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Original Contribution

Fitness Consequences of Infection by *Batrachochytrium dendrobatidis* in Northern Leopard Frogs (*Lithobates pipiens*)

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Abstract: The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been linked to amphibian declines and extinctions worldwide. The pathogen has been found on amphibians throughout eastern North America, but has not been associated with mass die-offs in this region. In this study, we conducted laboratory experiments on the effects of *Bd* infection in a putative carrier species, *Lithobates pipiens*, using two estimators of fitness: jumping performance and testes morphology. Over the 8-week study period, peak acceleration during jumping was not significantly different between infected and uninfected animals. Peak velocity, however, was significantly lower for infected animals after 8 weeks. Two measures of sperm production, germinal epithelium depth, and maximum spermatic cyst diameter, showed no difference between infected and uninfected animals. The width, but not length, of testes of infected animals was significantly greater than in uninfected animals. This study is the first to show effects on whole-organism performance of *Bd* infection in post-metamorphic amphibians, and may have important long-term, evolutionary implications for amphibian populations co-existing with *Bd* infection.

Keywords: *Batrachochytrium dendrobatidis*, chytrid fungus, fitness, northern leopard frog, *Lithobates pipiens*, whole-organism performance

Introduction

Pathogens are important and ubiquitous drivers of mortality in both plants and animals. Chytridiomycosis is a recently discovered, globally important, emerging amphibian disease that is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). In some parts of the world this pathogen has caused mass die-offs and even extinctions (Lips et al. 2005; Skerratt et al. 2007), whereas in others amphibians are heavily infected but show no acute signs of disease (Rothermel et al. 2008; Kinney et al. 2011). Amphibians in eastern North America fall into the latter category, where infection prevalence may be high (Longcore et al. 2007; Rothermel et al. 2008; Gaertner et al. 2009; Kinney et al. 2011; Chatfield et al. 2012) yet mass die-offs attributable to chytridiomycosis have not been observed. Acute infections and death have obvious fitness consequences for amphibians; however, sublethal and chronic infections may have important fitness consequences as well.

Total fitness is most clearly defined as the number of descendants produced by an individual relative to the average produced by other individuals in a population (Hunt et al.

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2004), and is affected by overall selection summed over several selective contexts (Hunt et al. 2009; Lailvaux and Kasumovic 2011). As such, understanding the effects of a selective factor such as a pathogen on a given trait or traits in each of these broad contexts (e.g., natural selection versus sexual selection) is necessary if we are to understand how that factor ultimately affects total fitness. A good candidate for a trait that is both linked to fitness and shaped by multiple selective contexts is locomotor performance, such as running, jumping or swimming (Arnold 1983; Ghalambor et al. 2003). Locomotory ability correlates positively with hunting success, escape from predators (Christian and Tracy 1981; Trombulak 1989), dispersal (Phillips et al. 2006), and mating success in several vertebrate species, the latter specifically through achieving victory in male combat via speed or agility (Able 1999; reviewed in Lailvaux and Irschick 2006; Husak and Fox 2008). Locomotor performance has also previously been used as an indicator of the health of an individual (Holem et al. 2006; Richard et al. 2010; but see Vervust et al. 2008), and declines prior to signs of morbidity or mortality associated with infection (Goater et al. 1993; Oppliger et al. 1996). Similarly, testis size, germinal epithelium depth, and spermatic cyst diameter are closely tied to reproductive success in several vertebrate taxa, and collectively may be used as a proxy for reproductive fitness among males (McCallum and Trauth, 2007). Together, locomotor performance and testis morphology therefore broadly capture different determinants of total fitness, and differences in these characteristics between infected and uninfected animals may thus reveal sub-lethal consequences of infection that are important to the long-term health of populations.

