Temperature effects on production of infectious zoospores from chytrid-infected *Xenopus laevis*

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ABSTRACT

A leading cause of global declines in amphibian populations is chytridiomycosis, the temperature-dependent pandemic disease, caused by the fungal pathogen *Batrachochydrium dendrobadtidis* (Bd). Temperature fluctuations caused by climate change could exacerbate this disease. Temperature-dependent models of Bd infections can provide important insights into Bd transmission dynamics in frog populations to help inform management actions. The Raffel lab has been working on a novel type of mechanistic model to predict Bd and host responses to fluctuating temperatures, based on a combination of metabolic theory and a type of dynamic model traditionally used to describe macroparasite dynamics. In this model shedding rate (i.e., the number of zoospore equivalents released per minute) of Bd zoospores from an infected host's skin constitutes an important parameter which we were able to measure. We infected 124 juvenile *Xenopus laevis* and subjected them to acclimation temperatures of 10 °C, 15 °C, or 20 °C for 35 days. All frogs were inoculated the day of performance temperature switch which happened at the end of the acclimation period; however, 38 were also inoculated 35 days before the switch. During the performance period frogs were subjected to temperatures of 10 °C, 15 °C, 20 °C, or 25 °C for 35 days. Shed zoospores were collected 7 days and 35 days post performance temperature switch. On day 7 of the performance period, shedding rate differed between each performance temperature, but not with acclimation temperature (Table 3). A higher shedding rate was also observed with lower performance temperatures. Shedding rate on day 35 of the performance period did not differ between each performance temperature and acclimation temperature. Shedding rate was also significantly lower on day 35 (Table 3). Frogs inoculated 35 days before the performance temperature switch did not differ between frogs inoculated the day of the switch. We also found Infection intensity and levels of shed zoospores were correlated

(Table 3), with filters having detected more zoospores day 7 of the performance period compared to swabs collected at the same time point. We conclude shedding rate from *X. laevis* is affected by temperature, and infection load. This research will help to inform the transmission parameter for the mathematical model our lab has been generating to untangle the complex temperature dependence of Bd disease dynamics in frog populations which will potentially help conservation programs.

INTRODUCTION

Amphibian populations, the most threatened vertebrate class on the planet, are declining worldwide (Fisher et al., 2009). *Batrachochydrium dendrobadtidis* (Bd) is a fungal pathogen that causes the skin disease chytridiomycosis in amphibians and is a driver for the decline and extinction of amphibian species (Berger et al., 1998; Longcorel et al., 1999; Van Rooij et al., 2015). Bd is present in almost all amphibian populations, and although some species are tolerant and may act as super shedders, other species are susceptible and build up a lethal amount of the pathogen and die (Van Rooij et al., 2015). Bd has two main life stages, a motile infectious stage, the zoospore, and a reproductive stage, the zoosporangium (Berger et al., 2005). Zoospores encyst in the host's skin and produce a germination tube that allows cyst contents to migrate into host tissue (Berger et al., 2005). Sporangia develop inside the skin until they finally occur in the stratum corneum – the outermost layer of skin – where zoospores are released in the environment (Van Rooij et al., 2015). Zoospores can persist in the environment for a long time, lake water cultures have shown zoospores present 1-7 weeks post inoculation (Johnson & Speare, 2003)

Amphibians are ectotherms, so their immune defenses are affected by temperature (Raffel et al., 2006; Robak et al., 2019). Their resistance to infection is also affected by unpredictable temperature fluctuations, which are hypothesized to increase as a result of climate change (Raffel et al., 2006). Frogs will experience thermal acclimation to counteract the effects of temperature fluctuations; however, delays in thermal acclimation following a temperature shift can lead to suboptimal immunity which increases susceptibility to infections (S. E. Greenspan et al., 2017; Raffel et al., 2006, 2013, 2015). Temperature also affects growth and reproductive characteristics of Bd (Stevenson et al., 2013). Due to the strong temperature dependence of Bd infection, the potential for temperature-dependent zoospore shedding is important to account for in models describing Bd infections in variable temperature environments (Briggs et al., 2005, 2010; Wilber et al., 2017, 2021).

In the Raffel Lab, we are working to develop a mathematical model of temperature dependent Bd transmission in *Xenopus laevis* populations. However, we currently lack the necessary data to estimate a key model parameter, the rate of zoospore production (i.e., "shedding") from infected hosts. *X. laevis* is utilized as our model frog because they are naturally tolerant of Bd infection and are able to maintain an active infection for multiple weeks without dying (Van Rooij et al., 2015). Shedding rate of zoospores (i.e., the amount of zoospores released per minute) has previously been measured for other frog other species (DiRenzo et al., 2014, 2018; Maguire et al., 2016; Reeder et al., 2012; Shin et al., 2014), but not for a fully aquatic species such as *X. laevis*. The previously studied species were also terrestrial, which may affect zoospore shedding dynamics compared to fully aquatic frogs such as *X. laevis*. Unlike aquatic frogs, zoospores produced by an infected terrestrial frog should build up on the frog's skin, rather than being constantly shed into the water column. This might result in higher levels

of zoospores being released from a terrestrial frog when placed in water, in comparison to an aquatic frog that should not have the same buildup of zoospores. Furthermore, prior authors did not incorporate different temperatures into their experiments, making the temperature-dependence of this process an open question.

I hypothesized that the rate of zoospore shedding into the water column would be directly proportional to a frog's Bd load (measured via qPCR of a skin swab), because Bd load is a direct measurement of the density of Bd zoospores on the skin which should be influenced by the same processes (e.g., temperature) as zoospore shedding. To evaluate this hypothesis, I quantified the zoospore shedding rate of the aquatic frog species *X. laevis* at different temperatures. I also describe the relationship between individual levels of infection and how it relates to shed zoospores. These measurements will help inform the zoospore production parameter of the host-parasite model our group is working on, leading to a more mechanistic understanding of Bd transmission and helping to inform conservation efforts for amphibian species.

AIMS AND OBJECTIVES

The purpose of this project was to quantify the shedding rate of Bd zoospores as functions of temperature. To accomplish this, *X. laevis* were acclimated to 10°C, 15°C, or 20°C before being switched to performance temperatures to investigate the effects of temperature on shedding rate. At the time of exposure, *X. laevis* were individually dosed with 1×10^6 Bd zoospores and housed at various performance temperatures, 10°C, 15°C, 20°C, and 25°C. My main goal with this project was to accomplish three main aims which helped parameterize the

model we are developing to describe temperature dependent population-level transmission of Bd Infections within *X. laevis* populations.

Aim 1: To measure the temperature dependence of zoospore shedding rate from infected *X. laevis* across relevant temperatures.

