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Batrachochytrium dendrobatidis exposure effects on foraging efficiencies and body size in anuran tadpoles

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ABSTRACT: Chytridiomycosis, the amphibian disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), is fatal to adults of many species. *Bd* is largely sublethal to amphibian larvae; however, it is known to reduce larval (i.e. tadpole) growth rates, with possible long-term effects on population dynamics and fitness. We conducted an experiment to test how *Bd* altered southern leopard frog *Lithobates sphenocephalus* tadpole mouthpart damage, percentage of food ingested, and subsequent body size. We examined our results using path analyses. We hypothesized that *Bd* would increase mouthpart damage, causing less food to be ingested, and ultimately reduce body size. In our model, both *Bd* exposure and increased mouthpart damage significantly reduced food ingested and subsequent body size. However, our study provides evidence against the long-standing hypothesis of mouthpart damage as a pathway for *Bd*-induced reductions in larval group. Here we provide evidence for reduced foraging efficiency (percentage of food ingested) as a mechanism for *Bd*-induced reductions in body size. This work highlights the importance of studying the sublethal effects of *Bd* on larval amphibians.

KEY WORDS: Chytrid fungus · Feeding · Larvae · Life history traits · Path analysis

INTRODUCTION

Chytridiomycosis is an emerging infectious amphibian disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998). *Bd* has been linked to amphibian population declines over the past 3 decades (Stuart et al. 2004) and is present on every continent where amphibians persist (Olsen et al. 2013). *Bd* has been shown to have dramatic negative effects on amphibian populations, causing mass die-offs in many regions in Central America (Lips et al. 2006) and parts of the United States (Vredenburg et al. 2010), due in large part to the infection pathway of the disease.

Bd attacks keratinized structures and is lethal to adults of many frog species because it causes thickening of the epidermis (which is primarily composed of keratin), disrupts osmotic regulation, and causes cardiac arrest (Voyles et al. 2009). As keratin occurs in the mouthparts of tadpoles in most species (McDiarmid & Altig 1999), *Bd* also infects tadpole mouthparts; however it is not usually a direct cause of mortality (but see Searle et al. 2013, Hanlon & Parris 2014). While non-lethal, *Bd*-induced damage to tadpole mouthparts has been shown to reduce foraging efficiencies (Venesky et al. 2009) and reduce tadpole growth (Venesky et al. 2009, 2013; but see Hanlon et al. 2012, Kleinhenz et al. 2012). Recently, Venesky et

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al. (2013) showed that *Bd* exposure reduced tadpole size, and mouthpart damage reduced the size of tadpole guts relative to body size. In other words, studies have shown that *Bd* causes mouthpart damage, damage reduces foraging efficiency, and reduced efficiency reduces growth. So it can be inferred that *Bd*-induced mouthpart damage potentially reduces growth. However, this pathway has never been proven. While previous studies have provided evidence for possible pathways by which *Bd*-induced reductions in tadpole growth may occur, a direct pathway from *Bd*-induced mouthpart damage to reduced growth has yet to be confirmed.

Much work has focused on the direct, lethal effects of *Bd* on host populations. While such research is imperative to our understanding of the disease, the sublethal effects of *Bd*, especially on larval amphibians, could have lasting carry-over effects that negatively alter natural populations. To address the sublethal impact(s) of *Bd* on tadpoles and identify a pathway whereby *Bd* reduces growth and development, we tested the hypothesis that *Bd* would cause mouthpart damage, food intake, and subsequent growth. We conducted a laboratory study using southern leopard frog *Lithobates sphenocephalus* tadpoles. Using path analyses, we tested how *Bd*, mouthpart damage, and percentage of food in the gut (hereafter gut content), directly or indirectly influenced body size (growth measure). We predicted that *Bd* would increase mouthpart damage, in turn reducing gut content and subsequent body size. Also, independent of *Bd* exposure, we predicted that increased mouthpart damage would negatively correlate with gut content, which would in turn positively correlate with body size.

