EVALUATING THE ROLE OF ZOOPLANKTON AND AQUATIC MACROINVERTEBRATES IN THE TRANSMISSION OF BATRACHOCHYTRIUM DENDROBATIDIS IN AN AMPHIBIAN DISEASE SYSTEM

Isha Amin, Maria Bonilla, Emily Hickey, Sydney Kim, Prisha Malik, Tharun Nathan, Jessica Pappas, Rahil Patel, Sania Patel, Gabriela Ramirez, Shalini Shankar, Annette Tran

Advisor: Jessica McQuigg, Ph.D
Assistants: Harris Naqvi B.S., Jonah Fine

ABSTRACT

Discovered in 1998, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has immensely decreased amphibian populations in the Americas, Europe, and Africa. Known for causing chytridiomycosis, the pathogen attacks the keratin on the skin of amphibians and inhibits their abilities to thermoregulate and remain hydrated. This study investigates the role of various zooplankton and aquatic macroinvertebrates in the transmission of *Bd*, and questions how, if at all, invertebrates affect the abundance of *Bd* in a closed system. *Callibaetis ferrugineus* (mayflies), Chironomidae *chironomus* (non-biting midges), *Ferrissia fragilis* (freshwater limpets), Dytiscidae (diving beetles), Cyclopoid copepod (copepods), and Hydrachnidae (water mites) were collected from Drew University's *Bd*-negative artificial ponds for experimentation. Each animal was then housed in either *Bd* spiked or *Bd* negative water for the duration of the experiment. Concentrations of zoospores, the motile and infectious form of *Bd*, were quantified using quantitative polymerase chain reaction (qPCR) after the introduction of invertebrates to the system. Control and experimental setups were prepared for each species to assess their impact on *Bd* zoospore abundance over a specific period. The zoospore expression in the controls averaged ~71. The average zoospore expression for *Ferrissia fragilis* was significantly greater with an average *Bd* expression of ~382. For *Callibaetis ferrugineus*, the average expression was ~236. Cyclopoid copepods significantly decreased *Bd* presence with an average expression of ~38. These findings suggest complex interactions between invertebrates and *Bd*, highlighting potential biotic factors influencing pathogen dynamics in aquatic ecosystems. Further research should explore the mechanistic basis of these interactions and evaluate the potential of invertebrates in controlling *Bd* spread and abundance.

INTRODUCTION

Pathogens, impacted by a plethora of biotic and abiotic factors, present enormous risks to natural ecosystems and severely limit their biodiversity and overall health (1). These factors often interact synergistically within an ecosystem, impacting pathogen abundance, virulence, and host susceptibility (2). In recent decades, there has been notable increase in fungal infectious diseases, many of which are drug-resistant and result in high mortality among infected species (3).

Considered to be the most damaging and invasive panzootic on Earth, the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a pathogenic fungus that was first discovered in 1998. The lineage of *Bd* indicates that the pathogen originated in the early 20th century in East Asia, and it is hypothesized that *Bd* initially spread through the intercontinental expansion of amphibian trading (4/5). Over 700 amphibian species have been reported to be

hosts of *Bd*, and many are now extinct (6). Concern surrounding *Bd* revolves around the fact that it is the first known chytrid to infect, attack, and kill vertebrate species, and has significantly damaged amphibian populations throughout the years (7).

Bd causes chytridiomycosis, a skin disease in amphibians that primarily infects frogs, toads, salamanders, and newts. *Bd* has both a motile and sessile form, which contributes to its success in infecting amphibians. The disease is first transmitted as aquatic, motile zoospores. The zoospores possess posterior flagella which facilitate their movements in aquatic environments. This enables them to infect amphibian hosts by attaching to the keratin layer of the amphibians' skin and penetrating it. Additionally, the zoospores can attach to the area around the mouth of a tadpole and inhibit its ability to feed, dramatically weakening the tadpole. Zoospores lack cell walls while motile, and instead maintain tonicity using water expulsion vacuoles. Only after locating a host do the zoospores encyst and form a cell wall, which propels them into the second sessile phase of their life cycle: zoosporangia. Within a two-day period, zoosporangium can release hundreds to thousands of zoospores (8/9). The zoosporangia cause skin thickening, erosions, and ulcerations capable of disrupting the feeding methods of frogs and other amphibians, as well as their ability to transport water, oxygen, and ions—functions integral to the survival of these organisms.

Thus far, research has largely been focused on determining how and where *Bd* is spread. Numerous factors such as the specific amphibian host species, the temperature of the environment, UV exposure, and precipitation have been shown to correlate to *Bd* frequency. Previous research has also found that there are no specific biotic or abiotic factors that directly correlate to *Bd* presence in an ecosystem (9).

Current literature is insufficient to fully understand why *Bd* is present in some ecosystems and not others, regardless of the commonality between environmental and biological factors. It is also unclear how other organisms and environmental factors can affect *Bd* abundance. This study is preceded by research completed by Strauss and Smith of Washington University in St. Louis in 2013. Their study aimed to identify biotic and abiotic causative agents that lead *Bd* to exist in certain pond systems while not existing in others. They found an indirect correlation between certain invertebrate taxa and *Bd* presence in ponds. At the conclusion of their experiment, Strauss and Smith were unable to identify a significant relationship between amphibian species that tested positive for *Bd* and the presence of *Bd* in the same pond. They also could not identify a correlation between the presence of *Bd* and any of their tested biotic and abiotic factors. However, their study does suggest a positive correlation between Central Newts (*Notophthalmus viridescens*) and the presence of *Bd*, along with a correlation between greater amphibian and invertebrate regional richness among non-*Bd* ponds. This finding specifically brings new attention to the role invertebrates could play in facilitating the spread of *Bd* within pond ecosystems and raises the question of how invertebrates interact with the pathogen. Their results also support direct correlations between physicochemical dissimilarity and amphibian-based dissimilarity, along with a correlation chain between physicochemical dissimilarity, invertebrate incidence-based dissimilarity, and *Bd* incidence. Strauss and Smith concluded that the observed patterns in the occurrence of *Bd* among ponds are mainly attributable to dissimilarities in invertebrate community structure, and they suggest that future research should focus on non-amphibian biota and *Bd* incidence.

