5-2010

Seasonality, variation in species prevalence, and localized disease for Ranavirus in Cades Cove (Great Smoky Mountains National Park) amphibians

Megan Todd-Thompson
mtoddtho@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Megan Todd-Thompson entitled "Seasonality, variation in species prevalence, and localized disease for Ranavirus in Cades Cove (Great Smoky Mountains National Park) amphibians." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.

Benjamin M. Fitzpatrick, Major Professor

We have read this thesis and recommend its acceptance:

Graham J. Hickling, Gary F. McCracken

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Megan Curry Todd-Thompson entitled "Seasonality, variation in species prevalence, and localized disease for *Ranavirus* in Cades Cove (Great Smoky Mountains National Park) amphibians." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.

                                  Benjamin M. Fitzpatrick, Major Professor

We have read this thesis
and recommend its acceptance:

Graham J. Hickling

Gary F. McCracken

                                  Accepted for the Council:
                                  Carolyn R. Hodges
                                  Vice Provost and Dean of the Graduate School

Seasonality, variation in species prevalence, and localized disease for *Ranavirus* in Cades Cove (Great Smoky Mountains National Park) amphibians

A thesis presented for the
Master of Science degree in
Ecology and Evolutionary Biology
University of Tennessee

Megan C. Todd-Thompson
May 2010

Acknowledgements

Abstract

World-wide amphibian declines sparked concern and encouraged investigation into potential causes beginning in the 1980's. Infectious disease has been identified as one of the major potential contributors to amphibian declines. For example, *Ranavirus* has caused amphibian die-offs throughout the United States. Investigators isolated *Ranavirus* from dead or moribund amphibians during large-scale die-offs of amphibians in the Cades Cove area of Great Smoky Mountains National Park in 1999-2001. In 2009, after nearly a decade without follow-up monitoring, I undertook an investigation to determine if the virus persisted in the area, and if so, to assess spatial, temporal, and taxonomic patterns in prevalence. Three amphibian breeding ponds, including Gourley Pond, the site of these earlier mortality events, were monitored for *Ranavirus* during the 2009 amphibian breeding season. A peak in prevalence occurred at Gourley Pond corresponding to a massive amphibian die-off. Prevalence varied among three different taxonomic groups during this mortality event with the highest prevalence, 84%, detected in larval Ambystomatids, 44.4% prevalence in adult Newts, and no virus detected in adult Plethodontids. I did not detect virus at either of the other two breeding ponds despite equivalent sampling effort, similar community composition, and close proximity to Gourley Pond. These results suggest that the severity and spatial extent of *Ranavirus* in Cades Cove remains unchanged since its initial detection a decade ago. Also, despite the observed massive die-offs there is no evidence of local amphibian extinction at Gourley Pond.

Table of Contents

List of Figures

List of Tables

Introduction

Amphibian declines have increased dramatically since the 1980's, and today nearly one third of amphibian species are threatened with extinction (Bailie *et al.* 2004). Suspected causes of these declines include habitat disturbance and destruction, introduced species, climate change, and disease (Collins & Storfer 2003). Disease is one of the main, but also least understood causes of amphibian declines (Cunningham *et al.* 1996, Daszak *et al.* 2003). Further understanding of disease ecology in natural populations of amphibians can provide insight into why amphibian populations experience disease outbreaks and how to lessen the impacts of disease.

Two main pathogens are thought to threaten amphibian populations. One is the chytrid fungal pathogen, *Batrachochytrium dendrobatidis*, associated with amphibian population declines and species extinctions in Central and South America (Daszak *et al.* 2003). The second, and the subject of this study, is a viral pathogen in the genus *Ranavirus*, family Iridoviridae. Amphibian die-off events attributed to *Ranavirus* occur sporadically throughout North America (Green *et al.* 2002), Australia, Great Britain, Europe, and South America (Hyatt *et al.* 2000). This virus has been documented in several diverse groups of amphibians including frogs, toads, salamanders, and newts (Chinchar 2002).

The double-stranded DNA viruses in the *Ranavirus* family cause systemic infection in fish, amphibians, and reptiles and have been detected in at least fifteen U.S. states (Green *et al.* 2002). Both naked and enveloped viral particles infect host cells, in the former through fusion with the host cell plasma membrane and in the latter through receptor mediated endocytosis (Chinchar *et al.* 2009). At the cellular level, *Ranavirus* inhibits cellular, protein, RNA, and DNA synthesis, and induces apoptosis (Chinchar *et al.* 2009). Virus particles show a strong

1

tropism for the proximal tubular epithelium of the kidney (Chinchar *et al.* 2009) but other organs are also infected. At the organismal level, infection manifests as hemorrhaging and necrosis in internal organs, often the kidney, liver, spleen, and intestines (Gray *et al.* 2009a). Affected animals often exhibit balance problems, erratic swimming, lethargy, lesions, and reddening of ventral skin and hindlimbs (Converse & Green 2005).

Two strains in the *Ranavirus* family, *Ambystoma tigrinum virus* (ATV) and Frog Virus 3 (FV3) cause most of the infections in amphibians in the United States (Chinchar *et al.* 2009). Mortality in pond-dwelling larval amphibians, where most virus-associated mortality is observed, can occur in excess of 90% during die-off events (Green & Converse 2005). In the Southwestern United States, viruses associated with mortality are typically identified as the ATV strain and affect mainly Tiger Salamanders (*Ambystoma tigrinum*) in die-off events (Jancovich *et al.* 2005). In the eastern United States, mortality events usually occur in communities with several amphibian species, most commonly Eastern Red-spotted Newts (*Notophthalmus viridescens*), Wood Frogs (*Rana sylvatica*), and Spotted Salamanders (*Amybstoma maculatum*) (Green *et al.* 2002). Viruses isolated from die-off events in this region are typically most similar to the Frog Virus 3 (FV3) strain (Chinchar *et al.* 2009).

Both FV3 and ATV strains of *Ranavirus* can infect multiple amphibian species and result in wide-ranging outcomes, from asymptomatic carrier states to lethal infections (Schock *et al.* 2008). Temperature (Rojas *et al.* 2005), host species (Schock *et al.* 2008), viral load , and host life-stage all influence infection outcome (Brunner *et al.* 2005). Additionally, exposure to toxins and pesticides (specifically atrazine) increase rates of mortality following *Ranavirus* exposure (Forson & Storfer 2006).

Transmission of the *Ranavirus* occurs through necrophagy and other types of direct contact with infected individuals, as well as contact with infected water or sediment (Harp & Petranka 2006). In the ATV/Tiger Salamander system, Brunner *et al.* (2004) documented that some of the adult Tiger Salamanders returning to their natal pond to breed were asymptomatic carriers for the virus; the authors propose that adults may serve as intraspecific reservoirs that infect naïve larvae and perpetuate the disease cycle year after year.

Since most studies of *Ranavirus* in amphibian populations have focused on extreme mortality events (Green *et al.* 2002) the mechanisms of viral persistence in a population outside of die-off events is not well understood. My study focuses on the Cades Cove area of the Great Smoky Mountains National Park (GSMNP), which has a documented history of this disease. A United States Geologic Survey (USGS) field crew conducting amphibian surveys as part of a Inventory and Monitoring Program documented mortality attributed to *Ranavirus* infection in amphibians at Gourley Pond, located in Cades Cove, in 1999, 2000, and 2001 (Green *et al.* 2002, Dodd 2003). A more thorough summary of their findings is presented in Appendix B During these events, affected species included marbled salamander (*Ambystoma opacum*) larvae, pickerel frog (*Rana palustris*) metamorphs, wood frog (*Rana sylvatica*) tadpoles, and Eastern Red-spotted Newts (*Notophthalmus viridescens*) (Green *et al.* 2002). During their extensive sampling, USGS personnel did not observe evidence of virus outbreaks at any other location in GSMNP (Dodd 2004). No die-off events have been reported at Gourley Pond since the conclusion on the USGS Inventory and Monitoring Program in 2001, but this most likely reflects lack of monitoring.

Given the past series of outbreaks, the Cades Cove amphibian community provides an ideal situation for investigating the potential of the virus to remain in a population following a

series of die-off events. During work completed in 2008, I did not detect virus in any samples

taken from salamanders collected at six streams in and around Cades Cove (176 individuals

sampled, corresponding to a prevalence of 5% or less with 95% confidence). This result was

surprising given that Gray *et al.* (2009b) detected virus in similar species in other areas of the

park in 2007. Lack of detectable virus in the 2008 samples also raised the question of whether

the virus was still present in the Cades Cove amphibian population. I targeted the location of

previous outbreaks during the 2009 amphibian breeding season in order to address the possibility

that *Ranavirus* might remain in the population and to assess the possibility that the virus was not

detectable elsewhere and may occur only in the Gourley Pond amphibian community.

