1-1-2009

The Occurrence of Batrachochytrium Dendrobatidis in Salamander Populations of West Virginia

Casey John Bartkus
casebkus87@yahoo.com

The occurrence of *Batrachochytrium dendrobatidis* in salamander populations of West Virginia

Thesis submitted to
The Graduate College of
Marshall University

In partial fulfillment of the
Requirements for the Degree of
Master of Science
Biological Sciences

By

Casey John Bartkus

Dr. Thomas K. Pauley, Committee Chair
Dr. Charles Somerville, Committee Member
Dr. Jayme L. Waldron, Committee Member

Marshall University

May 2009

ABSTRACT

The occurrence of *Batrachochytrium dendrobatidis* in salamander populations of West Virginia

Batrachochytrium dendrobatidis (*Bd*), otherwise known as the amphibian chytrid fungus, is a pathogen that causes the amphibian disease, chytridiomycosis. While *Bd* has been found in amphibian populations in other states across the country, little is known of its occurrence in West Virginia. *Batrachochytrium dendrobatidis* is highly associated with amphibians in montane habitats. Because West Virginia has the highest mean elevation of any state east of the Mississippi River, amphibians in the state may be at high risk of an outbreak. The goal of this study was to determine the status of *Bd* in salamander species of concern in West Virginia. I took 266 samples from 8 different species at 22 different sites and sent samples to Washington State University where real-time Taqman Polymerase Chain Reaction (PCR) assays were used to detect the presence of *Bd* zoospores. Only one site tested positive for the incidence of *Bd;* however, further testing is needed to adequately sample for statewide occurrence. Sites testing positive for *Bd* occurrence were mapped to show the possible distribution of the disease, which is essential for development of protocols that will aid managers in reducing the spread of *Bd*.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First, I would like thank my parents, Christopher and Lynn Bartkus, for their never-ending love and support. Without the constant encouragement from the both of them, as well as other family members, I would have never made it through some of the tough times I have encountered along the way. Next, I would like to thank Dr. Pauley, without his expertise and patience, I would have been lost numerous times throughout my graduate career. Dr. Pauley is truly one of the most genuine individuals I have ever met. I would like to thank my committee members, Dr. Somerville and Jayme Waldron, for their suggestions and assistance. Both were extremely helpful in the production of this manuscript. I would like to thank Joe Greathouse and The Good Zoo at Oglebay Park in Wheeling, West Virginia for letting me jump into this project and develop a thesis. It was a pleasure working with Joe and the zoo and their assistance is greatly appreciated. I must not forget the fellow graduate students that assisted in field work: Reid Downer, Casey Bradshaw, Scott Jones, Scott Albaugh, Kevin Saunders, Amanda Spriggs, and Sarah Miloski. I would like to thank Dr. Tom Jones and his lab for assistance with the caving aspect of my project as well as assistance on past projects. I would like to thank Amy Cimarolli and The Nature Conservancy for assistance in accessing General Davis Cave. Also, I would like to thank Andy Longenecker for assistance in locating other caves. I would also like to thank Washington State University for providing fast service in analyzing my samples. Funding was provided by the West Virginia Division of Natural Resources and the Marshall University Graduate College. This project was completed under Marshall University Animal Use Protocol Numbers 404, 404-1, 404-2, 404-3, 404-4, 404-5, and 404-6. All animals I collected were done so under West Virginia Collecting Permit #2008.056.

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Amphibians are declining at an alarming rate. Recent data suggests that approximately one-third of the 6,300 amphibian species are threatened with extinction (Wake and Vredenburg, 2008). Amphibians have survived past extinctions with limited loss of species; however, recent data show that amphibians are declining more rapidly than any other taxon of vertebrates (Stuart et al. 2004, Wake and Vredenburg, 2008). Researchers have had difficulty in pinpointing a common cause or causes for these declines. Alford and Richards (1999) noted the most widely accepted causes of decline included habitat modification and destruction, introduced predators, climate change, introduced toxicants, and emerging infectious diseases. Habitat modification and destruction are the best documented cases of widespread amphibian declines (Alford and Richards, 1999, Dimauro and Hunter, 2002). Clearcutting of forests can possibly expose amphibians of a particular, interior habitat type to new climatic factors such as higher temperature regimes and lower humidity and litter moisture levels (Dimauro and Hunter, 2002). Petranka et al. (1993) estimated an annual loss of approximately 14 million salamanders due to clearcutting in the Appalachian Mountains in western North Carolina. Habitat fragmentation becomes a problem when populations are "divided" by the destruction of a particular section of habitat. This division stops the movement of individuals and creates two, separate populations that are not able to interbreed, therefore, interrupting the genetic flow between populations. Wetland destruction is highly detrimental to amphibian populations because it eliminates breeding sites for some species while others experience complete habitat loss. In New York, loss of historical wetlands has been estimated at 60% (Gibbs et al. 2005). Across the United States there have been alarming rates of wetland destruction. It is estimated that over the past 200

years, the United States has lost nearly 53% of its original wetlands, with some states, such as California, reporting losses of 91% (Dahl, 1990).

Introduced predators and other exotics severely impact amphibian populations. For example, introduced predatory fishes can cause dramatic declines in larval amphibian abundance. Predatory fishes are considered the most detrimental predators to amphibians (Petranka et al. 1987). While larval amphibians may have evolved defenses against native predatory species, introduced fishes pose a new threat to which larval amphibians may not have adapted responses (Fisher and Shaffer, 1996, Gamradt and Kats, 1996, Petranka et al. 1987, Vredenburg, 2004). Vredenburg (2004) found that the widely introduced fish species, *Salvelinus fontinalis* (Brook Trout) and *Oncorhynchus mykiss* (Rainbow Trout) have very detrimental effects on amphibian populations in previously fishless habitats. The removal of such fish resulted in rapid reintroduction of native amphibian populations.

Much debate is occurring on whether or not climate change is a factor in amphibian declines, or furthermore, a global dilemma at all. However, many amphibian studies have linked climate change as either a direct or indirect cause of declines (Alexander and Eischeid, 2001, Pounds and Crump, 1994, Stuart et al. 2004, Wake and Vredenburg, 2008). Pounds and Crump (1994) found that the disappearance of *Bufo periglenes* (Golden Toads) and *Atelopus varius* (Harlequin Frogs) in Monteverde Cloud Forest, Costa Rica is tied to climate change. They developed two hypotheses that combine climate change with another limiting factor in the disappearance of these species. One hypothesis combines climate change and its impact to drive species into close quarters, therefore subjecting individuals to an infectious disease agent. The other hypothesis combines the dry weather event with higher concentrations of chemical agents. Through multiple years of surveying, they noted populations that historically showed high levels

of recruitment in consecutive years in several different areas had drastically declined to no individuals being observed at all. For instance, surveys that they conducted in April and May of 1987 produced abundant numbers of both species, however, after a warm, dry El Niño-caused climate shift, both species seemed to disappear from these areas. They documented climate fluctuations over time and noticed that the 1987 climatic event was the worst on record, therefore, more than likely attributing to the decline of *B. periglenes* and *A. varius*.

Amphibians can be exceptionally susceptible to toxins that have been introduced into water systems. Because amphibians undergo cutaneous respiration, chemical agents that are in the water or soils around the individual can easily pass through the skin and enter the blood stream. Also, most amphibians have biphasal life cycles, meaning that they possess a larval and adult stage. Most amphibian egg and larval stages are spent in water before becoming terrestrial adults, leaving these stages susceptible to the introduction of chemical agents. However, the effects on amphibians across a species scale or temporal scale has not been widely addressed (Bridges and Semlitsch, 2005, Hoppe, 2005).

Emerging infectious diseases are also known to be an important cause of amphibian declines. Infectious diseases began to alarm conservationists when they noticed massive die-offs and extinctions in areas that were heavily protected from disturbances. One such disease, chytridiomycosis, has had severe impacts on populations of amphibians throughout the world. Chytridiomycosis, an infection caused by the aquatic chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*), has been directly linked to amphibian mortality in Australia, North America, South America, and Europe (Berger et al. 1998, Bosch et al. 2000, Bradley et al. 2002, Lampo et al. 2006). Chytridiomycosis is considered to be the worst infectious disease ever recorded among vertebrates in terms of the number of species affected and the ability of the disease to

drive species to extinction (Bonaccorso et al. 2003, Briggs et al. 2005, La Marca et al. 2005, Stuart et al. 2004). Morehouse et al. (2003) found that little to no genetic diversity exists between strains extracted from outbreaks in different areas of the world. These data suggest that *Bd* may have been introduced into other parts of the world from a common source (Berger et al. 2005), or that the fungus may have been distributed worldwide, but only recently became pathenogenic. The source of origin is believed to be Africa, from the common trade of African Clawed Frogs (*Xenopus laevis*) (Weldon et al. 2004).

Members of the phylum Chytrdiomycota typically live in the soil or water and primarily act as decomposers. However, *Bd* is the only member of this phylum known to pathogenically infect vertebrates (Berger et al. 1998, Berger et al. 2005). *Batrachochytrium dendrobatidis* has three life stages that all occur within a single host organism. The first is an infectious zoospore stage, when the zoospores have a single flagellum and are mobile in the water or soil or on a carrier organism. Once on a host, zoospores develop into a growing organism, called the thallus, which grows subcutaneously. The thallus develops a sporangium that extends above the epidermis and discharges zoospores, restarting the cycle. Temperature seems to be an important factor in *Bd* development. Woodhams et al. (2008) found that *Bd* zoospores encyst and die at faster rates at higher temperatures. Zoosporangia release more zoospores when temperatures are within the range of 7°C and 10°C, and therefore remain infectious for a longer period of time. However, growth of the thallus and zoosporangia are fastest at temperatures between 17°C-25°C. Therefore, infection is greatest in cold weather events, or in high elevations, but warm temperatures are needed for further development into the zoospore releasing zoosporangium stage. Temperatures above 30°C are fatal to *Bd* at all stages.

Infection occurs when the zoospores attack the keratinized sections of the stratum granulosum and stratum corneum of the epidermis of susceptible amphibians, see Figure 1 (Berger et al. 1998, Berger et al. 2005). In anuran tadpoles, sporangia were only found around the mouth parts (Berger et al. 1998). It is not directly known how mortality results from infection, but it is hypothesized that chytridiomycosis may cause hyperplasia that impairs cutaneous respiration or that the fungus releases proteolytic enzymes that may be toxic (Bakal et al. 2007, Berger et al. 1998).

The effect of chytridiomycosis at the species level is not completely understood. *Batrachochytrium dendrobatidis* can be highly associated with species in montane, stream habitats and montane species with aquatic eggs and larvae are at the highest risk of extinction from the disease (Lampo et al. 2006). However, Cummer et al. (2005) detected the presence of *Bd* on a terrestrial species of salamander *Plethodon neomexicanus* (Jemez Mountains Salamander). Some species seem to be highly affected by the disease, while others may just be carriers. There are several causes for variation in resistance or susceptibility including the variation in the pathogenicity of *Bd*, the peptide secretions of amphibians, and differences in the microflora of the skin of amphibians, as well as differences in amphibian habits and life histories (Harris et al. 2006, Rollins-Smith et al. 2005). Harris et al. (2006) found that some of the bacteria inhabiting the skin of *Plethodon cinereus* (Eastern Red-backed Salamanders) and *Hemidactylium scutatum* (Four-toed Salamanders) inhibit the growth of *Bd*. It is hypothesized that these and other amphibian species that brood their eggs have cutaneous bacteria colonies that effectively inhibit the growth of many fungi, including *Bd*. In the United States, most die-offs due to chytridiomycosis infections are restricted to the west coast. Massive die-offs occurred in the Sierra Nevada Mountains of the northwest, most notably in populations of

Mountain Yellow-legged Frogs (*Rana muscosa*), and were directly attributed to chytridiomycosis (Briggs et al. 2005, Fellers et al. 2001). Because chytridiomycosis only affects the keratinized mouth parts of anuran larvae, it is not fatal at the larval stage (Fellers et al. 2001, Vredenburg and Summers, 2001). However, when infected anuran larvae metamorphose into adults and the skin begins to keratinize, mortality may occur, depending on the susceptibility of the species. Also, there are records of transmission between larval and adult *R. mucosa*, indicating the possibility of transmission between larvae and adults of all susceptible species (Rachowicz and Vredenburg, 2004). Some species may act as carriers for the disease, and are not directly affected (Davidson et al. 2003, Dazak et al. 2004, Cummer et al. 2005, Johnson et al. 2005). In the United States and Canada, infections date back to 1960 in museum specimens that were tested (Ouellet et al. 2005). Ouellet et al. (2005) found infections in wild-caught specimens from 7 states (California, Wyoming, Minnesota, Wisconsin, Missouri, Indiana, and Virginia), see Figure 1.

Figure 1. Distribution of *Batrachochytrium dendrobatidis* infection in Canada and the United States. Taken from Ouellet et al. (2005).

Most research involving chytridiomycosis in field specimens has focused on areas of die-offs and has typically involved anuran species. Very few studies have focused on the distribution of the disease over an area or the variability of the disease in caudates (Davidson et al. 2003). Davidson et al. (2003) detected *Bd* in populations of *Ambystoma tigrinum stebbinsi* (Sonora Tiger Salamanders). They found that *Bd* isolated from field-collected *A. t. stebbinsi* were able to be transferred to laboratory-kept anuran species, thus confirming the possible transfer of the disease between the two orders. Also, Brodman and Briggler (2008) documented the first case of *B. dendrobatidis* on larval salamanders in *Ambystoma jeffersonianum* (Jefferson Salamander). *Batrachochytrium dendrobatidis* infection has also been detected in wild-caught *Plethodon neomexicanus* (Jemez Mountains Salamander), documenting the first case of infection in a terrestrial caudate, therefore expanding the potential vulnerability to the terrestrial environment (Cummer et al. 2005). Infections from field-collected specimens have been widespread across the eastern United States, but there have not been any records of die-offs associated with infections from *B. dendrobatidis* in this area (Brodman and Briggler, 2008, Byrne et al. 2008, Grant et al. 2008, Longcore et al. 2007, Rothermel et al. 2008, Timpe et al. 2008).

West Virginia has 34 species of salamanders from a variety of habitats. Of these 34 species, 2 are endemic. These species, *Plethodon nettingi* (Cheat Mountain Salamander) and *Gyrinophilus subterraneus* (West Virginia Spring Salamander), have limited ranges only in West Virginia (Green and Pauley, 1987). *Plethodon nettingi* is a federally threatened species, and it is protected by the Endangered Species Act. *Gyrinophilus subterraneus* is found only in General Davis Cave in Greenbrier County. This cave is owned and managed by The Nature Conservancy, and the cave is completely closed to the general public and only selected

individuals may enter. However, the headwaters of the stream that passes through General Davis Cave are on private land, causing concern for managers. Some of West Virginia's other salamander species have also experienced declines throughout their ranges. These species, as well as the endemic species, are at a high risk of rapid decline if an extraneous factor such as a disease were to be introduced into populations. West Virginia is among several states that have not conducted distributional research of *Bd* in amphibian populations. To adequately understand whether chytridiomycosis is affecting populations of West Virginia amphibian species, it is important to recognize which populations or watersheds may contain *Bd*. These populations may then be monitored to establish whether or not chytridiomycosis is a factor in possible declines. Thus, my goal is to determine the occurrence of *Bd* in salamander populations of greatest conservation need in West Virginia, and to map locations of such occurrences.

