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Female Salamanders Experience Higher Parasitism Compared to Males: A Cost of Female Reproduction?

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ABSTRACT.—**Males tend to experience higher rates of parasitism compared to females, a phenomenon associated with ecological factors, the fact that males engage in risky behaviors, and because testosterone is known to be immunosuppressive. However, females could experience higher rates of parasitism if energy is allocated from costly immune responses towards producing eggs. We used pooled data sets from laboratory experiments to investigate sex-specific differences in salamander (*Plethodon cinereus*) resistance to the emerging fungal pathogen *Batrachochytrium dendrobatidis* ("*Bd*"). Contrary to our predictions, we found that female salamanders had a higher prevalence of infection (∼56%) and carried a higher *Bd* infection burden (455 zoospores equivalents per sample) compared to male salamanders (which had a *Bd* infection prevalence of ∼24% and an average infection burden of 58 zoospore equivalents per sample). We also found that female reproductive investment (i.e., mass of eggs) positively correlated with *Bd* infection burden, suggesting that females who previously invested more into reproduction carried a higher *Bd* infection burden. Collectively, our findings might indicate that female salamanders experience a cost of reproduction in the form of decreased disease resistance.**

Male vertebrates frequently experience higher rates of parasitism compared to females (Poulin, 1996), a pattern thought to exist because of ecological and hormonal differences between the sexes (Zuk and McKean, 1996). For example, males of some species of amphibians arrive to breeding sites earlier and spend more time in the water than do females, increasing their exposure to aquatic parasites (Tinsley, 1989). Males are also known to have a larger home range size than females (Perry and Garland, 2002; Dahle and Swenson, 2003), presumably allowing males to maximize mating opportunities but also possibly increasing their exposure to parasites. In conjunction with these ecological differences, androgens are known to be immunosuppressive (Mougeot et al., 2004; Oppliger et al., 2004) and testosterone can downregulate the activity of natural killer cells, macrophages, and antibody production (Klein, 2004). For instance, captive and free-living male Dark-Eyed Juncos (*Junco hyemalis*) treated with testosterone implants experience reduced cell-mediated and humoral immune responses, as measured by skin swelling responses to phytohemagglutinin and antibody production to sheep red blood cells (Casto et al., 2001).

The negative association between testosterone and immunity, coupled with the stimulatory effect that testosterone has on the development of male sexual characteristics, has led researchers to speculate that males could experience a trade-off between reproduction and immunity. Indeed, Folstad and Karter (1992) posited that only the males with the highest genetic quality are able to express their sexual characteristics fully without experiencing costly fitness-reducing effects of parasitism (i.e., the immunocompetence handicap hypothesis). Despite the generality of the immunocompetence handicap hypothesis, it is not always supported (reviewed in Roberts et al., 2004) and males do not always carry higher parasite burdens compared to females. Females can experience costs of reproduction that are manifested in reduced locomotor performance (Veasey et al., 2001), increased metabolism (Angilletta and Sears, 2000), and increased levels of stress hormones (Moore and Jessop, 2003). Life-history theory predicts that trade-offs between energetically costly functions such as reproduction and immunity occur because organisms differentially allocate limited energetic resources (Stearns, 1992). Thus, reproductively active females of some species experience reduced immune function and can thus carry high parasite burdens (Hanssen et al., 2005; Christe et al., 2012).

Understanding the ways in which females and males respond to pathogens might have no greater importance than for amphibians. Amphibians are the most threatened vertebrate taxon on the planet and their population declines are a driving force behind the current biodiversity crisis (Wake and Vredenburg, 2008). Of particular concern is an amphibian chytrid fungus, *Batrachochytrium dendrobatidis* ("*Bd*"), an emerging pathogen that causes amphibian chytridiomycosis. *Bd* has a near global distribution, infects many species of amphibians, and is associated with high amphibian mortality rates; thus, *Bd* is implicated in global amphibian population declines (Fisher et al., 2009). Opportunistic field sampling of *Bd* in amphibians has yielded conflicting results in relation to any sex bias in patterns of infection. In a field survey in Maine, USA, Longcore et al. (2007) sampled 390 adults from seven anuran species and did not find any differences in the percent infected between the sexes. In contrast, sampling of 379 field-collected adult anurans in Connecticut, USA revealed that female frogs had a higher probability of being infected with *Bd* compared to male frogs (Richards-Hrdlicka et al., 2013). Yet, in northern Queensland, Australia, male frogs from the genus *Litoria* had higher probabilities of being infected in the field compared to females (Rowley and Alford, 2013). When considering salamanders, a field survey of 86 adults from nine salamander species revealed that male and female salamanders did not differ in their prevalence of infection (Richards-Hrdlicka et al., 2013).

