Drew University

College of Liberal Arts

The influence of aquatic macroinvertebrates on the abundance of *Batrachochytrium dendrobatidis*

A Thesis in Environmental Science

by

Rachel Sirica

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ABSTRACT

Amphibian chytrid fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), poses a fatal threat to amphibians, but does not infect all aquatic ecosystems equally. The interactions between hosts (frogs and toads) and pathogen (*Bd)* are influenced by various abiotic and biotic factors. Prior studies have shown that certain aquatic macroinvertebrates and zooplankton species can limit the number of *Bd* zoospore equivalents in a controlled experiment and suggest other taxa may also have a significant effect on *Bd* in this way as well. There is a need to further research biotic factors including aquatic macroinvertebrates' impact on *Bd* to best inform land management decisions and wildlife conservation practices in amphibian habitats. In this study, I investigated the influence of five aquatic macroinvertebrates on the abundance of *Bd*. This included: large and small *Gammarus fasciatus* (freshwater amphipods), *Hydra vulgaris* (freshwater polyp), *Chaoborus* (glassworms), large and small Chironomid midge larvae as well as large and small Culicidae Mosquito larvae. I also tested Long Pond in Zuck Arboretum in Madison, New Jersey, for the presence of *Bd*. Taxa were placed into a controlled experiment and inoculated with *Bd* zoospores alongside a control group. DNA was extracted from the experimental samples as well as from Long Pond water samples and ran in Bio-Rad CFX Touch Real-Time PCR Detection System. The measured *Bd* zoospore equivalents were analyzed in R Studio to determine statistical significance of each taxa's impact on the presence of *Bd* in Long Pond. The analyzed data suggest that hydra and mosquito larvae do not have a significant impact on *Bd* abundance. Glassworms were found to significantly decrease the quantity of zoospore equivalents in experiment, which is consistent with prior studies. Large amphipods increased zoospore abundance while small amphipods did not. Large Chironomid midge larvae had a notable variance in zoospore abundance across samples, with some increasing the quantity of zoospore equivalents in experiment. Small Chironomid midge larvae did not have a significant impact on zoospore abundance. According to the analyzed qPCR data, Long Pond tested positive for the presence of *Bd.* The results from the macroinvertebrates studied suggest the need for further research on the mechanisms in which large amphipods and large Chironomid midge larvae harbor *Bd* zoospores, specifically when these animals are in their last larval instar. Evaluating the effects on the presence of *Bd* across a wider taxonomic range within the aquatic environment would further support the applicability of these findings. Effective land management and amphibian conservation strategies rely on a deeper understanding of *Bd* and its surrounding environment.

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INTRODUCTION

In the wake of radically changing ecosystems, biodiversity loss remains an incredible threat to food web stability, natural resources, and the conservation of vulnerable species (Tompkins et al. 2011). Climate change, habitat loss, and wildlife disease all pose threats to amphibians, with disease being their primary threat (J. E. Longcore, Pessier, and Nichols 1999; Crowl et al. 2008). This widespread loss must be mitigated in order to best conserve the most imperiled of species in the most productive of environments. The severity of this ecological concern has reached a new peak as we witness a sixth mass extinction unfold, involving a notable decline in amphibian populations (Stuart et al. 2004; Wake and Vredenburg 2008). The need to prioritize conservation of amphibians is a crucial step in mitigating global biodiversity loss as they are the most vulnerable vertebrate group to date (Wake and Vredenburg 2008; Luedtke et al. 2023).

Threatened and extinct species statistics are becoming well-documented, providing a comprehensive view of the fragile position amphibians are in (Luedtke et al. 2023). In 2004, it was published that approximately 43% of 6,600+ amphibian species were threatened with extinction globally (Stuart et al. 2004; Vredenburg et al. 2010). The second Global Amphibian Assessment in 2022 reported 40.7% of 8,011 amphibian species to be threatened with extinction (Luedtke et al. 2023). There has been an increase in species who hold the status of threatened and extinct over the last two decades as the global outbreak of fatal chytridiomycosis spreads and anthropological changes impact amphibians' access to clean water, air, and land (J. R. Longcore et al. 2007; Luedtke et al. 2023).

Chytridiomycosis is a disease induced when amphibians contract *Batrachochytrium dendrobatidis* (*Bd*), a novel unicellular pathogen that is a chytrid fungus (Berger et al. 1998). The effects of infected amphibians vary from individuals to individuals but typically amphibians will

first suffer pathophysiological symptoms in their skin (Voyles et al. 2009; Vredenburg et al. 2010). The transport of electrolytes and proper osmoses through their semi-permeable epidermis is interrupted, hindering proper bodily hydration and oxygen flow (Voyles et al. 2009; Vredenburg et al. 2010). Once in this fragile state, amphibians are exposed to skin deterioration, immune system failure, and heart attacks, all of which can lead to their death (Voyles et al. 2009; Strauss and Smith 2013).

Not only are amphibians vulnerable as a group because populations have considerably decreased, they are anatomically vulnerable as individuals. Amphibian skin is semi-permeable and highly sensitive to foreign bodies such as the infectious *Bd* (Wake and Vredenburg 2008). *Bd* threatens the life of amphibians on a global scale with many species facing serious decline and others rendered to extinction (Stuart et al. 2004; Wake and Vredenburg 2008; Tompkins et al. 2011).

Unfortunately, a universal immunity response to the threat of *Bd* does not exist in frogs and toads (Wake and Vredenburg 2008). *Bd* thrives on keratin-rich surfaces making the skin of post-metamorphic amphibians an optimal host for the pathogen (Berger et al. 1998; Longcore, Pessier, and Nichols 1999; McQuigg 2022). In order to infect its chosen host, *Bd* zoosporangia release *Bd* zoospores which infect amphibians via their skin (Berger et al. 1998; J. E. Longcore, Pessier, and Nichols 1999). Figure 1 depicts the life cycle of *Bd* from zoospore (A) to colony (E) (Berger et al. 2006). A zoospore is a single fungal cell with flagellum that allows the cell to be motile as shown in stage A of Fig. 1 (Berger et al. 2006). In the second stage of the *Bd* life cycle, labeled B in Fig. 1, a zoospore encysts and becomes a germling, the earliest formation of zoosporangium (Berger et al. 2006). Zoosporangium develop, as shown in stage C in Fig. 1, to form discharge papillae (Berger et al. 2006). Stage D in Fig. 1 shows that in response to a wet

environment, discharge papillae dissolve, releasing zoospores from the zoosporangium into the environment where they will come in contact with potential hosts (Berger et al. 2006). When cultured in a lab setting, *Bd* forms colonies of zoosporangia as seen in the final stage of the life cycle labeled E (Berger et al. 2006). The pathogen reproduces asexually in water, continuing its infection cycle on any vulnerable host within a given body of water (Wake and Vredenburg 2008).

