Chytridiomycosis in the Direct-developing Frogs of Puerto Rico

by

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ABSTRACT

Epidemiological theory normally does not predict host extinction from infectious disease because of a host density threshold below which pathogens cannot persist. However, host extinction can occur when a biotic or abiotic pathogen reservoir allows for density-independent transmission. Amphibians are facing global population decline and extinction from the emerging infectious disease chytridiomycosis, caused by the fungus *Batrachochytrium dentrobatidis* (Bd). I use the model species *Eleutherodactylus coqui* to assess the impact of Bd on terrestrial direct-developing frog species, a common life history in the tropics. I tested the importance of two key factors that might influence this impact and then used laboratory experiments and published field data to model population-level impacts of Bd on *E. coqui*. First, I assessed the ontogenetic susceptibility of *E. coqui* by exposing juvenile and adult frogs to the same pathogen strain and dose. Juveniles exposed to Bd had significantly lower survival rates compared with control juveniles, while adult frogs often cleared infection. Second, I conducted experiments to determine whether *E. coqui* can become infected with Bd indirectly from contact with zoospores shed onto vegetation by an infected frog and from direct exposure to an infected frog. Both types of transmission were observed, making this the first demonstration that amphibians can become infected indirectly in non-aquatic habitats. Third, I tested the hypothesis that artificially-maintained cultures of Bd attenuate in pathogenicity, an effect known for other fungal pathogens. Comparing two cultures of the same Bd strain with different passage histories revealed reduced zoospore production and disease-induced mortality rates for a susceptible frog species (*Atelopus zeteki*) but not for the less-susceptible *E. coqui*. Finally, I used a mathematical model to project the

population-level impacts of chytridiomycosis on *E. coqui*. Model analysis showed that indirect transmission, combined with either a high rate of zoospore production or low rate of zoospore mortality, is required for Bd to drive *E. coqui* populations below an extinction threshold. High rates of transmission plus frequent re-infection could lead to poor recruitment of infected juveniles and population decline. My research adds further insight into how emerging infectious disease is contributing to the loss of amphibian biodiversity.

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INTRODUCTION

Host extinction from infectious disease is not normally predicted by theory because of the density-dependent nature of most pathogen transmission (Anderson and May 1979). However, extinction can occur when a pathogen can persist in an environmental reservoir or a tolerant host species, allowing transmission to proceed in the declining host at low densities (De Castro and Bolker 2004). There is increasing evidence that the emerging infectious disease chytridiomycosis has led directly to the decline and extinction of amphibian populations in the Americas, Europe and Australia (Collins and Crump 2009). For species with restricted-ranges or small populations, these declines translate into global extinctions (La Marca et al. 2005). As such, the Bd-amphibian system is a good model for studying the role of infectious disease in biodiversity loss (McCallum 2012).

Research into the impacts of chytridiomycosis on the *Eleutherodactylus* frogs of Puerto Rico has been underway for more than a decade, prompted by the disappearance of three species—*E. karlschmidti*, *E. jasperi*, *E. eneidae*—and ongoing declines of extant species attributed to this emerging infectious disease (Burrowes et al. 2004). Field and laboratory studies have been used to describe the geographic distribution of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) in Puerto Rico and the host species infected (Burrowes et al. 2008a), variation in prevalence and infection intensity by season (Longo et al. 2010) and life stage (Longo and Burrowes 2010), and potential fitness costs of infection (Burrowes et al. 2008b). Lab work has shown that the common coqui (*Eleutherodactylus coqui*), the model species used in this study, can succumb to the pathogen in drought conditions (Longo et al. 2010).

My research adds to this body of knowledge in several areas. Juvenile *E. coqui* show higher prevalence and intensity of Bd infection compared with adults in the wild (Longo and Burrowes 2010), but it was unclear whether and to what extent juveniles are susceptible to chytridiomycosis. In Chapter 1, I tested if susceptibility to Bd varies with ontogeny by exposing *E. coqui* adults and juveniles to the same pathogen dose and strain under identical laboratory conditions. Adult frogs were collected from El Yunque, Puerto Rico and Hilo, Hawaii for this study, and and juveniles were reared in the laboratory (Appendix A provides a full list of frogs collected and used for each experiment). This is the first known study of ontogenetic susceptibility to Bd in direct-developing frogs and sheds light on a possible mechanism for observed pathogen-induced population declines in a species with relatively resistant adults.

