

*Temporal and interspecific variation of the amphibian
chytrid fungus Batrachochytrium dendrobatidis in
northwest Pennsylvania*

By
Logan Scott Billet
April 3, 2017



Allegheny College
Department of Biology

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**A Senior Comprehensive Project in Partial Fulfillment of the
Requirements for a Bachelor of Science Degree from Allegheny College**

*I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor
Code and to maintain the integrity of both myself and the College community as a
whole.*

Pledge

Approval by the Senior Thesis Committee:

Dr. Matthew Venesky 1st Reader

Dr. Casey Bradshaw-Wilson 2nd Reader

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Abstract

The abundance of parasites varies through time and among species, and is often associated with environmental factors (e.g. temperature) and host life-history traits (e.g. developmental rate). The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), is one of the primary drivers of amphibian population declines and extinctions around the world. Like many parasites, *Bd* has temperature-dependent growth and amphibian species vary in their resistance to *Bd*, both of which could affect *Bd* prevalence in populations. To understand seasonal *Bd* dynamics in populations of amphibians, I conducted field surveys throughout the fall in northwest Pennsylvania. I tested for temperature-driven temporal patterns of *Bd* prevalence and infection intensity in green frog (*Lithobates clamitans*) and bullfrog (*Lithobates catesbeianus*) tadpoles at 2 ponds from September 23 – November 27 2016. I also recorded body length of each tadpole to assess for variation in *Bd* infection throughout tadpole development. Water temperature was a significant negative predictor of *Bd* infection prevalence in bullfrogs and a marginally significant negative predictor of *Bd* infection prevalence in green frogs. Length was a significant positive predictor of *Bd* infection prevalence and intensity in bullfrogs but not in green frogs. These results emphasize the importance of considering species identity and developmental stage when surveying for *Bd* and suggest that like in other regions, temperature is a driver of *Bd* prevalence fluctuations in northwest PA.

Introduction

Anthropogenic stressors such as climate change, pollution, and habitat destruction have put ecosystems under tremendous pressure in recent decades. This is problematic not only because of the direct negative effects that these stressors have on many species, but also because these stressors have altered the distribution and virulence of many parasites (Patz et al. 2000). This means that parasites are gaining access to hosts that have never encountered them before. This is concerning from a conservation standpoint, because parasites often have a negative impact on host fitness. For example, infection by the parasitic gastrointestinal nematode *Ostertagia gruehneri* has a strong influence on reindeer body condition and results in reduced body mass and back fat depth (Stien et al. 2002). Over a longer period, *O. gruehneri* infection influences reindeer population dynamics by reducing the likelihood of reindeer reproduction (Albon et al. 2002). Though the effects of many parasites are sub-lethal, they can become lethal and lead to mass die-offs as has been the case for bats in North America suffering from white-nose syndrome (Blehert et al. 2009). Thus, it is important to study emerging parasites to understand where they occur and when they are most prevalent.

In most host-parasite systems, parasitic prevalence and infection intensity tend to vary temporally at any single location. Temporal variation of parasites is well documented and is present on all continents, though the patterns and trends of fluctuation can vary significantly (Phiri et al. 2007; Deviche et al. 2011; Moller et al. 2003). Parasites tend to vary temporally because many environmental factors that have an impact on their physiology vary through time as well (Strauss & Smith 2013, Fredensborg et al. 2005; Xiao et al. 2010; Byers et al. 2008). Understanding how environmental and physiological factors influence parasite abundance presents a challenge to the field of disease ecology because parasite abundance

tends to fluctuate through time and space. This is especially true in studying hosts with a broad geographic distribution since the impact of infection-driving factors can be highly variable from location to location. For example, warm-adapted amphibians are more susceptible to pathogenic infection at relatively cooler temperatures, while cold-adapted amphibians are more vulnerable to pathogenic infection at relatively warmer temperatures (Cohen et al. 2017). Because of this variability in response to environmental factors, it is important to study parasite dynamics in a wide range of ecosystems and environments as an understanding of parasite dynamics in one area may not be applicable in other regions with different environmental conditions.

One of the most studied environmental factors in the temporal distribution of parasites is temperature. Temperature can impact the prevalence and infection intensity of a parasite, as parasites are usually ectothermic and have ideal temperatures for survival and reproduction. Temperature can also affect the ability of a host to fight off infection, especially in ectotherms because their body temperature is reliant on the external temperature (Thomas & Blanford 2003). In *Daphnia magna*, the parasite *Pasteuria ramosa* has varying virulence depending on the temperature. At a warmer temperature of 20-25 °C, the parasite is extremely virulent and sterilizes its host (Mitchell et al. 2005). At a colder temperature of between 10-15 °C, the parasite is not very virulent, leaving the host uncastrated and able to reproduce (Mitchell et al. 2005). Though the ideal temperature for *D. magna* is between 18-22 °C, the parasite also survives better at these temperatures, overruling the temperature at which the daphnia's immune function is at its peak. Thus, because temperature differentially impacts hosts and parasites, the effect of temperature on the interaction between the two is context dependent.

It is important to understand the profound effects that temperature has on hosts and parasites given the fact that global temperatures are changing in unpredictable ways. In amphibians, some components of the immune system, such as the production of T lymphocytes and eosinophils, are downregulated in response to lower temperatures (Maniero & Carey 1997). The reason for this is twofold: downregulating the immune system allows organisms to save energy during the winter (Bonneaud et al. 2003; Raffel et al. 2006), and pathogen growth rates are reduced at colder temperatures, which reduces the risk of a deadly infection (Ratkowsky et al. 1982; Raffel et al. 2006). The adjustment of immune parameters to changing temperatures is not perfect, however, since hosts and parasites respond to temperature changes in different ways because of their differing physiology. In particular, the adjustment of immune parameters in ectothermic hosts to long-term temperature changes is often imperfect, and immunity may fall above or below optimal levels. In amphibians, this is referred to as the seasonal acclimation effect (Raffel et al. 2006), and is the result of slow acclimation of amphibians to seasonal temperature declines, causing overall immune function to fall below optimal levels for preventing parasitic infection.