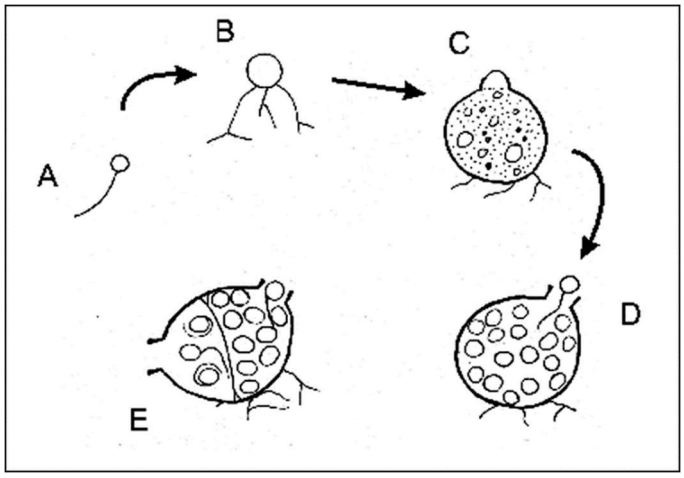

Figure 1. *Batrachochytrium dendrobatidis* life cycle in culture. Diagram from Berger et al., *Life cycle stages of the amphibian chytrid Batrachochytrium dendrobatidis* (Berger et al. 2006).

For a comprehensive understanding of *Bd*-amphibian interactions, it must be understood that the extent of said interactions are not limited to the influence of the host and the influence of the pathogen alone; environmental factors are also at play (Spitzen-van der Sluijs et al. 2017; McQuigg 2022). Though *Bd* is largely responsible for amphibian mortality, it impacts host environments at varying degrees (Strauss and Smith 2013; Spitzen-van der Sluijs et al. 2017). Research suggests that both abiotic and biotic factors, which may not be amphibian at all, are responsible for this (Strauss and Smith 2013; McMahon et al 2013; McQuigg 2022). The work of Alex Strauss and Kevin G. Smith included the survey and identification of invertebrates that coexist with amphibian populations in the presence of *Bd* (Strauss and Smith 2013). Their work

suggests possible mechanisms that vary host-pathogen relationships from habitat to habitat (Strauss and Smith 2013). Controlled laboratory experiments with common wetland macroinvertebrates and zooplankton have since been conducted in order to test the hypotheses of *Bd* abundance variance. Backswimmers, glassworms, crayfish, nematodes, and *Daphnia* are suggested to have a significant impact on decreasing the abundance of *Bd* in controlled experiments (Buck, Truong, and Blaustein 2011; Strauss and Smith 2013; McMahon et al. 2013). Being that these species are only a small sample of the complex communities that exist in ecosystems susceptible to *Bd*, further research is necessary to determine additional macroinvertebrates that are responsible for impacting *Bd* abundance. Although *Bd* is a well-studied pathogen, further research exploring the mechanisms which limit the abundance of *Bd* zoospores in a given environment is needed.

I chose to investigate how species that are common in Long Pond may interact with *Bd*. Previous research suggests glassworms as significant in lowering the abundance of *Bd* zoospores in experiment (McQuigg 2022). I wanted to repeat this experiment to verify the success of methods and provide further support to the initial hypothesis that macroinvertebrates are biotic factors that lower the quantity of zoospore equivalents in experiment. In addition to testing the impact of glassworms (phantom midge larvae) on *Bd* abundance, I also tested the impact of Chironomid midges because their larval stage mimics that of glassworms which were found to decrease the zoospore equivalents of *Bd* in a previous study (McQuigg 2022). Experiments in which crayfish were fed *Bd* zoospores also result in a decrease in zoospore abundance (T. A. McMahon et al. 2013). Amphipods, like crayfish, are a part of the Malacostraca class of crustaceans. Amphipods were not only abundant in my region of study, but also share evolutionary relationships with crayfish which have been successful in lowering *Bd* abundance, which prompted me to use them

in this study (McMahon et al. 2013). Hydra were of interest because of their diet and feeding habits. These freshwater hydrozoan feed on small organisms that swim in water such as algae and fungi (Deserti et al. 2017). Hydras' predation habits make them a great candidate for potentially eating *Bd* zoospores and lowering their abundance. Mosquito larvae became of interest for their wider implication on wildlife management and the excessive practice of mosquito control. In other words, if mosquitos are found to have more positive implications for our ecosystems, management practices surrounding the species should certainly be mitigated. Mosquitos were found at Drew University in a stagnant water source, an environment that *Bd* is known to thrive in. It was important to explore these specific taxa as they exist in wetland ecosystems in which the pathogen has been found.

The goal of this study is to investigate the impact of chosen macroinvertebrates on the abundance of *Bd* and conclude the current status of *Bd* in Long Pond in Zuck Arboretum in Madison, New Jersey on Drew University's campus. I hypothesized that the abundance of *Bd* zoospores would decrease in the presence of freshwater macroinvertebrates. I also hypothesized that Long Pond would test positive for *Bd* given the rapid spread of the pathogen along the northeast coast of the U.S. (J. R. Longcore et al. 2007). To prevent further spread of *Bd* and conserve amphibians, it is crucial to investigate the biotic factors that impact the fungal pathogen's abundance and determine which landscapes are already infected with the pathogen.

MATERIALS AND METHODS

Animal Collection and Husbandry

For this study, I chose to work with freshwater aquatic macroinvertebrate taxa that are abundant in my area of study, Long Pond. Long Pond is located in the Zuck Arboretum of Drew University in Madison, NJ. For both a preliminary collection to determine taxa abundance and my experimental collection, I dipnetted three times in three distinct locations (40.76235, -74.43034; 40.7625, -74.43081; 40.76287, -74.43012) in the leaf litter around the shallow edges of the pond to collect macroinvertebrates. Macroinvertebrates were stored in a 5-gallon bucket with lid for transport to the lab for up to 24 hours before their release back to Long pond or euthanasia. In order to limit possible contamination of *Bd* into the field, macroinvertebrates are euthanized via freezing in instances in which the pathogen has been present in the lab. To identify the species present in my samples, I sorted through the pond water and leaf litter separating species into their own ice cube trays. The species were kept individually in this temporary housing so I could evaluate their size class (Table 1) and keep count of how many individuals of each species were found.

Pond water and leaf litter were sorted through for macroinvertebrates of interest. The taxa chosen from this collection method to be used in this investigation included: *Gammarus fasciatus* (freshwater amphipods), *Hydra vulgaris* (freshwater polyp), *Chaoborus* (glassworms), and Chironomid midges. Culicidae Mosquito larvae used in this study were collected from a stagnant water source on Drew University campus. All animals to be used in experiment were stored in 2 L beakers with well water (amphipods) or filtered pond water (glassworms, midges, hydra and mosquito larvae). The pond water was filtered through a 0.7 μm filter to remove potential presence

of *Bd* and other microscopic organisms.

Well water and pond water were the chosen animal accommodations to prevent qPCR inhibition in the later analysis of the experiment while also maintaining the ion balances necessary for aquatic organisms to survive and thrive (Bickley et al. 1996). Inhibition in qPCR occurs when additional substances block the target DNA in samples from being measured effectively. Said inhibitors can be introduced into the sample not only during the plating process for qPCR, but also in the controlled experimental setting. In order to reduce the likelihood of possible containments to the qPCR process such as calcium ions, which are common in our water system, experiments relied on the use of untreated well water in the absence of filtered pond water and on filtered pond water when available.