Aim 2: To determine how the zoospore shedding rate from *X. laevis* relates to the level of Bd infection on an individual frog

Aim 3: To statistically describe the relationship between zoospore shedding rate from *X. laevis* and temperature

Objectives

1. I measured the zoospore shedding rate from Bd infected *X. laevis* at multiple temperatures by placing each frog in a small volume of clean water for a set period of time, then filtering zoospores from the water. Frogs were also held at varying performance temperatures before filtering of the water. I utilized quantitative PCR to measure the number of zoospores collected on the filters.
2. Each time I measured the rate of zoospore shedding from an individual frog, I also swabbed the frog for qPCR quantification of Bd infection on the skin. I compared these data with the shedding rate data to determine how the level of Bd infection on an amphibian's skin relates to the zoospore shedding rate. This step is necessary to estimate a key parameter in our model of temperature-dependent disease transmission in *X. laevis* frogs.

3. I used statistical modeling in the R programming language to determine how the zoospore shedding rate from *X. laevis* relates to both acclimation and performance temperatures, and to determine how the shedding rate relates to the level of infection on a frog's skin.

MATERIALS AND METHODS

Bd inoculates for infection experiments

We utilized a cryopreserved Bd sample of strain JEL 423 and followed the thawing protocol from Boyle et al., (2003). Bd samples were transferred to 1% Tryptone broth and were allowed to grow for one week at room temperature. During the 1-week period we monitored for growth and potential contamination before plating. After one week 1% Tryptone plates were inoculated with 1000 µL of cultured tryptone broth and were allowed to grow for 5-7 days at room temperature (Blooi et al., 2013; Boyle et al., 2004). After letting the Bd grow for 5-7 days, the tryptone plates were flooded with 3mL of artificial spring water (Cohen et al., 1980) using a 1000 µL pipette and collected into a beaker. Due to including a −35 Day exposure time point and two temporal blocks in the experimental design, inoculates were prepared at three differing time points throughout the experiment. This means there may have been variation in inoculate quality through time. To reduce potential variation in inoculate quality, zoospores were first confirmed to be viable (i.e., motile) via microscopy, then they were counted on a hemocytometer and diluted to an average zoospore density of 1×10^6 zoospores per 1 mL. Frogs were moved to their performance temperature incubators and inoculated on their dorsal side with 1×10^6 zoospores using a 1000 µL micropipette.

Bd infection experiment

We obtained 124 juvenile *X. laevis* and ensured they were clear of Bd infection by subjecting them to a seven-day heat treatment and a 6-day itraconazole treatment (Brannelly, 2014). Frogs were massed then swabbed for qPCR testing to confirm they were cleared of any prior Bd infection. Frogs were housed in 500 mL of water and randomly assigned to an incubator to acclimate at 10 °C, 15 °C, or 20 °C. Six spatial blocks (A-F) were utilized compromising 20 frogs each expect for one containing 24 frogs. The six spatial blocks made up two temporal blocks (A-C and D-F) which were separated by 1 day. We then randomly selected frogs in each spatial block to be inoculated with Bd at two time points. 28 were inoculated 35 days before and then again the day of the performance temperature switch. 96 were inoculated the day of the performance temperature switch. Performance temperatures were 10 °C, 15 °C, 20 °C, and 25 °C, and frogs were randomly assigned to each temperature within each spatial block. Acclimation and Performance periods lasted 35 days in length for both temporal blocks.

Zoospore collection and quantification

Filter samples were collected one- and five-weeks post inoculation. For soaking and filtering, methods from DiRenzo et al., (2014) were followed with slight modifications. Frog housing water was drained and refilled with 100 mL of new water. Frogs soaked for approximately 15 minutes and timed, after which they were removed from the water. 100 µL of BSA was added and the water was mixed before a 10 mL sample was collected using a 50 mL syringe. The sample was filtered through a 0.45 µm syringe filter to collect shed zoospores, using

an adapted caulk gun with a hydraulic pinch pressure gauge (Fig 7). This ensured the syringe filters never experienced pressures higher than 75 psi, which was the limit the manufacturer stated. To calculate the maximum force I could exert on the syringe piston, I took the area of the piston and multiplied it by 75 psi which equates to 51.8 lbs of force. This technique allowed for more efficient and consistent filtering than was possible without the caulk gun and pressure gauge. Filters were capped, stored, and refrigerated for no more than 4 days until extraction. Frogs were also swabbed using a sterile urethral swab five times across each of the ventral thighs and feet. Swabs were collected weekly throughout the experiment and stored in a -20 °C freezer until the DNA was extracted. I extracted DNA from swabs using standard procedures (Boyle et al., 2004) and from filters using procedures established by DiRenzo et al. (2014). Briefly, I added 200 µL of a 1:1 Prepman Ultra to 0.25x TE buffer to the filters. I then recapped the filters and heated them to 100 °C for 10 minutes. I pushed extract out into a microfuge tube using air from a 10 mL syringe. I added 60 µL of Prepman Ultra to the swabs, heated them to 100 °C for 10 minutes, and collected extract using a 20-200 µL micropipette. Extract from both filters and swabs was analyzed using qPCR methods, following methods from Boyle et al., (2004) and Hyatt et al., (2007).

Statistical Analysis

I performed all analyses using R Statistical Software version 4.2.1 (R Core Team 2022). The number of zoospores collected on filters was converted to a shedding rate by dividing each sample by the amount of time each frog spent soaking, multiplying by a 10× dilution factor to account for taking a 10 mL subsample of the 100 mL total water volume, then applying a natural

log transformation. I used linear regression to compare shedding rate to swab Bd levels, and to test for effects of timing of exposure, acclimation temperature, and performance temperature on shedding rate and swab Bd levels. Multiple regression was used to compare shedding rate to performance and acclimation temperature, Swab Bd to performance and acclimation temperature, and Swab Bd to performance temperature and day. When testing for effects of performance and acclimation temperature on the shedding rate, I limited each model to a single time point of collection to avoid pseudo replication.

RESULTS

Shedding rate on day 7 differed between performance temperatures, with lower mean shedding rates at lower temperatures (Table 3; Fig. 1). However, I did not detect a significant effect of thermal acclimation on day 7 (Table 3; Fig. 1*)*. There was no significant effect of either performance temperature or acclimation temperature on day 35 (Table 3). Shedding rate was significantly higher on day 7 than day 35 (Table 3; Fig. 2). The average ln Bd zoospores (± SE) per minute for each performance temperature is shown in Table 1. The average ln Bd zoospores (± SE) per minute for each acclimation temperature is shown in Table 2.