METHODS

Species Selection

I chose federally endangered or threatened species first because they represent the highest risk of rapid decline that may be attributed to chytridiomycosis. Only one federally threatened species occurs in West Virginia, *Plethodon nettingi* (Cheat Mountain Salamander). *Plethodon punctatus* (Cow Knob Salamander) also has protection throughout most of its range. The populations in George Washington National Forest are protected in a cooperative agreement with the U.S. Forest Service and the U.S. Fish and Wildlife Service. I chose other species based on their inclusion on the West Virginia Wildlife Diversity Program's Species in Greatest Need of Conservation list. *Batrachochytrium dendrobatidis* has been detected on populations of *Cryptobranchus alleganiensis alleganiensis* (Eastern Hellbenders) in other states such as Pennsylvania, Missouri, Arkansas, and North Carolina. Also, surveys in 2007 detected *Bd* on *C. a. alleganiensis* in the northern panhandle of West Virginia (J. Greathouse, pers. comm.). I also surveyed *Desmognathus quadramaculatus* (Black-bellied Salamander), *Desmognathus welteri* (Black Mountain Salamander), *Eurycea lucifuga* (Cave Salamander), and *Gyrinophilus subterraneus* (West Virginia Spring Salamanders). I surveyed *Gyrinophilus porphyriticus porphyriticus* (Northern Spring Salamanders) at General Davis Cave to supplement sample size because of the uniqueness of the site.

Table 1. List of species sampled for the presence of *Bd* in 2008, their state rank, global rank, and general habitat type. Table also indicates documented cases of *Bd* prior to 2008 surveys.

Species	**State Rank**[*A]	**Global Rank**[*B]	**Habitat**	***Bd* Documentation**
Plethodon nettingi	S2 G2		Terrestrial	Not documented
Plethodon punctatus	S1 G3		Terrestrial	Not documented
Cryptobranchus a. alleganiensis	S2	G3	Stream	WV, PA, MO, AR, NC
Desmognathus quadramaculatus	S3 G5		Stream	Not documented
Desmognathus welteri	S2 G4		Stream	Not documented
Eurycea lucifuga	S3 G5		Caves	Not documented
Gyrinophilus subterraneus	S1 G1		Caves	Not documented
Gyrinophilus p. porphyriticus	S5 G5		Caves/Stream	Not documented

A) S1= Extremely rare and critically imperiled; S2=Very rare and imperiled; S3=Twenty-one to 100 documented occurrences; S5=Very common and demonstrably secure.

B) G1=Extremely rare and critically imperiled; G2=Very rare and imperiled; G3=Either very rare and local throughout its range or found locally in a restricted range; G4=Common and apparently secure globally, though it may be rare in parts of its range, especially at the periphery; G5=Very common and demonstrably secure, though it may be rare in parts of its range, especially at the periphery.

Site Selection

I chose sites based on a prioritization method. I gave priority to sites for which recent data suggested that it was possible to capture numerous individuals. I then chose sites for which historical data suggested that the capture of numerous individuals was possible. Also, I tried to address a broad area each species' total range in West Virginia. I chose at least three sites per species, with the exception of *G. subterraneus*, which only occurs in one location. I located sites using topographic maps and GPS coordinates, or by personal communication. I recorded all sites using a Garmin eTrex Vista GPS system in UTM NAD83 zone 17 coordinates.

Site Surveys

I surveyed sites using a variety of methods, depending on the species. I conducted diurnal surveys for *Plethodon nettingi* by looking under cover objects until I was certain that the site had been exhausted. I surveyed for *Plethodon punctatus* using two methods: 1) Diurnal surveys consisting of searching in talus and under cover objects for individuals, and 2) Nocturnal surveys consisting of using a head lamp to examine the talus for individuals that were actively foraging on the surface. I conducted night surveys between dusk and 4:00 a.m. in either 100% humidity or during rain events. I surveyed for *Eurycea lucifuga* by visually examining cave walls and crevices throughout the entrance and twilight area of the cave until I believed the area had been exhausted. I surveyed for *D. quadramaculatus* and *D. welteri* by turning over cover objects in or near the stream edge, and used aquarium nets to assist in capture. Joe Greathouse and staff from The Good Zoo at Oglebay in Wheeling, West Virginia conducted all surveys for

C. a. alleganiensis. They turned over rocks, probed under rocks with a camera, and used scuba techniques to capture individuals in streams. Individuals were marked using a radio-frequency identification (RFID) tag so that streams could be sampled more than once without repeatedly sampling the same individual.

Sampling Protocol

I used the swabbing protocol from Brem et al. (2007). This technique involved using a sterile cotton swab to collect epidermal samples from individuals. This method is non-lethal and has been proven to be very sensitive for detection of chytrid zoospores, requiring only one zoospore to produce a positive result (Boyle et al. 2004). I captured individuals by hand and quickly placed them into a Zip-Loc® bag. Once in the bag, I used a cotton swab to stroke the entire ventral surface of the individual at least ten times. Also, the inside of the bag was swabbed to pick up any residue that was left behind. Next, I placed the swab into a 1.5 mL plastic snap-top vial (Fisher Scientific) that was pre-filled with 1 mL of 70% ethanol. The cotton tip was broken off into the vial and the vial was labeled according to species. I measured each individual's snout-to-vent length (SVL) and total length (TL) in millimeters using dial calipers that are accurate to 0.1 mm. Because temperatures above 30°C are fatal to *Bd*, I measured water temperatures to the nearest 0.5°C with an armored thermometer except when not applicable for the terrestrial species. In the case of terrestrial species, I measured soil temperatures with the armored thermometer. I also measured air temperatures to the nearest 0.5°C at each site with a digital thermometer.

Figure 2. Swabbing technique used to sample individuals for *Bd*.

Photo by: Sarah Miloski

Batrachochytrium dendrobatidis Hygiene Protocol

I followed hygiene protocols outlined by Brem et al. (2007) and NSW NPWS (2001) to impede the possible transfer of *Bd* between individuals and sites. Also, proper hygiene was essential in preventing a false positive, in which a sample may have been contaminated. To prevent the possible transfer of *Bd* between individuals and to prevent the creation of a false positive when sampling, I changed latex exam gloves (MaglaTouch) between the handling of individuals. Also, I placed individuals in Zip-Loc® plastic bags for processing. New bags were used for each individual. A new, sterile, wood shafted, cotton-tipped applicator (Dukal Corp.) was used for each individual and care was taken to not allow the applicator to come into contact with anything other than the individual being processed. Individuals were processed for *Bd* sampling before any other measurements were taken or any data were recorded.

When travelling between sites, I used caution to avoid the possible accidental spread of *Bd*. Any equipment that came into contact with water or an individual from a previous site was cleansed using nine parts water, one part bleach solution. This solution was applied to all field equipment including calipers, thermometers, and aquarium nets. Also, I cleaned boots of any organic matter and then used the aforementioned solution.

Sample Analyses

I sent all swab samples to a lab at Washington State University (WSU). I sent samples to the lab grouped together by species and also sent forms to correlate sample labels to field notes. The lab used the notes to group samples together by site. Once grouped by site, the samples were further grouped into sets of samples (when applicable) to be run as one analysis. This

method was chosen on the basis that it would greatly reduce costs, however, it does make detection at the individual level impossible.

The lab at WSU used the protocol outlined in Boyle et al. (2004) which uses real-time Taqman Polymerase Chain Reaction (PCR) assays to detect chytrid zoospores extracted from the samples. The assays were sensitive enough to detect as little as one zoospore of *Bd.* The lab extracted the possible DNA from the samples by homogenizing the swabs and extracting a supernatant from the homogenization after centrifuging. PCR uses a pair of primers that are complementary to a sequence of the target DNA. Real-time PCR was used to quantify the amount of *Bd* zoospores in each sample or group of samples. Real-time PCR detected the accumulation of double-stranded DNA by monitoring the change in fluorescence of an intercalating dye resulting from binding to the DNA (Higuchi et al. 1993). Therefore, the fewer cycles it took to produce a detectable change in fluorescence, the more zoospores that were present in the sample or group of samples. This method allowed the lab at WSU to quantify the level of infection, providing a mean zoospore count per sample within a group of samples.

SITE DESCRIPTIONS

Site descriptions and maps for federally protected species (i.e. *Plethodon nettingi* and *P. punctatus*) are vague due to protective regulations. Site descriptions also do not include locations for *Cryptobranchus a. alleganiensis* to protect the sensitivity of the locations.

Cabin Mountain, Monongahela National Forest, Tucker County

I chose Cabin Mountain based on historical and current records indicating sufficient populations of *P. nettingi* (T. Pauley, pers. comm.). The site consisted mainly of canopy cover from *Picea rubens* (Red Spruce) and *Betula alleghaniensis* (Yellow Birch). Ground cover was woody debris with some talus and rocks scattered throughout. Also, cover boards from a previous study were used as salamander refugia.

Figure 3. Typical *Plethodon nettingi* habitat.

Photo by: Casey Bartkus

Figure 4. An Adult *Plethodon nettingi*.

Photo by: Reid Downer

Dolly Sods Wilderness Area, Monongahela National Forest, Tucker County

I selected Dolly Sods based on historical and recent data suggesting sufficient populations of *P. nettingi* (T. Pauley, pers. comm.). The particular area of Dolly Sods that I surveyed consisted of mainly *Betula alleghaniensis* as canopy cover. Ground cover was downed timber with some scattered rocks.

Bear Heaven Campground, Monongahela National Forest, Randolph County

I chose Bear Heaven Campground based on historical and recent data suggesting that sufficient populations of *P. nettingi* were possible (T. Pauley, pers. comm.). Bear Heaven habitat consisted mainly of *Betula alleghaniensis* and *Picea rubens* as canopy cover with dense patches of *Rhododendron maximum* (Great Rhododendron). Ground cover was rocks with some downed timber.

Gaudineer Scenic Area, Monongahela National Forest, Pocahontas County

I selected Gaudineer Scenic Area on the basis of historical and recent data indicating that sufficient populations of *P. nettingi* were possible (T. Pauley, pers. comm.). Canopy cover was mainly *Picea rubens*. Most ground cover was downed timber with some scattered rock piles. The forest floor is mainly open with *Bazzania* spp. covering the majority of the ground and cover objects.

Reddish Knob, George Washington National Forest, Pendleton County

I chose sites at Reddish Knob that possessed suitable habitat for *P. punctatus* as outlined in Petranka (1998) and Green and Pauley (1987). All sites were steep slopes and consisted mainly of hardwoods as canopy cover. Ground cover was talus with some downed timber.

Shenandoah Mountain, George Washington National Forest, Pendleton County

I selected sites on Shenandoah Mountain based on suitable habitat descriptions for *P. punctatus* from Petranka (1998) and Green and Pauley (1987). Sites were talus slopes with mixed hardwoods and evergreens. Ground cover was talus with some downed timber.

Figure 5. An adult *Plethodon punctatus*.

Photo by: Casey Bartkus

Figure 6. An adult *Plethodon Virginia* (Shenandoah Mountain Salamander).

Photo by: Casey Bartkus

Bear Creek, Camp Creek State Forest, Mercer County

I chose Bear Creek based on recent data suggesting that sufficient populations of *D. welteri* were possible for sampling (Bond, 2007, Felix, 2001). Bear Creek is located near a campground within the borders of Camp Creek State Forest, near the town of Princeton. Bear Creek is a high-gradient, second-order stream with abundant rock cover and fast-moving water. It flows into Camp Creek, which is the major watershed of the state forest. Canopy cover mainly consists of various hardwood species and *Rhododendron maximum* is abundant on the stream bank.

Figure 7. Typical *Desmognathus welteri* habitat.

Photo by: Casey Bartkus

Figure 8. An adult *Desmognathus welteri*.

Photo by: Casey Bartkus

Figure 9. An adult female *Desmognathus welteri* with eggs.

Photo by: Casey Bartkus

Turkeywallow Branch, Wyoming County

I chose Turkeywallow Branch because recent data suggest the presence of healthy *D. welteri* populations (Felix, 2001). Turkeywallow Branch is a second-order stream that runs parallel to Turkey Wallow Road near State Route 16 south of the town of Pineville. The stream runs through a deep ravine and main canopy cover consists of a mixture of evergreens and hardwoods. There are abundant rocks scattered throughout the streambed for cover. Also, a copious amount of trash is present throughout the streambed and on nearby hillsides.

Unnamed Tributary to New River near Quinnimont, Fayette County

I selected this stream based on recent data suggesting that sufficient populations of *D. quadramaculatus* were possible (T. Bond and T. Pauley pers. comm.). This first-order stream is within the boundaries of New River Gorge National River (National Park Service) and empties into the New River. It is located approximately 1.5 km east of Quinnimont on State Route 41. The stream is high gradient with large amounts of rocks and boulders. Near the mouth of the stream, close to the road, copious amounts of trash are present. Canopy cover consists mainly of hardwood species.

Unnamed Tributary to New River near Prince, Fayette County

I chose this stream because historical data suggested that sufficient populations of *D. quadramaculatus* were possible from this site (Pauley, 1993). This first-order stream is located approximately 6.5 km northeast of the town of Prince and flows under Thurmond McKendree Road (County Road 25). The stream is high-gradient with abundant boulders and rocks. Canopy cover consists mainly of hardwood species.

Figure 10. Typical *Desmognathus quadramaculatus* habitat.

Photo by: Amanda Spriggs

Figure 11. An adult *Desmognathus quadramaculatus*.

Photo by: Amanda Spriggs

Tank Hollow, Fayette County

I selected this first-order stream based on historical records suggesting sufficient populations of *D. quadramaculatus* were possible (Pauley, 1993). The stream is located approximately 5 km east of the town of Quinnimont on the south side of State Road 41. It empties into Laurel Creek, which eventually empties into the New River. There are abundant rocks and boulders throughout the stream. Canopy cover is a mixture of hardwoods and evergreens.

Unnamed Stream near Brooklin, Raleigh County

I chose this first-order stream based on historical records suggesting sufficient populations of *D. quadramaculatus* were possible (Pauley, 1993). This first-order stream is located approximately 1.5 km northwest of the town of Hinton on County Road 26-3 and eventually drains into New River. The stream is high-gradient with canopy cover consisting of a mixture of evergreens and hardwoods.

Falls Branch, Raleigh County

I selected this stream based on historical records suggesting sufficient populations of *D. quadramaculatus* were possible (Pauley, 1993). This second-order stream is located approximately 11 km northwest of Hinton and eventually drains into New River under New River Road (County Road 26). The stream has a lower gradient relative to most other streams that I surveyed for *D. quadramaculatus*. Large rock outcroppings line both sides of the stream in some areas. Canopy cover consists mainly of hardwood species.

Buckeye Creek Cave, Greenbrier County

I selected this site because recent records indicated that sufficient numbers of *E. lucifuga* could be found (Longenecker, 2000). The cave is located on Buckeye Road (County Road 17-4) approximately 6.5 km north of Frankford. The entrance is on private land and I received permission before entering the cave. A stream flows through the cave and remains shallow throughout the areas that I surveyed. I surveyed approximately 60 meters of the main passage of the cave, as well as some small side passages. The ceiling of the cave was high enough to walk upright from the entrance to the farthest surveyed point. The floor of the cave is covered by rock rubble and gravel in the streambed. Approximately 15 meters from the entrance of the cave, there is a small rimstone pool that has historically contained *E. lucifuga* larvae (Longenecker, 2000).

Figure 12. Entrance to Buckeye Creek Cave.

Photo by: Casey Bartkus

Figure 13. An adult *Eurycea lucifuga* from Buckeye Creek Cave.

Photo by: Casey Bartkus

Higginbothams Cave, Greenbrier County

I selected Higginbothams Cave because recent records indicated that sufficient populations of *E. lucifuga* were possible (Longenecker, 2000, Osbourn, 2005). Higginbothams Cave system has 4 entrances in one area. I surveyed two separate entrances, entrance #1 (see Figure 14) and entrance #2 (not pictured), which are both on private land. I was granted permission before accessing either entrance. Both cave entrances are on a private driveway that extends from Williamsburg Road (County Road 17) approximately 1.5 km west of Frankford. Entrance #1 was large enough to walk into and was a steep grade down to a subterraneous stream. The floor of the cave is mainly broken shale and rock rubble. I surveyed approximately 50 meters of the main passage of the cave as well as some small side passages. Entrance #2 is approximately 25 meters from entrance #1. It is a small opening, requiring crawling, but quickly expands into standing room. The cave is a steep downward slope with some small side passages. There was not a stream in this passage at the time of the survey, however, a series of rimstone pools occur approximately 40 meters from the entrance. The floor of the cave is mostly soft clay with some rock rubble near the entrance.

Figure 14. Entrance #1 to Higginbothams Cave.