Beakers were kept at approximately 21-25 degrees Celsius and covered with bug screen. Animals were separated into specified beakers based on size class (large or small), when applicable, and kept until they were transferred into housing for experimentation (Table 1). All beakers were inoculated with 100-200 ml of unfiltered pond water approximately 3 times a week based on visible pond debris and algae growth as a food source until animals were to be fasted for experimentation.

Batrachochytrium dendrobatidis **Culturing**

Batrachochytrium dendrobatidis used in this study was commercially sourced from the Collection of Zoosporic Eufungi at the University of Michigan. *Bd* was first cultured in a tryptone broth made of 10 g tryptone and 1000 ml Mili-Q water in a screw-top glass flask. All materials brought into the work space were sterilized with 70% ethanol. I also sprayed my gloved hands throughout to process to limit any contamination. Working in a sterile tissue culture hood, the

screw top flasks were filled ¾ of the way using sterilized pipettes. Then, 2 mm chunks of the parent *Bd* culture were transferred into each flask. Flasks were capped but not sealed completely shut to allow for cell growth. I wrapped a piece of Parafilm around the caps to keep the culture sterile. I let these cultures develop at approximately 21-25 degrees Celsius for up to a week. After this point, cultures can be stored in the fridge for up to 3 months. This process moves the culture from solid to liquid in order to initiate zoospore release (J. E. Longcore, Pessier, and Nichols 1999).

I also transferred the culture from its liquid tryptone broth to solid tryptone-agar plates to establish new cultures and limit the possibility of contamination to my full *Bd* supply. A tryptone-agar solution was poured into petri plates to solidify in preparation for the addition of *Bd*. After the solution solidifies, I decanted the liquid solution containing *Bd* onto the plates, spreading it throughout the surface using a sterile cell spreader. The lids were placed onto the plates which are then wrapped in Parafilm. These cultures also developed at room temperature for up to a week before being moved to the fridge for up to three months. Moving the culture from solid to liquid and then liquid to solid allowed me to work with a selection of plates, considering not all cultured at the same rate.

Experimental Setup

Treatment groups were fully randomized and I used a haphazard process to assign animals to their housing. The amphipod experimental design differs from the rest in that there were six treatment groups: negative controls containing no *Bd* nor animal, *Bd* positive without animal, *Bd* negative with large animal, *Bd* positive with large animal, *Bd* negative with small animal, and *Bd* positive with small animal. In all experiments thereafter for this study, the variables manipulated were animal presence and size class, when applicable (Table 1). This was for the benefit of

efficiency in experimental breakdown and processing fewer samples in qPCR per experiment. Glassworms, large Chironomid midges, and small Chironomid midges shared the same control for their experiment as these experiments were conducted concurrently. Amphipods, hydra, and mosquito larvae all had control groups specific to the individual taxa and were not shared.

Amphipods were individually housed in 15 ml tubes filled with 14ml of well water. Hydra were house in 2 ml tubes filled with 1.5 ml of filtered pond water. Mosquito larvae were individually housed in 14ml of filtered pond water. Glassworms and Chironomid midges were housed individually in 100 ml beakers. All taxa were fasted overnight for 24 hours at room temperature, uncovered. After the fasting period, containers in *Bd* treatment were spiked with *Bd,* either 100 zoospores per ml (amphipods) or 1000 zoospores per ml (glassworms, Chironomid midges, hydra, mosquito larvae).

To spike, I chose *Bd* plates free of signs of contamination with visible zoospore density. I removed the Parafilm from the plates and released 10 ml of Milli-Q dechlorinated water to draw the zoospores away from the zoosporangia. After 30 minutes, I pulled up the liquid containing the zoospores in each plate to be expelled into a 50 ml tube to create a composite stock. To reach the desired number of zoospores for spiking, I counted 1 µl of the decant solution using a hemocytometer. To read the hemocytometer, I manually counted the number of zoospores in the cells of each of the four corners of the tool. I took the average of all four corners and multiplied this by 10,000 as I was looking at 1/10000 of a ml to determine the number of zoospores per ml within my solution. The same number of zoospores were added to each tube or beaker for *Bd* positive treatments per experiment.

Animals were left in experiment overnight for a total of 18 hours at room temperature. Mosquito larvae were left in experiment overnight for 16 hours. At the final hour mark, 10 ml of

water is removed from the animals' housing and placed into a new 15 ml tube. The new set of 15 ml tubes with the removed water, containing *Bd* zoospores, were centrifuged for 10 minutes at 3000 g. In the case of hydra, the animals were removed from their housing to be preserved and the remaining water was kept in the original 2 ml tubes to be centrifuged for 10 minutes at 3000 g. The excess water was vacuumed off the DNA pellet using glass Pasteur pipette until there was approximately 50 µl of sample remaining. Animals were preserved in 70% ethanol to be measured via microscopy for analysis later.

Pond *Batrachochytrium dendrobatidis* Analysis

In addition to studying the impact of various macroinvertebrates on *Bd* presence in controlled experiments, I also wanted to investigate the possible presence of *Bd* naturally occurring within my area of study, Long Pond. To do this, I sampled 50 ml of pond water from five distinct locations around the pond. I stored the water samples in the fridge until I was prepared to begin processing them for DNA extraction. Each sample was filtered through a 0.45 µm pre-sterile filter. This was a cellulose nitrate filter that was low binding and allowed for DNA to be freed easily off the surface during extraction. The filtering apparatus was cleaned with a 10% bleach solution in between samples to prevent cross contamination of present DNA. The filtered water was discarded and the filters were stored in 15 ml tubes and kept in the fridge for DNA extraction.

DNA Extraction, Dilution, and qPCR

Extraction from experiment water

My DNA extraction protocol for this study was adapted from Dr. Kerry Kriger and Dr. Taegan McMahon (Kriger, Hero, and Ashton 2006; T. McMahon et al. 2023). To perform DNA

extractions, first 30-40 mg of 0.5 mm silica beads were weighed and placed into sterile, 2 ml screw cap tubes. I then added 150 µl of PrepMan Ultra reagent into each tube containing microbeads and replaced the lids. I labeled all tubes with a sample number from the experiment. The DNA pellets formed in the initial centrifugation during experimental breakdown (50 µl of liquid sample) were transferred to their corresponding numbered tube with the PrepMan Ultra and beads. Samples and reagents were homogenized in two cycles of 60 seconds in the bead beater then 30 seconds at 13,000 g in the centrifuge. This process forced the zoospores to break open, allowing for *Bd* DNA to be extracted. All beaten and spun tubes were then boiled for 10 minutes in a heat-safe tube rack. After the boiling period, I removed the tube rack from the water and placed it into an ice bath for 5 minutes. The chilled samples were spun in the centrifuge for an additional 3 minutes at 13,000 g.