Populations of northern leopard frogs (*Lithobates pipiens*, formerly *Rana pipiens*) underwent declines in the 1970s along with other North American species, prompting Carey et al. (1999) to suggest that *Bd* was the causative agent of those declines. More recently, it has been suggested that *L. pipiens* may be a "carrier" species (Woodhams et al. 2008; Gahl et al. 2011), as it is currently not known to exhibit acute symptoms or mortality when infected by *Bd*. In a recent study of an endangered population of *L. pipiens* in British Columbia, *Bd* is thought to have swept through in an epidemic fashion leading to rapid declines and endangerment of that population (Voordouw et al. 2010). Although that population now appears to be coexisting with *Bd*, coexistence does not mean that the disease is not affecting fitness.

The goal of this laboratory study was to investigate the sublethal effects of *Bd* infection on the total potential fitness of *L. pipiens*, a species that is known to harbor *Bd* infections but that usually no longer succumbs to acute chytridiomycosis. To accomplish this goal, we incorporated measures of both components of organismal fitness—survival and reproduction. Specifically, we compared jumping performance and testis morphology (gross dimensions, germinal epithelium depth, and spermatic cyst diameter) between infected and uninfected male *L. pipiens* over a period of 8 weeks.

Methods

Study Subjects

Adult, male *L. pipiens* ($N = 36$) were acquired from Connecticut Valley Biological Supply Company (Southampton, MA). Upon receipt, frogs were uniquely color-marked using elastomer tags, weighed, measured and swabbed for the presence of *Bd* (described below). Snout–vent length (SVL) and left hind limb length were measured to the nearest 0.1 mm. All frogs were housed individually for 7 days in plastic tanks measuring 36 × 21 × 16 cm and filled with approximately 1 L of filtered tap water. During this period, swabs were analyzed via quantitative PCR (details below) to determine which, if any, of the animals arrived with a *Bd* infection. After the 7-day quarantine period, animals were housed communally in either the treatment ($N = 18$) or control ($N = 18$) tank at 16°C. Individuals that arrived with an infection were automatically assigned to the treatment group. Communal tanks measured 55 × 35 × 34 cm and were filled with approximately 10 L of filtered tap water. Except during inoculation periods, a dry perch was provided so that animals could freely exit the water.

The experiment was conducted over an 8-week period from June to August 2011. During this time, individuals were swabbed, weighed, measured and "jumped" (procedure described below) once a week. Animals from both tanks experienced high mortality through the first month of the project, presumably due to stress associated with shipment and captivity. For this reason, our statistical analyses include only those animals that survived until the end of the experiment (treatment, $N = 10$; control, $N = 10$). For this same reason, we limited our locomotory analyses to measurements taken during weeks 4–8 of the experiment.

Inoculations

Following the 7-day quarantine period, frogs in the treatment group were inoculated with *Bd* three times over a 5-day period by placing them in a bath containing *Bd*

zoospores (strain JEL423 obtained from J. Longcore, University of Maine). In brief, fungal cultures were grown on a tryptone-agar medium at 23°C for 5, 7, and 9 days for inoculations 1, 2, and 3, respectively (Table 1). Zoospores were harvested by flooding culture plates with 5 mL of 20% Holtfreter's solution for ten minutes. The number of zoospores per inoculation was estimated using a hemacytometer. The prepared inoculum was diluted in 3.25 L of 20% Holtfreter's solution. In each inoculation, animals remained in the zoospore bath for approximately 48 h. A control solution was prepared using identical tryptone-agar plates that had not been inoculated with *Bd*, and diluted in 3.25 L in 20% Holtfreter's solution. Control animals were bathed in this solution in a manner analogous to that of the treatment animals.

Since *L. pipiens* has been known to clear infection (Voordouw et al. 2010; Paetow et al. 2012), second and third rounds of inoculations were performed at weeks 3 and 6, respectively (Table 1). Each of these latter rounds consisted of two inoculations in a 3.25 L bath of 20% Holtfreter's solution over a 3-day period. Throughout the study we chose to inoculate the animals using high numbers of zoospores (3.05×10^8–9.92×10^9) so as to increase the likelihood and maintenance of infection.