Previous transmission studies focused on amphibian species with aquatic life stages (Rachowicz and Vredenburg 2004, Rachowicz and Briggs 2007, Mitchell et al. 2008, Greenspan et al. 2012), and there was no information on Bd transmission in terrestrial frogs. In Chapter 2, I describe experiments in which I assessed transmission dynamics in *E. coqui* and specifically whether frogs can become infected with Bd indirectly from contact with zoospores shed onto vegetation by an infected frog as well as through direct contact with infected animals. Chapter 3 reports a test of the hypothesis that Bd strains maintained *in vitro* attenuate in pathogenicity. This line of research was necessary to test an explanation for why three earlier experiments that I undertook to measure transmission rates in a variety of climate conditions and habitat types showed no transmission. I hypothesized that the Bd culture used to infect frogs in those failed experiments, in active culture for 6 years, lost the infective or pathogenic properties of

wild-type Bd. Using a cryopreserved sample of the same Bd isolate, I compared these two samples of the same isolate with different passage histories through strain phenotype and amphibian exposure experiments. This work has led to ongoing genomic analysis investigating the underlying genetic mechanisms of Bd pathogenicity and the immune responses of *Atelopus zeteki* to Bd infection.

In Chapter 4, I describe a mathematical model used to explore the population-level impact of Bd on *E. coqui* and describe conditions for pathogen-induced population extinction. The model is parameterized with lab data from Chapters 1 and 2 and long-term field data collected on *E. coqui*. In particular I evaluate the importance of high juvenile pathogen-induced mortality and the occurrence of both direct and indirect pathogen transmission in disease dynamics.

In a concluding chapter I draw together the main findings from each chapter, discuss conservation implications, and highlight priorities for future research. Chapters 1 and 2 are submitted for publication, Chapter 3 is published, and Chapter 4 will be submitted for publication early next year.

Chapter 1

SUSCEPTIBILITY TO THE AMPHIBIAN CHYTRID FUNGUS VARIES WITH ONTOGENY IN *ELEUTHERODACTYLUS COQUI*

ABSTRACT

Age-related differences in susceptibility to infectious disease are known from a wide variety of plant and animal taxonomic groups. For example, the immature immune systems of young vertebrates, along with limited prior exposure to pathogens and behavioral factors, can place juveniles at greater risk of acquiring and succumbing to a pathogen. This study investigated the ontogenetic susceptibility of terrestrial direct-developing *Eleutherodactylus coqui* frogs to the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which has the largest host range of any known vertebrate pathogen and is responsible for the decline of amphibian species worldwide. By exposing juvenile and adult frogs to the same dose and strain of Bd, ontogenetic differences in susceptibility were uncovered. Juvenile frogs exposed to the pathogen had significantly lower survival rates compared with control juveniles, while adult frogs largely cleared infection and had survival rates indistinguishable from control frogs, even when exposed to a much higher dose of Bd. The high disease-induced mortality rate of juveniles may explain ongoing population declines in eastern Puerto Rico, where Bd is endemic and juveniles experience higher prevalence and infection intensity compared to adults. These results have important implications for understanding and modeling the decline, possibly to extinction, of amphibian populations and species.

INTRODUCTION

Ontogenetic differences in susceptibility to infectious disease are known in a variety of organisms including plants (Ficke et al. 2002), insects (Bull et al. 2012), amphibians (Rohr et al. 2010), birds (Gavier-Widen et al. 2012), and mammals (Fallon et al. 2003, Burns-Guydish et al. 2005). Among the vertebrates, young animals frequently have immature immune systems and limited prior exposure to pathogens that stimulate adaptive immunity (Solomon 1978, Pabst and Kreth 1980, Du Pasquier et al. 1986), resulting in greater disease susceptibility compared to older juveniles and adults.