Background

Temporal variation

Multiple pathogens in a variety of communities exhibit seasonality in disease outbreaks

(Altizer *et al.* 2006). Green *et al.* (2002) note the seasonal nature of *Ranavirus* infections,

specifically that in the United States 88% of mortality events analyzed and caused by the virus

begin in June, July, or August. Seasonality of viral outbreaks may occur as a function of biotic

factors such as variation in susceptibility during different life stages or temporal variation in

amphibian host abundance (Brunner *et al.* 2004). In particular, larval amphibians and those

undergoing metamorphosis may experience increased disease susceptibility due to

immunological characteristics. Based on the *Xenopus* model, larval anurans may not have fully

developed immune systems (Carey *et al.* 1999), and amphibians undergoing metamorphosis

experience temporary immunosuppression as a result of high levels of hormones that mediate

this transitional stage (Rollins-Smith 1998). Abiotic factors such as temperature can likewise

4

affect infection rates and also produce seasonal patterns. For Tiger Salamanders exposed to *Ranavirus* at 10, 18, and 26°C, the group at 18°C experienced the highest rate of morality, potentially indicating an interaction between the efficiency of virus replication at different temperatures and host immune function (Rojas *et al.* 2005). Natural populations of *Ambystoma tigrinum* in Arizona demonstrate temporal variation in *Ranavirus* prevalence, typically observed as one to two peaks in prevalence each year corresponding to high larval abundance (Greer *et al.* 2009). I expected that if the virus were still present I would witness at least one peak in prevalence during the breeding season.

Species variation

Mortality events documented in nature most frequently involve ambystomatid or anuran larvae and recent metamorphs (Green *et al.* 2002). Co-occurring species not generally involved in die-offs are not usually assessed for viral infection (pers. obs.). Laboratory exposure experiments demonstrate that different species of amphibians have variable rates of mortality following exposure to *Ranavirus* (Schock *et al.* 2008). Because dead and moribund species are usually the target of analysis, little is known about the possibility that additional species also experience infection. Amphibians from several distinct phylogenetic groups utilize ponds and the surrounding area. These groups vary in their life history, especially in the amount of time they spend in the pond. Amphibian anuran, ambystomatid, and newt larvae are confined to the pond. Adult newts spend time as adults both in the pond and on land. Finally, Plethodontidae includes both stream-dwelling and terrestrial salamanders that can be found within one meter of pond shorelines. I expected to see variation in prevalence between these groups as a result of

either variation in exposure due to group-specific habitat preference/requirements or variation in the animals' susceptibility to the virus.

Site variation

Experimental and observational evidence has begun to give a more detailed understanding of the spatial dynamics of *Ranavirus,* but we still do not completely understand how the virus moves through the landscape and between water bodies. Extremely localized outbreaks of *Ranavirus* have been documented even for ponds within a wetland complex characterized by high connectivity via animal movement (Petranka *et al.* 2007). The complex spatial dynamics for this virus are demonstrated in the ATV/tiger salamander system on the Kaibab Plateau in Arizona, where ponds located closest to each other had less similar patterns than more spatially distant ponds (Greer *et al.* 2009).

In Cades Cove, the USGS crew documenting the amphibian mortality at Cades Cove, conducted visual encounter surveys for tadpoles and salamanders at several other amphibian wetland breeding sites, including Methodist Church Pond and Gum Swamp. They did not observe any signs of disease at these other locations (Dodd 2004). Sampling for disease surveillance in Cades Cove at locations other than Gourley Pond did not occur prior to my study in 2009. However, lack of documented disease in other locations by the USGS field crew suggests a localized die-off at Gourley Pond. I sampled three sites, including Gourley Pond in the Cades Cove area to better understand the spatial extent of the disease in the Cades Cove amphibian community.

In order to better understand the disease ecology of *Ranavirus* in Cades Cove amphbibians, this study address three questions: Does prevalence vary 1) across the season, 2)

between taxonomic groups, and 3) between amphibian breeding sites? Understanding whether species, locations, and seasonality influence viral prevalence will increase the broad understanding of this disease and will also help to inform management decisions to prevent or mitigate population-level impacts of this amphibian pathogen.

Methods

<u>Sampling</u>

Several amphibian pond-breeding sites occur within Cades Cove. I chose to monitor

three ponds: Gourley Pond, Methodist Church Pond, and Gum Swamp to discern whether

disease occurs in additional ponds or if the *Ranavirus* die-offs are a phenomenon localized to

Gourley Pond. The sites sampled in this study are all within a 3 kilometers radius (Figure A-1)[1].

All three ponds have similar amphibian species composition, including the species involved in

past *Ranavirus* mortality events in Cades Cove (Table A-1). I compared the prevalence data

obtained during the 2009 breeding season at each of the three sites in order to assess the

possibility of a localized outbreak at Gourley Pond.

To detect temporal variation in prevalence, I monitored each site once per month

beginning in February 2009 and then every 10-14 days from May through September 2009.

Except on days of low amphibian abundance, tail tissues were collected from the first thirty

salamanders (adults and larvae) or tadpoles encountered. Table A-2 summarizes sample dates

and sizes for the three ponds. On days with low abundance, I searched for at least two person

hours (one hour with two people) and collected tail tissue from the amphibians I encountered, if

any. Because infection status of an individual can change temporally, I sampled previously tested

individually if they were encountered. I obtained tail tissue by placing each animal into a plastic

bag and applying gentle pressure through the plastic bag with the straight edge of a ruler. This

procedure induces the natural adaptation of salamanders and tadpoles to shed a portion of their

tail in order to evade predation. Salamanders and tadpoles autotomize their tail resulting in

---

[1] All figures and tables are located in the Appendix.

minimal blood loss and the tail tissue regenerates naturally. The target sample size of thirty

allows for detection of disease (with 95% confidence) when prevalence is at or above 10%

(Dohoo *et al.* 2003). To document variation in species prevalence, I collected tissue samples

from several distinct phylogenetic groups of amphibians that inhabit the area in and around

Gourley Pond during a period of high observed amphibian mortality attributed to *Ranavirus* on

May 19 and 22 of the 2009 field season.

Using tail tissue to detect disease provides an alternative to lethal sampling methods and

is frequently used to detect *Ranavirus* in host populations (Brunner *et al.* 2004). This sampling

method was appropriate in this case because of concern with minimizing impacts on the local

amphibian population in a national park and because of the large sample size required to address

my objectives. Greer *et al*. (2007) do show that tail clips have lower sensitivity than whole-

animal sampling methods. Less than five days following exposure tail clip samples tend to

underestimate prevalence (Greer & Collins 2007), (Figure A-2). The prevalence reported here

may underestimate true viral prevalence in the population, particularly immediately following

exposure. However, because I sampled so frequently it was not imperative for me to detect virus

immediately.   Sampling took place under National Park system permit # GRSM-2008-

SCI-0056 and all methods were approved by IACUC at the University of Tennessee (protocol #

1763).

<u>Virus detection</u>

Tail tissue was stored in 95% ethanol at -20°C until processing. I extracted DNA from

up to 25mg of tissue using the standard protocol for DNeasy (Qiagen) tissue extraction or a

standard salt-extraction protocol (Sambrook & Russell 2001). The DNeasy protocol was

followed as outlined in the DNeasy Blood and Tissue Handbook, except I eluted the DNA with

100µl of buffer AE instead of the standard 200µl. To extract DNA using the standard salt

extraction protocol, I incubated approximately 10 mg of tissue in 300 µl cell lysis buffer

(100mM NaCl, 100mM Tris-Cl, 25mM EDTA and 0.5%SDS) with 1.5 µl proteinase K

(20mg/mL) at 37ºC overnight. Samples were vortexed briefly following this incubation.

Following the addition of 100µl protein precipitate solution (4M Guanidine thiocyanate, 100mM

Tris-Cl), I centrifuged the samples at 13,000 rpm for 5 minutes, repeating this step when the

protein pellet was not tight. The supernatant (containing DNA) was removed to a clean tube via

aspiration with a pipetter. To precipitate the DNA I added 300µl 100% isopropanol and mixed

the tubes by inverting them gently 50 times. I discarded the supernatant following centrifugation

at 13,000 rpm for 5 min. The DNA was washed using 300 µl 70% chilled ethanol, tubes were

then inverted and dried overnight. I resuspended the DNA in 50µl of buffer AE (Qiagen). In

order to confirm extraction success, I quantified the amount of DNA using a NanoDrop™. I

used buffer AE (Qiagen) to further dilute samples with a high quantity of DNA to a final

concentration between 5 and 250 ng/ul.

In order to detect virus in the tissues, I used MCP 4 ("forward") and MCP 5 ("reverse")

primers (Mao *et al.* 1997), which amplify a highly conserved portion of the viral genome. Each

10µl reaction used reagents from the *GoTaq* Promega system and contained 2µl *GoTaq* flexi

buffer, 0.8µl 25mM McCl$_2$, 0.2µl dNTPs (2.5mM each nucleotide), 0.4µl each primer at 2.5µM

concentration, 1 unit of *Taq* polymerase, 5.0µl DNA grade water, and 1µl template DNA. All

reactions were carried out in duplicate and alongside positive and negative controls. DNA

extracted from skin of a known *Ranavirus* positive (by culture and previous PCR) anuran

obtained from the Veterinary Diagnostic and Investigational Laboratory at the University of

Georgia, College of Veterinary Medicine in Tipton, Georgia served as the positive control. Negative controls used DNA extracted from animal tissue that had never been exposed to *Ranavirus* or tested negative for virus in previous PCR's. *Ranavirus* detection PCR used the following thermocycler conditions: initial denaturation of 5 minutes at 95˚C followed by 35 cycles of 94˚C for 30 seconds, 56˚C for 30 seconds, and 72˚C for 30 seconds, ending with a final annealing step at 72˚C for 2 minutes, and then held at 4˚C until tubes were removed and stored at -20˚C or visualized on an agarose gel.