MATERIALS AND METHODS

Twelve *Lithobates sphenocephalus* clutches were collected from ponds within the University of Memphis Edward J. Meeman Biological Field Station (MBS), Meeman-Shelby State Park, Shelby County, TN (35° 22′ N, 90° 01′ W), USA, and Shelby Farms Park, Shelby County, TN (35° 9′ N, 89° 51′ W) between March 6 and 9, 2013. Eggs were transported to the laboratory at the University of Memphis, Memphis, TN. Upon hatching, tadpoles were maintained in 8 l aquaria (filled with 4 l of aged tap water). Upon reaching the free-swimming stage (Stage 25; Gosner 1960), tadpoles were combined from the different clutches to minimize potential genetic effects of the measured traits. While in the laboratory tadpoles were maintained on a 12 h light:12 h dark photoperiod at 19°C.

Batrachochytrium dendrobatidis inoculation

The *Bd* isolate used in our experiment was locally isolated from an infected adult *L. sphenocephalus* captured from MBS in May 2010. The isolate was grown in the laboratory in tryptone broth (TGhL; 1.6% tryptone, 0.2% gelatin hydrolysate, and 0.4% lactose) according to standard protocol (Longcore et al. 1999). Stock cultures were transferred monthly, plated to TGhL plates, and all *Bd* inoculates were taken from these plates. This strain has resulted in successful infections in both laboratory and field experiments (Venesky et al. 2009, 2013, Hanlon et al. 2012).

We harvested *Bd* zoospores by flooding the plates with sterile water and collecting the zoospores that emerged from the zoosporangia after 45 min. All water from the flooded plates was combined, and we estimated the density of zoospores using a 0.100 mm deep hemocytometer (Fisher Scientific). The *Bd*-exposed group (N = 12) was inoculated with *Bd* through exposure to water baths containing fungal zoospores. Tadpoles were placed in 50 ml water baths (3 individuals per 50 ml in 500 ml containers) and zoospores (2.88×10^6 zoospores) was added to each bath for 48 h. The non-exposed group (N = 12) followed the same protocol but the water was added to plates with TGhL alone. This design simulates transmission by water, a possible mode of *Bd* transmission in natural environments (Johnson & Speare 2005), and has resulted in successful infections in previous studies (Venesky et al. 2009, 2013, Hanlon et al. 2012). After 48 h, tadpoles were removed from their exposure treatment, individually placed into containers containing clean water, and infection was allowed to develop over 7 d.

Experimental design

After *Bd* inoculation (or control exposure), tadpoles were individually placed into 1.5 l plastic containers filled with 1.0 l of aged tap water. We changed the water in the containers every 5 d, at which time tadpoles were fed 15 mg Sera® Micron fish food. Because we were interested in the effects of *Bd* on larval mouthpart damage, foraging efficiency, and subsequent growth, the experiment was ended at Day 50, prior to initiation of metamorphosis. Individ-

uals were sacrificed through lethal exposure to buffered MS-222 according to approved Institutional Animal Care and Use Committee (IACUC) protocols. Body size was calculated as a function of tadpole mass divided by snout-vent length. Each tadpole was weighed (to the nearest 0.01 g) and measured for length (to the nearest 0.01 mm). To calculate the gut content, we dissected each tadpole and removed and straightened the intestine on a dissecting pan. We measured the length of the intestine (to the nearest 0.01 mm) and length of food in the intestine (Sera Micron is green and easily recognizable), and determined the gut content by division (adapted from Venesky et al. 2009). The researcher was blind to treatment combinations when performing all measurements.