Age-related differences in disease susceptibility are of particular interest with chytridiomycosis, an emerging infectious disease implicated in the decline and extinction of amphibian species worldwide (Collins and Crump 2009). The aquatic fungus that causes chytridiomycosis, *Batrachochytrium dentrobatidis* (Bd), has the widest host range of any known vertebrate pathogen, with over 500 amphibian species known to be infected (Olson et al. 2013). Bd colonizes the keratinized tissue of amphibian skin (Longcore et al. 1999) and ultimately causes death through osmotic and electrolyte imbalance (Voyles et al. 2007). Species vary in susceptibility (Blaustein et al. 2005, Searle et al. 2011, Gahl et al. 2012, Gervasi et al. 2013), with responses ranging from mass mortality (Lips et al. 2006), to tolerance of subclinical infections (Garner et al. 2006), to resistance (Marquez et al. 2010). Researchers have recently shown that Bd inhibits the acquired immune response of some species (Fites et al. 2013).

Bd susceptibility can also vary across amphibian life history stages. Tadpoles of some species often show markedly lower susceptibility than post-metamorphic animals (Berger et al. 1998, Rachowicz and Vredenburg 2004, Tobler and Schmidt 2010, Gervasi

et al. 2013), presumably because keratinized tissue is restricted to the mouthparts in tadpoles (Berger et al. 1998). In contrast, tadpoles of other species show reduced survival from infection, particularly when exposed to high doses of Bd (Blaustein et al. 2005, Garner et al. 2009). Fitness impacts of infection on tadpoles include smaller metamorphic body mass, increased larval-period length, and reduced foraging performance (Parris and Cornelius 2004, Garner et al. 2009, Venesky et al. 2009, Venesky et al. 2011). These fitness costs can result in mortality of tadpoles or metamorphs, even in the absence of infectious disease (Garner et al. 2009).

Only one study has examined the developmental susceptibility of terrestrial amphibian life stages to Bd (Ortiz-Santaliestra et al. 2013). Researchers exposed American toad (*Anaxyrus americanus*) juveniles, a species with larval development, at the end of metamorphosis or 28 days following metamorphosis. Juveniles exposed to Bd showed significantly greater mortality than control animals at day zero but not at day 28, suggesting an ontogenetic difference in immune competence. The authors acknowledge that these results could be also explained by greater body size of juveniles at day 28, as other studies have shown that body size is an important determinant of survival following Bd infection (Carey et al. 2006, Burrowes et al. 2008b, Garner et al. 2009, Tobler and Schmidt 2010).

The ontogeny of the amphibian immune system has been studied in only one anuran, the African clawed frog (*Xenopus laevis*), a species with larval development (Du Pasquier et al. 1986, Rollins-Smith 1998). Innate immune responses, such as secretion of anti-microbial peptides, are one of several factors known to influence amphibian susceptibility (Rollins-Smith et al. 2011); other factors include symbiotic skin bacteria

that inhibit Bd (Harris et al. 2006), microhabitat preferences (Brem and Lips 2008), and thermoregulatory behavior (Richards-Zawacki 2010). The adaptive immune response of *X. laevis* appears to be inhibited by Bd (Fites et al. 2013, Rollins-Smith et al. 2011).

Although direct development of terrestrial eggs is prevalent in the tropics and has evolved independently in at least 12 clades of anurans (Duellman and Trueb 1986), there are no data indicating how and when the immune system of direct-developing frogs develops or matures. Development time of *Eleutherodactylus coqui* embryos ranges from 17-26 days and is qualitatively different from larval development with at least seven unique morphological features (Townsend and Stewart 1985). Because direct developers do not undergo metamorphosis with a corresponding reorganization of the immune system (Rollins-Smith 1998), it is unclear whether hatchlings of direct developing anurans may be more or less vulnerable to infection as the recently metamorphosed frogs of larval developing species.

This study represented the first known investigation of age-related susceptibility of direct-developing frogs to Bd. The model species is the common coqui (*E. coqui*) from Puerto Rico, a terrestrial direct-developing species that has been well studied in the laboratory and the field. Bd is suspected in the decline and extinction of three frog species from Puerto Rico in recent decades (Burrowes et al. 2004), and ongoing declines have been reported for *E. coqui* and other *Eleutherodacylus* species at mid-elevations in eastern Puerto Rico (Longo et al. 2010). Recent laboratory work indicates that adult *E. coqui* frogs may have low susceptibility to Bd, at least under normal climatic conditions (Longo et al. 2010, Langhammer et al. 2013), and moribund adults are rarely found in the field (Longo et al. 2013). Juvenile *E. coqui* show higher prevalence and intensity of Bd

infection compared with adults in El Yunque, PR (Longo and Burrowes 2010), but it is unclear whether juveniles are susceptible to chytridiomycosis.