Table 1. Invertebrate taxa relative abundance in *Bd*-positive or *Bd*-negative ponds (10)

Macroinvertebrate Taxa	**More Abundant In:**
Chironomid sp.	*Bd*-ponds
Physa gyrina	non-*Bd* ponds
Hesperocorixa spp.	*Bd*-ponds
Chaoborus sp.	*Bd*-ponds
Libellula semifasciata	*Bd*-ponds
Pachydiplax longipennis	non-*Bd* ponds
Notonecta (juvenile)	*Bd*-ponds
Buenoa spp.	*Bd*-ponds
Dineutus sp.	*Bd*-ponds
Callibaetes sp.	*Bd*-ponds

To explore the questions raised by Strauss' and Smith's research, Dr. Jessica McQuigg and 2023 GSNJS students conducted experiments regarding the ecosystemic interactions between *Bd* and invertebrate populations, and how these interactions impacted the abundance of *Bd* within the environment. This study tested two specific species: *chaoborus (*glassworms) and Chironomidae *chironomus*. There was a great deal of variation in the Chironomidae *chironomus* data pool regarding causative increases and decreases in zoospore abundance, suggesting that the Chironomidae *chironomus* must interact with *Bd* in some capacity. This paper's investigation of Chironomidae *chironomus* attempted to further explore this relationship to clarify the mechanisms through which they interact.

In this study, samples containing species of invertebrates were collected from artificial mesocosm ecosystems on Drew University's campus near Zuck Arboretum: *Callibaetis ferrugineus* (mayflies), Chironomidae *chironomus* (non-biting midges), *Ferrissia fragilis* (freshwater limpets), Cyclopoid copepods (copepods; specific species unknown), Dytiscidae (diving beetles; specific species unknown), and Hydrachnidae (water mites; specific species unknown).

Chironomidae *chironomus*

Chironomidae *chironomus*, also known as non-biting midges, are a type of fly with over 20,000 known species worldwide. This experiment focused on the larvae of Chironomidae *chironomus*, which are abundant within freshwater ecosystems including coastal areas,

riverbanks, and nutrient-rich ponds. The anatomical structure of larvae are long and cylindrical with a sclerotized head and a pair of prolegs on their thorax. Chironomidae *chironomus* larvae feed on plankton, algae, and decaying organic matter found in their habitats (11). As stated in the Strauss and Smith study, these larvae exist in large numbers, sometimes even thousands per square meter, and are also seen to be the most abundant invertebrate in *Bd*-infested ponds, as stated by Strauss and Smith (10).

Ferrissia fragilis

Ferrissia fragilis, or freshwater limpets, are mollusks found on and under dead leaves, rocks, and woody debris in still bodies of water (12). They are herbivores with diets consisting of mainly micro algae scraped from the surface of live plants or rocks, a possible site of *Bd* presence. *Ferrissia fragilis* has not been identified as a species that interacts with *Bd* at the time of this study.

Callibaetis ferrugineus

The taxa *Callibaetis ferrugineus*, a species of mayfly, are aquatic insects found in the United States. *Callibaetis ferrugineus* are unable to fly before metamorphosing, so juvenile nymphs were collected and investigated in this study. The larvae are detritivores and herbivores, having diets mainly containing detritus, algae, and other small aquatic plants.

Cyclopoid Copepods

Cyclopoid copepods are a type of zooplankton with over 13,000 species that are incredibly abundant in both freshwater and marine ecosystems, making them one of the most numerous multicellular animals on the planet (13, 14). The specific species of copepod utilized in this study remains unidentified, however, because it is incredibly challenging to identify individual species by observation. Nonetheless, there is not a large amount of ecological variation among copepod species, so its order will be used. Cyclopoid copepods were the order studied in this paper. These freshwater crustaceans are omnivores with diets consisting of detritus, cyanobacteria, and protists, such as diatoms, attached to sediment grains (14). Cyclopoid copepods can sometimes be carriers of pathogens such as the bacterium that causes cholera, and thus, a relationship with *Bd* that has yet to be explored is viable.

Hydrachnidae

The Hydrachnidae family, commonly referred to as water mites, lives in still, fresh water. They feed on insect larvae (15), many of which are suspected to correlate to the presence of *Bd* in ponds (10).

Dytiscidae

Larvae of the family Dytiscidae, also known as diving water beetles, can grow up to two inches in length and typically live at the edge of ponds. Adult Dytiscidae have the ability to fly to new bodies of water if need be, so it is possible that pathogens could be transmitted between

ecosystems. Although, their juvenile stages were studied as they do not yet have the ability to fly from ecosystem to ecosystem meaning that *Bd* transfer would not be due to cross contamination.

Figure 1. Top Row Left to Right: Chironomidae *chironomus* (Bertone 2018), *Ferrissia fragilis* (Son 2007); Middle Row Left to Right: *Callibaetis ferrugineus* (Hafele 2017), Cyclopoid copepod; Bottom Row Left to Right: Hydrachnidae (Hamersky), Dytiscidae (North Carolina State University 2015)

METHODS

Sampling Process

Our experimental samples were collected from 10 artificial mesocosms near Zuck Arboretum on Drew University's campus. These ponds, established on April 2, 2024, are managed by Dr. McQuigg. Each pond was able to successfully form its own ecosystem as invertebrates entered the mesocosms on their own (19).

We filled several large containers with water from these artificial ponds along with invertebrates, sediment, and decomposing organic matter. The larval cases of Chironomidae *chironomus* were carefully scraped from the side of the artificial pond and beetle larvae were collected from small foam ledges placed in each artificial pond. *Ferrissia fragilis*, *Callibaetis ferrugineus*, Hydrachnidae, and Cyclopoid Copepod were all within the leaf litter, and were later extracted in the lab. All samples were collected with standard precaution methods, which included using nets that were checked for the pathogen. After the samples were collected in buckets, they were transported to the lab and covered with a fine mesh, both to prevent the organisms from escaping and to protect their homeostatic environment.

Sample Filtration and eDNA Extraction

To identify if the artificial ponds had been infected with *Bd*, we extracted samples from each artificial pond for later eDNA analysis. We then assembled a vacuum pump and vacuum flask for filtration. A funnel cork was placed in the vacuum flask and we used bleached forceps to place a sterile filter on the platform. To ensure that there was no contamination during the filtration process, a funnel, prior to its assembly, was bleached in 10% bleach solution for 3 minutes, followed by a fresh water rinse for 2 minutes. The funnel was only then assembled, and after turning on the vacuum pump, we added the water sample. Once the filter was fully dried and the vacuum seal was broken, the filter was moved to a fresh foil square using bleached forceps. The filter was cut into eighths and 1/8th was placed into a 1.5 mL tube while the remaining was placed in a second 1.5 mL tube.