Photo by: Casey Bartkus

Norman Cave, Greenbrier County

I chose Norman Cave because recent records suggested that sufficient populations of *E. lucifuga* were possible (Longenecker, 2000, Osbourn, 2005). Norman Cave is considered an open cave, meaning permission is not needed to access the cave, even though it is on private land. The cave is located near a sharp bend on Brownstone Road (County Road 7) approximately 6.5 km east of Renick. The entrance of the cave is on a slight incline from a stream bed and is dry with rock rubble, but quickly declines into a pool of water. The main passage is narrow with rock ledges along the side of the water. I surveyed approximately 45 meters of the cave. Fifteen meters into the cave, there is a side passage containing a few rimstone pools. Twenty meters into the cave, a stream is located that feeds the pool of water at the entrance. The floor of the cave is mainly covered in rock rubble with some of the side passages having a soft clay floor.

Figure 15. The entrance to Norman Cave.

Photo by: Reid Downer

Figure 16. The main passage in Norman Cave.

Photo by: Reid Downer

Figure 17. An adult *Eurycea longicauda longicauda* (Long-tailed Salamander) from Norman Cave.

Photo by: Reid Downer

US-219 Cave, Greenbrier County

I selected this cave because recent records suggested that sufficient populations of *E. lucifuga* were possible (Osbourn, 2005, T. Jones, pers. comm.). The cave is located directly next to U.S. Route 219 approximately 1.5 km south of Renick. The cave entrance is very small and a stream flows through the entire main passage. The ceiling is low for approximately 5 meters but then opens up into a larger room. The floor of the cave is mainly cobble with some areas of soft clay. I surveyed approximately 20 meters of the main passage, but farther, it appeared parts of the soft clay from the ceiling had recently collapsed.

General Davis Cave, Greenbrier County

I selected General Davis Cave because it is the only location known to contain *G. subterraneus* (Conant and Collins, 1998; Green & Pauley, 1987; Petranka, 1998). The cave is owned by The Nature Conservancy and is completely closed year-round because of its unique fauna. The entrance is in a wooded area. Upon entering, the main passage declines slightly with soft clay on the cave floor. There are approximately 20 meters of passage before the gate that closes off the rest of the cave. After this point, the passage declines at a much steeper grade into a stream. The stream has cobble as its substrate and is lined with slopes of soft clay on both sides for most of the passage that I surveyed. I surveyed approximately 200 meters of the main passage as well as some small side passages.

Figure 18. An adult *Gyrinophilus subterraneus* from General Davis Cave.

Photo by: Amy Cimarolli, The Nature Conservancy

Figure 19. Formation in General Davis Cave.

Photo by: Amy Cimarolli, The Nature Conservancy

Figure 20. A rimstone pool in General Davis Cave.

Photo by: Amy Cimarolli, The Nature Conservancy

RESULTS

Between 15 June 2008 and 8 October 2008, I sampled 266 individuals from 8 species at 22 different sites. Results are broken down according to site and are discussed in chronological order.

West Fork Greenbrier River, Pocahontas County

Joe Greathouse and staff from the Good Zoo at Oglebay captured two *Cryptobranchus a. alleganiensis* on 15 June 2008, two on 10 July 2008 and four on 26 August 2008. All samples were grouped into one set and run as one analysis at Washington State University. All samples produced negative results for the presence of *Bd*. Mean water temperatures were 22.2°C, 19.4°C, and 20.1°C on their respective days. Air temperatures were 24.3°C, 21.2°C, and 20.3°C on their respective days.

Cabin Mountain, Monongahela National Forest, Tucker County

I hand-captured 23 *Plethodon nettingi* by turning over cover objects on 18 June 2008. Samples were grouped into three sets (two sets of 10, one set of three) and run as three analyses. All samples produced negative results for the presence of *Bd*. Mean soil temperature was 15.0°C and air temperature was 22.1°C.

Dolly Sods Wilderness Area, Monongahela National Forest, Tucker County

On 19 June 2008, I hand-captured 1 *P. nettingi* by turning over cover objects. The sample was run as one analysis which produced a negative result for the presence of *Bd*. Soil temperature was 14.8°C and air temperature was 21.7°C.

Bear Heaven Campground, Monongahela National Forest, Randolph County

I hand-captured three *P. nettingi* by turning over cover objects on 19 June 2008. Mean soil temperature was 15.2°C and air temperature was 21.5°C. I additionally captured 15 individuals on 29 July 2008. Average soil temperature was 13.7°C and air temperature was 22.5°C. Samples were grouped into one set of 10 samples (containing three samples from 6/19/08 and seven from 7/29/08) and one set of eight samples (all from 8/29/08). Both analyses produced negative results for the presence of *Bd*.

Buffalo Creek, Brooke County

Joe Greathouse and staff from the Good Zoo at Oglebay captured two *C. a. alleganiensis* on 25 June 2008, one on 15 August 2008, eight on 16 August 2008, five on 23 August 2008 and one on 3 September 2008. Samples were grouped into one set of 10 samples (containing two samples from 6/15/08, one from 8/15/08, and seven from 8/16/08) and one set of five samples (containing one sample from 8/16/08 and four from 8/23/08). Both sets produced negative results for the presence of *Bd*. Mean water temperatures were 25.2°C, 21.6°C, 21.2°C, 20.8°C, and 21.4°C, respectively. Air temperatures were 25.7°C, 24.4°C, 24.4°C, 20.9°C, and 23.1°C, respectively.

Reddish Knob, George Washington National Forest, Pendleton County

I hand-captured two *Plethodon punctatus* on 25 June 2008 by turning over cover objects during the daylight hours. Average soil temperature was 14.7°C and air temperature was 21.4°C. Additionally, on 9 July 2008, I captured four individuals on a nearby talus slope. Mean soil

temperature on this day was 14.2°C and air temperature was 23.2°C. Samples were run as one set which produced a negative result for the presence of *Bd*.

Shenandoah Mountain, George Washington National Forest, Pendleton County

On 26 June 2008, I hand-captured two *P. punctatus* by turning over cover objects on Shenandoah Mountain. Soil and air temperatures were not recorded on this date due to equipment malfunction. I hand-captured four *P. punctatus* during a visual night survey on 9 July 2008. Air temperature was 18.9°C. I captured an additional six *P. punctatus* on 10 July 2008 by turning cover objects. Mean soil temperature was 16.6°C and air temperature was 19.8°C. Samples grouped into one set of 10 (containing one sample from 6/26/08, four from 7/9/08, and five from 7/10/08). The other two samples (one from 6/26/08 and one from 7/10/08) were run as their own analyses. All three analyses produced negative results for the presence of *Bd*.

Bear Creek, Camp Creek State Forest, Mercer County

On 8 July 2008, I used aquarium nets to capture eight *Desmognathus welteri* by turning over cover objects on the stream edge. Water levels were extremely high because of a rain event. Mean water temperature was 12.0°C and air temperature was 18.4°C. All samples were run as one set and produced negative results for the presence of *Bd*.

Unnamed Tributary to New River near Quinnimont, Fayette County

I used aquarium nets to capture 15 *Desmognathus quadramaculatus* on 8 July 2008 by turning over cover objects on the stream edge. Mean water temperature was 13.1°C and air temperature was 22.0°C. On 23 August 2008, I captured an additional 10 individuals. Mean

water temperature was 13.0°C and air temperature was 22.8°C. The samples were grouped into one set of 10 (containing samples from 7/8/08), another set of 10 (containing five samples from 7/8/08 and five samples from 8/23/08), and a set of five (containing samples from 8/23/08). The two sets of 10 produced negative results, however, the set of five produced a positive result for the presence of *Bd*. The mean zoospore quantity for this set was 1.499 with a standard deviation of 0.1006.

Gaudineer Scenic Area, Monongahela National Forest, Pocahontas County

I hand-captured one *P. nettingi* by turning over cover objects on 29 July 2008. The sample was run as its own analysis and produced a negative result for the presence of *Bd*. Soil temperature was 16.2°C and air temperature was 24.8°C.

Helmick Rock, Private Land, Hardy County

On the night between 28 August 2008 and 29 August 2008, I searched the talus and hand-captured 20 *P. punctatus* during visual night surveys. Air temperature was 17.0°C. Analyses were run on two sets of 10. Both sets produced negative results for the presence of *Bd*.

Turkeywallow Branch near Pineville, Wyoming County

On 31 August 2008, I used aquarium nets to capture 12 *D. welteri* by turning over cover objects on the stream edge. Mean water temperature was 14.0°C and air temperature was 22.4°C. Samples were grouped into two sets of six. Both sets produced negative results for the presence of *Bd*.

Wheeling Creek, Marshall County

Joe Greathouse and staff from The Good Zoo at Oglebay captured three *C. a. alleganiensis* on 2 September 2008. All three samples were grouped into one set and run as one analysis. The set produced negative results for the presence of *Bd*. Mean water temperature was 22.5°C and air temperature was 23.7°C.

Unnamed Tributary to New River near Prince, Fayette County

On 7 September 2008, I used aquarium nets to capture 21 *D. quadramaculatus* by turning over cover objects on the stream edge. Mean water temperature was 14.2°C and air temperature was 20.6°C. Samples were grouped into three sets (two sets of 10 and one individual sample), all of which produced negative results for the presence of *Bd*.

Tank Hollow, Tributary to New River, Fayette County

On 7 September 2008, I used aquarium nets to capture three *D. quadramaculatus* by turning over cover objects on the stream edge. Mean water temperature was 13.9°C and air temperature was 21.9°C. The samples were grouped into one set and run as one analysis. The set produced negative results for the presence of *Bd*.

Buckeye Creek Cave, Private Land, Greenbrier County

On 12 September 2008, I examined the cave walls and crevices and hand-captured one *Eurycea lucifuga* approximately 15 meters from the cave entrance. The sample was run as one analysis and produced negative results for the presence of *Bd*. Water temperature was 11.0°C and air temperature was 14.5°C at capture location.

Higginbothams Cave, Private Land, Greenbrier County

On 12 September 2008, I examined the cave walls and crevices and hand-captured seven *E. lucifuga*. Mean water temperature in the cave was 7.9°C and air temperature ranged from 16.6°C at first capture near the entrance to the cave to 12.7°C at the deepest capture. Additionally, I captured 20 individuals from another entrance to Higginbothams Cave on 26 September 2008. This cave entrance did not have water near any of the captures. Air temperatures ranged from 19.6°C at the first capture near the entrance to 16.3°C at the deepest capture. Samples were grouped into three sets (two sets of 10 and one set of seven) and run as three analyses. All sets produced negative results for the presence of *Bd*.

Norman Cave, Private Land, Greenbrier County

I examined the cave walls and crevices and hand-captured 10 *E. lucifuga* on 26 September 2008. Water temperature was 8.0°C and air temperatures ranged from 16.5°C at the first capture near the entrance to 12.8°C at the deepest capture. Samples were grouped into one set. The set produced negative results for the presence of *Bd*.

US-219 Cave, Private Land, Greenbrier County

On 26 September 2008, I examined the cave walls and crevices and hand-captured 10 *E. lucifuga*. Also, two of the individuals were captured just outside of the cave entrance. Water temperature was 10.3°C and air temperatures ranged from 19.8°C just outside of the cave entrance to 15.6°C at the deepest capture. All samples were run as one analysis and produced negative results for the presence of *Bd*.

Unnamed Tributary to New River near Brooklin, Raleigh County

I captured two *D. quadramaculatus* on 27 September 2008 by turning over cover objects on the stream edge. Mean water temperature was 13.0°C and air temperature was 19.4°C. The samples were group together into one set and run as one analysis. The set produced negative results for the presence of *Bd*.

Falls Branch, Tributary to New River, Raleigh County

I captured 11 *D. quadramaculatus* on 27 September 2008 by turning over cover objects on the stream edge. Mean water temperature was 12.5°C and air temperature was 20.7°C. Samples were grouped into two sets (one set of 10 and one individual sample) and run as two analyses. Both sets produced negative results for the presence of *Bd*.

General Davis Cave, The Nature Conservancy, Greenbrier County

On October 8, 2008, I examined the cave walls and stream and hand-captured or used aquarium nets to capture 8 *Gyrinophilus subterraneus*, 12 *Gyrinophilus p. porphyriticus*, and 10 *E. lucifuga*. Water temperatures and air temperatures were not recorded due to time constraints. Samples were grouped into 3 sets of 10 and run as 3 analyses. All 3 sets produced negative results for the presence of chytrid.

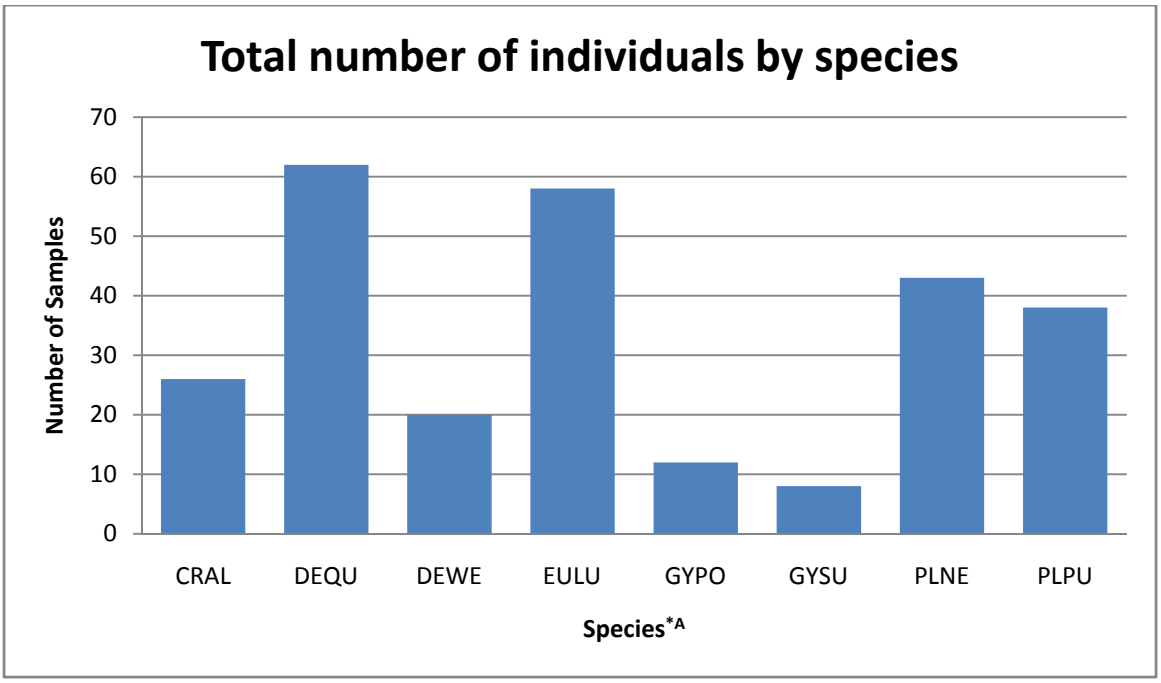

Figure 21. Total number of samples by species taken in 2008.

A) CRAL=*Cryptobranchus a. alleganiensis*; DEQU=*Desmognathus quadramaculatus*; DEWE=*Desmognathus welteri*; EULU=*Eurycea lucifuga*; GYPO=*Gyrinophilus p. porphyriticus*; GYSU=*Gyrinophilus subterraneus*; PLNE=*Plethodon nettingi*; PLPU=*Plethodon punctatus*

Table 2. Overall results for 2008 surveys.

