Salamanders increase their feeding activity when infected with the pathogenic chytrid fungus *Batrachochytrium dendrobatidis*

Alexandra Hess[#], Caroline McAllister, Joseph DeMarchi, Makenzie Zidek, Julie Murone, Matthew D. Venesky[#,*]

Department of Biology, Allegheny College, Meadville, PA, USA

[#]contributed equally to writing this manuscript

*Corresponding author: mvenesky@gmail.com

Running title: Feeding Activity of Chytrid Infected Salamanders

ABSTRACT: Immune function is a costly line of defense against parasitism. When infected with a parasite, hosts frequently loose mass due to these costs. However, some infected hosts (e.g. highly resistant individuals) can clear infections with seemingly little fitness losses and few studies have tested how resistant hosts mitigate these costly immune defenses. We explored this topic using salamanders (*Plethodon cinereus*) and a fungal pathogen (*Batrachochytrium dendrobatidis*; "*Bd*"). *Bd* is generally lethal for amphibians and stereotypical symptoms of infection include loss in mass and deficits in feeding. However, individuals of *P. cinereus* can clear their *Bd* infections with seemingly few fitness costs. We conducted an experiment in which we repeatedly observed the feeding activity of *Bd*-infected and non-infected salamanders. We found that *Bd*-infected salamanders generally increased their feeding activity compared to non-infected salamanders. The fact that we did not observe any differences in mass change between the treatments suggests that increased feeding might help *Bd*-infected salamanders minimize the costs of an effective immune response.

KEY WORDS: amphibian, *Batrachochytrium dendrobatidis*, behavior, chytridiomycosis, disease, *Plethodon cinereus*, resistance

INTRODUCTION

The immune system is an effective line of defense against parasitism. However, the development and activation of an immune response can have substantial genetic (McKean et al. 2008) and physiological (Lochmiller & Deerenberg 2000) costs. Because resources are often limited, theory in ecological immunology predicts that organisms face an allocation trade-off between an investment in immune function and other functions such as growth and development (Schmid-Hempel 2003), physiological capacity (Martin et al. 2008, Ardia et al. 2012), and traits associated with mating success (Hasselquist & Nilsson 2012). One common type of trade-off comes in the form of "sickness behaviors" (e.g., lethargy, decreased libido, and lack of social interactions; Johnson 2002), which are thought to be adaptive behavioral responses that allow hosts to direct energy away from non-vital functions and to promote immune responsiveness. For example, male Song Sparrows (*Melospiza melodia*) whose immune system was activated via lipopolysaccharide injection exhibited increased lethargy and decreased territoriality during the non-breeding season compared to non-injected birds (Owen-Ashley & Wingfield 2006), presumably an adaptive reallocation of energy towards immune function. However, lethargy associated with sickness behaviors can result in deficits that are not always adaptive in respect to the host and thus favor parasite success (Thompson et al. 2005). For instance, parasitism can decrease host feeding behavior and efficiency (Gegear et al. 2006, Venesky et al. 2009), which can cause hosts to become nutritionally deprived and thus further reduce the strength of their immune responses (Gasparini et al. 2006, Brzek & Konarzewski 2007).

To offset the some of the costs associated with activating the immune system, some parasitized hosts consume more food compared to non-infected individuals, thereby limiting the

52 trade-offs between immune function and other physiological processes. This concept is
53 illustrated by observations that organisms with access to high quality food have more effective
54 immune responses compared to low quality food (Diamond & Kingsolver 2011) and that food
55 consumption is positively associated with immune system activity (Tyler et al. 2006). Moreover,
56 there is evidence that infected individuals selectively choose a diet that is high in macronutrients
57 and/or anti-parasite compounds (de Roode et al. 2013). For example, protein synthesis and amino
58 acid production increase significantly during immune activity (Lochmiller & Deerenberg 2000).
59 In support of this, Lee et al. (2008) found that a high protein diet increased immune function in
60 African Cotton Leafworms (*Spodoptera exempta*) relative to a low protein diet. In addition,
61 when given a choice between isocaloric diets that were rich in either protein or carbohydrates,
62 infected individuals of *S. exempta* increased protein consumption whereas non-infected
63 individuals did not alter their dietary choice (Povey et al. 2009).
64 Because food acquisition and feeding behavior have direct effects on an individual's
65 resource pool, determining how infected hosts mitigate the costs associated with immunity has
66 emerged as a central principle in the field of ecological immunology. Understanding how
67 individuals or species offset the costs of immunity is of no greater importance for amphibians,
68 whose populations are declining rapidly on a global scale (Wake & Vredenburg 2008).
69 Chytridiomycosis, caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis*
70 (*Bd*), is an emerging infectious disease that is associated with amphibian population declines
71 (Rohr & Raffel 2010). Symptoms of chytridiomycosis in amphibians generally include
72 stereotypical sickness behaviors (e.g. lethargy and inappetence) as well as irregular skin
73 sloughing and loss of righting reflex (Voyles et al. 2011). Despite the fact that *Bd* can cause high
74 mortality in amphibians, some individuals and species are resistant (or tolerant) to infection

