycosis include cutaneous symbiotic bacteria and anti-microbial peptides, and proposed treatment measures include use of fungicides and bioaugmentation. Efforts to eradicate the fungus from localized areas of disease outbreak have not been successful. Instead, control measures to mitigate the impacts of the disease on host populations, many of which are already persisting with *Batrachochytrium* in an endemic state, may be more realistic. The infective stage of the fungus is an aquatic zoospore, 3-5µm in diameter. Here we show that zoospores of *Batrachochytrium* are consumed by the zooplankter *Daphnia magna*. This species inhabits amphibian breeding sites where *Batrachochytrium* transmission occurs, and consumption of *Batrachochytrium* zoospores may lead to effective biological control of *Batrachochytrium.*

Keywords: amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, biological control, *Daphnia magna*, zooplankton

Introduction

As part of an overall “biodiversity crisis”, amphibians are undergoing population declines and extinctions at unprecedented rates (Stuart et al. 2004; McCallum 2007). Emerging infectious diseases such as chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis*, are playing a prominent role in these declines (Mendelson et al. 2006). The impact of

chytridiomycosis on amphibians has been called “the most spectacular loss of vertebrate biodiversity due to disease in recorded history” (Skerratt et al. 2007). Efforts to treat chytridiomycosis *in vitro*, including treatment with anti-fungal compounds and supplementation of natural cutaneous microbes (bioaugmentation), have met with varied success (Lubick 2010; Woodhams et al. 2011). Although eradication of chytridiomycosis has been attempted in some natural populations (Lubick 2010; Woodhams et al. 2011), it has not been successful, and eradication may not be a realistic goal. Instead, Woodhams et al. (2011) proposed that control measures should seek to mitigate the effects of the pathogen on host populations, many of which are already persisting with *Batrachochytrium* in an endemic state.

Parasites commonly function as prey within ecosystems (Johnson et al. 2010), and we suggest that biological control through predation may be effective in controlling *Batrachochytrium*. The infective stage of *Batrachochytrium* is a free-living aquatic flagellated zoospore, 3-5μm in diameter (Longcore et al. 1999), which is within the size range of preferred prey items of cladocerans such as *Daphnia spp.* Abundant in lentic habitats globally, *Daphnia* are selective filter feeders consuming nanoplanktonic algae, bacteria, fungi, protozoa, and detritus 1-100μm in size (Thorp and Covich 2010). Kagami et al. (2004; 2007) showed that a *Daphnia galeata*×*hyalina* population benefitted from consumption of zoospores of a chytrid fungus, *Zygorhizidium planktonicum,* thereby protecting phytoplankton hosts from infection. Furthermore, Ibelings et al. (2011) suggest that zooplankton may also benefit from a mixed phytoplankton community if chytrid infection reduces the dominant inedible phytoplankton species. A negative correlation between *Daphnia* abundance and *Batrachochytrium* zoospore density over a 3-day experimental trial has been reported (Woodhams et al. 2011). Although this

suggests the possibility of predation of *Batrachochytrium* zoospores by *Daphnia*, this was not confirmed. Here, we experimentally tested the hypothesis that *Daphnia magna* consume *Batrachochytrium* zoospores.

Methods

Daphnia magna were collected from a self-contained covered outdoor culture and were transported to a laboratory maintained at 21.5-23.3ºC. *Batrachochytrium* was grown in pure culture on plastic Petri plates (10 cm-diameter) with standard TGhL nutrient agar medium (Longcore et al. 1999). Plates were inoculated with liquid culture of *Batrachochytrium* isolate JEL 274, originally isolated from *Anaxyrus boreas* toads from Colorado, and incubated at 22 ˚C for 9 d prior to use. We conducted two experiments to determine whether *Batrachochytrium* could be detected in the gut of *D. magna*.

Visual confirmation

Nile red (Fisher Scientific), a lipophilic fluorescent stain, was dissolved in dimethyl sulfoxide (DMSO) and added to standard TGhL nutrient agar medium at a concentration of 500 $\mu g\ L^{-1}$. *Batrachochytrium* was cultured on plates poured from this agar. Visual examination indicated that the stain was taken up by the fungus. A broth containing *Batrachochytrium* scraped from flooded plates was diluted to achieve a concentration of 1.2×10^5 zoospores mL^{-1}, and 1 mL of this broth was filtered through a Whatman GF/F filter to eliminate excess stain. To dislodge zoospores, the filter was washed in a 500mL plastic cup containing 200mL dechlorinated water. *D. magna* (n=10) that had been starved for 24h prior to the experiment were exposed individually in plastic cups at a concentration of 600 zoospores mL^{-1}. Starved control *D.*

magna were exposed to *Batrachochytrium* grown on standard TGhL nutrient agar medium lacking the stain (*Batrachochytrium* control, n=10), and to a control inoculation from plates containing Nile red (stain control, n=10), both filtered through a Whatman GF/F filter. After 3.5 hours, all individuals were preserved in 90% ethanol and viewed under an Olympus Vanox AH2 fluoroscope. Images were captured with an Olympus DP72 digital camera.