PCR products were visualized following standard protocol on a 2% agarose gel to detect the presence of a 500 base pair band. For ambiguous samples, when the duplicate reaction did not confirm the initial result, I re-extracted the DNA and ran a third PCR for that sample. A subset of negative samples (75) were tested in PCR reactions with the standard Eukaryote primers 1427F and 1616R (van Hannen *et al.* 1998) to verify that negative results were not caused by failed extraction or contamination with PCR-inhibiting chemicals.

To verify virus identification and evaluate whether different strains might be affecting different populations, a selection of positive PCR products were directly sequenced using the MCP-specific PCR primers. In addition, for the same samples, I sequenced two genome regions used by Jancovich *et al.* (2005) to evaluate variation in ATV strains (primer sets 16F and 16R and 100F and 100R). For the 100F/100R primer set, I used 1.8µl of 10x *GoTaq* flexi buffer, 2.16µl $MgCl_2$, 0.36µl dNTP's (10mM concentration each nucleotide), 1.5µl of each primer (at 10µM concentration), 1.5units of *TAQ* polymerase, 0.38µl BSA, 1.5µl of template DNA and DNA-grade water up to a 18µl total volume. The reagent volumes were identical for PCR reactions with the 16F/16R primer set except that I only added 1.44µl of $MgCl_2$. Thermocycler conditions for both regions were as follows: initial denaturation of 2 minutes at 94˚C followed by

11

30 cycles of 94˚C for 15 seconds, 55˚C for 15 seconds, and 72˚C for 15 seconds, ending with a final annealing step at 72˚C for 1 minute, and then held at 4˚C. All sequencing was performed at the University of Tennessee Molecular Biology Resource Facility on an ABI 3730.

Data analysis

Temporal variation in prevalence at Gourley Pond (the only location where I detected virus) was explored using a logistic regression model specifying a quadratic function of time as the predictor for prevalence. The following equation was used:

$$\log[p/(1\text{-}p)] = \beta_0 + \beta_1\,t + \beta_2\,t^2$$

Where p is the probability of an amphibian testing positive for virus and t is time expressed in Julian days (days after January 1, 2009). Logistic regression was fitted using the glm function in R (www.r-project.org). This is a purely phenomenological approach intended to distinguish constant prevalence (no relationship between time and virus detection), a steady increase or decrease in prevalence (significant linear regression or significant quadratic without a local maximum or minimum), or a temporal spike in prevalence (significant quadratic regression with a local maximum within the range of the data). The constant (β_0 only), steady change (β_0 and β_1), and quadratic (β_0, β_1, and β_2) models were compared using Akaike's Information Criterion (AIC). Comparison of the AIC values for these models provides support for the model with the minimum AIC value when the minimum AIC value is ten or more less than the AIC values for the other models (Burnham & Anderson 2002). This analysis was performed with all samples, and also with Plethodontids removed, because Plethodontids were collected on only one sampling date.

To test for variation in prevalence among ecological/phylogenetic groups, I categorized samples from the die-off period into one of three different groups: adult newts, larval ambystomatids, and plethodontids. I did not include anuran amphibians in this comparison because they did not occur in sufficient numbers during the dates of the die-off. Variation in prevalence between groups was analyzed using a contingency table randomization test, in place of a χ^2 test, to avoid the possibility of bias due to low expected counts (McDonald 2009). Fisher's exact tests were used post-hoc to explore variation between pairs of groups.

Finally, I used a contingency table randomization test to analyze the association between site and number of positive or negative amphibian samples. Similar to the methods specified above for detecting variation among phylogenetic groups, Fisher's exact tests were used to examine variation between pairs of sites.

. All analyses were carried out using R (http://www.R-project.org).

Results

<u>Mortality event</u>

I observed several hundred dead or dying larval ambystomatids on 19 and 22 May, 2009 at Gourley Pond and refer to these dates, hereafter, as the mortality event. Dead animals were also observed at Gourley Pond on June 4, although in smaller numbers (fewer than 100). This mortality coincided with a marked increase in amphibian abundance/activity (Tables A-2 & 3).

<u>Temporal variation</u>

I first detected virus at Gourley Pond in samples from Aril 30. Viral prevalence at this site reached 80% on May 19, 2009. Virus was detected at declining levels on 22 May and 4 June. I did not detect virus in samples collected after 4 June. Table A-3 provides a summary of individuals positive for each species sampled on each sampling date at Gourley Pond. Prevalence demonstrated temporal variation as seen in Figure A-3. A logistic regression with time as the predictor variable models the prevalence data well both with (Fig. A-3a) and without (Fig A-3b) including plethodontid salamanders in the analysis. In both cases, the date and the square of the date are significant predictors of infection status (all p-values <0.0001 Table 4c). Figure 3a shows the fitted predicted curve of the logistic regression with the actual data plotted as hollow circles. Error bars represent the 95% confidence interval for the predicted prevalence for each sampling date (from the predict.glm function in R). Table A-4a and A-4b list the AIC values for a null model, the model with time as a linear predictor, and with a quadratic function of time. The quadratic, which uses both time and the square of time to predict prevalence, has the lowest AIC. This pattern is true with and without Plethodontids. By the standards given in Burnham and Anderson (2002), when ΔAIC is greater than 10 (where $\Delta AIC_i = AIC_i - AIC_{minimum}$),

14

the model being considered, here a model without day or the square of day, has essentially no support and fails to explain substantial variation in prevalence. Parameter estimates for these variables in the quadratic regression model are given in Table A-4c (p-values all < 0.0001).

Species variation

During the course of this 2009 study I detected virus in tissues from larval Spring Peepers (*P. crucifer*), Upland Chorus Frogs (*P. feriarum*), Wood Frogs (*R. sylvatica*), Marbled Salamanders (*A. maculatum*), Spotted Salamanders (*A. oppacum*), and adult Eastern Red-Spotted Newts (*N. viridescens*) collected from Gourley Pond (Table A-3).

During the observed mortality event at Gourley Pond, three distinct phylogenetic groups: larval ambystomatids, adult Plethodontids, and adult newts were sampled. Figure A-4 displays the prevalence observed during the die-off differed among these three co-occurring groups (χ^2=25.7 randomization p-value < 0.0001). Fisher's exact test confirmed that each group differs significantly from the others (Fig. A-4). Prevalence was highest in the ambystomatid larvae at 84%. I did not detect virus in any of the ten individuals in the plethodontid group. Five of the nine adult newts sampled tested positive for the virus giving a prevalence of 44%. The other species in which I detected virus are listed in Table 3 but were not included in the comparison due to the small sample size for these species during the mortality event.

Site variation

Figure 5 displays the prevalence detected at the three different sampling sites during the mortality event at Gourley Pond. Virus was not detected in any of the samples from Methodist Church Pond or Gum Swamp. Prevalence intervals for the highest observed prevalence represent 95% Clopper-Pearson confidence intervals based on a binomial distribution.

15

Prevalence reached 80% in the pond-breeding amphibians tested on 19 May at Gourley Pond during the observed mortality event. The χ^2-value comparing prevalence at the three sites was 67.9 with a corresponding randomization p-value of <0.0001 (Fig. A-5). Fisher's exact test gives p-values < 0.0001 for comparing Gourley Pond prevalence to the other two sites.

Viral strain identification

I sequenced three different regions of the virus extracted from six tail clips taken from two Wood Frog tadpoles, a Spring Peeper tadpole, an adult Newt, and a Spotted Salamander larva at Gourley Pond. All homologous sequences were identical and matched closely with the FV3 virus reference genome (accessible on Genbank). Each sequenced region differed from the FV3 reference by exactly one base pair (Table A-5).

Amphibian community composition

Amphibian community composition was similar at all three sites. Table A-1 summarizes the species of pond-breeding amphibians found (and tested for *Ranavirus*) at each of the sites during the 2009 breeding season. Of the pond-breeding species documented from Gourley Pond based on their surveys from 1998-2001 (Dodd 2004), I did not find Northern Green Frogs (*R. clamitans*) or Pickerel Frogs (*R. palustris*), American Bullfrog (*R. catesbeiana*) or Fowler's Toad (*B. fowleri*), at one or more of the three sites sampled

Discussion

Surprisingly, the results of this study suggest the severity and spatial distribution of the impacts of *Ranavirus* on amphibians in Cades Cove have not changed over a decade. I detected virus only in Gourley Pond despite good statistical power to detect even modest prevalence at two neighboring breeding sites (Methodist Church Pond n=274 corresponding prevalence of ≤ 1.34%, and Gum Swamp n=185 corresponding prevalence of ≤ 2% with 95% confidence). Detection of virus was tightly associated with a brief die-off of hundreds of Ambystomatid and Anuran larvae. Following the documentation of dramatic amphibian die-offs in Gourley Pond in 1999-2001, to my knowledge monitoring of these ponds did not continue again until my work in 2009. The pattern reported here answers the three questions regarding temporal, species, and site variation in prevalence but also raises several additional questions critical for disease ecology, evolution, and conservation.

