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Ryan Huang April 17, 2011

Detecting the presence of Batrachochytrium dendrobatidis in amphibians in Piedmont vs Blue Ridge habitats in northern Georgia

by

Ryan Huang

Dr. Lawrence Wilson
Adviser

Department of Biology

Dr. Lawrence Wilson

Adviser

Dr. Chris Beck

Committee Member

Dr. Darrell Stokes

Committee Member

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An abstract of
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Abstract

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With countless species of amphibians under threat of extinction, the deaths of individuals from the chytrid fungus (Batrachochytrium dendrobatidis) are all the more worrisome. On top of this, the presence of chytrid in the Blue Ridge habitats of northern Georgia, known to be one of the world's hotspots of salamander diversity, is particularly alarming. This study is aimed at determining the extent to which chytrid has infected both anura and caudata species in habitats in both the Blue Ridge as well as the Piedmont physiographic provinces of Georgia. Ten different sites, five in Blue Ridge Province of north Georgia and five within the Piedmont region, were sampled using dip nets, and upon capture, individuals were swabbed to look for the presence of zoospores. Each swab sample was analyzed using real-time PCR to determine the presence or absence of chytrid. Two-hundred and seventy three individuals were sampled for chytrid from eleven different salamander species and four different frog species. Fifty-one of the samples tested positive for chytrid (18.7% prevalence) and susceptibility to chytrid infection was determined by what species an individual was. Overall, there has been a large increase in chytrid prevalence since the last known study in Georgia. As such, I recommend more extensive surveying to determine which, if any, species are susceptible to a population decline.

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Introduction

Missing Amphibians

After the disappearance of the golden toad in 1988 (Crump et al. 1992), attention was drawn towards countless other amphibian species that have been going extinct in protected and pristine areas throughout Australia, Central and North America, and Europe (Richards et al. 1993, Lips 1998, Bosch et al. 2001, Bradley et al. 2002). With over a third of the 6,500 known species of amphibians threatened with extinction, amphibians are the most threatened group of vertebrates today (Stuart et al. 2004). While only 15 species of amphibians have been declared extinct since 1500, nine of the extinctions have occurred since 1980 and in the same time period 168 species have not been seen in the same time period (IUCN 2010).

Several causes are responsible for this massive decline in amphibian populations and it is likely that no single factor is responsible (Blaustein and Kiesecker 2002). The first and most obvious cause is loss of habitat. Specific examples of habitat destruction include clear-cutting forests, draining wetlands, and altering vegetation. One study has estimated that a total of 14 million salamanders are lost every year in the United States alone due to clear cutting (Petranka et al. 1993). Another factor in the disappearance of many amphibians is the introduction of invasive species. These new species often compete, prey upon, or transmit disease to endemic species and can have severe detrimental consequences (Blaustein and Kiesecker 2002). Climatic changes of an environment can also have significant negative consequences for the native amphibian populations (Blaustein and Kiesecker 2002, Daszak et al. 2005). For example, changes in temperature or precipitation can considerably alter the amount of available water, which is of utmost importance to many amphibians.

While all of the above factors are causes for distress, the leading concern of today's conservation efforts is the mass mortalities of amphibians in protected areas. If we cannot protect species in preserves, where else would a safe haven exist? These protected areas are policed so that the impact of human development, pollution, and even invasive species are at a minimum. As such, there is likely another contributing factor at play in amphibian die-offs. The introduction of a novel pathogenic parasite to naïve populations is known as pathogenic pollution (Cunningham et al. 2003). These naïve populations often have not had time to develop immunity and severe population declines occur through outright mortality of infected individuals. Mortality can also occurs through indirect means such as a change in the competitive ability of two species or via the decline of a prey species affected by the pathogen. This phenomenon of pathogenic pollution appears to be responsible for the widespread occurrence of a highly virulent pathogenic fungus from the chytrid family known as *Batrachochytrium dendrobatidis*.

This chytrid fungus has been found at the locations of several mass die-offs of amphibians and several species appear to experience a 100% mortality rate upon infection (Berger et al. 1998, Berger et al. 2004). However, not all amphibian species share the same susceptibility to the fungus and are often asymptomatic. It is not currently known what characteristics determine how severe an infection will be on an individual, but there are several features of a species' ecological guild that may make them more prone to a population decline (Daszak et al. 1999). Species that are regionally endemic, have a low fecundity, stream-breeding, and exist at high altitudes appear to be the most vulnerable for severe population declines.

History, Identification, and Spread of Chytrid

During the period from September 1996 to October 1997, 31 individual frogs from three different species (two of which were poison dart frogs from the genus *Dendrobates*) at the National Zoological Park in Washington, D.C., mysteriously died (Longcore et al. 1999, Pessier et al. 1999). The only indications of possible illness seen in the individuals were anorexia and lethargy noted the day before their death. Necropsies found only a slight discoloration of the ventral surface and hind legs and feet. Histological examinations found consistent skin lesions in the pelvic areas and between the toes of the deceased individuals. Closer examination of the epidermis found single-celled eukaryotic individuals within the stratum corneum. Ultrastructural analysis of the apparent zoospores placed the species in the fungal class Chytridiomycetes of the phylum Chytridiomycota (Pessier et al. 1999). As a result, the amphibian disease afflicting the individuals was termed cutaneous chytridiomycosis. In 1999, the morphological features and thalli development were recorded and determined to be taxonomically different enough from existing chytrid fungi to be given a new genus and species, *Batrachochytrium dendrobatidis*, named after the genus of poison dart frogs from which the organism had been isolated (Longcore et al. 1999). During this time when *B. dendrobatidis* was being discovered in the United States, the same species of chytrid was found involved in mass mortalities of frogs species in the montane riparian habitats of Australia and Central America (Berger et al. 1998).