Understanding how male and female amphibians respond to *Bd* could have important implications for amphibian conservation. For example, if male amphibians are more susceptible to *Bd*, sex differences in patterns of mortality could exacerbate existing female-biased sex ratios that have resulted from endocrine-disrupting agrochemicals (Hayes et al., 2003). How-

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ever, there is no clear pattern whether male and female amphibians differ in their response to *Bd*. We addressed this inconsistency in the literature by using data sets from a series of laboratory experiments in which male and female Eastern Red-Backed Salamanders (*Plethodon cinereus*) were exposed to *Bd*. We chose *P. cinereus* as a disease model for a number of reasons: (1) despite being fully terrestrial, adults of this species have been found with *Bd* infections in nature (Richards-Hrdlicka et al., 2013); (2) it is very similar ecologically to a number of congeners that are in decline (e.g., *Plethodon shenandoah*, *Plethodon hubrichti*, *Plethodon nettingi*); and (3) the socioecology of *P. cinereus* is well described (Jaeger et al., 2016), allowing us to interpret our results in the context of energy expenditures made by each sex. We used our pooled data set to test whether males or females differ in their resistance to *Bd* by evaluating *Bd* prevalence and infection abundance. If male salamanders experience immunosuppressive effects of testosterone, we predicted that males should have higher infection prevalence and abundance compared to females. If females experience a cost associated with reproduction, we predicted that females should have higher metrics of infection as measured by *Bd* prevalence and abundance compared to males. We then conducted an experiment in which we tested for an association between reproductive effort (as measured by total egg mass) and *Bd* resistance in female salamanders.

Materials and Methods

Salamander Collection and Husbandry.—One hundred forty-six adult *Plethodon cinereus* (female, $n = 84$; male, $n = 62$) were field collected from Pennsylvania and Ohio, USA during their breeding season between 2013 and 2017 and used in six independent experiments. The salamanders used in data sets 1–4 were collected in Summit County, Ohio, USA (41.228022; −81.51607). The salamanders used in data sets 5–6 were collected in Crawford County, Pennsylvania, USA (41.65395; −80.14896). All salamanders were collected by hand under rocks and logs from forested areas. Both the Ohio and Pennsylvania populations are genetically similar (Cameron et al., 2019) and belong to the same mitochondrial clade (Radomski, 2017). We used a combination of external morphology (e.g., the presence of prominent nasolabial cirri in males; Anthony and Pfingsten, 2013), dissections, visual examination of eggs through the ventral body wall, and laboratory oviposition to determine sex. See the Electronic Supplementary Material for specific details related to each of the six experiments (Table S1).

Data set 4 came from an experiment that was conducted at John Carroll University (Ohio, USA) and all the other data sets came from experiments were conducted at Allegheny College (Pennsylvania, USA). During these experiments, salamanders were housed individually in vented plastic containers (75 mm deep, 110 mm diameter) on nonbleached paper towels soaked with approximately 12 mL of aged and de-chlorinated tap water. Throughout the experiments, we provided the salamanders with paper towel substrates and we fed each salamander approximately 25 wingless fruit flies (*Drosophila melanogaster*) that were dusted with Rep-Cal Herptivite Multivitamin on a weekly basis. All salamanders were housed at 18°C (±1°C) under a 12:12 light:dark photoperiod during the experiments.

Exposure to Batrachochytrium dendrobatidis.—After being acclimated to laboratory conditions, salamanders were exposed to *Bd*. *Bd* (JEL 660/JS OH-1, isolated from an infected amphibian in Ohio, USA) was grown in the laboratory in 1% tryptone broth or on 1% tryptone agar plates. In the experiments using broth, broth containing *Bd* zoospores were pipetted directly onto the dorsum of each salamander and the excess broth was allowed to trickle into the bedding. In the experiments using agar plates, each plate was flooded with ~15 mL of aged tap water and the zoospores that emerged from the zoosporangia were harvested after approximately 45 min. This inoculate was then pipetted onto the dorsum of each salamander as described above. Experiments 1, 5, and 6 used 1.0×10^6 zoospores. Salamanders were exposed to *Bd* for 24 or 48 h. Experiment 2 used a total of 5.5×10^5 zoospores. Experiment 3 used 6.0×10^5 zoospores. The salamanders in Experiment 4 were exposed to *Bd* in two experimental blocks over two consecutive days; the first used 1.6×10^6 zoospores and the second used 1.2×10^6 zoospores. After the exposure period, salamanders were placed in new containers with *Bd*-free bedding to eliminate the possibility of detecting remnant DNA of *Bd* during swabbing (see below). After this initial bedding change, the bedding was changed weekly. These doses and concentrations are within the range of doses that have been previously used to cause infections in individuals of this species (Hess et al., 2015; Venesky et al., 2015).