After filtration was completed, we extracted the DNA and tested it for *Bd*. A 2.0 mL conical bottom tube containing silica zirconia beads was labeled for the sample number and 200 µL of PrepMan Ultra reagent was added using a micropipette. We then added the sample to its designated tube using flamed forceps and the tubes were placed on ice. To homogenize the samples and physically break the spores open, we placed the tube in the bead beater for 60 s, cooled it, and then transferred it to the centrifuge, running for 30 s at 13,000 G. We repeated this process once and then placed it in a floating, heat-safe rack in boiling water for 10 minutes. After the tubes were removed from the water, we chilled them for 5 minutes and spun again for 3 minutes at 13,000 G. The remaining liquid should contain *Bd* DNA if it is present. The sample was then diluted at a 1:100 ratio. This was done by adding 990 µL of DEPC water to a labeled 1.5 mL tube, vortexing the sample, and adding 10 µL of the extracted DNA to the tube. We then placed the tube in the refrigerator to await qPCR. We repeated these processes for each water sample taken from each of the sample ponds.

Experimental Setup Protocol

After a 48 hour period, the collection buckets were uncovered. We removed each invertebrate from their collection buckets and placed them into ice cube trays separated by species. We labeled a limited number of trays as Chironomidae *chironomus* and *Callibaetis ferrugineus*. Any other trays were labeled ‘other’ and filled with miscellaneous organisms, to be later identified. From these miscellaneous organisms, there was an adequate amount of collected *Ferrissia fragilis*, Cyclopoid copepods, Hydrachnidae, and Dytiscidae larvae for the experiment.

Organisms were removed from the sample using narrow and wide droppers, as well as forceps. Once caught, we identified the organisms with the use of magnifying glasses and

dissecting microscopes. Fifteen Chironomidae *chironomus*, *Callibaetis ferrugineus*, Dytiscidae larvae, and Hydrachnidae, along with thirty *Ferrissia fragilis* and 150 Cyclopoid copepod were used in the study, each approximately equal in size, color, and instar to the rest of their species. Organisms of the same species were transferred, using wide droppers, into deli containers containing ~500 ml of spring water and their respective foods, to allow for their survival until the next experimental period.

Identification of Invertebrate Species

Ninety-five clear plastic cups were labeled 1-95 with 100 ml of spring water added to each cup. 1-15 were designated for the *Ferrissia fragilis*, 16-30 for the Chironomidae *chironomus*, 31-45 for the *Callibaetis ferrugineus*, 46-60 for the Cyclopoid copepod, 61-70 for the Dytiscidae larvae, 71-80 for the Hydrachnidae, and 81-95 were controls with just spring water. For the first fifteen cups, two *Ferrissia fragilis* were randomly assigned to each of the cups. They were diligently transferred using droppers, ensuring that no pond water was inadvertently transferred with them. This same protocol was utilized for the Chironomidae *chironomus*, the *Callibaetis ferrugineus*, the Dytiscidae larvae, and the Hydrachnidae, but with only one organism transferred to each cup. For the Cyclopoid copepod, 10 organisms were placed within each cup. Of each grouping within the fifteen cups, ten were experimental and infected with the *Bd* pathogen, while five were procedural controls, free of the *Bd* pathogen. For the organisms with only ten cups, 7 were designated as experimental with the pathogen, while three were procedural controls. Any waste leaf litter or water was placed into waste litter buckets. The organisms in these samples were fasted for 24 hours before being exposed to *Bd*.

Cell Counting and Spikes

Kept sealed, six *Bd* culture plates from the *Bd* culture line JSOH1 were placed under a microscope capable of 4x magnification. These cells were isolated in 2011 from the infected Northern Leopard Frogs in Ohio (20). We recorded observations regarding the appearance of the cells, characteristics of the cells relative to the other cells, and the actions of the cells on the culture plate.

We carefully removed the lids of the *Bd* plates and 5 ml of Milli-q water were pipetted evenly along the plates in order to allow the zoospores the ability to traverse on the surface of the plates. Milli-q water is deionized, demineralized, and filtered in order to ensure that no contaminants can impact the sample. The lids were replaced and were left aside for 10 minutes in order for the zoosporangia to release zoospores. Once the 10 minutes passed, we collected the water on the plates using micropipettes and washed the surface of the plates. We repeated this process 3 times and added all liquids to 15 mL Falcon tubes.

We removed 100 μL from the tube using a micropipette and loaded it into the well of a hemocytometer. Then, we counted the number of zoospores in each corner of the 16-square grids using a hand clicker and recorded the results. The four measurements were averaged and multiplied by 10,000 in order to get the measurement in units of 1,000 zoospores/ml.

At 4:00 pm, 24 hours after the invertebrates began to fast, we added 100,000 zoospores to each experimental cup for 18 hours. The amount of microliters of the zoospore solution that was added was based on the calculations described earlier.

Invertebrate Measurements

After a 24 hour period, we labeled flip-cap tubes with their animal numbers, 1-95. Using a new dropper for each organism, we carefully moved the sample animals to their designated flip cap tubes, the *Ferrissia fragilis* being the only species with 2 organisms in 1 tube. Attempts were made to transfer as little water as possible. If water spilled while transferring, *Bd*-infected water was immediately cleaned with ethanol to prevent cross-contamination between samples. Once all animals were transferred, the organisms were euthanized by ethanol. After 5 minutes, we transferred the euthanized samples to petri dishes one at a time using forceps. After observing the organisms under the dissecting microscope, we measured the lengths of the organisms in millimeters to the nearest tenth and recorded the results in a data table. We referenced online resources to confirm the suspected species and stages of life for each organism. After every inspection, the animals were placed back into their tubes and placed aside for preservation.

qPCR

DNA extraction and qPCR (quantitative Polymerase Chain Reaction) methods were then used in order to determine if the invertebrates interacted with the pathogen, compared to relative controls. Once 18 hours had passed since the samples were first exposed to the microbe, we weighed out 30-40 mg of 0.5 mm zirconium beads for each sample and added them to sterile, flip cap tubes. We then spun the samples for 10 minutes at 3,000g, and the pellets were removed to leave behind 50 ul of liquid, which were then added to the flip cap tubes with the zirconium beads. 150 µl PrepMan™ Ultra Reagent was then added for rapid preparation and purification of the DNA template. The reagent inactivates PCR inhibitors, allowing researchers to better analyze these pathogens through amplification techniques. Next, we gently agitated the samples three times through a micropipette, after which 150 µl of each sample was transferred into the tube. Throughout this process, finished tubes were put into ice to maintain sample integrity.