Temporal Variation

I observed seasonal variation in prevalence of *Ranavirus* in Gourley Pond over the 2009 amphibian breeding season (Fig. A-3). Qualitatively, prevalence followed a typical infectious disease epidemic curve. In my study, the highest observed prevalence of virus coincided with the presence of large numbers of larval amphibians in the community. Fluctuations in larval abundance may account for the observed variation in prevalence because larvae tend to have greater susceptibility to *Ranavirus* than adults (Brunner *et al.* 2004), and transmission rates are likely density-dependent (Greer *et al.* 2008). Temperature may also contribute to the seasonality of the virus. Average daily ambient temperature is plotted along with the prevalence pattern

17

observed at Gourley Pond (Fig. 5). A notable temperature effect would require further monitoring to reveal patterns in temperature and peaks in prevalence.

Species variation

This study also documents variation in observed viral prevalence between ecological/phylogenetic groups (Fig. 4. The statistically significant variation in prevalence between the three distinct phylogenetic groups at Gourley Pond may reflect the groups' different rates of exposure or susceptibility to the virus. Plethodontid salamanders were found outside the pond (within one meter) and did not have detectable levels of virus. In contrast, larval ambystomatids are confined to the pond and would have much higher rates of exposure to infected water or sediment in a pond with virus than the stream-dwelling or terrestrial plethodontids. Prevalence among adult newts was 0.44 during the mortality event, although no gross signs of disease or mortality were observed in this group. Newts were, however, observed eating decomposing larval ambystomatids and presumably had levels of exposure to the virus similar to the larval ambystomatids. None of the Newts sampled had gross signs of disease and, as mentioned previously, may be good candidates as asymptomatic carriers of the virus.

Variation in detected prevalence between species may reflect both variation in exposure and susceptibility to *Ranavirus*. Amphibian species have different breeding times and those breeding either before or after virus outbreaks would have much lower rates of exposure. Also, exposure experiments conducted in the laboratory indicate that species vary both in their susceptibility to the virus and in their ability to recover from infection (Schock *et al.* 2008).

The observed die-off confirms that the virus remains active in the Cades Cove area and prompts investigation into what conditions allow for the persistence of virus between outbreaks.

Harp and Petranka (2006) showed that the virus remained viable in moist sediment and water but dried infected sediment did not cause disease in Wood Frogs. The hydrology of Gourley Pond makes viral persistence in the environment an unlikely possibility. Gourley Pond was dry from July through December 2009. This dry period likely occurs every year and makes survival of the virus more likely in an animal reservoir than in the environment.

Identifying a potential reservoir candidate is an interesting and complex problem in such a speciose amphibian community. Newts have several characteristics that suggest they may fulfill the role of asymptomatic carriers. Newts have detectable levels of virus at least seasonally, they have not been observed dying from the virus in large numbers, and unlike ambystomatids and anurans that leave the ponds following metamorphosis, they tend to remain in ponds throughout the breeding season.

Site variation

Although a decade has passed since the initial detection of viral presence in Cades Cove, the pattern I observed during the 2009 breeding season suggests that the spatial distribution of disease remains the same. As summarized in Smith and Green (2005), during their lifetimes Marbled Salamanders can travel at least 1 km, Eastern Red-Spotted Newts at least 1 km, and Northern Green Frogs 4.8 km, and Wood Frogs 2.5 km. The neighboring breeding ponds exist within the range of pond-breeding amphibian dispersal and I tested species susceptible to *Ranavirus* at these sites as well (Table A-1). Even though animals almost certainly move between these ponds, the disease remains localized to Gourley Pond (Fig. A-5).

Although sampling did not take place at Gum Swamp during the month of May, if the virus had been present at this site and followed a pattern similar to that observed for Gourley

Pond I would have detected virus in that community as well. These other ponds may be almost entirely free of *Ranavirus*, or it might be that the virus is common throughout Cades Cove but detectable only in very sick animals. In the latter case, we are still left with the question of why disease-associated die-offs appear to occur only at Gourley Pond. Investigation into abiotic factors that increase susceptibility, particularly factors that may cause increased stress and reduced immune function, would help to explain why some ponds are affected while others are not. For example, in Tiger Salamanders, vegetation patterns influence disease dynamics (Greer & Collins 2008) and pesticide exposure increases the susceptibility of Tiger Salamander larvae *Ranavirus* (Forson & Storfer 2006). Understanding viral persistence and spread in addition to what mediates its acute health effects can inform management to reduce population and community-level impacts.

Documenting a viral outbreak at Gourley Pond ten years following initial reports of mortality suggests that these die-offs may have occurred annually for the past decade. The USGS crew that documented the mortality events in the early part of the decade concluded their work in 2001. Although Gourley Pond is located in a highly visited area of the park, it sits far enough from the road that the casual tourist would rarely happen upon it. Also, because amphibians frequently engage in necrophagy and dead amphibians decompose quickly in aquatic environments, evidence of mortality lasts only for a short duration.

Observation of the 2009 die-off and the lack of monitoring between 2001 and 2009 raise the possibility that die-offs of hundreds of amphibians have occurred at this site on an annual basis for over 10 years. Yet, amphibian populations at Gourley Pond persist. Specific monitoring of population dynamics must be carried out in order to understand the population-level effects of recurring disease in this ecosystem. It is possible that the virus does not reduce

populations enough to cause local extinction, or that the system functions as part of a metapopulation, and animals from unaffected ponds colonize and maintain the location population at Gourley Pond. Pond-breeding amphibians have incredibly high rates of pre-metamorphic mortality. Studies summarized by Wells (2007), demonstrate that Wood Frogs and Spotted Salamanders frequently experience premetamorphic mortality rates of 95% or greater. Thus, the mortality rates caused by *Ranavirus* may not exceed typical rates in virus-free years or ponds.

Discriminating declines from natural population fluctuations of amphibians requires long-term study. In fact, during twelve years of monitoring at a pond in South Carolina several species of Ambystomatids exhibited low to negligible levels of juvenile recruitment for several years (Pechmann *et al.* 1991). The authors analyzed annual precipitation in addition to amphibian demographic information, concluding that the population was experiencing natural fluctuations caused by drought rather than population decline (1991). A model testing the risk of extinction at the same site studied by Pechman *et al.* demonstrated that rates of terrestrial survival in Marbled Salamanders of 0.6 can maintain local populations even when the model includes frequent catastrophic reproductive failure (Taylor *et al.* 2005).

The virus most certainly acts as a strong selective force, at least locally. Possible outcomes of recurring infection include eventual reduction in virulence of the pathogen or increased resistance in the host population. Greer *et al.* (2009) speculate that increased resistance in hosts or reduced pathogen virulence has occurred in Arizona leading to detection of virus without any observed mortality in the host population. Continued monitoring of the amphibian populations will help answer questions about what happens when a population endures repeated pathogen outbreaks.

Using tail tissue for viral detection is a limitation in this study. I detected virus in tail clips only a few weeks prior to observing a massive mortality event. Using tail clips to detect infection is not ideal as the test can detect virus only once the virus becomes systemic in the animal. Testing liver and kidney gives a more accurate indication of infection status particularly during early stages of infection when tail clips can yield false negative results (Greer & Collins 2007). Unfortunately testing liver and kidney tissue requires lethal sampling and is not realistic for conducting surveillance when amphibian conservation is a priority. The observed prevalence may not reflect the actual viral prevalence in the population especially if animals have sublethal infections and only very low levels of virus. Inability to detect low levels of virus also makes it difficult to identify potential reservoirs that may carry the virus and infect other animals without showing signs of disease. Actual prevalence may be higher than that reflected in these results and more sensitive whole animal sampling or more sensitive molecular techniques such as quantitative PCR could give a more accurate picture of true prevalence. Nevertheless, my study demonstrated that variation in relative detection rates is highly informative regarding the association of *Ranavirus* with disease and variation in impact across species, time, and space.

Future studies could benefit from the use of antibody tests to detect virus exposure, potentially answering some of the questions about whether or not lack of prevalence in other communities reflects variation in exposure or variation in resistance. Currently, amphibian immune responses to *Ranavirus* are not well studied, with the exception of the well-characterized and understood laboratory organism *Xenopus laevis* (Gantress *et al.* 2003). While *X. laevis* does produce detectable levels of antibodies when exposed to *Ranavirus*, using this antibody test in a natural population would require an understanding of immune function in each amphibian species and life-history phase to know that the test would accurately demonstrate viral exposure.

This study suggests that the spatial extent of mortality events has not changed over a decade. Secondly, the continued presence of amphibian populations at Gourley Pond suggests that no species have experienced obvious local extinction. The factors that preclude disease manifestation in proximal ponds remain unclear. Questions also remain about the abiotic or biotic factors that may make Gourley Pond amphibians more susceptible to *Ranavirus* and the population-level impact of the virus on these amphibians.

