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## PATHOGENICITY OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN LARVAL AMBYSTOMATID SALAMANDERS

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**Abstract.**—Chytridiomycosis is a disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), which colonizes keratinized tissues in adult and larval amphibians. Considerable progress has been made in understanding the host-pathogen ecology of *Bd* in larval anurans, yet little is known about how *Bd* affects larval salamanders. Because the structure of keratinized jaw sheaths in *Ambystoma* larvae have not been thoroughly documented, we first described the structure in three species of larval *Ambystoma*. We then conducted a laboratory experiment to test if *Bd* affects growth and developmental rates of larval Marbled Salamanders (*Ambystoma opacum*). We observed keratinized jaw sheaths in all three species of *Ambystoma*, but the sheath was not present in all individuals. In our exposure experiment, none of the *A. opacum*, whose mouthparts were screened for *Bd*, tested positive, nor was there an effect of *Bd* on larval life-history responses. A cautionary note, however, is that although our method of *Bd* infection has been successful in other amphibian-*Bd* experiments in our laboratory, our exposure experiment did not include a positive control of other taxa known to become infected with *Bd*. We are uncertain why none of the larval *A. opacum* became infected with *Bd*, given that we observed keratinized jaw sheaths in this species. Two possible explanations are the keratinized jaw sheaths of larval *Ambystoma* differ among species in structure or keratin type so that *Bd* may not be able to successfully infect them or, *A. opacum* larvae may have cleared low intensity *Bd* infections prior to metamorphosis.

**Key Words.**—*Ambystoma opacum*; *Batrachochytrium dendrobatidis*; jaw sheath; life history

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### INTRODUCTION

There is increasing evidence for the sudden global decline of many species of organisms. Of the declining taxa, amphibian population declines are occurring at a startling rate (Stuart et al., 2004). Chytridiomycosis, an emerging infectious disease of amphibians caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), has played a major role in the decline of amphibian populations and communities (Berger et al. 1998; Lips et al. 2006). Understanding the etiology of *Bd* infection is a critical component of the biology of this emerging infectious disease; however, little is known about how species specific differences affect susceptibility to *Bd* infection, particularly during larval stages (but see Blaustein et al. 2005; Woodhams and Alford 2005; Venesky et al. 2010). If intra- or interspecific differences in larval morphology (Pfennig et al. 1998) or life-history traits (Rowley and Alford 2007) affect susceptibility, this variation may be crucial for understanding host-pathogen disease dynamics in amphibian communities.

There has been considerable progress in understanding the host-pathogen ecology of *Bd* in larval anurans, especially in how host ecology and life-history traits interact to affect susceptibility to *Bd* (see Daszak et al. 2003). However, data on the host-pathogen ecology of

*Bd* in larval salamanders are lacking, although both adult (Bosch and Martinez-Solano 2006; Briggler et al. 2007; Davidson et al. 2003; Pasmans et al. 2004; Vazquez et al. 2009) and larval (Brodman and Briggler 2008; Padgett-Flohr and Longcore 2007) salamanders are susceptible to *Bd* infections. Nonetheless, studies on the pathogenicity of *Bd*, specifically how infection impacts fitness in the larval stage, remains largely unknown. In addition, compared to anurans, little is known about the mechanism of infection in larval salamanders. Considering the evidence that *Bd* is capable of causing population declines in some salamander taxa (Bosch and Martinez-Solano 2006; Lips et al. 2003), understanding the mechanisms of pathogen-induced population declines is needed. Specifically, data are lacking on what, if any, effects *Bd* may have on life-history responses of salamanders. For example, in anuran larvae, *Bd* has been shown to reduce growth and developmental rates (Parris and Baud 2004; Parris and Cornelius 2004; but see Smith et al. 2007). Any perturbations in the timing of metamorphosis may negatively impact fitness by increasing the chance of death by desiccation in a pond (Semlitsch 1987), decreasing the ability to escape gape-limited predators (Kurzava 1998), increasing intraspecific competition (Semlitsch et al. 1988), or increasing the time spent in the water, thereby increasing the potential for exposure

to aquatic pathogens.

One potential reason that larval salamanders have not been tested thoroughly for pathogen-induced life history changes is that compared to larval anurans, relatively few urodeles have keratinized tissues as larvae (Frost et al. 2006). *Bd* infects keratinizing tissue, or those tissues fated to keratinize (Fellers et al. 2001), and colonization of *Bd* in amphibians is restricted to areas where keratinized cells are present. Because most anuran larvae have keratinized labial teeth and jaw sheaths (Altig and McDiarmid 1999), they are often susceptible to *Bd*. Larvae in Ambystomatidae represent one family of salamanders in which species have a keratinized jaw sheath (Petranka 1998), but detailed descriptions of this structure are lacking. Noble (1931) documented that larvae of some *Ambystoma* have a horny beak, similar to the beak of tadpoles; however, no further description of the cellular make-up of the structure was provided. Altig (1973) observed a loose network of keratin (presumably the jaw sheath) close to the jaw in larval *A. mexicanum*, but the structure of the entire sheath was not described. In addition, Petranka (1998) used the presence of a keratinized jaw sheath as a key morphological character shared by all larval *Ambystoma*, but did not provide further descriptive information of the sheath. More information on the structure of the sheath and the extent to which keratin is present is needed to assess the full potential impact of *Bd* exposure in larval *Ambystoma*.