All of the tubes were placed on the bead beater for 60 seconds, 3 tubes at a time to physically break the spores open and release the DNA from inside the nuclear envelope of the cell. They were then cooled on ice, and then all spun simultaneously in the centrifuge for 30s at 13,000 G to bring the DNA down to the bottom of the tube and condense it. Each sample was beaten and spun twice and then placed in a floating, heat-safe rack in boiling water for 10 minutes to activate the Prepman solution. The tubes were then chilled for five minutes and spun for 3 minutes at 13,000 G.

Due to the extreme sensitivity of quantitative polymerase chain reactions, we followed special laboratory procedures to avoid contamination. The lab was regularly wiped with ethanol and only qPCR-designated pipettes were used.

Using the extracted DNA, we utilized a Nanodrop 2000 to equalize the concentration of DNA in each sample. Then, we ran qPCR to quantify the number of copies present of the target *Bd* DNA sequences in each well relative to the control samples. This was first completed by creating a mastermix containing 2 ul DEPC water, 10 ul Taqman, and 1 ul of the following primers: ITS1-3 Chytr, 5.85 Chytr, and Chytra MGB2. We multiplied each amount by the number of wells that needed to be filled, plus an additional few wells in case of loss during transfer. These primer sequences and the minor groove binder probe sequence were noted by Boyle et. al. in 2004 to target the ribosomal RNA and in which a PCR Taqman assay that could quantify one zoospore in a sample of *Bd* was developed (21). These primers target the ribosomal

RNA of ITS-1 regions, which are conserved regions in most fungal pathogens. We ran each reaction in triplicate to allow for averaging. First, 15 ul of mastermix was pipetted into each well of a PCR plate for ninety-six wells total. We ran four triplicate trials by adding 5 ul of solutions containing different concentrations of zoospores, including 10^1, 10^2, 10^3, and 10^4, which acted as our standard curve. Another triplicate trial was run by adding 5 ul of DEPC water to act as a control for contamination. For the rest of the wells containing the mastermix, 5 ul of each standard from their respective water samples were added. Once all the required wells were filled, we used thermoseal to cover the entire PCR plate. The plate was then centrifuged for two minutes at 500 G and a standard PCR protocol was used. Based on the intensity of fluorescence signals, Minor Groove Binder (MGB) probes during qPCR cycles quantified the number of copies of the target DNA that were present relative to the controls.

RESULTS

Our qPCR yielded a comparison of the zoospore count between the control, *Ferrissia fragilis*, Chironomidae *chironomus*, *C. ferrugineus*, Cyclopoid copepod, Dytiscidae Beetle Larvae, Hydrachnidae Water Mite.

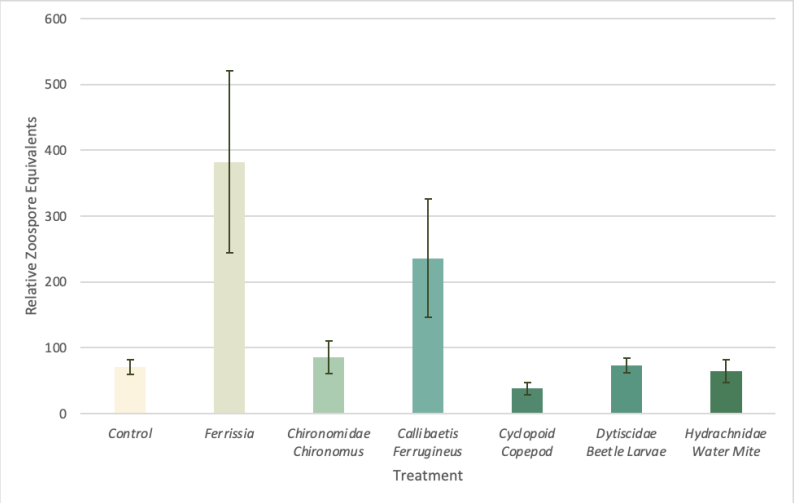


Figure 2. Untransformed Mean Abundance of *Bd* Zoospores by Treatment - Every organism was run triplicate through qPCR. The mean values for each sample within the seven treatments are represented by the bars. The error bars represent the standard error of the sample data.

Table 2. Variation in *Bd* zoospore expression levels by species

Speices	Maximum	Minimum	Range

Control	127.02	27.19	99.83
F. fragilis	1155.62	48.52	1107.10
Chironomidae *chironomus*	186.63	0.00	186.63
Callibaetis ferrugineus	739.73	8.52	731.21
Cyclopoid Copepods	93.66	12.17	81.49

Control number 84 was an outlier and was removed from the data. The biological $\alpha = 0.05$, and if the p-value is below 0.050, then the alternative hypothesis is accepted.

The data indicates that *Ferrissia fragilis* had a significantly higher zoospore expression than the control group (*Ferrissia fragilis*: 382.41 ± 138.40; control: 71.29 ± 11.07). *Ferrissia fragilis* were found to have a p-value of ~0.049,which is less than 0.050, indicating statistically significant results.

Cyclopoid Copepods were found to have a significantly lower zoospore expression than the control group (Cyclopoid Copepods: 38.16 ± 9.34; control: 71.29 ± 11.07). Samples 51 and 53 of the Cyclopoid Copepods were removed from the data, in order to remain conservative, as those samples had zero zoospore expression. Cyclopoid Copepods were found to have a p-value of ~ 0.046, which is statistically significant.

Callibaetis ferrugineus increased zoospore expression compared to the control (*Callibaetis ferrugineus*: 236.14 ± 89.67, control: 71.29 ± 11.07). The p-value for *Callibaetis ferrugineus* was ~ 0.087, which is marginally significant since it falls between 0.050 and 0.100.

The data indicates that Chironomidae *chironomus* had a greater zoospore expression than the control group (Chironomidae *chironomus*: 85.78± 24.70; control:71.29 ± 11.07)(Figure 2). Subject 22 of the Chironomidae *chironomus* was removed from the analysis due to an erroneous amplification likely as a result of contamination. The p-value for Chironomidae *chironomus* was ~0.603, indicating not statistically significant results.