In addition to the effects of temperature on host-parasite interactions, differences in host life-history traits also affect whether an individual will be infected. There are points in an organism's development where they are more vulnerable to pathogens because of changes in energy allocation due to growth, reproduction, metamorphosis, or other behaviors and physiological processes. In cotton aphids, mortality from treatment with the fungal parasite *L. attenuatum* varies significantly by host developmental stage; third-instar nymphs and adults are more likely to succumb to infection than first-instar nymphs due to a combination of developmental factors (Kim & Roberts 2012). Similar variations in susceptibility are

present in *D. magna* populations, in which younger organisms are more susceptible to parasitic infection than older ones (Izhar & Ben-Ami 2015). This variation in susceptibility by life stage is present across many taxa and can result in an uneven spread of disease within a population and a disproportionate rate of infection in certain developmental classes.

Different host species can also vary in their susceptibility to infection because of differences in their developmental rates. Developmental rates tend to vary along a slow to fast continuum, and often have an impact on immunological defenses. For example, rodents that develop faster have stronger innate, non-specific immune responses, which are less energetically costly to produce and are quicker to respond (Previtali et al. 2012). Conversely, rodents with slower developmental rates mount stronger adaptive, pathogen-specific immune responses, which are more energetically costly but more robust against pathogens (Previtali et al. 2012). These trends follow the predictions of the pace of life continuum, which predicts that fast-living organisms invest little energy into defenses and more energy into reproduction and growth, while slow-living organisms invest much more energy into defenses since they are expected to encounter more pathogens through their lifetime (Johnson et al. 2012). This same trend has been supported by studies of other taxa (Martin et al. 2008; Sparkman & Palacios 2009; Johnson et al. 2012).

Batrachochytrium dendrobatidis (hereafter *Bd*) is a fungal pathogen that infects and feeds on the skin of live amphibians. Specifically, *Bd* infects the keratinized tissues of amphibians, which are usually found on the feet and ventral surfaces of adults and around the mouths of tadpoles (Fellers et al. 2001). Because amphibians breathe and absorb nutrients through their skin, severe *Bd* infections cause death, usually by cardiac arrest (Voyles et al. 2009). Since its discovery, *Bd* has had a devastating impact on amphibian populations around

the world as it can infect most species of amphibian (Berger 1998). Because *Bd* is considered a driver of amphibian population declines, understanding what factors affect the spatiotemporal variation of *Bd* in amphibian ecosystems is of the utmost importance; amphibians are currently one of the most threatened taxa in the world, with nearly 50% of species threatened with extinction (Fisher et al. 2009). Studying *Bd* infection, among other extinction factors, is important because amphibians can succumb to infection rapidly and the fungus disproportionately kills rare and endemic species (Lips et al. 2006; Smith et al. 2009). Though there are studies that assess the spatiotemporal dynamics of *Bd*, many of these studies are lab studies or take place in regions that *Bd* is causing mass amphibian extinctions (Kriger & Hero 2007; Bustamante et al. 2010). Because of this, there are limited data on the spatiotemporal distribution patterns of *Bd* in areas where *Bd* is not causing population declines.

Pennsylvania is one area where *Bd* is not known to be causing amphibian declines. A field study by Groner & Relyea (2010) found that out of 14 total sample ponds in northwest Pennsylvania, 7 were *Bd*-positive. Although this study serves as a good indicator of the presence of *Bd* in ponds in northwest PA, each pond in the study was sampled only a single time, and only a small number of samples were taken from each pond. No temporal trends of *Bd* can be drawn from Groner & Relyea (2010) because of this. To follow up on these findings, I performed a preliminary survey as part of a summer research project from May through July of 2015 to test for any spatial or temporal variation of *Bd* prevalence in northwest PA. I sampled for eight weeks at four ponds in the greater Meadville area. My results showed that there is marked spatiotemporal variation of *Bd* in northwest PA during the summer months. Spatially, *Bd* ranged from an average prevalence of 17% at one pond to

78% at another (Billet & Venesky, *unpublished*). Temporally, *Bd* prevalence decreased relatively linearly through the summer at all sampling sites (Billet & Venesky, *unpublished*). Although my previous research demonstrated spatiotemporal variation in *Bd* prevalence in PA, that study spanned only the summer, and thus the spatiotemporal trends of *Bd* during other seasons are unknown.

The main objective of this study was to expand my previous field study of *Bd* dynamics in the summer into the fall. Sampling took place through the fall because previous studies in other regions have found that *Bd* prevalence varies seasonally, and specifically that *Bd* prevalence tends to increase as temperatures decrease and thus fall may be a time when amphibians are most susceptible to infection (Berger et al. 2004; Lenker et al. 2014). Two sites were sampled to allow me to discern the general *Bd* infection trends of northwest PA better than could be determined from a single site. I focused my sampling efforts on bullfrog tadpoles (*Lithobates catesbeianus*) in one pond and green frog tadpoles (*Lithobates clamitans*) in the other. Both species are susceptible to *Bd* and are present in high densities locally and throughout the northeastern United States, making them excellent study species (Gahl et al. 2012). These two species vary in their larval developmental rate, with bullfrogs typically taking between 12 to 36 months to metamorphose, and green frogs taking between 3 to 12 months to metamorphose (Collins 1979; Martof 1956). Like other taxa, developmental rate has been found to be an important factor in infection dynamics in many amphibian species, with faster developing species being more prone to infection (Johnson et al. 2012). Intraspecifically, studies have found that larger tadpoles at more advanced developmental stages (i.e. closer to metamorphosis) are more susceptible to some parasites (Haislip et al. 2011; Smith et al. 2007).