In our study, we first sought to observe and further describe the keratinized jaw sheath of larval *Ambystoma*. Specifically, we tested for the presence of the sheath and described patterns of variation in the structure in Marbled Salamanders (*A. opacum*), Smallmouth Salamanders (*A. texanum*), and Streamside Salamanders (*A. barbouri*). Because a detailed description of the formation of the keratinized jaw sheath throughout ontogeny was beyond the scope of this study, we focused our observations on late-staged larvae when keratinized jaw sheaths were likely to be fully developed so we could describe the entire structure. Secondly, we conducted a *Bd* exposure experiment with larval *A. opacum* to test whether *Bd* infections affected growth and developmental rates. Larval *A. opacum* have a wide geographic range in the United States (Petranka 1998) and occur sympatrically with other *Bd* infected amphibians (Venesky and Brem 2008), and thus are likely exposed to *Bd* in natural amphibian communities. Because susceptibility to *Bd* is associated with the development of keratinized structures (e.g., jaw sheaths), we exposed larval *A. opacum* to *Bd* at three different developmental stages to test for differential susceptibility throughout ontogeny and also for *Bd* induced changes in life-history traits. Specifically with our exposure experiment, we tested: (1) if *A. opacum* larvae are susceptible to *Bd*; (2) if developmental stage

of larvae has an effect on susceptibility to infections; and (3) if *Bd* affects larval size, mass, survival, or length of larval period. It is important to note that in the first experiment, we focused our observations on late-staged larvae when keratinized jaw sheaths were likely to be fully formed. However, it is possible that the keratinized jaw sheath begins to form earlier in development, possibly indicating that larval *Ambystoma* are susceptible to *Bd* at various stages in ontogeny.

### MATERIALS AND METHODS

**Collection of larvae.**—We used larvae in this study from eggs of *Ambystoma opacum*, *A. texanum*, and *A. barbouri* we collected in the field. On 23 October 2006, we collected approximately 120 eggs from three female *A. opacum* (N~40 per female) from the margins of a pond in Shelby Co., Tennessee, USA (35°21'43"N; 90°0'41"W). On 2 March 2007, we collected six distinct masses of *A. texanum* eggs from two ponds in Livingston Co., Kentucky, USA (39°22'23"N; 88°27'56"W). On 11 March 2007, we collected five masses of *A. barbouri* eggs from the underside of five rocks from two streams (Boone Co., Kentucky, USA; 38°26'07"N; 84°57'14"W). A “mass” of eggs refers to either a monolayer of eggs beneath a rock or a discrete cluster of adjoining eggs attached to a single twig or piece of vegetation. Each egg mass for *A. texanum* and *A. barbouri* likely represents a separate female parent (Maurer and Sih 1996; Petranka 1984).

We combined eggs from the clutches of each species to evenly distribute potential genetic effects on the larval traits we measured. Upon hatching, we maintained larvae in 4.2 L plastic containers at a density of five larvae/L for approximately two weeks. During this period, we fed larvae brine shrimp (*Artemia* spp.) *ad libitum* every three days. After this period, we removed all larvae that had not begun foraging regularly and placed larvae individually in 1.5 L plastic containers filled with 1 L of aged tap water. After larvae reached the minimum size required to eat larger prey, we fed them Blackworms (*Lumbriculus variegates*) through the remainder of the experiment. Throughout the experiment, we fed larvae and performed water changes every three days.

**Test for keratinized jaw sheaths.**—We tested for the presence of the sheath and described patterns of variation in the structure in larval *A. opacum*, *A. texanum*, and *A. barbouri*. We raised larval *A. opacum* ( $n = 22$ ), *A. texanum* ( $n = 13$ ), and *A. barbouri* ( $n = 10$ ) as described above until they had developed their hind limbs (post-Harrison stage 46; Harrison, 1969). The larval *A. opacum* used in this portion of the study were not used in the pathogen exposure experiment (below). After larvae of each species developed hind limbs, we

ethanized them, fixed them in formalin, and stored them in 70% ethanol for observations. To determine if larvae had keratinized jaw sheaths, we dissected the head of each larva and observed the upper and lower jaw with a Nikon® SMZ800 dissecting scope with 10–60X magnification. Larvae with keratinized jaw sheaths were photographed under 40X with a Meiji® MX5300L microscope mounted with a ProgReg® C3 digital camera.