Site Number	Site Name	County	Species[*A]	Number Surveyed	Date Surveyed	*Bd* Survey Results
1	West Fork Greenbrier River	Pocahontas	*CRAL*	8	6/15, 7/10, 8/26/2008	Negative
2 C	abin Mountain	Tucker	*PLNE*	23 6/	18/2008	Negative
3	Bear Heaven Campground	Randolph	*PLNE*	18	6/19, 7/29/2008	Negative
4 D	olly Sods	Tucker	*PLNE*	1 6	/20/2008	Negative
5 Buffalo	Creek	Brooke	*CRAL*	15	6/25, 8/15, 8/16, 8/23/2008	Negative
6 Red	dish Knob	Pendleton	*PLPU*	6	6/25, 7/9/2008	Negative
7 Sh	enandoah Mountain	Pendleton	*PLPU*	12	6/26, 7/9, 7/10/2008	Negative
8 Bear	Creek	Mercer	*DEWE*	8 7	/8/2008	Negative
9	Unnamed Tributary near Quinnimont	Fayette	*DEQU*	25	7/8/, 8/23/2008	Positive
10 Gau	dineer Knob	Pocahontas	*PLNE*	1 7	/29/2008	Negative
11 Helmick	Rock	Hardy	*PLPU*	20 8/	28/2008	Negative
12 Tur	keywallow Branch	Wyoming	*DEWE*	12 8/	31/2008	Negative
13 Wheel	ing Creek	Marshall	*CRAL*	3 9	/2/2008	Negative
14	Unnamed Tributary near Prince	Fayette	*DEQU*	24 9/	7/2008	Negative
15 Tank	Hollow	Fayette	*DEQU*	3 9	/7/2008	Negative
16	Buckeye Creek Cave	Greenbrier	*EULU*	1 9	/12/2008	Negative
17 Hi	gginbothams Cave	Greenbrier	*EULU*	27	9/12, 9/26/2008	Negative
18 Norm	an Cave	Greenbrier	*EULU*	10 9/	26/2008	Negative
19 US-	219 Cave	Greenbrier	*EULU*	10 9/	26/2008	Negative
20	Unnamed Stream near Brooklin	Raleigh	*DEQU*	2 9	/27/2008	Negative
21 Falls	Branch	Raleigh	*DEQU*	11 9/	27/2008	Negative
22	General Davis Cave	Greenbrier	*GYSU* *GYPO* *EULU*	8 12 10	10/8/2008	Negat ive

A) CRAL=*Cryptobranchus a. alleganiensis*; DEQU=*Desmognathus quadramaculatus*; DEWE=*Desmognathus welteri*; EULU=*Eurycea lucifuga*; GYPO=*Gyrinophilus p. porphyriticus*; GYSU=*Gyrinophilus subterraneus*; PLNE=*Plethodon nettingi*; PLPU=*Plethodon punctatus*.

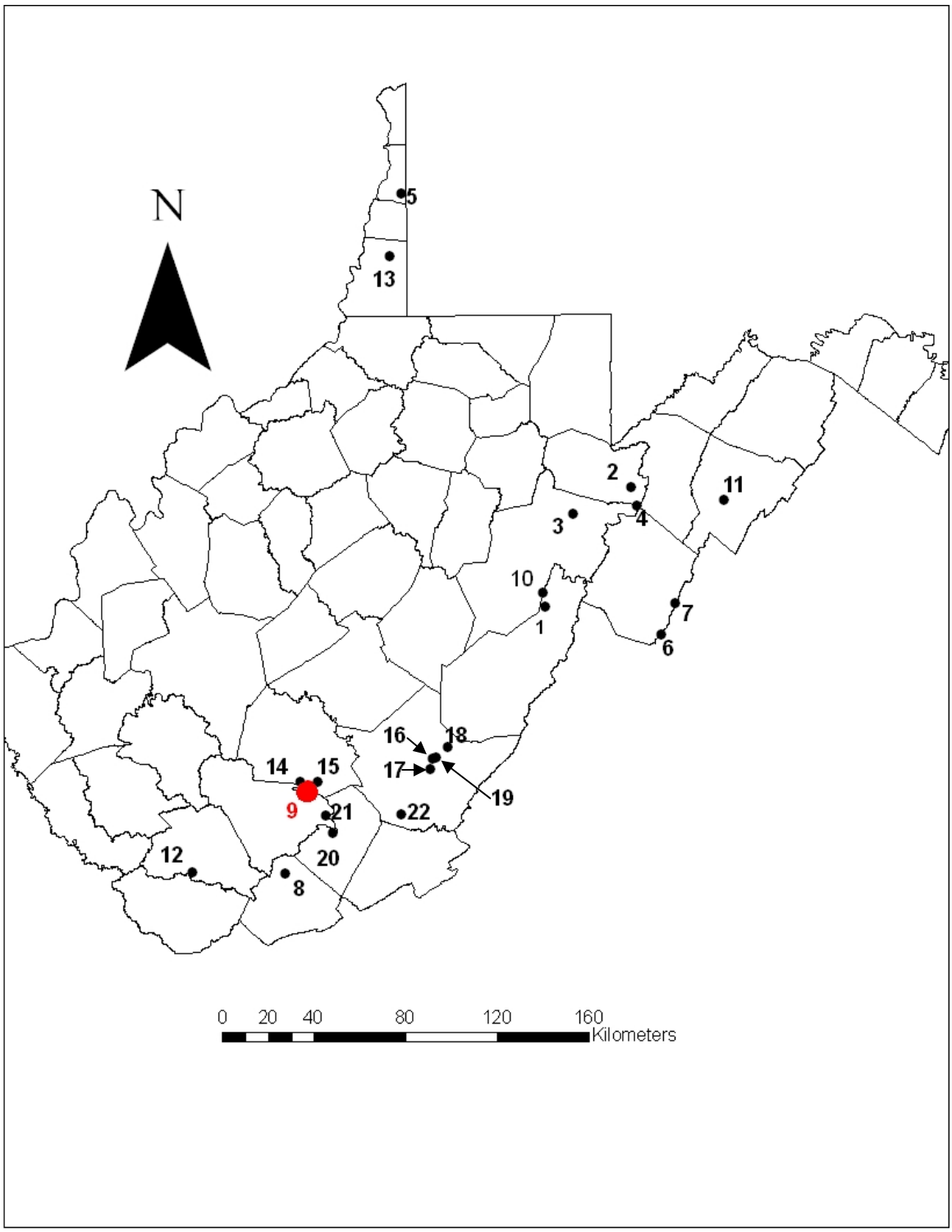

Figure 22. Overall locations for 2008 chytrid surveys, see Table 2 for site information.

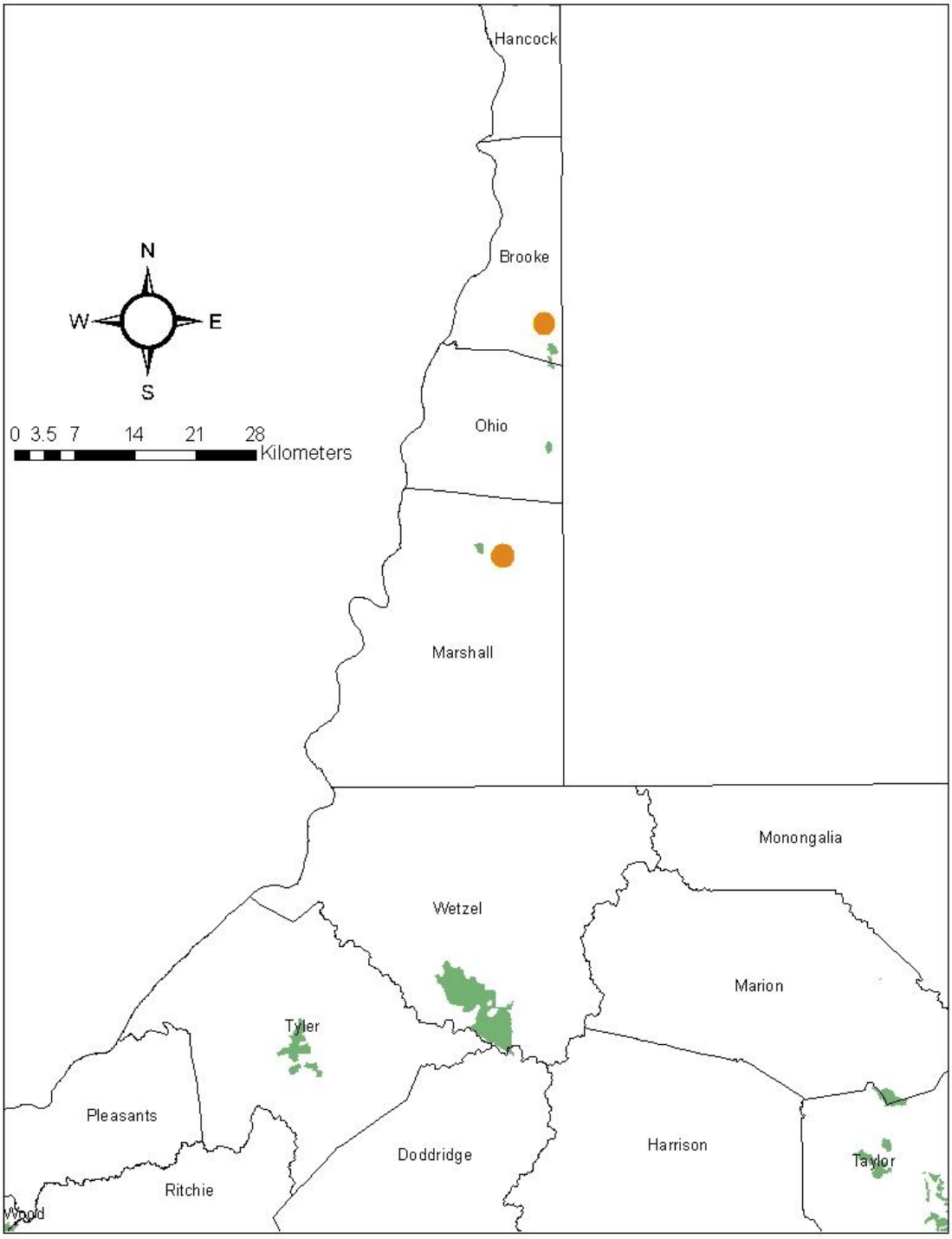

Figure 23. Locations of Buffalo Creek and Wheeling Creek.

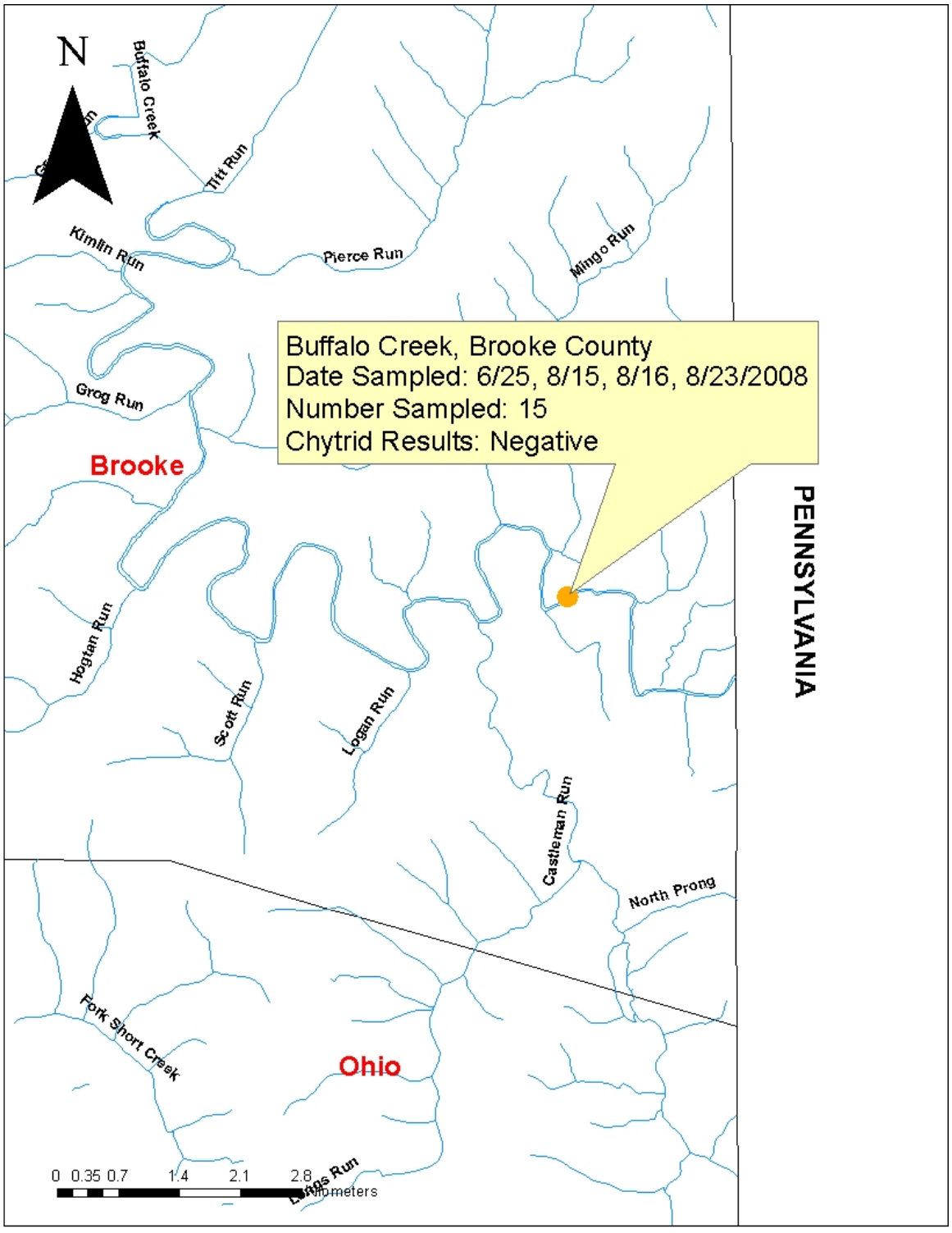

Figure 24. Close-up map of Buffalo Creek, surveyed for *C. a. alleganiensis*.

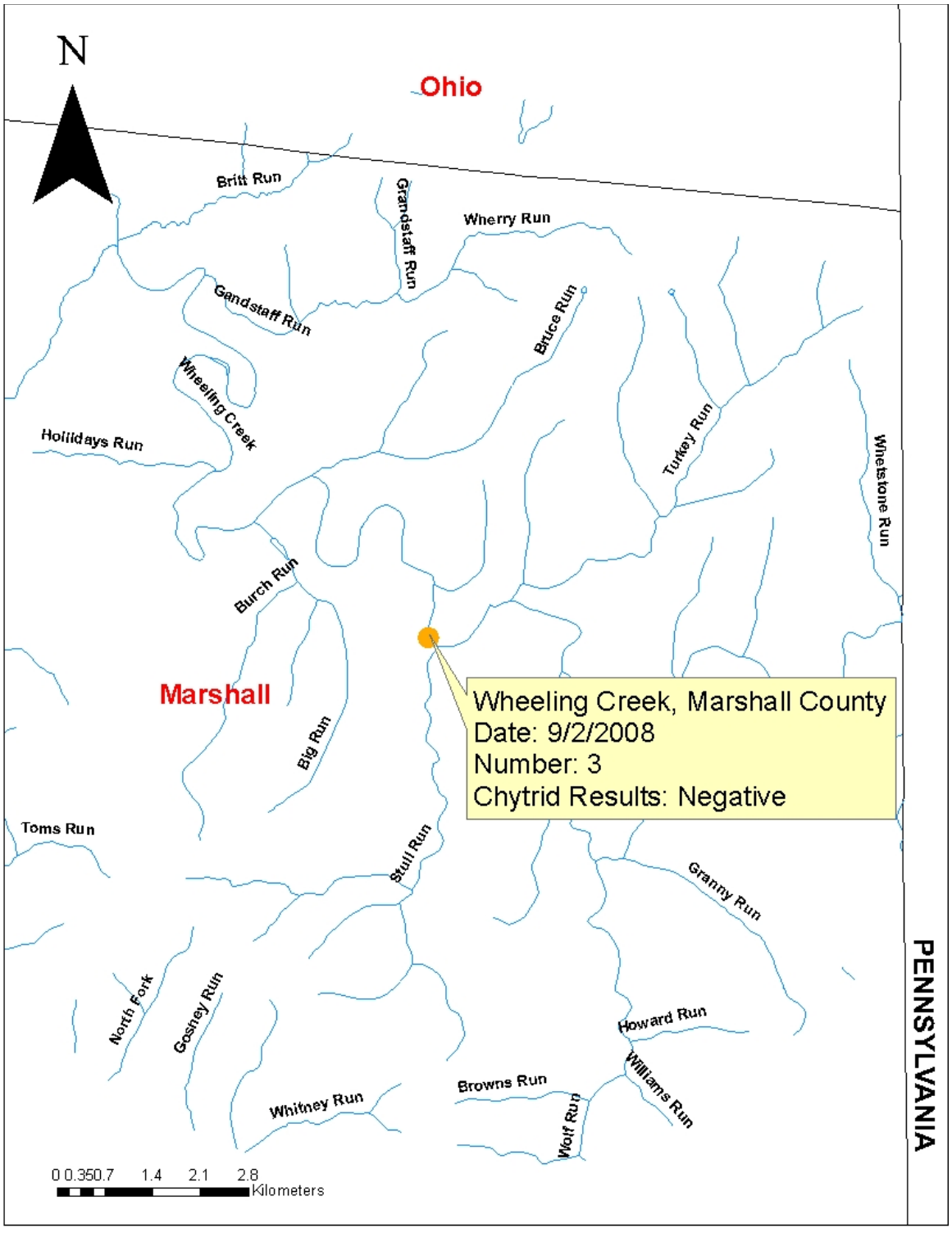

Figure 25. Close-up map of Wheeling Creek, surveyed for *C. a. alleganiensis*.

