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Dynamics of an Emerging Infectious Disease of Amphibians: From Individuals to Communities

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DYNAMICS OF AN EMERGING INFECTIOUS DISEASE OF AMPHIBIANS: FROM INDIVIDUALS TO COMMUNITIES

by

Matthew Damien Venesky

A Dissertation

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ABSTRACT

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Chytridiomycosis is an infectious disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), and it has played an important role in the worldwide decline of amphibians. My dissertation research examined the consequences of *Bd* infections on tadpole feeding biomechanics and activity, pathogen transmission, and host immunology. The keratinized labial teeth of *Bd*-infected Fowler's Toad (*Anaxyrus* [= *Bufo*] *fowleri*) and Grey Treefrog (*Hyla versicolor*) tadpoles slipped off of surfaces on which they grazed and were in contact with an algal-covered surface for a shorter duration of time during each feeding cycle. During feeding trials, *Bd*-infected *A. fowleri* and *H. versicolor* tadpoles obtained significantly less food and were less active during feeding relative to non-infected tadpoles. Collectively, these data show that *Bd*-infected tadpoles are less efficient and less active while feeding and provide a potential mechanism for reduced growth and development in *Bd*-infected tadpoles of these species.

In artificial ponds, *A. fowleri* tadpoles raised in the presence of *Bd* aggregated significantly more relative to controls, whereas *H. versicolor* aggregated significantly less. In addition, ponds with *A. fowleri* tadpoles supported higher *Bd* prevalences and infection intensities relative to ponds with *H. versicolor*, suggesting that aggregation behavior may impact intraspecific *Bd* transmission. Independent of species, tadpoles raised in the presence of *Bd* were smaller and less developed than tadpoles raised in disease-free conditions, even when *Bd* prevalence was low. Although *A. fowleri* tadpoles

seem more susceptible to *Bd* and carrying heavier infections, our results suggest that *Bd* can negatively impact larval life history traits associated with fitness.

To test possible mechanisms related to differential *Bd* susceptibility, I raised Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles on diets that differed in their protein content. Tadpoles fed a low-protein diet had less effective immune responses (PHA-induced skin-swelling response and the ability of tadpole blood to kill *E. coli*), increased susceptibility to *Bd*, and were less developed relative to tadpoles fed a high-protein diet. However, the immune responses of tadpoles infected with *Bd* were similar, suggesting that neither T cell recruitment nor cytotoxicity of tadpole blood (i.e., PHA and bacterial killing ability, respectively) specifically inhibit *Bd* infections.

Preface

Prior to arriving at The University of Memphis and joining the lab of Dr. Matthew Parris, I had strong research interests in the ecology and behavior of amphibians. Shortly into my M.S. degree (at John Carroll University under the guidance of Dr. Carl Anthony), reports of global amphibian declines were dominating the popular and scientific press. Sometime during the middle of my M.S. research, I decided that in order to research amphibians in the future, I needed to contribute to their conservation. Thus, I switched my future research focus from ecological separation in polymorphic terrestrial salamanders to pursue a research program that focused on identifying mechanisms of amphibian declines. While at The University of Memphis, I used a multidisciplinary approach to examine one cause of amphibian declines—emerging infectious diseases. My dissertation research focused the consequences of chytridiomycosis, a disease of amphibians caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), on tadpole behavior and survival. I integrated my training in amphibian behavior with the resources in disease ecology at The University of Memphis to examine a variety of topics related to host-pathogen biology, such as functional morphology, host behavior, host immune function, and community interactions. The results of these research projects are detailed within this dissertation, broken into a brief introduction on my study system, four research chapters (which are written as separate manuscripts that are either in print, in review, or in preparation for submission), and a conclusion. Each of the four research chapters represents an independent research project in which I was the lead investigator and author. However, the work presented in this dissertation could not have been

completed without the help of other investigators. Their contribution to my research efforts is reflected with co-authorship on published/in review manuscripts.

Chapter 1 is the introduction to my research topic and establishes how my research projects contributed to the gap in our understanding of amphibian-*Bd* interactions. This chapter will not be submitted as a manuscript and has been formatted according to the guidelines for dissertations set forth by the APA Checklist provided by the Graduate School at the University of Memphis. Chapter 2, entitled "Fungal Pathogen Changes the Feeding Kinematics of Larval Anurans" has been published in *Journal of Parasitology* (Venesky et al., 2010, 96: 552-557). Chapter 3, entitled "Effects of *Batrachochytrium dendrobatidis* infections on larval foraging performance" has been published in *EcoHealth* (Venesky et al., 2009, 6:565-575). Chapter 4, entitled "Can Differences in Host Behavior Drive Patterns of Disease Prevalence in Amphibian Communities?" has been written in manuscript form and is currently in review for the journal *Animal Conservation.* Chapter 5, entitled "Dietary protein content affects immune response and disease resistance in anuran tadpoles" has been written in manuscript form for *Journal of Experimental Biology* and will be submitted soon. Finally, the conclusion (Chapter 6) ties together the findings of my Chapters 2–5 and discusses how the results from Chapter 5 will direct my postdoctoral research in the lab of Dr. Jason Rohr at the University of South Florida. Chapter 6 will not be submitted as a manuscript and has been formatted according to the guidelines set forth by the APA Checklist provided by the Graduate School at the University of Memphis.

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Chapter 1 Introduction Disease-Induced Amphibian Declines

Determining the consequences of emerging infectious diseases is a pressing issue facing human and wildlife health (National Resource Council, 2001; Patz et al., 2005). This challenge comes, in part, because most diseases are zoonotic and thereby affect humans and wildlife (Woolhouse, 2002). While emerging diseases are an increasing hazard to humans, they also place tremendous pressures on wildlife populations and threaten animal conservation efforts by causing rapid population declines and/or extinctions of threatened taxa (Daszak et al., 2008). To meet this current environmental challenge, conservation strategies need to go beyond assessing the risk of infection and integrate ecology with a mechanistic understanding of the etiology of disease.

Amphibians have experienced significant population declines in the last three decades (Lips et al., 2006), with approximately 40% of 6,000+ species threatened with extinction (Stuart et al., 2004), placing amphibian conservation at the forefront of conservation science. Amphibians are exposed to a diverse assemblage of parasites and pathogens (hereafter, "pathogens"), several of which are implicated in population declines (Daszak et al., 2003), making this a tractable study system to explore host-pathogen interactions. My research focused on the consequences of *Batrachochytrium dendrobatidis* (hereafter "*Bd*") infection on tadpoles. *Bd* is a pathogenic fungus that causes the emerging infectious disease chytridiomycosis, which has been implicated in global amphibian population declines. In metamorphs, *Bd* infections are located in the keratinized epithelium and usually results in mortality (Voyles et al., 2009). In tadpoles, *Bd* infects the keratinized mouthparts (labial teeth and jaw sheaths) and often results in

reduced growth and development (Parris and Cornelius, 2004). Prior to my dissertation research, there were three major gaps in our understanding of *Bd*-tadpole biology: (1) the mechanism for reduced growth and development in *Bd*-infected tadpoles; (2) the ways in which *Bd* affected tadpoles throughout the span of their larval stage; and (3) how species-specific differences in behavior affect *Bd* transmission. The focus of my dissertation research was to test hypotheses relating to each of these topics and integrate my results into the larger picture of host-pathogen biology.

In Chapters 2–4, I used two species of anuran tadpoles as my amphibian models—Fowler's toads (*Anaxyrus* [= *Bufo*] *fowleri*) and grey treefrogs (*Hyla chrysoscelis*). These two species are ideal focal taxa because they are sympatric in ponds yet differ in their aggregative behavior, which may differentially impact pathogen transmission. Generally, *Anaxyrus* tadpoles aggregate on the substrate of ponds (Beiswenger, 1977), whereas *Hyla* tadpoles typically occur solitary in the middle of the water column (Wilbur and Alford, 1985). I capitalized on this variation in host behavior to assess how *Bd* affects tadpoles through ontogeny and if host-specific behaviors facilitated differential *Bd* transmission. In Chapter 2, I used high-speed videography to test how *Bd*-induced mouthpart deformities affected the feeding kinematics of *A. fowleri* and *H. chrysoscelis* tadpoles. I hypothesized that the teeth of *Bd*-infected tadpoles would slip off surfaces in which they grazed and would contact with an algal-covered substrate for a shorter duration relative to non-infected tadpoles.

In Chapter 3, I conducted behavioral observations to test whether *Bd* infections reduced foraging activity and efficiency of *A. fowleri* and *H. chrysoscelis* tadpoles

through ontogeny. I hypothesized that *Bd*-infected tadpoles would forage less often and less efficiently than non-infected tadpoles.

In Chapter 4, I examined how differences in aggregation behavior of *A. fowleri* and *H. versicolor* tadpoles and tested for differences in transmission of *Batrachochytrium dendrobatidis* (*Bd*) and host-specific fitness consequences of infection in single-species amphibian mesocosms. I hypothesized that mesocosms with *A. fowleri* tadpoles would support higher *Bd* prevalences and infection intensities relative to mesocosms with *H. versicolor* tadpoles and that the duration of time in which tadpoles were exposed to *Bd* would impact their growth and developmental rates.

One consistent result from Chapters 2–4 was the variation in susceptibility of tadpoles to *Bd*. This observation, although already known by scientists, was influential in determining my future research efforts (at the graduate and postdoctoral levels). I became interested in the ways in which hosts reduce pathogen burden (i.e., resistance) or minimize harm caused when infected by a pathogen (i.e., tolerance). In Chapter 5, I examined some of the immunological defenses that tadpoles might use to resist *Bd* infections. I switched my model taxon to Southern Leopard Frog (*Lithobates sphenocephalus* [=*Rana sphenocephala*]) tadpoles, which are larger in body size and easier to manipulate for immunological assays. I used a fully factorial design to examine effects of nutritional status (high and low protein) and *Bd* infection (infected and non-infected) on immune responses and infection success of tadpoles.

Chapter 2 Fungal Pathogen Changes the Feeding Kinematics of Larval Anurans

ABSTRACT: Pathogens can alter host life history traits by affecting host feeding activities. In anuran tadpoles, keratinized mouthparts (teeth and jaw sheaths) are essential for feeding. *Batrachochytrium dendrobatidis* (*Bd*) is a pathogenic fungus of amphibians that can infect these mouthparts and reduce tadpole survival. However, the precise way that *Bd*-induced changes in tadpole mouthparts impact tadpole feeding is unknown. We use high-speed (500 frames/sec) videography to study how *Bd*-induced mouthpart deformities affect the feeding kinematics of Fowler's toad (*Anaxyrus* [=*Bufo*] *fowleri*) and Grey treefrog (*Hyla chrysoscelis*) tadpoles. We tested for species-specific patterns of *Bd*-induced mouthpart deformities to assess how deformations to specific areas of tadpole mouthparts alter feeding kinematics. The teeth of tadpoles from the *Bd*-exposed treatment slipped off of surfaces on which tadpoles graze and are in contact with an algal-covered substrate for a shorter duration in each gape cycle compared to teeth of control tadpoles. We also found that the jaw sheaths had significantly more deformations than labial teeth; however, how this relates to feeding kinematics is unclear. Our data show explicitly how *Bd* infection reduces foraging efficiency of anuran tadpoles by altering feeding kinematics and elucidate a mechanistic link between the pathogen infection and reduced host fitness.

Ecologists increasingly recognize the impacts of parasites and pathogens (hereafter "pathogens") on host life history traits (Poulin, 1994; Altizer et al., 2003; Forson and Storfer, 2006). Because pathogens can cause mortality in hosts (deCastro and Bolker, 2005), there are strong selective forces for the shifting of host life history traits to

mitigate the negative effects of infection (Minchella, 1985; Perrin et al., 1996). For example, because pathogens can reduce the reproductive success of their hosts, hosts may alter certain life history traits, such as growth and developmental rates (Goater, 1994; Kiesecker and Skelly, 2001) or specific schedules of reproduction (Agnew et al., 1999), in order to diminish these negative effects. Thus, pathogen-induced changes to host life history traits can have profound impacts on hosts by reducing host fitness (Anderson and May, 1978; Price et al., 1998).

One general pattern in which pathogens alter host life history traits is by affecting growth and developmental rates through reduced feeding abilities (Crowden and Broom, 1980; Levri and Lively, 1996; Ottershatter et al., 2005). By reducing energetic resources devoted to activities related to feeding and metabolism, hosts can reallocate energy to immunological functions (Connors and Nickol, 1999), potentially reducing growth and development. Although this overall effect may be similar across taxa, the mechanism(s) underlying this response may be specific to an individual host-pathogen system. For example, common dace (*Leuciscus leuciscus*) infected with an eye-fluke (*Diplostomum spathaceum*) forage less efficiently because of visual impairment caused by the parasite (Crowden and Broom, 1980). In comparison, bumblebees (*Bombus impatiens*) infected with tracheal mite (*Locustacarus buchneri*) exhibit decreased foraging efficiency because they visit fewer flowers per min than uninfected bees (Otterstatter et al., 2005). A full understanding of the pathogenicity of an infective organism requires understanding how the organism alters the behavior of its host.

Understanding how pathogens affect amphibian populations and communities is especially important considering worldwide amphibian declines, and growing evidence

indicates that infectious diseases play a role in these declines (Daszak et al., 1999; Daszak et al., 2003). *Batrachochytrium dendrobatidis* (*Bd*) is the causal agent of chytridiomycosis, an emerging infectious disease of amphibians, which has been identified as having played a major role in the decline of amphibian populations over the past 2 decades (Berger et al., 1998; Daszak et al., 2003). This host-pathogen system is ideal for studying disease impacts on hosts for numerous reasons. First, in anuran tadpoles, *Bd* infections are site-specific, i.e., *Bd* infects keratinizing tissue, or tissues fated to keratinize (Fellers et al., 2001; Rachowicz and Vredenburg, 2004), and colonization of *Bd* is restricted to the keratinized mouthparts (jaw sheaths and labial teeth). Jaw sheaths and labial teeth play an important functional role in tadpole feeding. In short, during a single gape cycle (i.e., the opening and closing of the jaw sheaths and labial teeth), the labial teeth anchor the oral apparatus to the substrate as the keratinized jaw sheaths close and bite material off the substrate (Wassersug and Yamashita, 2001). After the jaw sheaths are closed, the labial tooth rows sequentially release and rake over the substrate again (Wassersug and Yamashita, 2001), creating a suspension of food that is brought into the tadpole's mouth during the next gape cycle. In addition, tadpoles exhibit considerable ecomorphologic diversity in their keratinized mouthparts and behavioral specializations related to foraging modes (Altig, 2006), providing an opportunity to compare different host responses to a single pathogen. Second, some species of tadpoles succumb to *Bd* infections (Blaustein et al., 2005), while others survive but exhibit reduced growth and developmental rates (Parris 2004; Parris and Baud, 2004; Parris and Cornelius 2004; but see Smith et al., 2007). Therefore, this host-pathogen system

provides an opportunity to study how intrespecific differences in host life history patterns and morphology influence host-pathogen ecology.

Surprisingly, few studies have examined how *Bd* reduces growth and development in tadpoles. A recent study found that *Bd* infections reduce foraging efficiency in *Anaxyrus* (= *Bufo*) and *Hyla* tadpoles (Venesky et al., in press a) and inefficiency at obtaining food can negatively affect growth rates. Given the functional role of keratinized jaw sheaths and labial teeth in tadpole foraging (Wassersug and Yamashita, 2001; Venesky et al., in press b) and that *Bd* infections often deform these structures (Fellers et al., 2001), it is possible that *Bd*-induced oral deformities may contribute directly to reduced foraging efficiency. In this regard, data related to feeding kinematics of tadpoles with deformed, or missing, keratinized mouthparts are relevant in assessing the impact mouthpart abnormalities have on overall larval survival. Additionally, few comparative studies have examined how species with diverse life history traits or morphology respond to *Bd* (but see Parris et al., 2004; Blaustein et al., 2005; Han et al., 2009; Venesky et al., in press a). Identifying the specific *Bd* mediated effects on host life history traits, and how these differ among species, can advance our understanding of how *Bd* affects amphibian fitness.

The purpose of the present study was to evaluate experimentally the impact(s) of *Bd* on the feeding performance of tadpoles. We used two species of tadpoles—Fowler's toads (*Anaxyrus* [= *Bufo*] *fowleri*) and Grey treefrogs (*Hyla versicolor*). These two species are ideal focal taxa because they differ in behaviors associated with how and where they forage within the water column of ponds. Generally, *Anaxyrus* tadpoles aggregate in small groups and scrape food off the substrate of ponds (Beiswenger, 1977),

whereas *Hyla* tadpoles forage actively from the middle portion of the water column (Wilbur and Alford, 1985). Both species are susceptible to *Bd* in field and laboratory settings (Parris and Cornelius, 2004); however, neither species is experiencing *Bd*-related population declines. First, we tested if *Bd*-induced changes in host morphology affected feeding kinematics in *A. fowleri* and *H. chrysoscelis* tadpoles. We used high-speed (500 frames/sec) videography of *Bd*-exposed and nonexposed *A. fowleri* and *H. chrysoscelis* tadpoles to test the hypothesis that *Bd*-induced deformations to the keratinized jaw sheaths and labial tooth loss result in a reduced amount of time that tadpoles attach to, and graze upon, an algal covered substrate. We measured the duration of the full gape cycle (opening and closing of the jaws) and the closing phase (the duration of time that the labial teeth were attached to the algal covered substrate). These two kinematic variables are metrics of the effectiveness of a tadpole feeding. We considered the duration the full gape cycle a measurement of overall speed of each gape cycle and the duration of the closing phase of the gape cycle an estimate of raking efficiency. We predicted that mouthparts of tadpoles from our *Bd* treatment would slip while foraging and that the duration of their gape cycle and the amount of time their labial tooth rows were in contact with an algal substrate would be shorter than for control tadpoles. Second, we tested for species-specific differences in *Bd*-induced mouthpart deformations by calculating a deformity index, which estimated the degree of mouthpart deformation to the labial teeth, jaw sheaths, and both keratinized regions. We then used regression analysis to assess whether deformations to specific areas of the mouthparts related to inferior feeding kinematics.

MATERIALS AND METHODS

Animal collection and husbandry

Anaxyrus fowleri and *H. chrysoscelis* egg masses (N = 8 and 6, respectively) were collected from natural ponds within the Meeman Shelby State Park in Tennessee (Shelby County, Tennessee) between 26–29 April 2009. Immediately after collection, eggs were transported to the laboratory at The University of Memphis. Upon hatching, tadpoles were maintained in 37.85-L glass aquaria (filled with 35 L of water, which was continually, but gently, aerated) at a density of 10 tadpoles/L, until they reached the free-swimming stage (stage 25; Gosner, 1960). We then combined the tadpoles from the different clutches to evenly distribute potential genetic effects and haphazardly selected our test subjects out of the remaining stock of tadpoles. Test subjects were placed individually in 1.5-L plastic containers filled with 1 L of aged tap water. Throughout the experiment, tadpoles were maintained on a 12 light: 12 hr dark photoperiod at 19 C (±1 C) and were fed Sera Micron® daily.