**Pathogen effects on larval *Ambystoma opacum*.**—In amphibians, the colonization of *Bd* is restricted to areas where keratinized cells are present (Fellers et al. 2001). At the start of this experiment, it was unknown at what point in ontogeny the keratinized jaw sheath begins to form in ambystomatid larvae. Because one of our objectives was to test if larval *A. opacum* can become infected with *Bd*, we exposed larval *A. opacum* to *Bd* zoospores at early (Harrison 38–40), middle (Harrison 41–42), and late (Harrison 45–46) stages, which span the entirety of larval ontogeny. We assigned larvae into three groups based on their date of hatching: individuals in group 1 (early) hatched 13–17 November, individuals in group 2 (middle) hatched 18–22 November, and individuals in group 3 (late) hatched 25–27 November. Thus, the date of hatching created cohorts that matched our developmental stage treatment and each developmental group contained larvae from each of the three clutches we collected. We considered the median date of hatching for each group as experimental day 0; thus, day 0 for the early group was 15 Nov., for the middle group was 20 Nov., and for the late group was 26 Nov. The diet and feeding schedule of the larvae used in this experiment were similar to those described above. We also took the following precautions during water changes to avoid accidental *Bd* transmission between treatments. During each water change, an experimenter wore latex gloves to prevent the transmission of *Bd*. We used a piece of mesh screen to remove the focal larva from its container, and placed the larva 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. We then placed the focal larva back into the experimental container. To minimize the possibility of *Bd* transmission between *Bd* exposed or unexposed treatments, we always performed water changes on unexposed larvae before exposed larvae. We also used different laboratory equipment (gloves, transfer container, mesh screen) between each developmental stage in both of the pathogen treatments. We changed water every three days.

We used a fully factorial design to test the effect of *Bd* (exposed or unexposed) and developmental stage (early, middle, or late) on body size at metamorphosis, length of

larval period, and survival of larval *A. opacum*. We randomly assigned 90 1.5 L containers (18 X 14 X 9 cm) to the six treatment combinations, filled them with 1 L of aged tap water, and placed them on laboratory shelves, each containing a random arrangement of each treatment. We inoculated larvae with *Bd* isolate #277 (isolated from a wild caught individual of *A. tigrinum*; Davidson et al. 2003) by exposing them to fungal zoospores. We grew *Bd* in the laboratory on tryptone-gelatin hydrolysate-lactose (TGhL) agar in 9 cm Petri dishes according to standard protocol (Longcore et al. 1999). On 19 December 2006, we harvested zoospores by adding 10.0 mL of sterile water to the cultures and collected the zoospores that emerged from the zoosporangia after 30 min. The inoculate from all the Petri dishes was combined into a single container and zoospore density was estimated by using a hemacytometer and counting the number of active zoospores. We placed larvae in 100 mL waterbaths and exposed them to an infectious concentration of zoospores (2,500 zoospores/mL; Padgett-Flohr 2008) for 48 hours. This method of *Bd* infection has been successful in other amphibian-*Bd* experiments in our laboratory (Parris et al. 2006; Venesky et al. 2010). As a control, we followed the same protocol but used plates with only TGhL instead of plates with *Bd*. Our design simulated transmission by water, one of the possible modes of *Bd* transmission in natural environments (Pessier et al. 1999). 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*. Throughout the experiment, all equipment and water was disinfected in a similar fashion.

**Detection of *Batrachochytrium dendrobatidis* infections.**—Four weeks after exposure to *Bd* (18 January 2007), we removed each larvae from the *Bd* exposed treatment and swabbed the external and internal portions of the mouth with sterile, cotton-tipped applicators. Immediately after swabbing each individual, we placed the swab in a sterile centrifuge tube with 70% EtOH to be analyzed with real-time quantitative polymerase chain reaction (qPCR) following methods in Boyle et al. (2004). Additionally, we included a cotton-tipped swab that was rubbed on a Petri dish of *Bd*, which was included as an exogenous internal positive control. Each sample was run in triplicate against a *Bd* standard titration using relative qPCR. Of the 45 larvae in the *Bd* exposed treatment, we selected a subset of 20 swabs that contained samples from each of the three developmental groups for qPCR analysis to test if larval *A. opacum* were infected with *Bd*. For each sample, the pathogen treatment (*Bd* exposed or control) was unknown to the experimenter.

**TABLE 1.** Summary of the MANOVA of length of larval period, snout-vent length (SVL), and mass for *Ambystoma opacum* larvae either unexposed or exposed to *Batrachochytrium dendrobatidis* in laboratory containers. Stage refers to the developmental stage at which larvae were exposed to *B. dendrobatidis*.