For this study, we calculated a deformity index of mouthpart damage (adapted from Hanlon et al. 2013). Deformities were assessed in 10 zones of the oral disc: labial teeth (anterior tooth rows [3 zones], posterior tooth rows [3 zones]) and jaw sheaths (4 zones). Using a Nikon® SMZ800 dissecting scope with ×10 to ×60 magnification, one observer estimated the proportion of damage per zone for each tadpole on a scale of 0 to 1. For example, if 30% of a zone was missing, that damage score was 0.30. This process was repeated for all 10 zones for each tadpole. Mouthparts were then removed and stored in a −18°C freezer until qPCR analysis to quantify *Bd* load (see below).

Confirmation of *Batrachochytrium dendrobatidis* infection

Infection status (*Bd*-positive or negative) of all experimental animals was determined using real-time quantitative polymerase chain reaction (qPCR) following the method used by Boyle et al. (2004). DNA was extracted from the mouthparts of the tadpoles. This extraction technique is known to be reliable for extracting *Bd* DNA and the same exposure protocols have resulted in successful infections in previous experiments (e.g. Cheng et al. 2011, Hanlon et al. 2012, Prunier et al. 2012).

For qPCR analysis, standards were obtained from Commonwealth Scientific and Industrial Research Organisation (CSIRO) labs in Australia and were the same as those used in Boyle et al. (2004). The standards used in the qPCR reaction served as the positive controls on each plate and each plate contained a negative control; all samples were run in triplicate. The negative controls tested negatives on all plates. Samples were considered positive when a majority (2 out of 3) of replicates tested positive. This 'fast' qPCR technique has proven successful, especially in regards to accounting for false negatives (Kerby et al. 2013). For calculations of prevalence, mouthparts were categorized as *Bd*-positive when zoospore equivalents were ≥1 in a majority of each replicate per sample (as used by Vredenburg et al. 2010).

Statistical analysis

We assessed the effect of *Bd* on tadpole survival using a generalized linear model (GLM) with a binomial error distribution using the 'car' package in R.

We used path analysis with maximum-likelihood estimation to examine the relationship between *Bd*, mouthpart damage, gut content, and tadpole body size. We tested the hypotheses that (1) mouthpart damage, (2) gut content, and (3) body size were directly (e.g. *Bd* to body size) or indirectly (e.g. *Bd* to mouthpart damage to food in gut to body size) influenced by *Bd* exposure. The path analyses were conducted using the 'lavaan' package in R. The relative strength of each path was assessed by comparing the standardized coefficients: a larger absolute (i.e. farther from zero) value indicated a more significant path.

We ran 2 path analyses to account for differing treatments of the *Bd* predictor variable. Path 1 used *Bd* exposure as a categorical predictor (exposed or unexposed). Path 2 used *Bd* load as a continuous predictor. Using both methods allowed us to determine the best use of *Bd* as a predictor.

RESULTS

Bd infection was confirmed in tadpoles in 100% of exposed individuals with loads that ranged from 1.7 to 433 zoospore equivalents (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d112p061_supp.pdf). Additionally, no tadpoles from our control treatments tested positive for *Bd* infection.

Our analysis did not detect an effect of *Bd* ($F_{1,23}$ = 1.430, p = 0.232) on tadpole survival.

Our comparison between the 2 models (*Bd* exposure versus *Bd* load) revealed that *Bd* exposure was a more suitable predictor than *Bd* load. When considering *Bd* load as a predictor, we found a significant effect of the full model (p < 0.001); however, we found a non-significant effect of the model when using *Bd* exposure (p = 0.100). In these analyses, a

value of $p < 0.050$ indicates that a model is a poor fit (Beaujean 2014). Accordingly, the results presented here use *Bd* exposure as a predictor (see Table S2 in the Supplement for analysis using *Bd* load as a predictor).

In our model that examined the effects of *Bd* exposure on body size, multiple paths were significant (Fig. 1). *Bd* exposure was a negative predictor of gut content (Fig. 2A). Additionally, mouthpart damage was a negative predictor of gut content (Fig. 2B) and gut content was a positive indicator of body size (Fig. 2C). Summary statistics are presented in Fig. S1 in the Supplement.