Samples were diluted to a 2ng per 1 µl ratio in order to limit the possibility of qPCR inhibition. DNA dilution protocol to reach this stock solution strength required quantifying DNA present in each sample via use of the NanoDrop 2000. From the found DNA quantity, calculations were performed to find the necessary volume of sample and Diethylprocarbonate treated (DEPC) water to reach 2ng per 1 µl (McQuigg, 2022). The diluted samples were refrigerated or frozen immediately to be plated for qPCR at a later date.

Extraction from filter of eDNA

I followed a similar protocol as describe above but instead of working with liquid DNA from my samples, I was extracting DNA off of the filters. In order to decrease likelihood of qPCR inhibition, I increased the volume of Prepman Ultra reagent used in my DNA extraction protocol to 200 µl for pond water samples. Prepman Ultra was added to the 2ml screw cap tubes containing

30-40 mg of 0.5 mm silica beads. I labeled all tubes with a sample number corresponding to the 5 pond locations. I flamed my scissor and forceps used in cutting and transferring the filter between each sample. I cut out 1/8 of the filter to go in each tube. Filters containing DNA and reagents were homogenized in two cycles of 60 seconds in the bead beater then 30 seconds at 13,000 g in the centrifuge. All beaten and spun tubes were then boiled for 10 minutes in a heat-safe tube rack. After the boiling period, I removed the tube rack from the water and placed it into an ice bath for 5 minutes. The chilled samples were spun in the centrifuge for an additional 3 minutes at 13,000 g. To account for the abundance of environmental DNA (eDNA) present on the filters in addition to the unknown presence of *Bd*, the extracted DNA samples were diluted with DEPC water (Ficetola et al. 2008). Samples were serially diluted by the following factors: 1:50 (10 µl sample to 490 µl DEPC water), 1:100 (100 µl sample to 900 µl DEPC water) and 1:1000 (100 µl sample to 900 µl DEPC water). The serial dilution factors were necessary to limit qPCR inhibitors and determine the optimal sample ratio for analysis (Ficetola et al. 2008).

Quantitative Polymerase Chain Reaction (qPCR)

The use of Quantitative Polymerase Chain Reaction (qPCR) in laboratory study allows for sensitive, accurate detection of target DNA abundance in a series of samples (Boyle et al. 2004). The real-time monitoring associated with qPCR not only identifies the presence of DNA, as done by PCR, but also quantifies it. For the sake of experiments that are studying potential changes in DNA abundance, such as my own, this quantitative step is crucial. In inoculating samples with a known number of *Bd* zoospores at the start of my experiments, I am able to determine the changes in *Bd* abundance when measuring the remaining DNA at the end of the experiments by utilizing qPCR.

The qPCR assay used to quantify remaining *Bd* DNA was adapted from Boyle et al. and Kriger (Boyle et al. 2004; Kriger, Hero, and Ashton 2006). To begin preparing for this process, I created a master mix of *Bd* specific primers, aFAM probe, and TaqMan universal mix (containing the necessary agents to amplify DNA). The FAM probe will fluoresce at a known wavelength so that we can estimate the amount of *Bd* DNA present at each amplification cycle. I used a 96 well plate in all of my qPCR runs and haphazardly placed samples into triplicate groups onto the plate. Each well was pipetted with 5 µl of the diluted sample and 15 µl of the master mix. I included the four-points of my standard curve (1×10^1 to 1×10^4) as samples on the plate as well as DEPC water as my no template control so I could monitor any possible contamination to the whole of the plate. Plates were sealed with a clear qPCR adhesive seal and centrifuged for 2 minutes at 500 rpm. I ran the plates in the Bio-Rad CFX Touch Real-Time PCR Detection System. The qPCR assay used required samples to be brought up to temperature in the first 2 minutes and then denatured sample DNA for the next 15 minutes. After these initial stages, the 50 cycles of amplification take place at 75 seconds per cycle. From the completion of this run in qPCR for each experiment, triplicate groups were averaged and this quantity is the relative DNA per sample used in analysis.

Statistics

The goal of statistically analyzing the aforementioned experiments was not to create comparisons between them, but rather to study the internal significance of the animal treatments on the presence of *Bd* as well as their size class when applicable. All analyses were conducted in R version 4.3.0. Analyses in R included a t-test of experimental controls against the species. The t-tests were two-tailed with no prior assumptions. For experiments with three or more treatment groups due the variable of size class (amphipods, Chironomid midges, mosquitos), I analyzed the

data with an ANOVA to quantity variance among the groups. For post hoc analysis to make pairwise comparisons among groups, I used a Tukey test to compare size class impact to experimental controls and to one another for each species (mosquitos, amphipods, and Chironomid midges). The figures produced in the results section of this study were created in Excel.

RESULTS

Table 1 Macroinvertebrates size class as animals were measured post experiment

Taxa	Size Range (mm)	Standard Deviation	Average (mm)
Large Amphipods	5 – 8	1.03	6.67
Small Amphipods	3 – 4	0.5	3.75
Large Midge	10 – 20	3.67	15.3
Small Midge	6 – 7	0.55	6.4
Glassworms	6 – 8	0.84	7.2
Large Mosquito Larvae	5 – 9	1.67	6.4
Small Mosquito Larvae	3 – 4	0.45	3.8

Table 2 Impact of macroinvertebrates on *Bd* quantity. Significance of taxa analyzed via ANOVA are shown by F-values and significance of taxa analyzed via t-test are shown by t-values. Significant effects are indicated in bold.

Treatment	Test	Test Statistic (t-value/ F-value)	P-value
Amphipods	**ANOVA**	**3.8942**	**0.04522**
Chironomid Midge Larvae	ANOVA	0.904	0.4247
Glassworms	**t-test**	**4.5188**	**0.001342**
Mosquito Larvae	ANOVA	1.3794	0.2785
Hydra	t-test	0.2789	0.7837

Glassworms

Glassworms (Fig. 2) had a significant impact on the quantity of *Bd* when compared to the experimental control (Fig. 3). There were significantly fewer *Bd* zoospores in the beakers with glassworms (Table 2).

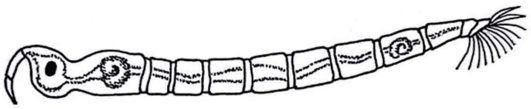

Figure 2 *Chaoborus*, glassworm illustration by Margaret Blewett.