(Venesky et al. 2014, Ellison et al. 2015, Rollins-Smith et al. 2015). Although *Bd* is known to evade components of the amphibian immune response (Fites et al. 2013), amphibians can deploy effective immune responses towards *Bd*. The first line of innate immune defenses against *Bd* is generally thought to come from antimicrobial peptides and other skin microbiota (Voyles et al. 2011, Bletz et al. 2013). Furthermore, as a result of sublethal X-irradation, frogs had reduced leukocyte numbers in the spleen associated with increased *Bd* infection intensity, which suggests that an adaptive lymphocyte response contributes to the control of *Bd* skin infections (Ramsey et al. 2010). In corroboration of Ramsey et al (2010), recent evidence demonstrates that frogs can acquire resistance to *Bd*, possibly mediated through changes in lymphocyte abundance and proliferation (McMahon et al. 2014).

To explore the relationship between feeding activity and *Bd* resistance, we conducted a laboratory experiment in which we compared the feeding behavior of *Bd*-infected and non-infected *P. cinereus* throughout the progression of an infection. Terrestrial plethodontid salamanders appear generally resistant to *Bd* (Muletz et al. 2014), lack stereotypical symptoms of chytridiomycosis, and individuals of the eastern red-backed salamander (*Plethodon cinereus*) can clear *Bd* infections in laboratory conditions thought to be ideal for growth of many *Bd* isolates (Becker & Harris 2010, Venesky et al. 2015). Because individuals of *P. cinereus* are relatively resistant to *Bd* and *Bd* resistance is presumably costly, we predicted that *Bd*-infected salamanders would attack and consume more fruit flies (*Drosophila melanogaster*) than non-infected salamanders. Because the costs of immune function should be greatest when the salamanders are infected with *Bd*, we also predicted that *Bd*-infected salamanders would increase their feeding activity early in the experiment (when they were still infected) relative to non-infected salamanders but that the *Bd*-infected and non-infected salamanders would not differ in their

feeding activity late in the experiment (after the *Bd*-infected salamanders cleared their infections).

MATERIALS AND METHODS

Salamander Collection and Husbandry

We hand collected adult red-striped *Plethodon cinereus* (n = 21 in May for Experiment 1; n = 24 in October for Experiment 2) from under rocks and logs from a single location in an oak/maple forested area in Crawford County, Pennsylvania, USA. Upon collection, the salamanders were transported to a laboratory and housed individually in vented plastic containers (75 mm deep, 110 mm diameter) on non-bleached paper towels soaked with 12 mL of aged and de-chlorinated tap water. Throughout the experiments, we provided the salamanders with fresh bedding and fed each salamander approximately 20 wingless fruit flies (*Drosophila melanogaster*) once per wk. Throughout the duration of the experiment, the salamanders were housed in an environmental chamber at 18.5°C with a 12:12 light:dark photoperiod. Exposure to *Bd* occurred within 2-4 wk. after the salamanders were collected.

In Experiment 1, we were interested in obtaining the rate at which salamanders could clear *Bd* and thus this experiment utilized only *Bd*-exposed salamanders. In Experiment 2, we were interested in comparing feeding behavior of *Bd*-infected and non-infected salamanders, so we randomly assigned the salamanders into one of two treatment groups: *Bd*-exposed (n = 12) and control (n = 12). We then weighed the salamanders (to the nearest 0.01g) prior to being exposed to the appropriate *Bd* treatment to confirm that the two treatment groups did not differ in

their mass (because larger salamanders might generally eat more food than smaller salamanders). At the start of Experiment 2, the mean mass of the salamanders in each treatment group were similar (*Bd*-exposed: 0.70 g ± 0.08, control: 0.74 g ± 0.05; mean ± SE).