References

Altizer S, Dobson A, Hosseini P, Hudson P, Pascual M, Rohani P (2006) Seasonality and the dynamics of infectious diseases. Ecology Letters 9:467-484

Bailie JEM, Hilton-Taylor C, Stuart SNe (2004) 2004 IUCN Red List of Threatened Species: A Global Species Assessment

Brunner JL, Richards K, Collins JP (2005) Dose and host characteristics influence virulence of ranavirus infections. Oecologia 144:399-406

Brunner JL, Schock DM, Davidson EW, Collins JP (2004) Intraspecific reservoirs: Complex life history and the persistence of a lethal ranavirus. Ecology 85:560-566

Burnham KP, Anderson DR (2002) Model selection and multimodel inference: a practical information-theoretic approach. Springer Science+Business Media, Inc., New York

Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. Developmental and Comparative Immunology 23:459-472

Chinchar VG (2002) Ranaviruses (family Iridoviridae): emerging cold-blooded killers - Brief review. Archives of Virology 147:447-470

Chinchar VG, Hyatt A, Miyazaki T, Williams T (2009) Family Iridoviridae: Poor Viral Relations No Longer. In: Lesser Known Large Dsdna Viruses, Vol 328, p 123-170

Collins JP, Storfer A (2003) Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9:89-98

Converse KA, Green DM (2005) Diseases of salamanders. In: Majumdar SK, Huffman JE, Brenner FJ, Panah AI (eds) Wildlife diseases: landscape epidemiology, spatial distribution, and utilization of remote sensing technology. The Pennsylvania Academy of Science, Easton, PA, p 118-130

Cunningham AA, Langton TES, Bennett PM, Lewin JF, Drury SEN, Gough RE, MacGregor SK (1996) Pathological and microbiological findings from incidents of unusual mortality of the common frog (Rana temporaria). Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 351:1539-1557

Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. Diversity and Distributions 9:141-150

Dodd KC (2003) Monitoring amphibians in Great Smoky Mountains National Park. USGS Circular 1258

Dodd KC (2004) The Amphibian of Great Smoky Mountains National Park. University of Tennessee Press, Knoxville

Dohoo I, Martin W, Stryhm H (2003) Veterinary epidemiologic research. AVC Inc., Charlottetown, Prince Edward Island, Canada

Forson DD, Storfer A (2006) Atrazine increases ranavirus susceptibility in the tiger salamander, Ambystoma tigrinum. Ecological Applications 16:2325-2332

Gantress J, Maniero GD, Cohen N, Robert J (2003) Development and characterization of a model system to study amphibian immune responses to iridoviruses. Virology 311:254-262

Gray MJ, Miller DL, Hoverman JT (2009a) Ecology and pathology of amphibian ranaviruses. Diseases of Aquatic Organisms 87:243-266

Gray MJ, Miller DL, Hoverman JT (2009b) First report of *Ranavirus* infecting lungless salamanders. Herpetological Review 40:316-319

Green DE, Converse KA, Schrader AK (2002) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. In: Domestic Animal/Wildlife Interface:

Issue for Disease Control, Conservation, Sustainable Food Production, and Emerging Diseases, Vol 969, p 323-339

Green DM, Converse KA (2005) Diseases of amphibian eggs and embryos. In: Majumdar SK, Huffman JE, Brenner FJ, Panah AI (eds) Wildlife diseases: landscape epidemiology, spatial distribution, and utilization of remote sensing technology. Pennsylvania Academy of Science, Eaton, p 118-130

Greer AL, Briggs CJ, Collins JP (2008) Testing a key assumption of host-pathogen theory: density and disease transmission. Oikos 117:1667-1673

Greer AL, Brunner J, L., Collin JP (2009) Spatial and temporal patterns of *Ambystoma tigrinum* virus (ATV) prevalence in tiger salamanders *Ambystoma tigrinum nebulosum*. Diseases of Aquatic Organisms 85:1-6

Greer AL, Collins JP (2007) Sensitivity of a diagnostic test for amphibian ranavinus varies with sampling protocol. Journal of Wildlife Diseases 43:525-532

Greer AL, Collins JP (2008) Habitat fragmentation as a result of biotic and abiotic factors controls pathogen transmission throughout a host population. Journal of Animal Ecology 77:364-369

Harp EM, Petranka JW (2006) Ranavirus in wood frogs (Rana sylvatica): Potential sources of transmission within and between ponds. Journal of Wildlife Diseases 42:307-318

Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J, Coupar BEH (2000) Comparative studies of piscine and amphibian iridoviruses. Archives of Virology 145:301-331

Jancovich JK, Davidson EW, Parameswaran N, Mao J, Chinchar VG, Collins JP, Jacobs BL, Storfer A (2005) Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. Molecular Ecology 14:213-224

Mao J, Hedrick RP, Chinchar VG (1997) Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229:212-220

McDonald JH (2009) Handbook of biological statistics (2nd ed.). Sparky House Publishing, Baltimore, MD

Pechmann JHK, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons JW (1991) Declining Amphibian Populations: The Problem of Separating Human Impacts from Natural Fluctuations. Science 253:892-895

Petranka JW, Harp EM, Holbrook CT, Hamel JA (2007) Long-term persistence of amphibian populations in a restored wetland complex. Biological Conservation 138:371-380

Rojas S, Richards K, Jancovich JK, Davidson EW (2005) Influence of temperature on Ranavirus infection in larval salamanders Ambystoma tigrinum. Diseases of Aquatic Organisms 63:95-100

Rollins-Smith LA (1998) Metamorphosis and the amphibian immune system. Immunological Reviews 166:221-230

Sambrook J, Russell DW (2001) Molecular Cloning: a laboratory manual, 1. Cold Springs Harbor Laboratory Press, Cold Spring Harbor

Schock DM, Bollinger TK, Chinchar VG, Jancovich JK, Collins JP (2008) Experimental evidence that amphibian ranaviruses are multi-host pathogens. Copeia:133-143

Smith MA, Green DM (2005) Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? Ecography 28:110-128

Taylor BE, Scott DE, Gibbons JW (2005) Catastrophic reproductive failure, terrestrial survival, and persistence of the marbled salamander. CONSERVATION BIOLOGY 20:792-801

van Hannen EJ, van Agterveld MP, Gons HJ, Laanbroek HJ (1998) Revealing genetic diversity of eukaryotic microorganisms in aquatic environments by denaturing gradient gel electrophoresis. Journal of Phycology 34:206-213

Wells KD (2007) Amphibians and their predators. In: The ecology and behavior of amphibians. University of Chicago Press, Chicago, p 684
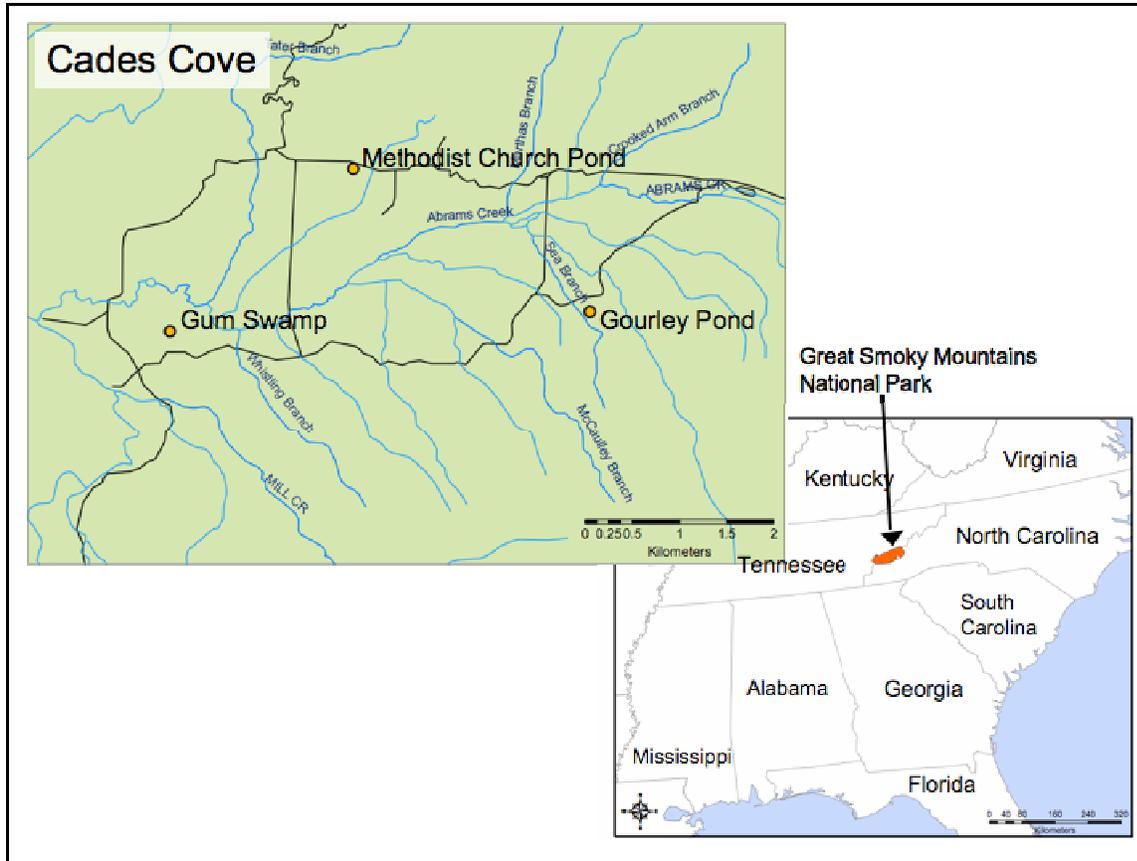
Appendix A

Figure A- 1.