The response of both juvenile and adult *E. coqui* to Bd infection was tested under identical laboratory conditions, including exposure to the same Bd strain and dose. The decline of *E. coqui* populations in northeastern Puerto Rico may be a result of high disease-induced mortality of juveniles (Longo and Burrowes 2010). Thus, juveniles exposed to Bd were predicted to show significantly lower survival rates compared to adults. Theoretical work with larval developing species has highlighted the importance of juvenile survival rate in determining population persistence (Hels and Nachman 2002, Hyman 2012), and this work represents a first step in understanding the response of direct-developing juvenile frogs to Bd.

METHODS

Arizona State University Institutional Animal Care and Use Committee and the University of Maryland Institutional Animal Care and Use Committee approved this research. Permission to collect and export *E. coqui* frogs was obtained from the Department of Natural and Environmental Resources in Puerto Rico (permits 2009-IC-015, 2009-IC-014) and the Department of Land and Natural Resources in Hawaii (permit EX10-08).

Frog acquisition and rearing

In 2010, 29 adult *E. coqui* frogs were collected from El Yunque, Puerto Rico and 14 from an introduced population near Hilo, Hawaii for a total of 43 frogs (>23 mm snout-vent length [SVL]). Each animal was measured, sexed, and toe clipped for

individual identification. Ventral skin surfaces were swabbed to determine Bd infection status (Hyatt et al. 2007). A new pair of disposable nitrile gloves was used for each frog to prevent cross-contamination.

After all frogs were housed individually for at least 10 weeks and repeatedly tested Bd-negative, they were placed in large naturalistic terraria in groups of 4-6 to initiate breeding. The Puerto Rican and Hawaiian *E. coqui* frogs were kept separate. Frogs that were collected at the same time as the 43 used in this study were also involved in breeding. More than 15 clutches of 1-34 juvenile froglets were produced by at least nine different breeding pairs.

Just as in earlier attempts to breed *E. coqui* in the lab (Michael 1995), overall survival of juveniles was low. Poor survival of juveniles is attributed to the difficulty in maintaining a constant supply of tiny food items. The unavailability of termites, for both juveniles and adults, was particularly problematic (J. Stabile, pers. comm.). However, enough juveniles survived beyond six weeks to test overall juvenile susceptibility to Bd. At six weeks of age, most juveniles were large enough so that they could be handled briefly without causing inadvertent mortality. A total of 105 juveniles were used in this experiment. Most juveniles (n = 92) were 6-10 wks old, and the remaining (n = 13) were 18-21 wks old. The size range of all experimental juveniles was 6.9-13.9 mm.

Animal husbandry

For the experiment, adult frogs were housed individually in acrylic terraria (20.3 x 17.8 x 12.7 cm) with vented lids. Each terrarium had a moist sphagnum moss substrate with *Cercropia* sp. leaves as hiding places. Terraria were sprayed daily with purified water and changed bi-weekly. Frogs were fed crickets *ad libitum* twice a week. The

laboratory was maintained at a constant temperature of 20-22°C on a 12-hour day-night cycle.

Juvenile *E. coqui* were treated similarly except that they were housed individually in 473 ml deli cups or plastic Ziploc containers lined with moss and vented lids. The smallest juveniles were fed springtails and older juveniles were fed both springtails and wingless fruit flies 5-6 times per week.

Experimental set-up

Juvenile *E. coqui* were randomly assigned to one of two treatment groups: control (n=54) or exposure to Bd at a dose of 10^5 zoospores/ml (n=51). Mean SVL (+/- 1 SE) and age of juveniles was 11.1 $\pm$ 0.2 mm and 9.6 $\pm$ 0.4 weeks, respectively. There was no significant difference between the treatment groups in length ($F=1.76$, $p=0.19$) or age ($F=0.87$, $p=0.35$).