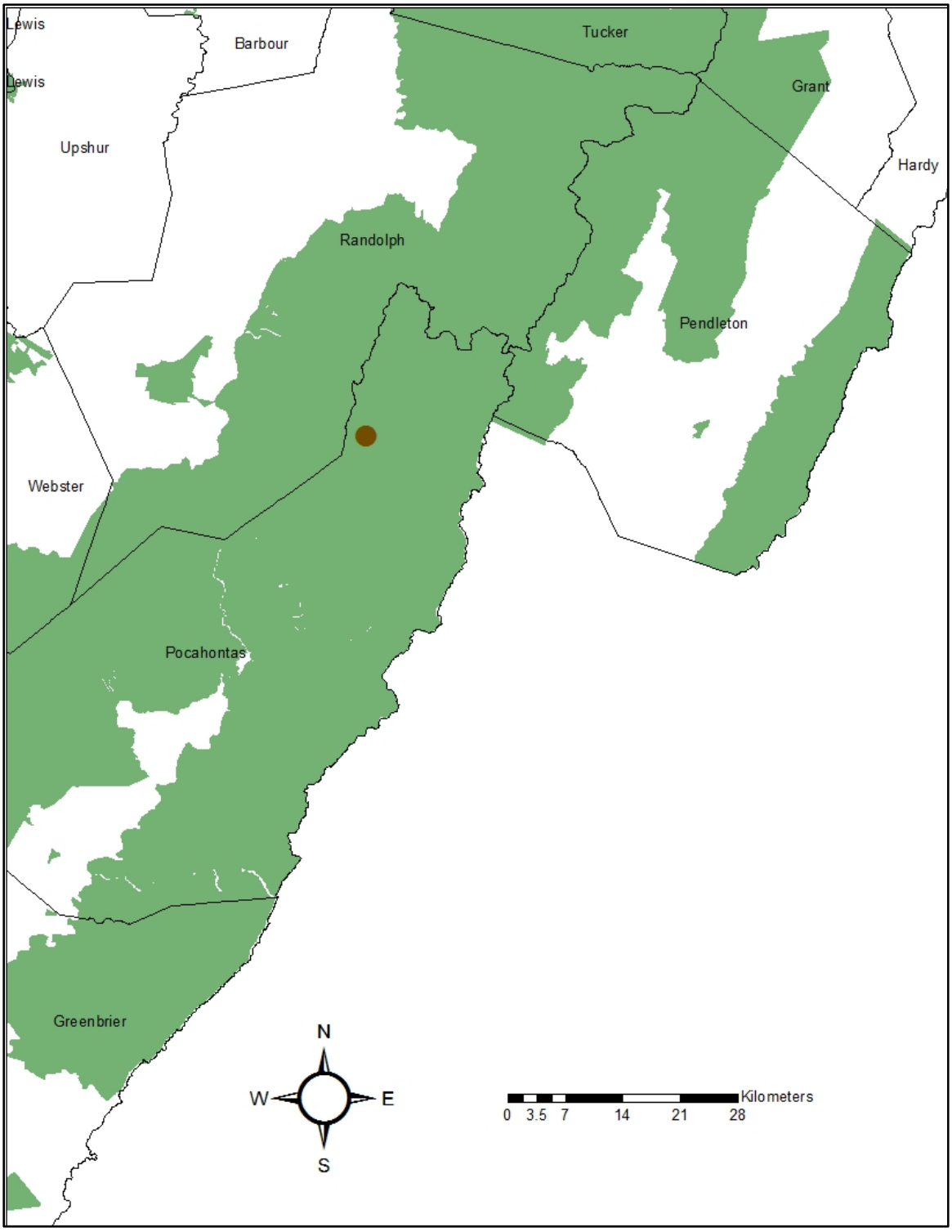

Figure 26. Location of West Fork Greenbrier River.

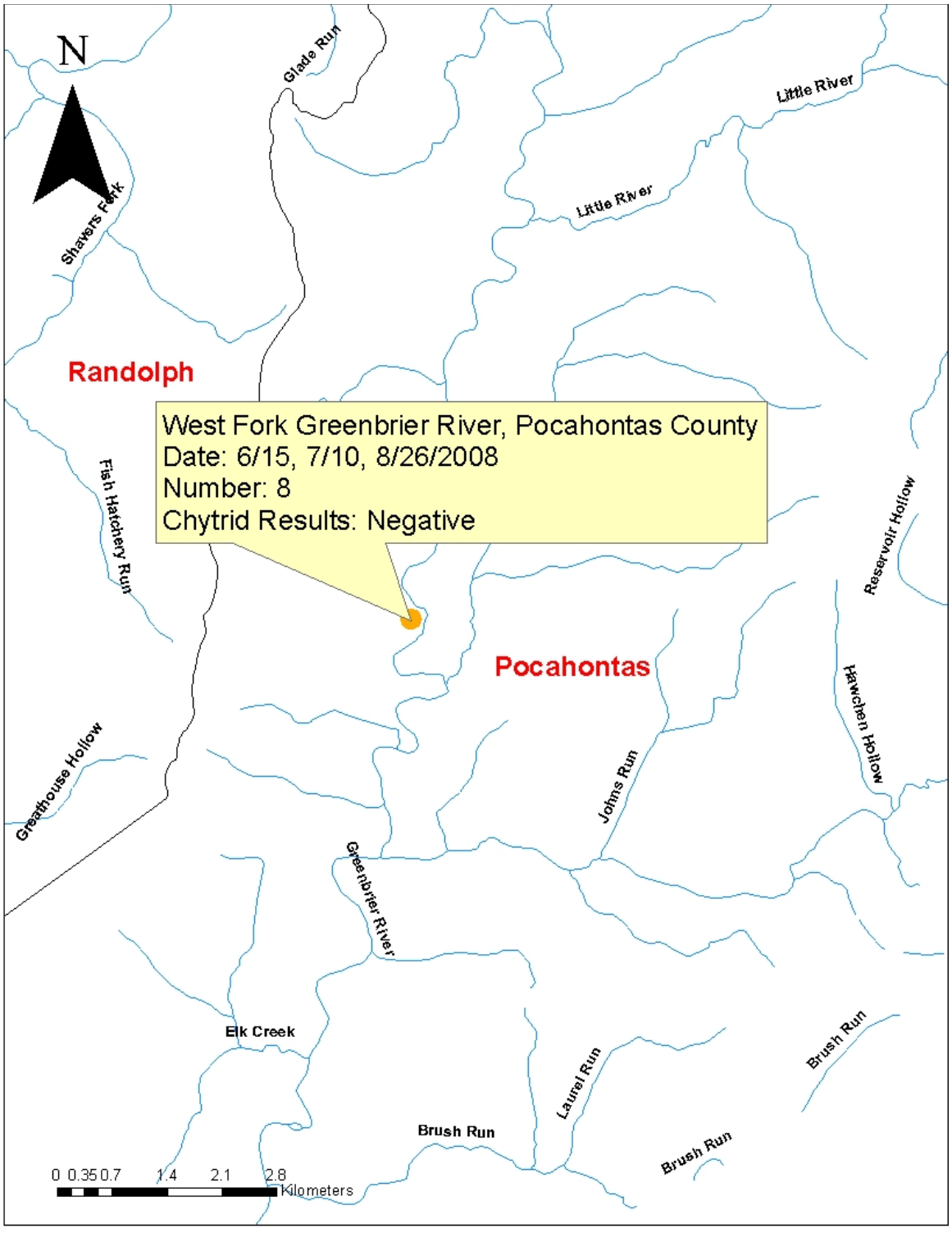

Figure 27. Close-up map of West Fork Greenbrier River, surveyed for *C. a. alleganiensis*.

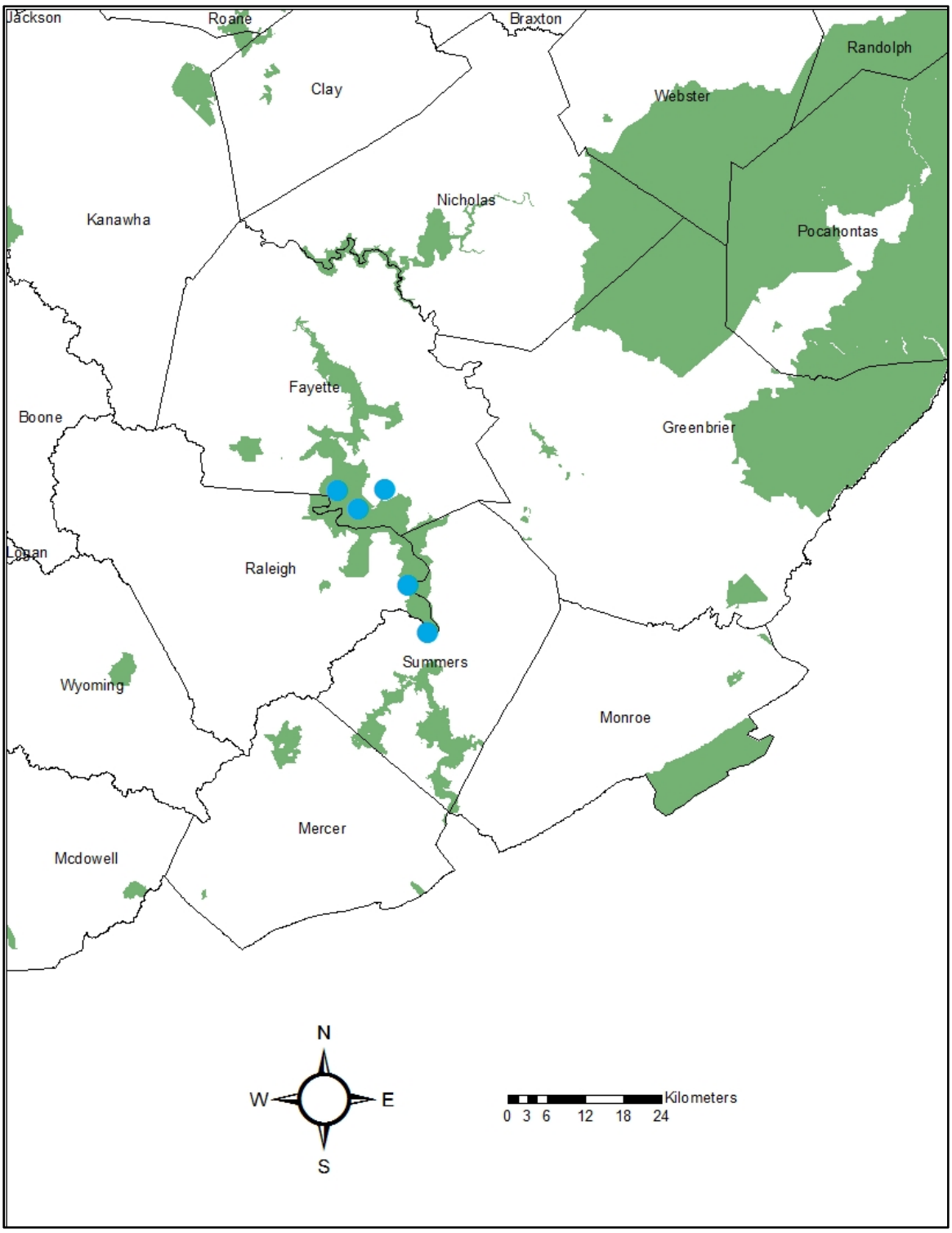

Figure 28. Locations of sites surveyed for *Desmognathus quadramaculatus*.

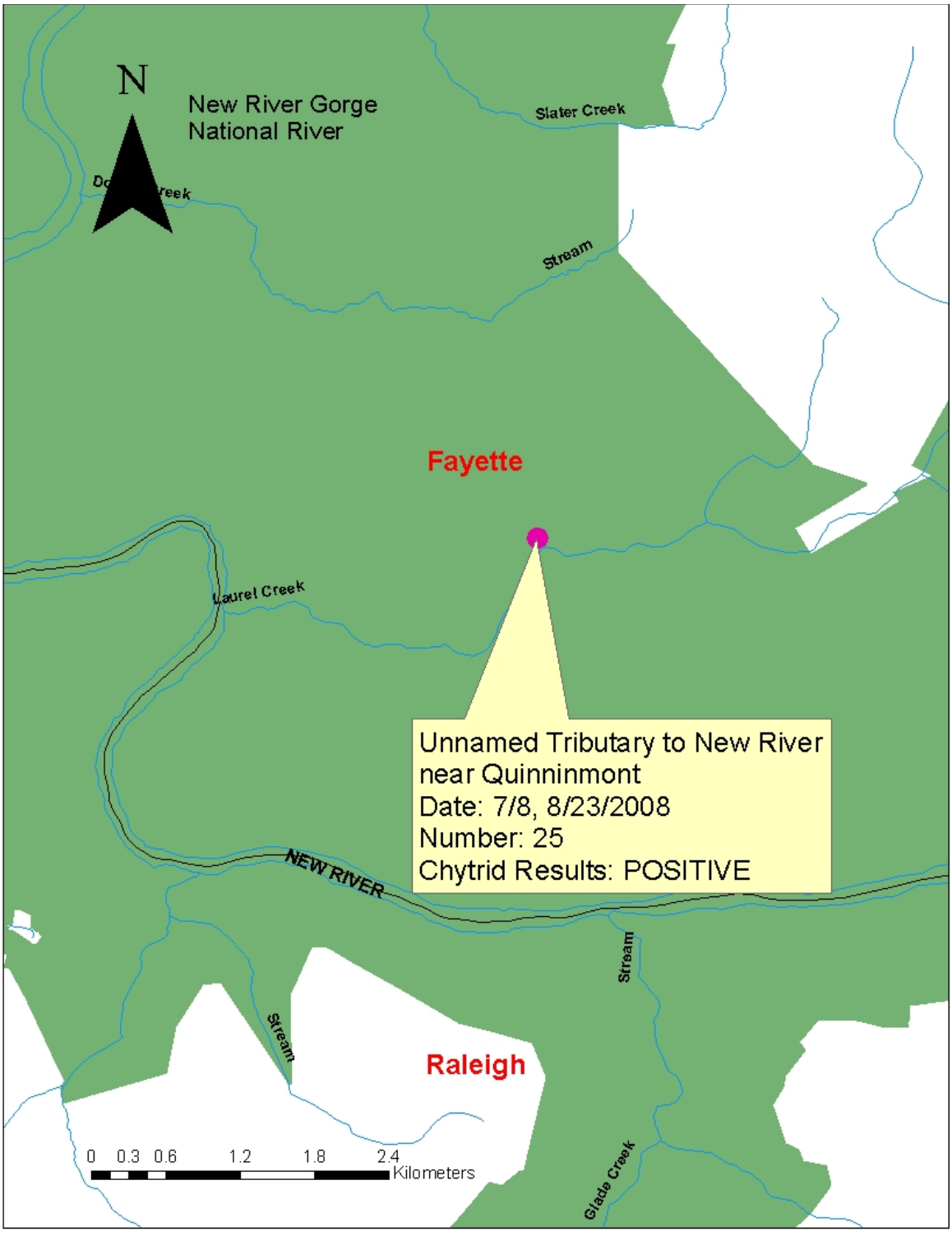

Figure 29. Close-up map of unnamed tributary near Quinnimont, surveyed for *D. quadramaculatus*.