Dytiscidae larvae and Hydrachnidae had the greatest p-values (Dytiscidae larvae: 0.901; Hydrachnidae: 0.729); thus, the results are not statistically significant.

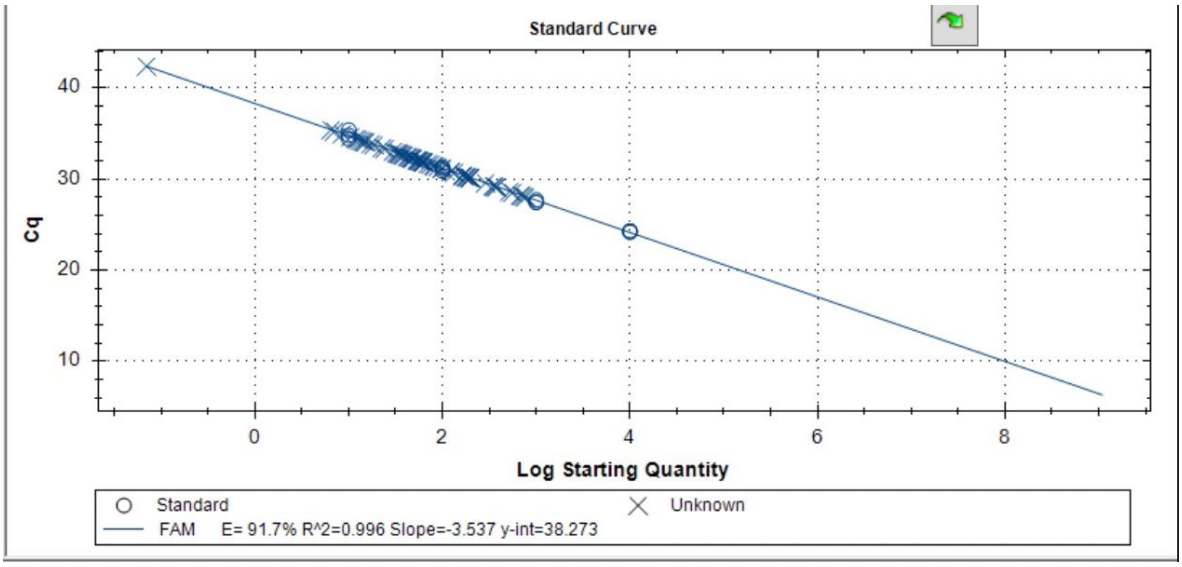


Figure 3, Log-transformed qPCR analysis results of *F. fragilis*, Hydrachnidae, and controls adjusted to a standard curve.

Via qPCR analysis, all samples were plotted along a standard curve in order to better visualize the data concerning relative *Bd* DNA presence in the samples.

DISCUSSION

This study aimed to identify a relationship between invertebrates and *Bd* abundance, hypothesizing that invertebrates would directly impact the amount of *Bd* zoospores. Six organisms were analyzed: *Ferrissia fragilis*, Chironomidae *chironomus, Callibaetis ferrugineus*, Dytiscidae larvae, Cyclopoid copepod, and Hydrachnidae.

Consistent with the study's hypothesis, the experimental data from *Ferrissia fragilis* showed a statistically significant difference in the amount of zoospores present. Exhibiting a p-value of 0.049, this species notably increased *Bd* abundance over the 18 hour period, thus suggesting that there exists a relationship between *Ferrissia fragilis* and zoospore abundance.

The data from the Cyclopoid copepods demonstrates a statistically significant difference in zoospore expression (38± 9.34), a value significantly lower than the control group (71.29 ± 11.07). The low zoospore expression can likely be attributed to the Cyclopoid copepod preying on *Bd* zoospores, resulting in a decreased zoospore abundance in the samples. This supports the alternative hypothesis: there is an interaction between Cyclopoid copepod and *Bd*. This species was not specifically mentioned in Strauss and Smith's paper, but zooplankton species in general were mentioned as not having a significant effect on *Bd* abundance. However, copepods were not mentioned specifically, so it could be possible they never tested the species in their research. Additionally, it is important to note that they may also act as a host for *Bd,* as it is known that fungi often attach to the bodies of Cyclopoid copepods. However, the lower zoospore abundance value relative to the control shows that Cyclopoid copepod behavior of feeding on zoospores is the more likely interaction.

Callibaetis ferrugineus increased zoospore expression when compared to the control group. However, because the *p*-value (~0.087) is in between the range of 0.050 and 0.100, the results are marginally significant. Thus, it is difficult to conclusively reject or fail to reject this study's null hypothesis (22). Additionally, the high average of the *Callibaetis ferrugineus* zoospore concentration in Figure 2 is due to highly varied data points, hence the high p-value. Samples 3, 6, 7, 8, 9 have zoospore expression levels below 60 while samples 1, 2, 3, 5 display expression levels exceeding 300.

The Chironomidae *chironomus*, in comparison to the control group, showed no statistically significant difference in the amount of zoospores (p=0.603). However, unpublished research from the NJGSS students and Dr. McQuigg in 2023 found large variation in the amount of zoospore DNA when Chironomidae *chironomus* were studied, a pattern not identified in this year's data. They proposed that the Chironomidae *chironomus* could be possible reservoirs or alternative hosts for *Bd*. They believed that the Chironomidae *chironomus* may have been shedding excess *Bd* into the environment because zoospores are able to attach to skin of infected organisms, multiply the zoospore population, and then release *Bd* into water. Earlier this year it was found that those Chironomidae *chironomus* were collected from a *Bd* positive environment, unlike the specimens in this year's study, which were only collected from *Bd* negative environments. (23). This could indicate that the Chironomidae *chironomus* only assumes the position of alternate hosts or reservoirs in environments with *Bd*. Additionally, the full cycle of *Bd* infection ranges from 24 to 48 hours, and experimental design restricted this study to a time period of 18 hours. Therefore, the Chironomidae *chironomus* were not exposed to *Bd* for an adequate, extended period of time, which, in theory, prevented them from ascertaining the role of multiplying or reducing the amount of *Bd*.