Shedding rate on day 7 of the performance temperature period did not differ between frogs exposed on day -35 versus day 0 (Table 3) (Fig. 3). Shedding rate on day 35 of the performance temperature period also did not differ between frogs exposed on day -35 and day 0 (Table 3).

There was a significant positive relationship between shedding rate and swab Bd load (Table 3; Fig. 5). We also found that the average shedding rate was higher compared to the

average swab Bd load for all performance temperature on day 7 (Fig 6.). On day 35, however, average shedding rates were lower when compared to the average Bd load collected on swabs (Table 1; Figure 1).

The amount of Bd zoospores collected on swabs (swab Bd) was also significantly affected by performance temperature but not by acclimation temperature, at least when swab data from day 3 was included (Table 3). If data from day 3 is excluded, there were significant effects of both performance temperature and acclimation temperature on levels of Bd zoospores collected on swabs (Table 3). Looking at data collected on day 3, Bd Load was significantly affected by performance temperature but not by acclimation temperature, and this same pattern is seen with data collected on day 7 (Table 3). Bd load decreased with increasing performance temperature, with highest loads in the 10 °C group (Fig. 2). The average ln Bd load for swabs (± SE) for each performance and acclimation temperatures is shown in Table 1 and Table 2.

There were significant negative effects of both performance temperature and time since the temperature shift on swab Bs load, even if data from day 3 was excluded (Table 3). Swab Bd load decreased over time for all performance temperatures (Fig. 2). The average ln Bd load (± SE) collected by swabs for performance temperatures on day 7 and 35 of the performance period can be seen in Table 1 and Table 2.

DISCUSSION

X. laevis shed a large number of zoospores on day 7 of the performance period, compared to day 35 which had very low levels. More zoospores were shed at lower performance temperatures compared to higher ones with infection intensity following the same trend. This

highlights the importance of temperature in the host-parasite dynamics of Bd which can be seen in other studies (Raffel et al., 2006, 2013; Ribas et al., 2009; Stevenson et al., 2013). The level of zoospore production was highly correlated with infection intensity as measured via qPCR of skin swabs, supporting my hypothesis. This is also consistent with results observed in prior studies (DiRenzo et al., 2014, 2014, 2018; Maguire et al., 2016; Reeder et al., 2012). Analysis of filter samples detected higher levels of Bd zoospores compared to swabs on day 7 of the performance period. In contrast, analysis of filter samples detected lower levels during day 35 of the performance period compared to swabs. However, the day 35 Bd levels were generally much lower than on day 7 for both sampling methods, which might make the day 35 data less reliable. These results are generally consistent with prior studies that observed higher detectability of Bd zoospores from filtered-water samples than from swab samples (DiRenzo et al., 2018; Maguire et al., 2016; Shin et al., 2014).

Previous studies in our lab have confirmed infection intensity decreasing with increasing performance temperature seen in the literature (Raffel et al., 2013, 2015; Ribas et al., 2009). However, our previous studies did not include measuring Bd load 3 days after inoculation. Looking at the data, we believe this time point does not truly represent zoospore production by mature zoosporangia and is thus not biologically comparable to swab data from later time points. A previous study of Bd infection in *X. laevis* found that 3 days post inoculation, most zoosporangia had not finished developing yet and therefore would not be producing zoospores (Van Rooij et al., 2012). They also found that most of these immature zoosporangia were still associated with the outer layers of epidermis at this time (Van Rooij et al., 2012). Based on this published evidence, we think that the high levels of Bd DNA detected from skin swabs on day 3 were largely driven by these immature zoosporangia, unlike in later time points when swab data

is more likely to reflect zoospore production by mature zoosporangia. Therefore, these early data are likely not comparable to later time points and should be interpreted with caution, or disregarded, in analyses of Bd population dynamics post-exposure. Future studies could test this further by soaking and filtering *X. laevis* at this early time point to compare shed zoospores with Bd load.

Our results also show that pre-exposure to Bd, 35 days prior to a secondary exposure (on Day 0), did not change the number of zoospores shed at subsequent time points. Pre-exposure was also seen to not affect the amount of zoospores shed in another study (DiRenzo et al., 2014). Frogs in our experiment also showed a reduction in Bd loads over time. These results are interesting, because they may relate to whether and to what extent *X. laevis* mount an acquired immune response to Bd infection (McMahon et al., 2014). Bd is also able to effectively suppress or evade the immune system of many host species, which could also influence the temporal dynamics of infection (Fites et al., 2013). The decline in Bd load through time suggests the possible effect of increasing acquired resistance through time, but the absence of a pre-exposure effect tends to contradict this hypothesis.

Acclimation effects were observed to influence swab Bd load but were not observed to affect shedding rates at 7 days post-exposure. Previous experiments in our lab and other studies have shown acclimation effects with infection intensity, specifically animals acclimated to warmer temperatures having higher infection intensities when exposed at cooler temperatures ((Bradley et al., 2019; Raffel et al., 2013, 2015, p. 20). This pattern was seen in infection intensity in our experiment; however, this pattern was not present in the shedding rate data from the same time point. We predicted acclimation effects would be seen with both infection

intensity and shedding rate, based on the assumption that both swabbing and filtering are detecting the same process, i.e., zoospores production by mature zoosporangia. The lack of an acclimation effect on shedding rate, despite a significant acclimation effect on Swab Bd, suggests the possibility these two assays measure response variables that are biologically distinct from each other. This further implies that the Bd shedding rate may not always by directly proportional to Bd infection intensity, contrary to a common assumption of dynamic disease models (Briggs et al., 2010). Our results suggest that this assumption might be an oversimplification. The relationship of what makes zoospore shedding rate and infection intensity distinct from one another is unclear, but it is possible that immature zoosporangia make a greater DNA contribution to Swab Bd measurements than has previously been assumed. Further studies will be needed to clarify the relationship, and to explore thermal acclimation effects on shedding rate versus infection intensity. In this study we only collected filter data at two time points, the earliest being 7 days post inoculation, i.e., late enough to provide zoosporangia time to mature and start producing zoospores. It would be interesting for future studies to collect filter data at various timepoints throughout the performance period, including earlier time points, for comparison with Swab Bd measurements.

In conclusion, my results highlight the importance of temperature as a driver of Bd infection and zoospore shedding, with important implications for among-frog transmission. They also highlight the potential role of fluctuating temperatures and host acclimation responses on Bd transmissibility. Temperature has also revealed a possible difference between infection intensity and shedding rate as measures of Bd infection, in particular revealing differences in how host thermal acclimation influences these variables. This means that Bd infection intensity and zoospore shedding rate may not have as a direct relationship as previously thought. This shows

more research is needed to truly understand shedding rate at a host and population level. Finally, the data collected will contribute to our future research goals by allowing us to estimate the relationship between swab Bd load and zoospore shedding rate, a key parameter for modeling Bd transmission in amphibian populations.