Bd Assays

Individuals were tested for *Bd* infection by gently rubbing a cotton-tipped swab (MWE113, Advantage Bundling SP, LLC, Durham, NC) five times over the dorsum, five times on each side, five times on the venter, and five times on the bottom of each foot. DNA was extracted from the swabs using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA), with a final elution volume of 200 µL. Extracted DNA was then analyzed using quantitative, real-time PCR (qPCR, 7500 system, Applied Biosystems, Carlsbad, CA) following Boyle et al. (2004) with the following minor modifications: each sample was diluted 1:10 with double-deionized water and 0.7 µL of bovine serum albumin (BSA, Applied Biosystems, Carlsbad, CA) was added to each reaction well prior to amplification (following Garland et al. 2010). We ran each sample in triplicate, with a positive and negative control, and a series of dilution standards. One of the three replicates for each swab contained an internal positive control (VIC_{TM} IPC, Applied Biosystems, Carlsbad, CA) to insure that PCR inhibition was not affecting our results. Samples were scored as positive if *Bd* was detected in one or more of the triplicate wells.

Jumping Performance

To measure jumping performance, we used a Kistler Z17097 piezoelectric force plate connected to a Kistler 9685 charge amplifier to measure the three dimensional forces during jumping. Digital traces were read from a Kistler 5691 DAQ-book into a Windows computer using Bioware version 4.1.0.2. Body mass was first subtracted from the vertical forces (Z), and the resultant force vector calculated using the vector sum of the individual X, Y, and Z forces. We obtained the acceleration of the center of mass by dividing the resultant 3-D ground reaction force by the body mass of the animal. Instantaneous velocity of the center of mass was calculated by numerical integration of the acceleration profile using Simpson's rule. The angle of take-off was determined by calculating the angle the cosine of which corresponds to the dot product of the horizontal ($X + Y$) and vertical (Z) jump ground reaction forces (as in Toro et al. 2003, 2004). We extracted both the peak acceleration (m/s^2) and the peak instantaneous velocity (m/s) during take-off from these force traces.

Prior to jumping, animals were acclimated to room temperature (approximately 22°C) for at least 30 min. To control for differences in body temperature, *Bd*+ and

Table 1. Number of *Bd* zoospores used in inoculations of *Lithobates pipiens*.

	Inoculation	Total Zoospores	*N*	Zoospores per Frog
Round 1 (week 0)	1	3.05×10^8	18	1.69×10^7
	2	3.34×10^9	18	7.43×10^8
	3	9.92×10^9	18	5.51×10^8
Round 2 (week 3)	1	2.42×10^9	10	2.42×10^8
	2	5.94×10^8	10	5.94×10^7
Round 3 (week 6)	1	3.34×10^8	10	3.34×10^7
	2	3.26×10^8	10	3.26×10^7

control animals were jumped in groups of five individuals, alternating between *Bd*+ and control groups. Animals were placed on the force plate and encouraged to jump by the approach of a hand or gently touching their hindquarters. Each animal was jumped five times (Losos et al. 2002; Adolph and Pickering 2008), with 15 min between jumps. For each animal the jump with the greatest peak acceleration and peak instantaneous velocity during take-off was used in the analyses (c.a. Losos et al. 2002).

Reproductive Fitness

Individuals from *Bd* + ($N = 10$) and control ($N = 10$) groups were euthanized after 8 weeks by immersion in a bath of MS-222 (500 mg/L) for 1 h. After death, the testes were removed, measured (length and width) to the nearest 0.1 mm, and fixed in 10% neutral-buffered formalin (NBF) for light microscopy. Only left testes were used for histological analyses. Tissues were fixed in NBF for at least 1 week, after which they were rinsed in de-ionized water, dehydrated through a series of ethanol solutions (70, 80, 95, and 100%), cleared in two changes of toluene, and placed in melted paraffin under vacuum for a period of 24 h. Tissues were then embedded in paraffin blocks and allowed to harden. Sections with a thickness of 8 μm were cut with a rotary microtome (RMC Instruments, Tucson, AZ) and affixed to albuminized slides. Slides from each specimen were stained with hematoxylin-eosin for general histology (Hayat 1993).