Although the fungus was not classified until 1999, a review of archived specimens has found infected individuals as early as 1938 on African Clawed frog (*Xenopus laevis*) samples and as such *X. laevis* is now thought to be the endemic host (Weldon et al. 2004). However, there are no known reports of *X. laevis* dying from chytridiomycosis and thus this species is believed to act as a natural carrier. Within southern Africa, overall prevalence and geographic

distribution of chytrid infections has not significantly changed over the years. This stability in infection rates supports the hypothesis that this area is the origin of chytridiomycosis. While southern Africa is the site of the earliest known infection by *B. dendrobatidis*, it is still possible that chytridiomycosis first originated elsewhere in Africa and spread through multiple closely-related hosts.

During the 1930s, people started to use *X. laevis* as a pregnancy test (Shapiro and Zwarenstein 1934) and as such, mass capture and exportation of the frogs became profitable, with just under 5,000 individuals exported in the year 1970 alone (Weldon et al. 2004). Even after non-amphibian pregnancy tests became commercially available, *X. laevis* is still used today as an important model organism for developmental biology in laboratories across the world. Within the importing countries, escaped infected individuals or water infected with zoospores may come into contact with endemic amphibian populations, starting the transmission of the pathogen.

In today's global economy, both domestic and international trade has enhanced the spread of *B. dendrobatidis* over large geographic ranges via the transportation of infected individuals (Fisher and Garner 2007). In every instance of a population decline as a result from chytrid, there is a likely mode of dispersal. In some instances, frogs are farmed for food and exported internationally. Indonesia alone exports roughly 28,000 frogs for consumption per year, and yet several exported species are known to experience chytrid infections (Gratwicke et al. 2009). At other times amphibians are introduced as a biocontrol agent such as the cane toad (*Rhinella marinus*) in Australia (Easteal 1981). It is also thought that scientists may act as carriers if their equipment comes into contact with zoospores in one environment and are then transported to a new naïve population. The popular capturing of poison dart frogs in Central

America by zoological gardens that had *B. dendrobatidis* during the 1970s and onward is thought to be the main introduction of chytrid to this area (Pessier et al. 1999, Fisher and Garner 2007).

Much like *X. laevis*, other amphibian species may act as carriers regardless of whether or not they are the endemic host. The American bullfrog (*Lithobates catesbeiana*) is thought to be one species that acts as an important reservoir host for the pathogen since they appear to be asymptomatic in that they experience low levels of zoospores and thus are not killed by chytridiomycosis (Berger et al. 1999, Daszak et al. 2004). Therefore, established populations of the endemic host are not necessary for continual transmission of the fungus. This is of major concern since constant release of zoospores by infected individuals would allow *B. dendrobatidis* to travel large geographic ranges distances since it can be carried by its host for the remainder of the host's lifespan. In addition, these carriers allow the fungus to persist in an environment long after susceptible species have experienced population declines (Dobson and Foufopoulos 2001).

In addition to a wide variety of hosts, it is possible that *B. dendrobatidis* persists in the environment as a free-living saprophyte, and thus exists in the environment as a decomposer rather than as a parasite, much like many other members of the chytrid family (Daszak et al. 1999). Similarly, in insect models in which pathogenic bacteria can reproduce saprophytically, the host threshold that is needed to maintain the pathogen's population is significantly lowered (Godfray et al. 1999). Therefore, in combination between saprophytic reproduction and a vast suite of asymptomatic host reservoirs, more susceptible hosts may be easily driven to extinction instead of a fluctuating population decline that might be typical of host-parasite interactions.

Lastly, the lack of genetic variation between individuals of chytrid over a large geographic range suggests that world-wide distribution of the fungus has occurred rapidly enough so that few mutations have occurred (Morehouse et al. 2003). This, in combination with

low host specificity, support the ongoing idea that *B. dendrobatidis* has been introduced into new, naïve populations rather than a change in a pre-existing relationship between amphibians and fungus due to abiotic factors such as climate change.

Life History of Chytrid

Species of chytrid are highly ubiquitous with species being found everywhere from the tundra to the tropics and in both aquatic and terrestrial habitats (Powell 1993). Most known chytrid species live in the soil or water and are decomposers of organic matter. However, some chytrid fungi are known to be either obligate or facultative parasites of algae, vascular plants, invertebrate animals, as well as other fungi. Of the chytrid fungi, *B. dendrobatidis* is the only known species to be pathogenic to vertebrates (Berger et al. 1998).

The life cycle of *B. dendrobatidis* starts with a spherical, unwalled, motile zoospore with a single posterior flagellum (Longcore et al. 1999, Berger et al. 2005) (Figure 1). The period of zoospore motility appears to last between 18 and 24 hours after release and does not vary with environment (Piotrowski et al. 2004). Locally, chytrid is most likely dispersed through individual-to-individual transmission as evidenced by the inability for zoospores to travel long distances and epidemic fronts of introduction (Piotrowski et al. 2004, Lips et al. 2006). There also appears to be no evidence of chemotaxis that would increase the distance that zoospores would travel. In addition, the lack of a resilient resting stage and the evidence that drying *B. dendrobatidis* kills the entire culture suggest that zoospore dispersal mainly occurs over short periods of time and solely through an aqueous medium (Berger et al. 1999, Daszak et al. 1999, Morehouse et al. 2003).

After dispersal, the zoospore encysts, a process that includes reabsorbing the flagellum and creating a cell wall (Berger et al. 2005). When the zoospore encounters amphibian

epidermis and first infects the individual, encystment occurs within the deeper, more immature cells of the stratum corneum. A single cell can be infected by multiple zoospores. The actual method of penetrating the cell is unknown, but encystment could occur on the surface of the epidermal cell and then the fungus would empty its contents into the cell through a germ tube (Longcore et al. 1999). Once an amphibian is infected with chytrid zoospores, the epidermis starts becoming more keratinized, a condition known as chytridiomycosis (Daszak et al. 1999). Due to the combination of the fungus's ability to live saprophytically on keratin (Longcore et al. 1999) and the induction of hyperkeratosis in the host, some believe that *B. dendrobatidis* uses keratin as a nutrient (Daszak et al. 1999).