DISCUSSION

While previous studies have shown relationships between *Bd* exposure and mouthpart damage (Venesky et al. 2013), amount of food ingested (Venesky et al. 2010), and growth (Parris & Cornelius 2004), our study shows the interrelatedness of these responses. Two specific paths were observed in the *Bd* exposure group, one from *Bd* to gut content to body size, and one from mouthpart damage to gut content to body size. Counter to our hypothesis, *Bd* exposure did not affect body size through the *Bd* to mouthpart damage to gut content to body size pathway, likely due to high mouthpart damage in both *Bd*-exposed and unexposed treatments.

In our experiment, significant mouthpart damage was observed in both *Bd* exposed and unexposed subjects, a results that has been observed previously (Venesky et al. 2013). Because the loose food that was added to the containers often sank, tadpoles were forced to scour the bottoms of the containers, likely contributing to mouthpart damage in both treatments. However, even in the absence of a *Bd* to mouthpart damage path, the *Bd* to gut content path was significant. One explanation for this observation

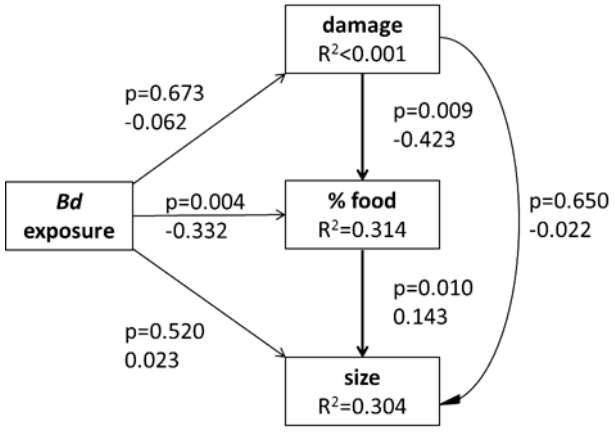

Fig. 1. Path analysis of the relationships between *Batrachochytrium dendrobatidis* (*Bd*) exposure, mouthpart damage, percentage of food in gut, and body size in *Lithobates sphenocephalus* tadpoles. p-values (significant at $p \leq 0.05$) and standardized coefficients (indicating strength and direction of each relationship) are provided for each pathway