Six slides were randomly selected from each testis, and three randomly chosen histosections from each slide were analyzed. Germinal epithelium depth and spermatic cyst diameter were measured on the largest spermatic cyst in each histosection (following McCallum and Trauth 2007) using an ocular micrometer and microscope (Olympus SZH) at 124X magnification. Sperm were considered present if at least one sperm was identified on each randomly selected histosection.

Statistical Analyses

SVL and leg length at week 0 were compared between the *Bd*+ and control groups using two-tailed *t* tests. As an index of body condition, mass/SVL was compared between the *Bd*+ and control groups over the course of the experiment using ANCOVA. Raw acceleration and velocity scores were divided by leg length and analyzed using ANCOVA (weeks 4–8) and one-tailed *t* tests (at week 8). At the completion of the experiment, testes length and width were compared between the *Bd* + and control groups using two-tailed *t* tests, and germinal epithelium depth and spermatic cyst diameter were compared using general linear models (GLMs). We controlled for body size by dividing testes measurements by SVL. All statistical analyses were done in SPSS version 17.

Results

Bd Infection and Body Condition

Of 36 frogs, three arrived with *Bd* infections, were subsequently incorporated as treatment animals, and were among those used in the final analysis. Inoculations were successful, with all treatment animals testing positive 1 week after inoculation (Fig. 1). In addition, the average zoospore load per *Bd*+ animal (as measured in zoospore equivalents) at week 1 was high (11980.7, SE 2290.5). Additional rounds of inoculations proved necessary as some individuals appeared to clear their infection as soon as 2 weeks post-inoculation. One week after the second and third round of inoculations there were 10 and 9 infected treatment animals (round 2: 422.1 SE 46.7, round 3: 33.7 SE 6.7 average zoospore equivalents), respectively.

There was no difference in leg length (two-tailed *t* test: $t_{18} = 0.48$, $P = 0.64$) or SVL (two-tailed *t* test: $t_{18} = 0.60$, $P = 0.56$) between *Bd*+ and control animals at the start of

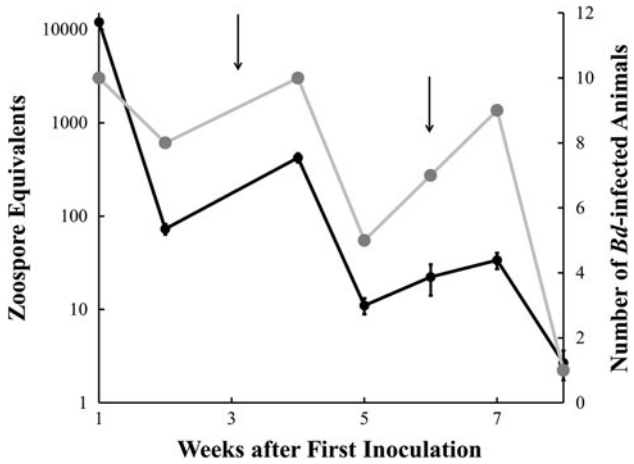

Fig. 1. Number of *Bd*-infected individuals in the treatment group for the duration of the experiment and zoospore equivalents averaged over the treatment animals for the duration of the experiment. The *arrows* at week 3 and 6 indicate re-inoculations. Zoospore equivalents: *black line*, Number of *Bd*-infected animals: *grey line*. *Error bars* indicate standard error.