Source	df	Wilks' $\lambda$	F	P
Pathogen	3, 59	0.901	2.17	0.102
Stage	6, 118	0.532	7.29	<0.0001
Path. $\times$ Stage	6, 118	0.978	0.22	0.970

three dependent variables, larval period length, snout-vent length (SVL), and body mass at metamorphosis ( $\alpha = 0.05$ ). We then used Bonferroni-adjusted (significance level of 0.016 for three response variables) univariate analysis of variance (ANOVA) contrasts on each response variable to test for significant contributors to the multivariate effects (SAS version 9.1, SAS Institute, Inc., Cary, NC). Our data met the assumptions of the statistical analyses used. We calculated *Bd* infection prevalence and 95% Clopper-Pearson binomial confidence intervals (CI) for *A. opacum* larvae exposed to *Bd*.

**RESULTS**

**Test for keratinized jaw sheaths.**—The keratinized jaw sheath appeared structurally similar in each of the four *Ambystoma* taxa examined (Fig. 1). A sheath was observed only on the lower jaw and extended for a variable distance around the curvature of the jaw. Generally, the sheath was attached to the medial surface of the jaw, spanned the transverse width of the jaw, and had an appearance of a dense layer of keratin. Differences in the general integrity and extent of the sheath varied among specimens and species. For example, in some specimens, the sheath did not span the entire width of the jaw (Fig. 1C). In addition, parts of the sheath seemingly differed in keratin density (Fig. 1A, left side). It is unknown if these variations are species specific or are developmental or ecological differences.

Because we made observations only on late stage larvae, we cannot evaluate when and how the sheaths form ontogenetically, but we know that the structures are not always present in similarly staged larvae within and between taxa. Eighteen percent of the larval *Ambystoma opacum*, 30 % of the larval *A. texanum*, and 10 % of the larval *A. barbouri* we examined had keratinized jaw sheaths.

**Pathogen effects on larval *Ambystoma opacum*.**—Of the 20 animals screened, all were negative for *Bd* (0.00, 95% CI 0.00–0.16). The positive test result of a blind swab rubbed on a plate of *Bd* confirmed that the qPCR reaction worked properly. In addition, no larvae from the pathogen treatments died during the

**Statistical analyses.**—We used multivariate analysis of variance (MANOVA) to test for effects of two independent factors, disease (*Bd* exposed or unexposed) and developmental group (early, middle, and late), on experiment, nor did any metamorphs exhibit clinical signs of chytridiomycosis infection typically observed in metamorphic anurans and salamanders (e.g., skin sloughing or skin lesions).

MANOVA indicated non-significant effects of pathogen and pathogen  $\times$  developmental stage interaction effects on combined larval responses, but there was a significant effect of developmental stage on multivariate responses (Table 1). Similarly, all univariate ANOVAs revealed non-significant effects of pathogen on life-history responses. Developmental stage had a significant effect on the length of larval period in the univariate analysis (Table 2).

Clopper-Pearson binomial confidence interval revealed that, given our sample size, we would be unable to detect with 95% confidence an actual *Bd* prevalence up to 16%.

**DISCUSSION**

Understanding which species are susceptible to pathogens and the interspecific patterns of pathology are important in developing our understanding of how *Bd* affects amphibian populations. Experimental evidence has revealed some of the interactions between anurans and *Bd*, especially in how host life-history traits affect susceptibility to *Bd* (see Daszak et al. 2003); however, data on the host-pathogen ecology of *Bd* in larval salamanders are primarily observational (e.g., Lips et al. 2003; Pasmans et al. 2004; Bosch and Martinez-Solano 2006; Briggler et al. 2007; Brodman and Briggler, 2008). To date, data on the mechanisms of *Bd*

**TABLE 2.** Summary of the ANOVAs of length of larval period, snout-vent length (SVL), and mass for *Ambystoma opacum* larvae either unexposed or exposed to *Batrachochytrium dendrobatidis* in laboratory containers. Stage refers to the developmental stage at which larvae were exposed to *B. dendrobatidis*.

Response	Source	df	squares	F	P
Larval Period	Pathogen	1	0.0010	3.03	0.087
	Stage	2	0.0061	18.21	<0.0001
	Path. $\times$ Stage	2	0.0002	0.07	0.933
	Error	61	0.0204		
SVL	Pathogen	1	0.0005	0.41	0.526
	Stage	2	0.0001	0.04	0.961
	Path. $\times$ Stage	2	0.0001	0.12	0.881
	Error	61	0.0719		
Mass	Pathogen	1	0.0007	0.29	0.589
	Stage	2	0.0011	0.48	0.620
	Path. $\times$ Stage	2	0.0030	0.62	0.543
	Error	61	0.1432		