ACKNOWLEDGEMENTS

This project and my overall growth in the field of research would have not been possible without Dr. Raffel. During the summer of my freshman year, I had the privilege to work under Dr. Raffel for my SURP project which kicked off my continuous involvement in his lab ever since. Dr. Raffel through his hard work and personality has provided a constant atmosphere of engagement, compassion, curiosity, and warmness which has allowed me to grow as a student and a person. I am extremely thankful to be a member of the Raffel lab, and the memories I have made in the lab and from this project will follow me for the rest of my life.

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TABLE 1. Mean ± SE for log number of Bd zoospore equivalents (ln(Bd Zoospore equivalents + 1), for filters and swabs for different performance temperature treatments and days following the temperature shift. Filter Bd zoospore equivalents was measured as a rate (zoospores produced per minute) and log-transformed for analysis.

Performance Temperature (°C)	*Method of Bd Collection*	*Mean ln(Bd Zoospore equivalents + 1)*
10	Day 7 Filter	3.75 ± 0.38
	Day 35 Filter	0.07 ± 0.05
	Day 7 Swab	2.22 ± 0.39
	Day 35 Swab	0.61 ± 0.33
15	Day 7 Filter	1.53 ± 0.40
	Day 35 Filter	0.00 ± 0.00
	Day 7 Swab	0.41 ± 0.26
	Day 35 Swab	0.00 ± 0.00
20	Day 7 Filter	0.57 ± 0.17
	Day 35 Filter	0.00 ± 0.00
	Day 7 Swab	0.24 ± 0.11
	Day 35 Swab	0.05 ± 0.05
25	Day 7 Filter	0.13 ± 0.09
	Day 35 Filter	0.00 ± 0.00
	Day 7 Swab	0.00 ± 0.00
	Day 35 Swab	0.00 ± 0.00

TABLE 2. Mean ± SE for shedding rate for different acclimation temperature treatments and days following temperature shift. Filter Bd zoospore equivalents was measured as a rate (zoospores produced per minute) and log-transformed for analysis.

Acclimation Temperature (°C)	*Performance Temperature (°C)*	*Day*	*Mean ln(Bd Zoospore equivalents + 1)*
10	10	7	3.40 ± 0.52
		35	0.00 ± 0.00
	15	7	1.34 ± 0.68
		35	0.00 ± 0.00
	20	7	0.28 ± 0.19
		35	0.00 ± 0.00
	25	7	0.23 ± 0.23
		35	0.00 ± 0.00
15	10	7	4.29 ± 0.68
		35	0.00 ± 0.00
	15	7	1.35 ± 0.53
		35	0.00 ± 0.00
	20	7	1.02 ± 0.43
		35	0.00 ± 0.00
	25	7	0.14 ± 0.14
		35	0.00 ± 0.00
20	10	7	3.59 ± 0.78
		35	0.27 ± 0.19
	15	7	2.08 ± 1.04
		35	0.00 ± NA
	20	7	0.45 ± 0.24
		35	0.00 ± 0.00
	25	7	0.00 ± 0.00
		35	0.00 ± 0.00

TABLE 3. t value, degrees of freedom, and P value for various predictor and response variables utilizing linear and multiple linear regression

Response	*Predictor*	*t*	*df*	*P*
Ln Shedding rate (full dataset)	Ln Swab Bd	6.334	183	< 0.001
Ln Shedding rate (Day 7)	Timing of exposure	0.016	122	0.987
Ln Shedding rate (Day 35)	Timing of exposure	0.016	60	0.987
Ln Shedding rate (full dataset)	Day	-5.724	184	< 0.001
Ln Shedding rate (Day 7)	Performance Temperature	-8.985	121	< 0.001
	Acclimation Temperature	0.516	121	0.607
Ln Shedding rate (Day 35)	Performance Temperature	-1.313	59	0.194
	Acclimation Temperature	1.356	59	0.18
Ln Swab Bd (including day 3)	Performance Temperature	-8.552	740	< 0.001
	Acclimation Temperature	1.78	740	0.0754
Ln Swab Bd (excluding day 3)	Performance Temperature	-8.008	614	< 0.001
	Acclimation Temperature	2.674	614	0.008
Ln Swab Bd (Day 3)	Performance Temperature	-11.851	123	< 0.001
	Acclimation Temperature	0.545	123	0.587
Ln Swab Bd (Day 7)	Performance Temperature	-6.009	121	< 0.001
	Acclimation Temperature	1.224	121	0.223
Ln Swab Bd (including day 3)	Performance Temperature	-9.738	740	< 0.001
	Day	-15.693	740	< 0.001
Ln Swab Bd (excluding day 3)	Performance Temperature	-8.057	614	< 0.001
	Day	-3.979	614	< 0.001