I hypothesized that throughout the study period, 1) bullfrogs would have a higher infection prevalence and intensity than green frogs, 2) water temperature would be negatively correlated with *Bd* infection prevalence and intensity, and 3) tadpole size would be positively correlated with infection prevalence and intensity.

Methods

Study site

I quantified *Batrachochytrium dendrobatidis* (*Bd*) infection intensity and prevalence through the fall in amphibian communities at 2 ponds in Crawford County, Pennsylvania, USA. The first study site is a permanent open pond located on State Game Lands 146 (hereafter “SGL”; 41.733718, -79.974207) (Fig. 1). SGL is an isolated winterkill pond containing no fish and is rich in macroinvertebrate and amphibian life. Most of the detritus is autochthonous. SGL is managed by the Pennsylvania Game Commission and experiences little degradation from human activities. Pond SGL is approximately 0.9 hectares in area. The second study site is a permanent pond with a partial canopy located on the property of a private landowner (hereafter “PL”; 41.695549, -80.015023) who gave permission for sampling (Fig. 2). PL has a small inflow and outflow and receives more allochthonous inputs than pond SGL. PL contains fish and other amphibians and is a relatively undisturbed habitat. Pond PL is approximately 0.15 hectares in area. PL and SGL have been sampled previously in field studies that took place in the summer of 2015, fall of 2015, and summer of 2016, and both ponds are known to harbor amphibians infected with *Bd* (Billet & Venesky, *unpublished*).

SGL and PL both contain dense but different tadpole populations. SGL consists almost exclusively of green frog (*Lithobates clamitans*) tadpoles, whereas PL consists of

mostly bullfrog (*Lithobates catesbeianus*) tadpoles. The difference in amphibian species assemblages between these two ponds is due in large part to the presence of fish predators at PL but not at SGL (Werner & McPeck 1994). Due to these differences, green frogs were sampled exclusively from SGL and bullfrogs were sampled exclusively from PL. Both species are commonly infected with *Bd* but have not been found to readily die off from *Bd* infections like other species (Gahl et al. 2012; Schloegel et al. 2012; Korfel & Hetherington 2014; Richards-Hrdlicka et al. 2013), which suggests they have a high tolerance to *Bd*. This pathogenic tolerance suggests that green frog and bullfrog tadpoles should serve as a useful indicator species for the presence of *Bd* in these water bodies.

Field Survey Methods

I surveyed field sites from September 23, 2016, through November 28, 2016. Five sampling sessions took place at each pond and 10 samples were collected at each session for

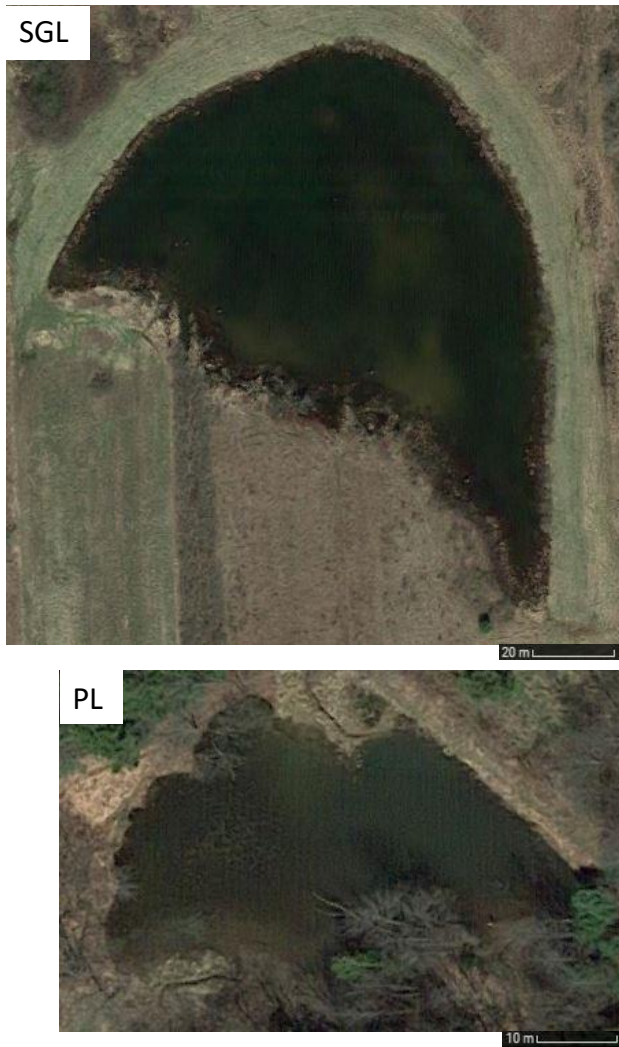


Figure 1: Overhead images of the study sites. Top image is of State Game Lands 146 (SGL) site, bottom image is of private landowner (PL) site. Appropriate scale bars are located under each image. Both images were provided by Google maps (Imagery ©2017 Google, Map Data ©2017 Google).

a total of 50 samples at each location. Sampling sessions typically took place every two weeks; the time between sessions, however, was occasionally shortened or extended due to inclement weather and limitations during the hunting season. Surveys typically took place between 14:00-18:00 in order to keep sampling time uniform between sampling dates. To survey the tadpole populations, I collected tadpoles from around the margins of each pond through random sampling by sweeping through the water column with a dip net. I then swabbed the mouth parts of captured tadpoles 10 times with sterile fine-tip swabs, as described by Hyatt et al. (2007). Swabbing the mouthparts of living tadpoles is an effective way of detecting *Bd*, and if any chytrid is present, it is transferred from the keratinized tissue in the mouth to the swab tip (Retallick et al. 2006). Each swab tip was then placed in 1.5 ml microcentrifuge tube and stored on ice. I changed nitrile gloves between handling each organism to prevent DNA contamination between samples. I took a picture of every captured individual with a metric ruler in the image for reference when measuring body lengths with computer software. I collected samples until 10 organisms of the target species were swabbed at each site. Between sampling sites, boots and nets were soaked in a 10% bleach solution to prevent the potential spread of *Bd* from site to site (Johnson et al. 2003).