Full water changes were conducted once per wk. We used a piece of mesh screen to remove the focal tadpole from its container and placed it in a temporary transfer container with the same dimensions and volume of water as the experimental container. Water from the experimental container was poured into a bucket and 1.0 L of aged tap water was then added to that container. The focal tadpole was then placed back into the experimental container. To minimize the possibility of *Bd* transmission between *Bd*-exposed and nonexposed (control) treatments, we always performed water changes on nonexposed tadpoles before exposed tadpoles. Additionally, we used different laboratory equipment between nonexposed and pathogen treatments. At the end of each water

change, and upon completion of the experiment, we thoroughly disinfected all water and laboratory equipment by adding bleach (6% sodium hypochlorite) to yield a 10% solution, which kills *Bd* (Johnson and Speare, 2003).

Batrachochytrium dendrobatidis inoculation

Batrachochytrium dendrobatidis (USA isolate 284) was grown in the laboratory on tryptone-gelatin hydrolysate-lactose (TGhL) agar in 9-cm Petri dishes according to standard protocol (Longcore et al., 1999). We harvested *Bd* zoospores by adding 10.0 ml of sterile water to the cultures, collected the zoospores that emerged from the zoosporangia after 30 min, and counted them with a hemacytometer. At Gosner 25, all tadpoles of each species were randomly split into 2 experimental groups—a *Bd*-exposed group and a nonexposed (control) group. For the *Bd*-exposed group, we administered *Bd* infections by exposing *A. fowleri* (N = 6) and *H. chrysoscelis* (N = 6) to 50 ml water baths containing infectious concentrations of fungal zoospores (120,000 zoospores/ml) for 24 hr. For the nonexposed group, we followed the same protocol, but used plates with only TGhL, and exposed an additional group of tadpoles (*A. fowleri*, N = 6; *H. chrysoscelis*, N = 6) to water baths with no *Bd* zoospores. Our design simulated transmission by water, one of the likely modes of *Bd* transmission in natural environments (Pessier et al., 1999). Tadpoles were given 30 days to develop clinical symptoms of *Bd* infections (deformaties of the keratinized mouthparts) prior to filming. No mortality occurred during the experiment.

Feeding kinematics

To produce a standardized substratum on which the tadpoles could graze, we submerged microscope slides in a 4.5-L plastic container with aged tap water and placed

the container by a window for 2 wk to allow epiphytic algae to colonize the microscope slides (following Wassersug and Yamashita, 2001). Before the start of a video trial, we scrubbed one side of an algal slide to remove the algae and mounted the cleaned side of the slide against the inside wall of a Plexiglas container (12.7 X 11.4 X 10.2 cm) where the tadpoles were videographed. The container was filled with approximately 750 ml of aged tap water, which was continually, but gently, aerated during each videography trial. For greatest resolution, we prefocused the camera on the inside surface of the microscope slide, which was covered with a dense layer of epiphytic algae. Different algal covered slides were used for each videography trial and slides were equally covered with algae. To encourage grazing, the tadpoles were not fed the day before filming.

During our experiments, the tadpoles ranged in size from 16.0–22.1 mm (total length) and in Gosner (1960) stage from 32–35. We filmed the tadpoles in individual trials while they grazed on an algal covered slide, recording a single feeding bout for each tadpole. Herein, a feeding bout consisted of the point where the mouth of the tadpole first touched the algal covered slide until the tadpole fully released from the slide and swam away. Each feeding bout consisted of a continuous rapid series of at least 5 gape cycles (7.58 ± 0.45; mean ± standard error), during which the tadpoles scraped algae from the slide. As per Wassersug and Yamashita (2001), we define 1 gape cycle as: (1) starting with the jaw sheaths fully closed and the anterior and posterior tooth rows in closest proximity; (2) proceeding to the point where the mouth is fully open and the labial tooth rows reached maximum gape; and (3) ending with full closure of the jaw sheaths and anterior and posterior tooth rows in closest proximity to each other. We randomly distributed the order of filming between species (Hurlbert, 1984) and filmed all control

tadpoles first to avoid contamination between treatments. Filming concluded after 4 consecutive trial dates.

We quantified the kinematics of 2 aspects of foraging related to the ability of the tadpole to obtain food. We recorded the duration of time (in millisec) of the full gape cycle and also the duration of time (in milliseconds) of the closing phase of the gape cycle. We chose to focus our observations on the outermost labial tooth rows because they are the first tooth rows to contact, and last to release from, the substratum when feeding, and they play an important role in anchoring a tadpole to a surface and raking food off it (Wassersug and Yamashita, 2001; Venesky et al., in press b). Because the feeding bout contained varying numbers of gape cycles, we considered the mean time for each kinematic measurement during the foraging bout as a datum in our analysis. We used 2-way Analysis of Variance (ANOVA) to test for differences in size (total length) and developmental stage (Gosner) between species and treatments. We then used ANCOVA (with size as a covariate) to test for differences between treatment groups (*Bd*-exposed and nonexposed) in the duration of time for the full gape cycle and also the duration of time for the closing phase of the gape cycle. Our data met all assumptions of these statistical tests.

Mouthpart evaluations

For each tadpole, we calculated a deformity index, adapted from a similar index presented by Drake et al. (2007). Deformities were tabulated in 14 zones of the oral apparatus, i.e., the left and right sides of each jaw sheath (=4 zones) and the left and right sides of each labial tooth row (=10 zones). We then estimated the % damage to each zone under a Nikon® SMZ800 dissecting scope with 10–60X magnification. For example, if

30% of the left anterior labial tooth row was missing, that tadpole was given a score of 0.30. We then calculated a deformity index for the jaw sheaths (summing the total score of all 4 jaw sheath zones and dividing the total by 4), labial teeth (summing the total score of all 10 labial teeth zones and dividing the total by 10), and a total deformity index (summing the total score of all 14 zones and dividing the total by 14). We used ANOVA to test for species-specific and location differences in mouthpart deformations. We also used regression analyses to evaluate the relationships between the deformity index and the independent variables duration of the total gape cycle and the closing phase of the gape cycle (a measure of the effectiveness in raking the surface as the jaw closed).

RESULTS

Taxonomic patterns of damage

Overall, there were relatively few mouthpart deformities to *A. fowleri* and *H. chrysoscelis* tadpoles in the nonexposed treatment (Figs. 1A, B). However, tadpoles of both species exhibited considerable *Bd*-induced mouthpart deformities (Figs. 1C, D).

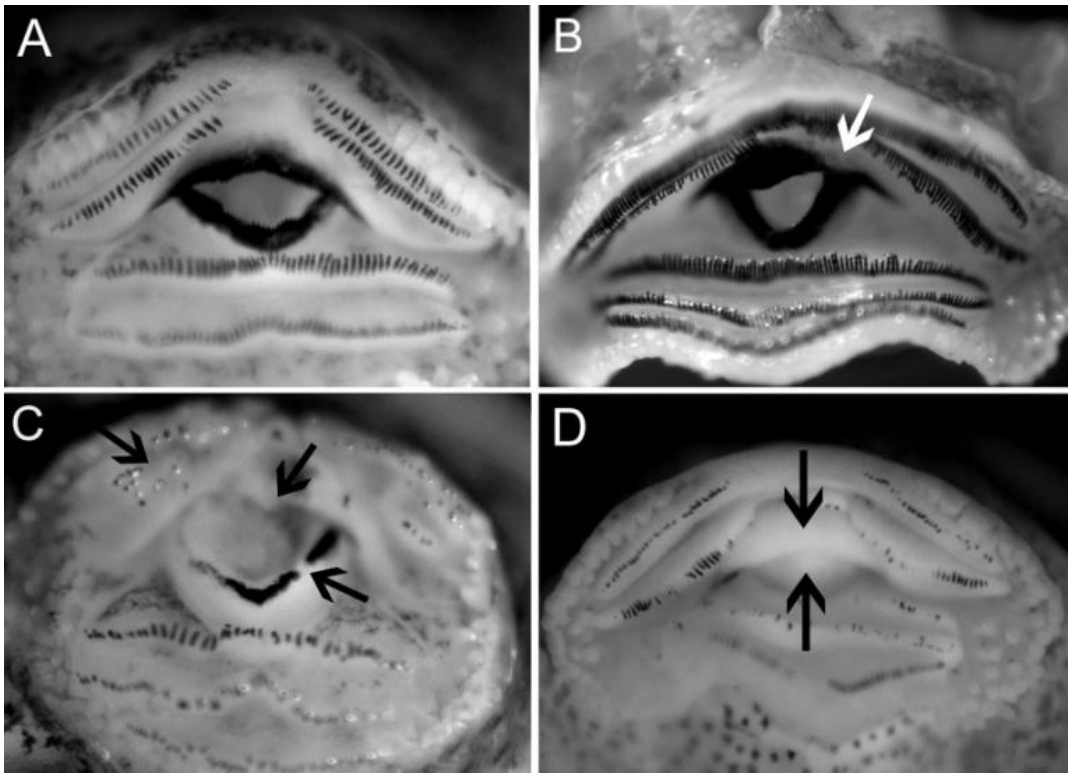

Figure 1. Examples of mouthpart deformities to keratinized jaw sheaths and labial teeth of Grey treefrog (*Hyla chrysoscelis*) tadpoles. Black arrows point to specific areas with deformations. Tadpoles from the control treatment showing natural deformities. (A) A small gap in the labial teeth in anterior tooth row A-1. (B) The loss of a small piece of keratin to the left side of the anterior jaw sheath. Tadpoles from the *Batrachochytrium dendrobatidis* (*Bd*) treatment showing *Bd*-induced oral deformities. (C) The loss of the majority of labial teeth in anterior tooth rows A-1 and A-2, loss of the keratin in the anterior jaw sheath, gap in the left side of the posterior jaw sheath. (D) Complete loss of the keratin in both jaw sheaths.

Within tadpoles from the *Bd*-exposed treatment, we detected an overall significant effect of the location where mouthpart deformities occurred ($F = 5.572$, $P = 0.009$), with Sidak's post hoc analysis indicating that jaw sheaths had significantly more damage than the labial teeth rows. Additionally, within *Bd*-exposed tadpoles, there was a significant species by location interaction ($F = 3.474$, $P = 0.044$), with Sidak's post hoc analysis

indicating there were more deformations to the jaw sheaths of *H. chrysoscelis* tadpoles compared to jaw sheaths of *A. fowleri* tadpoles.

Effects of *Bd* on feeding kinematics

Two-way ANOVA revealed that tadpoles from the *Bd*-exposed treatment 10.5% smaller than tadpoles from control treatment ($F = 7.404$, $P = 0.013$); however, there was no significant difference in Gosner stage between treatments ($F = 0.372$, $P = 0.549$). Because of the significant difference between *Bd*-exposed and control treatments in body size, we used an analysis of covariance (ANCOVA) to remove the potentially confounding effects of tadpole size on response to pathogen exposure. Although the covariate analysis demonstrated a non-significant effect of size on the full gape cycle ($P = 0.397$) and closing phase of the gape cycle ($P = 0.632$), there were significant effects of species and treatment on the duration of time of the full gape cycle (species: $F = 5.636$, $P = 0.028$; treatment: $F = 5.747$, $P = 0.027$) and on the closing phase of the gape cycle (species: $F = 5.047$, $P = 0.037$; treatment: $F = 4.475$, $P = 0.048$). Overall, tadpoles from the *Bd*-exposed treatment had a shorter duration of gape cycle and closing phase than nonexposed tadpoles (Figs. 2, 3). Interestingly, we also observed species specific differences in both kinematic variables we measured. *Anaxyrus* tadpoles had a shorter closing phase compared to *Hyla* tadpoles (Figs. 2, 3), suggesting that the feeding kinematics of the *2* tadpole species differ.

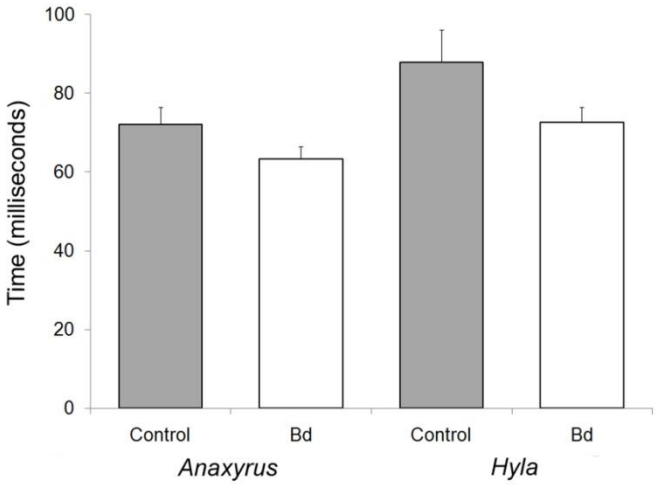

Figure 2. The duration of time (in milliseconds) of the full gape cycle of Fowler's toad (*Anaxyrus fowleri*) and Grey treefrog (*Hyla chrysoscelis*) tadpoles from *Batrachochytrium dendrobatidis* (*Bd*) exposed and nonexposed (control) treatments. Error bars are 1 S.E. of the mean. Overall, tadpoles from the *Bd*-exposed treatment had a shorter gape cycle than tadpoles from the control treatment ($P = 0.027$). Additionally, *A. fowleri* tadpoles had a shorter gape cycle compared to *H. chrysoscelis* tadpoles, irrespective of treatment ($P = 0.028$).

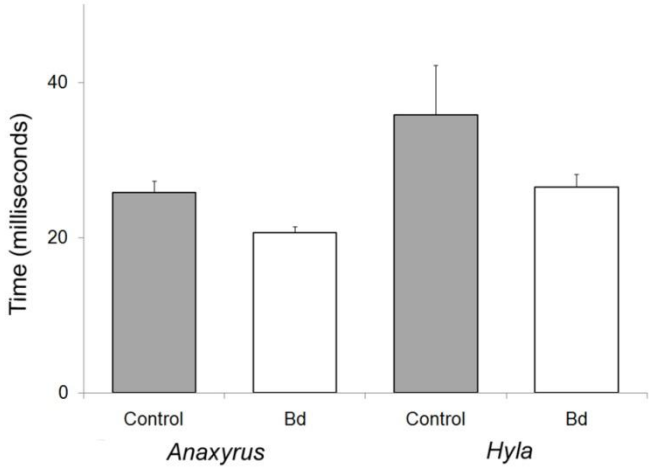

Figure 3. The duration of time (in milliseconds) of the closing phase of the gape cycle of Fowler's toad (*Anaxyrus fowleri*) and Grey treefrog (*Hyla chrysoscelis*) tadpoles from *Batrachochytrium dendrobatidis* (*Bd*) exposed and nonexposed (control)

treatments. Error bars are 1 S.E. of the mean. Overall, tadpoles from the *Bd* exposed treatment had a shorter closing phase of the gape cycle than tadpoles from the control treatment ($P = 0.048$). Additionally, *A. fowleri* tadpoles had a shorter gape cycle compared to *H. chrysoscelis* tadpoles, irrespective of treatment ($P = 0.037$).

Although there were clear effects of *Bd* infection on the feeding kinematics of both *A. fowleri* and *H. chrysoscelis* tadpoles, we were unable to detect how specific patterns of damage directly contributed to feeding kinematics. For the full gape cycle, there were no relationships between the three deformity indices and this kinematic measurement ($r^2 < 0.04$). However, for the closing phase of the gape cycle, there was a stronger relationship between the overall deformity index and the duration of time of the closing phase of the gape cycle. Linear regression analysis revealed a negative relationship between the overall deformity index and the duration of the closing phase of the gape cycle in both species (*Anaxyrus*: $r^2 = 0.232$, $P = 0.113$; *Hyla*: $r^2=0.160$, $P = 0.198$; Figure 4). Although not statistically significant, these data suggest that as the overall deformity index increases, the duration of time it takes for tadpoles to close their mouth decreases.

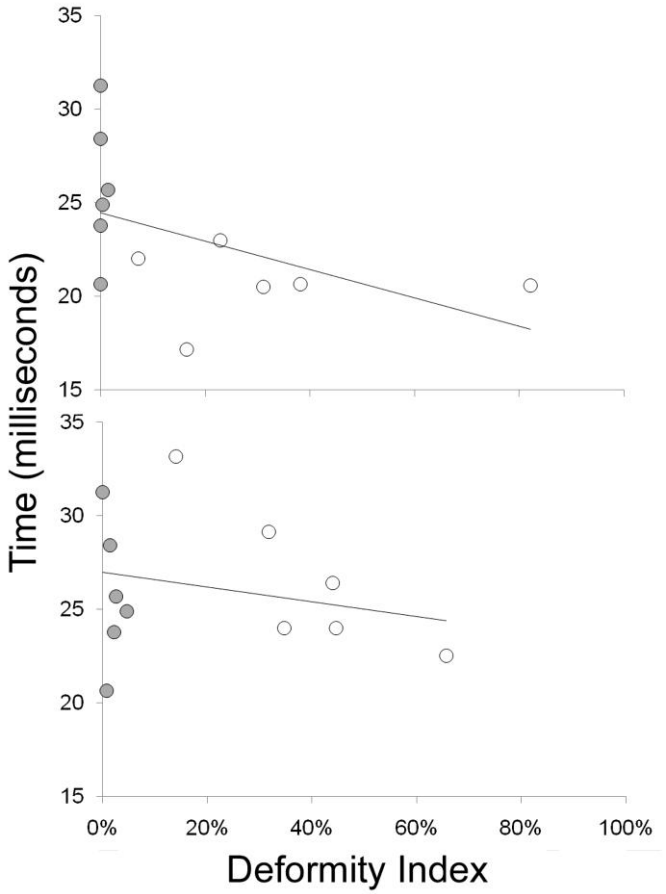

Figure 4. The relationship between overall deformity index and the duration of the closing portion of the gape cycle in *Batrachochytrium dendrobatidis* (*Bd*) exposed Fowler's toad (*Anaxyrus fowleri*) and Grey treefrog (*Hyla chrysoscelis*) tadpoles. Each data point represents the mean duration of the cycle for an individual tadpole regressed with the deformity index. Grey circles represent tadpoles from the control treatment; open circles represent tadpoles from the *Bd*-exposed treatment. (Top) Overall deformity index for *A. fowleri* tadpoles: r^2=0.232, *P*=0.113. (Bottom) Overall deformity index for *H. chrysoscelis* tadpoles: r^2=0.160, *P*=0.198.