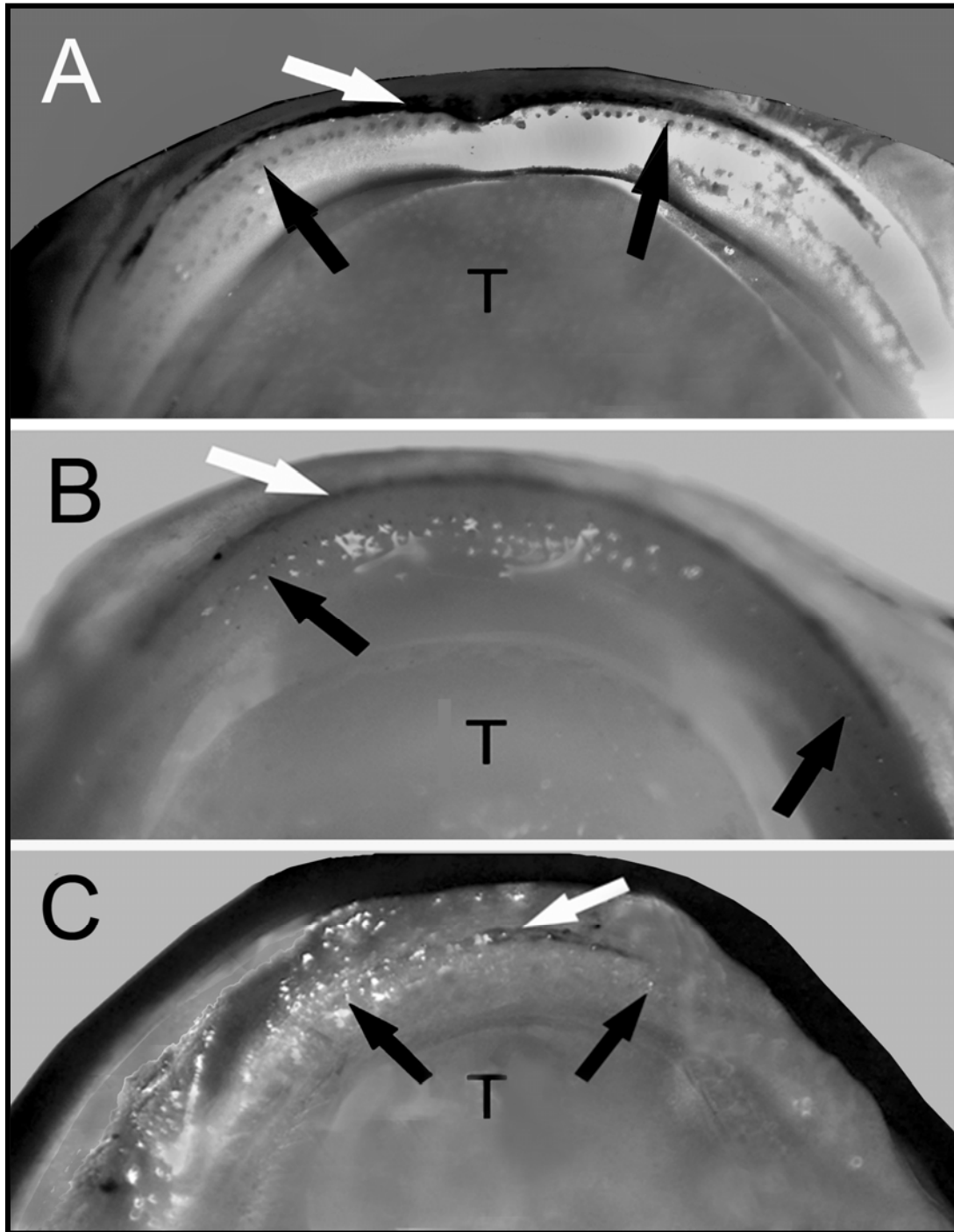


FIG. 1.—Photographs of the ventral jaw of larval (A) *Ambystoma californiense*; (B) *A. opacum*; and (C) *A. texanum*. T = tongue; black arrows point to teeth; white arrow points to keratinized jaw sheath. (Photographed by M. Venesky)

infections, and *Bd* induced life-history responses in larval salamanders are lacking. Given that *Bd* can cause population declines in salamander taxa (Bosch and Martinez-Solano 2006; Lips et al. 2006), it is important to understand how hosts respond to *Bd*.

We observed keratinized jaw sheaths in three species of larval *Ambystoma*, indicating that the species we examined should be susceptible to *Bd*, yet none of the *A. opacum* we screened tested positive for *Bd*, regardless at which developmental stage they were exposed. In

addition, larval *A. opacum* exposed to *Bd* did not respond to the pathogen with changes in growth or developmental rates, indicating that *Bd* exposure had no significant effect on growth or development. Significant developmental effects likely are caused by differences in the starting age of the larvae among the three developmental stage groups. Our data are similar to those collected in a preliminary experiment, in which larval Spotted Salamanders (*A. maculatum*) exposed to *Bd* were not detrimentally affected in terms of their growth and developmental rates (Parris, unpublished data). Although Brodman and Briggler (2008) found late-staged field collected larval *A. jeffersonianum* infected with *Bd*, our results suggest: (1) that not all larval *Ambystoma* are susceptible to *Bd*, (2) larvae may only be susceptible to *Bd* immediately prior to metamorphosis when they express keratin in their epidermis, or (3) the larval *A. opacum* used in our experiment cleared their infection prior to metamorphosis.