Swabbing, DNA Extractions, and qPCR.—Individuals of this species are relatively resistant to *Bd* and can clear their infections within 28 d postexposure (Hess et al., 2015). Thus, we swabbed the entire dorsal surface of each salamander (a total of 10 times in Experiments 1, 2, 3, 5, and 6 and a total of 15 times in Experiment 4) with a sterile fine-tipped swab (Advantage Bundling) 7–10 d postexposure (salamanders from experiments 4 and 6 were swabbed 7 d post exposure; salamanders from experiments 1, 2, 3, and 5 were swabbed 10 d postexposure). To prevent cross-contamination with *Bd* or *Bd* DNA, the experimenter used a different pair of latex gloves while handing each salamander. Swabs were frozen at −20°C until DNA extractions were completed. We extracted DNA from each swab using a PrepMan Ultra (ThermoFisher Scientific).

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, Foster City, California, USA). Our DNA extractions and qPCR analyses followed the methods of Boyle et al. (2004) and those modified by Hyatt et al. (2007). Our qPCR protocol uses an activation stage of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Test samples were run singly instead of in 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 a synthesized 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. None of our samples were inhibited and thus did not require any further dilution. We used the number of *Bd* zoospore equivalents per swab as an estimate of infection intensity. Zoospore equivalents per swab 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 DNA extraction and qPCR preparation. We considered a sample *Bd* positive when zoospore equivalents were ≥ 1.

Salamander Egg Mass.—In addition to the above, our final experiment (Experiment 6) used exclusively gravid female salamanders and tested for a trade-off between previous

TABLE 1. Summary statistics for the results of the statistical models on *Batrachochytrium dendrobatidis* ("*Bd*") prevalence and *Bd* abundance in *Plethodon cinereus*.

Response	χ^2 statistic	df	*P*
Bd prevalence			
Sex	4.260	1	0.039
Mass	2.282	1	0.131
Experiment	18.040	5	0.003
Color	0.125	1	0.723
Response	*F*	df	*P*
Bd abundance			
Sex	5.189	1	0.019
Mass	0.484	1	0.470
Experiment	62.000	5	<0.001
Color	0.921	1	0.320

investment in reproduction and *Bd* resistance. After assessing *Bd* infection burden, we first weighed each female and then dissected and weighed the eggs of the salamanders. We used a topical application of benzocaine to anesthetize and euthanize each salamander. Eggs were removed under a dissecting scope and placed in a plastic weigh dish with a drop of deionized water. Excess fat was carefully removed from the surface of the eggs with forceps and iridectomy scissors. The eggs were dried by gently rolling them on a KimWipe® and then they were placed in a second, pre-tared, plastic weigh dish. We recorded the number of eggs dissected from each salamander and also the mass (to the nearest 0.001 g) of the entire clutch of eggs dissected from each female, each of which was used as a proxy for investment in reproduction.

Statistical Analyses.—There are three parameters frequently used to quantify parasite infection: prevalence, infection intensity, and infection abundance. Upon exposure to a parasite, individual hosts will become either infected or remain noninfected (the percentage of hosts exposed to a parasite that get infected is termed "prevalence"). Hosts that get infected carry a parasite burden (termed "infection intensity," which is measured by the number of parasites on the exposed and infected individuals). Infection abundance unifies the parameters of prevalence and infection intensity because it measures the number of parasites found in all hosts that were pathogen-exposed, including the zero values of the hosts that were exposed to but not infected with a parasite. Because plethodontid salamanders are relatively resistant to *Bd* (Hess et al., 2015; Fonner et al., 2017), many of our salamanders had an infection value of "0"; thus we had limited statistical power to analyze infection intensity. As such, we assessed sex-specific differences in resistance by examining *Bd* prevalence (i.e., the percentage of exposed salamanders that were infected) and *Bd* infection abundance (i.e., the number of *Bd* zoospore equivalents of the salamanders exposed to *Bd*, including those whose value was "0"). We were interested in testing for sex differences in *Bd* resistance; thus we did not need a nonexposed "control" group because the salamander infection burden would be "0" (*Bd* has not been documented in individuals of *P. cinereus* from either collection locality used in this study). All statistical tests were performed using R version 3.4.2 (R, 2017).