DISCUSSION

Theoretical models of host-pathogen dynamics predict that pathogens can regulate host populations (Anderson and May, 1978). Indeed, a growing body of literature details

specific mechanisms by which pathogens affect a diverse range of host species by causing modifications to host life history traits (Poulin, 1994; Altizer et al., 2003; Forson and Storfer, 2006), or behaviors, or both (Crowden and Broom, 1980; Reed and Dobson, 1993), which can have cascading effects throughout food webs (Lafferty, 2006). In particular, pathogens can negatively affect the feeding behavior, or efficiency of hosts, or both (Crowden and Broom, 1980; Levri and Lively, 1996; Gunn and Irvine, 2003; Ottershatter et al., 2005). Despite the reports of *Bd*-induced mouthpart deformities in anuran tadpoles (Fellers et al., 2001), few studies have tested how these deformities impact larval survival (but see Venesky et al., in press a). Our results suggest that: (1) there are species specific patterns of *Bd*-induced mouthpart deformations; (2) *Bd*-induced mouthpart deformations negatively affect the feeding kinematics of *A. fowleri* and *H. chrysoscelis* tadpoles; and (3) an overall negative relationship between mouthpart deformities index and duration of time tadpoles attach to an algal substratum. Our kinematic data (this study), in corroboration with *Bd*-induced reductions in food consumption (Venesky et al., in press a), are some of the first to provide a mechanistic link between pathogen-induced negative effects and fitness in anuran tadpoles.

Overall, our results show that tadpoles from the *Bd*-exposed treatment exhibited considerable damage to the labial teeth and jaw sheaths compared to relatively few deformities to tadpoles in the nonexposed treatment (Fig. 4). Although all tadpoles from the *Bd*-exposed treatment had deformities to their labial teeth and jaw sheaths, we found significantly more overall damage to jaw sheaths than labial teeth. Additionally, we found significantly more deformities to the jaw sheaths of *H. chrysoscelis* tadpoles than of *A. fowleri* tadpoles. Our results differ from a recent survey of oral deformities of field

collected tadpoles in which tooth row deformities comprised 95.9% and 84.2% of the total deformities of the 2 tadpole species, respectively (Drake et al., 2007). Interestingly, our data suggest that in both species of tadpoles, *Bd*-induced deformities are found more frequently in jaw sheaths than in labial teeth. However, we recognize that Drake et al. (2007) surveyed tadpoles from natural environments potentially exposed to many pathogens or pollutants, which may have contributed to differences between the studies. Our results, however, corroborate the findings of Rachowicz and Vredenburg (2004), who found that in mountain yellow legged frog (*Rana muscosa*) tadpoles, *Bd*-induced infections start in the jaw sheaths and progress through these structures before deforming labial teeth.

In addition, our results show that *Bd*-induced oral deformities can significantly handicap tadpoles by altering their feeding kinematics. Although tadpole jaw sheaths had more deformations than labial teeth, it is important to note that tadpoles from the *Bd*-exposed treatment also had severely deformed labial teeth (Figs. 1 C, D), which are important in feeding. Generalized pond-type anuran tadpoles that forage by grazing on algae have an elaborate feeding mechanism. For each gape cycle, i.e., the opening and closing of the jaw sheaths and labial teeth, the labial teeth anchor the oral apparatus to the substrate and also function in concert with the closing of the jaw sheaths to rake material off the substratum (Johnston, 1982; Arens, 1994; Wassersug and Yamashita, 2001; Venesky et al., in press b). Relative to nonexposed tadpoles, the keratinized labial teeth of *Bd*-exposed the 2 tadpole species slipped when attached to an algal substratum, thereby reducing the duration of their full gape cycle and also the closing phase of their gape cycle. The duration of time that the keratinized structures are attached to an algal

substratum and the amount of food they can remove per gape cycle are likely correlated. Thus, *Bd*-induced oral deformities may directly compromise the feeding efficiency of tadpoles by either directly reducing the amount of food removed from a substratum per foraging bout (Venesky et al., in press b) or indirectly by reducing their net energy intake per foraging bout (Venesky et al., in press b). Inefficiency at obtaining maximum energy gain from food resources can negatively affect growth rates, which can reduce host survival in larvae (Kurzava, 1998; Semlitsch et al., 1988) and adults (Berven, 1990; Smith 1987).

It is important to note that *Bd*-exposed tadpoles were smaller than tadpoles from the control treatment. This result was expected given the negative impact(s) *Bd* has been shown to have on feeding efficiency (Venesky et al., in press a) and growth and development (Parris and Cornelius, 2004). However, our results corrected for differences in initial body size and unambiguously demonstrate that the treatment effects we observed on feeding kinematics were caused by differences between species and *Bd* exposure.

Although our primary aim was not to compare overall feeding kinematics between species (a topic to be addressed more extensively in a subsequent manuscript), we detected an overall species-specific difference in the 2 kinematic variables we measured. *Hyla chrysoscelis* tadpoles had a significantly longer gape cycle and closing phase of the gape cycle than *A. fowleri* tadpoles (Figs. 2, 3). These data corroborate the known inter-specific behavioral differences in feeding behaviors of these species (Beiswenger, 1977; Wilbur and Alford, 1985) and likely relate to different spatial patterns of feeding within ponds. Interestingly, when exposed to *Bd,* both species

responded similarly to *Bd* with reduced feeding kinematics (Figs. 2, 3) but there was not a significant species x treatment interaction. Given the differences in feeding efficiency observed in *Bd*-exposed *Anaxyrus* and *Hyla* (Venesky et al., in press a), our data suggest that *Bd*-induced mouthpart deformations do not singly contribute to reduced feeding efficiency in these species. As suspected, the interactions between *Bd* and amphibian hosts are dynamic and reductions in host survival are likely mediated by multiple factors. However, the data from the present study are the first to document the mechanism in which *Bd*-induced mouthpart deformations negatively impact fitness of *A. fowleri* and *H. chrysoscelis* tadpoles.

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Chapter 3 Impacts of *Batrachochytrium dendrobatidis* Infection on Tadpole Foraging Performance

Abstract: Pathogen induced modifications in host behavior, including alterations in foraging behavior or foraging efficiency, can compromise host fitness by reducing growth and development. Chytridiomycosis is an infectious disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), and it has played an important role in the worldwide decline of amphibians. In larval anurans, *Bd* infections commonly result in reduced developmental rates; however, the mechanism(s) responsible are untested. We conducted laboratory experiments to test whether *Bd* infections reduced foraging performance of Grey Treefrog (*Hyla chrysoscelis*) and Fowler's Toad (*Anaxyrus* [= *Bufo*] *fowleri*) tadpoles. In the first experiment, we observed foraging behavior of *Bd* infected and uninfected tadpoles to test for differences in foraging activity. In a second experiment, we tested for differences in the ingestion rates of tadpoles by examining the amount of food in their alimentary track after a 3 hour foraging period. We hypothesized that *Bd*-infected tadpoles would forage less often and less efficiently than uninfected tadpoles. As predicted, *Bd* infected larvae forage less often and were less efficient at obtaining food than non-infected larvae. Our results show that *Bd* infections reduce foraging efficiency in *Anaxyrus* and *Hyla* tadpoles, and that *Bd* differentially affects foraging behavior in these species. Thus, our results provide a potential mechanism of decreased developmental rates of *Bd* infected tadpoles.

Key words: *Anaxyrus fowleri*, *Bufo*, chytridiomycosis, foraging behavior, *Hyla chrysoscelis*, pathogen

INTRODUCTION

There is increasing theoretical and empirical evidence of pathogen-induced modifications in host behavior (Altizer et al., 2003; Kluger et al., 1975; Moore, 2002). Pathogens can reduce survival in host species by directly causing mortality (Daszak et al., 1999; deCastro and Bolker, 2005) or indirectly by promoting behaviors that decrease host fitness while simultaneously increasing pathogen transmission (Dobson, 1988; Tierney et al., 1993). For example, snails (*Potamopyrgus antipodarum*) infected with the parasitic trematode (*Microphallus*) spend more time on rocks than uninfected snails, which leads to reduced foraging efficiency for snails and greater parasite transmission to the definitive host (Levri, 1999). In addition to causing mortality in host species, pathogen-induced changes in host behavior may negatively affect host life history traits, including growth and development (Forson and Storfer, 2006; Goater, 1994), and can alter population or community dynamics by disrupting intra- or interspecific interactions (Kohler and Wiley, 1997; Parris and Cornelius, 2004). Ultimately, pathogen impacts on host mortality or life history can reduce host fitness and even regulate host population size (Anderson and May, 1978).

One pathway by which pathogens alter host life history traits is through behavioral changes involving reductions in foraging performance (Levri and Lively, 1996). When pathogens reduce the foraging ability of individuals, host fitness may be compromised despite a sub-lethal infection (Gunn and Irvine, 2003). Although host species' behavioral responses to pathogens may be similar, the mechanisms behind reduced foraging performance may differ. For example, pathogens can directly reduce foraging performance by decreasing the host's foraging efficiency through impaired

visual performance (Crowden and Broom, 1980), a reduced stomach capacity (Wright et al., 2006), or damage to foraging structures (Drake et al., 2007). Pathogens can also indirectly reduce foraging performance of hosts by reducing their activity levels so that infected individuals forage less often than uninfected individuals. For example, bumble bees (*Bombus impatiens*) infected with an intestinal protozoan (*Crithidia bombi*) forage less often than uninfected bees (Ottershatter et al., 2005).

The role that pathogens play in amphibian populations and communities is especially important considering worldwide amphibian declines (Daszak et al., 2003; Lips et al., 2006). Although the loss of amphibian species are associated with multiple factors (Collins and Storfer, 2003; Dodd and Smith, 2003; Pounds et al., 2006), evidence is accumulating for the role that infectious diseases play in population regulation (Daszak et al., 2003; de Castro and Bolker, 2005). Chytridiomycosis is an emerging infectious disease of amphibians caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), which has played a major role in the decline of amphibian populations over the past two decades (Berger et al., 1998; Daszak et al., 2003). *Bd* infects keratinizing tissue, or those tissues fated to keratinize (Fellers et al., 2001), and colonization of *Bd* in tadpoles is restricted to the keratinized mouthparts. Although *Bd* does not directly cause mortality in anuran tadpoles, *Bd* often reduces growth and developmental rates (Parris 2004; Parris and Baud, 2004; Parris and Cornelius 2004; Garner et al., 2009; but see Smith et al., 2007). However, the mechanism(s) underlying this result are untested. Rapid growth and development is critical for survival of the majority of amphibian species which are dependent on temporary aquatic habitats (Werner and Anholt, 1993; Wilbur and Collins, 1973). That is, reaching a minimum size for metamorphosis before pond drying

increases the probability of successfully metamorphosing (Kiesecker and Skelly, 2001; Wilbur and Collins, 1973). Reductions in growth and development can also compromise larval fitness by decreasing the ability to escape gape-limited predators (Kurzava, 1998), and increasing intraspecific competition (Semlitsch et al., 1988).

Given the potential impacts that host behavior has on the disease transmission process, understanding the effects of *Bd* on amphibians with different behaviors and life histories is a key component in understanding the etiology of *Bd*. Between amphibian life stages (i.e., tadpoles and metamorphs), there are apparent differences in the pathogenicity of *Bd* (Carey et al., 2006; Parris and Cornelius, 2004). In the larval stage of anurans, there is interspecific variation in susceptibility to *Bd* (Blaustein et al., 2005; Woodhams and Alford, 2005) and *Bd* induced changes in growth and developmental rates (Parris and Cornelius, 2004); however, it is unknown whether *Bd* differentially affects species' behaviors. If differing host foraging strategies or morphologies are differentially impacted by *Bd* infections, the overall impact of *Bd* may be species specific. Intraspecific effects of *Bd* also remain unclear; specifically, little is known as to how *Bd* affects tadpoles through ontogeny (but see Smith et al., 2007). For example, natural ponds often contain many species of amphibian larvae in various developmental stages due to variation in timing of the breeding season. Given the evidence that tadpoles respond to environmental stressors differently during ontogeny (e.g., Audo et al., 1995), disease may influence larval performance at some developmental stages more than others. To elucidate the mechanism for reduced growth rates observed in *Bd* infected tadpoles, we conducted a series of laboratory experiments investigating the foraging behavior and foraging efficiency of anuran tadpoles through ontogeny. We used two

species of tadpoles—Grey Treefrogs (*Hyla versicolor*) and Fowler's Toads (*Anaxyrus* [= *Bufo*] *fowleri*). These two species are ideal focal taxa because they differ in behaviors associated with how and where they forage within the water column of ponds. Generally, *Anaxyrus* tadpoles aggregate in small groups and scrape food off the substrate of ponds (Beiswenger, 1977), whereas *Hyla* tadpoles forage actively from the middle portion of the water column (Wilbur and Alford, 1985). These differences in behavior are a critical component of our research design because differences in foraging behavior may differentially impact host fitness.

In the first experiment, we made repeated observations of foraging behavior at three stages during larval development in *Bd*-infected and uninfected *A. fowleri* and *H. chrysoscelis* tadpoles to test if *Bd* reduced foraging activity. In the second experiment, we tested the effects of *Bd* on foraging efficiency through development. We hypothesized that *Bd*-infected tadpoles would forage less often and less efficiently than uninfected tadpoles. If *Bd* differentially affects foraging performance at different stages in larval ontogeny, we also hypothesized that the effects of *Bd* on foraging performance would vary according to developmental stage.

MATERIALS AND METHODS

Animal Collection and Husbandry

Anaxyrus fowleri and *H. chrysoscelis* eggs were collected from natural ponds within the Meeman Shelby State Park in Tennessee, USA (Shelby County, TN, USA). All tadpoles

used in our experiments were derived from field-collected egg masses of each species. On 25 April 2008, we collected five *A. fowleri* egg masses from three ponds; on 2 May 2008, we collected six *H. chrysoscelis* egg masses from two ponds. Immediately after collection, eggs were transported to the laboratory at The University of Memphis. Upon hatching, tadpoles were maintained in 4.2 L plastic containers at a density of 5 tadpoles/L for two days. After this period, we removed all tadpoles that had reached the free-swimming stage (stage 25; Gosner, 1960) and combined the tadpoles from the different clutches to evenly distribute potential genetic effects on the larval traits we measured. We then randomly selected our test subjects out of the remaining stock of tadpoles. Test subjects were placed individually in 1.5 L plastic containers filled with 1 L of aged tap water. Throughout the experiment, tadpoles were maintained on a 12 h light: 12 h dark photoperiod at 19 C (±1 C) and were fed a mixture of ground rabbit chow and Sera Micron® daily.

Full water changes were conducted once per week. We took the following precautions to avoid accidental *Bd* transmission between treatments. For each water change, we used a piece of mesh screen to remove the focal tadpole from its container and placed it in a temporary transfer container with the same dimensions and volume of water as the experimental container. Water from the experimental container was poured into a bucket and 1.0 L of aged tap water was then added to that container. The focal tadpole was then placed back into the experimental container. During each water change, an experimenter wore latex gloves to prevent the transmission of *Bd*. To minimize the possibility of *Bd* transmission between *Bd* exposed and nonexposed (control) treatments, we always performed water changes on nonexposed tadpoles before exposed tadpoles.

Additionally, we used different laboratory equipment (gloves, transfer container, mesh screen) between each developmental stage in both of the pathogen treatments. At the end of the experiment, we thoroughly disinfected all containers by adding bleach (6% sodium hypochlorite) to yield a 10% solution, which kills *Bd* (Johnson and, Speare 2003). Throughout the experiment, all equipment and water was disinfected in a similar fashion.

Batrachochytrium dendrobatidis inoculation

Bd was grown in the laboratory on tryptone-gelatin hydrolysate-lactose (TGhL) agar in 9 cm Petri dishes according to standard protocol (Longcore et al., 1999). We harvested *Bd* zoospores by adding 10.0 mL of sterile water to the cultures and collected the zoospores that emerged from the zoosporangia after 30 min. At Gosner 25 (Day 0), all tadpoles of each species were randomly split into two experimental groups—a *Bd* exposed group and a nonexposed (control) group. For the *Bd* exposed group, we administered *Bd* by exposing *A. fowleri* (N = 40) and *H. chrysoscelis* (N = 37) to water baths containing infectious concentrations of fungal zoospores. Tadpoles were placed individually in 50 mL waterbaths and exposed to an infectious concentration of zoospores (2,500 zoospores/mL) for 24 hours. For the nonexposed group, we followed the same protocol but used plates with only TGhL, and exposed an additional group of tadpoles (*A. fowleri*, N = 40; *H. chrysoscelis*, N = 37) to water baths with no *Bd* zoospores. Our design simulated transmission by water, one of the possible modes of *Bd* transmission in natural environments (Pessier et al., 1999). Six days after exposure to *Bd*, we conducted the two foraging experiments which ran concurrently.

Experiment 1: tadpole foraging behavior

In Experiment 1, we tested whether *Bd* infections negatively affect foraging behavior of *A. fowleri* and *H. chrysoscelis* tadpoles through ontogeny. We made repeated observations of the foraging behavior of *Bd* exposed (N = 10, per species) and nonexposed (N = 10, per species) tadpoles at three developmental stages—early (Gosner 28–29), middle (Gosner 30–33), and late (Gosner 36–38). At each developmental stage, we made a series of three behavioral observations of foraging activity on each individual on three consecutive days. In total, nine behavioral observations were made on each larva. On each observation date, the order of the observations was randomized to minimize the potential for any diel effects on tadpole foraging behavior. In addition, the infection status of each larva was unknown during the behavioral observations.

Prior to the start of the behavioral trial, the focal container of each test subject was removed from a laboratory shelf and placed on an observation table. 0.1 grams of the rabbit chow/SeraMicron® mixture was dispensed into the water and then the test subject was allowed to acclimate for 3 minutes to any disturbances associated with the movement of the container. After the acclimation period, an experimenter observed the foraging behavior of the test subject during a 12 minute behavioral trial. Each experimental trial was divided into 20 second intervals and we recorded whether the test subject foraged at any point during the 20 second interval. The proportion of intervals during which the test subject foraged during the 12 minute experimental trial was calculated as an estimate of foraging activity. Similar measures have been used in other larval anuran behavior

experiments, and are accurate indices of overall activity level (Parris et al., 2006; Han et al., 2008).

Because of the differences in foraging behavior and mode among the two study species, we conducted a series of preliminary observations on uninfected tadpoles to qualify foraging behavior in our experiment. In general, *Anaxyrus* tadpoles foraged exclusively on food that sank to the floor of the experimental container and foraged with their bodies parallel to the floor of the container. In contrast, *Hyla* generally foraged on food that remained floating on the surface of the water with their bodies' perpendicular to the floor of the container. However, some *H. chrysoscelis* tadpoles foraged on large pieces of food that had sunk to the floor of the container. For both species, we qualified the test subject as foraging if it performed either of these behaviors. In addition, we qualified these behaviors as foraging only when the test subject was in close proximity to food in order to avoid erroneous classification of breathing as foraging behavior. A preliminary analysis of variance (ANOVA) revealed no significant differences in the percentage of time spent foraging between each of the three trial dates at each developmental stage ($P > 0.100$ in all cases). Accordingly, for each developmental stage, we took the grand mean of the three replicate trials and analyzed those data with repeated measures ANOVA. For each species, we used two way repeated measures ANOVA to test for an effect of pathogen treatment (*Bd* exposed or control) and developmental stage (early: Gosner 28–29; middle: Gosner 30–33; and late: Gosner 36–38) on tadpole foraging behavior. When appropriate, we used Holm-Sidak post-hoc analyses. All statistical analyses were performed in SPSS (version 17).