The zoospore then becomes a single-celled thallus that develops multiple nuclei through mitotic divisions (Berger et al. 2005). Cleavage results in mature zoospores still encased in the thallus, which by now is known as the sporangium. Discharge papilla form towards the distal surface of the host and upon dissolution of a plug that blocks the opening, the mature zoospores are released. Release of the zoospores appears to be triggered in part by some moisture threshold. Timing of the release also appears to be synchronized with the maturation of the epidermal cell and thus release occurs during the sloughing of these outer layers. If the maturation of the sporangium occurs before more distal layers have been sloughed, the zoospores will simply be released into the intracellular spaces. No new zoospores will develop within the thallus after this initial release but empty sporangium will often be colonized by bacteria. It is currently believed that *B. dendrobatidis* reproduces clonally, which is supported by multilocus sequence genotyping as well as the lack of evidence for a sexually produced resting stage common for sexually reproducing fungi (Morehouse et al. 2003).

Three hypotheses exist as to how chytridiomycosis specifically causes the death of a host. The first hypothesis is that the fungus indirectly kills the host through the induction of hyperkeratosis in the pelvic area, which makes the epidermis more impermeable thereby preventing the crucial uptake of water that allows for proper osmoregulation (Pessier et al. 1999). This would result in a severe electrolyte imbalance, dehydration, and even possible suffocation since limited respiration also occurs through the ventral surface of amphibians. The second hypothesis is that the fungus is directly responsible for the host's death by releasing toxins that are absorbed by the host through its epidermis (Berger et al. 1998). The third hypothesis is simply that there is a combination of effects of the previous two hypotheses. Regardless, susceptible species may die from chytridiomycosis anywhere between 18 and 70 days after exposure to zoospores (Berger et al. 2005). Clinical signs of an infection include lethargy, skin discoloration, and loss of a righting complex (Berger et al. 1999). Unfortunately, these symptoms appear no more than a few days before death.

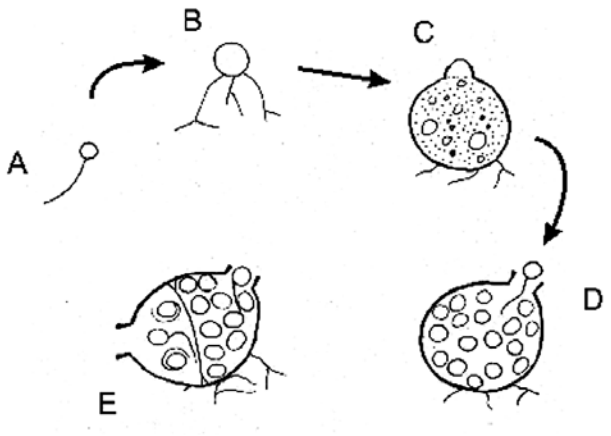

Figure 1: Diagram of the life cycle of *B. dendrobatidis*. The individual starts as a motile zoospore (A) and then encysts after 18-24 hours (B). What is now a thallus enlarges and undergoes mitotic division to become a zoosporangium (C). Upon the dissolution of a plug in the discharge tube, the zoospores are released (D). Sometimes the thalli develop into multiple sporangium through the creation of a septa dividing the contents (E). Modified from Berger *et al.* (2005).

Impact on Georgia

Recently, *B. dendrobatidis* has also been found in the state of Georgia but the full extent of its distribution in the area is not well understood (Timpe et al. 2008, Hossack et al. 2010) Previous studies of the area have found a very low incidence of chytrid and more in depth surveys are needed to establish locations of chytrid infections. It is essential that surveys be conducted to determine which species are susceptible to chytridiomycosis and may experience a population decline in the future. Within the southeastern United States, a few amphibian species have experienced population declines (Daszak et al. 2005). While chytrid was present in these populations, the direct cause of these declines appears to be a climatic drying of the habitats in question. However, fungal infections could place additional stress on the host so that they become more susceptible to dying from changes in the environment. So although chytrid has not necessarily caused mass die-offs in Georgia, the evidence of other environmental stressors in conjunction with *B. dendrobatidis* illustrates the importance of understanding the distribution of the fungus in order to help protect the biodiversity of the region. Keeping the amphibian species alive and healthy is important since these species account for the majority of the vertebrate biomass in the area and thus are responsible for supplying higher predators with energy by acting as prey (Wright et al. 2001). There can also be shifts in the macroinvertebrate community structure and disruption of the nutrient cycle due to the loss of amphibians as a important predator of invertebrate detritivores (Petranka 1998, Colon-Gaud 2009). This means that if these amphibian populations were to decline, many other species that depend on them could collapse as well.

Purpose and Hypothesis

The purpose of this study is to determine where and what amphibian species are experiencing infections. Although most of the chytridiomycosis research has been focused on anuran species (frogs and toads), there have been several documented reports of population declines in caudata species (salamanders and newts) (Davidson et al. 2003, Bosch and Martínez-Solano 2006). For this reason, all amphibian species encountered during the study were sampled for chytrid infections. Two different habitat types were surveyed for chytrid infections, the Blue Ridge habitats of the Southern Appalachians and the Piedmont plateau around the Atlanta region. This is the first known chytrid survey of the Atlanta area. Each habitat is home to a variety of salamander and frog species. I hypothesize that more chytrid infections would be found in the montane Blue Ridge habitats since montane areas have been the most affected by chytrid in the past and because *B. dendrobatidis* development is slower in the warmer lowlands (Daszak et al. 1999, Longcore et al. 1999).