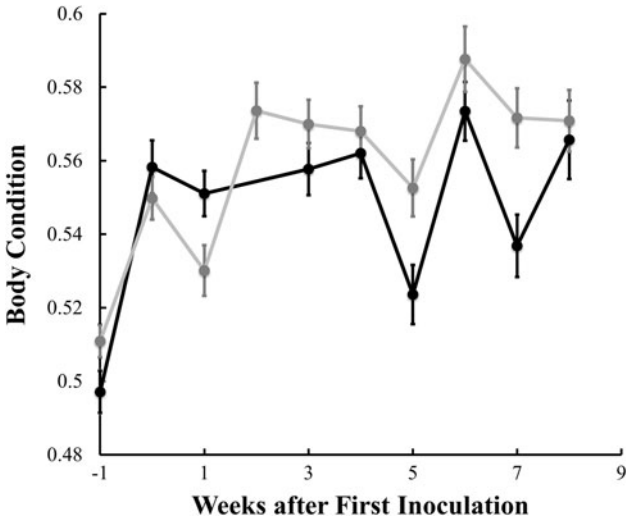

Fig. 2. Body condition (mass in g/SVL in mm) over the course of the experiment. Week 0 is arrival and week 1 is the date of first inoculation. Control animals: *black line*, treatment animals: *grey line*. *Error bars* indicate standard error.

the experiment (week 0). Furthermore, over the course of the experiment, body condition (mass/SVL) did not vary significantly between treatment and control animals (ANCOVA: $F_{1,18} = 0.121$, $P = 0.732$, Fig. 2).

Jumping Performance

There was a consistent trend toward greater peak velocity and peak acceleration of control versus *Bd*+ animals from weeks 4–8; however, these trends were not significant (ANCOVA: acceleration, $F_{1,18} = 1.941$, $P = 0.181$; velocity, $F_{1,18} = 3.472$, $P = 0.079$; Fig. 3). At week 8 of the experiment, however, control animals jumped with significantly greater velocity than *Bd*+ animals (one-tailed t test: $t_{18} = 2.443$, $P = 0.0125$), although the difference in acceleration between *Bd*+ and control animals was marginally non-significant (one-tailed t test: $t_{18} = 1.512$, $P = 0.074$).

Reproductive Fitness

There was no difference in length in either the right or left testes; however, the width of both the left and the right testes were significantly larger in the *Bd*+ than the control animals (Table 2). Neither germinal epithelium depth nor spermatic cyst diameter was significantly different between *Bd*+ and control groups (GLM: epithelium, $F_{1,11} = 0.564$, $P = 0.468$; cyst, $F_{1,11} = 0.018$, $P = 0.895$). Sperm were visible in at least one histosection from all animals; however, while sperm were present in all histosections from *Bd*+ animals, only 77.8% of histosections from control animals contained sperm.

Discussion

Bd has been detected, often with high prevalence, in amphibians of eastern North America (Longcore et al. 2007; Rothermel et al. 2008; Gaertner et al. 2009; Kinney et al. 2011; Chatfield et al. 2012). However, despite the fungus being common, no mass die-offs have been attributed to chytridiomycosis in the region. Therefore, the trend in eastern North America is one of widespread occurrence of the fungal pathogen, but with a relatively low prevalence of acute disease symptoms. There might, however, be important fitness effects occurring in the absence of declines and acute terminal infections, an idea that has not been well addressed for post-metamorphic amphibians. The central goal of this study was to elucidate the effects that *Bd* has on species that are known to harbor the pathogen, but that do not succumb to lethal infection.

Table 2. Summary of testes measurements between control and *Bd*-infected *Lithobates pipiens*.

Measure	Testis	Treatment	*N*	Mean ± SD (mm)	*t*	*P*
Width	Right	Control	10	4.01 ± 0.35	2.61	0.0177
		Bd+	10	4.47 ± 0.42		
	Left	Control	10	4.12 ± 0.41	2.33	0.0324
		Bd+	10	4.55 ± 0.39		
Length	Right	Control	10	6.54 ± 0.75	1.96	0.0658
		Bd+	10	7.40 ± 1.00		
	Left	Control	10	6.92 ± 0.72	1.41	0.1756
		Bd+	9	7.64 ± 1.34		