In addition to possible explanations from the host perspective, alternative explanations from the pathogen perspective warrant discussion. First, the lack of a positive control in our exposure experiment precluded us from concluding that our *Bd* inoculation methods were sufficient to cause *Bd* infection. By exposing, and infecting, another host species with *Bd* at the time of exposure to *A. opacum*, larvae would have confirmed that experimental error was not responsible for the lack of *Bd* positive samples. Related to this topic, the possibility exists that our *Bd* inoculation methods were not appropriate for larval salamanders. Our *Bd* inoculation protocol was developed for infecting larval anurans and we believed should infect susceptible larval salamanders. Exposure to higher concentrations of *Bd* zoospores for a longer duration may affect susceptibility in larval salamanders. We are confident that at the time of inoculation, *Bd* zoospores were viable. Approximately five minutes prior to inoculation, zoospore density was estimated by using a hemacytometer, during which all zoospores were still living and appeared to move the same in the inoculate as in culture. Finally, it is possible that the *Bd* isolate was no longer pathogenic. Attenuation of fungal virulence has been reported (Choi et al. 1995); however, Daszak et al. (2004) found that despite many passages of *Bd* outside an amphibian host, their strain of *Bd* had not become attenuated.

We are uncertain why none of the larval *A. opacum* we screened tested positive for *Bd* in our experiment. We use this model with caution in that it assumes that the starting prevalence in the population is 0%. Given that each individual in our sample population was purposefully exposed to *Bd*, the confidence interval calculated by the Clopper-Pearson method may not indicate the actual power of missing a positive sample.

Considering that all animals in the *Bd* exposure group were intentionally exposed to *Bd*, it is unlikely that we missed a positive infection in the unscreened animals in that treatment group. More data on the pathogenicity of *Bd* in larval salamanders or estimates of prevalence levels of *Bd* in larval salamanders in natural populations would be useful in determining minimum natural levels for *Bd* occurrence in larval salamanders. Also, we only swabbed the mouthparts of each individual and some larval salamanders have keratinized tissue on their feet (Whitear 1977) and *Bd* infections have been detected in the feet of larval *Taricha* (Padgett-Flohr and Longcore 2007) and *A. jeffersonianum* (Brodman and Briggler, 2008). Therefore, *Bd* may have colonized the keratinized tissues of the feet of the larval *A. opacum* used in our experiment, but we would not have detected it. However, because larval *A. opacum* have keratinized jaw sheaths, we would have expected to detect *Bd* on their mouthparts if *Bd* infections were present. Still, if *Bd* infections were restricted to the feet of larval *A. opacum*, it did not impact the life-history responses that we measured in our experiment.

Potential structural, chemical, or developmental differences between the jaw sheaths of larval *Ambystoma* and anurans may explain why we were unable to detect *Bd* infections on the mouthparts of larval *A. opacum*. Although the keratinized jaw sheaths of larval *Ambystoma* and larval anurans appear similar, it is unclear if they are formed the same way or constructed of similar keratins. In larval anurans, the sheath covers the cartilaginous jaw (Altig and McDiarmid 1999) whereas in *Ambystoma*, it overlies dermal bones and little is known about the types of keratin in either anuran or *Ambystoma* jaw sheaths. Although no comparative studies have been done on interspecific variation in the expression of keratin types, both type I and type II keratin genes are expressed in larval and adult skin of *Xenopus laevis* (Miyatani et al. 1986). If *Bd* responds differently to type I and II keratin, and if some amphibians have only one type of keratin at a given stage, pathogenicity of *Bd* may vary across taxa. Future studies could focus on a histological description of the jaw sheath of larval *Ambystoma* and compare it to the sheath of larval anurans.

Studies on the timing of development of the oral apparatus are available for a few taxa of anurans (Bonacci et al. 2008; Thibaudeau and Altig 1988), but none of these examined the conditions that influence the formation of keratinized structure. In general, the jaw sheath forms and keratinization starts at about Gosner stage 23 in anuran development (Bonacci et al. 2008). If the sheath keratinizes within a similar timeframe in larval *Ambystoma*, it may occur as early as Harrison stages 42–43; however, this hypothesis remains untested. It is important to note that in our exposure experiment, larval *A. opacum* from the late developmental stages

were in Harrison stages 45–46 when exposed to *Bd*. We observed keratinized jaw sheaths in *A. opacum*, and others ambystomatids from these developmental stages, indicating that the sheaths should have been susceptible to *Bd* exposure. However, because late staged larval *A. opacum* were not susceptible to *Bd* in our experiment, it appears that the development of a keratinized jaw sheath may not affect their susceptibility. Because species specific differences in susceptibility are apparent in *Ambystoma* (larval *A. jeffersonianum* are susceptible to *Bd* infections [Brodman and Briggler 2008] but larval *A. opacum* are not), other factors such as immunological differences may affect susceptibility.