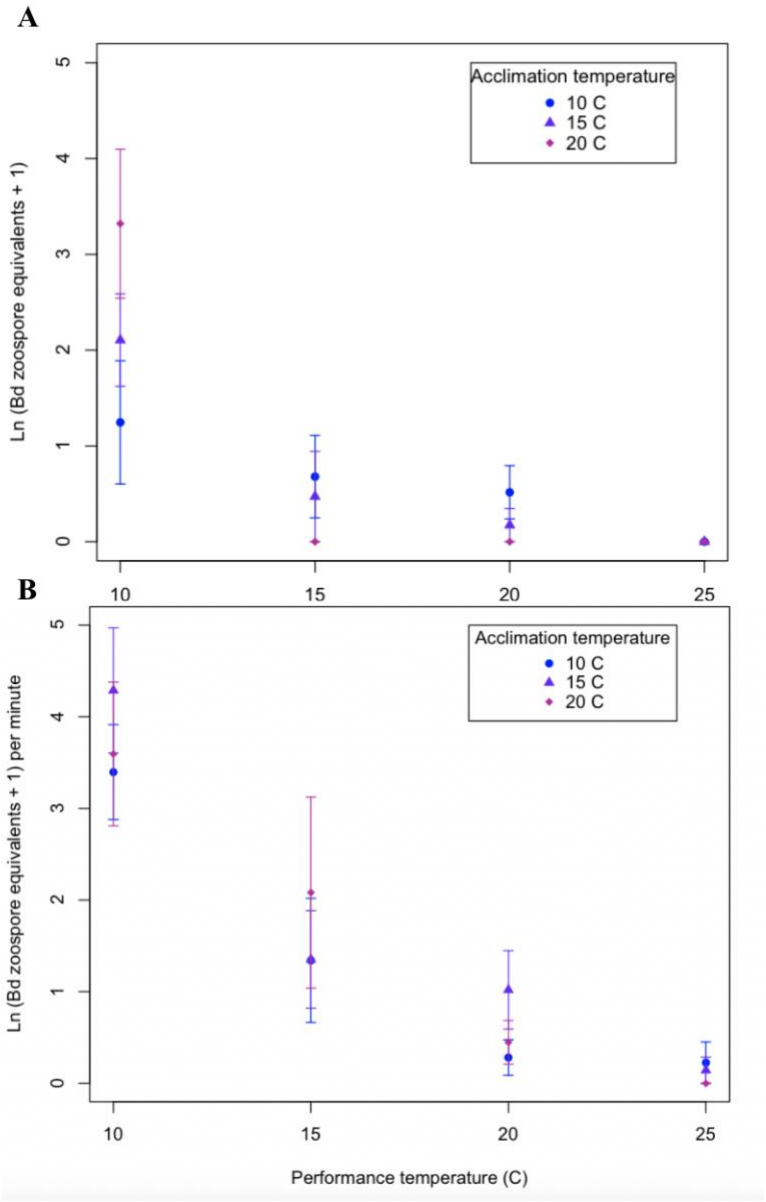

Figure 1. (A) This graph represents the average Bd load (Ln (Bd zoospore equivalents + 1)) collected on swabs of each acclimation temperature on day 7 of the performance period of the experiment. (B) This graph represents the average shedding rate (Ln (Bd zoospore equivalents + 1) per minute) collected on filters of each acclimation temperature on day 7 of the performance period of the experiment.

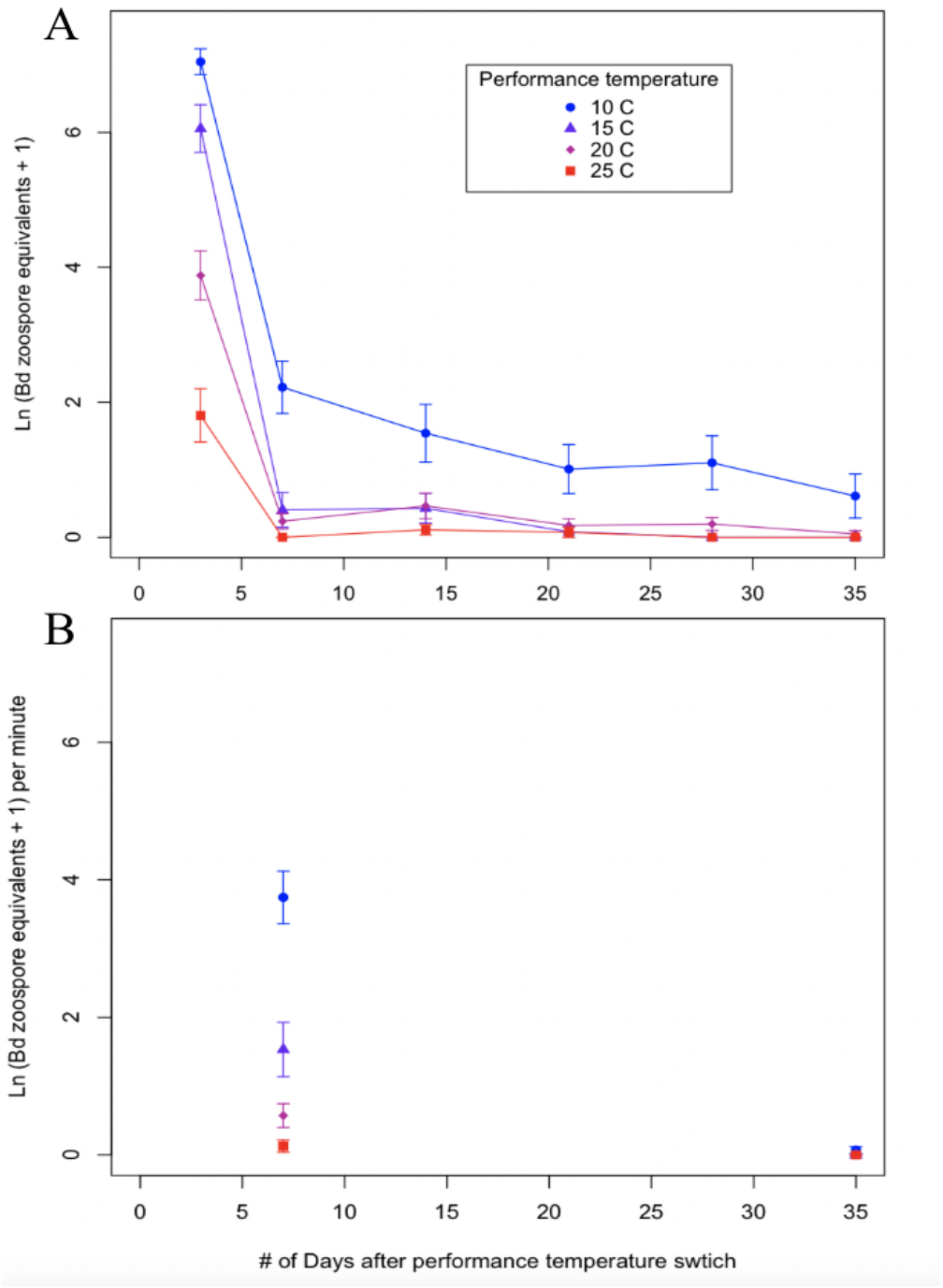

Figure 2. (A) represents the average zoospore equivalents (Ln (Bd zoospore equivalents + 1)) collected on swabs at each performance temperature over the course of the performance period.

(B) represents the average shedding rate (Ln (Bd zoospore equivalents + 1) per minute) collected on filters at each performance temperature over the course of the performance period.