Methods

Study Sites

A total of ten different sites were selected, five sites located around Cooper's Creek in north Georgia in the Blue Ridge habitat of the southern Appalachians; the other five sites were located around Atlanta, Georgia, in the Piedmont habitat. Specifically, the fives Piedmont sites were located at Fernbank Forest, Lullwater Preserve, Stone Mountain Park, Arabia Mountain Heritage Area, and Panola Mountain State Park (Figure 2). The average elevation of the five Blue Ridge sites was 630m above sea level while the average elevation of the five Piedmont sites was 275m above sea level. At each site a permanent stream and the immediate banks were sampled since species restricted to these areas appear to be most prone to infection (Kriger and Hero 2007). Roughly 50m of bank on both sides of the river was considered a single site.

Sampling Protocol

Sampling lasted from September to the first week of January with each site being sampled roughly once a month. Sites were sampled by flipping over rocks and dead logs as well as dip netting in the water. Once an individual was caught, overall health, snout to vent length, and mass were recorded as standard measurements. Each individual was swabbed for *B. dendrobatidis* following the methodology laid out by Brem *et al*. (2007) using polyester swabs. No individuals were released until sampling of a site was finished in order to ensure individuals were not being recaptured.

Although immature individuals (tadpoles and larval salamanders) may carry the fungus on the keratinized areas such as the mouthparts (Berger et al. 1998), immature individuals were not sampled in this study due to the difficulty in identifying the species of immature individuals

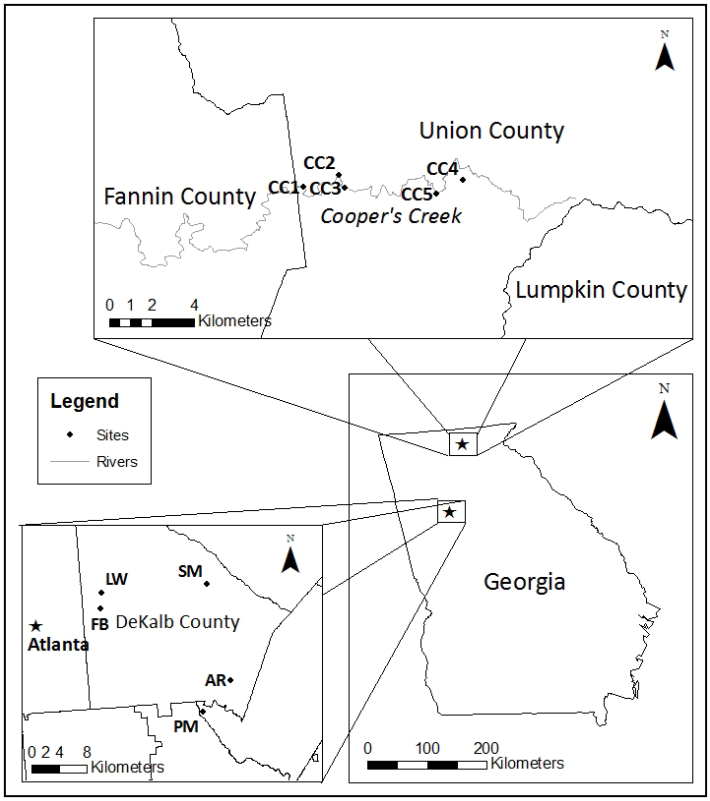

Figure 2: Map of ten study sites in two different habitats in northern Georgia, Blue Ridge mountains and Piedmont plateau. CC1-5 are five different sites around Cooper's Creek and are part of the Blue Ridge habitat of the Southern Appalachians. AR (Arabia Mountain Heritage), FB (Fernbank Forest), LW (Lullwater Peserve), PM (Panola Mountain), and SM (Stone Mountain) are all a part of the Piedmont habitat.

in combination with the fact that immature individuals must be killed and have their mouthparts excised for identification of chytrid.

After the measurements were taken, I released individuals at the point of capture. Swabs were subsequently stored in a freezer at -20˚C. Once a site was sampled, all nets and shoes were disinfected with a 10% solution of bleach to prevent the spread of chytrid from site to site. Furthermore, new non-powdered gloves were used whenever an individual was handled, and each individual was kept in a new zip-loc bag to ensure that I was not artificially dispersing the chytrid fungus. The pH of the water was not sampled since chytrid is able to survive and grow within the pH range of freshwater systems (Piotrowski et al. 2004).

Molecular Protocol

All swab DNA extractions were done at Emory University. The DNA from each swab was extracted using a DNeasy Blood and Tissue kit (Qiagen). To determine if chytrid was present or absent, we used real-time PCR (polymerase chain reaction) assays were conducted using SYBR green and an Applied Biosystems StepOnePlus Real-Time PCR System. The primers were chosen based upon those designed by Boyle *et al* (2004) to amplify the internal transcribed spacer (ITS-1) and 5.8S junction in the *B. dendrobatidis* genome. Reactions (10 μL) containing 5 μL of SYBR green master mix (Applied Biosystems), primers at a concentration of 5 pmol/mL, and 1 μL of DNA were prepared in duplicate and used for the PCR assays. Each plate was run with a negative and positive control, molecular water and known chytrid DNA at a concentration of roughly 10 ng/μL, respectively. Melting curve analysis after amplification was used to confirm that positive test samples were of a similar template as the positive control. To quantify the amount of chytrid in positive samples relative to a control of known chytrid DNA concentration, each positive sample was run again, in triplicate, alongside a diluted standard series. Chytrid DNA concentration of each positive was then estimated relative to these standards.

Data Analysis

Bar graphs (Microsoft Excel 2007) were created to compare chytrid prevalence between species, habitats, and month of collection. Data was displayed as percentages to account for variation in sample sizes. All statistical tests were run using JMP (SAS Institute Inc., 2010, ver. 9.0.0). Generalized linear models using a binomial logit distribution were used to run logistical regressions, which allowed for simultaneous comparisons of multiple variables and allowed me to determine which variables were mainly responsible for the prevalence of chytrid.