Because Dytiscidae larvae and Hydrachnidae have p-values greater than 0.100 (0.901 and 0.729, respectively) it is concluded that they do not increase or decrease *Bd* to a statistically significant degree. Therefore, they do not warrant future research if they are collected from *Bd* negative environments. Nonetheless, this information still offers evidence for Strauss and Smith's theory that *Bd* positive ponds are associated with certain macro invertebrates. This is illustrated by Figure 2, where the Dytiscidae larvae and Hydrachnidae are shown to not interact with *Bd*.

LIMITATIONS

Since the invertebrates tested were not bred for this experiment and were instead collected from naturally forming ecosystems, there are a small number of limitations in this study that could have impacted the results. When we left the mayflies to interact with the *Bd*, 3 of the mayflies died. So, in this population, the sample size was unintentionally smaller than expected and we had less power in our findings. Additionally, a mayfly metamorphosed and so we lost some uniformity among our samples. Moreover, this was a joint study with a plethora of scientists completing each procedure. This creates the possibility that the samples were not all uniform and increases the chance of potential contamination.

CONCLUSIONS AND FUTURE DIRECTIONS

As a result of the findings in this study, there is evidence of interactions between *Bd* and certain invertebrates. In the case of this experiment, interactions were observed in *Ferrissia fragilis* and Cyclopoid copepods. Possible interactions were observed in *Callibaetis ferrugineus* and Chironomidae *chironomus*, although further experimentation is needed to confirm the involvement of these organisms in the spread of *Bd*. A larger sample size of *Callibaetis ferrugineus* may increase the power of the experiment and lead to a statistically significant result. Additionally, Chironomidae *chironomus* can be kept in contact with *Bd* for longer periods of time and see if they are able to become an alternative host.

Thus, future research should aim to further understand the mechanisms by which the pathogen interacts with its host utilizing visualization techniques. For example, Nile Red has been used to artificially stain the lipid components of zoospores and help track their location on or inside the host (21). Additionally, Chironomidae *chironomus* have previously been shown to increase *Bd* abundance when sourced from a *Bd* positive pond. However, those results were not significant. Any data regarding Chironomidae *chironomus* from *Bd*-negative ponds was also insignificant. However, there was great variation in both data sets with the Chironomidae *chironomus*. Thus, Chironomidae *chironomus* should be studied as a possible alternative host of the pathogen. A challenge for Chironomidae *chironomus* is that they are red in color just like the Nile Red (24,25), so an alternative method of fluorescent probes, BODIPY FL and BODIPY 558/568 can be utilized to investigate the pathogen's path in the organism (26).

Furthermore, this experiment was conducted with the highly controlled environment of the laboratory and therefore results may vary in a natural ecosystem. Thus, future research should be conducted while accounting for temperature, pH levels, etc in order to replicate the natural environment and further understand how invertebrates interact with the pathogen.

ACKNOWLEDGEMENTS

This research was conducted as a part of the Governor's School of New Jersey in Science at Drew University. We would like to thank certain organizations for their gracious donations which allowed us to complete this endeavor, including the Office of the Secretary of Higher Education of New Jersey, The Overdeck Family Foundation, Novartis, and GSNJS Alumni & Parents of Alumni. Next, we would like to extend our sincerest thanks to Dr. Jessica McQuigg, who generously guided us with their extensive expertise and support throughout our research. In addition, we would like to thank Dr. McQuigg's team of dedicated undergraduate students, Chloe Dudonis, Breena Hezel, Brynn McCarthy, and Danny Walker, who assisted us in completing our procedure within the given time frame. We would also like to recognize Harris Naqvi and Jonah Fine whose contributions were an integral part of our research process. Finally, we would like to thank Dr. Adam Cassano, Dr. Stephen Dunaway, the counselors of the GSNJS, and Drew University faculty for this opportunity.

REFERENCES

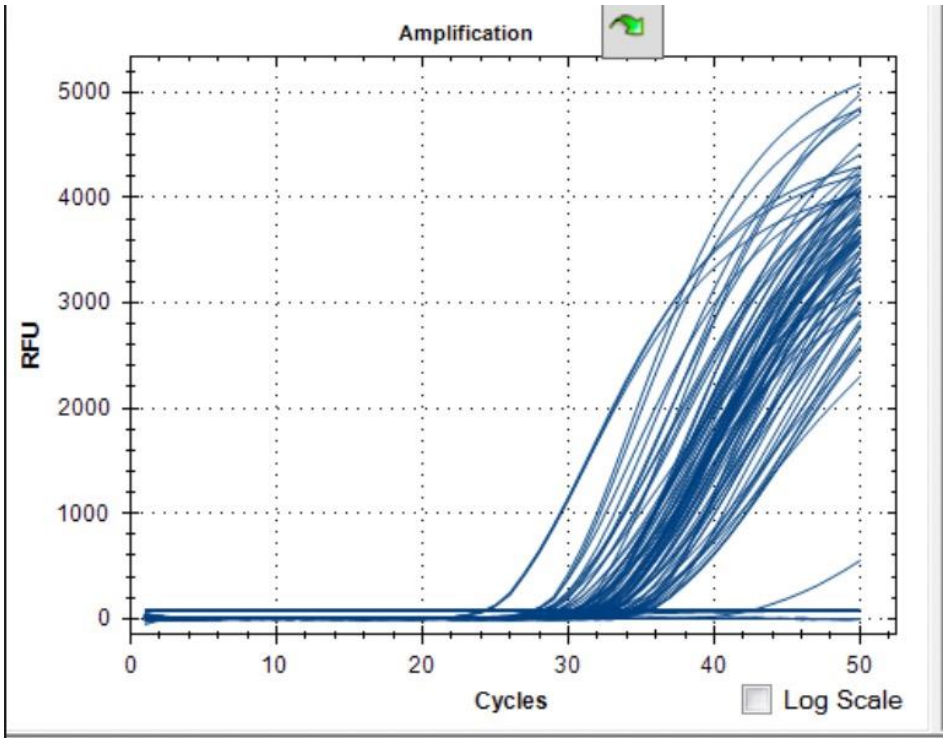
1. Bernardo-Cravo AP, Schmeller DS, Chatzinotas A, Vredenburg VT, Loyau A. 2020. Environmental Factors and Host Microbiomes Shape Host–Pathogen Dynamics. Trends in Parasitology. 36(7):616–633. doi:https://doi.org/10.1016/j.pt.2020.04.010. https://www.cell.com/trends/parasitology/fulltext/S1471-4922(20)30107-0?rss=yes&utm_source=researcher_app&utm_medium=referral&utm_campaign=RESR_MRKT_Researcher_inbound
2. Mcquigg JL, Boone M. EVALUATING THE INFLUENCE OF ABIOTIC AND BIOTIC ENVIRONMENTAL CHARACTERISTICS IN AN AMPHIBIAN DISEASE SYSTEM [PhD Thesis]. Oxford (OH): Miami University. 2022. https://etd.ohiolink.edu/acprod/odb_etd/ws/send_file/send?accession=miami1657724856475325&disposition=inline
3. Pappas G, Vrioni G. 2024 The Last of Us and the Question of a Fungal Pandemic in Real Life. Emerging Infectious Diseases journal. doi:10.3201/eid3003.230684. https://wwwnc.cdc.gov/eid/article/30/3/23-0684_article#:~:text=In%20recent%20years%2C%20a%20rising.
4. O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, Kosch TA, Murray KA, Brankovics B, Fumagalli M, et al. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. Science. 360(6389):621–627. doi:https://doi.org/10.1126/science.aar1965. https://science.sciencemag.org/content/360/6389/621.
5. Gladieux P, Byrnes E.J., Aguileta G, Fisher M.C., Billmyre R.B., Heitman J.H., Giraud T. 2017. 4 - Epidemiology and Evolution of Fungal Pathogens in Plants and Animals. https://doi.org/10.1016/B978-0-12-799942-5.00004-4. https://www.sciencedirect.com/science/article/abs/pii/B9780127999425000044
6. Lips KR. 2016. Overview of chytrid emergence and impacts on amphibians. Philosophical Transactions of the Royal Society B: Biological Sciences. 371(1709):20150465. doi:https://doi.org/10.1098/rstb.2015.0465. https://royalsocietypublishing.org/doi/10.1098/rstb.2015.0465
7. Rosenblum E, Fisher M, James T, Stajich J, Longcore J, Gentry L, Poorten T. 2009. A molecular perspective: biology of the emerging pathogen Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms. 92(3):131–147. doi:https://doi.org/10.3354/dao02179. https://pubmed.ncbi.nlm.nih.gov/21268975/
8. Searle CL, Mendelson JR, Green LE, Duffy MA. 2013. Daphnia predation on the amphibian chytrid fungus and its impacts on disease risk in tadpoles. Ecology and Evolution. 3(12):4129–4138. doi:https://doi.org/10.1002/ece3.777. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3853558/