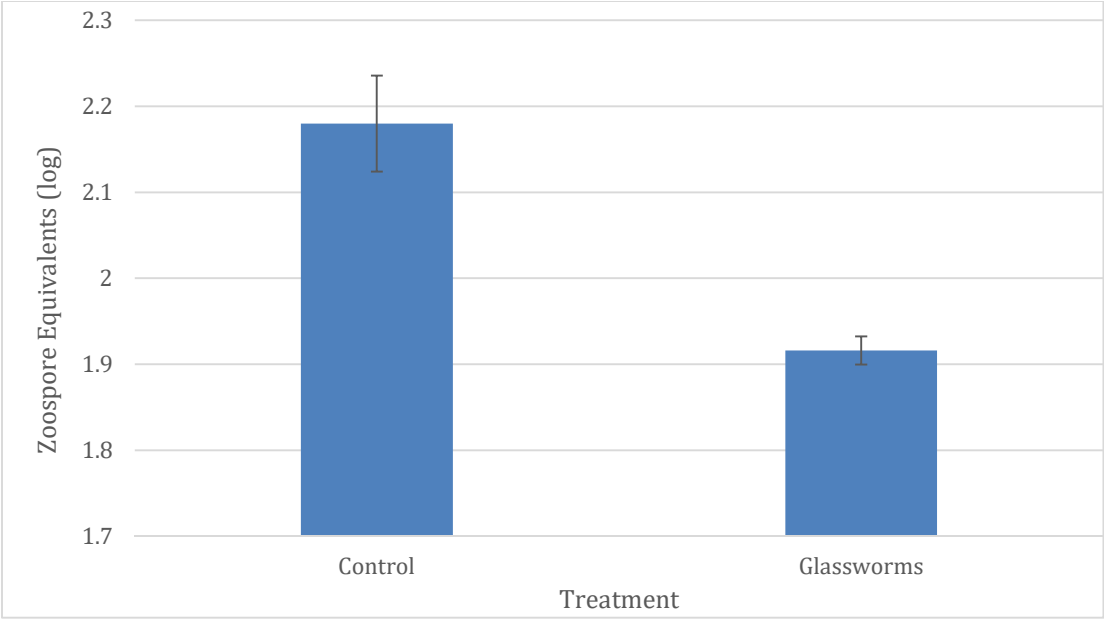

Figure 3 Impact of glassworms on the quantity of *Bd* zoospores from experiment on July 26, 2023. Error bars represent ± 1 standard error.

Amphipods

There were notably more zoospore equivalents measured from the treatment group of large amphipods (5 – 8 mm) when compared to the treatment group of small amphipods (Table 2). Large amphipods did not have significant impact on the quantity of *Bd* when compared to that of the control treatment group (Table 2). Small amphipods (3 – 4 mm) also did not have a significant impact on the quantity of *Bd* present when compared to that of the control group (Table 2). When compared to the small amphipod treatment group, large amphipods (Fig. 4) averaged to have higher zoospore equivalents. There was a wider range of zoospores measured among samples in the large treatment group (Large = 150.16 to 420.52 zoospore equivalents) when compared to the range of zoospores measured among samples in the small treatment group (Small = 156.70 to 249.23 zoospore equivalents) as evidenced by the large standard error in the number of zoospore equivalents in the presence of large amphipods shown in Figure 5.

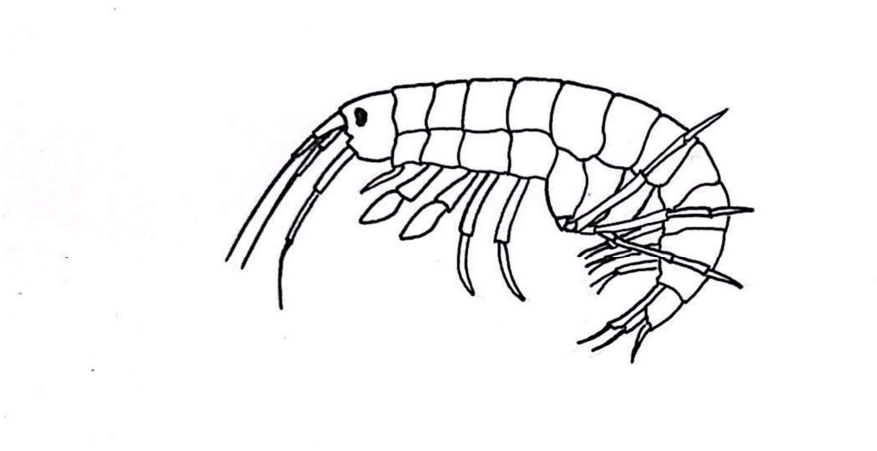

Figure 4 *Gammarus fasciatus,* freshwater amphipods illustration by Margaret Blewett.

Table 3 Summary findings for amphipod data in Tukey's test for post-hoc analysis. Significant effects are indicated on bold.

Treatment Group Comparison	p-value
Small vs Large	**0.0406373**
Small vs Control	0.7174124
Large vs Control	0.2004707

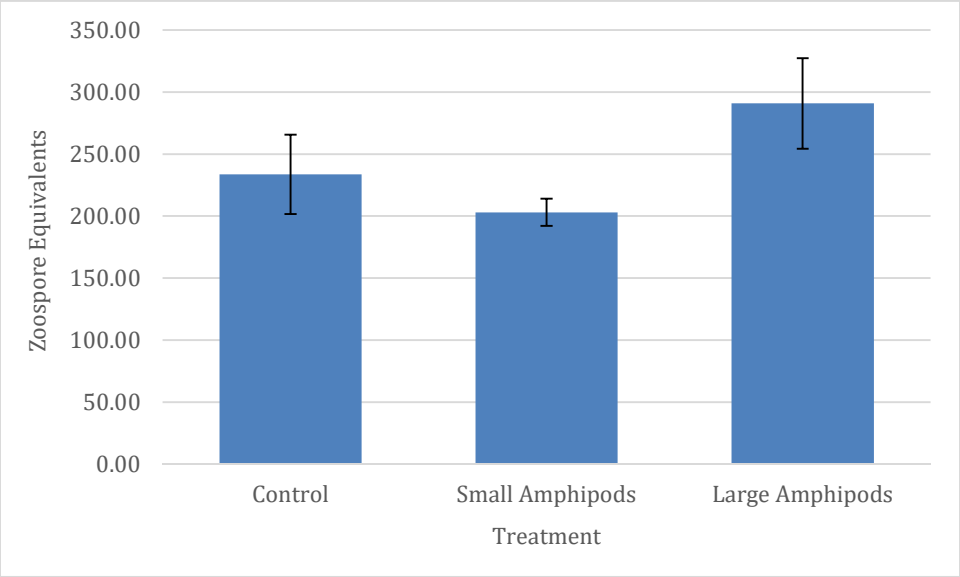

Figure 5 Impact of amphipods on quantity of *Bd* zoospores from experiment on July 12, 2023. Error bars represent ± 1 standard error.

Chironomid Midges

A similarly interesting phenomenon occurred between large Chironomid midges (10 – 20 mm) and small Chironomid midges (6 – 7 mm) in which each size class displays different trends when compared to each other (Table 4). Though, there were no significant effects seen in Chironomid midge larvae (Fig. 6), there is a larger range of zoospore equivalents across the large

midge size class (Large = 1.36415 to 1230.846 zoospore equivalents) when compared to the range of zoospores measured among samples in the small midge size class (Small = 39.61735 to 714.5532 zoospore equivalents). Some large Chironomid midges were found to have 10 times as many zoospore equivalents as the average the experimental control (Fig 7). The small Chironomid midge treatment group did not have a significant impact on the quantity of *Bd* present when compared to the control group (Table 4).