Bd Exposure

Our *Bd* exposure protocols were similar for both experiments. *Bd* (JEL 660/JS OH-1, isolated from an infected amphibian in Ohio, USA) was grown in the laboratory in 1% tryptone broth. For salamanders in the *Bd*-exposed treatment, we pipetted a 5.0 mL dose of *Bd* that contained approximately 7×10^5 zoospores onto the dorsal surface of each salamander. Excess broth and zoospores were allowed to trickle onto the bedding of salamander's container. In Experiment 2, we also exposed salamanders from the control treatment to 5.0 mL of 1% tryptone broth that did not contain any *Bd* zoospores. In both experiments, we transferred each salamander from the container in which they were exposed to *Bd* (or a tryptone control) to a new container with fresh bedding (as described above) after 24 hrs.

Experiment 1: *Bd* clearance

Throughout the 28 d experiment, salamanders were maintained in the laboratory and exposed to *Bd* as described above. We swabbed the dorsal surface, including the tail, of each salamander 10 times with a sterile fine-tipped swab (product MW113; Advantage Bundling) on 5, 10, 17, and 28 d post exposure. We stored the swabs at -20°C until further analysis and

assessed the infection burden via qPCR (described below). Throughout this experiment, we monitored salamanders daily for mortality.

Experiment 2: Salamander Feeding and Mass Change

We observed salamander feeding behavior on 7, 14, and 28 d post-exposure. Prior to each behavioral observation, we randomized the order of the observations so that we did not confound treatment with time of day. The feeding observations were conducted blind such that the researcher did not know the treatment of the salamander that they were observing. The order of the observations was randomized prior to the start of each trial date, and the researcher did not necessarily observe the same salamander on each of the 3 trial dates.

Immediately prior to each observation, an individual salamander was removed from its container and placed in a 25 mm deep x 140 mm diameter Petri dish (hereafter "behavioral arena"). After a 3 min acclimation period, a researcher placed 10 wingless fruit flies in the behavioral arena, marking the start of a 10 minute trial. During the behavioral trial, we recorded the number of attacks (successful and unsuccessful) made on each fruit fly. Immediately following the behavioral trial, we weighed each salamander (to the nearest 0.01 g) and swabbed its dorsal surface, including the tail, 10 times with a sterile fine-tipped swab (product MW113; Advantage Bundling). We stored the swabs at -20°C until further analysis and assessed the infection burden via qPCR (described below). Throughout this experiment, we monitored salamanders daily for mortality.

To prevent cross-contamination with *Bd* or *Bd* DNA, experimenters used a different pair of nitrile gloves whenever they handled a salamander. In addition, we used a different behavioral

arena for each salamander. Throughout the experiment, we sterilized all of the equipment by either soaking it in 10% bleach or placing it in an autoclave (Johnson et al. 2003).

Detection of *Bd*

The number of genome equivalents on each swab was measured using quantitative polymerase chain reaction (qPCR) on an Applied Biosystems Step One Real-time PCR System (Applied Biosystems). Our DNA extractions and qPCR analyses followed the methods of Boyle et al. (2004) and those modified by Hyatt et al. (2007). Test samples were run singly instead of triplicate to control costs (Kriger et al. 2006). We added TaqMan® Exogenous Internal Positive Control (Exo IPC) Reagents (Applied Biosystems) to every reaction well to assess inhibition of the PCR reaction (Hyatt et al. 2007). The Exo IPC system uses a standardized concentration of an artificial DNA sequence that is added to each reaction well with its own set of primers and a separate fluorescent probe. The strength of this reaction is used to assess overall reaction inhibition.

We considered infection intensity as the number of *Bd* zoospore equivalents per sample. Zoospore equivalents were calculated by multiplying the genome equivalent values generated by the qPCR assay by 80, which accounts for the 80-fold dilution of DNA from the swabs during extraction and qPCR preparation. We considered a sample *Bd* positive when zoospore equivalents were ≥ 1.