9. Kilpatrick AM, Briggs CJ, Daszak P. 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends Ecol Evol. 25(2):109–118. doi:10.1016/j.tree.2009.07.011.
10. Strauss A, Smith KG (2013) Why Does Amphibian Chytrid (Batrachochytrium dendrobatidis) Not Occur Everywhere? An Exploratory Study in Missouri Ponds. PLoS ONE 8(9): e76035. doi:10.1371/journal.pone.0076035 https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0076035
11. Bay E.C. 2003. Chironomid Midges. https://s3.wp.wsu.edu/uploads/sites/408/2015/02/PLS-45-Chironomid-Midges.pdf
12. Ponder, W. F., Hallan, A., Shea, M. E., Clark, S. A., Richards, K., Klunzinger, M. W., and Kessner, V. 2023. Australian Freshwater Molluscs. Revision https://keys.lucidcentral.org/keys/v3/freshwater_molluscs/key/australian_freshwater_molluscs/Media/Html/entities/ferrissia.html.
13. About the COPEPOD Project. [accessed 2024 Jul 25]. https://www.st.nmfs.noaa.gov/copepod/about/#:~:text=A%20%22copepod%22%20is%20a%20type,habitat%2C%20even%20in%20underground%20caverns.
14. Potter G. 2020. Cyclopoid Copepods (Apocyclops panamensis). Aquatic Live Food | Aqua Cultured Aquarium Foods. [accessed 2024 Jul 25]. https://www.aquaticlivefood.com.au/cyclopoid-copepods-apocyclops-panamensis/.
15. Hamrsky J. Water mites - LIFE IN FRESHWATER. LIFE IN FRESHWATER - Macro photography of aquatic insects and other freshwater invertebrates. [accessed 2024 Jul 25]. https://lifeinfreshwater.net/water-mites/.
16. Hafele R. 2017. Rick Hafele. [accessed 2024 Jul 23]. https://www.rickhafele.com/mayflies
17. Bertone M. 2018. NC State Extension. Raleigh (NC): NC State Extension Publications; [accessed 2024 Jul 23]. https://content.ces.ncsu.edu/biology-and-control-of-non-biting-aquatic-midges
18. Son MO. 2007. North American freshwater limpet Ferrissia fragilis (Tryon, 1863) (Gastropoda: Planorbidae) – a cryptic invader in the Northern Black Sea Region. In: Aquatic Invasions. Ukraine: European Research Network on Aquatic Invasive Species; [accessed 2024 Jul 23]. Vol. 2, Issue 1, p.55-58. https://www.semanticscholar.org/paper/North-American-freshwater-limpet-Ferrissia-fragilis-Son/790e22a37753d1dbe3fd070489fd5a00872910c6
19. https://www.semanticscholar.org/paper/North-American-freshwater-limpet-Ferrissia-fragilis-Son/790e22a37753d1dbe3fd070489fd5a00872910c6
20. McQuigg J, Kissner K, Boone M. 2023 28. Exposure to Amphibian Chytrid Fungus Alters Terrestrial Growth and Feeding Rate in Metamorphic Anurans. meridianallenpresscom. https://meridian.allenpress.com/journal-of-herpetology/article/57/1/36/491752/Exposure-to-Amphibian-Chytrid-Fungus-Alters.