To test for differences in *Bd* prevalence, we used a generalized linear model ('glm function') with a binomial error distribution because salamanders were either infected or not infected. We considered sex a categorical predictor and used salamander mass on the swab date as a covariate. We also included the categorical variable of color morphology (striped or unstriped) as a covariate because color morphs differ in their *Bd* resistance (Venesky et al., 2015). Lastly, we considered each data set as a temporal block in the analysis to control for the subtle differences between the experimental procedures that produced each data set statistically (see above). To test for differences in infection abundance, we used a linear model ('lm' function) with the same predictor variables described above. Unlike above, we log-transformed our response variable (*Bd* infection + 1) for normality. Because some of the experiments included only a single sex, we conducted two additional analyses using a trimmed data set of experiments that used both sexes. The details of these statistical procedures can be found in Appendix 2. In our final statistical models, we used a linear model ('lm' function) to test whether the total number of eggs per salamander or log-transformed continuous variables egg mass were significant predictors of log-transformed the *Bd* infection intensity of only the infected female salamanders from Experiment 6 (i.e., we excluded the salamanders that were exposed to, but not infected with, *Bd*). We also included salamander mass as a covariate in each statistical model. Significant differences ($P < 0.05$) were determined using the 'Anova' function in the 'car' package for each of the statistical analyses described above.

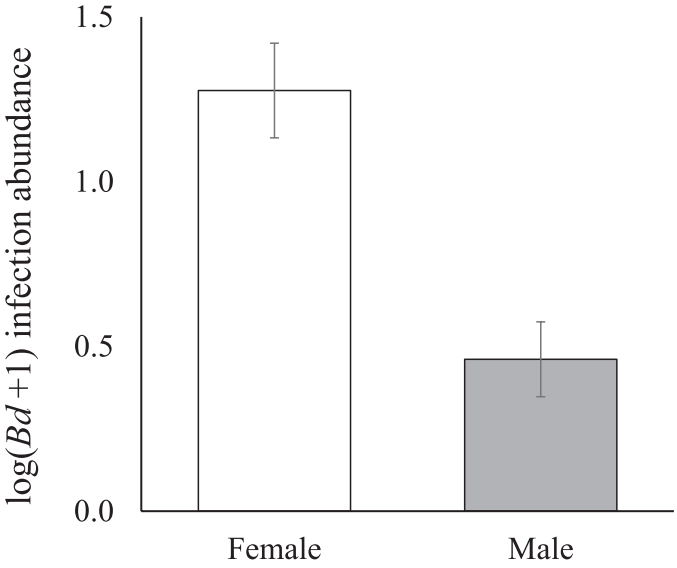

FIG. 1. *Batrachochytrium dendrobatidis* ("*Bd*") infection abundance of female and male *Plethodon cinereus*. Values are presented as the mean of the log-transformed (*Bd* zoospore equivalent +1) for each sex (calculated as the grand mean from all individuals used). Error bars are (±) are 1 standard error.

RESULTS

All the salamanders survived the duration of each experiment. Females had a significantly higher prevalence of infection (55.95%) compared to male infection prevalence (24.19%; Table 1). Similarly, infected females had a significantly higher *Bd* infection abundance compared to males (Table 1). Average *Bd* infection abundance on females was approximately 455 zoospores but was approximately 58 zoospores on males (Fig. 1). It is important to note that sex was a significant predictor of both infection metrics even after statistically controlling for the experimental block. These results are similar to those obtained from the trimmed data set (Appendix 2).