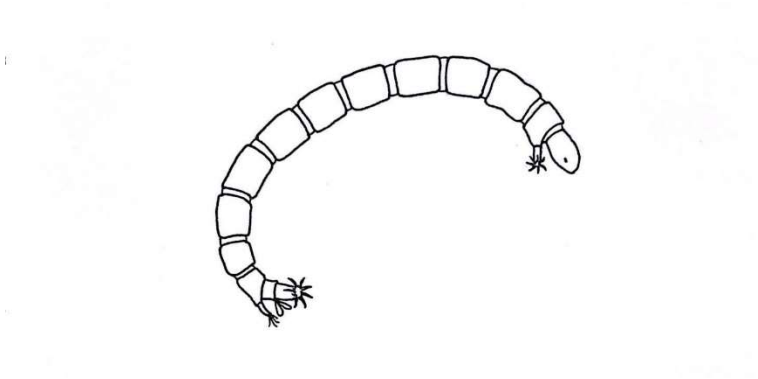

Figure 6 Chironomid midge larvae illustration by Margaret Blewett.

Table 4 Summary findings for midge data in Tukey's test for post-hoc analysis.

Treatment Group Comparison	p-value
Small vs Large	0.6369357
Small vs Control	0.9552937
Large vs Control	0.3998465

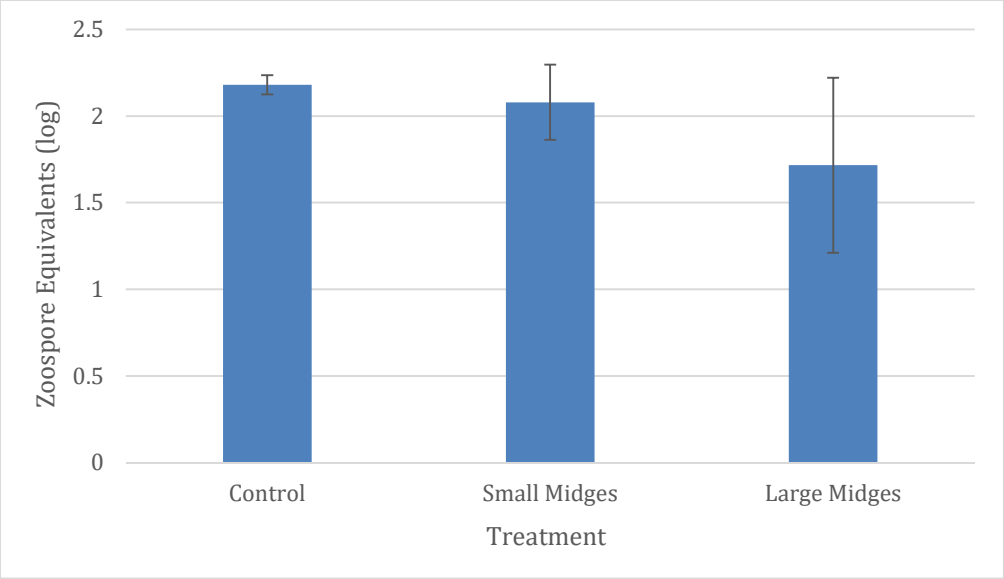

Figure 7 Impact of small midges and large midges on the quantity of *Bd* zoospores from experiment on July 26, 2023. Error bars represent ± 1 standard error.

Mosquitos

Mosquito larvae (Fig. 8), from both large and small size classes, did not have a significant effect on the quantity of *Bd* when compared to the control (Table 5). The large and small mosquito treatment groups do not suggest behavior in this experiment varied between size class (Fig. 9).

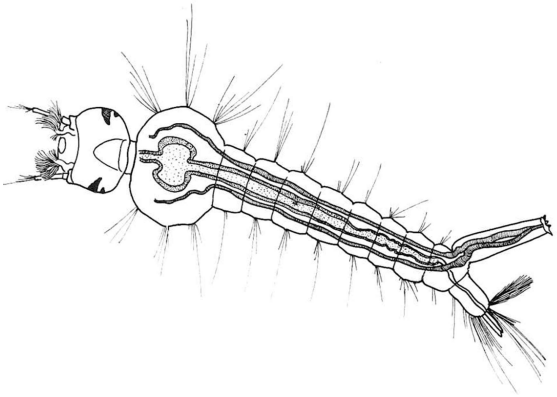

Figure 8 Culicidae Mosquito larvae illustration by D.G. Mackean (Mackean 2024).

Table 5 Summary findings for mosquito data in Tukey's test for post-hoc analysis.

Treatment Group Comparison	p-value
Small vs Large	0.9910703
Small vs Control	0.4192127
Large vs Control	0.9910703

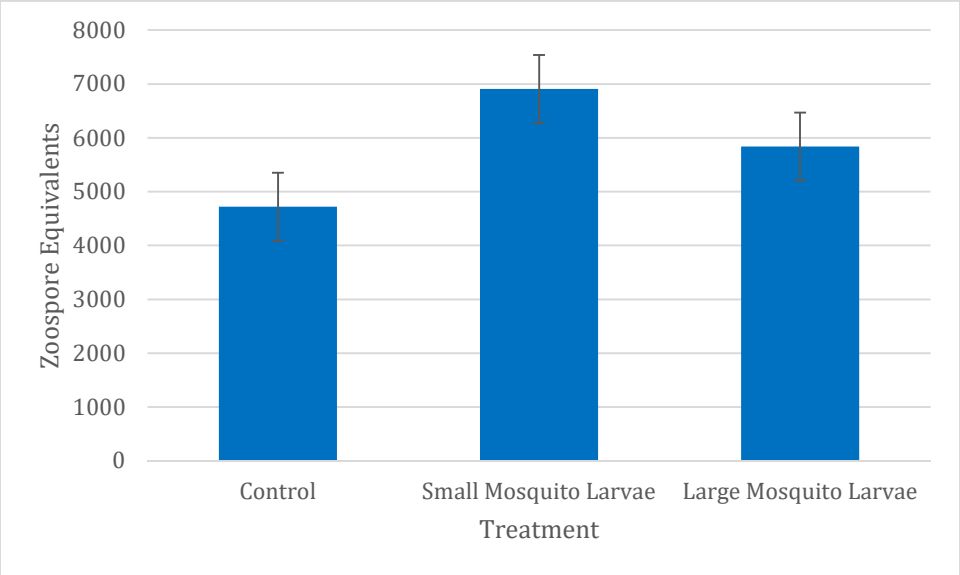

Figure 9 Impact of mosquito larvae on quantity of *Bd* zoospores from experiment on October 11, 2023. Error bars represent ± 1 standard error.

Hydra

Hydra (Fig. 10) did not have a significant effect on the quantity of *Bd* when compared to the experimental control (Fig. 11). Two samples were deemed outliers and removed from analysis due to a magnitude of difference of 10.

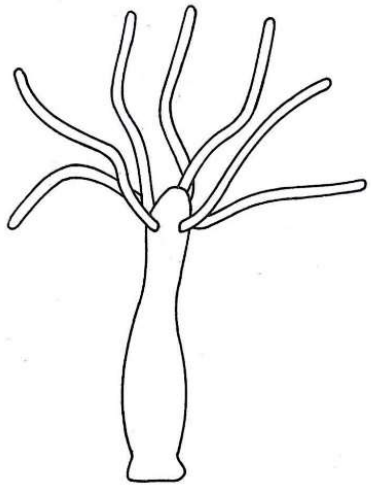

Figure 10 *Hydra vulgaris,* freshwater polyp illustration by Margaret Blewett.