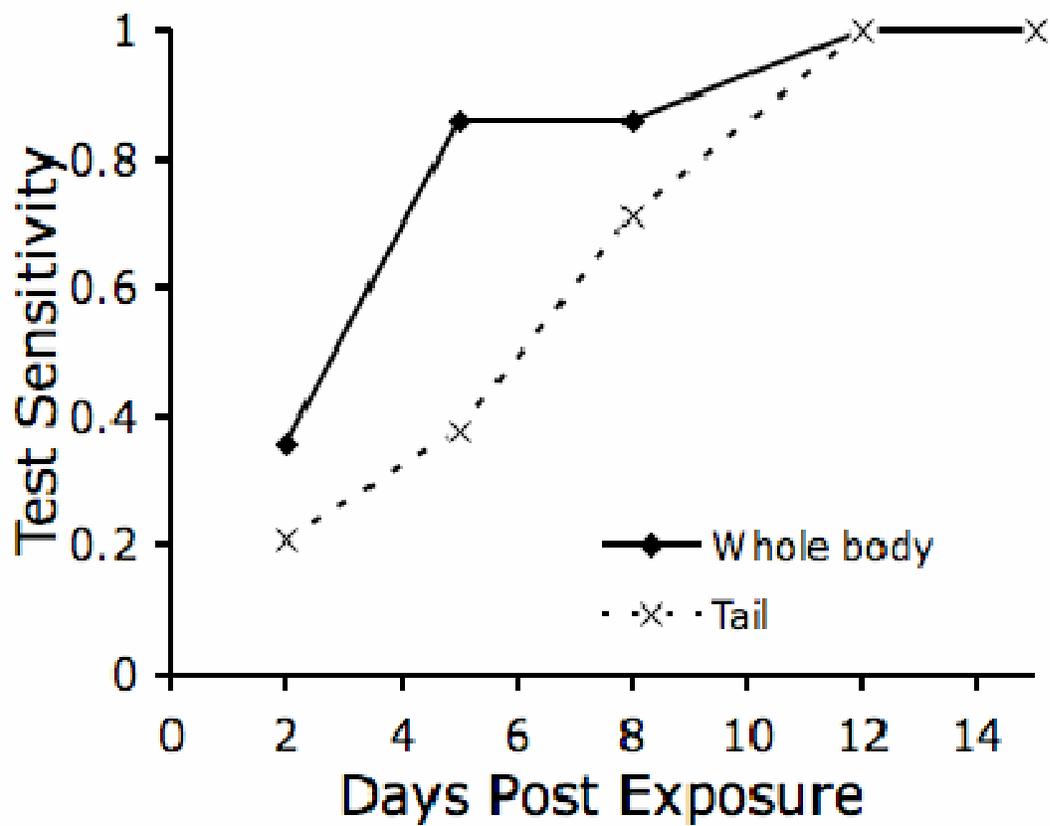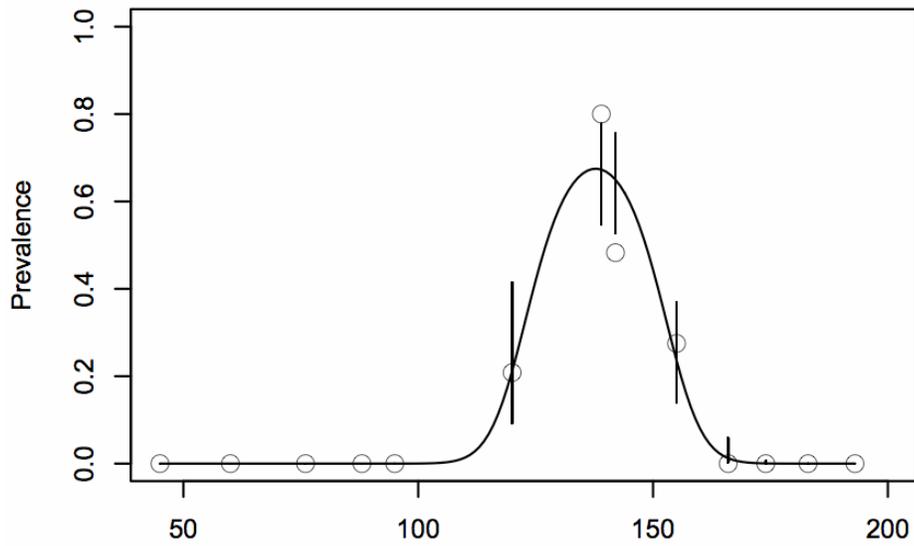Map of sample sites in the Cades Cove region of Great Smoky Mountains National Park.

Figure A- 2.

Adapted from Greer and Collins (2007). Sensitivity of using PCR to detect *Ranavirus* in tail tissue versus using a lethal, whole body sampling protocol.

a.)

b.)
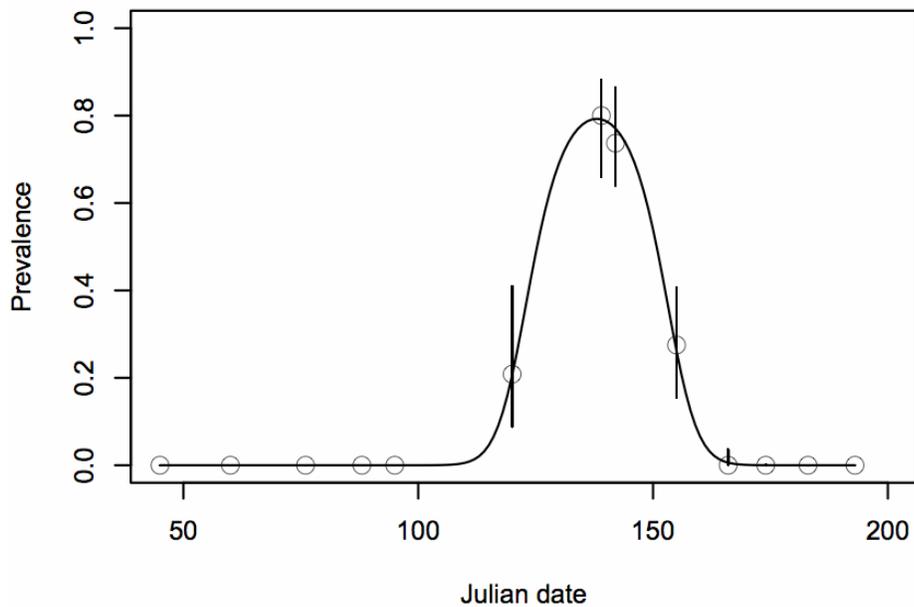
Figure A- 3.

The fitted predicted curve of the logistic regression using the quadratic of time as the predictor variable for infection status, with the predicted peaking occurring at Julian day 139 (May 19). Observed data are plotted as hollow circles. Error bars represent 95% confidence intervals for predicted prevalence for each sample date. Graph a.) includes plethodontid salamanders whereas b.) includes only pond-dwelling amphibians. Equation and associated AIC values are given in table 2a and 2b. Parameter estimates for the variables in the logistic regression equations are given in table 2c.
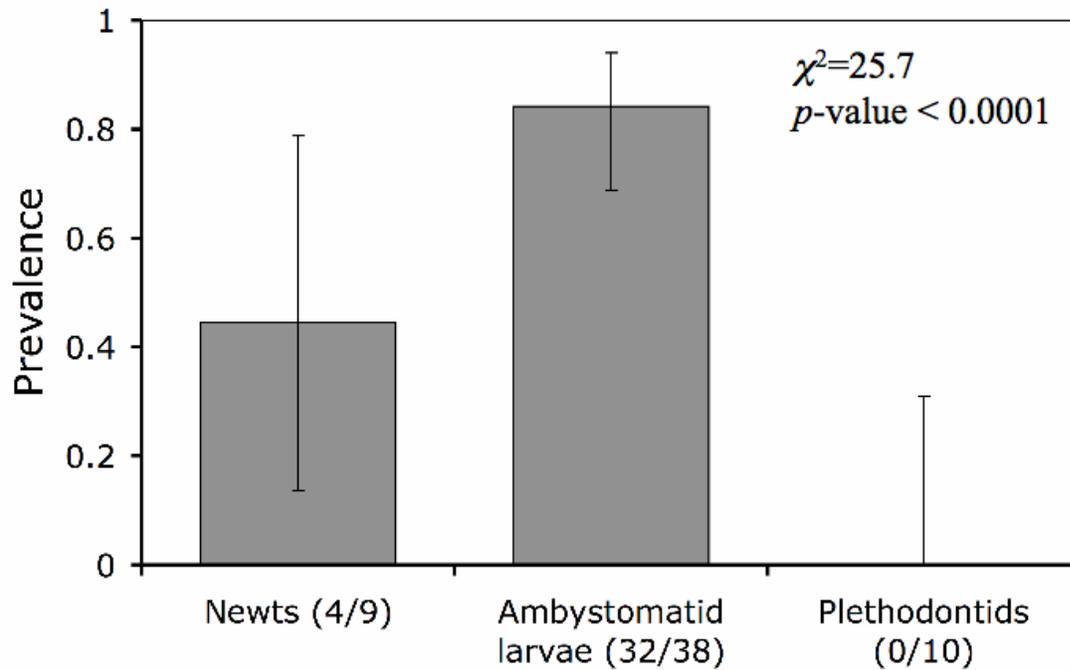
Figure A- 4.