Only a subset of the data in which species were found in both habitat types was used for habitat analyses.

For the chytrid quantity data, only samples that tested positive were analyzed. Since the data were not normal according to a Kolmogorov-Smirnov test, all values were log-transformed for all analyses. To account for the possibility that larger individuals were able to harbor more chytrid, I first ran a regression analysis. Then generalized linear models similar to those performed in the chytrid presence/absence data were run, except using analysis of variance (ANOVA) to account for the continuous data rather than the binomial distribution used for the categorical data used in the presence/absence analyses.

Lastly, a scatter plot was created (Microsoft Excel 2007) to illustrate possible differences in health between infected and non-infected individuals using mass: length ratios as a proxy for health. Length values were also not normally distributed according to a Kolmogorov-Smirnov test and were thus also log transformed. This difference was then statistically tested by using a t-test to compare mass: length ratios to presence/absence of infection by species.

Results

A total of 273 individuals were tested for the presence of chytrid, of which 51 samples tested positive (18.7% prevalence). These individuals came from 14 different species, the most plentiful being black-bellied salamanders (*Desmognathus quadramaculatus*), spotted dusky salamanders (*Desmognathus conanti*), two-lined salamanders (*Eurycea cirrigera*), and seal salamanders (*Desmognathus monticola*) (Table 1). The most prevalent anuran species caught was the Northern cricket frog (*Acris crepitans*), of which almost all were exclusively found at the Arabia Mountain Heritage Area. In fact, *A. crepitans* was the only species caught at Arabia. Of the species that were caught, a subset of four species were caught in both habitat types: bullfrogs (*L. catesbeiana*), seal salamanders (*D. monticola*), red salamanders (*Pseudotriton ruber*), and spring salamanders (*Gyrinophilus porphyriticus*).

The species with the highest incidence of infection was the North American bullfrog (*L. catesbeiana*) with 80% of caught individuals testing positive. The second most infected species was the southern two-lined salamander (*E. cirrigera*) (57.4% positive). However, the individual that was host to the greatest amount of chytrid was a seepage salamander (*D. aeneus*) (99.1 ng/µL) while the second most severely infected individual was a North American bullfrog (*L. catesbeiana*) (12.8 ng/µL). Most individuals had less than 1 ng/µL of chytrid. As for locations, the most infected site was Fernbank Forest (38.9%) and the second most infected site was Lullwater Preserve (35.1%), both of which are in the Piedmont habitat. Conversely, the only site that had no incidence of chytrid was Arabia, also in the Piedmont habitat.

Table 1: Overview of the numbers of each species caught in each of two different habitats in northern Georgia over the period from September to December 2010. The second number in parentheses is the number of individuals that tested positive for chytrid for the habitat columns. The second number in parentheses for the quantity column is standard deviation.

Species	Blue Ridge	Piedmont	% Incidence	Quantity (ng/µL)
Acris crepitans	0	23 (0)	0	0
Desmognathus aeneus	6 (2)	0	20	16.7 (40.4)
Desmognathus conanti	0	50 (4)	8	$2.1x10^{-3}$ (0.01)
Desmognathus monticola	31 (4)	7 (0)	10.5*	$6.1x10^{-3}$ (0.03)*
Desmognathus ocoee	30 (5)	0	16.7	$1.4x10^{-2}$ (0.05)
Desmognathus quadramaculatus	57 (5)	0	8.8	$1.8x10^{-3}$ (0.01)
Eurycea cirrigera	0	47 (27)	57.5	$8.3x10^{-1}$ (1.9)
Eurycea wilderae	4 (0)	0	0	0
Gyrinophilus porphyriticus	3 (0)	1 (0)	0	0
Lithobates catesbeiana	1 (0)	4 (4)	80*	2.7 (5.7)*
Lithobates clamitans	0	1 (0)	0	0
Plethodon serratus	0	1 (0)	0	0
Plethodon glutinosus complex	2 (0)	0	0	0
Pseudacris crucifer	0	1 (0)	0	0
Pseudotitron ruber	1 (0)	3 (0)	0	0
Total	**135 (16)**	**138 (35)**	**18.68**	**0.5334 (5.925)**

* Denotes that all positives were found in only one habitat type.

There appears to be an effect of habitat on chytrid infections when considering all species together, with more infected individuals found in the Piedmont area (Figure 3A). However, a subset of the data was needed, which included only those species caught in both habitat types, to statistically separate habitat and species effect. The analysis of this data set revealed no statistical effect of habitat or species on the prevalence of chytrid; therefore, habitat was excluded for all subsequent analyses. However, for the entire data set, species was a significant factor associated with chytrid prevalence ($\chi^2 = 71.8166$, df = 14, $p < 0.0001$) (Figure 3B).

Additionally, an apparent seasonal trend in chytrid prevalence is apparent, with more infections occurring in September even though most species were caught in all months (Figure 3C), but this trend was only significant for some species. Specifically, upon further analysis of the time data, two species were found to be solely responsible for the large prevalence of chytrid