qPCR confirmation

Live *D. magna* that had been starved for 24h prior to the experiment (n=12) and starved *D. magna* killed with 90% ethanol (n=12) were exposed individually in 500 mL plastic cups filled with 200 mL of dechlorinated water to *Batrachochytrium* at a concentration of 600 zoospores mL^{-1}. Starved *D. magna* (n=12) were exposed to a control inoculation. After 3.5 hours, all animals were preserved in 90% ethanol. The guts of all individuals exposed to *Batrachochytrium* and three randomly chosen control individuals were extracted with the use of a dissecting microscope. 30-40mg of Zirconium/silica beads measuring 0.5mm diameter (Biospec products) were added to a vial containing the gut, and the vial was alternately homogenized in a BBX24W-Bullet Blender (Next Advance) for 45 sec and centrifuged 5 times. 60µL Prepman Ultra (Applied Biosystems) was added and vials were heated to 100ºC for 10 min, cooled for 2 minutes, and the supernatant was extracted and diluted to a 10% solution. Real-time quantitative PCR (qPCR) was conducted on an Applied Biosystems StepOne Plus real-time PCR machine (Applied Biosystems, Inc., CA, USA) according to methods of Boyle et al. 2004. Each sample was analyzed in triplicate against a *Batrachochytrium* standard titration from 10^{-1} to 10^{2} zoospores, and the average number of genome equivalents per individual was calculated.

Results

Visual confirmation

When viewed under a fluoroscope, the gut of individual *Daphnia* exposed to *Batrachochytrium* grown on plates containing the stain appeared intensely fluorescent red (Fig1). Guts of individuals from the *Batrachochytrium* control and stain control treatments did not fluoresce.

qPCR confirmation

A Wilcoxon rank-sum test indicated that guts of *D. magna* exposed to *Batrachochytrium* while alive contained significantly more zoospore equivalents than guts of those exposed to *Batrachochytrium* after death ($W=191$, $p=0.0001$, Fig 2). qPCR confirmed that guts of unexposed individuals contained no *Batrachochytrium.*

Discussion

Our study demonstrates consumption of *Batrachochytrium* zoospores by the zooplankter *D. magna* and supports the potential for biological control of *Batrachochytrium* by zooplankton as discussed in Woodhams et al. (2011). Vredenburg et al. (2010) and Briggs et al. (2010) suggested that *Batrachochytrium* infection results in host mortality once a threshold density of sporangia (infection intensity) is reached, implying that control may be achieved by limiting the number of *Batrachochytrium* zoospores. We suggest that zooplankton such as *D. magna* may effectively limit the number of infective *Batrachochytrium* zoospores and may be a useful means of biological control for chytridiomycosis. Moreover, we suggest that the threat of

Batrachochytrium to amphibians would be lower in systems containing dense populations of zooplankton if the species of zooplankton fed on *Batrachochytrium*. Furthermore, it may be possible to augment the numbers of *Batrachochytrium*-eating zooplankton in natural systems for effective biological control, although previous species introductions for biological control have met with varied success (Cory and Myers 2000). These suggestions should be examined in natural systems for a more thorough understanding of how *Batrachochytrium* may be controlled via zooplankton.

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Figure 1. *Daphnia magna* after consuming zoospores stained with Nile red. (a) Brightfield image. (b) Fluorescent image with sensitivity = 204.78ms.