Prevalence of *Ranavirus* in three distinct taxonomic groups sampled during the 19 and 22 May mortality event at Gourley Pond. Error bars were calculated separately for each group using a Clopper-Pearson 95% confidence interval. Each group differs significantly from the others (*p*-value < 0.05 for each comparison using Fisher's Exact Test).
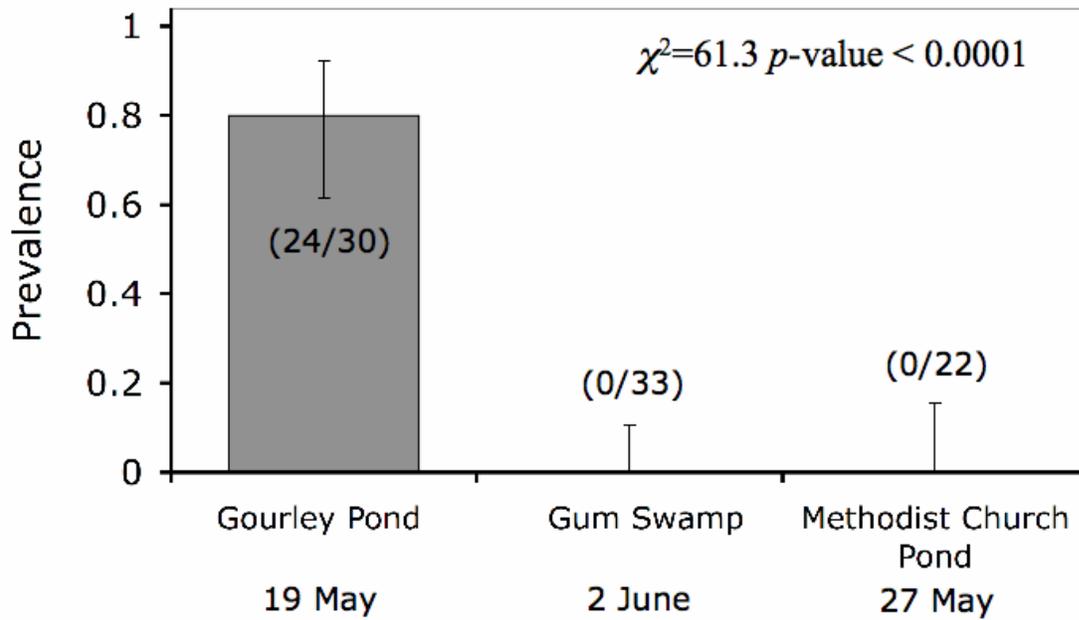
Figure A- 5.

Highest observed prevalence for the 2009 amphibian breeding season at each of the three sites monitored. Prevalence at Gourley Pond peaked at 80% but virus was not detected at either of the other two sites. The displayed 95% confidence intervals are Clopper-Pearson confidence intervals based on the sample size for the specified date at each site. Prevalence at Gourley Pond differed significantly from the other two ponds (Fisher's Exact Test all $p < 0.0001$).
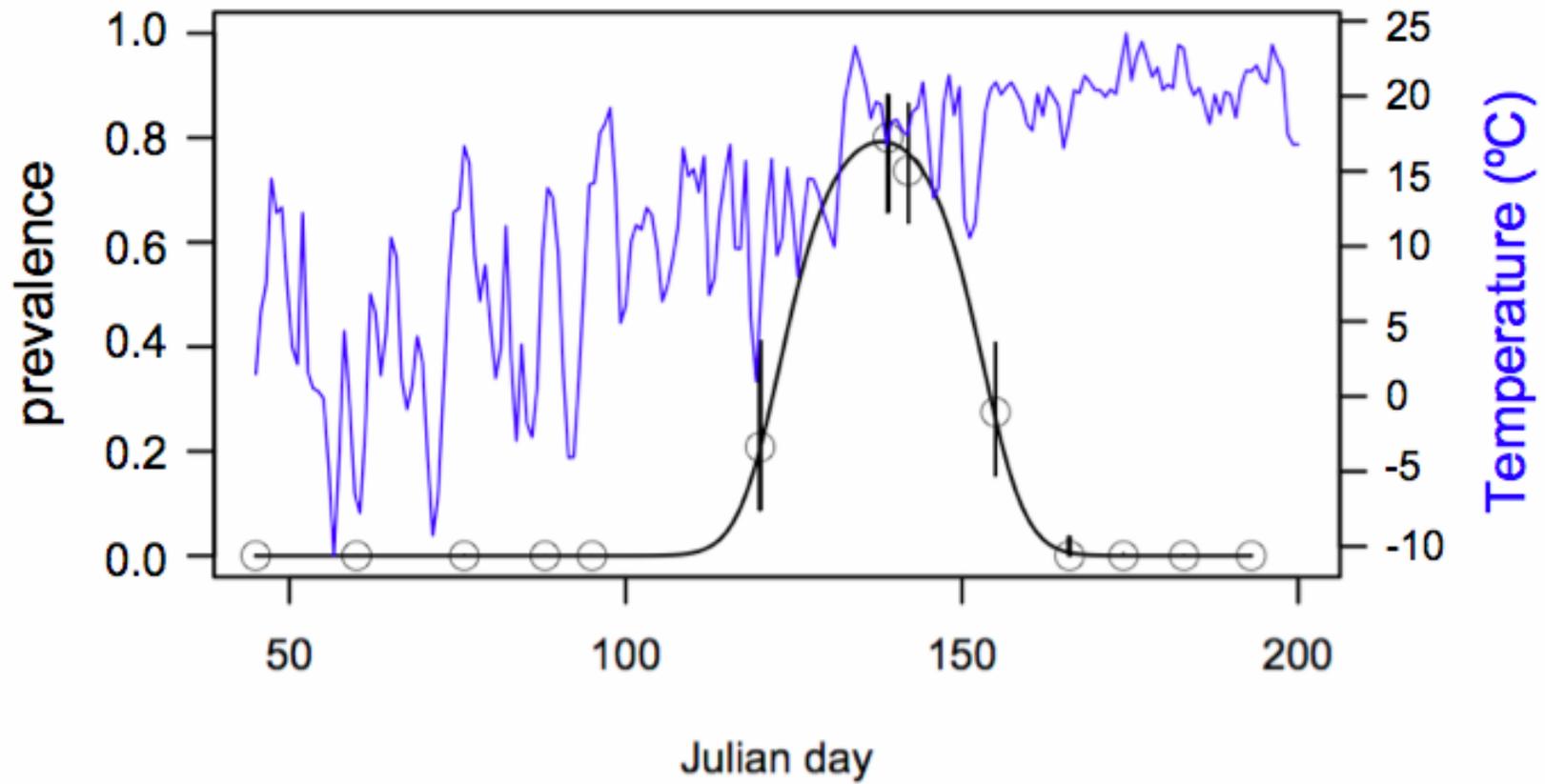
Figure A- 6

Average ambient daily temperature plotted in blue along with *Ranavirus* prevalence at Gourley Pond from mid-February through July 2009.

Table A- 1

Pond-breeding species occurrence by site. An X designates individuals from this species were both observed and tested for Ranavirus. An O indicates that the species was observed but not sampled for virus, and R indicates that this species has been documented at the location (Dodd 2004) but was not observed during the 2009 sampling period.

	Gourley Pond	Gum Swamp	Methodist Church Pond
A. maculatum	X	X	X
A. opacum	X	X	O
P. crucifer	X	X	X
P. feriarum	X	R	R
N. viridescens	X	X	X
R. sylvatica	X	X	X
H. chryoscleris	X	X	X
R. catesbiana	R		O
R. clamitans	R	X	X
R. palustris	R	X	X
B. americanus	O	O	R
B. fowleri	R	R	R

Table A- 2

Sample sizes and dates for each of the three sites. Numbers are [individuals tested positive]/[total number collected] for that date.

Pond Site	Sample Date		Pos/Total
Gourley Pond	Feb	14	0/5
	Mar	1	0/2
		17	0/22
		29	0/1
	Apr	5	0/1
		30	5/24
	May	19	24/30
		22	14/29
	June	4	11/40
		15	0/34
		23	0/30
	July	2	0/30
		12	0/3
	Total		**54/251**
Methodist Church Pond	Mar	1	0/3
		17	0/2
		29	0/1
	Apr	5	0/6
	May	5	0/19
		27	0/22
	June	11	0/34
		27	0/37
	July	15	0/32
		25	0/30
	Aug	8	0/28
		22	0/30
	Sept	13	0/30
	Total		**0/274**
Gum Swamp	Feb	14	0/4
	Mar	29	0/1
	Apr	5	0/5
	June	2	0/33
		16	0/32
		30	0/28
	July	15	0/20
		25	0/29
	Aug	8	0/30
		23	0/3
	Sept	13	0/0
	Total		**0/185**

Table A- 3

For each sampling date [number individuals teseted positive for *Ranavirus*]/[total number individuals sampled] at Gourley Pond in 2009.