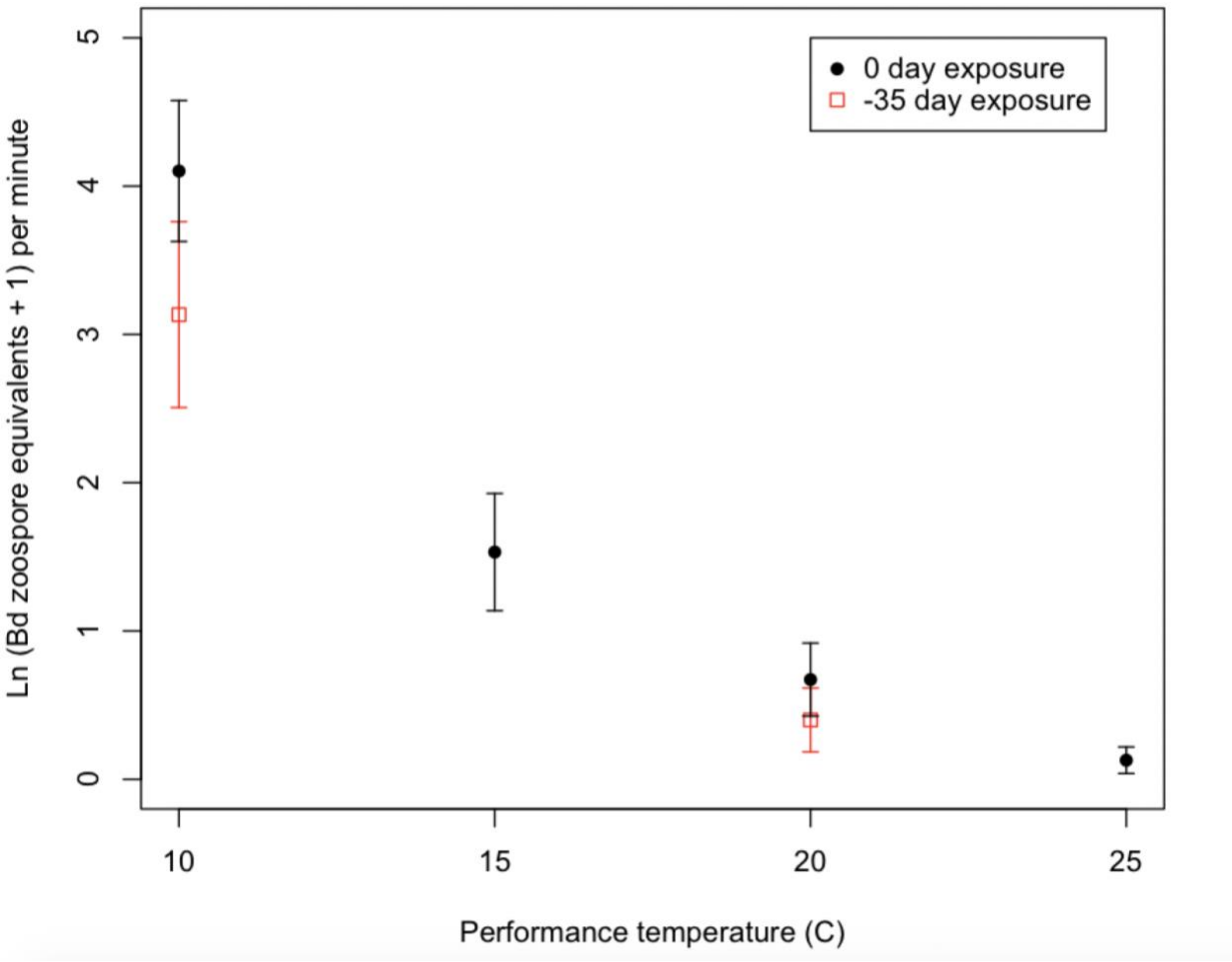

Figure 3. This graph represents the average shedding rate (Ln (Bd zoospore equivalents + 1) per minute) collected on filters from each performance temperature for frogs exposed to Bd -35 days and 0 days before performance temperature switch. Data is from day 7 post performance switch

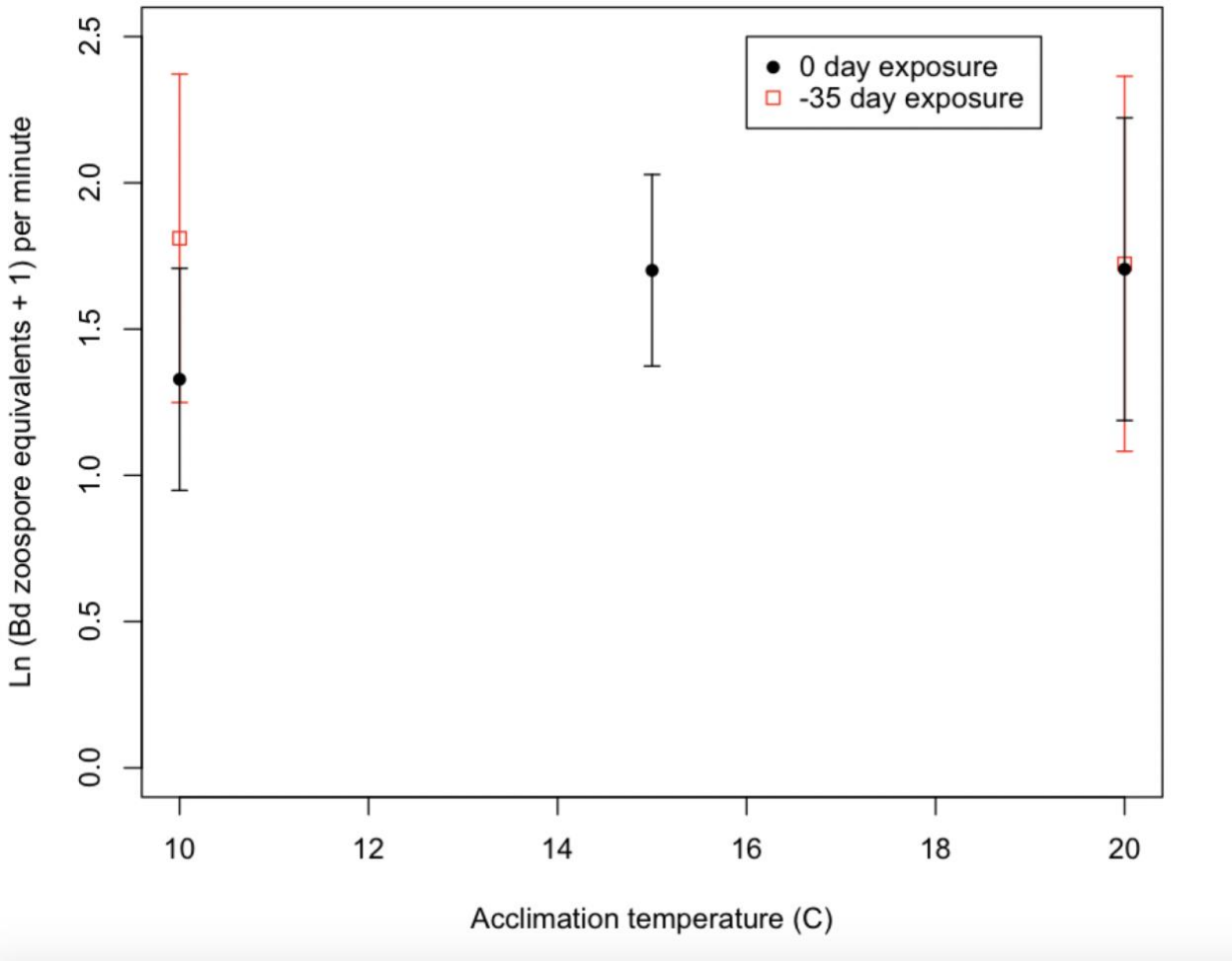

Figure 4. This graph represents the average shedding rate (Ln (Bd zoospore equivalents + 1) per minute) collected on filters from each acclimation temperature for frogs exposed to Bd -35 and 0 days before performance temperature switch