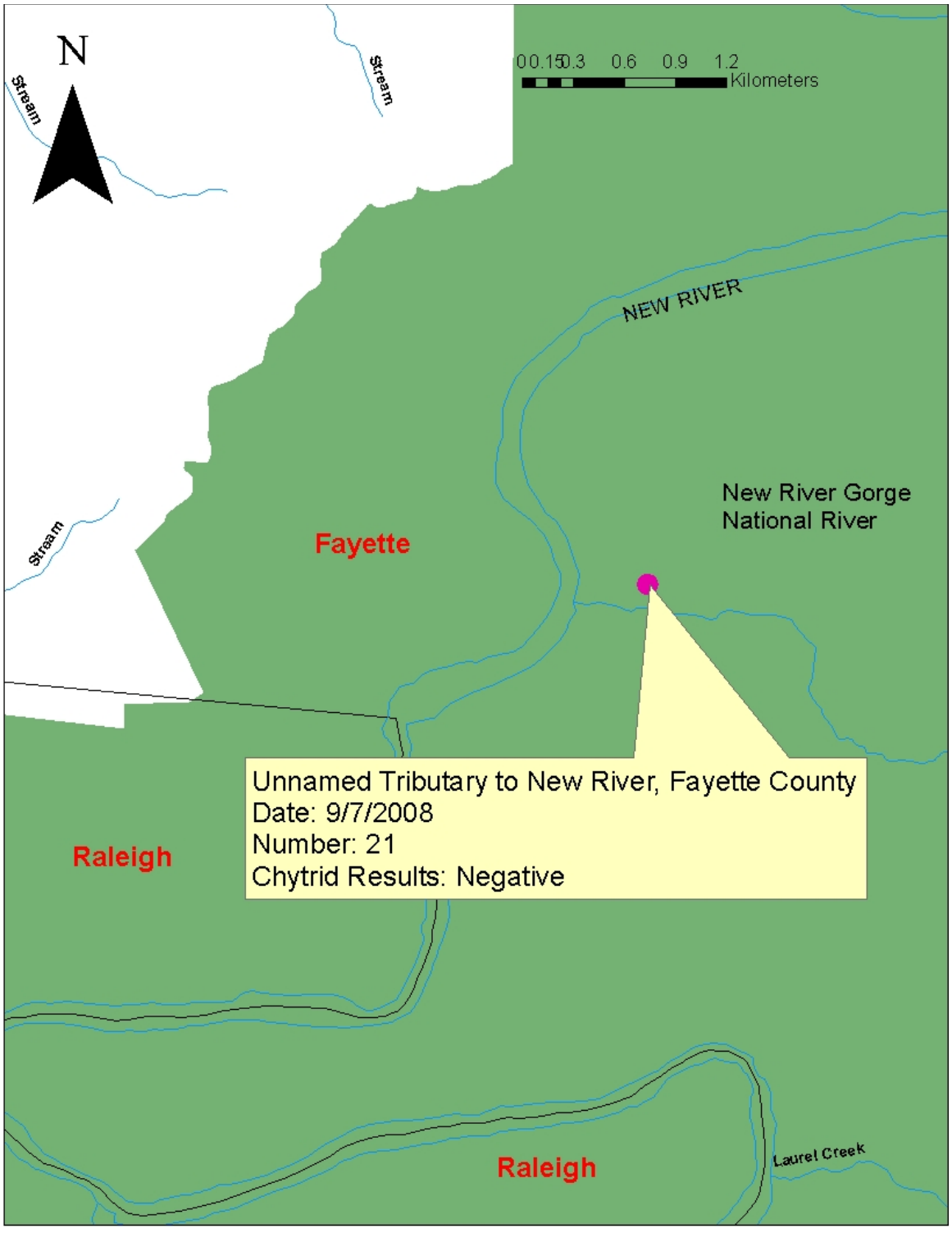

Figure 30. Close-up map of unnamed tributary near Prince, surveyed for *D. quadramaculatus*.

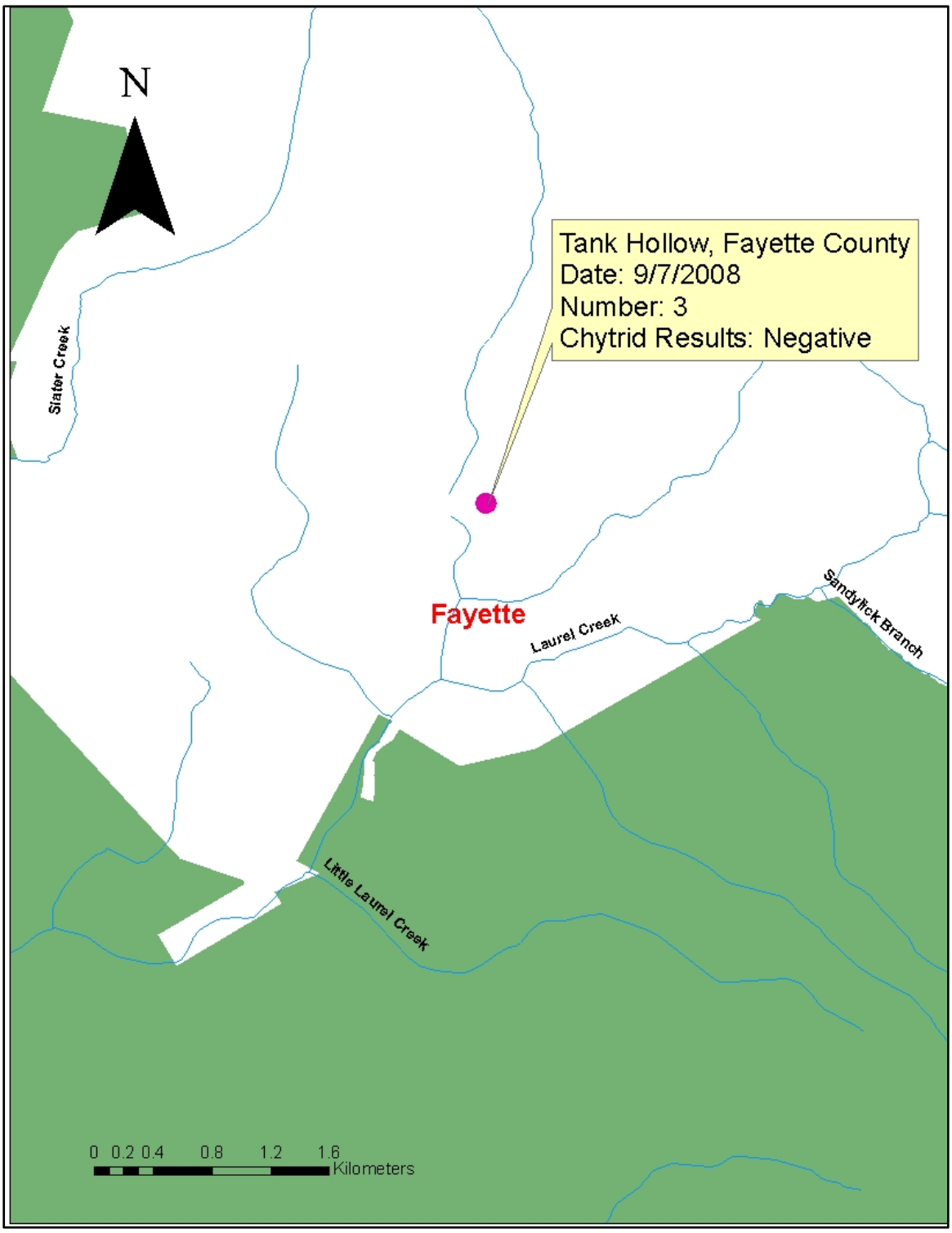

Figure 31. Close-up map of Tank Hollow, surveyed for *D. quadramaculatus*.

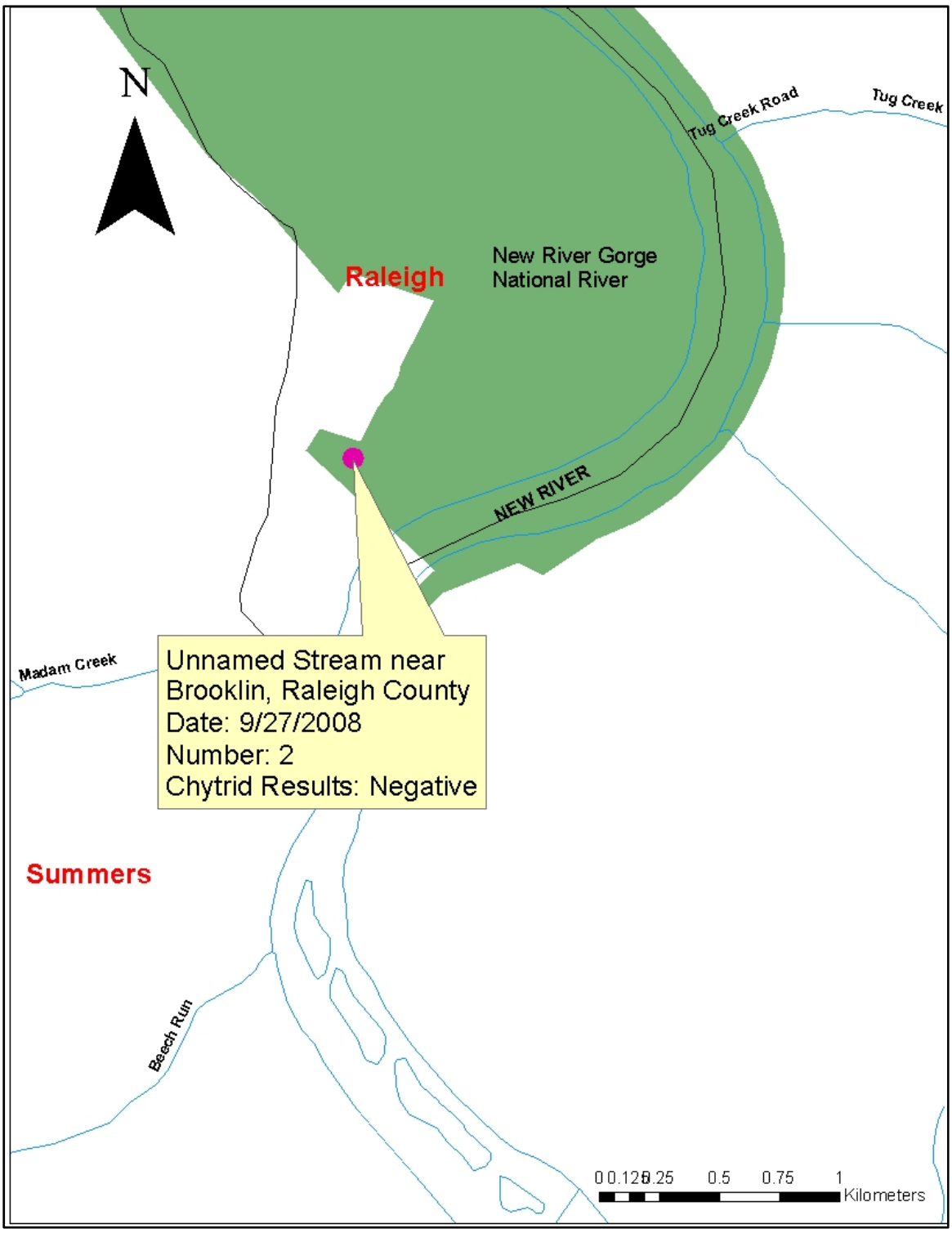

Figure 32. Close-up map of unnamed stream near Brooklin, surveyed for *D. quadramaculatus*.

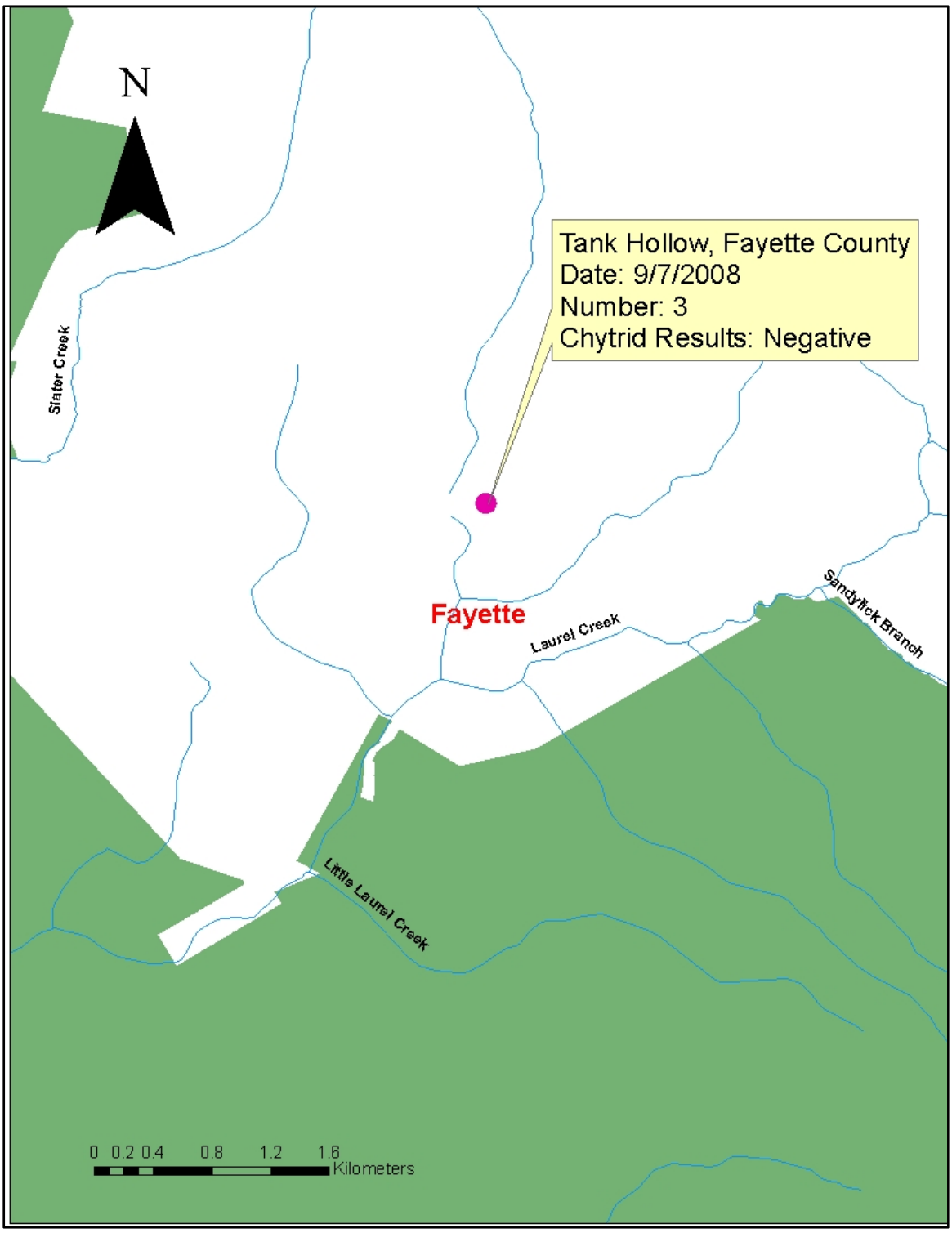

Figure 33. Close-up map of Falls Branch, surveyed for *D. quadramaculatus*.