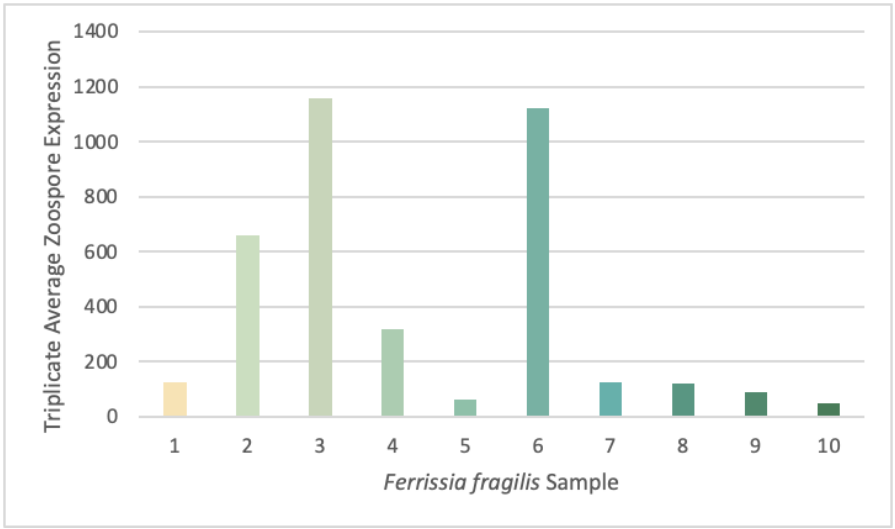
21. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, Zhao S, Wangen C, Ferreira CM, Hipolito M, Davies AJ, Cuomo CA, Daszak P, James TY. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. 21(21):5162-77. doi: 10.1111/j.1365-294X.2012.05710.x.
22. Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms.
23. Pritschet L, Powell D, Horne Z. 2016. Marginally Significant Effects as Evidence for Hypotheses: Changing Attitudes Over Four Decades. In: Psychological Science. 7th ed. Sage Publications; [accessed 2024 Jul 23]. Vol. 27, p.1036-1042. Available from Sage Journals; https://journals.sagepub.com/doi/10.1177/0956797616645672
24. Sirica R. The influence of aquatic macroinvertebrates on the abundance of Batrachochytrium dendrobatidis. [Bachelor's Thesis]. Madison(NJ): Drew University. 2024.
25. Galindo LJ, Milner DS, Gomes SL, Richards TA. 2022. A light-sensing system in the common ancestor of the fungi. Current Biology. 32(14):3146-3153.e3. doi:10.1016/j.cub.2022.05.034. [accessed 2024 Jul 26]. https://www.sciencedirect.com/science/article/pii/S0960982222007965.
26. Herbert, Sm, Tlf Leung, and Pj Bishop. 2011. "Fluorescent Probes as a Tool for Labelling and Tracking the Amphibian Chytrid Fungus Batrachochytrium Dendrobatidis." Diseases of Aquatic Organisms. https://doi.org/10.3354/dao02377.

APPENDICES

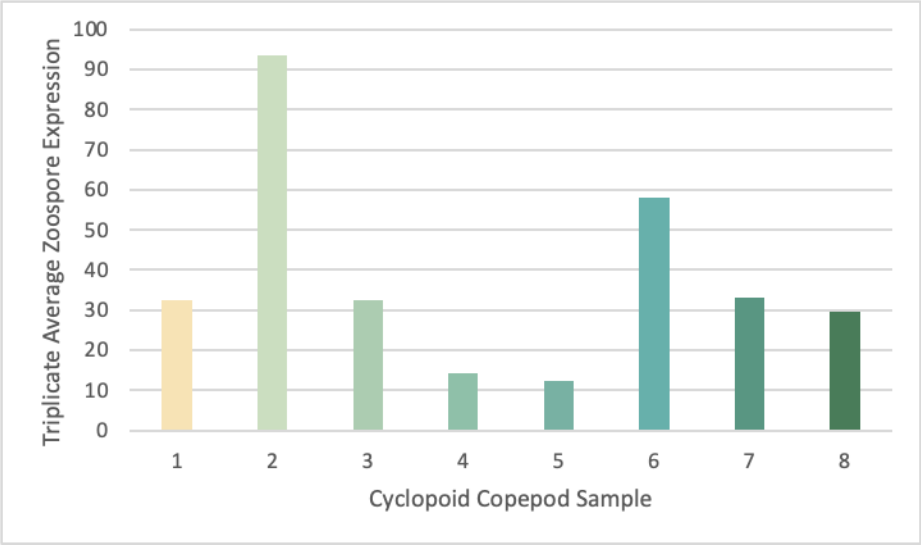
Supplementary Figure 1. 50 cycles of qPCR amplification readings from control group, *Ferrissia*, and Dytiscidae beetle larvae samples, represented as Relative Fluorescent Units.



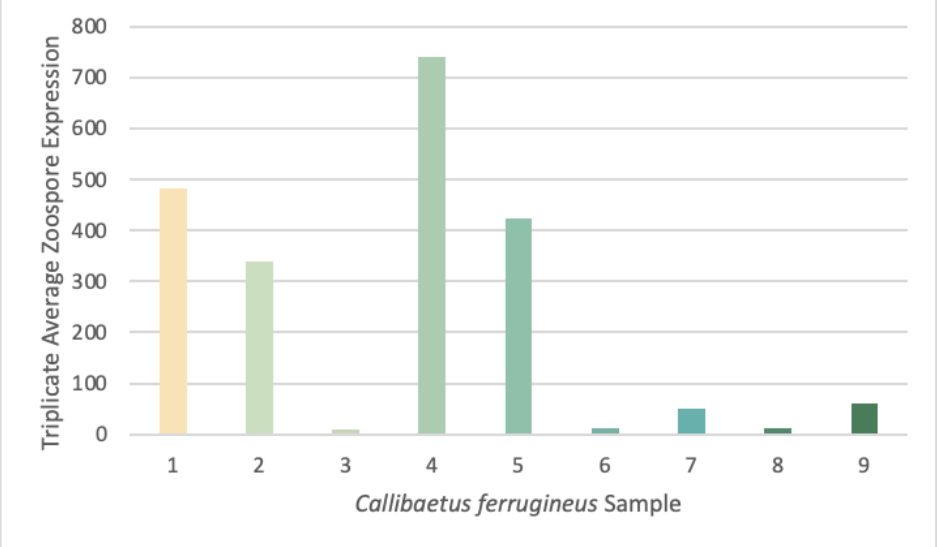
Supplementary Figure 2. Untransformed zoospore abundance of *Ferrissia fragilis* individual samples.



Supplementary Figure 3. Untransformed Zoospore Abundance of Cyclopoid copepod individual samples.



Supplementary Figure 4. Untransformed Zoospore Abundance of *Callibaetis ferrugineus* Individual Samples.



Supplementary Figure 5. Untransformed Zoospore Abundance of Chironomidae *chironomus* Individual Samples.