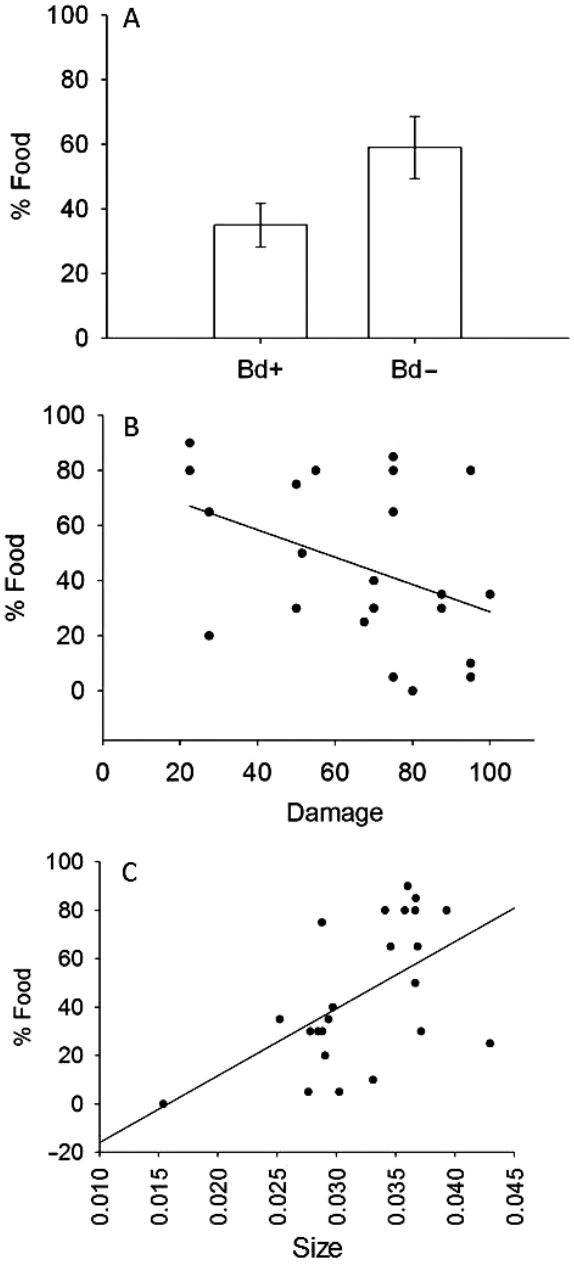

Fig. 2. (A) Effects of *Batrachochytrium dendrobatidis* (*Bd*) exposure on percent food in gut, and relationships between (B) mouthpart damage and percentage of food in gut, and (C) percent of food in gut and body size. Values plotted for A are least-squares means ± 1 SE

could be *Bd*-induced lethargy in exposed tadpoles. Numerous studies have shown that *Bd* causes lethargy and reduced swimming abilities in tadpoles (e.g. Blaustein et al. 2005, Venesky et al. 2009, Kleinhenz et al. 2012) and specifically in *Lithobates sphenocephalus* tadpoles (Parris et al. 2006). While reduced activity could lead to a suite of negative effects including increased predation rates and reduced competitive advantage (Boone & Semlitsch 2003, Boone et al. 2007), reduced foraging activity could result directly in less food being consumed by tadpoles (Venesky et al. 2009). Such effects have been observed previously as a result of *Bd* exposure (Venesky et al. 2009); thus, it is likely that a similar mechanism occurred in our study.

Mouthpart damage also affected gut content, to a greater degree than *Bd*. Independent of *Bd* treatment, tadpoles with greater mouthpart damage consumed less food. This observation is supported by previous research that has shown that mouthpart damage directly relates to foraging efficiencies (Venesky et al. 2009). There was also a significant gut content to body size relationship. In support of our hypothesis, less food in the gut resulted in poorer body size, independent of the mechanism (*Bd* exposure or mouthpart damage).

CONCLUSION

While previous work has shown that *Bd* causes reduced growth (i.e. body size) in tadpoles (Parris & Cornelius 2004, Venesky et al. 2013), our study is the first to quantitatively show reduced foraging success (i.e. percentage of food in gut) as a potential mechanism. While we were unable to show *Bd*-induced mouthpart damage as a potential mechanism for reduced gut content (likely due to high damage in control subjects), *Bd*-induced lethargy provides a plausible alternative scenario. Independent of *Bd* exposure, we also showed a pathway from mouthpart damage to gut content to body size. This pathway is important for future studies as a potential mechanism for any observed reductions in tadpole growth.

Determining the pathway(s) whereby *Bd*-induced reductions in larval growth occur is a necessary step in furthering our understanding of the *Bd* host–pathogen relationship. While a majority of *Bd*-related research has focused on the lethal effects of the fungus, our study shows that *Bd* can alter foraging efficiencies and reduce tadpole growth. Such alterations to the larval stage of amphibians (anurans and salamanders) can affect larvae and carry over into adulthood. Smaller larvae that cannot obtain sufficient nutrients are likely to be less mobile, potentially resulting in greater predation and poorer competitive advantages (Asquith & Vonesh 2012). These results are likely to carry over into adulthood and be further compounded by reductions in mating success (Bowcock et al. 2013). Smaller, 'weaker' adults are less likely to mate, resulting in reduced fitness and recruitment to subsequent generations (Allentoft & O'Brien 2010). Thus, the sublethal effects of *Bd* on individual larvae could have lasting effects that would influence entire populations. Our work highlights the importance of investigating the sublethal role of *Bd* on larval amphibians.

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