Statistical Analyses

For Experiment 1, we used a linear mixed-effects model (R statistical software, package: 'lme4', function 'lmer') to test whether *Bd* abundance (log (*Bd* infection + 1), including animals with a zero infection) changed throughout the duration of the experiment as measured on the four swab dates. In this analysis, we nested salamander within trial to account for the repeated sampling of each salamander. We assessed significant differences ($p < 0.05$) using the 'Anova' function in the 'car' package in R statistical software.

For Experiment 2, we conducted two separate analyses to test how *Bd* affected salamander feeding. In our first analysis, we used a generalized linear mixed-effects model (R statistical software, package: 'lme4', function: 'glmer') with a Poisson error distribution to test for the main and interactive effects of treatment (*Bd*-exposed, control) and behavioral observation (Day 7, 14, and 28) on the number of attacks that each salamander made on fruit flies. In this analysis, we nested salamander within trial to account for the repeated observations on each salamander. We also used salamander mass as a covariate in this analysis. In our second analysis, we used a generalized linear mixed-effects model (R statistical software, package: 'lme4', function: 'glmer') with a binomial error distribution to test for the main and interactive effects of treatment (*Bd*-exposed, control) and behavioral observation (Day 7, 14, and 28) on the number of successful captures that each salamander made on fruit flies. To do this, we converted our dataset into binomial dataset such that each of the ten flies was given a value of "captured" or "not captured". In this analysis, we nested fly within trial which was nested within salamander. As with our first statistical model, we used salamander mass as a covariate. We assessed significant differences ($p < 0.05$) in both statistical models using the 'Anova' function in the 'car' package in R.

Next, we used a linear model to test whether *Bd*-exposed and control salamanders differed in the percentage of mass change by the end of the experiment. We treated pathogen treatment as a categorical predictor and arc sin transformed our percentage data. We conducted this analysis using the 'lm' function and assessed significant differences ($p < 0.05$) using the 'Anova' function in the 'car' package in R statistical software.

RESULTS

Experiment 1: *Bd* clearance

There was no salamander mortality in this experiment. *Bd* abundance changed significantly across the four swab dates ($\chi^2_3 = 165.99$, $p < 0.001$). One hundred percent of the salamanders tested positive for *Bd* on Day 5 and had an average *Bd* abundance of 23,446.44 (± 7,361.47) (Fig. 1). By Day 17, *Bd* prevalence decreased to 38% and the average *Bd* abundance was 174.61 (± 69.71) (Fig. 1). Upon termination of the experiment on Day 28, none of the salamanders tested positive for *Bd* (Fig. 1).

Experiment 2: Salamander Feeding and Mass Change

Salamanders that were infected with *Bd* attacked fruit flies significantly more often than non-exposed salamanders ($\chi^2_1 = 3.941$, $p = 0.047$; Fig. 2a). The variables of trial and mass, as well as the interaction between pathogen treatment and trial, did not vary between *Bd*-exposed and non-exposed salamanders (trial: $\chi^2_2 = 4.621$, $p = 0.099$; treatment x trial: $\chi^2_2 = 1.249$, $p =$

234 0.536; mass: $\chi^2_1 = 1.821$, p = 0.177). We observed a trend suggesting an increase in the number
235 of successful captures as a function of pathogen treatment and trial (Fig. 2b); however, these
236 results were all non-significant (treatment: $\chi^2_1 = 2.763$, p = 0.096; trial: $\chi^2_2 = 2.255$, p = 0.390;
237 treatment x trial: $\chi^2_2 = 1.320$, p = 0.573; mass: $\chi^2_1 = 0.642$, p = 0.423).

238 In addition to the effects of *Bd* on feeding, we found that *Bd* infection did not affect
239 survival (100% of the salamanders survived throughout the experiment) and *Bd* infection did not
240 affect salamander growth. There was no difference in the percentage of mass change between
241 *Bd*-infected and non-infected salamanders when comparing 28 day mass to the pre-experiment
242 mass ($F_{1,22} = 0.202$, p = 0.658; Fig. 3).