I also collected environmental data at each sampling session. The temperature of each pond was recorded at each sampling session via a glass thermometer (Fisher Scientific B607100220). The thermometer was placed in the water and allowed to acclimate near where tadpoles were collected from the pond in two separate locations. The two temperature values were then averaged to give a composite temperature for the site.

Laboratory methods

After samples were collected, the swab samples were processed to determine the number of *Bd* genome equivalents on each swab. DNA extractions were performed using Prepman Ultra Sample Preparation Reagent and were diluted 1:10 to reduce sample inhibition. Real-time quantitative polymerase chain reaction (qPCR) analyses were performed following the methods outlined by Boyle et al. (2004) and those modified by Hyatt et al. (2007). TaqMan and Exogenous Internal Positive Control (Exo IPC) were added to each reaction to assess PCR reaction inhibition (Hyatt et al. 2007). To keep costs down, samples were run singly instead of in triplicate (Kriger et al. 2006). Analyses were performed with an Applied Biosystems Step One Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Infection intensity is measured as the number of *Bd* zoospore equivalents per sample, which is calculated by multiplying the genome equivalent values generated by qPCR by 8 to account for the dilution of the DNA during preparation. Any sample containing greater than 1 zoospore equivalent was considered positive.

Tadpole body lengths were measured using ImageJ 1.50i software (Schneider et al. 2012). The measurement scale was set using a reference metric ruler that was in all pictures. A body length measurement (e.g. Van Buskirk & McCollum 2000) of each tadpole was then taken and recorded in millimeters. No pictures were taken of green frog tadpoles on the final sampling date, and thus there is no length data for these individuals.

Statistical analyses

I ran a series of statistical models to test for the effects of water temperature and tadpole length on *Bd* prevalence and infection intensity. All statistical analyses were

conducted using JMP software (JMP®, Version 12.0.1. SAS Institute Inc., Cary, NC, 1989-2007).

For my first statistical model, I ran a generalized linear model on the prevalence data to look at the main effects of temperature and length on infection prevalence when controlling for the effects of the other variable. I used a generalized linear model because it allows for the analysis of binomial data, as is the case with infection prevalence data (0 = uninfected, 1 = infected). Separate analyses were conducted for each species to determine the main effects of tadpole length and water temperature on *Bd* infection prevalence. Analysis was also conducted on the entire population of tadpoles, regardless of ID (i.e. entire dataset), to determine the main effects of tadpole length and water temperature on infection prevalence in general.

For my second statistical model, I ran a fit least squares regression model on the intensity data to look at the main effects of temperature and length on infection intensity (*Bd*+1 of all infected and non-infected tadpoles) when controlling for the effects of the other variable. Intensity data was log transformed to normalize it since it has a high range of variability. Analysis was only run on “infected” individuals (*Bd* intensity > 0) to determine if either variable affects how intensely (i.e. how many genome equivalents present) an infected individual is infected. Separate analyses were conducted for each species to determine the main effects of tadpole length and water temperature on *Bd* infection prevalence. Analysis was also conducted on the entire population of tadpoles, regardless of ID (i.e. entire dataset), to determine the main effects of tadpole length and water temperature on infection intensity in general.

Results

Summary Data

Table 1: Summary statistics for sampled bullfrogs and green frogs through the entirety of the study $\pm$ SE. When necessary, sample sizes are in indicated parenthesis. Significant differences between groups are in bold.

<i>Parameter</i>	<i>Bullfrog</i>	<i>Green frog</i>	<i>P</i>
Total sampled	50	50	---
<i>Bd</i> prevalence	52% (26/50)	42% (21/50)	0.4230
Avg. <i>Bd</i> intensity	3715.4 $\pm$ 1101.7 (26)	744.6 $\pm$ 257.8 (21)	0.0218
Avg. length (mm)	25.29 $\pm$ 0.514 (47)	28.76 $\pm$ 0.572 (38)	< 0.0001

Throughout the entirety of the study period, the infection prevalence between the two species was not significantly different, with 52% of bullfrog tadpoles infected with *Bd* and 42% of green frog tadpoles infected with *Bd* ($P = 0.4230$) (Table 1). The average *Bd* infection intensity, measured in genome equivalents (GE), between the two species was significantly different ($t_{1,45} = -2.38$, $P = 0.0218$), with higher infection intensity in infected bullfrog tadpoles ($M = 3715.4$ GE, $SE = 1101.7$ GE) than in infected green frog tadpoles ($M = 744.6$ GE, $SE = 257.8$ GE) (Table 1). There was a significant interspecific difference in average tadpole body length, with green frog tadpoles being significantly longer ($M = 28.76$ mm, $SE = 0.572$ mm) than bullfrog tadpoles ($M = 25.29$ mm, $SE = 0.514$ mm) ($t_{1,83} = 4.51$, $P < 0.0001$), (Table 1).