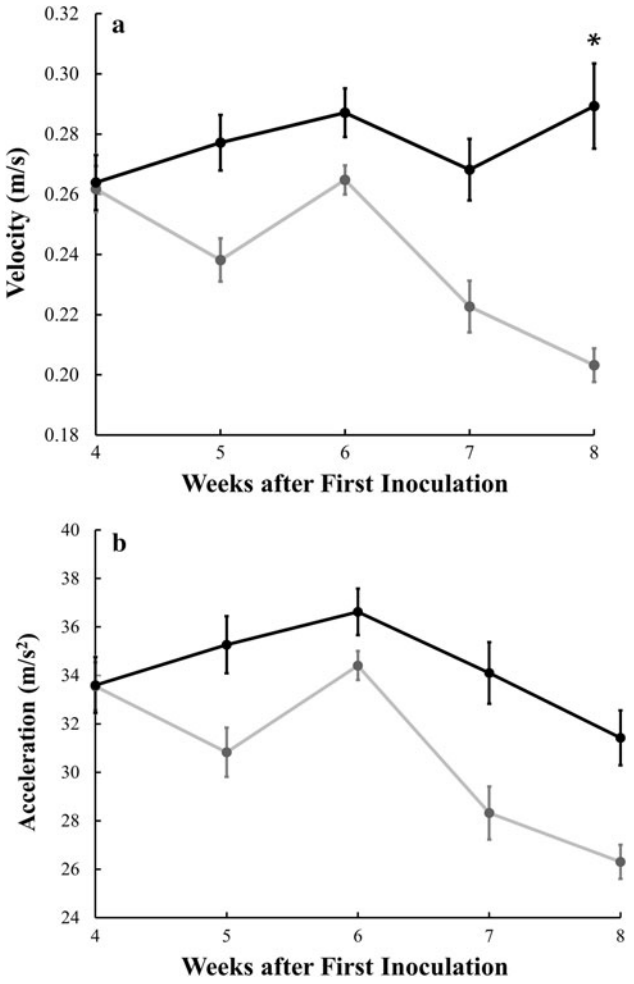

Fig. 3. a) Velocity and b) acceleration of amphibian jumps. Control animals: *black line*, treatment animals: *grey line*. *Error bars* indicate standard error. The *asterisk* in **a** indicates a statistically significant difference between control and treatment animals at week 8.

While acute infections resulting in death are often viewed as maladaptive for pathogens, sublethal consequences of disease are well-documented in a variety of animal species. Sublethal effects of infection may take the form of reduced developmental rates and body mass (Parris and Cornelius 2004; Latorre-Margarlef et al. 2009; Hesketh et al. 2012), reduced activity levels (Parris et al. 2006), diminished feeding and migratory ability (van Gils et al. 2007; Venesky et al. 2009), or reduced reproductive output (Grooms 2004). With respect to *Bd* and amphibians, previous studies on tadpoles have also documented altered interspecific interactions (Parris and Cornelius 2004), predator–prey dynamics (Parris et al. 2006), and foraging behavior (Venesky et al. 2009). In addition, previous studies have identified an effect of *Bd* on body condition and weight change in post-metamorphic frogs (Carey et al. 2006; Retallick and Miera 2007; Ramsey et al. 2010).

Locomotion is an important fitness indicator, and if locomotory ability is reduced due to *Bd* infection, an animal's ability to hunt, escape from predators, and mate may also be reduced. Our results indicate that jumping performance is negatively affected by *Bd* infection, even if the infection is light, with infected animals having a reduced peak velocity 8 weeks post-inoculation. Peak acceleration was also lower in animals infected with *Bd*, although this trend was not statistically significant. The mechanism underlying such a reduction is unclear, although one possible explanation lies in life-history trade-offs (Stearns 1989). Previous studies have shown that immune system function is costly; for example, immune challenge reduces reproductive growth and output in the lizard *Ctenophorus fordi* (Uller et al. 2006). If activation of the immune system during *Bd* infection draws on energetic resources that might otherwise be allocated toward muscle function or maintenance, then this could explain why some aspects of locomotor capacity decline in infected *L. pipiens*. Indeed, this explanation seems likely given that locomotor ability has previously been shown to be susceptible to resource allocation trade-offs in other animal species (see Zera and Harshmann (2001) for discussion; Saglam et al. (2008) for an example). Given these two findings, repeating this experiment for a longer duration may uncover an even more pronounced effect of *Bd* infection on measures of whole-organism performance, particularly if immune activation is able to be maintained for a longer period of time than in this study.