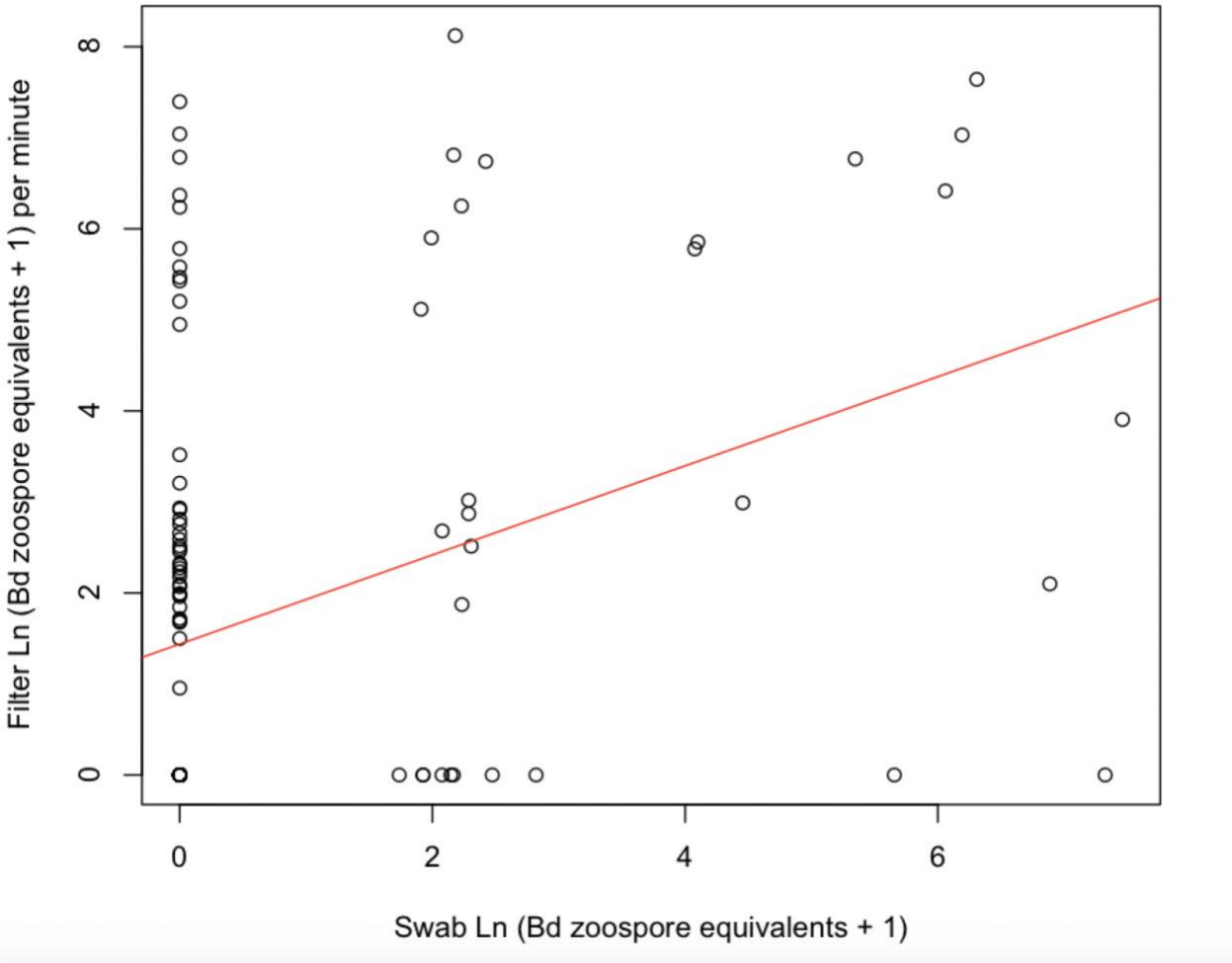

Figure 5. This scatterplot shows the shedding rate (Ln (Bd zoospore equivalents + 1) per minute) collected on filters and zoospore equivalents (Ln (Bd zoospore equivalents + 1)) collected on swab samples for each frog on day 7. The red line represents the line of best fit using a linear model.