Experiment 2: tadpole foraging efficiency

In Experiment 2, we tested whether *Bd* infections negatively affect foraging efficiency of *A. fowleri* and *H. chrysoscelis* tadpoles throughout ontogeny. We assessed the short-term foraging efficiency of *Anaxyrus* (N = 30; 10 at each developmental stage) and *Hyla* (N = 27; 9 at each developmental stage) tadpoles by examining the quantity of food consumed during one 3 hour trial. Test subjects in this experiment were different than the subjects used in Experiment 1, although both experiments ran concurrently. Trials were conducted at the same developmental stages as those used in Experiment 1—early (Gosner 28–29), middle (Gosner 30–33), and late (Gosner 36–38). Each subject was sacrificed immediately following the trial; therefore, different individuals were used at each of the three developmental stages. As in Experiment 1, the infection status of each larva was unknown to the experimenter during data collection.

Prior to the start of the foraging efficiency trial, tadpoles were fasted for two days to empty the intestine. In addition, we checked the focal containers periodically during the two days prior to the experiment to remove any fecal matter, which could be a food source for tadpoles. A preliminary experiment with *H. versicolor* tadpoles confirmed this method of emptying the intestine (M. Venesky, *unpublished data*). At the start of the trial, we placed 0.30 g of cultured *Anabaena* in the container of each test subject. At the end of the trial, each test subject was removed from the container, sacrificed, and stored in 70% EtOH. All tadpoles were dissected on the same date of the experiment to accurately quantify *Anabaena* consumption during the trial.

To quantify the amount of *Anabaena* ingested during the experimental trial, we dissected and straightened the entire intestine on a dissection pan. We measured the length of the intestine in mm (to the nearest 0.05 mm) with calipers. We also measured the diameter (mm) of the intestine in three locations—the midpoint, and the anterior and posterior ends. We took the mean of the three diameter measurements and then estimated the total volume of the intestine. *Anabaena* is green and provides a sharp contrast to an empty intestine, allowing us to calculate the percentage of the intestine filled with food that was consumed during the 3 hour trial. At each developmental stage, we used independent samples *t* tests to assess species specific foraging responses to *Bd.* In each analysis, we used the percentage of intestine with food as the independent variable and the pathogen treatment (*Bd*-exposed or control) as the dependent variable. Our data met all assumptions of parametric statistics. All statistical analyses were performed in SPSS (version 17).

Confirmation of *Batrachochytrium dendrobatidis* infections

We confirmed the infection status of all *Bd* exposed and control tadpoles used in both experiments using real-time quantitative polymerase chain reaction (qPCR) following methods in Boyle et al. (2004). DNA was extracted from the tissue of the entire oral apparatus, which was dissected from all tadpoles immediately after their respective experimental trials. All tissues were stored in 100% EtOH until qPCR analyses. Each sample was run in triplicate against a *Bd* standard titration (from 10^5 to 10^1 zoospores) using relative qPCR on an ABI 7300 real-time PCR machine, and the pathogen treatment

(*Bd* exposed or control) was unknown to the experimenter. However, because quantification of infection intensity was beyond the scope of these experiments, we considered an animal as "infected" if it tested positive in at least two of the three qPCR replicates.

RESULTS

No tadpoles from our control treatment tested positive for *Bd* infection; however, qPCR analyses revealed considerable interspecific variation in the infection status of *Bd* exposed tadpoles. For *Anaxyrus,* 27.5% of the *Bd* exposed tadpoles tested positive: of the 11 *A. fowleri* infected with *Bd,* 4 were from the foraging behavior experiment and 7 were from the foraging efficiency experiment (4 tadpoles in the early and 3 tadpoles in the late developmental stages). For *Hyla*, 72.5% of the *Bd* exposed tadpoles tested positive: of the 29 *H. chrysoscelis* infected with *Bd,* 7 were from the foraging behavior experiment and 22 were from the foraging efficiency experiment (5, 9, and 8 individuals from the early, middle, and late developmental stages, respectively).

We designed our experiments to test for the effects of *Bd* infection on tadpole life history traits. However, the relatively high numbers of *Bd* exposed, but not infected, tadpoles allowed us to opportunistically test if *Bd* exposure, in the absence of infection, reduces tadpole foraging performance. When appropriate (when $N > 3$ within treatments), we used two way repeated measures ANOVA to test for an effect of pathogen treatment (*Bd* exposed and infected, *Bd* exposed but uninfected, or control) and developmental stage (early: Gosner 28–29; middle: Gosner 30–33; and late: Gosner 36–

38) on tadpole foraging behavior. For the foraging efficiency experiments, we were unable to statistically analyze the three treatment groups because of low sample sizes. Thus, we excluded the data from tadpoles in the *Bd* exposure treatment that did not test positive for *Bd* infection and tested for differences between *Bd* infected and control tadpoles (as described previously).

Experiment 1: tadpole foraging behavior

For *A. fowleri,* repeated measures ANOVA indicated a significant main effect of pathogen treatment on foraging behavior ($F_{2,59} = 10.71$; $P < 0.001$), with *Bd* infected animals foraging significantly less often than control and *Bd* exposed but uninfected animals (Fig. 1). Additionally, there was no significant difference between foraging activity of control and *Bd* exposed but uninfected animals. There was no effect of developmental stage ($F_{2,59} = 2.890$; $P = 0.069$) or a pathogen treatment by developmental stage interaction ($F_{4,59} = 0.611$; $P = 0.657$) on foraging behavior. Holm-Sidak post-hoc analysis indicated that *Bd* exposed and infected tadpoles were significantly less active than tadpoles from the control treatment in the early ($P = 0.006$), middle ($P < 0.0001$), and late ($P = 0.009$) developmental stages (Fig. 1). Additionally, *Bd* exposed and infected tadpoles were significantly less active than tadpoles from the *Bd* exposed and uninfected treatment in the early ($P = 0.009$) and middle ($P = 0.008$) developmental stages but not during the late developmental stage ($P = 0.091$). There were no significant differences in activity levels of tadpoles from the control and *Bd* exposed but not infected treatments.

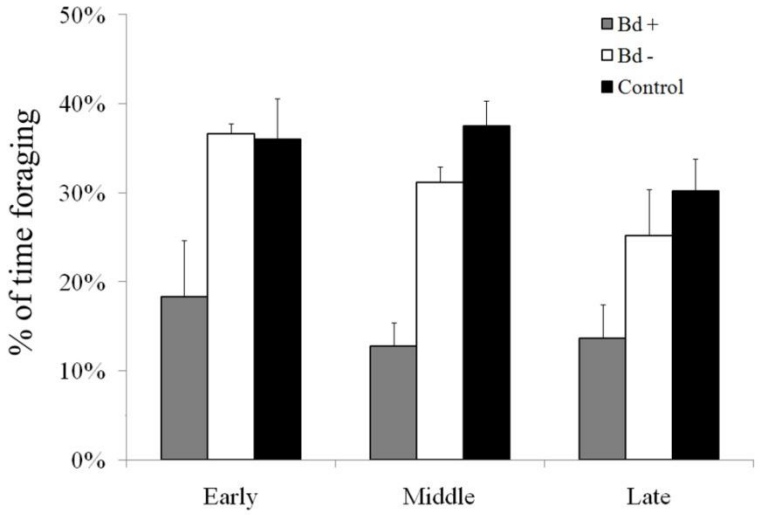

Figure 1. Percentage of time spent foraging for *Batrachochytrium dendrobatidis* exposed and infected (*Bd* +), *B. dendrobatidis* exposed and uninfected (*Bd* -), and uninfected (Control) Fowler's Toad (*Anaxyrus fowleri*) tadpoles observed through ontogeny. Asterisks indicate significant differences ($p < 0.05$) between pathogen treatments within early (Gosner 28–29), middle (Gosner 30–33), and late (Gosner 36–38) developmental stages. Values at each developmental stage are the treatment means of three observations + 1 SE.

In contrast to *A. fowleri*, *Bd* differentially affected tadpole foraging behavior in *H. chrysoscelis.* Repeated measures ANOVA revealed no main effect of pathogen treatment on foraging behavior ($F_{2,59} = 1.97$; $P = 0.170$), with *Bd* exposed and infected, *Bd* exposed but uninfected, and control animals spending similar amounts of time foraging (Fig. 2). There was a significant effect of developmental stage on time spent foraging ($F_{2,59} = 4.038$; $P = 0.027$); however, there was no pathogen treatment x developmental stage interaction ($F_{4,59} = 0.406$; $P = 0.803$).

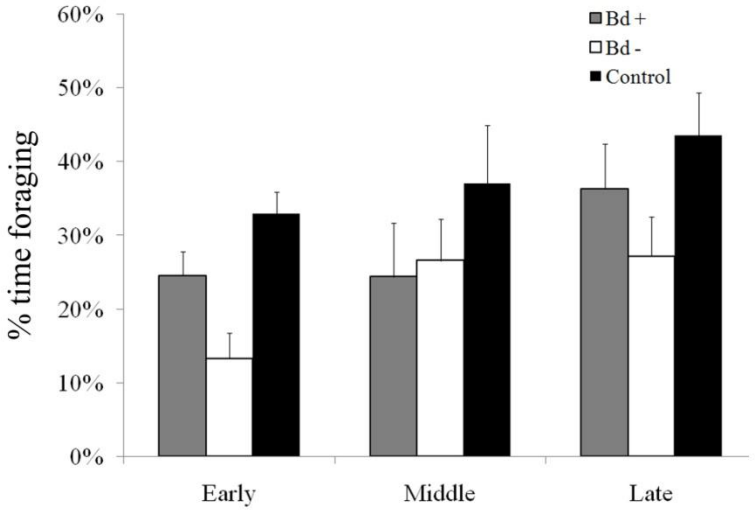

Figure 2. Percentage of time spent foraging for *Batrachochytrium dendrobatidis* exposed and infected (*Bd* +), *B. dendrobatidis* exposed and uninfected (*Bd* -), and uninfected (Control) Grey Treefrog (*Hyla chrysoscelis*) tadpoles observed within early (Gosner 28–29), middle (Gosner 30–33), and late (Gosner 36–38) developmental stages. Values at each developmental stage are the treatment means of three observations + 1 SE.

Experiment 2: tadpole foraging efficiency

In general, *Bd* infection had similar overall effects on tadpole foraging efficiency as it did on foraging behavior; however, there were clear intra- and interspecific differences in the foraging efficiency of *Bd*-infected tadpoles. *Bd* infected and uninfected *A. fowleri* did not exhibit differences in foraging efficiency early in development (t_{12} = 1.07; P = 0.308, Fig. 3 A) but *Bd* significantly reduced foraging efficiency late in development (t_{10} = 4.68; P = 0.001, Fig. 3 B). *Bd* infection in *H. chrysoscelis* reduced foraging efficiency in the early and middle developmental stages (early: t_{12} = 3.65; P = 0.003; middle: t_{16} = 4.50; P = 0.003; Fig. 4 A, B) but had no effect late in development (t_{15} = 1.47; P = 0.162; Fig. 4 C).

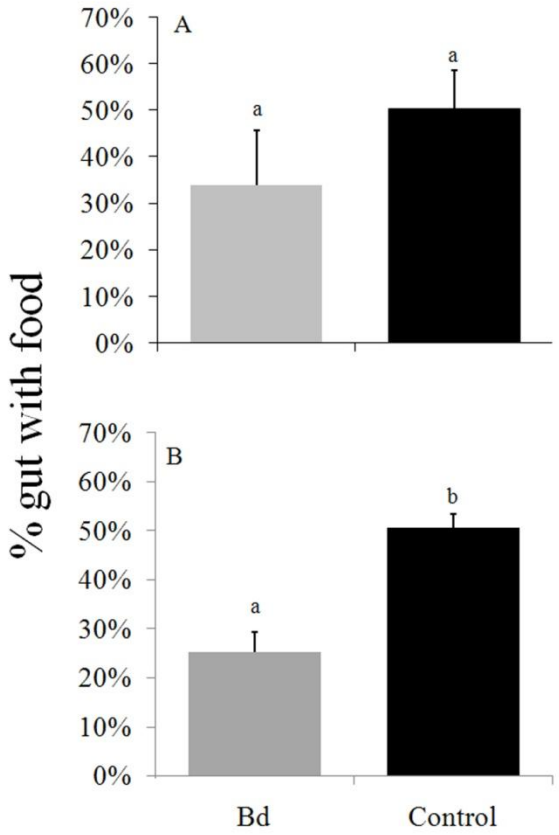

Figure 3. Percentage of food consumed for uninfected (Control) and *Batrachochytrium dendrobatidis* infected (*Bd*+) Fowler's Toad (*Anaxyrus fowleri*) tadpoles during (A) early and (B) late developmental stages. Different letters above histograms indicate significant differences between pathogen treatment groups ($p < 0.05$). Values are treatment means + 1 SE.

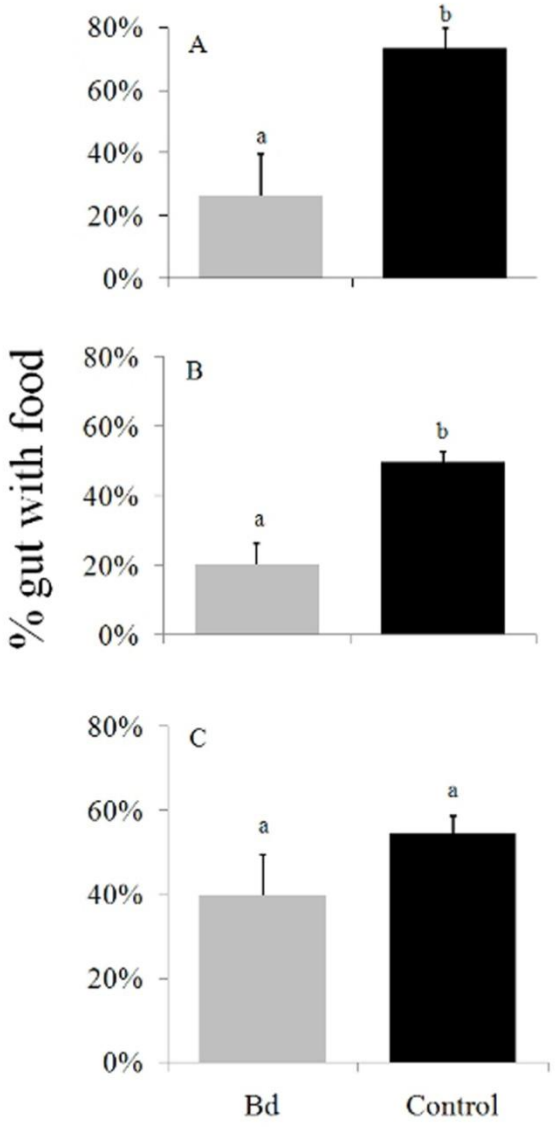

Figure 4. Percentage of food consumed for uninfected (Control) and *Batrachochytrium dendrobatidis* infected (*Bd*+) Grey Treefrog (*Hyla chrysoscelis*) tadpoles during (A) early, (B) middle, and (C) late developmental stages. Different letters above histograms indicate significant differences between pathogen treatment groups ($p < 0.05$). Values are treatment means + 1 SE.

DISCUSSION

Ecologists increasingly recognize the potential for pathogens to modify host behavior which can disrupt predator-prey dynamics (Parris et al., 2006; Reed and Dobson, 1993), food webs (Lafferty, 2006), and compromise foraging abilities of infected hosts (Levri and Lively, 1996). Recent evidence suggests that tadpoles exhibit reduced growth and developmental rates when infected with *Bd* (Parris and Cornelius, 2004), yet the mechanism underlying this response was previously untested. Our results provide evidence that *Bd* infections reduce foraging efficiency in *Anaxyrus* and *Hyla* tadpoles and that *Bd* differentially affects foraging behavior in these species. Namely, *Bd* infections reduce the foraging activity of *Anaxyrus* tadpoles but not *Hyla*. In addition, uninfected tadpoles exposed to *Bd* were as active as control tadpoles.

Prevention of pathogenesis is costly to the host (Woodhams et al., 2007) and may reduce the host's ability to maintain normal somatic growth and development. Given the high energetic costs of metamorphosis (Steiner and van Buskirk, 2008), inadequate growth rates during the larval period can negatively affect adult fitness. Therefore, with or without infection, exposure to a pathogen may impose physiological costs on the host and thereby negatively affect performance or survival. For example, Garner et al. (2009) found that *Bd* exposure always reduced growth and developmental rates of *Bufo bufo* tadpoles, even if they did not exhibit infection at the time of death. However, we found no evidence that *Bd* exposure, without pathogenesis, negatively affects foraging behavior in these species. Thus, *Bd* infections negatively impact the foraging performance of infected tadpoles of some species and may decrease growth and developmental rates,

effects which may have cascading effects on host survival in larval (Kurzava, 1998; Semlitsch et al., 1988) and adult (Berven, 1990; Smith 1987) phases.

In temporary aquatic habitats, the optimal strategy for tadpoles is to obtain the most food in the shortest period of time within the biotic and abiotic constraints of the given habitat (Wilbur and Collins, 1973). Ultimately, low activity levels and reduced foraging behavior in tadpoles may lead to reduced food intake (Anholt et al., 1996; Skelly, 1994), which can impact growth and developmental rates and cause larval mortality due to pond drying (Skelly and Werner, 1990) or decrease adult fitness in terrestrial environments because of smaller metamorphic sizes (Goater, 1994). In our foraging behavior experiments, *Bd* infected *Anaxyrus* tadpoles reduced the amount of time they spent foraging compared to uninfected tadpoles; however, *Bd* had no effect on foraging time in *Hyla* tadpoles. The general reduction in overall tadpole activity levels we observed is consistent with recent experiments examining pathogen- induced changes in host behavior. For example, Parris et al. (2004) examined activity levels of *Bd* infected Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles within an antipredatory context and found that *Bd* infected tadpoles decreased their activity levels across all treatments, regardless of whether a predator was present or absent. Low activity levels may be a host's general response to infection (Giles, 1983) rather than a specific response to a predator (Parris et al., 2004) or reduced foraging ability (this study). It is important to note that the link between foraging activity and growth rates is complex (Steiner, 2007) and factors such as metabolic rate (McPeek, 2004), gut morphology (Relyea and Auld, 2004), and immune response (Giles, 1983; Roy and

Kirchner, 2000) may interact to mediate the tradeoffs between foraging activity and growth rates.