Date	Ambystomatid larvae	Ambystomatid adult	*R. sylvatica* tadpoles	*N. viridescens* adults	*N. viridescens* larvae	*Pseudacris* tadpoles	*Hyla* tadpoles	Total Pond-breeding amphibians	Plethodontids
14-Feb		0/3		0/1				0/4	
1-Mar		0/1						0/1	0/1
17-Mar	0/4			0/18				0/22	
29-Mar				0/1				0/1	
5-Apr				0/1				0/1	
30-Apr	0/4		5/8	0/6				5/18	0/6
19-May	19/25			3/3		2/2		24/30	
22-May	13/13			1/6				14/19	0/10
4-Jun	7/8		0/1	2/4	0/6	2/21		11/40	
15-Jun	0/1			0/8		0/24	0/1	0/34	
23-Jun	0/2				0/7	0/20	0/1	0/30	
2-Jul	0/8				0/15	0/4	0/2	0/29	
12-Jul	0/2			0/1				0/3	
TOTALS	39/67	0/4	5/9	6/49	0/28	4/71	0/4	54/233	0/17

Table A- 4

Values for AIC and associated degrees of freedom in a model using prevalence data from all taxonomic groups (a), and with Plethodontids excluded (b). Parameter estimates for quadratic regression model are given in c.

a.)

Model (all taxonomic groups) n=251	AIC	df
$z = \beta_0$ (model with no parameters)	263.3848	1
$z = \beta_0 + \beta_1\, x$ (linear)	264.3785	2
$z = \beta_0 + \beta_1\, x + \beta_2\, x^2$ (quadratic all taxonomic groups)	154.7717	3

b.)

Model (plethodontids excluded) n=241	AIC	df
$z = \beta_0$ (model with no parameters)	258.4272	1
$z = \beta_0 + \beta_1\, x$ (linear)	259.3806	2
$z = \beta_0 + \beta_1\, x + \beta_2\, x^2$ (plethodontids excuded)	130.1201	3

c.)

	β_0	β_1	β_2
All taxanomic groups	-0.0121*	1.776*	-0.006442*
Plethodontids removed	-0.01562*	2.282*	-0.008267*

* indicates corresponding p-values<0.0001

Table A- 5

Viral sequence isolated from Gourley Pond samples shown in black, reference FV3 genome (GenBank Accession no. AY548484) sequence shown in blue. Sequence shown in are from a.) the MCP region, b.) the region amplified with the 100F/100R primers and c.) the region amplified with the 16F/16R primers.

a.)

	TCTTGAGAGAGCAATGTACGGGGGTTCGGACGCCACCACGTACTTTGTCAAGGAGCACTACC TCTTGAGAGAGCAATGTACGGGGGTTCGGACGCCACCACGTACTTTGTCAAGGAGCACTACC
	CCGTGGGGTGGTTCACCAAGCTGCCGTCTCTGGCTGCCAAGATGTCGGGTAACCCGGCTTTCG CCGTGGGGTGGTTCACCAAGCTGCCGTCTCTGGCTGCCAAGATGTCGGGTAACCCGGCTTTCG
	GGCAGCAGTTTTCGGTCGGCGTTCCCAGGTCGGGGGATTACATCCTCAACGCCTGGTTGGTGC GGCAGCAGTTTTCGGTCGGCGTTCCCAGGTCGGGGGATTACATCCTCAACGCCTGGTTGGTGC
	TCAAGACCCCCGAGGTCGAGCTCCTGGCTGCAAACCAGCTGGGAGACAATGGCACCATCAGG TCAAGACCCCCGAGGTCGAGCTCCTGGCTGCAAACCAGCTGGGAGACAATGGCACCATCAGG
	TGGACAAAGA ACCCCATGCACAACATTGTGGAGAGCGTCACCCTCTCATTCAACGACATCAG TGGACAAAGA ACCCCATGCACAACATTGTGGAGAGCGTCACCCTCTCATTCAACGACATCAG
	CGCCCAGTCCTTTAACACGGCATACCTGGACGCCTGGAGCGAGTACACCATGCCAGAGGCCA CGCCCAGTCCTTTAACACGGCATACCTGGACGCCTGGAGCGAGTACACCATGCCAGAGGCCA
	AGCGCACAGGCTACTATAACATGATAGGCAACACCAGCGATCTCATCAACCCCGCCCCGGCC AGCGCACAGGCTACTATAACATGATAGGCAACACCAGCGATCTCATCAACCCCGCCCC**T**GCC
	ACAGGCCAGGACGGAGCCAGGGTCCTCCCGGCCAAGAACCTGGTTCTTCCCCTCCCATTCTTC ACAGGCCAGGACGGAGCCAGGGTCCTCCCGGCCAAGAACCTGGTTCTTCCCCTCCCATTCTTC
	TTCTCCAGAGACAGCGGCCTGGCCCTGCCAGTCGTCTCCCTCCCCTACAACGAGATCAGG TTCTCCAGAGACAGCGGCCTGGCCCTGCCAGTCGTCTCCCTCCCCTACAACGAGATCAGG

b.)

	GCAATGTGTGACGAAAAGTCTTTTGAGAACAAAAAGGTTTTACAGCCGGACGTTGGGGTTAT GCAATGTGTGACGAAAAGTCTTTTGAGAACAAAAAGGTTTTACAGCCGGACGTTGGGGTTAT
	GCACGCAAAGATGTAGACTCTTTCCGAATATAAGGAGAGTGTCGTAACATTTATAGAATATC GCACGCAAAGATGTAGACTCTTTCCGAATATAAGGAGAGTGT**G**GTAACATTTATAGAATATC
	TTTACTCTTTCCGACGAGTGTACCAACATTTATACCAGATACGCTTTCAGAAGATATGGCCAG TTTACTCTTTCCGACGAGTGTACCAACATTTATACCAGATACGCTTTCAGAAGATATGGCCAG
	TAAAGATTGGTGTACCAACATTTATACTAGATGTAATCTTTACTCTTTCAGGCGATATGGAGA TAAAGATTGGTGTACCAACATTTATACTAGATGTAATCTTTACTCTTTCAGGCGATATGGAGA
	GTAAAGA GTAAAGA

c.)

	AGAAATCTTGCGAGACCGTCAAGCCTATTACTATCGTAAGATATCTTCGCTAGAAAGTTTCAG AGAAATCTTGCGAGACCGTCAAGCCTATTACTATCGTAAGATATCTTCGCTAGAAAGTTTCAG
	GACAAGAGTTAGATGTAACAACGTCTTGAGATACTATTATCTTAAGATACTATTATCTTAAGA GACAAGAGTTAGATGTAACAACGTCTT**A**AGATACTATTATCTTAAGATACTATTATCTTAAGA
	TACTATTATCTTAAGATACTATTATCTTAAGATACTTTCTCACACTCCTCATTTCCACTCGCAG TACTATTATCTTAAGATACTATTATCTTAAGATACTTTCTCACACTCCTCATTTCCACTCGCAG
	AGCGAGTAGAAACGTCTCATCACTTGCTTTTTCTCTTGGTGGAAAATAGGGCTGCAATCACCA AGCGAGTAGAAACGTCTCATCACTTGCTTTTTCTCTTGGTGGAAAATAGGGCTGCAATCACCA
	ACA ACA

Appendix B

Summary of previous *Ranavirus* accounts and sampling as part of Great Smoky Mountains National Park amphibian survey conducted by USGS as part of the Inventory and Monitoring Program.

In late June of 1999, a USGS field crew observed several dead or dying amphibians at Gourley Pond in Cades Cove. They collected three dead juvenile Pickerel Frogs from the pond margin and submitted them to the USGS National Wildlife Heath Center (NWHC) in Madison, Wisconsin. The crew also noted dead or dying recently metamorphosed Spotted Salamanders at Gourley Pond on July 12, 1999 and submitted three to the National Wildlife Health Center. No explicit testing was conducted to confirm the presence of *Ranavirus* in these animals but it was the presumed cause of mortality.

In 2000 twenty-five animals of four species, Wood Frog and Spring Peeper tadpoles, and Marbled Salamanders and Easter Red-Spotted Newts, were collected from Gourley Pond on May 9 during a similar mortality event. Of the specimens collected and analyzed, several tested positive for *Ranavirus*, including Wood Frog tadpoles (5/10), Marbled Salamander larvae (3/3), and an Eastern Red-spotted Newt (1/3). The Spring Peeper tadpole collected appeared normal. On May 16 of that year, the field crew searched Gourley Pond as well as other amphibian pond-breeding sites, Finley Cane Sinkholes, Cane Creek and the Sinks and did not observe any signs of disease or dead or dying amphibians. On June 20, following reports of dying Bullfrog tadpoles in North Carolina, the USGS field crew searched Abrams Creek and Gourley Pond and all Bullfrog tadpoles appeared healthy.

In 2001 the field crew again observed mortality at Gourley Pond and sent fifteen animals to the USGS NWHC. Southern Red-backed Salamanders and Wood Frog tadpoles were tested for *Ranavirus* which was found only in the Wood Frog tadpoles. Drying of Gourley Pond on the 21 May resulted in mass mortality.

During the extensive sampling throughout Great Smoky Mountains National Park, from 1998-2001, including Methodist Church Pond and Gum Swamp no virus outbreaks were observed anywhere else in the park.

Exact sampling dates and frequency are not clear from USGS reports so I do not know how extensively the three sites of my study were sampled.

Vita

Megan Todd-Thompson grew up in Austin, TX. She attended Grinnell College in Iowa earning her degree in Biology in 2004. She began her Master of science degree in Ecology and Evolutionary Biology in 2007 and has enjoyed learning about the mountains and amphibians of East Tennessee.