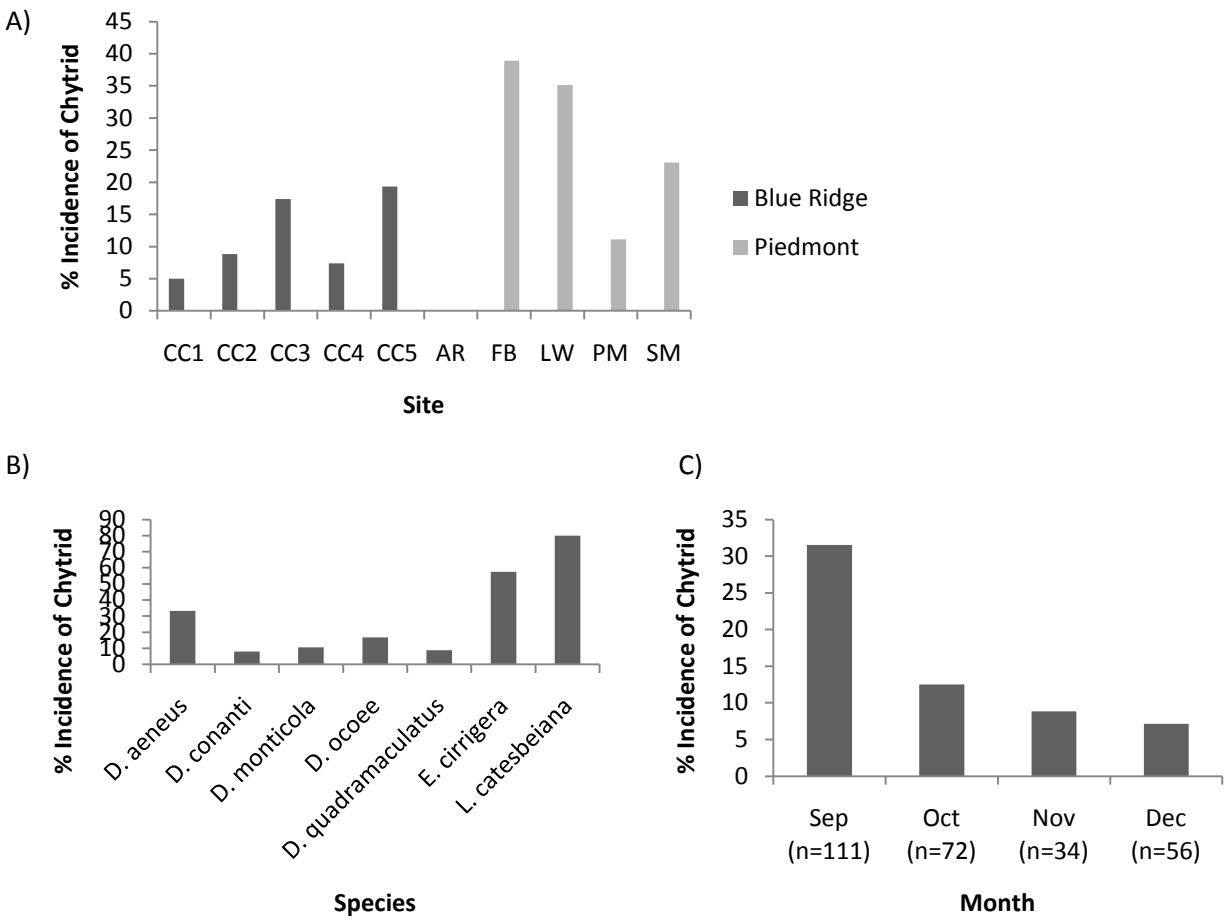

Figure 3: (A) Prevalence of chytrid infections found across ten different sites from two different habitats in Northern Georgia. CC1-5 are five different sites around Cooper's Creek and are part of the Blue Ridge habitat of the Southern Appalachians. AR (Arabia Mountain Heritage), FB (Fernbank Forest), LW (Lullwater Preserve), PM (Panola Mountain), and SM (Stone Mountain) are all a part of the Piedmont habitat. (B) Seven of the fourteen species surveyed were found to be positive for chytrid infections. These include the black-bellied salamander (*Desmognathus qudramaculatus*), the North American bullfrog (*Lithobates catesbeiana*), the Ocoee salamander (*Desmognathus ocoee*), the southern two-lined salamander (*Eurycea cirrigera*), the seal salamander (*Desmognathus monticola*), the seepage salamander (*Desmognathus aeneus*), and the spotted dusky salamander (*Desmognathus conanti*). (C) Chytrid infections occurred all months of sampling (Spetmeber to December 2010) but were most prevalent during September. Standard deviations were used for the error bars in all figures.

in September rather than any other month, *D. ocoee* (χ^2 = 14.00, df = 1, n = 30, p = 0.0002) and *E. cirrigera* (χ^2 = 12.53, df = 1, n = 47, p = 0.0004). For both species there were more individuals that tested positive for chytrid in September than all other months combined. The September prevalence of *D. ocoee* is of particular interest since four of the nine individuals caught in September had chytrid, yet none of the 21 individuals caught in the following three months were chytrid positive.

Length had no influence on the amount of chytrid on an individual when analyzing the quantity of zoospores per sample on an individual (Linear regression, t = 0.03, df = 50, p = 0.97) (Figure 4). Neither habitat nor time of year influenced the severity of infection (habitat, One-way ANOVA, $F(1,49)$ = 2.63, p = 0.11, month, One-way ANOVA, $F(3, 47)$ = 1.08, p = 0.37) (Figure 5). Species on the other hand significantly affected the amount of chytrid on an individual (One-way ANOVA, $F(6, 44)$ = 3.17, p = 0.011) (Table 1). However, this significance is due to an outlier of a single *D. aeneus* (99.09 ng/µL) that had over seven times as much chytrid as the next highest individual which was *L. catesbeiana* (12.84 ng/µL). Upon removal of this individual, species was no longer significant (One-way ANOVA, $F(6, 43)$ = 2.10, p = 0.073).

Health of an individual using mass: length ratio as a proxy was also examined to look for differences between infected and uninfected individuals for each species. Only in *E. cirrigera* was there a marginally statistically significant difference in health between those positive for *B. dendrobatidis* and those that were negative (One-way ANOVA, $F(1, 40)$ = 4.15, p = 0.05). As such, it appears that there are no dramatic differences in the mass: length ratios between individuals experiencing infection and individuals who are not.