Many temperate zone tadpoles have keratinized jaw sheaths and labial teeth (Altig and McDiarmid, 1999) which allow them to facultatively forage on attached or suspended phytoplankton (Seale and Wassersug, 1979). These specializations in feeding morphology are critical for successful tadpole foraging. Oral deformities in tadpoles occur in response to *Bd* (Fellers et al., 2001), environmental contaminants (Blaustein and Johnson, 2003), and eutrophication (Johnson and Chase, 2004). Although the relationship between the occurrence of oral deformities and the effect(s) of these deformations on food acquisition is unclear, damaged foraging structures (i.e., keratinized jaw sheaths and labial teeth) likely compromise the foraging abilities of tadpoles (Rowe et al., 1996). In our experiments, *Bd* infected *Anaxyrus* and *Hyla* tadpoles consumed less food during experimental trials than uninfected tadpoles, suggesting that *Bd* infected tadpoles are less efficient at foraging. Recent experiments have documented a strong association between *Bd* infection and the loss of keratinized jaw sheaths and labial teeth (Drake et al., 2007; Fellers et al., 2001; Marantelli et al., 2004) and we documented these deformities in a subset of the tadpoles we observed. In general, *Bd* infected tadpoles were missing portions of labial tooth rows. Given the functional role that labial teeth have in the foraging process (Wassersug and Yamashita, 2001), damage to labial tooth rows likely affects how tadpoles grasp and rake food while foraging.

Indeed, *Bd* infected *Anaxyrus* and *Hyla* tadpoles with missing teeth have inferior feeding kinematics compared to uninfected tadpoles. The keratinized labial tooth rows of *Bd* infected *Anaxyrus* and *Hyla* tadpoles spend less time raking over an algal covered

substrate relative to tadpoles with undamaged dentition (Venesky et al., *in review*). To compensate, *Bd* infected tadpoles increase the rate in which their mouths open and close while foraging. Thus, *Bd* infected tadpoles may suffer a reduced potential to obtain sufficient food or may expend more energy while foraging—both of which can reduce growth and developmental rates. It is important to note that we did not test for the degree of oral deformities in *Bd* infected tadpoles and are unable to quantify how specific patterns of mouthpart damage may influence foraging efficiency.

There is strong evidence that food availability can influence the size at and timing of metamorphosis (Blouin 1992; Semlitsch and Caldwell, 1982) and some experiments have tested the effect of food deprivation on growth and developmental rates through ontogeny (Audo et al., 1995; Hensley, 1993; Leips and Travis, 1994). These studies confirm that food availability affects size at metamorphosis, but developmental rates become fixed during late stages in ontogeny (Hensley, 1993; Leips and Travis, 1994). Food deprivation early in ontogeny reduces carbohydrate stores in tadpoles (Audo et al., 1995), which can reduce growth and developmental rates if food availability remains low. Therefore, it is possible that decreased foraging abilities and food acquisition in *Bd* infected tadpoles has a direct effect on growth and developmental rates.

One key finding of our experiments was that *Bd* differentially affected the foraging performance of *Anaxyrus* and *Hyla*. In our behavior experiments, *Bd* reduced the amount of time *Anaxyrus* spent foraging but did not affect the amount of time *Hyla* spent foraging. In our efficiency experiments, both *Anaxyrus* and *Hyla* responded to *Bd* with reduced foraging efficiency. Without an understanding of other components of the tadpole response to *Bd* (i.e., physiological), it is unclear why *Bd* reduced the foraging

behavior of *Anaxyrus* tadpoles but not *Hyla.* When considering the behavioral differences between tadpoles of these two species, *Anaxyrus* tadpoles differ from *Hyla* in that they are more social and are often found in aggregations (Beiswenger, 1977; Wilbur and Alford, 1985). Thus, the response observed in individual host behaviors may differ from behaviors exhibited when hosts are in groups (Han et al., 2008). Additional experiments testing the potential of pathogens to alter social cues would contribute to the biology of infectious diseases.

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Chapter 4 Can Differences in Host Behavior Drive Patterns of Disease Prevalence in Amphibian Communities?

Abstract

Differences in host behavior and susceptibility to disease can influence the outcome of community interactions. We capitalized on the variation in aggregation behavior of Fowler's toads (*Anaxyrus* [= *Bufo*] *fowleri*) and grey treefrogs (*Hyla versicolor*) tadpoles and tested for differences in transmission of *Batrachochytrium dendrobatidis* (*Bd*) and host-specific fitness consequences of infection in single-species amphibian mesocosms. On average, *A. fowleri* mesocosms supported higher *Bd* prevalences and infection intensities relative to *H. versicolor* mesocosms. Higher *Bd* prevalence in *A. fowleri* mesocosms may result, in part, from higher transmission rates due to the aggregation behavior observed in tadpoles raised in *Bd* treatments. We also found that, independent of species, tadpoles raised in the presence of *Bd* were smaller and less developed than tadpoles raised in disease-free conditions. Our results indicate that aggregation behavior might increase *Bd* transmission in semi-natural environments and that *A. fowleri* tadpoles appear more susceptible to infection relative to *H. versicolor* tadpoles. Although our results suggest that *A. fowleri* tadpoles are more susceptible to *Bd* and carry heavier infections, our results also demonstrate that *Bd* appears to negatively impact larval life history traits of *A. fowleri* and *H. versicolor* similarly.

Keywords *Anaxyrus fowleri*, chytridiomycosis, *Batrachochytrium dendrobatidis*, *Hyla versicolor*, life history

Introduction

Traditionally, ecologists have focused on the effects of disturbance, competition, and predation on community processes (Morin 1983; Menge and Sutherland 1987). However, recent studies underscore the impacts that parasites and pathogens (hereafter "pathogens") have on communities (Hatcher et al. 2008; Lafferty et al. 2008; Johnson et al. 2009). For example, pathogens can directly affect host populations by causing mortality (Brunner et al. 2004) or indirectly by altering life history traits, such as growth and developmental rates (Goater 1994). Additionally, pathogens can alter normal host behaviors such as feeding (Otterstatter et al. 2005), antipredator (Thiemann and Wassersug 2000), and thermoregulatory (Elliot et al. 2002), each of which can have regulatory effects in host populations and communities in the absence of host mortality.

Certain host behaviors, in the absence of infection, can increase disease susceptibility and thereby increase pathogen transmission rates. For example, hosts with promiscuous mating strategies may increase the probability of contact with an infected host (Altizer et al. 2003). Alternatively, pathogens can alter host behavior such that pathogen transmission is increased. For example, snails (*Potamopyrgus antipodarum*) infected with the parasitic trematode (*Microphallus* spp.) spend more time on rocks than non-infected snails, which leads to reduced foraging efficiency in snails and greater parasite transmission to the definitive host (Levri 1999). Since pathogen transmission is one of the driving forces behind pathogen regulation of host populations (de Castro and Bolker 2005), identifying how pathogen-induced changes in host behavior affect pathogen transmission is a central component of host–pathogen ecology.

Determining the role that pathogens play in amphibian populations is especially important given their worldwide population declines (Daszak et al. 2003; Lips et al. 2006). Chytridiomycosis, an infectious disease of amphibians, is caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) and has been implicated as a causal agent in amphibian population declines over the past three decades (Berger et al. 1998; Daszak et al. 2003). *Bd* is spread through aquatic environments by free-swimming zoospores (Longcore et al. 1999; Pessier et al. 1999). In metamorphosed individuals, *Bd* infects epidermal tissues (Longcore et al. 1999). *Bd* infections inhibit electrolyte transport in amphibian epidermis and ultimately result in mortality from cardiac arrest (Voyles et al. 2009). In tadpoles, *Bd* infections are restricted to, or around, the keratinized labial teeth and jaw sheaths. *Bd* does not generally cause mortality in anuran tadpoles (but see Blaustein et al. 2005); however, *Bd* infections reduce larval feeding efficiency (Venesky et al. 2009; Venesky et al. 2010a) and growth and developmental rates (Parris 2004; Parris and Baud 2004; Parris and Cornelius 2004; Garner et al. 2009).

Given the potential impacts that interspecific differences in host behavior and life history traits have on disease dynamics in host populations, understanding the effects of *Bd* on amphibians with different behaviors and life histories are a key component in understanding the etiology of *Bd*. However, host variation to susceptibility and pathogen-induced life history tradeoffs are poorly understood (but see Parris and Cornelius 2004; Blaustein et al. 2005; Woodhams and Alford 2005; Venesky et al. 2009). For example, Venesky et al. (2009) found that *Bd* infection reduced the foraging activity of *A. fowleri* tadpoles but not *H. chrysoscelis* tadpoles. Additionally, few data exist on how far zoospores can travel and infect a susceptible host. If *Bd* transmission is limited by how

far zoospores can travel, species-specific life history, or behavioral, strategies may differentially impact certain species. For example, species that aggregate may have an increased potential of disease transmission (Blaustein and Bancroft 2007). Indeed, recent evidence suggests that tadpoles of species that are highly social increase their aggregative behavior when infected with *Bd* (Han et al. 2008).

In our study, we used two species of anuran tadpoles—Fowler's toads (*Anaxyrus* [= *Bufo*] *fowleri*) and grey treefrogs (*Hyla versicolor*). These two species are ideal focal taxa because they are sympatric in ponds yet differ in their aggregative behavior, which may differentially impact pathogen transmission. Generally, *A. fowleri* tadpoles aggregate on the substrate of ponds (Beiswenger 1977), whereas *H. versicolor* tadpoles typically occur solitary in the middle of the water column (Wilbur and Alford 1985). In our study, we assessed pathogen effects on larval life history traits in replicated, single–species, outdoor experimental mesocosms. First, we estimated aggregation behavior of tadpoles reared in the *Bd* treatments to test if species-specific differences in behavior facilitated transmission rates. We then inferred *Bd* transmission rates from infected tadpoles to non-infected tadpoles and tested for species-specific differences in prevalence and infection intensity. We predicted that aggregation of *A. fowleri* tadpoles would increase *Bd* transmission and result in higher *Bd* prevalence and infection intensity. Lastly, we tested if susceptible host tadpoles reared in the presence of *Bd* affected their growth and development rates.

Materials and methods

Animal Collection and Husbandry

All tadpoles used in our experiments were derived from field-collected egg masses of *A. fowleri* and *H. versicolor* from within the Meeman Shelby State Park in Tennessee, USA (Shelby County, TN, USA) in 2009. Immediately after collection, eggs were transported to the laboratory at The University of Memphis. Upon hatching, tadpoles were maintained in 37.85 l glass aquaria (filled with approximately 20 l of aged tap water that was continually aerated) containers at a density of 5 tadpoles/l until they had reached the free-swimming stage (stage 25; Gosner 1960). We haphazardly selected a subset of tadpoles from all the clutches and combined the tadpoles from the different clutches to evenly distribute potential genetic effects on the larval traits we measured. We then randomly selected a subset out of the remaining stock of tadpoles to expose to *Bd*, which served as "pathogen source" tadpoles (see below). We kept the remainder of the tadpoles in 37.85 l glass aquaria by species in the laboratory for 7 days. Tadpoles were maintained on a 12 h light: 12 h dark photoperiod at 19 C (±1 C) and were fed a mixture of ground rabbit chow and Sera Micron® daily.

Batrachochytrium dendrobatidis inoculation

Bd was grown in the laboratory on tryptone-gelatin hydrolysate-lactose (TGhL) agar in 9 cm Petri dishes according to standard protocol (Longcore et al. 1999). We harvested *Bd*

zoospores by adding 10.0-ml of sterile water to the cultures and collected the zoospores that emerged from the zoosporangia after 30 min. We exposed the pathogen source tadpoles (N = 38 per species) to an infectious dose of *Bd* by placing individual tadpoles in 50-ml water baths containing infectious concentrations of fungal zoospores (120,000 zoospores/ml) for 48 hours. Our design simulated transmission by water, one of the possible modes of *Bd* transmission in natural environments (Pessier et al. 1999). After the exposure period, tadpoles were held in 37.85 l glass aquaria (filled with approximately 20 l of aged tap water that was continually aerated) by species in the laboratory. Most of the tadpoles (N = 24 per species) were held in the laboratory for 5 days post exposure (7 days total) before placing them in outdoor experimental tanks (see below); the remaining tadpoles (N = 14 per species) were held individually in the laboratory for 30 days to confirm our infection protocol.

Experimental Design

We manipulated the presence of *Bd* in replicated experimental mesocosms. We reared tadpoles in 32 polyethylene tanks (1.83 m diameter) positioned in an array at the University of Memphis Edward J. Meeman Biological Field Station (Shelby County, Tenn., United States; 35°22’ N, 90°1’W). Tanks were positioned in a fenced field adjacent to natural breeding habitats of both *A. fowleri* and *H. versicolor*. We prepared tanks prior to the breeding season of anurans in early April 2009. Each tank was filled with tap water to a depth of 30.5 cm (~ 613 l) and 1.0 kg of air dried leaf litter collected from a nearby deciduous forest was added. One 500-ml aliquots of concentrated plankton

suspension was collected from several unused experimental tanks that were set up during August 2008. We placed a 65.58 x 121.92 cm piece of fiberglass siding in each tank at a 30° angle which simulated the margin of a pond. This is an important component of our design because larval *A. fowleri* do not have lungs and are negatively buoyant (Wassersug and Feder 1983); thus, it allowed tadpoles to position themselves in the water column without expending extra energy to respirate and provided a location for *A. fowleri* tadpoles to aggregate as observed in natural ponds. We then securely fashioned fiberglass mesh screens (1-mm mesh) to each tank to prevent colonization of feral predators and competitors and to provide shading and allowed tanks to condition for 14 days before adding tadpoles. All tank preparations followed approved IACUC protocols and no further permits were necessary.

For each species (*A. fowleri* and *H. versicolor*), we used a 2 X 2 fully factorial design consisting of tadpoles reared in two pathogen treatments (*Bd* and control) for two trial durations (10 and 15 days). The resulting 8 treatment combinations were replicated 4 times and assigned randomly to the 32 experimental tanks. On 0800 on 04-May-2009 (Day 0), we brought the pathogen source and non-exposed tadpoles from the laboratory and allowed them to acclimate to the ambient temperature for 4 hours prior to placing them in the experimental tanks. For the control treatment, we placed 30 conspecific tadpoles in each experimental tank (N = 8 per species). For the *Bd* treatment, we first placed 27 non-exposed tadpoles of each species in the remaining experimental tanks (N = 8 per species). We then placed 3 *Bd* exposed tadpoles of each species in the tanks of the *Bd* treatment, equalizing the density of the two pathogen treatments. For our pathogen treatment, starting *Bd* prevalence was 10%. Since we were unable to individually mark

the pathogen source tadpoles by tail clipping or elastomer dyes because of their small body size and color of the tadpoles, we considered any prevalence per tank above 10% a conservative estimate for transmission.

Throughout the experiment, we placed all laboratory materials in a containment tank with bleach (6% sodium hypochlorite) to yield a 10% solution, which kills *Bd* (Johnson and Speare 2003). At the completion of the experiment, we also added bleach to each tank to yield a 10% solution. The lids were then securely fashioned on the tanks and we allowed the bleach solution sit for 30 days prior to emptying the water from each tank.

Bd transmission, prevalence and life history traits

On Day 10, we destructively sampled 16 experimental tanks by individually removing every larva from each tank with a small dipnet. To prevent accidental contamination of samples, we first collected all tadpoles from the control tanks first. In the *Bd* tanks, we collected each larva individually, rinsed it with aged tap water, and placed it in an individual screw top vial containing MS222. Before using the dipnet again, the dipnet was thoroughly rinsed with aged tap water and briefly soaked in the mesocosm to remove any potential zoospores the previous larva deposited on the net. These methods prevented accidental transfer of *Bd* between tadpoles in, and between, each experimental tank. On Day 15, we destructively sampled the remaining 16 experimental tanks as described previously. None of the tadpoles metamorphosed, thus justifying the 15 day experimental duration in assessing pathogen effects on tadpoles. All tadpoles were stored in 100%

EtOH and brought to the laboratory where we measured the total length (TL) and determined the Gosner stage of each larva. After collecting data on growth and development, we dissected the oral apparatus for quantitative PCR analysis to confirm *Bd* infection.

Following methods in Boyle et al. (2004), we used real-time quantitative polymerase chain reaction (qPCR) to confirm the infection status of all tadpoles from *Bd* mesocosms and three randomly chosen control mesocosms. We also used qPCR on laboratory held tadpoles (N = 28) to confirm our *Bd* exposure method. In brief, DNA was extracted from the tissue of the entire oral apparatus, which was dissected from all tadpoles immediately after collection, and stored in 100% EtOH until qPCR analyses. Each sample was run in triplicate against a *Bd* standard titration (from 10^5 to 10^1 zoospores) using relative qPCR on an ABI 7300 real-time PCR machine, and the pathogen treatment (*Bd* exposed or control) was unknown to the experimenter. We considered an animal as "infected" if the *Bd* genome equivalent was greater than 0.1 (Searle et al. 2010).

Behavioral Observations

We monitored our experimental array every five days from 0800–1000 to estimate the aggregation behavior of tadpoles by counting the number of tadpoles that co-occurred on the artificial fiberglass pond margin (a standardized area in the mesocosm). We focused on tadpole co-occurrence on the fiberglass pond margins because *A. fowleri* tadpoles are unlikely to aggregate on the floor of the tank because they avoid deep water. We counted

the number of tadpoles that co-occurred on the fiberglass pond margin and considered them to be "aggregating". In general, tadpoles on the fiberglass pond margin were approximately 15 cm apart. We considered this measure an effective measure of aggregation behavior because of the close proximity of tadpoles to one another on a standardized area within the mesocosm.

Statistical Analyses

To obtain *Bd* prevalence within each mesocosm, we calculated the proportion of tadpoles infected with *Bd* at the termination of the experiment. Additionally, we obtained the zoospore equivalents of *Bd*-infected tadpoles as a measure of infection intensity. For this measure, we considered mean values per tank as the unit of analysis because measurements from individuals within tanks were not independent. We used analysis of variance (ANOVA) to test for the effects of independent variables species (*A. fowleri* and *H. versicolor*) and experimental duration (10 and 15 days) on the dependent variables proportion of tadpoles infected and their corresponding zoospore equivalents. We used arcsine plus square root and log transformations to normalize the prevalence and intensity data (respectively).

We measured growth (TL) and developmental rates (Gosner) as metrics of larval performance. We considered mean values per tank as the unit of analysis because measurements from individuals within tanks were not independent. We used multivariate analysis of variance (MANOVA) to test for the effects of independent variables species (*A. fowleri* and *H. versicolor*), pathogen treatment (*Bd* and control), and experimental

duration (10 and 15 days) and their interactions on the dependent variables size (TL) and stage (Gosner). Because of significant correlations between TL and Gosner, we then used reciprocal univariate analysis of covariance (ANCOVA) on those dependent variables.

For each pathogen treatment, we averaged the proportion of tadpoles that we observed aggregating (number of tadpoles aggregating/total number of tadpoles per mesocosm) across both observation dates. We used two-way ANOVA to test for the effects of the independent variables species (*A. fowleri* and *H. versicolor*), pathogen treatment (*Bd* and control), and their interaction on the dependent variable proportion of tadpoles aggregated. We used arcsine transformation to normalize our data.