Because the adult *E. coqui* were so much larger than the juveniles, and potentially immunologically stronger, some frogs were exposed to a higher Bd dose (10^6 zoospores/ml) in addition to the treatment dose used for the juveniles (10^5 zoospores/ml). To determine if previous Bd infection affected survival, since *E. coqui* in the wild are likely to experience frequent re-infection in the field, a treatment group of frogs that were Bd-positive at the time of collection and either remained Bd-positive or cleared their infections by the time of the experiment was included. All other frogs were Bd-negative upon collection in the field and remained Bd-negative until the start of the experiment. Frogs were thus randomly assigned to treatment groups as follows: control (n=10), exposure to 10^5 zoospores/ml (n=7), exposure to 10^6 zoospores/ml (n=13), or previously-infected frogs exposed to 10^5 zoospores/ml (n=13). The average length of adults was 37.3

± 0.8 mm, and there was no significant difference in average length between treatment groups ($F=2.72$, $p=0.10$).

Bd exposure

A sample of Bd strain JEL427 was obtained from Dr. Joyce Longcore at the University of Maine. This strain was isolated in 2005 from a sick *E. coqui* frog in El Yunque, Puerto Rico. The isolate was continuously maintained at 4°C and transferred into new media 3-4 times per year. Upon receipt of this culture at ASU, it was held in 1% tryptone broth at 18°C and transferred into new media monthly.

One week prior to frog exposure, fast-growing broth culture was transferred to 1% tryptone-agar plates, which were then sealed and incubated at 23°C. To harvest zoospores, plates were flooded with 1 ml of sterile 1% tryptone broth and allowed to sit for 30 minutes. The zoospore solution was collected into a 50 ml sterile tube, and zoospore concentration was quantified using two counts on a hemacytometer (Hausser Scientific Bright-Line), which were then averaged. The zoospore stock solution was diluted with purified water to achieve the desired inoculation concentration of either 10^5 or 10^6 zoospores/ml.

For the Bd exposure, adult frogs were placed individually into 236 ml plastic deli cups with a fresh solution of Bd in 10 ml of zoospore solution for 10 hrs per day, on two consecutive days. Control frogs were treated identically, except that they were exposed to a sham solution of purified water containing 1% tryptone broth lacking zoospores.

Juveniles from both Puerto Rican and Hawaiian parents were exposed to 0.5-1.0 ml of zoospore (or sham) solution in 12 mm (height) x 32 mm (diameter) SKS Natural Polypro Hinge Top Vials. These shallow containers prevented tiny juveniles from

climbing the sides and escaping the Bd solution. The Bd inoculate or sham solution was added to the vial with a pipette through a small hole cut into the lid. Juveniles were exposed for 8 hrs on one day and then for another 8 hrs a week later. The risk of mortality from stress was considered to be too great to expose juveniles on two consecutive days, since juveniles are much more fragile than adults.

Behavior, morbidity, and mortality of adult and juvenile frogs were monitored daily for 77 days. To minimize suffering, frogs were euthanized by immersion in 300 mg/l tricaine mesylate (MS-222) when their righting reflex was lost, the only reliable indicator of imminent death thus far observed with *E. coqui*. At the conclusion of the experiment, frogs surviving were euthanized with MS-222.

Pathogen detection

Bd infection was detected by swabbing ventral skin surfaces of adult and juvenile frogs using a standardized technique (Hyatt et al. 2007) employing fine-tip cotton swabs (Medical Wire & Equipment #113). Adults were tested on several occasions before the experiment to ensure that they were Bd-negative. Juveniles were not swabbed prior to the experiment, because they had never been exposed to Bd in the lab. Following Bd exposure, adult frogs were swabbed on days 9, 17, 30, and upon death, or the end of the experiment (day 77). Juveniles were swabbed upon death or at the end of the experiment, and for roughly half of the juveniles, Bd infection was also assessed 20 days post-inoculation. Juvenile sampling was kept to an absolute minimum because it was easy to cause accidental mortality through swabbing or handling. For this reason, the second set of juveniles was not tested 20 days post-inoculation.

Swabs were stored at room temperature in sterile 2 ml screw-top vials until DNA extraction roughly two weeks later. DNA was extracted with PrepMan Ultra and infection status and intensity was determined using real-time quantitative PCR following standard protocol for detecting Bd (Boyle et al. 2004) as modified by Hyman and Collins (2012).