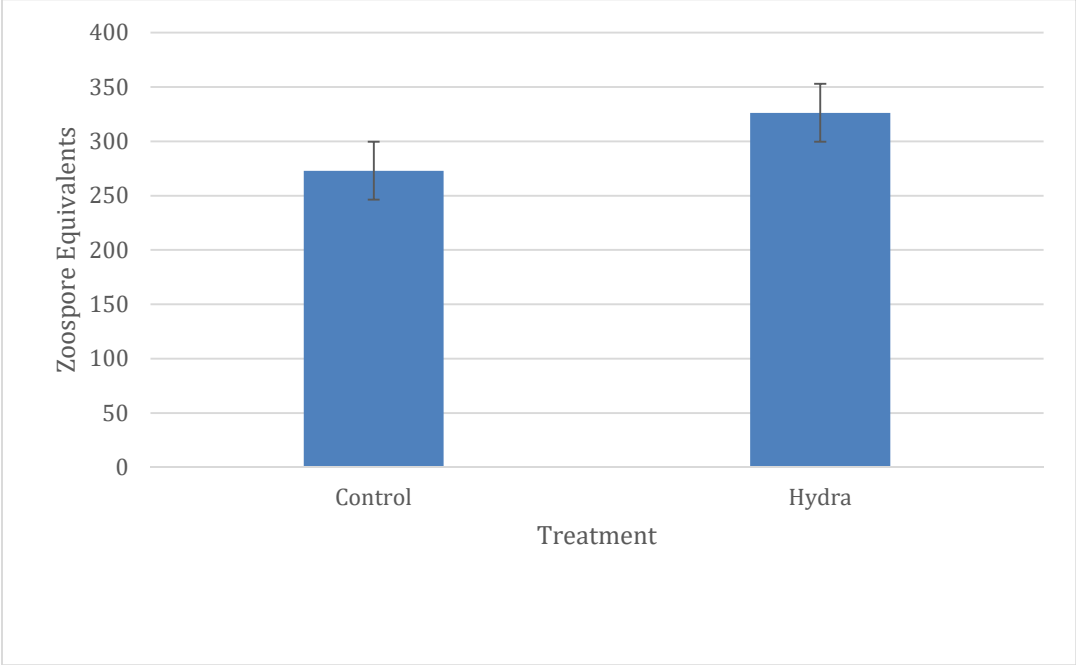

Figure 11 Impact of hydra on the quantity of *Bd* zoospores from experiment on July 26, 2023. Error bars represent ± 1 standard error.

Long Pond

According to the qPCR results of water sampled from Long Pond, all five locations are positive for *Bd* in varying capacities (Table 6). Locations 1-3 hit positively in triplicate at the 1:100 dilutions. Location 4 and 5 indicated the presence of *Bd* in one of the three replicates, suggesting but not conclusively indicating, a positive result (Table 6).

Table 6 Instances in which Long Pond samples from five distinct locations (at three dilution ratios) amplified in qPCR assay.

Location	Dilution Factor		
	1:50	**1:100**	**1:1000**
1	0	3	0
2	1	3	1
3	0	3	2
4	0	1	0
5	0	0	1

DISCUSSION

Of the selected taxa, glassworms were the only treatment group to decrease *Batrachochytrium dendrobatidis* (*Bd)* zoospore abundance compared to controls, which was also seen in previous studies (McQuigg 2022). Amphipods, Hydra, Chironomid midges, and mosquito larvae did not significantly decrease the abundance of *Bd* zoospores compared to controls. These macroinvertebrates, though present in an aquatic environment in which *Bd* would thrive, do not limit its presence. Though Culicidae mosquito larvae were a species of interest in this study for the purpose of addressing insect management concerns, there is no evidence to suggest their presence in aquatic ecosystems decreases the abundance of *Bd* zoospores. It was of specific interest to investigate the role of mosquitos as a biotic factor that may influence *Bd* abundance due to the current management practices that rid our ecosystems of mosquitos purposefully.

While mosquito larvae did not have a significant impact on *Bd*, they serve as an essential food source in their larval stage to fish in aquatic ecosystems (Luna-Figueroa et al. 2019). It is important to consider that complex freshwater communities host a wide variety of taxa that serve a wide variety of ecological services. Though the data from this study on mosquitos does not serve as argument against excessive eradication policies, their important role in complex communities in which *Bd* is also present must still be acknowledged (Strauss and Smith 2013; Luna-Figueroa et al. 2019).

Despite the lack of significance suggesting certain macroinvertebrates limit the quantity of *Bd* zoospores in this study, there is an important phenomenon suggested by the data. The trend observed in trials of large Chironomid midges and large amphipods was that both treatment groups increased *Bd* zoospore abundance in some treatments with great variance among samples

which does not support my hypothesis. Large Chironomid midges have a range of approximately 1 – 1230 zoospore equivalents at the end of the experiment, compared to approximately 87 – 251 zoospore equivalents in the experimental control. The substantial increase in some experimental units is a particularly interesting finding.

The experimental design of this study did not anticipate that *Bd* zoospores would replicate so quickly that there would be an increase in abundance within the 16-18-hour period. Rather, the experiment was designed to see if the selected macroinvertebrate would decrease *Bd* abundance or have no effect at all. The increase of *Bd* zoospores was unprecedented and cannot be explained by the context of the laboratory methods and macroinvertebrate qPCR results alone. The origin of these macroinvertebrates, however, may provide some insight.

The analyzed eDNA from pond water samples suggest that Long Pond is *Bd* positive. All animals used in the experiment, with the exception of mosquito larvae, were collected from Long Pond. Therefore, any animal that came into experiment from Long Pond would have been from an environment in which the pathogen was already present. In the case of large amphipods and large Chironomid midges, data suggests some animals came into the experiment already harboring *Bd* from Long Pond. The mechanism(s) as to how *Bd* affects the macroinvertebrates cannot be determined from this study. Fortunately, previous studies offer some insight on how this could occur.

It is possible that the animal, acting as a reservoir or alternate host for *Bd,* came into the experiment contaminated from Long Pond so that the pathogen was transported from one environment to the next (Strauss and Smith 2013; McMahon et al. 2013). Alternatively, the animal could have served as a host for the pathogen so that *Bd* infected the host allowing for zoospores to reproduce (Strauss and Smith 2013). *Bd* is suggested to cause mortality in nematodes by infection

through their cuticle lining, made up of collagen-based proteins (Strauss and Smith 2013; T. A. McMahon et al. 2013). Crayfish are also considered a potential alternative host for *Bd* shown to be infected via the keratin-based proteins that line their digestive system (Strauss and Smith 2013; T. A. McMahon et al. 2013). Both of these macroinvertebrates suggest that, like frogs and toads, *Bd* is able to thrive on the protein rich portions of their bodies. To guide the future macroinvertebrate research in regard to *Bd*, the physiological makeup of potential study subjects should be considered. In addition to the habitat, feeding patterns, and evolutionary relationships of aquatic organisms that co-exist with *Bd*, their physical makeup is likely what attributes to the ability for infection to take place.