All statistical analyses were performed in SPSS. Our data met the assumptions of the statistical tests used.

Results

Infection prevalence of the subset of tadpoles (N = 14 per species) that were held in the laboratory was 50% (35.7% for *A. fowleri* and 64.2% for *H. versicolor*), confirming that our infection protocol was capable of infecting susceptible hosts. One experimental mesocosm (*H. versicolor*, *Bd*, 15 day duration) developed an algal bloom that killed all the tadpoles, which was subsequently excluded from analyses. No tadpoles (N = 90) from the 3 randomly selected control mesocosms tested positive for *Bd*.

Bd transmission, prevalence, and infection intensity

Although *Bd* transmission was low in the pathogen treatments, we detected transmission ($N > 3$ *Bd* infected tadpoles per mesocosm) in 5/15 mesocosms—4 in *A. fowleri* and 1 in *H. versicolor* mesocosms. In addition, we found species differences in *Bd* prevalence, the total number of *Bd* zoospores per mesocosm, and the average infection intensity per individual. Additionally, *Bd* prevalence significantly differed between species ($F_{3,11} = 15.23$, $P = 0.0025$) where *A. fowleri* mesocosms had higher prevalence relative to *H. versicolor* mesocosms (11.7% ± 1.54 and 4.8% ± 1.60, respectively). Irrespective of species, tadpoles exposed to *Bd* for 15 days had marginally higher *Bd* prevalence ($F_{3,11} = 4.54$, $P = 0.0566$); however, there was no species x duration interaction.

The total number of *Bd* zoospores detected, calculated as a sum of zoospore equivalents among infected individuals in the same replicate, varied as a function of species. Irrespective of trial duration, the average *Bd* zoospores in *A. fowleri* mesocosms was significantly higher than those recovered in mesocosms with *H. versicolor* tadpoles ($F_{1,11} = 5.48$, $P = 0.036$; Fig. 1a). We observed a similar pattern for average infection intensities per tadpole. Overall, *A. fowleri* tadpoles had higher average infections relative to *H. versicolor* tadpoles ($F_{1,11} = 13.47$, $P = 0.004$; Fig. 1b). Additionally, we found a significant species x duration interaction, where at the 15 day trial duration, *A. fowleri* tadpoles had significantly higher average infections relative to *H. versicolor* tadpoles ($F_{1,11} = 5.74$, $P = 0.035$).

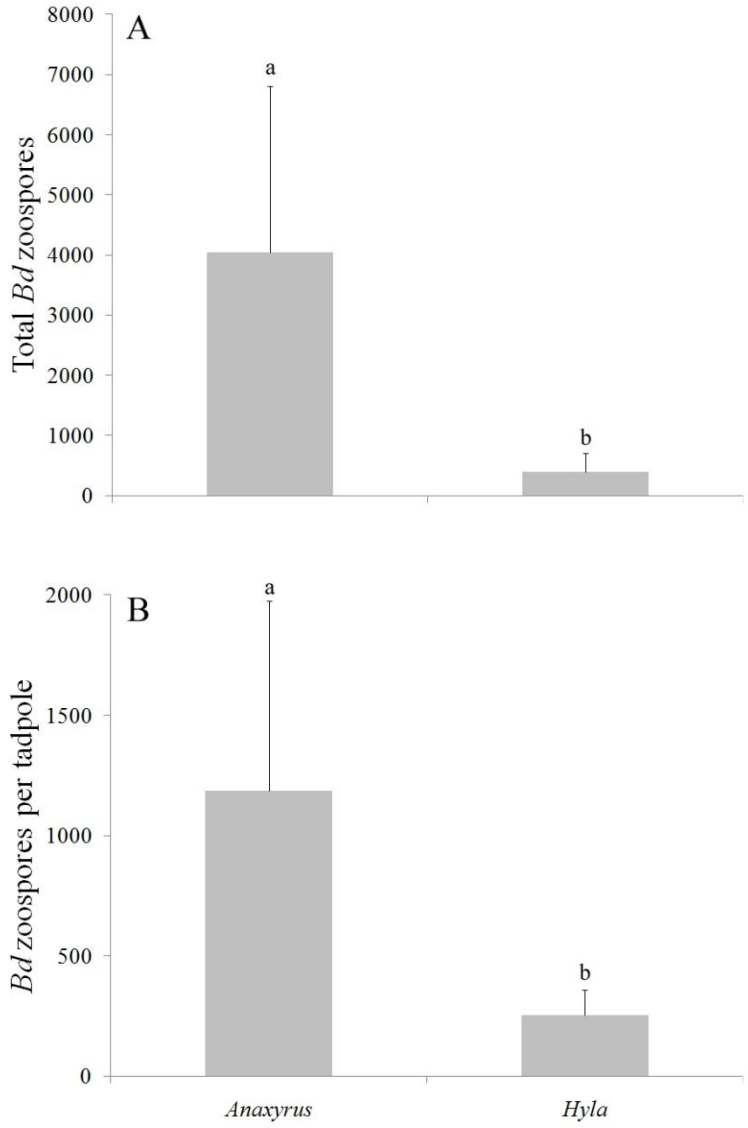

Fig. 1. The number of *Batrachochytrium dendrobatidis* (*Bd*) zoospores detected from whole mouthparts of *Anaxyrus fowleri* and *Hyla versicolor* tadpoles pooled across both trial durations. Different letters above histograms indicate a significant difference among treatments. (A) The total *Bd* zoospores detected (+1 SE), calculated as a sum of zoospores among infected individuals in the same replicate. On average, *A. fowleri* mesocosms supported more *Bd* zoospores than *H. versicolor* mesocosms ($F_{1,11} = 5.48$, $P = 0.036$). (B) The total *Bd* zoospores detected (+1 SE) on individual tadpoles within each replicate. On average, *A. fowleri* tadpoles had higher infections relative to *H. versicolor* tadpoles ($F_{1,11} = 13.47$, $P = 0.004$).

Behavioral Observations

Neither species ($F_{1,31} = 2.63$, $P = 0.116$) nor pathogen ($F_{1,31} = 0.149$, $P = 0.702$) significantly affected the proportion of tadpoles aggregating on the artificial fiberglass pond margin compared. However, we found a significant species x pathogen interaction ($F_{1,11} = 13.16$, $P = 0.001$) on the proportion of tadpoles aggregating. Holm-Sidak post hoc analyses revealed that *A. fowleri* tadpoles aggregated significantly more ($t = 2.84$, $P = 0.008$) in *Bd* mesocosms relative to control mesocosms whereas *H. versicolor* tadpoles aggregated significantly less ($t = 2.29$, $P = 0.030$) in *Bd* mesocosms relative to control mesocosms.

Life History Traits

There were significant MANOVA effects of species, pathogen treatment, duration, and species x duration interaction effects on the combined larval responses (Table 1). ANCOVA revealed significant effects of *Bd* on the size and stage of tadpoles developing in pathogen tanks. Under disease conditions, tadpoles of both species were smaller and less developed than tadpoles reared in disease-free conditions, irrespective of the experiment duration (Table 1, Fig. 2, Fig. 3). Additionally, at the 15 day trial duration, tadpoles of both species reared in disease conditions were significantly smaller than tadpoles in disease-free conditions for the same duration of time (Table 2, Fig. 3).

Table 1. Summary of the MANOVA and univariate ANCOVAs for larval stage (Gosner) and size (total length) for single species mesocosms of *Anaxyrus fowleri* and *Hyla chrysoscelis* tadpoles reared in the presence of absence of *Batrachochytrium dendrobatidis* infected tadpoles at two trial durations. Both independent variables were used as reciprocal covariates in ANCOVAs. Significance levels for univariate tests were interpreted at 0.05.

Source	Wilks' λ	*F*	*P*
MANOVA: stage, size			
Species	0.032	330.57	<0.001
Pathogen	0.629	6.490	0.006
Duration	0.294	26.47	<0.001
Species x pathogen	0.987	0.149	0.863
Species x duration	0.472	12.30	<0.001
Pathogen x duration	0.772	3.248	0.058
Species x pathogen x duration	0.939	0.720	0.498

Source	*F*	*P*
ANCOVA: stage		
Species	377.26	<0.001
Pathogen	12.57	0.002
Duration	0.08	0.781
Species x pathogen	0.29	0.596
Species x duration	21.89	<0.001
Pathogen x duration	2.10	0.161
Species x pathogen x duration	0.42	0.522
Size (covariate)	146.22	<0.001
ANCOVA: size		
Species	265.59	<0.001
Pathogen	8.65	0.008
Duration	1.44	0.244
Species x pathogen	0.29	0.599
Species x duration	23.70	<0.001
Pathogen x duration	4.47	0.046
Species x pathogen x duration	0.94	0.342
Stage (covariate)	172.19	<0.001

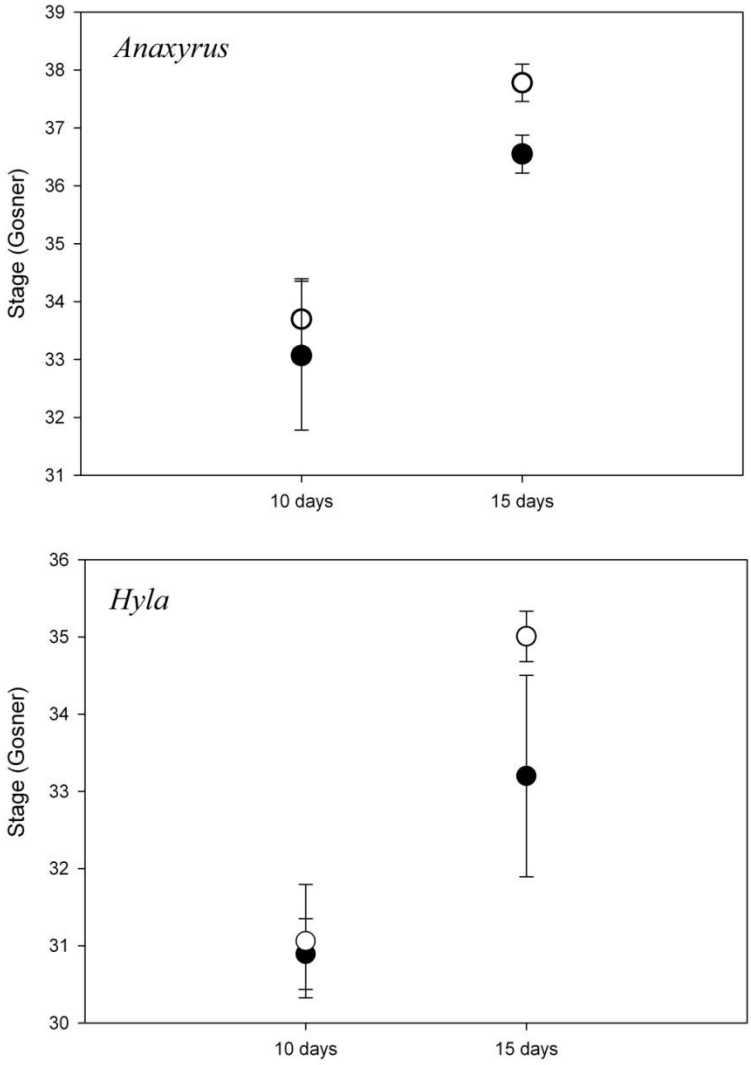

Fig. 2. Developmental stage (Gosner) of *Anaxyrus fowleri* and *Hyla versicolor* tadpoles raised for 10 and 15 days. Data points represent tank means (±1 SE). Open circles are tadpoles raised in the absence of disease; filled circles are tadpoles raised in the presence of *Batrachochytrium dendrobatidis* (*Bd*). Overall, tadpoles raised in the presence of *Bd* were less developed compared to tadpoles raised without *Bd* ($F_{1,22} = 12.57$, $P = 0.002$).

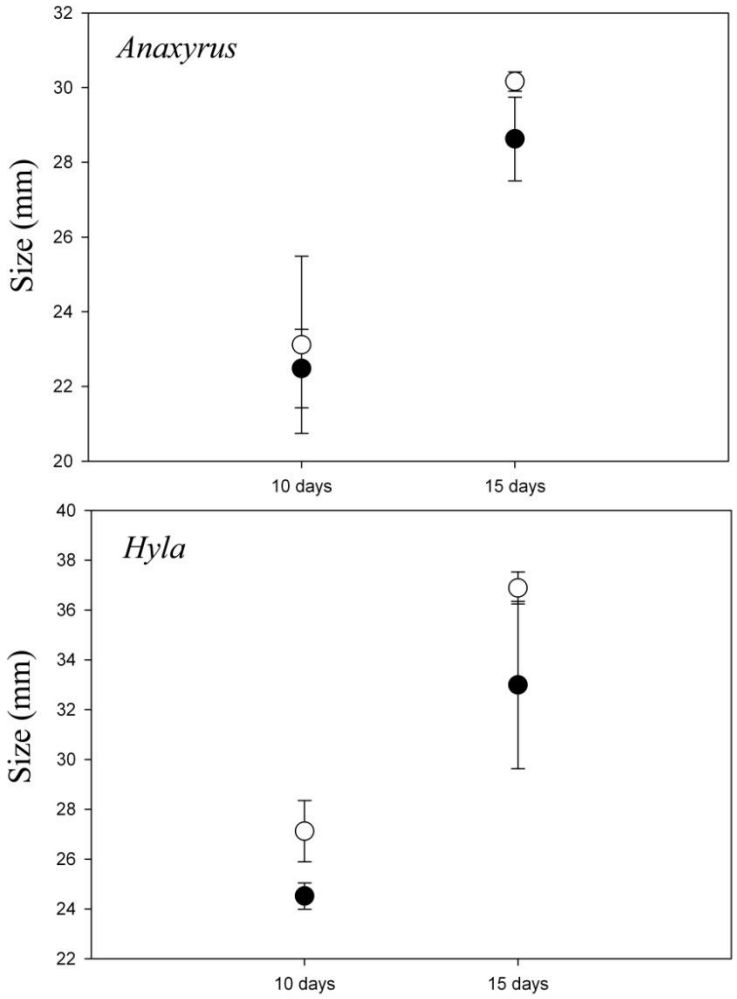

Fig. 3. Size (mm) of *Anaxyrus fowleri* and *Hyla versicolor* tadpoles raised for 10 and 15 days. Data points represent tank means (±1 SE). Open circles are tadpoles raised in the absence of disease; filled circles are tadpoles raised in the presence of *Batrachochytrium dendrobatidis* (*Bd*). Overall, tadpoles raised in the presence of *Bd* were smaller compared to tadpoles raised without *Bd* ($F_{1,22} = 8.65$, $P = 0.008$).

Discussion

Quantifying patterns and outcomes of host-pathogen interactions are essential for understanding not only the ecological implications of pathogens on their hosts, but also on ecosystem processes such as food web dynamics (Lafferty et al. 2006) and community

interactions (Kohler and Wiley 1997). Determining host specific rates of transmission and infection are key challenges of host-pathogen ecology because differential transmission rates can significantly impact disease dynamics. This is especially true in systems where a single pathogen infects multiple, sympatric, host species. Our experiment revealed species differences in *Bd* transmission between conspecific tadpoles. We detected transmission in four of eight *A. fowleri* mesocosms and only one of seven *H. versicolor* mesocosms. We also observed similar effects of species on *Bd* prevalence, where *A. fowleri* mesocosms had greater prevalences relative to *H. versicolor* mesocosms. Moreover, *A. fowleri* mesocosms supported approximately 10x more total zoospores than *H. versicolor* mesocosms and infected *A. fowleri* tadpoles were infected with approximately 4x more *Bd* zoospores relative to *H. versicolor* tadpoles. Collectively, our results provide a possible mechanism underlying rapid population declines of other bufonids. For example, tadpole and adult stages of bufonids are generally highly susceptible to *Bd* (Blaustein et al. 2005; Carey et al. 2006) and *Bd* can reduce survival in wild populations of boreal toads (*A. boreas*; Scherer et al. 2005). Although we did not observe any appreciable mortality (~2% across all *A. fowleri*, Bd^+ treatments), higher infection intensities and prevalences within populations could ultimately reduce survival in *A. fowleri*, even at low-level infections (Pilliod et al. 2010).

Ultimately, pathogen-induced behavioral changes, along with natural differences in host life history patterns, may lead to differential pathogen transmission within natural populations. For example, dense aggregations of larval amphibians increase the probability of contacting an infected host (Blaustein and Bancroft 2007) and can increase transmission rates within a population. Accordingly, we hypothesized that larval *A.*

fowleri, which aggregate in ponds, would transmit *Bd* at a higher rate compared to *H. versicolor* tadpoles. As predicted, we observed a higher proportion of *A. fowleri* tadpoles reared in the presence of *Bd* aggregating, which could lead to higher pathogen transmission and prevalence. However, since we are unsure of the infection status of the individuals observed aggregating, we are unsure if aggregation facilitated transmission in our experiment. Han et al. (2008) found that Bd^+ *A. boreas* tadpoles associated with Bd^+ conspecifics significantly more than towards Bd^- conspecifics; however, Bd^- *A. boreas* tadpoles associated with both Bd^- and Bd^+ conspecifics, which suggests that the aggregations of tadpoles observed in our experiment contained Bd^+ and Bd^- *A. fowleri* tadpoles. Alternatively, differences in the physiological makeup of *A. fowleri* and *H. chrysoscelis* tadpoles may have contributed to the species differences we observed. In terms of disease resistance, Rohr et al. (2010) hypothesized that *A. fowleri* tadpoles should have lower resistance (i.e., higher infection) to aquatic pathogens given their larval life history traits (e.g., highly terrestrial adults, fast larval developmental period, etc.). Indeed, they found that American toad (*A. americanus*) tadpoles suffered from higher mortality when infected with different trematode species (Rohr et al. 2010). Additionally, Johnson and Hartson (2009) revealed that relative to *H. versicolor*, *A.americanus* tadpoles exhibited higher susceptibility, mortality and degree of limb malformations when exposed to the digenetic trematode, *Ribeiroia ondatrae*.

Our results also demonstrate that tadpoles reared in the presence of *Bd* have reduced larval life history traits related to fitness. Overall, *A. fowleri* and *H. versicolor* tadpoles reared under disease conditions were less developed than tadpoles within disease-free conditions. Our results are similar to Paris and Cornelius (2004) but differ in

that the present study reveals some insights behind the mechanism of reduced growth and development in *Bd* infected tadpoles. Tadpoles respond to environmental stressors differently during ontogeny (e.g., Audo et al. 1995); thus, disease may influence larval performance at some developmental stages more than others, especially since developmental rates become fixed during late stages in ontogeny (Hensley 1993; Leips and Travis 1994). Thus, one key finding from our field experiment was that *Bd* reduces developmental rates early in development and that undersized individuals may not be able to "catch-up" in their developmental trajectory (Radder et al. 2007). In general, slower developmental rates can carry fitness consequences for larval amphibians. For example, rapid growth and development is critical for surviving temporary aquatic habitats because reaching a minimum size for metamorphosis before pond drying increases the probability of successfully metamorphosing (Wilbur and Collins 1973; Werner and Anholt 1993). Reaching a large size early in development can also increase larval survival by decreasing predation rates from gape-limited predators (Kurzava 1998) and decreasing intraspecific competition (Semlitsch et al. 1988). However, tradeoffs between growth and mortality exist in tadpoles (Werner 1986) and advantages for rapid development ultimately depends interactions between aquatic and terrestrial environments of amphibians.