243

244 **DISCUSSION**

245

246 The results of our first experiment (Fig. 1) corroborate previous research demonstrating
247 that individuals of the eastern red-backed salamander (*Plethodon cinereus*) are generally
248 resistant to *Bd* and that they can clear their *Bd* infections within 28 d (Becker & Harris 2010) at a
249 temperature that is thought to be optimal for *Bd* growth in culture (Piotrowski et al. 2004). In
250 addition, we found evidence that *Bd*-infected salamanders increase their feeding activity (Fig. 2a)
251 and tended to increase their food consumption (Fig. 2b) relative to non-infected salamanders.

252 Deploying an immune response is generally energetically demanding and it frequently
253 involves an increase in an organism's resting metabolic rate (Sheldon & Verhulst 1996,
254 Lochmiller & Deerenberg 2000). One way to reduce the energy losses from the redistribution of
255 resources from anabolic daily processes (e.g. growth and reproduction) to immune deployment is
256 to increase feeding activity. Although some studies have shown that infected hosts generally

decrease their food intake (reviewed in Lochmiller & Deerenberg 2000), recent research has demonstrated that infected hosts can sometimes shift their feeding behavior so that they offset the costs associated with immune activation (Lee et al. 2006, Lee et al. 2008). Our result that *Bd*-infected salamanders attacked more flies than non-infected salamanders (Fig. 2a) and tended to capture more flies (Fig. 2b) supports the hypothesis that increasing feeding activity might limit the extent of the nutritional deficits associated with immune deployment. These results are also similar to a recent study that demonstrated that increased dietary protein enhanced *Bd* resistance in anuran tadpoles (Venesky et al. 2011). Understanding the energetic costs of immune maintenance and deployment in amphibians that are generally resistant versus those that succumb to *Bd* would help put these findings into a broader context and should be a focal point of future research in amphibian disease ecology.

Determining the exact immune parameters that confer *Bd* resistance in *P. cinereus* was beyond the scope of our study. For *P. cinereus* (and other species of amphibians), commensal skin bacteria work in association with components of the innate immune system (e.g. antimicrobial peptides) to reduce the infection burden of cutaneous pathogens such as *Bd* (Myers et al. 2012, Colombo et al. 2015). Recent research has highlighted the fact that the cutaneous microbial community found on some species of amphibians can minimize *Bd*-induced mortality and other sub-lethal effects of chytridiomycosis (reviewed in Bletz et al. 2013). For instance, in *P. cinereus* infected with *Bd*, salamanders with intact microbial communities suffered lower mass loss than salamanders stripped of their cutaneous bacteria (Becker & Harris 2010). However, the removal of commensal skin microbes, does not appear to affect *Bd* infection intensity and salamanders with reduced skin microbiota can still clear their *Bd* infections within 28 d after exposure (Becker & Harris 2010). The findings of Becker & Harris (2010) suggest that

components of the innate immune system can clear *Bd* infections independent of the skin microbiota. It is our hypothesis that salamander resistance is driven, in part, by antimicrobial peptides, which are known to confer resistance to *Bd* in some species (Woodhams et al. 2007). Antimicrobial peptide synthesis in vertebrates is either constitutive or inducible (Ganz 2003) and is presumably costly; increasing feeding activity during these times is a plausible host response that could offset the costs associated with this type of immune activity.

We also found that 100% of the *Bd*-infected salamanders cleared their infections while experiencing a similar change in mass compared to the non-exposed salamanders (Fig. 3). This finding is important because it further supports our hypothesis that increased feeding activity of *Bd*-infected salamanders relative to non-infected salamanders can be used to reduce any nutritional deficits that resulted from immune activity. Based on this logic, *Bd*-infected and food-deprived salamanders should not clear their infections as quickly and should exhibit mortality compared to *Bd*-infected salamanders that are given higher quantities of food. Alternatively, the immune defenses might not be costly enough in this species to affect mass because the infections are cleared in such a short duration (Fig. 1). Future experiments that test these hypotheses would help elucidate how food quantity relates to susceptibility to *Bd*.