Bd Prevalence Results

Water temperature was a significant negative predictor of *Bd* infection among all individuals when controlling for the effects of length ($X^2 = 8.92$, $P = 0.0028$) (Table 2).

Tadpole body length was a significant positive predictor of *Bd* infection among all

Table 2: Generalized linear model used to test the effect of water temperature and tadpole body length on *Bd* infection prevalence in tadpoles. A dollar-sign (\$) beside a variable indicates a source of variation calculated without controlling for other variables. Significant predictors are in bold.

Species	Source of Variation	X^2	P
Combined	Water Temp. (°C)	8.92	0.0028
	Length (mm)	6.23	0.0126
Bullfrog	Water Temp. (°C)	6.42	0.0113
	Length (mm)	19.0	< 0.0001
Green Frog	Water Temp. (°C)	38.2	0.327
	Water Temp.\$ (°C)	3.36	0.0668
	Length (mm)	0.000679	0.979

individuals when controlling for the

effects of water temperature ($X^2 = 6.23$, $P = 0.0126$) (Table 2).

Interspecifically, water temperature was a significant negative predictor of *Bd* infection in bullfrogs ($X^2 = 6.42$, $P = 0.0113$) but not in green frogs ($X^2 = 38.2$, $P = 0.327$) when controlling for the effects of body length (Table 2; Fig. 2). When not controlling for effects of length, however, water temperature was a marginally significant negative

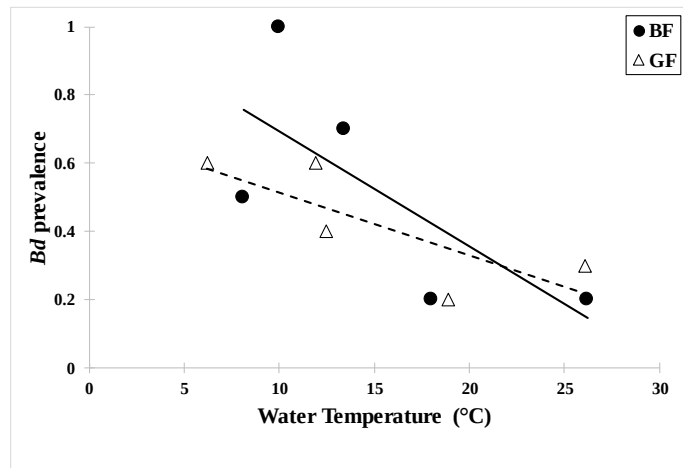


Figure 2: The relationship between temperature and *Bd* prevalence. Each data point is the average of 10 binomial data points from the respective sampling session (1 = infected; 0 = uninfected). Error bars were omitted for visual clarity. Solid symbols and solid line represent bullfrog (BF) data ($N = 5$), open symbols and dashed line represent green frog (GF) data ($N = 5$). Generalized linear models showed a significant negative relationship between temperature and *Bd* infection in bullfrogs ($X^2 = 6.42$, $P = 0.0113$) and a marginally significant negative relationship between temperature and *Bd* infection in green frogs ($X^2 = 3.36$, $P = 0.0668$).

predictor of *Bd* infection in green frogs ($X^2 = 3.36$, $P = 0.0668$) (Table 2; Fig. 2). Body length was a significant positive predictor of *Bd* infection in bullfrogs when controlling for the effects of water temperature ($X^2 = 19.0$, $P < 0.0001$) (Table 2; Fig. 3). Length was not a significant predictor of *Bd* infection in green frogs when controlling for the effects of water temperature ($X^2 = 0.000679$, $P = 0.979$) (Table 2; Fig. 3).

Bd Infection Intensity results

Table 3: Fit least squares models used to examine the effect of variables on *Bd* infection intensity in tadpoles. Significant predictors are in bold.

Species	Source of Variation	<i>t</i>	<i>df</i>	<i>P</i>
Combined	Water Temp. (°C)	-1.09	2,36	0.2846
	Length (mm)	2.68	2,36	0.0109
Bullfrog	Water Temp. (°C)	-1.44	2,22	0.1644
	Length (mm)	4.03	2,22	0.0006
Green Frog	Water Temp. (°C)	-0.16	2,11	0.8726
	Length (mm)	0.04	2,11	0.9724

Water temperature was not a significant predictor of *Bd* infection intensity among all infected individuals when controlling for the effects of body length ($t_{2,36} = -1.09$, $P = 0.2846$) (Table 3). Tadpole body length, however, was a significant positive predictor of *Bd* infection

intensity among all infected individuals when controlling for the effects of water temperature ($t_{2,36} = 2.68$, $P = 0.0109$) (Table 3).

By individual species, water temperature was not a predictor of *Bd* intensity for either bullfrogs ($t_{2,22} = -1.44$, $P = 0.1644$) or green frogs ($t_{2,11} = -0.16$, $P = 0.8726$) when controlling for the effects of length (Table 3; Fig. 4). Length was a significant positive predictor of *Bd* intensity in bullfrogs ($t_{2,22} = 4.03$, $P = 0.0006$) but not in green frogs ($t_{2,11} = 0.04$, $P = 0.9724$) when controlling for the effects of water temperature (Table 3; Fig. 5).