A second key finding related to growth and developmental rates was an interactive effect of trial duration and pathogen treatment on tadpole growth. Compared to 10 day trial duration, tadpoles reared in disease conditions for 15 days were significantly smaller than tadpoles reared in the absence of disease. These results suggest two alternative hypotheses. First, pathogen load (zoospore density) at the individual and

mesocosm levels was not high enough to affect growth rates until after 10 days, resulting in increased pathogen effects at the longer trial duration. Alternatively, *Bd* was transmitted quickly between pathogen source and susceptible tadpoles, increasing the duration of time that tadpoles were exposed to *Bd*, thereby increasing the effects of *Bd*. Since we do not know when susceptible tadpoles in both trial durations were infected with *Bd*, we are unable to reject either hypothesis. However, our data from the 10 day trial duration revealed transmission in 4/8 of the mesocosms but only 1/7 at the 15 day duration, providing some support for the second hypothesis. The duration of exposure to a pathogen can increase the probability of infection (e.g., Hajek 2001) and/or increase the duration the hosts' immune response, which can reallocate energy originally devoted to somatic maintenance and development to immune function (Sheldon and Verhulst 1996).

Given the strong effects of pathogen treatments on larval life history traits that we measured, the low prevalence of *Bd*-infected tadpoles was unexpected. Considering that tadpoles have few keratinized tissues (i.e., tissue that can harbor *Bd*-infections), it is probable that pathogen loads of *Bd*-infected pathogen source tadpoles in our field experiment were low. Accordingly, susceptible tadpoles would only be exposed to relatively low quantities of *Bd* zoospores. Garner et al. (2009) proposed two hypotheses on tradeoffs between immune activity and growth when tadpoles exposed to low doses of *Bd*. First, low intensity *Bd*-infections may be successfully cleared by the host shortly after infection. Although no immunopathologies have been reported for *Bd*, recent experimental evidence suggests that tadpoles exposed to a low dose of *Bd* exhibited reduced growth although only 40% of the tadpoles were infected with *Bd* (Garner et al. 2009). Second, *Bd*-infection is prevented at low doses but energy is reallocated from

growth and development to prevention. Although the exact mode of *Bd* infection of anuran tadpoles is unknown, other chytridiomycete fungi attach to host specific cells prior to entering the host (Deacon and Saxena 1997; Ibelings et al. 2004). Specific host responses, such as the activation of lymphocytes and antibodies of the larval immune system (Rollins-Smith 1998) or shedding keratinized mouthparts or infected stratum corneum (Marantelli et al. 2004) may prevent *Bd* infection. Whether infection is cleared or prevented, host responses to *Bd* are likely traded off against larval growth and development. Our data, along with Garner et al. (2009) emphasize the necessity of testing for *Bd* induced effects in hosts in the absence of infection.

Interestingly, we found reduced growth and developmental rates of tadpoles reared in disease conditions in the absence of severe mouthpart deformations. Two recent experiments have proposed that *Bd*-induced damage to the keratinized mouthparts of tadpoles reduce their growth and developmental rates by altering the feeding kinematics (Venesky et al. 2010a) and decreases their feeding efficiency (Venesky et al. 2009). During feeding, the keratinized rows of labial teeth and jaw sheaths of tadpoles work together to remove material from a substrate (Wassersug and Yamashita 2001) and are essential for effective feeding (Venesky et al. 2010b). We examined the mouthparts of each tadpole reared in disease conditions and found a low incidence of mouthpart deformation (< 5%; Venesky, unpublished data). The low incidence of *Bd*-induced mouthpart deformation is not surprising, given the length of our experiments. Larval *Rana muscosa* infected with *Bd* begin to lose keratin 49 days post-infection and have no keratinized jaw sheaths 147 days post-infection (Rachowicz and Vredenburg 2004), which is much longer than maximum duration of time tadpoles from our longest trial

were infected with *Bd* (i.e., 15 days). In the absence of severe mouthpart deformations, we observed strong pathogen effects on larval life history traits. Thus, our data suggest that perturbations to life history traits in this host-pathogen system are complex and that *Bd*-induced structural damage may not be the only mechanism behind reduced growth and development.

Our results underpin the context dependency of *Bd*-amphibian interactions, especially when considering pathogen transmission and prevalence. For example, when an infected frog was used as a pathogen source, *Bd* prevalence ranged from 86–100% in *A. fowleri* and *H. versicolor* mesocosms (Parris and Cornelius 2004). However, under our experimental conditions with the same focal taxa, *Bd* prevalence was considerably lower. We suspect that the low prevalence in our experiment is due, in part, to relatively low *Bd* infections in tadpoles. *Bd* infections are restricted to keratinized tissues (Fellers et al. 2001), which are present in the mouthparts of tadpoles but throughout the epidermis of metamorphs. Thus, our pathogen source tadpoles likely had significantly lighter *Bd* infections compared to the adult frogs used in Parris and Cornelius (2004). Indeed, Searle et al. (2010) suggested that low *Bd* levels in the pathogen source tadpoles prevented *Bd* transmission to heterospecific tadpoles.

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Chapter 5 Dietary protein content affects immune response and disease resistance in anuran tadpoles

SUMMARY

The immune system is a necessary, but potentially costly, defense against infectious diseases. When nutrition is limited, immune activity may consume a significant amount of an organism's energy budget. The nutrient content of diet, namely high dietary protein, optimizes immune system development and can influence disease resistance. Changing environments due to global climate change may dramatically affect access to sufficient high protein food sources for developing tadpoles in marginal habitats. We examined the effects of low dietary protein on disease resistance and immune responsiveness in Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles. Specifically, we tested: (1) if dietary protein content influenced their resistance to *Batrachochytrium dendrobatidis* (*Bd*) infection; (2) the singular and combined effects of diet and parasite treatments on tadpole skin-swelling in response to phytohaemagglutinin (PHA) injection (a measure of the T cell-mediated response of the adaptive and innate immune systems) and the bacterial killing ability (BKA) of tadpole blood (a measure of the complement-mediated cytotoxicity of the innate immune system); and (3) for the singular and combined effects of diet and parasite treatments on tadpole growth and development. Tadpoles raised on a low-protein diet had reduced PHA and BKA responses and were smaller and less developed relative to tadpoles on a high-protein diet. Importantly, high dietary protein increased resistance to *Bd*. Although we did not find an effect of *Bd* on the tadpole immune responses, our results suggest that

restricted dietary protein can impact the overall condition of a host such that they are less resistant to infecting parasites.

INTRODUCTION

Animals use a complex suite of behavioral and immunological responses to parasite and pathogen (hereafter "parasite") exposure, ranging from parasite avoidance (Behringer et al., 2006) to the production of lymphocyte and antibody-mediated defenses (reviewed in Janeway and Medzhitov, 2002). Although the immune system is a critical line of defense against invading parasites, significant costs are associated with its maintenance and operation. For example, it is generally thought that development of immune responses can use a significant amount of an organism's energy budget (reviewed in Schmid-Hempel, 2003). Thus, hosts that are in poor condition (i.e., nutritional state) may suffer a reduced potential to mount an effective immune response. For example, independent of gonadal regression, seasonally breeding rodents that are food-deprived and losing body fat generally exhibit reduced immunity (reviewed in Demas, 2004). While decreased food consumption can compromise immune function, immune maintenance and operation is strongly associated with dietary protein (Lee et al., 2006). Not only does high protein enhance parasite resistance (Lee et al., 2006), recent evidence shows that hosts can optimize their dietary protein intake. For instance, when challenged with a parasite infection, African armyworms (*Spodoptera exempta*) alter their feeding behavior such that they increase their protein intake (Povey et al., 2008).

Understanding the interplay between host condition and immune function is of great importance for amphibians. Compared to other vertebrates, amphibian populations

are declining at a startling rate—nearly one-third of the described species threatened with extinction (Stuart et al., 2004). Although a myriad of factors contribute to amphibian decline (Blaustein et al., 2003; Collins and Storfer, 2003), growing evidence links amphibian population declines and extinctions with infectious diseases (Daszak et al., 2003; Lips et al., 2005). *Batrachochytrium dendrobatidis* (*Bd*) is a widespread parasitic fungus which causes chytridiomycosis in amphibians and has been implicated in global population declines (Berger et al., 1998; Daszak et al., 2003). *Bd* is an aquatic parasite which infects the keratinized epithelial tissues of metamorphic anurans (Longcore et al. 1999). Severe *Bd* infections inhibit electrolyte transport in the epidermis of metamorphs and generally result in mortality from cardiac arrest (Voyles et al., 2009).

Field and experimental data show that amphibian species differ in their resistance to *Bd* (Blaustein et al., 2005; Woodhams and Alford, 2005). Although regional environmental differences (e.g., temperature; Piotrowski et al., 2004) and/or host behavioral patterns (e.g., aggregation; Blaustein and Bancroft, 2007) can lead to different patterns of disease outbreaks, they do not explain intra- and interspecific differences in resistance of sympatric hosts. A growing body of evidence points towards the role of amphibian immune defenses in preventing and mitigating *Bd* infections (reviewed in Richmond et al., 2009), which could help explain species-specific variation in resistance. Amphibians have a well-developed innate and adaptive immune response, with their skin acting as the primary defense from parasite infection (Carey et al., 1999). Cutaneous antimicrobial peptides from certain anuran families differentially inhibit the growth of *Bd* in vitro (Rollins-Smith and Conlon, 2005), supporting the hypothesis that species-specific differences in *Bd* resistance may be caused by differences in their innate immune

response. Apart from antimicrobial peptides, little is known about specific immune responses against *Bd* (reviewed in Richmond et al., 2009). In addition, the majority of the work on amphibian immune responses to *Bd* has been examined in metamorphic anurans. For example, Woodhams et al. (2007) found changes in circulating leukocytes following *Bd* infection of Red-eyed tree frog adults (*Litoria chloris*). Additionally, Ramsey et al. (2010) documented *Bd*-specific innate and adaptive immune responses of adult African clawed frogs (*Xenopus laevis*) which may contribute to *Bd* resistance. Combined, these results underpin the role of host immune responses as key factors in determining species differences in resistance to *Bd*.

Tadpoles provide an excellent model to examine host-*Bd* interactions. *Bd* infections do not generally cause tadpole mortality as they often do in metamorphs, rather they reduce growth and developmental rates (Parris and Cornelius 2004; Garner et al., 2009; but see Blaustein et al., 2005). Tadpoles are also good candidates to study the effects of nutritional stress on immune function and *Bd* resistance. *Bd* infections in tadpoles are restricted to the tissues around the keratinized labial teeth and jaw sheaths (Fellers et al., 2001) and diseased tadpoles exhibit reduced feeding efficiency (Venesky et al., 2009; Venesky et al., 2010). Thus, *Bd*-infected tadpoles may be in poor nutritional condition relative to non-infected individuals and may be unable to mount an adequate immune response to reduce, or resist, a *Bd* infection. Life-history theory predicts that as organisms develop, they will differentially invest in life-history traits, which could lead to immune-based trade-offs at different life stages (reviewed in Sandland and Minchella, 2003). Although recent studies support the role of immune defense in preventing *Bd* infection in metamorphic anurans, little is known about tadpole immune responses to *Bd*.

To date, the only published report of tadpole immune responses to *Bd* found that Bullfrog (*Lithobates catesbeianus*) tadpoles infected with *Bd* had higher numbers of circulating neutrophils (Davis et al., 2010).

In our experiment, we examined the effects of nutritional stress (i.e., low dietary protein) on disease resistance and immune responsiveness in Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles. First, we raised tadpoles on low- and high-protein diets and tested if dietary protein content influenced their susceptibility to *Bd*. We then compared aspects of the innate and adaptive immune responses among parasite treatment groups (non-exposed, exposed but not infected, and infected) by conducting two standardized challenges of the non-specific immune system: (1) tadpole skin-swelling in response to phytohaemagglutinin (PHA) injection (a measure of the T-cell mediated response of the adaptive and innate immune systems) and (2) the bacterial killing ability of tadpole blood (a measure of the complement-mediated cytotoxicity of the innate immune system). We predicted that tadpoles from the low protein treatment would have reduced immune capacities, ultimately resulting in decreased resistance to *Bd*. Also, if *Bd* is generally immunosuppressive, we expected to observe a weaker PHA response and a reduced bacterial killing ability for tadpoles exposed and/or infected with *Bd*, irrespective of diet treatment. Finally, we tested for the singular and combined effects of diet and parasite treatments on tadpole growth and development.

METHODS

Animal Collection and Husbandry

Southern leopard frog (*Lithobates sphenocephalus*) tadpoles used in our experiments were derived from eggs collected from natural ponds within Shelby County, TN, USA (35°08'16.52 N; 89°48'39.76"W). On 05 March 2010, we collected half of the eggs from each of 12 *L. sphenocephalus* egg masses from 3 ponds within a 1 mile radius. Immediately after collection, eggs were transported to the laboratory at The University of Memphis. Eggs and hatchling tadpoles were amalgamated and then separated into four 37.85 L glass aquaria filled with approximately 18.5 L of aged tap water, which was continually aerated, until all tadpoles that had reached the free-swimming stage (stage 25; Gosner, 1960). We then haphazardly sampled tadpoles (N=80) from the four aquaria to evenly distribute potential genetic effects on the larval traits we measured. Test subjects were placed individually in 1.5 L plastic containers filled with 1 L of aged tap water. Throughout the experiment, tadpoles were maintained on a 12 h light: 12 h dark photoperiod at 21 C (±1 C) and were fed approximately 0.20 grams of either low or high protein diet every three days (see below).

Full water changes were conducted every three days. For each water change, we used a piece of mesh screen to remove the focal tadpole from its container and placed it in a temporary transfer container. 1.0 L of aged tap water, along with the focal tadpole, was placed back into the container. After the inoculation of *Bd* (see below), we took the following precautions to avoid accidental parasite transmission between treatments. First, we always performed water changes on nonexposed tadpoles before exposed tadpoles. Second, we used different laboratory equipment (container, mesh screen) between each

parasite treatment. At the end of the experiment, we thoroughly disinfected all containers by adding bleach (6% sodium hypochlorite) to yield a 10% solution, which kills *Bd* (Johnson and Speare 2003). Throughout the experiment, all equipment and water was disinfected in a similar fashion.

Diets

On 15 March 2010 (Day 0), tadpoles were haphazardly assigned to two groups (N=40 per group) and were fed one of two experimental diets that varied in their nutrient content. The isocaloric diets were custom ordered (Harlan Teklad) and were made of natural ingredients (e.g., fish and corn meal) and varied in their protein and digestible carbohydrate content. The high protein diet consisted of 47.6 protein, 22.5 carbohydrate, and 10.1 fat (% by weight). In comparison, the low protein diet consisted of 13.8 protein, 48.6 carbohydrate, and 10.0 fat (% by weight). Other constituents of the diet were calcium (~1.7%), phosphorous (~1.2%) sodium (~0.35 %), and potassium (~1.0%).

***Batrachochytrium dendrobatidis* inoculation**

During February 2010, we isolated *Bd* from a recently dead *L. sphenocephalus* adult found in Shelby Co, TN, USA. *Bd* was grown in the laboratory on tryptone-gelatin hydrolysate-lactose (TGhL) agar in 9 cm Petri dishes according to standard protocol (Longcore et al., 1999). Prior to the use of *Bd* in the current experiment, we confirmed the pathogenicity of this strain by experimentally infecting *L. sphenocephalus* adults (M. Venesky, unpublished data).

On Day 30 (14 April 2010), tadpoles from both diet treatments were randomly split into two parasite groups—a *Bd* exposed group and a nonexposed (control) group. For the *Bd* exposed group, we administered *Bd* by exposing *L. sphenocephalus* (N = 40) to water baths containing infectious concentrations of fungal zoospores. We harvested *Bd* zoospores by adding 10.0 mL of sterile water to the cultures and collected the zoospores that emerged from the zoosporangia after 30 min. Tadpoles were placed individually in 100 mL waterbaths and exposed to 4,000 zoospores/mL for 72 hours. Because the *Bd* inoculate was lower than expected, we removed the tadpoles from the first inoculate and re-exposed them to a second inoculate of containing 40,000 zoospores/mL for an additional 48 hours. For the nonexposed group, we followed the same protocol but used plates with only TGhL, and exposed an additional group of tadpoles (N = 40) to water baths with no *Bd* zoospores. Our design simulated transmission by water, one of the possible modes of *Bd* transmission in natural environments (Pessier et al., 1999).

Phytohaemagglutinin (PHA) challenge

On 10 May 2010 (Day 56), we measured the immune response of a subset of the tadpoles from the *Bd* exposed and non-exposed treatments (N=20 per treatment) by challenging their immune system with a single phytohaemagglutinin (PHA) injection (Sigma-Aldrich, St Louis, MO). PHA is a lectin derived from the kidney bean, *Phaseolus vulgaris*, which represents a non-specific challenge to the immune system. Injection with PHA induces swelling around the injection site that is likely due to infiltration of T lymphocytes and other immune effector cells. A larger inflammatory response to PHA indicates a more roust immune response.

Prior to our PHA challenge, we made 1 L of amphibian phosphate-buffered saline (APBS) by adding the following ingredients to 1 L of sterilized (with a Whatman 25 mm GD/X 0.2 µm pore filter) DI water: 6.6 g NaCL, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4. We then made 5 mL of PHA by diluting 5 mL of APBS into 5 mg of crystallized PHA. On 10 May 2010 (Day 56), we placed individual tadpoles in approximately 500 mL of MS222 (diluted to approximately 0.06 g/L) until the tadpole was anesthetized and lost righting ability. For accuracy, we made all measurements and injections under a Nikon® SMZ800 dissecting scope with 10–60X magnification. We determined our injection location on each tadpole by measuring 20 mm from the posterior tip of the tail. At that location, we used a fine-gauged spessimeter (Mitutoyo, Precision Graphic Instruments, Inc., Spokane, WA) to measure the skin thickness prior to PHA injection. The average of three consecutive measurements was taken to minimize measurement error. We then injected 20 µL of PHA into the right side of a tadpole's tail, at the junction of the tail and muscle using a 100 µL Hamilton glass syringe with a 30 G, 5/16' disposable insulin needle. Contralateral injections of PHA were not possible given the size of anuran tadpoles. However, preliminary trials showed that the PHA dose we used could elicit a measurable inflammatory response that was significantly greater than that of tadpoles injected with APBS alone ($P < 0.05$, *unpublished data*). Data from a preliminary experiment showed that swelling response to PHA was greatest at 48 h post-injection. Accordingly, 48 h after the PHA, we anesthetized individual tadpoles and measured the skin swelling at the injection site as described previously. We considered the difference between the pre- and post-PHA injection swelling response as an indicator of the immune response in our analysis.