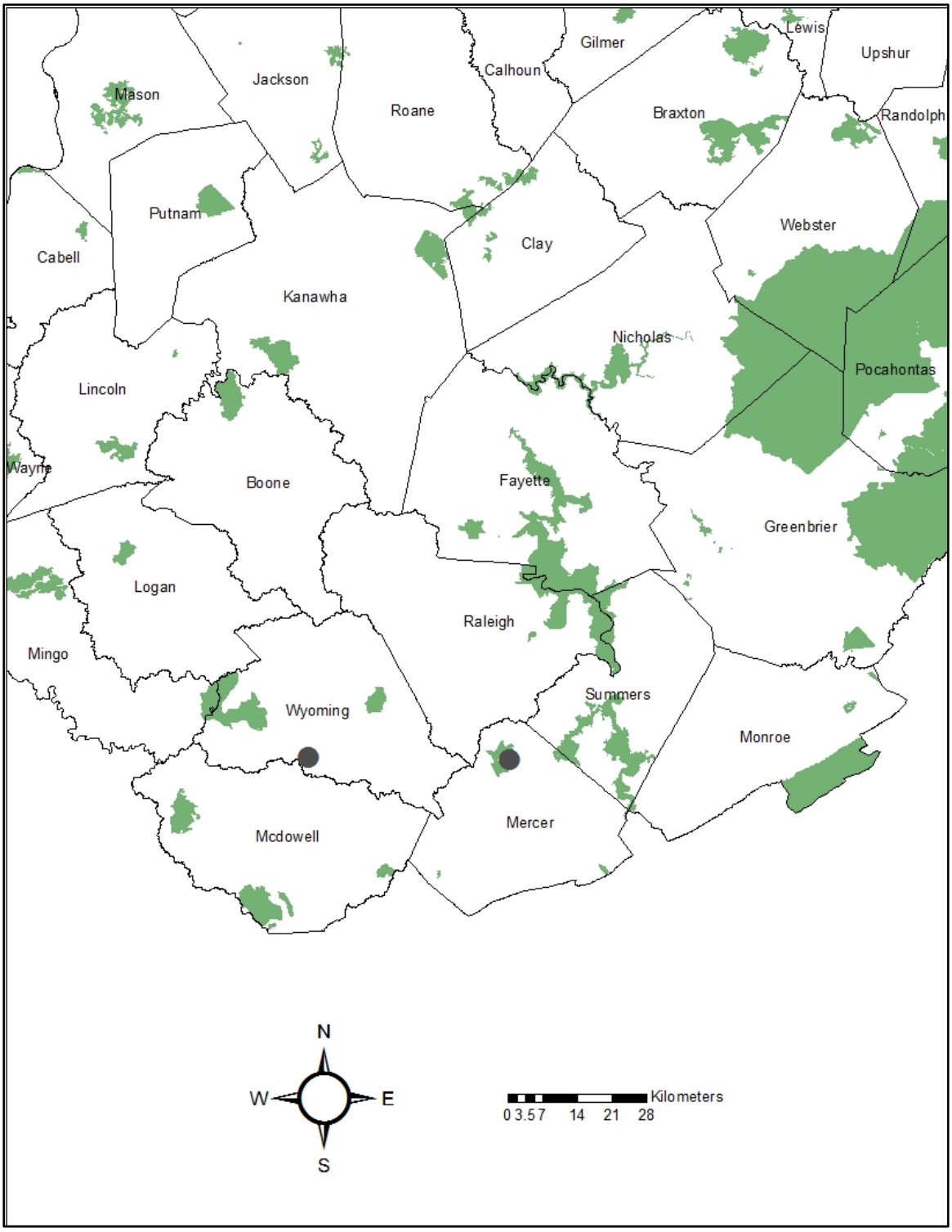

Figure 34. Locations of Bear Creek and Turkeywallow Branch.

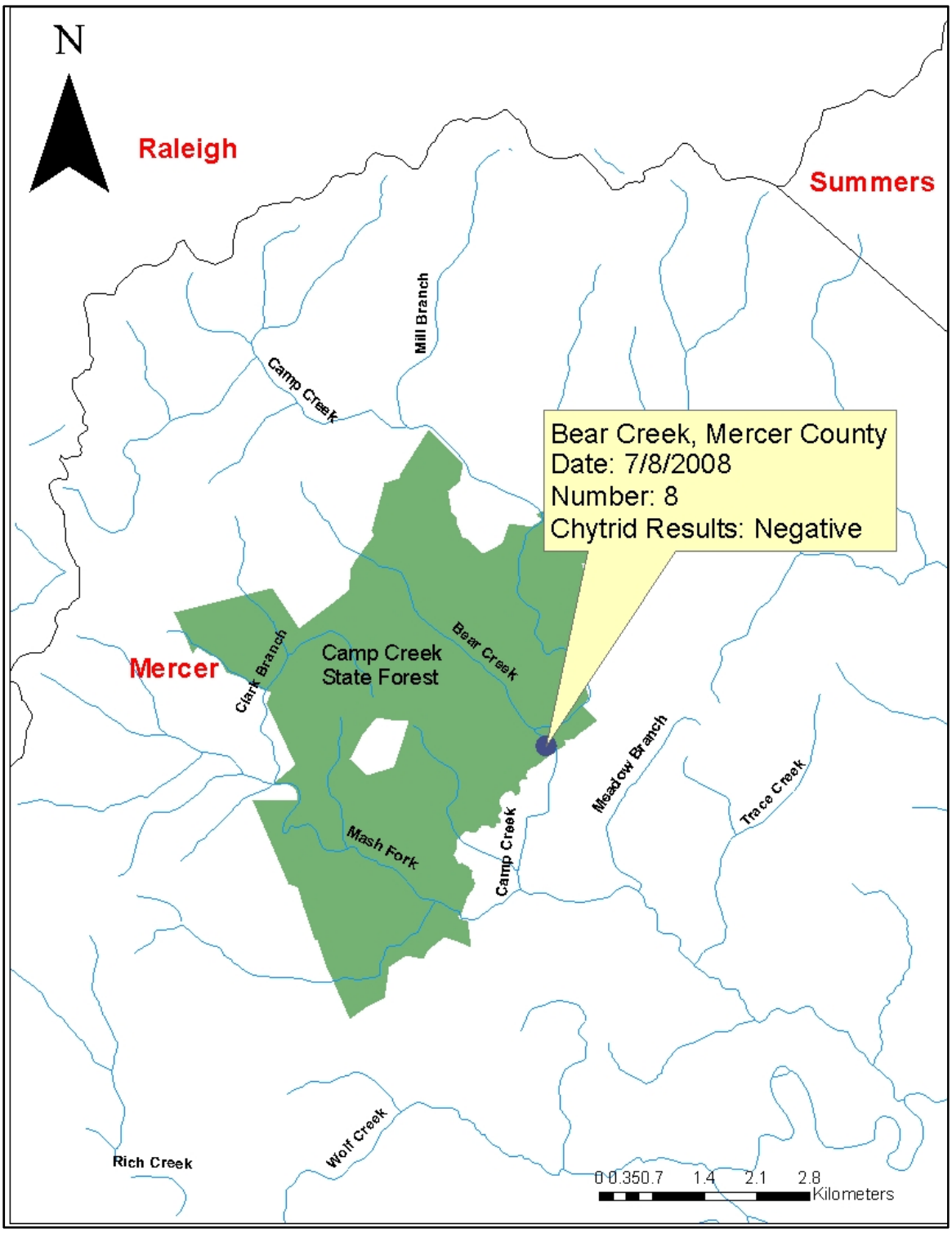

Figure 35. Close-up map of Bear Creek, surveyed for *Desmognathus welteri*.

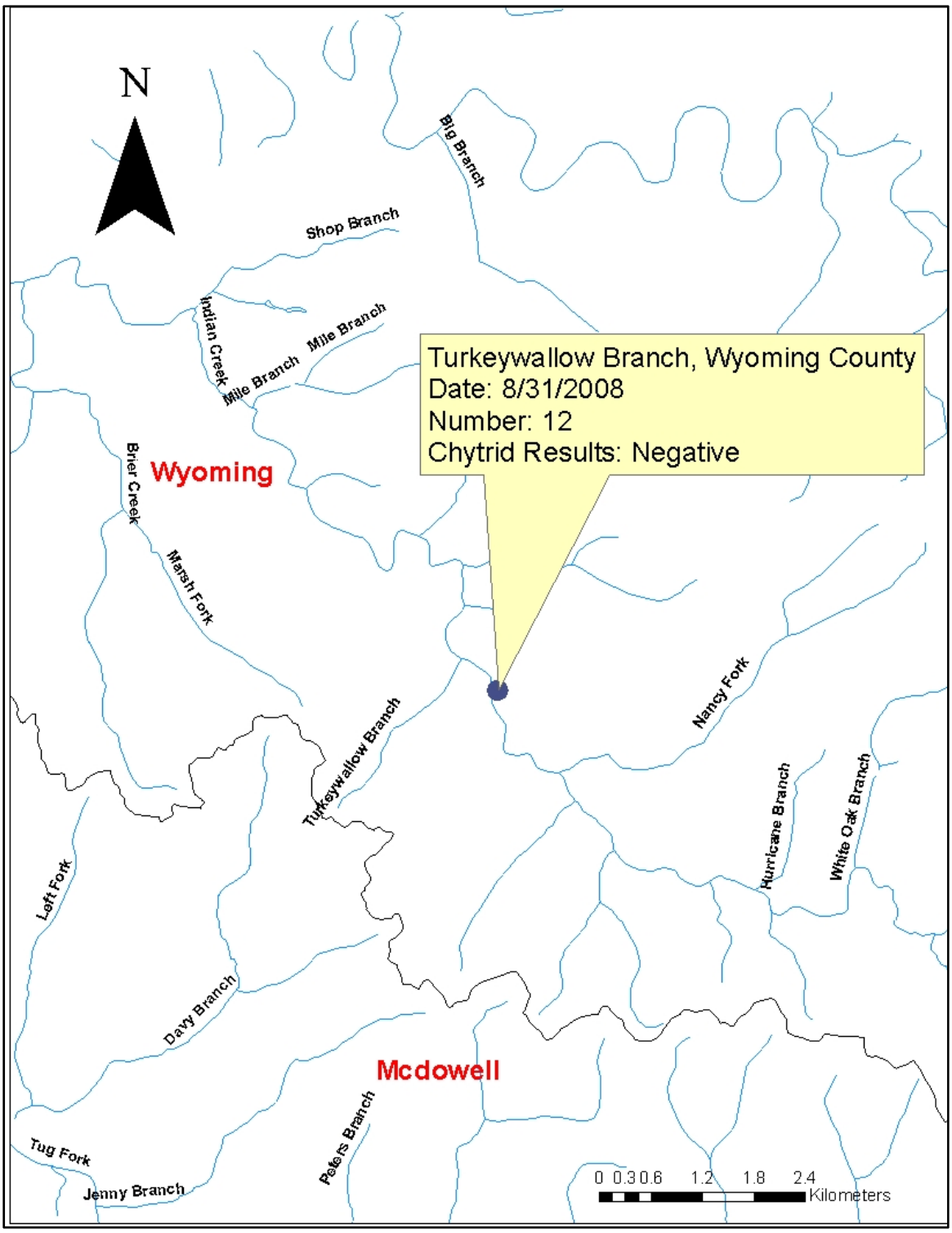

Figure 36. Close-up map of Turkeywallow Branch, surveyed for *D. welteri*.

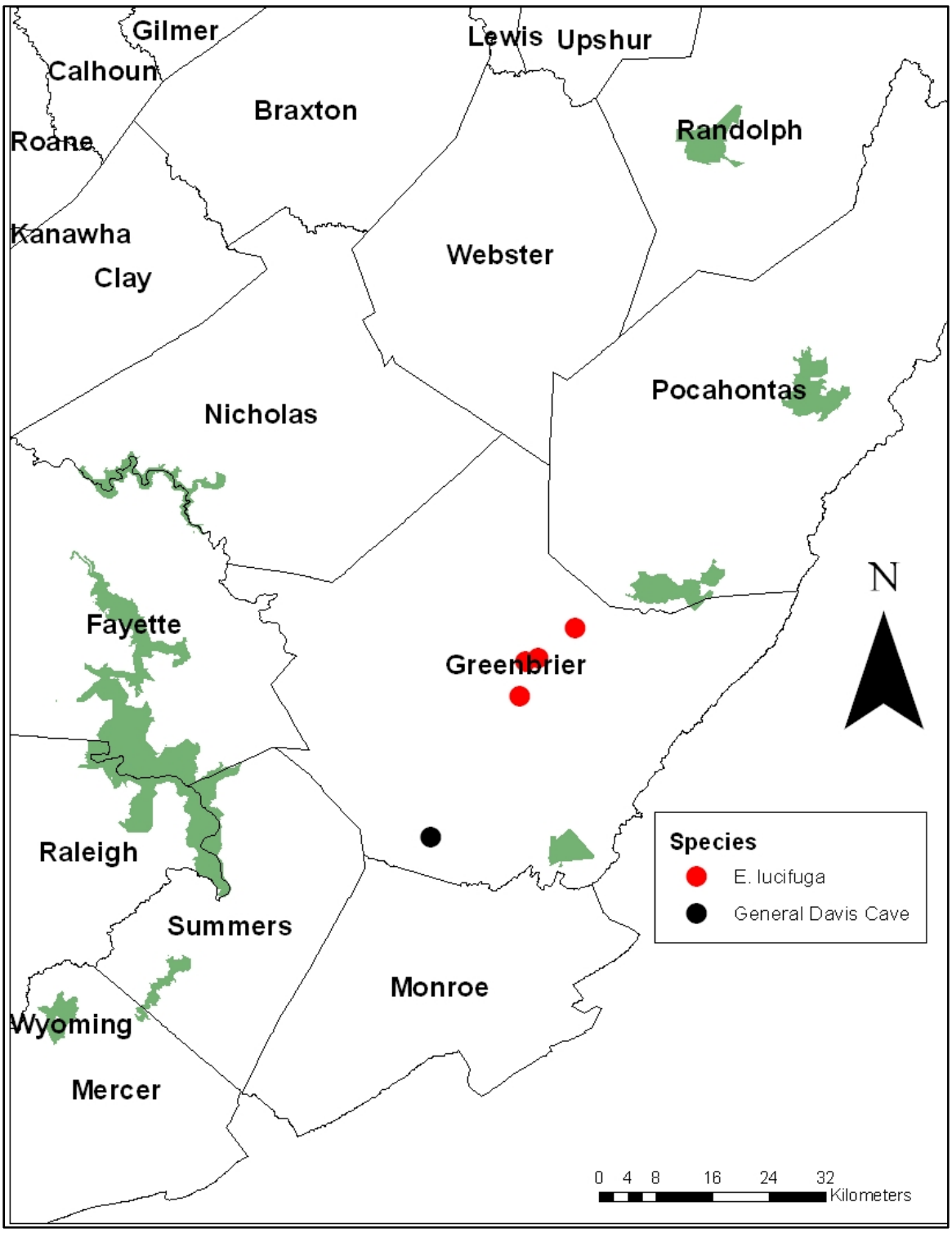

Figure 37. Locations of sites surveyed for *Eurycea lucifuga* as well as General Davis Cave.

DISCUSSION

Batrachochytrium dendrobatidis has been detected in populations of salamanders in other areas of the eastern United States. Byrne et al. (2008) found infections on *E. cirrigera* (Southern Two-lined Salamanders) in Alabama, documenting the first infection on this species. They documented that infected individuals were lethargic and had tails with little structural integrity. Grant et al. (2008) documented the first known *Bd* infection on stream-associated salamanders of the genera *Desmognathus* and *Eurycea*. Their observations expand the known range of *Bd* and include species *D. fuscus* (Northern Dusky Salamander) and *E. bislineata* (Northern Two-lined Salamander). They did not notice any physical signs of infection. Rothermel et al. (2008) found infections on *Notophthalmus viridescens* (Red-spotted Newts) in the southeastern United States, but also did not report any physical signs of infection. Timpe et al. (2008) found infections in *Desmognathus conanti* (Spotted Dusky Salamanders) in northwest Georgia. *Batrachochytrium dendrobatidis* has also been found to be prevalent in the Ozark Highlands of southern Missouri and northern Arkansas. Both *C. a. alleganiensis* (Eastern Hellbender) and *C. a. bishopi* (Ozark Hellbender) individuals were found to be infected with *Bd*, causing concern for both declining species (Briggler et al., 2008). Brodman and Briggler (2008) found infections on larvae of the pond-breeding *A. jeffersonianum* in Indiana. This documentation is the first of its kind in that it was found on larval salamanders. They noted lesions around the joints of the larvae, but could not directly attribute the lesions to *Bd* infection. Most salamander larvae do not have keratinized jaw sheaths, however, they do accumulate some keratin later in development. *Batrachochytrium dendrobatidis* was detected in West Virginia in 2007 (J. Greathouse, pers. comm.). Greathouse and staff detected *Bd* on three *C. a. alleganiensis* from two separate streams while conducting surveys in the northern panhandle of West Virginia (Marshall and Brooke counties). These sites

were surveyed again in 2008, however, both sites produced negative results. This situation is unique because the exact pools that were sampled in 2007 were re-sampled in 2008.