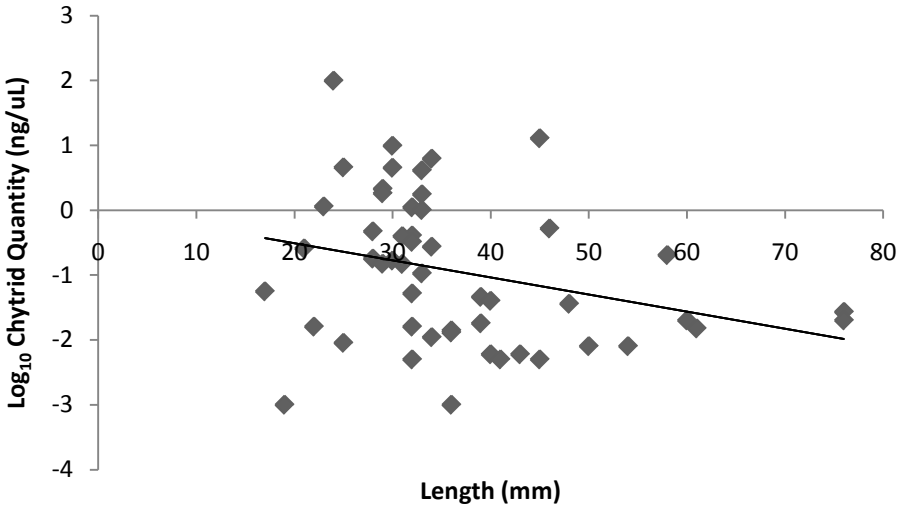

Figure 4: Scatter plot and regression of length of an individual against the $\log_{10}$ of the relative quantity of chytrid ($y = -0.03x + 0.02$, $R^2 = 0.09$, $p = 0.97$) from salamander and frog populations of Northern Georgia from two different habitats (Blue Ridge and Piedmont) collected from September 2010 to December 2010.

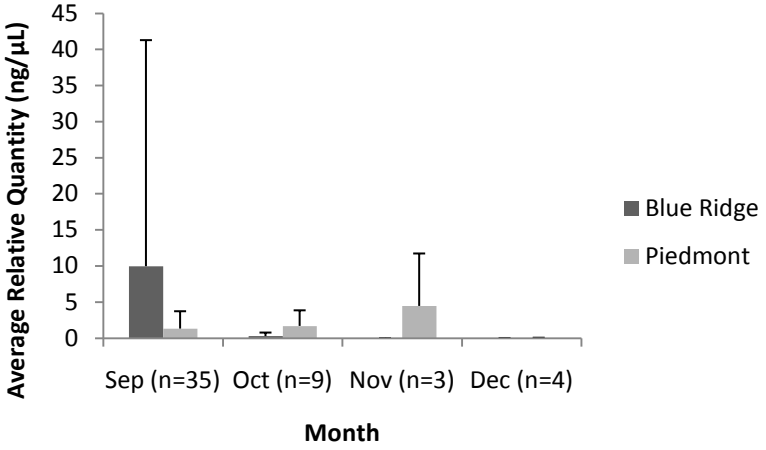

Figure 5: Average relative quantity of *B. dendrobatidis* on individuals captured in two different habitats in northern Georgia, the Blue Ridge mountains of the Southern Appalachians and the Piedmont plateau around Atlanta. Individuals were caught over several months, from September to December 2010. Only those samples known to be positive for chytrid were quantified. All error bars were calculated using standard deviations.

Discussion

The data presented here do not support our earlier hypothesis that there would be a higher prevalence of chytrid in the cooler mountains. Altogether, there were more incidences of infection in both the warmer lowlands as well as the warmer months. The contradiction is initially surprising due to the slow development of *B. dendrobatidis* in temperatures above 23ºC (Longcore et al. 1999) and lower prevalence of chytrid infections in lowland areas (Berger et al. 1998). However, upon review of our hypothesis, there are two factors not considered at the beginning of this study. The first is that the hypothesis was based upon the comparison of Georgian habitats to the tropical habitats of Central America and northern Australia. The difference in temperatures is such that the lowlands of Georgia are more similar to the montane habitats of the tropics than are the montane habitats of Georgia. The second factor is that surveying only occurred through the fall and winter months, well after the warmest months in Georgia. In fact, the average temperature for the Piedmont area during the month of September is 22˚C (National Weather), very close to the ideal temperature for the development of *B. dendrobatidis*. After September, as the months became colder, the number of infections decreased (Figure 3C) suggesting that there is a possible seasonal effect.

This is not the first study to find evidence of a seasonal affect on chytridiomycosis. In both Australia and Arizona, there have been reports of more amphibian mortalities in the colder winter months due to chytridiomycosis (Bradley et al. 2002, Berger et al. 2004). As the environment gets colder, *B. dendrobatidis* appears to be able to reproduce more effectively to reach adequate numbers to cause disease. However, once the temperature decreases past a certain point, chytrid development may slow so that its virulence is decreased and consequently so are mortalities. So while the coldest months in areas such as Australia and Arizona may be

the time of year where the prevalence of chytrid is at its highest, this may not always be the case in all areas. These Georgian habitats may be one such example where rate of amphibian mortalities during the coldest months are similar to the rate of mortalities during the warmest months. Thus the extreme climatic fluctuations in temperature in a habitat may benefit amphibians susceptible to chytridiomycosis.

Additionally, it is possible that the same trend of winter mortalities found elsewhere is also portrayed in Georgia. Our study did not look at numbers of deaths caused by chytrid, only the numbers of infected individuals. The lack of infected individuals during winter could be due to the deaths of those infected in the fall months. Stress from overwintering in combination with chytrid infections might increase the rate of mortalities. During winter, some aqueous environments experience a decrease in O_2 levels and some amphibians are known to survive this hypoxia through reliance on cutaneous gas exchange (Boutilier et al. 1997). However, the ability of *B. dendrobatidis* to impair cutaneous respiration would significantly decrease the host's ability to survive winter. Stress from overwintering could also come in the form of a compromised immune system, as seen in leopard frogs (*Lithobates pipiens*) (Maniero and Carey 1997). A weakened immune system in combination with a pathogenic fungus could easily cause mortalities as well.