Bacterial Killing Assay

We adapted the protocol described by Millet et al. (2007) to assay *Escherichia coli* (lyophilized pellets, ATCC #8739; Microbiologics, St. Cloud, MN) killing ability in each tadpole. Because the effectiveness of this technique in comparing bacterial killing ability among treatments could be influenced by blood storage time after removal from the body of individual tadpoles (as in birds; Wilcoxen et al. 2010), we performed four individual assays, one each following collection of blood from all individuals in each of the four treatments.

On 12 May 2010 (Day 58), we used the remaining subset of the tadpoles from the *Bd* exposed and non-exposed treatments (N=20 per treatment). To obtain blood samples, tadpoles were patted on medical gauze to reduce the amount of water and mucosal secretions on the body, and the tail was cut off with scissors that had been sterilized with 75% ethanol. After removing the tail, we collected a blood sample of 3–7µl (mean = 5.82µl) in a microhematocrit capillary tube and the blood was immediately transferred by a pipettor with disposable tip to a small snap-top vial for each tadpole. Phosphate buffered saline (PBS) was then added to each sample to bring the total volume to 113µl for use in an *in vitro* bacterial killing assay (BKA, see below). Blood samples were kept at laboratory temperature until use in a bacterial killing assay. The scissors were sterilized with ethanol prior to each cut and a new pipette tip was used for each sample. We were unsuccessful at obtaining sufficient blood from 3 of the 40 tadpoles.

Following collection of blood from all individuals in a treatment, we challenged the tadpole blood by adding 7µl of *E. coli* stock solution, prepared by hydrating a single

lyophilized pellet in 40mL of PBS the day before the assays (per the manufacturer's instructions). The 120µl suspension of bacteria and diluted blood was kept at laboratory temperature for 30 minutes to allow the challenge to proceed at near-body temperature for the tadpoles. Samples were vortex mixed and two 50µl aliquots of the mixture were spread onto separate trypticase soy agar plates and incubated overnight at 37˚C. Controls consisted of 7µL of the reconstituted bacteria culture diluted in 113µL of PBS, and paired control plates were prepared for each of the four assays. For each assay, colonies were counted 24 hours after placement of plates in the incubator. Bactericidal activity was calculated as the proportion of bacterial colonies killed in samples as compared to controls by dividing the difference in colony number on treatment plates and control plates by the total number of colonies on the control plates.

Because this immune response may be influenced by the number of enzymes present in the blood, we corrected for blood volumes that were lower than 7 µl (the maximum blood volume used in the assay). We considered the percentage of *E. coli* colonies killed (total colonies on the control plate minus corrected total colonies on the test plate divided by the total colonies on the control plate) as our indicator of tadpole immune response.

Life history responses and resistance to *Batrachochytrium dendrobatidis*

At the time of our immune challenges, we also recorded the total length (TL) (to the nearest 0.01 mm) and Gosner (1960) stage of all individuals. After completing the immune challenges, we sacrificed all tadpoles, dissected their mouthparts, and stored mouthparts in 100% EtOH for qPCR analysis to confirm tadpole infection status. We

screened all of the tadpoles from the *Bd*-exposed treatment and randomly selected half of tadpoles (N = 20) from the non-exposed treatment.

Statistical Analyses

Within the *Bd*-exposed treatment, we used Chi-square analysis to test if dietary protein content affected resistance to *Bd*. For the PHA challenge, we used ANCOVA (with Gosner stage as a covariate) to test for the effects of the independent variables diet (low- and high-protein) and parasite treatment (non-exposed, exposed but non-infected, and infected) on the dependent variable skin swelling. We used log transformations to normalize skin swelling responses. For the BKA assay, we used ANCOVA (with Gosner stage as a covariate) to test for the effects of the independent variables diet (low- and high-protein) and parasite treatment (non-exposed, exposed but non-infected, and infected) on the dependent variable % of *E. coli* colonies killed. We used MANOVA to test for the effects of independent variables species diet (low- and high-protein) and parasite treatment (non-exposed, exposed but non-infected, and infected) and their interactions on the dependent variables size (TL) and stage (Gosner). We then used Bonferroni-adjusted (significance level of 0.025 for each response variable) ANOVA contrasts on each response variable to determine significant contributors to multivariate effects.

RESULTS

Effect of diet on disease resistance

No individuals from the sub-sample of tadpoles in the control treatment tested positive for *Bd*. Among tadpoles exposed to *Bd*, dietary protein content significant affected resistance to *Bd* (Chi-square: χ^2=10.42, P<0.001), with 65% of the tadpoles raised on a low-protein diet infected with *Bd* compared to 15% of tadpoles raised on a high-protein diet.

Effects of diet on immune responses

Overall, diet and *Bd* treatments had similar effects on the immune responses of tadpoles. We found a significant effect of diet on PHA (ANCOVA: $F_{1,33}$=4.21, P=0.048) and BKA (ANCOVA: $F_{1,32}$=6.18, P=0.018) responses, where tadpoles raised on high-protein exhibited a stronger PHA-induced swelling response and bacterial killing ability relative to tadpoles raised on the low-protein diet (Figs. 1, 2). However, neither infection nor exposure to *Bd* significantly affected PHA or BKA responses (ANCOVA: $F_{1,33}$=1.68, P=0.203 and $F_{1,32}$=0.32, P=0.726, respectively) or the diet x parasite treatment interaction in either response. Additionally, there was no effect of the covariate (Gosner stage) on either of the response variables (ANCOVA, PHA: $F_{1,33}$=1.83, P=0.624; BKA: $F_{1,32}$=0.88, P=0.355).

Effects of diet on larval life history traits

There was a significant effect of diet on the combined life history responses we measured (MANOVA: $F_{2,73}$=31.49, P<0.0001). Additionally, there were significant effects of diet

on both TL (ANOVA: $F_{1,74}$=36.30, P<0.0001) and Gosner ($F_{1,74}$=63.84, P<0.0001), where tadpoles raised on the high-protein diet were longer and more developed than tadpoles raised on the low-protein diet. There was no effect of parasite treatment on life history responses (MANOVA: $F_{2,73}$=1.20, P<0.312).

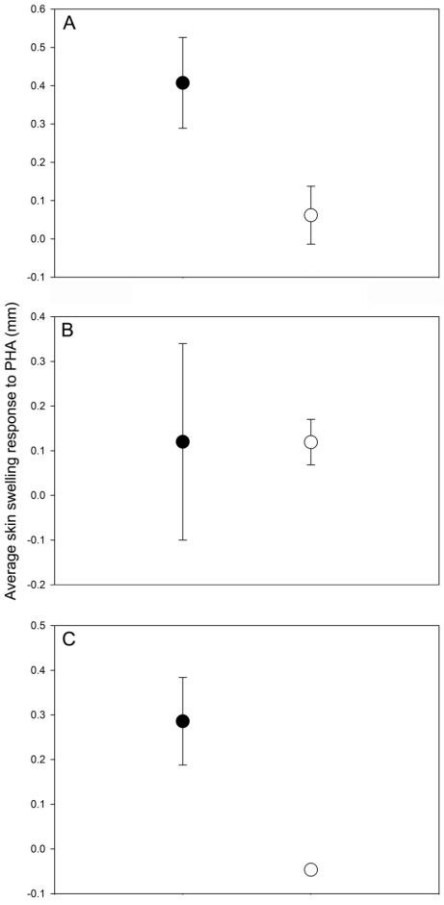

Fig. 1. The average skin swelling responses of Southern Leopard frog (*Lithobates sphenocephalus*) tadpoles to phytohaemagglutinin (PHA) injection. Different panels represent different parasite treatments: (A) control tadpoles not exposed to *Bd*; (B) tadpoles infected with *Bd*; and (C) tadpoles exposed but not infected with *Bd*. In each panel, filled circles represent tadpoles fed a high-protein diet; open circles represent tadpoles fed a low-protein diet. Error bars are 1 S.E. of the mean.

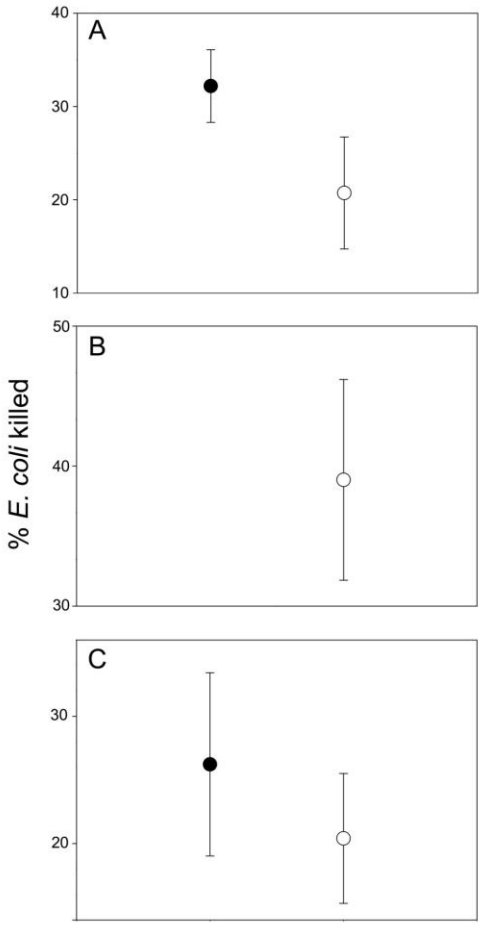

Fig. 2. The average *E. coli* killed by Southern Leopard frog (*Lithobates sphenocephalus*) tadpole blood. Different panels represent different parasite treatments: (A) control tadpoles not exposed to *Bd*; (B) tadpoles infected with *Bd*; and (C) tadpoles exposed but not infected with *Bd*. In each panel, filled circles represent tadpoles fed a high-protein diet; open circles represent tadpoles fed a low-protein diet. Error bars are 1 S.E. of the mean.

DISCUSSION

Our study demonstrates that reduced dietary protein content can impair immune function and decrease resistance to *Bd* in Southern Leopard frog (*Lithobates sphenocephalus*) tadpoles. In addition, tadpoles raised on a low-protein diet were smaller and less developed than tadpoles raised on a high-protein diet. Although we did not find an effect

of *Bd* on the nonspecific immune responses that we measured, our results suggest that restricted dietary protein can impact the overall condition of a host such that they are less resistant to *Bd* infections.

Ultimately, poor host immune function can lead to decreased resistance to parasitic infections (Houdijk et al., 2000; Povey et al., 2008) and higher parasite-induced mortality (Peck et al., 1992). As predicted, we found similar effects of dietary protein-content on tadpole resistance to *Bd*—65% of tadpoles raised on a low-protein diet were infected with *Bd* compared to 15% of tadpoles raised on a low-protein diet. This result is consistent with findings in other taxonomic groups. Ruminant mammals are often exposed to a great diversity of parasitic trematodes and nematodes and it has been shown that high protein dietary supplementation often increases parasite resistance in livestock (reviewed in Coop and Kyriazakis, 2001). In addition to low dietary protein, other factors can influence host condition and ultimately lead to differences in resistance. For example, Garner et al. (2009) found that metamorphs in poor condition (i.e., smaller body size) were less resistant to *Bd*-induced mortality. Thus, it appears that amphibian immunological defenses against parasites incur high energy costs and that hosts in poor nutritional condition are less resistant to *Bd*.

The overall condition of a host, namely their nutritional condition, greatly impacts their immune deployment because inadequate nutrients or low energy can suppress the resources devoted to immune maintenance and deployment (Lee et al., 2006). In general, decreased dietary protein can suppress specific immune functions (Lochmiller et al., 1993). In our study, tadpoles raised on a low-protein diet had reduced PHA-induced skin-swelling response and bacterial killing ability relative to tadpoles raised on a high-protein

diet. Tadpoles have a well-developed immune system that includes lymphocyte activity (Rollins-Smith, 1998); however, the impacts that host nutritional status has on specific tadpole immune responses have not been explored. An effective immune response (e.g., lymphocyte recruitment and antibody production) requires an increase in metabolic costs due to increased protein synthesis (Borel et al., 1998). Thus, if tadpoles raised on a low-protein diet were protein deficient, they would not have been able to deploy an effective response to the PHA injection. In addition, several components of the innate immune system mediate the ability of tadpole blood to kill *E. coli*, which may also be influenced by dietary protein content. Since several responses of innate immunity (e.g., phagocytes, natural antibodies, and complement; Millet et al. 2007) are measured in the BKA, we cannot ascertain which specific component(s) drove the observed patterns of dietary protein content in this assay. However, our data support the findings of other studies in that organisms that have a strong bacterial killing ability can increase disease resistance (Townsend et al., 2010) and ultimately survival (Wilcoxen et al., 2010).

Contrary to our predictions, we did not observe an effect of *Bd* exposure or infection on the nonspecific immune functions that we measured. Recent studies show that amphibians use components of their innate and adaptive immune system in response to *Bd* infection (e.g., Rollins-Smith et al., 2002; Rollins-Smith et al., 2005; Woodhams et al., 2007). For example, (Ramsey et al., 2010) found that metamorphic African clawed frogs (*Xenopus laevis*) have *Bd*-specific antibodies (IgM and IgY) and antimicrobial skin peptides, both of which are effective immune defenses against *Bd*. Additionally, Davis et al. (2010) examined Bullfrog (*L. catesbeianus*) tadpoles with clinical signs of *Bd* infection (e.g., severe mouthpart depigmentation) and found an increase in circulating

neutrophils, but no effect on lymphocyte production. Thus, it appears that *Bd* resistance in tadpoles is likely mediated by another component of the immune system, such as antibody production (e.g., Ramsey et al., 2010).

Additionally, our data show that there are high constitutive costs of maintaining immune function, in the absence of parasitism. Although we did not measure other aspects of tadpole physiology, it is possible that poor nutrition elevated corticosterone ("CORT", the primary anuran stress hormone) levels. For example, when nutritionally restricted, Couch's Spadefoot Toad (*Scaphiopus couchii*) tadpoles exhibited higher levels of whole-body CORT (Ledon-Rettig et al., 2009). Because the hypothalamic-pituitary-interrenal (similar to the hypothalamic-pituitary-adrenal axis in mammals) and immune system function are coupled (Rollins-Smith, 2001), it would not be surprising if increased CORT mediated the either of the immune responses that we measured. Specifically, increased CORT reduces circulating lymphocytes in *Xenopus* tadpoles (Rollins-Smith et al., 1997), which could ultimately inhibit PHA-induced skin swelling. For example, a recent study found that Wood frogs (*Lithobates sylvaticus*) exposed to a desiccation regime during their larval period had shorter developmental times and had weakened PHA-induced skin-swelling responses as metamorphs (Gervasi and Foufopoulos, 2008).

It is important to note that because we assessed infection status at the end of the experiment, we are unsure if non-infected tadpoles were resistant to *Bd* or lost their infection during the duration of the experiment. For example, immune defenses of tadpoles may have prevented *Bd* from infecting their keratinized mouthparts or reduced parasite load by mounting an immune response against *Bd* after infection. Although no data are available on tadpole resistance or tolerance (i.e., reducing parasite burden or

minimizing harm done by parasites; Clarke, 1986) to *Bd*, a recent study found age-dependent and host-specific variation in trematode resistance and tolerance in two species of anuran tadpoles (Rohr et al., 2010). Along with testing for *Bd*-specific immune responses in tadpoles, decoupling host resistance and tolerance in tadpoles should disentangle the specific ways in which amphibians respond to *Bd*.

In addition to the effects of nutrition on tadpole immune function and resistance to *Bd*, our results provide key insights into how specific components of tadpole diet contribute their growth and development. We found that tadpoles raised on the high-protein diet were larger and more developed than tadpoles raised on the low-protein diet. While detailed information on diets is lacking, tadpoles have access to a variety of food items in the aquatic environment that likely differ in nutritional quality. For example, nitrogen-fixing blue-green algae (Pryor, 2003) and animal products (Altig et al., 2007), each of which are high in dietary protein, can make up a significant amount of a tadpoles' diet. Thus, species-differences in immune responses of tadpoles and their resistance to infectious diseases may be driven, in part, by the biotic make-up of their aquatic environments.

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Chapter 6 Conclusion

Batrachochytrium dendrobatidis (*Bd*) infection in tadpoles often results in reduced growth and developmental rates; however, the mechanism(s) responsible were unknown. My first three research projects focused on the impacts of *Bd* infection on tadpole behavior and life history traits. I found that *Bd*-induced damage to the feeding structures of tadpoles slipped from algal covered substrates during tadpole feeding (Chapter 2), resulting in reduced feeding efficiency (Chapter 3). Additionally, I found species-specific differences in their feeding activity when infected with *Bd* (Chapter 3). Specifically, *Bd*-infected Fowler's Toad (*Anaxyrus fowleri*) tadpoles exhibited reduced feeding activity relative to non-infected tadpoles, whereas *Bd* infection did not affect the feeding activity of Grey Treefrog (*Hyla versicolor*) tadpoles. To determine the consequences of pathogen-induced behavioral modifications at a larger scale, I tested for differences in *Bd* transmission, prevalence, and infection intensity in single-species mesocosms. I found that in the presence of disease, tadpoles of *A. fowleri* aggregated significantly more than in control mesocosms. In contrast, in the presence of disease, tadpoles of *H. versicolor* aggregated significantly less than in control mesocosms (Chapter 4). I also found that mesocosms with *A. fowleri* supported higher *Bd* prevalences and infection intensities relative to mesocosms with *H. versicolor* (Chapter 4).

Together, these results suggest that: (a) the structural damage caused by *Bd* may sufficiently inhibit feeding such that *Bd*-infected tadpoles have less energy reserves than non-infected tadpoles and ultimately reduce their growth and development and (b)

species differences in pathogen-induced changes can affect *Bd* transmission in ponds and ultimately result in patterns of *Bd* outbreaks observed in natural habitats.

In my previous three projects, a large proportion of the tadpoles I exposed to *Bd* did not become infected and I wanted to elucidate some possible mechanisms. My final research project explored the consequences of pathogen exposure and pathogen infection and considered the immunological defenses that tadpoles use against *Bd.* I found that Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles that were nutritionally stressed (i.e., low-protein diet) were less resistant to *Bd* than tadpoles raised on high-protein diet (Chapter 5). I also found that irrespective of *Bd* infection status, tadpoles raised on a low-protein diet had inferior immune responses relative to tadpoles on a high-protein diet (Chapter 5), suggesting that nutritional stress can suppress immune function and ultimately lead to decreased resistance to pathogens. It is important to note that *Bd* did not affect the nonspecific immune responses that I measured, suggesting that other immune responses (e.g., antibody production) may directly respond to *Bd* infection in tadpoles.

For my postdoctoral research in the laboratory of Dr. Jason Rohr (The University of South Florida), I will continue to examine host physiological/immunological responses to *Bd* (and other amphibian pathogens).