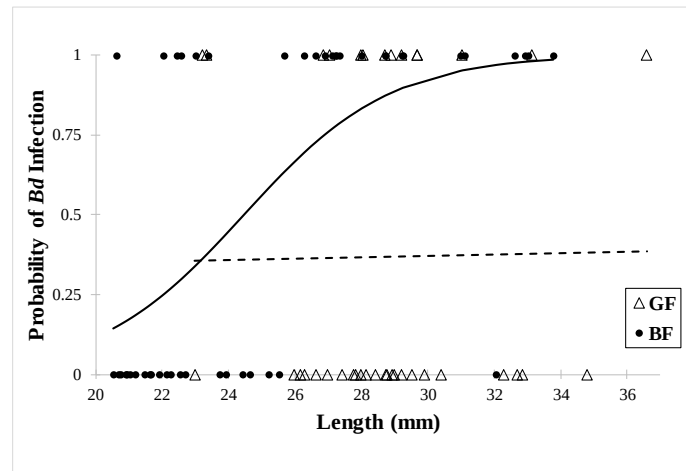


Figure 3: The relationship between tadpole body length and *Bd* infection status (raw data). Solid symbols and solid line represent bullfrog (BF) data ($N = 47$), open symbols and dashed line represent green frog (GF) data ($N = 38$). Linear regression trendlines represent the probability of infection by length and correlates with the y-axis. Binary (1,0) data points represent whether an individual was infected or not and do not correlate with the y-axis. Generalized linear models showed a significant positive relationship between length and *Bd* infection in bullfrogs ($X^2 = 19.0$, $P < 0.0001$) but not in green frogs ($X^2 = 0.000679$, $P = 0.979$).

Discussion

With increases in the emergence of pathogens globally due to climate change, habitat destruction, animal trades, and other factors, it is important to understand the dynamics of pathogens in natural systems, both where a pathogen is virulent and where it is seemingly benign. The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been identified in northwest Pennsylvania, but its temporal trends in the region are still unclear. In a field study, I examined the relationship between amphibian infection by the fungal pathogen *Bd* and two variables: tadpole body length and water temperature. I analyzed infection dynamics in two different larval amphibian species, green frogs and bullfrogs, at two separate ponds in

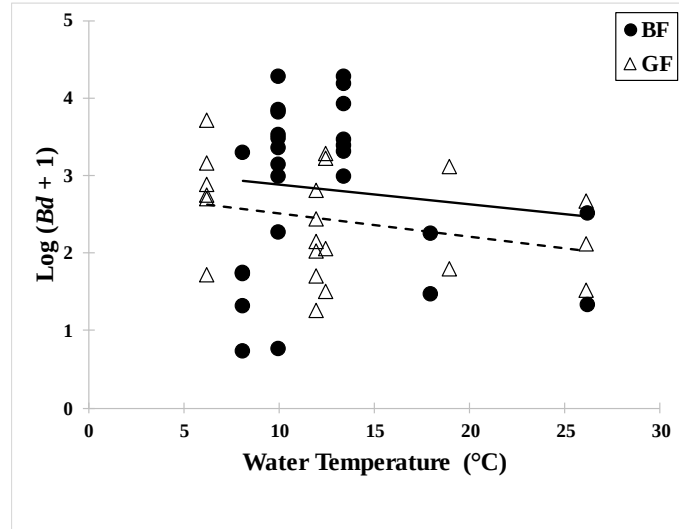


Figure 4: The relationship between water temperature and average *Bd* infection intensity via linear regression (raw data). Solid symbols and solid line represent bullfrog (BF) data (N = 26), open symbols and dashed line represent green frog (GF) data (N = 21). Fit least squares models showed no significant relationship between water temperature and infection intensity in bullfrogs ($t_{2,22} = -1.44$, $P = 0.1644$) or green frogs ($t_{2,11} = -0.16$, $P = 0.8726$).

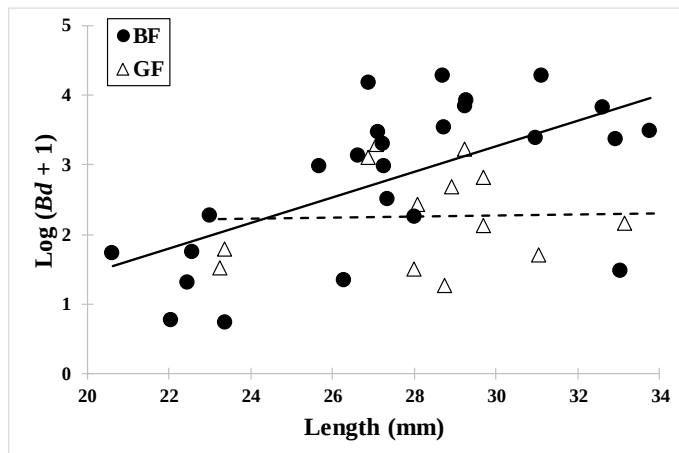


Figure 5: The relationship between tadpole body length and *Bd* intensity via linear regression (raw data). Solid symbols and solid line represent bullfrog (BF) data (N = 25), open symbols and dashed line represent green frog (GF) data (N = 14). Fit least squares models showed a significant positive relationship between tadpole length and infection intensity in bullfrogs ($t_{2,22} = 4.03$, $P = 0.0006$) but not in green frogs ($t_{2,11} = 0.04$, $P = 0.9724$).

northwest Pennsylvania where *Bd* is relatively benign and not the cause of mass die-offs. I found that in bullfrogs, pond temperature was a significant negative predictor and tadpole body length was a significant positive predictor of *Bd* infection. Length was also a significant positive predictor of *Bd* infection intensity in bullfrogs. In green frogs, water temperature was a marginally significant negative predictor of *Bd* infection.