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#### LITERATURE CITED

- Altig, R. 1973. Preliminary scanning electron observations of keratinized structures of amphibian larvae. *Herpetological Information Search Service* 1:129–131.
- Altig, R., and R.W. McDiarmid. 1999. Body plan: development and morphology. Pp. 24–51 *In* Tadpoles: The Biology of Anuran Larvae. McDiarmid, R.W., and R. Altig (Eds.). University of Chicago Press, Chicago, Illinois, USA.
- Berger, L., R. Speare, P. Daszak, D.E. Green, A.A. Cunningham, C.L. Goggin, R. Slocombe, M.A. Ragan, A.D. Hyatt, K.R. McDonald, H.B. Hines, K.R. Lips, G. Marantelli, and H. Parkes. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences, USA* 95:9031–9036.
- Blaustein, A.R., J.M. Romansic, E.A. Scheesseele, B.A. Han, A.P. Pessier, and J.E. Longcore. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conservation Biology* 19:1460–1468.
- Bonacci, A., E. Brunelli, E. Sperone, and S. Tripepi. 2008. The oral apparatus of tadpoles of *Rana dalmatina*, *Bombina variegata*, *Bufo bufo*, and *Bufo viridis* (Anura). *Zoologischer Anzeiger* 247:47–54.
- Bosch, J., and I. Martínez-Solano. 2006. Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park (Central Spain). *Oryx* 40: 84–89.
- Boyle, D.C., D.B. Boyle, V. Olsen, J.A.T. Morgan, and A.D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60:141–148.
- Briggler, J.T., J. Ettl, M. Wanner, C. Schuette, and M. Ducan. 2007. *Cryptobranchius alleganiensis*. Chytrid fungus. *Herpetological Review* 38:174.
- Brodman, R., and J.T. Briggler. 2008. *Batrachochytrium dendrobatidis* in *Ambystoma jeffersonianum* larvae in southern Indiana. *Herpetological Review* 39:320–321.
- Choi, G.H., B. Chen, and D.L. Nuss. 1995. Virus-mediated or transgenic suppression of a G-protein  $\alpha$  subunit and attenuation of fungal virulence. *Proceedings of the National Academy of Sciences, USA* 92:305–309.
- Daszak, P., A.A. Cunningham, and A.D. Hyatt. 2003. Infectious disease and amphibian population declines. *Diversity and Distributions* 9:141–150.
- Daszak, P., A. Strieby, A.A. Cunningham, J.E. Longcore, C.C. Brown, and D. Porter. 2004. Experimental evidence that the Bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* 14:201–207.
- Davidson, E.W., M. Parris, J.P. Collins, J.E. Longcore, A.P. Pessier, and J. Brunner. 2003. Pathogenicity and transmission of chytridiomycosis in Tiger Salamanders (*Ambystoma tigrinum*). *Copeia* 2003:601–607.
- Fellers, G.M., D.E. Green, and J.E. Longcore. 2001. Oral chytridiomycosis in the Mountain Yellow-legged Frog (*Rana muscosa*). *Copeia* 2001:945–953.
- Frost, D.R., T. Grant, J. Faivovich, R.H. Bain, A. Haas, C.F.B. Haddad, R.O. De Sá, A. Channing, M. Wilkinson, S.C. Donnellan, C.J. Raxworthy, J.A. Campbell, B.L. Blotto, P. Moler, R.C. Drewes, R.A. Nussbaum, J.C. Lynch, D.M. Green, and W.C. Wheeler. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History* 297:1–370.
- Harrison, R.G. 1969. Harrison stages and description of the normal development of the Spotted Salamander, *Ambystoma punctatum* (Linn.). Pp. 44–66 *In* Organization and Development of the Embryo. Wilens, S (Ed.). Yale University Press, New Haven, London.
- Kurzawa, L.M. 1998. Tests of functional equivalence:

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- complementary roles of salamanders and fish in community organization. *Ecology* 79:477–489.
- Lips, K.R., F. Brem, R. Brenes, J.D. Reeve, R.A. Alford, J. Voyles, C. Carey, L. Livo, A.P. Pessier, and J.P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences, USA* 102:3165–3170.
- Lips, K.R., J.D. Reeve, and L.R. Witters. 2003. Ecological traits predicting amphibian population declines in Central America. *Conservation Biology* 17:1078–1088.
- Longcore, J.E., A.P. Pessier, and D.K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen. et sp. Nov., a chytrid pathogenic to amphibians. *Mycologia* 91:219–227.
- Maurer, E.F., and A. Sih. 1996. Ephemeral habitats and variation in behavior and life history: comparisons of sibling salamander species. *Oikos* 76:337–349.
- Miyatani, S., J.A. Winkles, T.D. Sargent, and I.B. Dawid. 1986. Stage-specific keratins in *Xenopus laevis* embryos and tadpoles: the XK81 gene family. *Journal of Cell Biology* 103:1957–1965.
- Noble, G.K. 1931. *The Biology of the Amphibia*. Dover Publications, New York, New York, USA.
- Padgett-Flohr, G.E. 2008. Pathogenicity of *Batrachochytrium dendrobatidis* in two threatened California amphibians: *Rana draytonii* and *Ambystoma californiense*. *Herpetological Conservation and Biology* 3:182–191.
- Padgett-Flohr, G.E., and J.E. Longcore. 2007. *Taricha torosa* (California Newt). Fungal infection. *Herpetological Review* 38:176–177.
- Parris, M.J., and D.R. Baud. 2004. Interactive effects of a heavy metal and chytridiomycosis on Gray Treefrog larvae (*Hyla chrysosecelis*). *Copeia* 2004:343–349.
- Parris, M.J., and T.O. Cornelius. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. *Ecology* 85:3385–3395.
- Parris, M.J., E. Reese, and A. Storfer. 2006. Antipredator behavior of chytridiomycosis-infected Northern Leopard Frog (*Rana pipiens*) tadpoles. *Canadian Journal of Zoology* 84:58–65.
- Pasmans, F., P. Zwart, and A.D. Hyatt. 2004. Chytridiomycosis in the Central American bolitoglossine salamander (*Bolitoglossa dofleini*). *Veterinary Record* 154:153.
- Pessier, A.P., D.K. Nichols, J.E. Longcore, and M.S. Fuller. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's Tree Frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation* 11:194–199.
- Petranka, J.W. 1984. Sources of interpopulational variation in growth responses of larval salamanders. *Ecology* 65:1857–1865.
- Petranka, J.W. 1998. *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington DC, USA.
- Pfennig, D.W., W.G. Ho, and E.A. Hoffman. 1998. Pathogen transmission as a selective force against cannibalism. *Animal Behavior* 55:1255–1261.
- Rothermel, B.B., S.C. Walls, J.C. Mitchell, C.K. Dodd, L.K. Irwin, D.E. Green, V.M. Vazquez, J.W. Petranka, and D.J. Stevenson. 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Diseases of Aquatic Organisms* 82:3–18.
- Rowley, J.J.L., and R.A. Alford. 2007. Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms* 77:1–9.
- Semlitsch, R.D. 1987. Relationship of pond drying to the reproductive success of the salamander *Ambystoma talpoideum*. *Copeia* 1987:61–69.
- Semlitsch, R.D., D.E. Scott, and J.H.K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* 69:184–192.
- Smith, K.G., C. Weldon, W. Conradie, and L.H. du Preez. 2007. Relationships among size, development, and *Batrachochytrium dendrobatidis* infection in African tadpoles. *Diseases of Aquatic Organisms* 74:159–164.
- Thibaudeau, D.G., and R.A. Altig. 1988. Sequence of ontogenetic development and atrophy of the oral apparatus of six anuran tadpoles. *Journal of Morphology* 197:63–69.
- Whitear, M. 1977. A functional comparison between the epidermis of fish and of amphibians. Pp. 291–313 *In Comparative Biology of the Skin*. Spearman, R.I.C. (Ed.). Academic Press, London, UK.
- Woodhams, D.C., and R.A. Alford. 2005. Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conservation Biology* 19:1449–1459.
- Vazquez, V.M., B.B. Rothermel, and A.P. Pessier. 2009. Experimental infection of North American plethodontid salamanders with the fungus *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 84:1–7.
- Venesky, M.D., and F.M. Brem. 2008. Occurrence of *Batrachochytrium dendrobatidis* in southwestern Tennessee, USA. *Herpetological Review* 39:319–320.
- Venesky, M.D., M.J. Parris, and A. Storfer. 2010. The impacts of *Batrachochytrium dendrobatidis* infections on tadpole foraging performance. *EcoHealth*. DOI: 10.1007/s10393-009-0272-7.

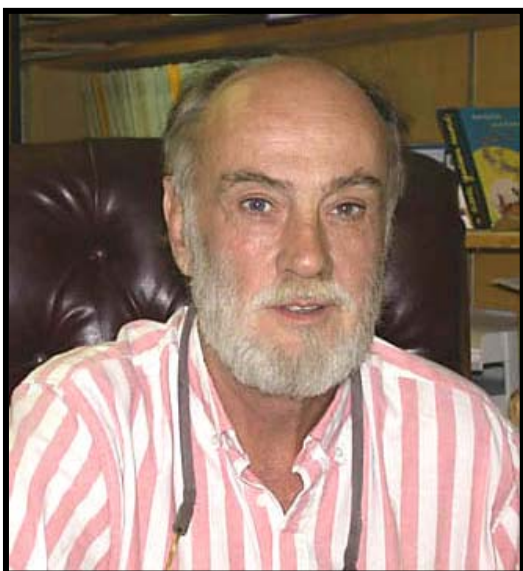


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