Unlike most amphibian species which exhibit stereotypical pathologies during chytridiomycosis (e.g. mass loss, inappetence, lethargy), we found that *Bd*-infected *P. cinereus* are generally resistant to *Bd* and cleared moderate infection levels within 28 d post-exposure. During the experiment, *Bd*-infected salamanders generally increased their feeding activity compared to non-infected salamanders, and they did not lose any mass during the course of an infection. Although we did not detect a significant interaction between pathogen treatment and time, it appears that the significant main effect of pathogen treatment is being driven primarily

by salamander feeding responses earlier in the experiment compared to later in the experiment (i.e. Day 7 versus Day 28, as indicated by the error bars in Fig. 2). Although resistance to parasites is generally costly, our results suggest that increased feeding might help *Bd*-infected salamanders minimize the costs of an effective immune response. These results provide an opportunity to improve our understanding of the relationship between host condition and *Bd* resistance and offer hope for amphibian management programs.

Although *Bd* is widely regarded as one of the deadliest pathogens on the planet (Rohr et al. 2008), not all amphibians are equally susceptible to *Bd* (Searle et al. 2011b), and differences in host resistance are particularly important because this variation can affect disease outcomes in amphibian communities (Searle et al. 2011a, Venesky et al. 2014). Our results, coupled with the evidence that field collected plethodontid salamanders rarely test positive for *Bd* from natural environments (Muletz et al. 2014), suggest that *Plethodon* spp. have traits that promote resistance to *Bd*. From an ecological perspective, knowing whether *Plethodon* spp. generally dilute disease risk for other amphibian species might help us understand regional patterns of *Bd*. The incidence of *Bd* in the United States is generally low in regions where there is high plethodontid salamander diversity (Olson et al. 2013), which supports the possibility that *Plethodon* spp. generally dilute *Bd* risk. Nonetheless, many other biotic and abiotic factors that are known to affect the distribution of *Bd* (Liu et al. 2013, Venesky et al. 2014) could be correlated with the geographic distribution of *Plethodon* spp., further emphasizing the need to understand whether salamanders dilute the risk of chytridiomycosis in natural settings.

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Figures and Figure Legends

Fig. 1. *Batrachochytrium dendrobatidis* ("*Bd*") abundance (left y-axis and circles) and prevalence (right y-axis and squares) of the eastern red-backed salamander (*Plethodon cinereus*) throughout the course of the experiment. *Bd* was detected using qPCR and swabs of the salamanders were obtained 5, 10, 17, and 28 days post exposure. By 17 days post exposure, the *Bd* abundance and prevalence decreased by more than 50% of their original values. 100% of the salamanders cleared their *Bd* infection by Day 28.