Another reason for the higher prevalence of chytrid at some of the Piedmont sites could be due to an artificial inflation of amphibian density. Both Fernbank Forest and Lullwater Preserve are small fragmented sections of wilderness within the larger metropolitan area of Atlanta, Georgia. Thus, dispersal out of these areas is difficult at best for amphibians and thus individuals cannot spread out as they could in the larger areas of the Blue Ridge sites. This

increase in density would likely increase the amount of individual-to-individual transmission that would occur and thus increase the prevalence of fungal infection.

What is even more surprising than the difference in chytrid prevalence between the two habitats is the lack of the habitat's significance in chytrid infections as compared to the species composition of the areas. Habitat type appears to only matter in that it determines species composition, which appears to be the main determinant of chytrid infections. What species an individual was is also important in influencing the severity of infection, with some species having more chytrid on them than others. Together, this suggests that certain species are more prone to experiencing varying levels of infection by *B. dendrobatidis*. This supports the idea that only certain species are prone to population declines (Daszak et al. 1999). However, the factors that make a population likely to experience a decline as a result of *B. dendrobatidis* include low fecundity, which prevents a population from easily rebounding, as well as being a stream-breeding species since chytrid is more easily dispersed through moving water rather than still water or across terrestrial habitats. Nonetheless, the specific reason for why one species is more likely to experience the initial infection is still somewhat unclear. Most of the species surveyed here were stream-breeding species and all were collected along the banks of moving water and yet some species were more likely to be infected than other species living in the same spot. Since *B. dendrobatidis* was present at virtually every site, it is reasonable to assume that every species surveyed here is at risk of experiencing some level of chytrid infection (with the exception of *A. crepitans* since they were only found at the only site where there were no infections detected). It is possible that there is a life history strategy difference or some other behavioral cause for differences in infections between species. Many amphibians are known to perform behaviors such as grouping to overwinter or aggregate breeding, both of which would

increase the likelihood of transmitting chytrid (Ultsch et al. 2000, Myers and Zamudio 2004). This implies that continued monitoring of these surveyed species is needed in order to establish which species are most at risk for a population decline.

Differences in susceptibility to infection may also result from differences in immunity between species (Woodhams et al. 2007). There is growing evidence that as part of an amphibian's innate immunity, there are defensive peptides in the amphibian's skin which have antimicrobial properties and inhibit the growth of *B. dendrobatidis*. Those species that seem to be the least susceptible to chytrid infections have the strongest antifungal defense. In combination with this initial line of defense, there is also evidence that some species are able to develop acquired immunity against *B. dendrobatidis* (Richmond et al. 2009). This means that if an individual survives an infection, they are more likely to survive a second infection. Our data could be indirect support of this idea of differences in immunity because all species caught from the genus *Desmognathus* were found to have some incidence of chytrid infections. Therefore these closely related species would most likely lack the proper gene regulation required for strong prevention of chytrid infection. Lastly, in some rare cases, amphibian species may have a symbotic bacteria, *Janthinobacterium lividum*, that lives in their skin and inhibits chytrid growth though the production of violacein (Harris et al. 2009). One such species is the redback salamander (*P. cinereus*) (Becker and Harris 2010). While no *P. cinereus* was caught in the study, the closely related *P. serratus* was. The *P. serratus* individual was in fact chytrid negative and is support of the species' decreased susceptibility to infection by chytrid.

With this study, I can neither confirm nor dismiss the possibility that the endemic amphibians are experiencing a population decline. No population analyses were done to look at the possibility of declining populations. These surveys are meant to be an initial determination

of what species and which areas are most susceptible to infections by chytrid. That being stated, it is difficult to determine which species are most prone to a population decline. Though it may seem that the species found to be positive for *B. dendrobatidis* are the most susceptible, it is also possible that the species found to have experienced chytrid infections are actually those that are asymptomatic. Consequently, the severity of infection found would actually be below the threshold that causes disease. This would mean that the species positive for chytrid are actually acting as carriers of chytrid and could therefore be spreading it to other populations.

It needs to be stated that the level and distribution of chytrid infections reported here are probably an underestimate of the true levels of chytrid. This is likely for two reasons. The first is that between testing each sample in duplicate and in combination with the melting curve analysis, the chance of false positives has been reduced as much as possible. However, there is still a chance for false negatives since some samples had such a small amount of chytrid that it became difficult to detect. In addition, at 4°C *B. dendrobatidis* will still survive but at a very low prevalence (Piotrowski et al. 2004). This is highly suggestive of chytrid's ability to overwinter on a host and thus in many of the sites sampled, individuals may have been positive for chytrid, but the number of zoospores was so low that chytrid was undetectable. This is an alternative explanation for the lack of infections found in the later months.

Altogether, there appears to be only a minimal impact of the habitat on prevalence of chytrid infections in Georgia. Instead, the time of year and an area's species composition play larger roles in influencing the rate of infection. At this point in time, there is no evidence to suggest that a population decline for any species is imminent since there was an overall low prevalence of chytrid (18.7%) from all samples collected and most species were still seen by the end of the survey. One exception to this may be *D. aeneus* since this species in particular had a

high prevalence of chytrid (Figure 3B), was not seen throughout the second half of the survey, and one individual had significantly more severe infection than any other sample. One point of concern is the increase of chytrid infections since the previous survey in 2008, which found a 1.88% prevalence (Timpe et al. 2008). The survey in 2008 was broader in scope but not as deep as my survey since they searched more sites over a larger geographic range, but not as thoroughly. So regardless of searching the same locations and sampling the same species, there was a much lower prevalence of chytrid infections. This significant increase suggests that chytrid is spreading throughout these areas and is rapidly becoming more and more prevalent. For this reason, I highly recommend population surveys to monitor infections in these areas and therefore help prevent continued extinctions.

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