My work is the first to document *Bd* infection in *D. quadramaculatus*. While *Bd* occurrence was only found at one site, it is of concern because of the locality and species infected. *Desmognathus quadramaculatus* are very commonly used as bait for fishermen throughout southern West Virginia. Their range has even been shown to be expanding via the transfer and release of individuals into streams that have not contained populations of the species in the past (T. Pauley, pers. comm.). Also, the New River is a popular fishing location. The stream that contained *Bd* infected individuals passes under a county road, therefore making the stream very accessible to fishermen seeking bait. Regulations may need to be modified to stop the use of *D. quadramaculatus* as a bait product and to stop the use of amphibians as bait.

Positive results indicate that *Bd* does occur in West Virginia; however, we still do not know how many species may be affected. My study focused on only species of conservation need, because these species are at the highest risk of rapid decline if a chytridiomycosis outbreak would occur. A broad range of habitat types were sampled, including high elevation talus slopes, high elevation red spruce forests, high gradient streams, large streams, and caves. More species need to be surveyed in these locations to determine if *Bd* would have been detected in larger sample sizes. Also, more research needs to be conducted to determine the detection probability in different temperature regimes and different seasons. The site that tested positive was sampled on two different dates, in early July and late August. All of the individuals sampled in July were negative. Fifteen individuals were sampled approximately 10 meters from the road, near the mouth of the stream. Another 10 individuals were sampled in August approximately 50 meters upstream from the area sampled in July. The samples were grouped in two groups of 10 and a

group of five. One group contained only samples from July with water temperatures averaging 13.1°C, another contained samples from July and late August with water temperatures averaging 13.1°C and 13.0°C, respectively, and the last group contained five samples taken only in late August with water temperatures averaging 13.0°C. The last group was the only group that tested positive for the occurrence of *Bd* zoospores. The mean zoospore count for the group was only 1.499, meaning that only seven to eight zoospores were present in the sample. It may be that sampling at this time of the year is inopportune for detecting presence of *Bd,* because fewer zoospores are released at higher temperatures, and zoospores are required to detect *Bd* using the alcohol-preserved swab method because the other life stages are subcutaneous. All sampling was done when water and soil temperatures were above the optimum zoospore temperature range of 7°C - 10°C, except for samples taken in caves. Temperature variance should not be a factor in caves, however, there is not a warm temperature period where water or soil temperatures would be within the 17°C-25°C range optimum for zoosporangium development, therefore conditions may not be appropriate for *Bd*. This is important especially for General Davis Cave, because of the unique fauna found in the system (e.g. *Gyrinophilus subterraneus*). Even without temperatures optimum for *Bd* development, it is possible that chytridiomycosis infections could occur in the subterraneous environment because external watersheds may transport zoospores from other areas. Also, troglophilic amphibians such as *Rana palustris* (Pickerel Frogs) may be pathways to spread *Bd* zoospores into the subterraneous environment.

Terrestrial salamanders (i.e. *Plethodon*) are at a low risk for infection, however, there are accounts of *Bd* infection in montane, terrestrial species (Cummer et al., 2005). Both terrestrial *Plethodon* that were sampled produced negative results, but continued monitoring is necessary because both species prefer moist, high elevation slopes. Also, it is possible that the movement

of other species, such as *Rana clamitans melanota* (Northern Green Frogs) and *Desmognathus ochrophaeus* (Allegheny Mountain Dusky Salamanders), from aquatic or semiaquatic environments to high elevation slopes may be a pathway to spread infection. *Batrachochytrium dendrobatidis* can survive on moist soil so amphibians exposed to this soil may contract the zoospores (Johnson and Speare, 2005).

Some amphibians in West Virginia have been experiencing noticeable declines, such as *Rana pipiens* (Northern Leopard Frog) and *Pseudotriton ruber ruber* (Northern Red Salamander) (T. Pauley, pers. comm.). While it is not known whether chytridiomycosis, or another emerging infectious disease is a causative agent in these declines, further research should focus on declining amphibian species. Also, monitoring should focus on other amphibian species of conservation need, but all species sympatric with these species should also be sampled to determine whether or not *Bd* is in the environment. It is difficult to make assumptions about possible declines because baseline population data are lacking for almost all amphibian species in West Virginia, however, if *Bd* is detected in a population, immediate long-term monitoring efforts should be made. Also, quarantines should be placed on areas that have tested positive for *Bd*, such as the case for the stream containing infected *D. quadramacultus*.

LITERATURE CITED

Alexander, M.A. and J.K. Eischeid. 2001. Climate variability in regions of amphibian declines. Conservation Biology. 15(4): 930-942.

Alford, R.A. and S.J. Richards. 1999. Global amphibian declines: a problem in applied ecology. Annual Review of Ecology, Evolution, and Systematics. 30: 133-165.

Bakal, R., L. Ball, C. Carey, J. Collins, E. Garcia, J. Mendelson, P. Mitchell, R. Moore, D. Olson, J. Reaser, and T. Woodward. 2007. Amphibian Declines and Chytridiomycosis: Translating Science into Urgent Action Symposium Proceedings. Tempe, AZ.

Berger, L., R. Speare, P. Daszak, D.E. Green, A.A. Cunningham, C.L. Goggin, R. Slocombe, M.A. Ragan, A.D. Hyatt, K.R. McDonald, H.B. Hines, K.R. Lips, G. Marantelli, and H. Parkes. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America. 95: 9031-9036.

Berger, L., A.D. Hyatt, R. Speare, and J.E. Longcore. 2005. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms. 68: 51-63.

Bond, T. 2007. A study of the genus *Desmognathus* in West Virginia, with emphasis on *Desmognathus welteri*, the Black Mountain Salamander. Master's Thesis, Marshall University, Huntington, WV.

Bonaccorso, E., J.M. Guayasamin, D. Mendez, and R. Speare. 2003. Chytridiomycosis as a possible cause of population declines in *Atelopus cruciger* (Anura: Bufonidae). Herpetological Review. 34(4): 331-333.

Bosch, J., I. Martinez-Solano, and M. Garcia-Paris. 2000. Evidence of a chytrid fungus involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation. 97: 331-337.

Boyle, D.G., D.B. Boyle, V. Olsen, J.A.T. Morgan, and A.D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms. 60: 141-148.

Bradley, G.A., P.C. Rosen, M.J. Sredl, T.R. Jones, and J.E. Longcore. 2002. Chytridiomycosis in native Arizona frogs. Journal of Wildlife Diseases. 38(1): 206-212.

Brem, F., J.R. Mendelson III, and K.R. Lips. 2007. Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians using alcohol preserved swabs. Version 1.0 (18 July 2007). Electronic document accessible at http://www.amphibians .org. Conservation International, Arlington, VA.

Bridges, C.M. and R.D. Semlitsch. 2005. "Variation in Pesticide Tolerance". Amphibian Declines: The Conservation Status of United States Species. Ed. M. Lannoo. University of California Press, Berkeley, CA, Los Angeles, CA, and London, England. 93-95.

Briggs, C.J., V.T. Vredenburg, R.A. Knapp, and L.J. Rachowicz. 2005. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. Ecology. 86(12): 3149-3159.

Brodman, R. and J.T. Briggler. 2008. *Batrachochytrium dendrobatidis* in *Ambystoma jeffersonianum* larvae in southern Indiana. Herpetological Review. 39(3): 320-321.

Byrne, M.W., E.P. Davie, and J.W. Gibbons. 2008. *Batrachochytrium dendrobatidis* occurrence in *Eurycea cirrigera*. Notes of the Southeastern Naturalist. 7(3): 551-555.

Conant, R. and J.T. Collins. 1998. Reptiles and Amphibians: Eastern/Central North America. 4th Edition. Houghton Mifflin Company, New York, NY. 616 pp.

Cummer, M.R., D.E. Green, and E.M. O'Neill. 2005. Aquatic chytrid pathogen detected in terrestrial plethodontid salamander. Herpetological Review. 36(3): 248-249.

Dahl, T.E. 1990. Wetland losses in the United States 1780's to 1980's. U.S. Department of the Interior, Fish and Wildlife Service, Washington, D.C. 13pp.

Davidson, E.W., M. Parris, J.P. Collins, J.E. Longcore, A.P. Pessier, and J. Brunner. 2003. Pathogenicity and transmission of chytridiomycosis in Tiger Salamanders (*Ambystoma tigrinum*). Copeia. 2003(3): 601-607.

Dazak, P., A. Strieby, A.A. Cunningham, J.E. Longcore, C.C. Brown, and D. Porter. 2004. Experimental evidence that the Bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal. 14: 201-207.

DiMauro, D. and M.L. Hunter, Jr. 2002. Reproduction of amphibians in natural and anthropogenic temporary pools in managed forests. Forest Science. 48(2): 397-406.

Felix, Z.I. 2001. A natural history study of *Desmognathus welteri* in West Virginia. Master's Thesis, Marshall University, Huntington, WV.

Fellers, G.M., D.E. Green, and J.E. Longcore. 2001. Oral Chytridiomycosis in the Mountain Yellow-legged Frog (*Rana muscosa*). Copeia. 2001(4): 945-953.

Fisher, R.N. and H.B. Shaffer. 1996. The decline of amphibians in California's Great Central Valley. Conservation Biology. 10(5): 1387-1397.

Gamradt, S.C. and L.B. Kats. 1996. Effect of introduced crayfish and mosquitofish on California Newts. Conservation Biology. 10(4): 1155-1162.

Gibbs, J.P., K.K. Whiteleather, and F.W. Schueler. 2005. Changes in frog and toad populations over 30 years in New York State. Ecological Applications. 15(4): 1148-1157.

Grant, E.H.C., L.L. Bailey, J.L. Ware, and K.L. Duncan. 2008. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in stream and wetland amphibians in Maryland, USA. Applied Herpetology. 233-241.

Green, N.B. and T.K. Pauley. 1987. Amphibians and Reptiles in West Virginia. University of Pittsburgh Press, Pittsburgh, PA. 241 pp.

Harris, R.N., T.Y. James, A. Lauer, M.A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. Ecohealth. 3: 53-56.

Higuchi, R., C. Fockler, G. Dollinger, and R. Watson. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology. 11(9): 1026-1030.

Hoppe, D.M. 2005. "Malformed Frogs in Minnesota: History and Interspecific Differences". Amphibian Declines: The Conservation Status of United States Species. Ed. M. Lannoo. University of California Press, Berkeley, CA, Los Angeles, CA, and London, England. 103-108.

Johnson, M.L. and R. Speare. 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of Aquatic Organisms. 65: 181-186.

La Marca, E., K.R. Lips, S. Lotters, R. Puschendorf, R. Ibanez, J.V. Rueda-Almonacid, R. Schulte, C. Marty, F. Castro, J. Manzanilla-Puppo, J.E. Garcia-Perez, F. Bolanos, G. Chaves, J.A. Pounds, E. Toral, and B.E. Young. 2005. Catastrophic population declines

and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). Biotropica. 37(2): 190-201.

Lampo, M., C. Barrio-Amoros, and B. Han. 2006. *Batrachochytrium dendrobatidis* infection in the recently rediscovered *Atelopus mucubajiensis* (Anura, Bufonidae), a critically endangered frog from the Venezuelan Andes. EcoHealth. 3(4): 299-302.

Leenders, T. 2001. A Guide to Amphibians and Reptiles of Costa Rica. Zona Tropical, S.A., Miami, FL. 305 pp.

Longcore, J.R., J.E. Longcore, A.P. Pessier, and W.A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management. 71(2): 435-444.

Longenecker, A.J. 2000. The life history of the cave salamander, *Eurycea lucifuga* Rafinesque, in West Virginia. Master's Thesis, Marshall University, Huntington, WV.

Morehouse, E.A., T.Y. James, A.R.D. Ganley, R. Vilgaly, L. Berger, P.J. Murphy, J.E. Longcore. 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. Molecular Ecology. 12: 395-403.

New South Whales National Parks and Wildlife Service. 2001. Hygiene protocol for the control of disease in frogs. Information Circular Number 6. NSW NPWS, Hurstville NSW.

Osbourn, M.S. 2005. The Natural History, Distribution, and Phenotypic Variation of Cave-dwelling Spring Salamanders, *Gyrinophilus* spp. Cope (Plethodontidae), in West Virginia. Master's Thesis, Marshall University, Huntington, WV.

Ouellet, M., I. Mikaelian, B.D. Pauli, J. Rodrigue, and D.M. Green. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology. 19(5): 1431-1440.

Pauley, T.K. 1993. Report of the Upland Vertebrates in the New River Gorge National River. Volume I-III. 1,119pp.

Petranka, J.W., L.B. Kats, and A. Sih. 1987. Predator-prey interactions among fish and larval amphibians: use of chemical cues to detect predatory fish. Animal Behavior. 35: 420-425.

Petranka, J.W., M.E. Eldridge, and K.E. Haley. 1993. Effects of timber harvesting on southern Appalachian salamanders. Conservation Biology. 7(2): 363-370.

Petranka, J.W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C. and London, England. 587 pp.

Pounds, J.A. and M.L. Crump. 1994. Amphibian declines and climate disturbances: the case of the golden toad and the harlequin frog. Conservation Biology. 8: 72-85.

Rachowicz, L.J. and V.T. Vredenburg. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. Diseases of Aquatic Organisms. 61: 75-83.

Rollins-Smith, L.A., L.K. Reinert, C.J. O'Leary, L.E. Houston, and D.C. Woodhams. 2005. Antimicrobial peptide defenses in amphibian skin. Integrative and Comparative Biology. 45: 137-142.

Rothermel, B.B., S.C. Walls, J.C. Mitchell, C.K. Dodd Jr., L.K. Irwin, D.E. Green, V.M. Vazquez, J.W. Petranka, D.J. Stevenson. 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. Diseases of Aquatic Organisms. 82: 3-18.

Stuart, S.N., J.S. Chanson, N.A. Cox, B.E. Young, A.S.L. Rodrigues, D.L. Fischman, and R.W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science. 306: 1783-1786.

Timpe, E.K., S.P. Graham, R.W. Gagliardo, R.L. Hill, M.G. Levy. 2008. Occurrence of the fungal pathogen *Batrachochytrium dendrobatidis* in Georgia's amphibian populations. Herpetological Review. 39(4): 447-449.

Vredenburg, V.T. 2004. Reversing introduced species effects: Experimental removal of introduced fish leads to rapid recovery of a declining frog. Proceedings of the National Academy of Sciences of the United States of America. 101(20): 7646-7650.

Vredenburg, V.T. and A.P. Summers. 2001. Field identification of chytridiomycosis in *Rana muscosa* (Camp 1915). Herpetological Review. 32: 151-152.

Wake, D.B. and V.T. Vredenburg. 2008. Are we in the midst of a sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences of the United States of America. 105(1): 11466-11473.

Weldon, C., L.H. du Preez, A.D. Hyatt, R. Muller, and R. Speare. 2004. Origin of the amphibian chytrid fungus. Emerging Infectious Diseases. 10(12): 2100-2105.

Woodhams, D.C., R.A. Alford, C.J. Briggs, M. Johnson, and L.A. Rollins-Smith. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. Ecology. 89(6): 1627-1639.