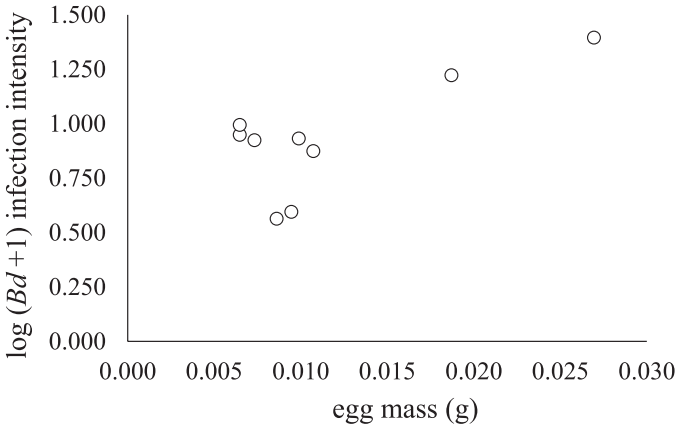

FIG. 2. The relationship between *Batrachochytrium dendrobatidis* ("*Bd*") infection intensity (log-transformed) and egg mass of female *Plethodon cinereus* exposed to, and infected with, *Bd*.

In our final experiment in which we used only gravid females (Experiment 6), 9 of the 21 salamanders exposed to *Bd* were infected (42.9%). Of those infected, total egg mass was a significant positive predictor of *Bd* infection intensity ($F_{1,6} = 10.485$, $P = 0.018$), indicating that *Bd* infection intensity increased with salamander egg mass (Fig. 2). Total number of eggs did not predict infection intensity ($F_{1,6} = 0.5311$, $P = 0.494$). The covariate of salamander mass was not a significant predictor of *Bd* infection intensity in either statistical model (egg mass $P = 0.214$; egg number $P = 0.908$).

DISCUSSION

Contrary to much of the literature that reports a male bias in disease prevalence (Poulin, 1996; Schalk and Forbes, 1997; Sheridan et al., 2000), including salamanders (Anthony et al., 1994) and our study species (Davis et al., 2009), we found that female salamanders have poor resistance to *Bd*. The prevalence of infection in female salamanders was approximately double that of males, and females carried an average infection abundance that was approximately eightfold higher than did males (Fig. 1). Although we did not directly measure any immune parameters, our data suggest that male salamanders are not immunosuppressed compared to female salamanders.

There are two plausible hypotheses that could explain why males do not seem to suffer any immunosuppressive effects of testosterone. The first is related to amphibian skin defenses against *Bd*. Individuals of *P. cinereus* are thought to utilize antifungal metabolites (Becker et al., 2009) produced from the mutualistic skin bacterium *Janthinobacterium lividum* to prevent *Bd* from infecting them (Brucker et al., 2008). If individuals of *P. cinereus* utilize the antifungal metabolites or antimicrobial peptides (Rollins-Smith et al., 2002) as a first line of defense, they might only use cellular or humoral components of the amphibian immune system (McMahon et al., 2014) to clear the relatively few *Bd* zoospores remaining. As such, any negative effect of testosterone on the immune system of male salamanders would likely be diminished. The second hypothesis is that testosterone does not downregulate immune activity in plethodontid salamanders as it does in with other amphibians (Crespi et al., 2015). In a recent study, Thomas and Woodley (2017) experimentally increased the levels of circulating testosterone in male Allegheny Dusky Salamanders (*Desmognathus ochrophaeus*; a salamander in the same family as our study organism) and found that testosterone did not affect salamander wound healing (a proxy for immunocompetence). Their data, coupled with our results, might indicate that the association between testosterone and immunity in this clade of salamanders is not strong. Future studies that manipulate testosterone levels and expose multiple amphibian species to *Bd* (and other pathogens) are needed to test the generality of our findings.

This begs the question of why females have poor resistance to *Bd*. Eggs are presumably costly to produce (Trivers, 1972); thus gravid females (or females who have recently laid eggs) should have low energetic reserves compared to females in nonbreeding condition (Carey, 1996). In *P. cinereus*, egg brooding is necessary for offspring survival (Highton, 1960), is energetically expensive (Yurewicz and Wilbur, 2004), and females forgo feeding during the brooding period (Ng and Wilbur, 1995). The female salamanders used in our experiments were either gravid or recently oviposited and may have had fewer available resources for immune responses because they previously allocated limited energetic resources towards reproduction. The results from Experiment 6, in which we found a significant and positive relationship between reproductive investment (i.e., females with a larger total mass of eggs) and *Bd* infection intensity, reinforces the notion that egg production is costly to females. Future research that measures metabolic costs of egg production and immune responses, and their possible interaction, would help us understand energetic trade-offs in salamanders.