Long Pond was the source for taxa with significant effects on the presence of *Bd* (glassworms) as well as taxa with notable differences among size class (amphipods and Chironomid midges). The variability among *Bd* abundance in what are seemingly similar environments could very well be dictated by the taxa within the environment. This study is not meant to create cross analyses among species but instead sole analyses of how one taxa impacts the presence of *Bd* when compared to its control or in some cases, compared to other size classes. Though the size class of large amphipods and large Chironomid midges do not relate to each other, the fact this phenomenon occurred in the large size class of both animals suggests that the adult phase (amphipods) or last larval instar (midges) of their life cycle could be relevant to their ability to serve as a reservoir or host to *Bd*. Animals in the large size classes are adults or in the case of Chironomid midge larvae, near metamorphosis.

The exoskeletons of freshwater amphipods are made up of chitin (Cauchie 2002). Though chitin is not a protein, it is a long-chain polymer that exhibits protein like characteristics such as those found in collagen and keratin (Cauchie 2002). Chitin can be found in many aquatic

macroinvertebrates, including many larval species (Cauchie 2002). Thus, Chrionomid midge larvae in last larval stage may be comprised of higher quantities of chitin which would be a suitable environment for *Bd* to infect. Studies suggest that chitin-binding module expansions are pathways specific to fungal disease infestation, including that of *Bd* (Abramyan and Stajich 2012). The presence of chitin in the studied macroinvertebrates should be analyzed further as a potential mechanism for the increase in abundance of *Bd* in adult amphipods and the wide range of *Bd* zoospores associated with Chrionomid midges in their last larval instar (Abramyan and Stajich 2012). Aquatic macroinvertebrates serving as alternative hosts or reservoirs to the pathogen could account for an increase in *Bd* abundance in environments that have a greater abundance of large amphipods and large Chironomid midges.

In order to further understand the mechanisms by which Chironomid midges and amphipods interact with *Bd*, future research is needed. Preliminary literature suggests Nile Red as an effective method to visualize how the pathogen infects a given host or lives in a given reservoir when used to dye *Bd* (Galindo et al. 2022). A challenge arises with this methodology for species like the Chironomid midge. The Chironomid midge larvae referenced in this study are bright red in color and therefore would nullify the use of Nile Red to signal live zoospores. Fortunately, there are fluorescent fatty acid probes that can be used to visualize zoospores: BODIPY FL and BODIPY 558/568 at 10 μM (Herbert, Leung, and Bishop 2011). These fluorescent dyes keep the cells viable and therefore could be utilized in experiment with various macroinvertebrates such as Chironomid midges and amphipods in order to visualize how the zoospores interact with the animals and determine the presence of *Bd* in chitin-based tissues (Herbert, Leung, and Bishop 2011; Abramyan and Stajich 2012).

BODIPY dyes could also provide further context as why some aquatic macroinvertebrates

do not have a significant impact on the abundance of *Bd*, specifically in terms of eating the zoospores. Despite feeding strategies consistent with mechanisms employed in other species to eat *Bd* (*Daphnia*), hydra did not have a significant impact on *Bd* zoospore abundance (Hamilton, Richardson, and Anholt 2012; Strauss and Smith 2013). Given the small size of hydra and our inability to visualize *Bd* zoospores without supplemental measures, further studies dying zoospores in a controlled experiment with hydra as a treatment group could provide more insight. Visualizing the mechanisms hydra use to feed when in the presence of *Bd* may suggest the need for adjustments to the experimental procedural of this study in order optimize their ability to feed on *Bd* zoospores.

Though here are grounds for further research from this study which need experimental redesign, there are certainly successful techniques resulting from this study as well. The 1:100 dilution ratio is optimal for diluting eDNA samples from Long Pond in order to detect *Bd* while diluting out qPCR inhibitors. Samples diluted at 1:50 did not amplify. This suggests the lower dilution factor did not dilute out a sufficient quantity of qPCR inhibitors so the target DNA could not be quantified nor detected at this level. The lack of amplification for samples diluted 1:1000 suggests that this dilution is too dilute for possible *Bd* DNA to be measured. One sample from location 5 did amplify at this dilution ratio, but because this amplification lacks statistical strength, I cannot be certain it is an accurate measure of *Bd* in this specific location in Long Pond.

In light of the community interactions in aquatic ecosystems, managing a given region to support biodiversity is an essential step in disease management. Land management measures in aquatic and barrier terrestrial systems should acknowledge the presence of *Bd* in order to implement practices that do not worsen the pathogen's behavior. Habitat fragmentation, water pollution, and customs that exacerbate climate change through the emission of Green House Gases would have negative ramifications on the food sources, breeding grounds, and diversity needed

for complex communities to thrive. The focus for amphibian conservation efforts in regard to chytridiomycosis should be on the maintenance of healthy, productive ecosystems with biodiversity at every trophic level. For frogs and toads that come in contact with *Bd*, their survival can be influenced by a well-managed habitat and the access to clean resources needed for all life phases.

In addition to limiting negative land management practices, there are also measures that can be put into place in order to support amphibian conservation. This includes maintaining the populations of glassworms in aquatic environments as they are suggested to lower *Bd* zoospore abundance. As the mechanisms in which large amphipods and large Chrionomid midge larvae harbor *Bd* are further studied, these are likely populations that should also be maintained in freshwater ecosystems. Though *Bd* replicates rapidly, there is potential to lessen the burden of disease on amphibians by ensuring the pathogen also be harbored in non-amphibian hosts (T. A. McMahon et al. 2013). Determining the taxa that do not lower *Bd* zoospore abundance (amphipods, Chironomid midges, hydra, mosquito larvae) provides insight that there may be alternative environmental factors influencing host-pathogen relationships, whether they be biotic or abiotic.

The threat of *Bd* renders amphibians critically challenged in their own habitat since the pathogen cannot be completely eradicated from ecosystems once infected (Stuart et al. 2004; J. R. Longcore et al. 2007). Fortunately, frogs and toads are not the only organisms that interact with the pathogen. The environment's relationship to host-pathogen interactions is an exceedingly crucial point of wildlife disease intervention. It is evident that there are complex community level interactions in effect between *Bd,* amphibians, and aquatic macroinvertebrate (Strauss and Smith 2013). From this study, it can be concluded that the magnitude at which macroinvertebrates impact

the abundance of novel unicellular pathogen, *Batrachochytrium dendrobatidis* varies from species to species, but said macroinvertebrate remain a biotic factor of interest in amphibian conservation. By studying the environmental factors that manipulate said relationship, we can better assess the need for various land, wildlife, and disease management measures. When caring for aquatic habitats in which macroinvertebrates, amphibians, and *Bd* are known to reside, it is important to consider the ecological value of said macroinvertebrates in terms of disease management. This study contributes to expanding the vision of the amphibian-*Bd*-environment relationship beyond the siloed idea of a host-pathogen relationship. The future of chytridiomycosis studies will provide amphibian populations with not only reactionary measures to the spread of *Bd* but also precautionary measures as well.

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