Through the study period, water temperature was a significant negative predictor of the presence of *Bd* infection in bullfrogs and a marginally significant negative predictor of *Bd* infection in green frog when not controlling for the effects of length (Fig. 2; Table 2). These results agree with the literature consensus, which has found that *Bd* prevalence increases with decreasing temperatures (Korfel & Hetherington 2014; Whitfield et al. 2012; Lenker et al. 2014; Andre et al. 2008). There are several dynamics at play in the relationship between water temperature and *Bd* prevalence through fall, most of which involve changing interactions between host immunity and pathogen physiology (Piotrowski et al. 2004; Raffel et al. 2006; Cohen et al. 2017). Studies have found that cold-acclimated ectotherms maintain higher levels of immune cell activities than warm-acclimated ectotherms that have been recently moved to cold conditions (Pxytycz & Józkowicz 1994). Raffel et al. (2006) have also described a seasonal acclimation effect in the fall, which refers to a change in immune cell production to below optimal rates following seasonal temperature decreases. This suggests that amphibians likely have difficulty adjusting their immune systems when temperatures change, making them more vulnerable to infection by *Bd*.

Along with sub-optimal levels of amphibian immunity, *Bd* also experiences changes in response to temperature decreases that may increase its chances of infecting a new host. Although *Bd* growth rates are decreased with decreasing temperatures, at colder temperatures

each zoosporangium produces a higher number of zoospores (Woodhams et al. 2008). Additionally, when *Bd* experiences a cold-shock, a sudden increase in zoospore production occurs (Woodhams et al. 2008). This suggests that though *Bd* may grow slower in colder temperatures, there is an increased chance of infection in cold temperatures due to the sudden increase in *Bd* zoospores. *Bd* has also been found to have longer periods of zoospore activity at lower temperatures, which suggests each zoospore has a higher likelihood of finding a host at colder temperatures (Voyles et al. 2012).

Parasites also tend to acclimate more rapidly and be more tolerant to changing temperatures than their hosts (Brown et al. 2004; Kingsolver & Huey 2008). Between amphibian hosts and *Bd*, there is evidence for a thermal mismatch, in which cold-adapted and warm-adapted amphibians are most susceptible to parasites in relatively warmer condition and cooler conditions, respectively (Cohen et al. 2017). This suggests that in response to changing temperatures, amphibians are likely more vulnerable to infection since parasites are less impacted by temperature variations away from the optimum. Thus, as temperatures fluctuate and drop in the fall, *Bd* likely has a physiological advantage, enabling it to infect more hosts during this time of increased susceptibility. With increased shifts in climate and an increase in the prevalence of extreme weather events, this effect may become even more pronounced and detrimental (Cohen et al. 2017). The increase in *Bd* reproduction and activity and decrease in amphibian immunity together with the more rapid adjustment of parasites to temperature changes may be a main driver of increases in *Bd* prevalence with decreasing temperatures.

Water temperature was a significant negative predictor of the presence of *Bd* in both bullfrogs and green frogs but was not a predictor of infection intensity in either species (Fig.

4). This suggests that temperature impacts whether an individual is infected or not, but it has no noticeable effect on the infection burden of infected individuals. A field study by Korfel & Hetherington (2014) found similar trends in a population of green frogs in Ohio, with temperature predicting *Bd* prevalence trends but not infection intensity trends. There are several logical explanations for this inconsistency in temperature-driven infection trends. The first explanation is that infection intensity is a better representation of when an individual became infected rather than the infection's response to temperature. Though there is a higher chance of becoming infected late in the fall, the infected population accounts for both individuals that have been infected for an extended time and newly infected individuals. *Bd* intensity in previously infected individuals would be expected to stay relatively consistent in colder temperatures while the proportion of individuals with low infection in the population would be expected to increase. This, in turn, would likely lead to an overall drop in average infection intensity through the winter rather than an increase. By this logic, we would also expect to see a relative increase in *Bd* intensity with warming weather because of some low-infected individuals clearing infection, a reduction in the rate of new infections, and an increase in infection intensity in high infection individuals because of increased *Bd* growth rate and an increased tolerance to infection in tadpoles. Korfel & Hetherington (2014) found results in green frog tadpoles that support this theory, with *Bd* infection intensity at its highest in the summer rather than the winter.

Infection intensity data in tadpoles may also be confounded by tadpoles metamorphosing. This study, as well as others, found that larger tadpoles are more likely to be infected by *Bd*. Larger tadpoles are also closer to metamorphosis and have been found to harbor higher infection intensities in this study and others (Conradie et al. 2011). As winter

approaches, tadpoles have two options: overwinter or metamorphose. Pathogenic infection has also been found to reduce time to metamorphosis, which may tip the scales more towards metamorphosis in infected tadpoles (Warne et al. 2010). Since larger tadpoles have higher infection intensity and some of them inevitably metamorphose, they are removed from the sampling population. Thus, as winter approaches there may not only be an increase in low-infection individuals but a decrease in high-infection individuals.

Host life-history traits are often just as important as environmental factors in host infection dynamics. In this study, tadpole length was a significant predictor of *Bd* infection and intensity of infection, particularly in bullfrogs (Fig. 3; Fig. 5). Longer bullfrog tadpoles were more likely to be infected and harbored higher infection loads than shorter individuals. Though tadpole Gosner developmental stage was not quantified for each tadpole, it was assumed that within a population, larger tadpoles tend to be farther along in development and closer to metamorphosis, although there is some variation in size at metamorphosis (Collins 1979). Other studies have found similar trends to the one found in bullfrogs in this study, with individuals at a later developmental stage having a higher infection prevalence (Smith et al. 2007; Vieira et al. 2013). Older tadpoles tend to have higher *Bd* prevalence and intensity for several reasons. The keratinized mouthparts, which *Bd* grow on, do not develop in tadpoles until between Gosner stages 23 and 25 (Vieira et al. 2013). The absence of these mouthparts acts as a barrier to infection, and chances of infection are slim before their development. Additionally, the amount of time that a tadpole has had keratinized mouthparts should be associated with its chances of becoming infected since it has more time to become exposed, again leading to larger tadpoles having a higher chance of infection (Smith et al. 2007). Infection of the keratinized mouthparts may also reduce feeding behavior (Venesky et

al. 2009; DeMarchi et al. 2014), which could cause an increase in infection intensity by reducing tadpole energy reserves to fight it off.