We did not detect an effect of *Bd* infection on germinal epithelium depth or maximum spermatic cyst diameter. Possible explanations for this result are that reproduction in males may not be hampered by *Bd* infection or there may be a lag time greater than 8 weeks before an effect is manifested. It is also possible that the lack of significance in these two measures of sperm production is related to the time of year. Sperm were visible in the testes histosections; however, as *L. pipiens* breeds in the spring, our ending date in August may have been too late in the year to detect an effect on sperm production had one existed.

In this study, we detected a difference in testes width between infected and uninfected frogs. Contrary to our expectations, testes of *Bd*+ animals were larger than those of control animals. In addition, the testes from *Bd*+ animals contained more sperm than those of control animals. One possible explanation for these results is that infected frogs may be making a terminal investment (Clutton-Brock 1984),

that is, investing more in reproduction compared to their uninfected counterparts as their reproductive lifespan may be limited by the pathogen. Terminal investment has been empirically documented in other systems. For example, house sparrows injected with Paramyxo virus vaccine were more likely to lay a second clutch in a single season (Bonneaud et al. 2004), male blue-footed boobies treated with liposaccharides to elicit an immune response had increased reproductive success (Velando et al. 2006), and Tasmanian devil populations afflicted with devil facial tumor disease showed precocial reproduction (Jones et al. 2008).

Although we were able to detect a difference in jumping ability between infected and uninfected frogs, only a single frog tested *Bd*+ by the end of the experiment. We postulate that the ability to shed a *Bd* infection may have come at a cost of reduced jumping ability, perhaps via life-history trade-off with muscle function. Similar trade-offs between immune defense and performance have been documented previously in vertebrates (see Lochmiller and Deerenberg 2000 and Schmid-Hempel 2003 for reviews), although the relationship between immune defense and locomotor performance is less well understood.

Although our results indicate possible sub-lethal effects of *Bd* infection on overall fitness in *L. pipiens*, it is important to note that we have not measured fitness in this study directly. Indeed, the relationships between jumping and fitness and testis morphology and fitness have yet to be empirically determined in this species. Nonetheless, a decline in fitness due to reduced locomotory ability is consistent with findings from previous studies investigating the evolutionary and ecological relevance of whole-organism performance in other vertebrate species (e.g., Husak et al. 2006; Phillips et al. 2006). Our data suggest that future studies measuring the form and intensity of selection acting on jumping performance in *L. pipiens* over longer time periods would be valuable for understanding the impact of such infections on evolutionary trajectories in this species.

It is also important to note that, although all animals in this study were acquired from the same supply company and were of a similar size (i.e., age group), they were wild-caught. We cannot, therefore, know with certainty the level of exposure or infection history in the study group. It is known, however, that *Bd* has been detected in wild amphibians throughout much of New England (Longcore et al. 2007) where the animals used in this study were likely collected. Given that 3 out of 36 animals tested positive for *Bd* upon arrival and the *Bd* is known from the region, it is likely that most or all animals have been previously exposed to *Bd*. This study underscores the need to understand the sublethal effects of pathogens on whole-organism fitness in wild populations. With respect to amphibian populations co-existing with *Bd* infection in the wild, our results suggest that there may be important long-term, evolutionary implications. In many parts of the world, *Bd* is now considered endemic (e.g., Murray et al. 2009) and elucidating the chronic effects on amphibian populations in these areas is crucial to understanding the impact that *Bd* will ultimately have on amphibian populations. Lastly, our results contribute to a growing understanding of the fitness consequences of disease and their potential to alter evolutionary trajectories through reduced locomotor performance.

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