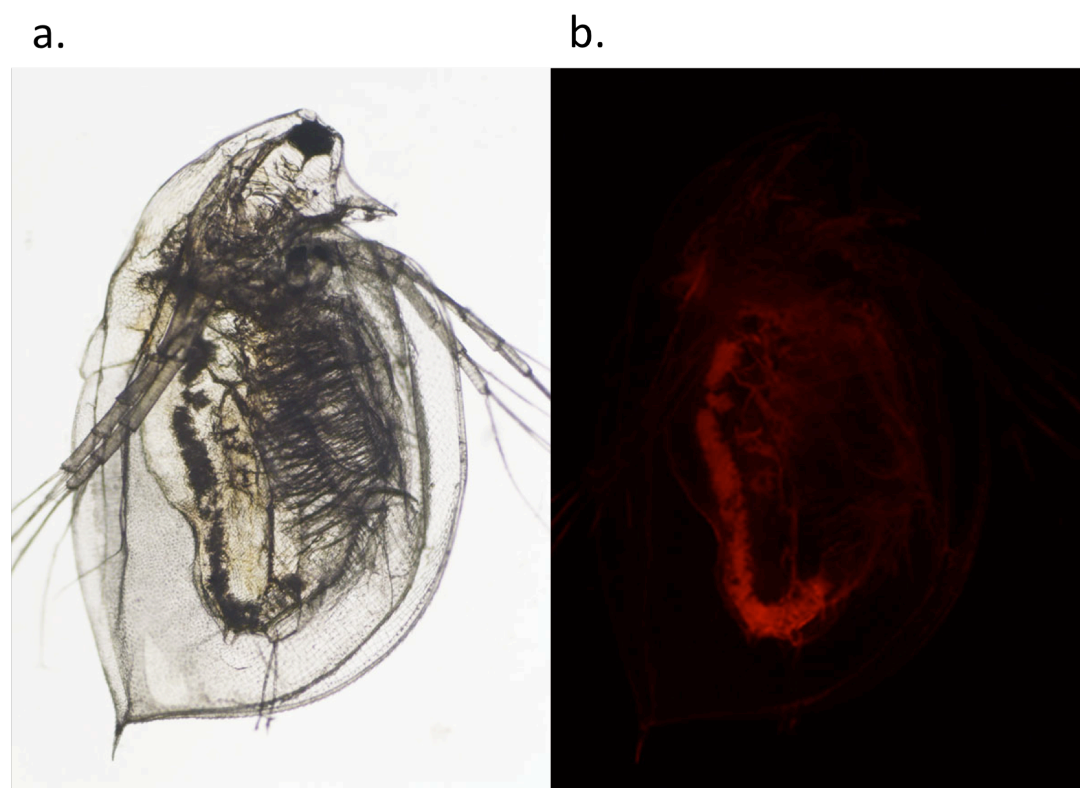
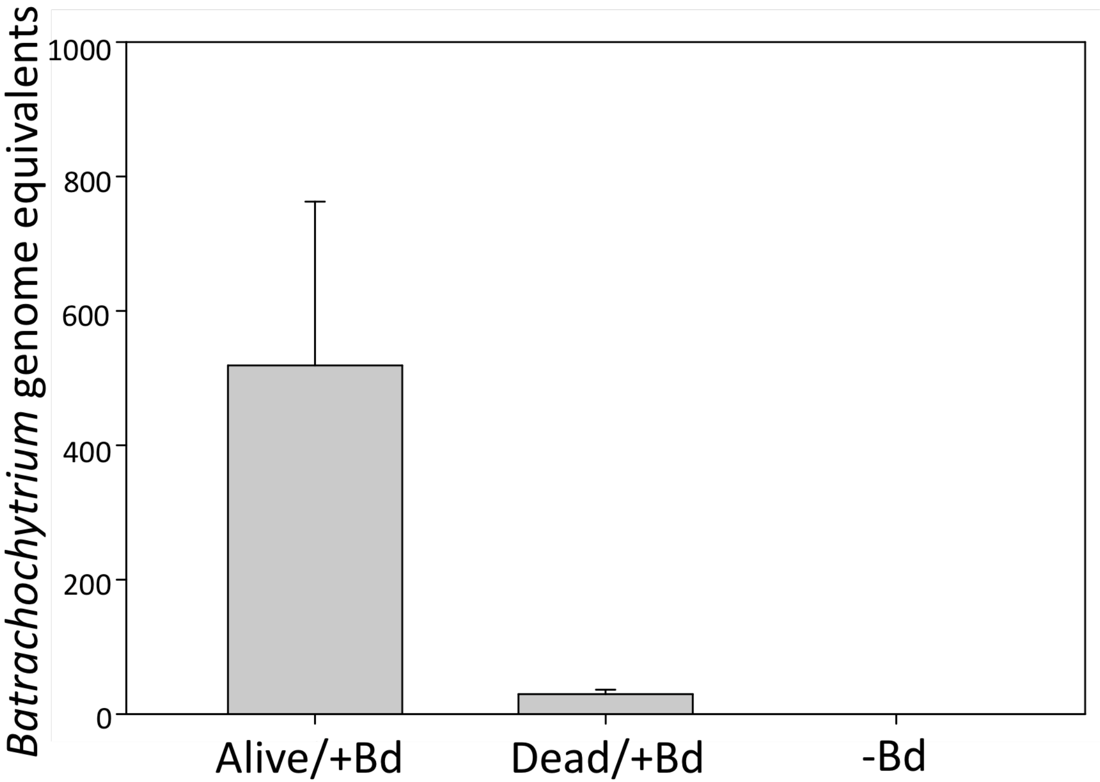


Figure 2. Zoospore equivalents in the extracted guts of live *D. magna* exposed to *Batrachochytrium*, killed *D. magna* exposed to *Batrachochytrium*, and live *D. magna* exposed to a sham inoculation (control). Wilcoxon rank-sum test: W=191, p=0.0001.


Batrachochytrium genome equivalents
1000
800
600
400
200
0
Alive/+Bd
Dead/+Bd
-Bd