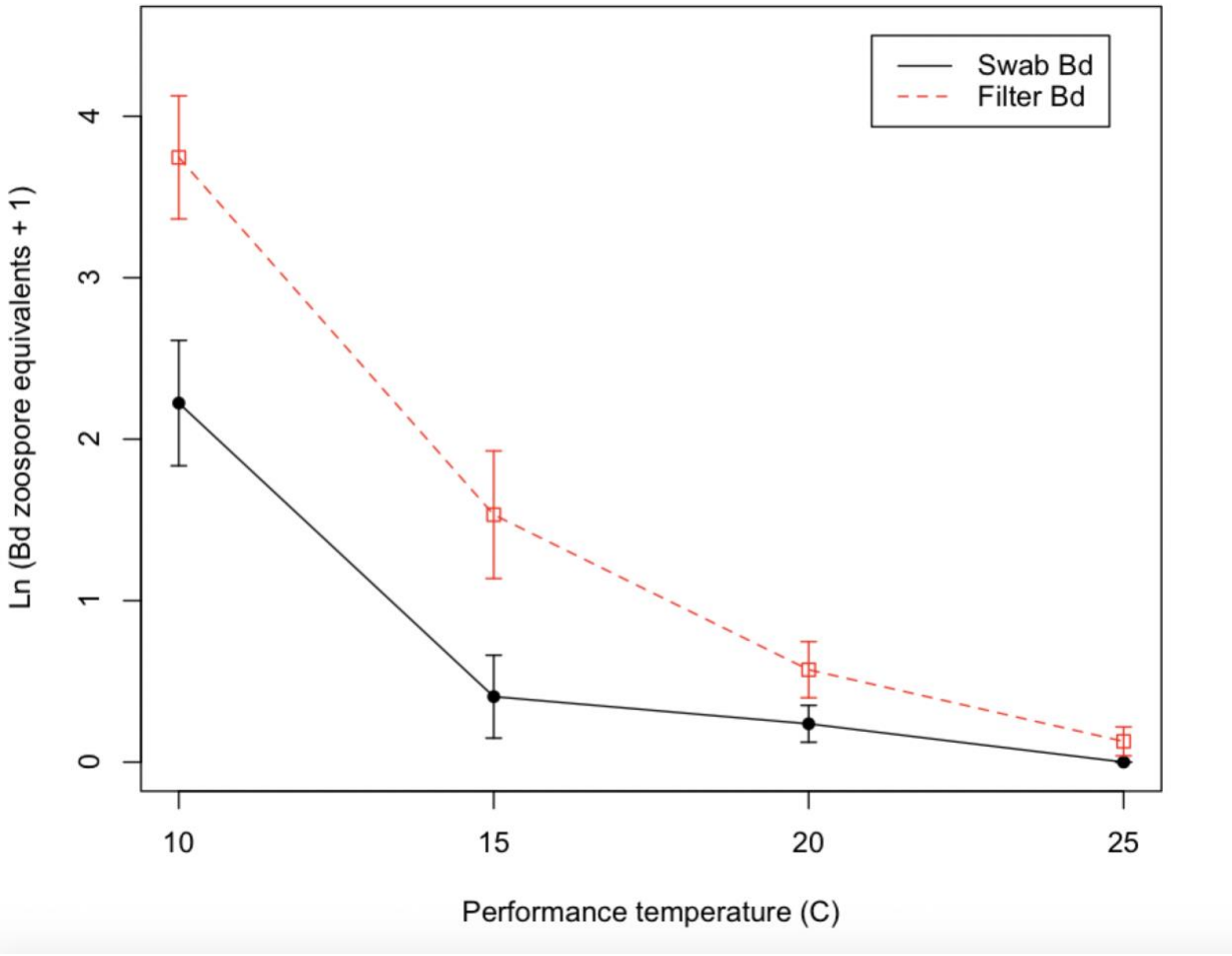

Figure 6. The graph shows average number of Bd zoospores equivalents (Ln (Bd zoospore equivalents + 1)) collected on swab and filter samples at each performance temperature 7 days post performance temperature shift. Filters are measure in zoospore equivalents per minute

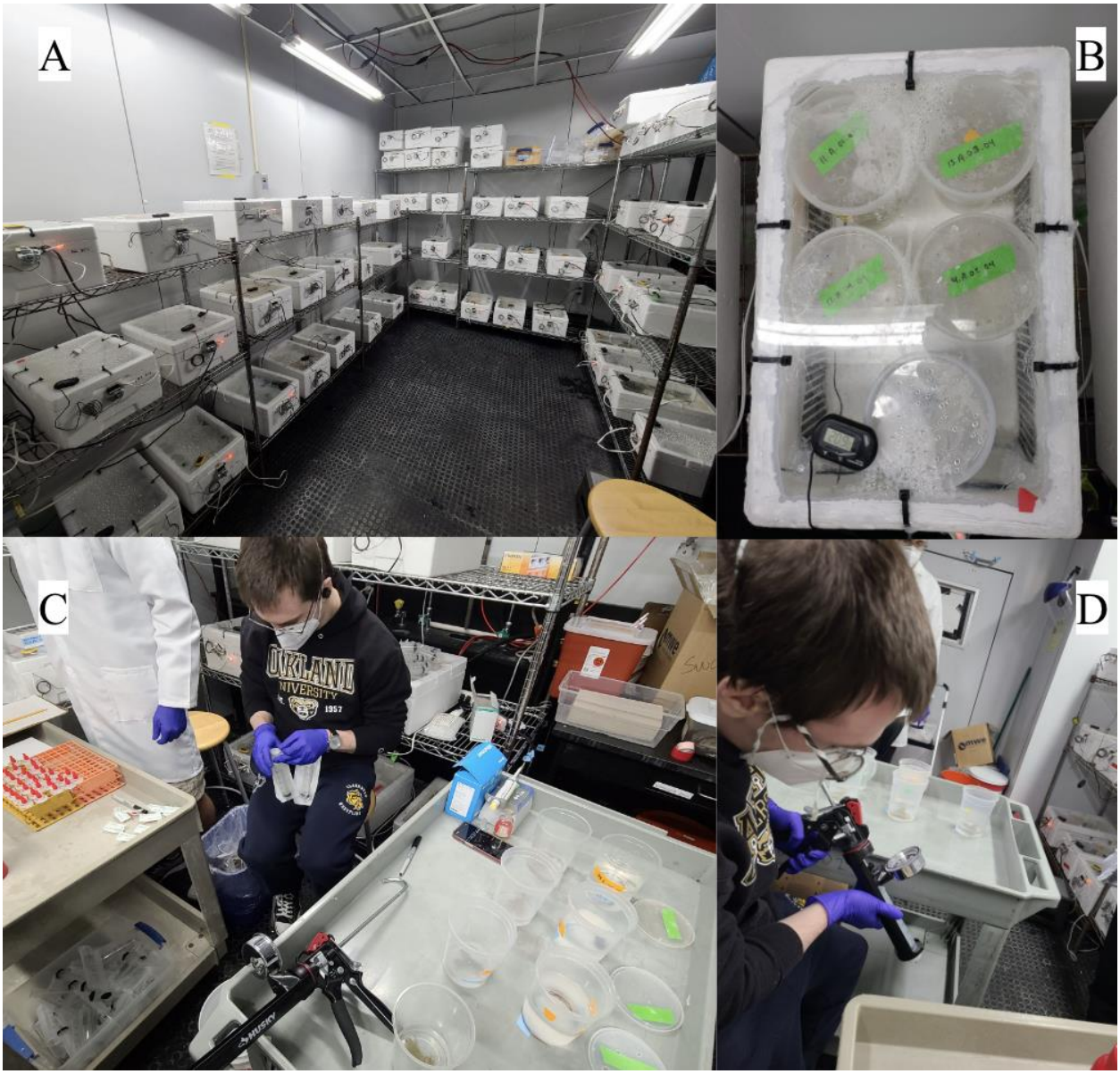

Fig 7. (A) This picture shows the array of incubators we utilized for the experiment. (B) This picture shows how frogs were incubated throughout the experiment. A thermometer was utilized to make sure frogs were kept at their appropriate temperature. (C) This picture shows the general set up for the soaking procedure we utilized. Frogs (on the right) soaked for 15 minutes, after which they were removed. BSA was then injected into the water, swirled, and a 10 mL sub sample was collected and filtered. Filters were then capped (on the left). (D) This picture shows

me using the adapted caulk gun with a pinch pressure gauge to filter a subsample of water a frog-soaked in.

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