Although we present multiple lines of evidence that demonstrate female salamanders have poorer resistance to *Bd* compared to male salamanders, our pooled data set combined projects that differed in collection dates, time in the laboratory, *Bd* exposure doses, and even whether male and female salamanders were both used in the same experiment (Table S1). Given these differences, it is not surprising that the experimental blocking factor we used in our statistical models was a significant predictor of both *Bd* infection metrics (Table 1). Although we cannot rule out the fact that any of these factors (e.g., the duration of time in the lab before being used in an experiment) affected our results, sex was still a significant predictor of both metrics of *Bd* infection despite the significant effect of the experimental block. When we trimmed our data set and analyzed the experiments in which male and female salamanders were both used in the same experiment (Experiments 2–4), we found support for the hypothesis that females have a higher *Bd* infection than males (Appendix 2). Moreover, the statistical support for a significant experimental blocking factor was either eliminated or reduced (Appendix 2), despite the fact that they were exposed to different concentrations of *Bd* and swabbed for *Bd* 7 or 10 d postexposure. Collectively, this suggests that the highly significant effect of experimental block in our primary statistical analyses reflected the fact that some experiments used either sex whereas other experiments used both sexes.

In species with complex social behaviors such as *P. cinereus* (Jaeger et al., 2016), females bear additional costs that may contribute to their poor resistance to *Bd*. For example, unlike other species of salamanders, females of *P. cinereus* are about equally likely to initiate courtship and engage in high-energy behaviors during the early courtship stages (Dyal, 2006). *Plethodon cinereus* is territorial, but it is not just males that engage in aggressive contests; females use aggressive behavior to codefend territories with males (Lang and Jaeger, 2000). *Plethodon cinereus* may present a special case where reproductive

costs beyond egg production may leave females especially vulnerable to energetic deficits, and this may result in decreased resistance to *Bd*. As such, males of *P. cinereus* (and perhaps other species of plethodontid salamanders) might not have higher parasite burdens compared to females. Our study highlights the importance of considering life history traits such as parental care and social behaviors when making broad generalizations about the effects of sex on disease resistance.

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APPENDIX 2

A description of the statistical models, and results, from the trimmed data set that included experiments in which both sexes were tested simultaneously (i.e., Experiments 2, 3, and 4).

We trimmed our full data set to include data from Experiments 2, 3, and 4. We tested for differences in *Bd* prevalence by using a generalized liner model ('glm function') with a binomial error distribution. We considered sex a categorical predictor and used salamander mass on the swab date as a covariate. We also included the categorical variable of color morphology (striped or unstriped) as a covariate because color morphs differ in their *Bd* resistance (Venesky et al., 2015). Lastly, we considered each experiment as a temporal block in the analysis to control for the subtle differences between the experiments statistically (see Appendix 1). To test for differences in infection abundance, we used a linear model ('lm' function) with the same predictor variables described above. Unlike above, we log-transformed our response variable (*Bd* infection + 1) for normality.

The results of these analyses are qualitatively similar to the global analyses that we presented in the main text of the manuscript. Female salamanders had a higher prevalence of infection compared to male salamander infection prevalence (female: 50.0%; male: 29.0%; $P = 0.056$). Similarly, infected female salamanders carried more than 2× the infection abundance than males: average *Bd* infection abundance on females was approximately 288 zoospores but was approximately 108 zoospores on males ($P = 0.054$). In the analysis for prevalence, the experimental block was no longer statistically significant ($P = 0.309$), but $P = 0.051$ for the experimental block in the analysis for infection abundance.

APPENDIX 1. A summary of the collection dates, sample sizes, method of sexing each salamander, and the sex ratios of the *Plethodon cinereus* used in the six experiments presented in this manuscript.

Experiment	Sex	*n*	Month and year collected	Date used in experiment	Method of sexing
1	Female	23	Apr 2013	Feb 2014	Females oviposited in the lab
2	Female and male	Female = 11; male = 8	Oct 2014	Jan–Feb 2015	Nasolabial cirri, appearance of snout, and presence of eggs
3	Female and male	Female = 13; male = 8	Apr 2015	June 2015	Nasolabial cirri, appearance of snout, and presence of eggs
4	Female and male	Female = 16; male = 15	Sept–Oct 2016	Jul–Aug 2017	Nasolabial cirri, appearance of snout, and presence of eggs
5	Male	31	Oct 2015	Oct–Nov 2015	Dissection looking for presence of testes and absence of eggs
6	Female	21	Oct 2016	Nov 2016	Dissection and presence of eggs