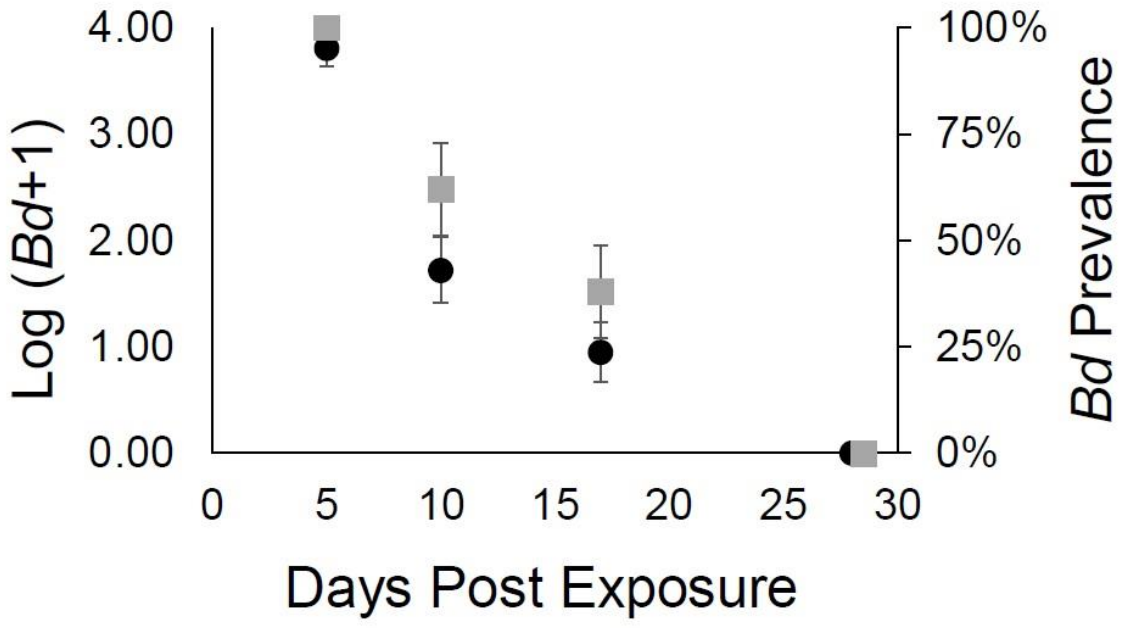

Fig. 2. Treatment differences in the number of fruit flies attacked (a) and captured (b) between eastern red-backed salamanders (*Plethodon cinereus*) exposed and non-exposed to *Batrachochytrium dendrobatidis* ("*Bd*"). Salamanders exposed to *Bd* attacked significantly more flies ($p = 0.047$) and tended to capture more flies ($p = 0.096$) compared to non-exposed salamanders, irrespective of the date of the behavioral trial. Error bars indicate 1 SE.

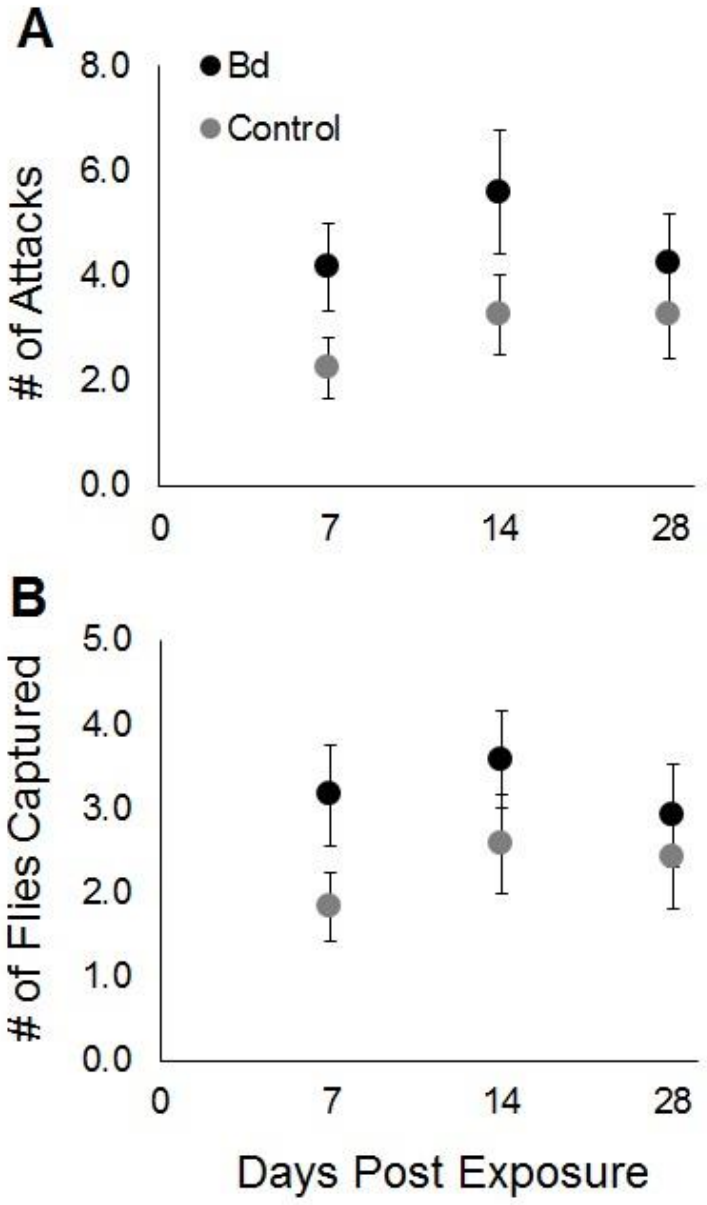

Fig. 3. The percentage of mass change (Day 0 versus Day 28 mass) between eastern red-backed salamanders (*Plethodon cinereus*) exposed and non-exposed to *Batrachochytrium dendrobatidis* ("*Bd*"). Error bars indicate 1 SE. There was no statistically significant difference between the two treatment groups (p = 0.658).