There are several explanations for why a relationship between length and *Bd* infection was found in bullfrogs but not in green frogs. First, bullfrog tadpoles have a much longer developmental period than green frogs (Collins 1979; Martof 1956), which may mean that the effects of time on the chances of becoming infected are amplified. The amount of time spent infected is likely amplified as well, leading to increased infection intensity at larger sizes. Whereas green frogs usually metamorphose in under a year and often metamorphose before winter, bullfrog tadpoles usually spend several years in the larval phase before metamorphosis. Second, there may be differences in the immune responses in bullfrogs and green frogs to *Bd* infection that amplify *Bd* prevalence in larger bullfrog tadpoles and reduce *Bd* prevalence in larger green frogs. Previous studies have found that bullfrogs can be highly infected by *Bd* but experience no adverse effects (Garner et al. 2006; Schloegel et al. 2010; Eskew et al. 2015). Green frogs, on the other hand, have been found to be generally tolerant to localized *Bd* strains but succumb to infection by foreign *Bd* strains (Gahl et al. 2012). This suggests that bullfrogs utilize more general tolerance strategies in immunity and green frogs utilize more pathogen-specific resistance strategies in immunity. Resistance refers to an organism's ability to clear an infection and tolerance refers to the ability of an organism to limit damage from a parasitic infection while maintaining an infection (Ayres & Schneider 2008). My results support this theory, as green frogs were less infected and had significantly lower infection loads than bullfrogs. It is important to note that the lack of relationship found between green frog tadpole body length and *Bd* infection should be interpreted

conservatively due to the lack of length data from green frogs on the final sampling day. This reduced the sample size compared to the bullfrog data, which reduced its statistical power.

When comparing trends between bullfrogs and green frogs in this study, there is a possibility that study site confounds the results. Though temperature and development have been identified as two driving factors in infection trends, trends may have been influenced by other factors that differed between the sites such as species assemblage, pollution, canopy cover, and water chemistry (Blaustein et al. 2005; Strauss & Smith 2013; Raffel et al. 2010; Searle et al. 2011). Studies on the influence of many of these environmental factors, however, have been inconclusive in determining how they impact the spatial variation of *Bd*. Additionally, a study by Strauss & Smith (2013) found that there was no relationship between the spatial variation of *Bd* and any one biotic or abiotic factor. They did, however, find that ponds containing *Bd* were more similar than by chance. In other words, *Bd*-harboring ponds tended to share similar characteristics, specifically in their macroinvertebrate assemblages and water chemistry. Between pond differences in the SGL and PL may have played a role in differing *Bd* trends, but that role was likely marginal. Thus, the results of this study are valuable for assessing general *Bd* dynamics in natural systems in the fall and for assessing how temperature and developmental stage differentially impact infection trends in bullfrogs and green frogs. The differences found between the two species, however, should be considered conservatively until a more controlled study can be conducted to assess the findings of this study.

Though *Bd* has not been found to cause amphibian die-offs in northwest Pennsylvania or the northeastern United States, having a general understanding of *Bd* dynamics in this region is important. Tadpoles play a role in the cycling of nutrients and the breakdown of

detritus (Iwai et al. 2009), and *Bd* infection may impede their ability to do this effectively (Venesky et al. 2009). Additionally, with the global animal trade, there is the possibility of the introduction of a novel strain of *Bd* in the northeast, as has happened elsewhere (Schloegel et al. 2012). Although most species in the northeast have been found to be tolerant to local *Bd* strains, foreign *Bd* strains can be more deadly (Gahl et al. 2012). The virulence of the local strain of *Bd* could also increase due to human-induced climate changes (Raffel et al. 2012; Cohen et al. 2017). Additionally, my results suggest that *Bd* more heavily impacts tadpoles closer to metamorphosis, which could negatively impact the health of post-metamorphic individuals and make them more vulnerable to death (Marantelli et al. 2004).

In the future, the results from this study should be followed up on and tested in a more controlled setting, such as in a mesocosm experiment. This will allow for better control of confounding environmental variables to provide a more concrete idea of seasonal *Bd* trends. Studies should also be expanded to other seasons to get a stronger understanding of *Bd* dynamics throughout the year. Additionally, I recommend conducting several spatial-oriented studies to identify what environmental factors influence variation in *Bd* across locations. I also suggest that future studies look closer at the non-lethal effects of *Bd* in regions where it is not causing mass die-offs. Though *Bd* may not be directly killing amphibians in this region, there may still be detrimental impacts of infection that should be studied to give a better understanding of how *Bd* impacts natural ecosystems.

In conclusion, my study suggests that there is variation in *Bd* prevalence and intensity as a result of both water temperature and host developmental traits. There was a significant negative effect of water temperature on bullfrog tadpole *Bd* prevalence and a marginally negative effect of temperature on green frog tadpole *Bd* prevalence. There was also a

significant positive effect of tadpole body length on *Bd* prevalence and intensity in bullfrogs. These results emphasize the differences in infection dynamics between species in a natural system, and also provide a general model of *Bd* infection trends in northwest PA in the fall. This data is valuable not only for future conservation efforts locally but also for conservation efforts in areas with similar environmental conditions by identifying times of the year when disease mitigation efforts may be most and least effective (i.e. when disease prevalence is lowest and highest, respectively). Long-term monitoring of deadly infectious diseases is one of the most important steps in conservation efforts and should continue in the local *Bd* system to increase our understanding of its trends and impacts.

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