RESULTS

Juvenile *E. coqui* exposed to Bd had a significantly lower rate of survival compared to the control group (Log-rank test χ^2=9.58, p=0.002, Fig. 1.1). Only 9.8% (5/51) of juveniles exposed to Bd survived the 77 day experiment, compared to 35.2% (19/54) in the control group.

Of the inoculated juveniles that died during the experiment 80% (37/46) tested Bd-positive upon death. Of the five inoculated juveniles that survived the experiment only one tested Bd-positive. None of the control juveniles tested Bd-positive upon death or at the end of the experiment, confirming that there was no cross-contamination.

In contrast to the juveniles, there was no significant difference in survival rate between Bd-exposed and control adult frogs (Log-rank χ^2=3.4, p=0.06, Fig. 1.1). One frog in the previously-infected 10^5 zoospores/ml treatment group died with a low pathogen load, but it had a high infection intensity following inoculation. Two control frogs died for unknown reasons.

All but one inoculated adult frog tested Bd-positive following inoculation (32/33, 97%). Average infection intensity of adult frogs on day 9, the first sampling date following inoculation, was 1336 $\pm$ 341 zoospore genomic equivalents. Both average

infection intensity and prevalence fell sharply by day 30 (Fig. 1.2). Swab samples for adult frogs taken at the end of the experiment (day 77) were lost in shipment and could not be analyzed. All control adult frogs tested Bd-negative at each sampling date.

DISCUSSION

A field survey of both juvenile and adult *E. coqui* in eastern Puerto Rico showed that Bd prevalence and average infection intensity was greater among juveniles compared to adults (Longo and Burrowes 2010). The present study corroborates their findings and demonstrates experimentally that juveniles are not only more susceptible to Bd infection but also more likely to die following infection. Juveniles exposed to Bd had a significantly lower rate of survival compared to control juveniles.

In contrast, adult *E. coqui* frogs in the three different Bd-exposure treatment groups had survival rates indistinguishable from control frogs. Exposure to a higher Bd dose and prior infection with Bd did not affect survival, and most frogs cleared their infections or reduced them to low levels. This finding aligns with other research showing that adult *E. coqui* can survive and clear infection when exposed to Bd under normal climate conditions (Longo et al. 2010, Langhammer et al. 2013) and the observation that adult frogs can eliminate natural Bd infections within a few weeks of arriving in the lab (P.F. Langhammer, unpublished data).

However, the fact that one adult frog died with signs of chytridiomycosis in this study suggests that the ability to clear infection varies among individuals (Tobler and Schmidt 2010). Analysis of mark-recapture data showed that the proportion of recaptured adult frogs was lower for infected adult *E. coqui* than for uninfected frogs in the Palo

Colorado Forest of eastern Puerto Rico (Longo and Burrowes 2010). Although populations of *E. coqui* persist in the wild with mild Bd infections, some individuals die from chytridiomycosis (Longo et al. 2013). Vulnerability of *E. coqui* adults to Bd is thought to be affected by seasonal climatic variables, specifically, a reduced ability to resist the pathogen in cooler, drier conditions or increased transmission in a limited number of humid retreat sites (Longo et al. 2010).

Previous work with two species of *Anaxyrus* toadlets showed that size was an important predictor of survival time following Bd infection, with larger individuals surviving longer (Carey et al. 2006, Burrowes et al. 2008b, Garner et al. 2009). Thus, the high susceptibility of juveniles observed may have been due to their small size (and greater skin surface-area to volume) or to their immature immune systems as suggested by Longo and Burrowes (2010). Also, the fact that nearly all Bd-exposed juveniles regardless of size died in this study indicates that juveniles younger than 21 weeks are highly vulnerable to Bd. It is unclear how juveniles older than 21 weeks would respond to Bd. Future work aiming to tease apart size and ontogenetic immune function will require a larger sample of juveniles of a given age and locality that vary in size naturally or due to diet manipulation. Either way, models of host-pathogen interactions for Bd and amphibians must account for the possibility of ontogenetic variation in Bd susceptibility.

In nature, adult *E. coqui* may exhibit behaviors that allow them to clear infection (Richards-Zawacki 2010), or they may experience reduced Bd transmission in terrestrial habitats compared with stream- or pond-associated frogs (Rowley and Alford 2007). However, the resistance to Bd observed in the lab suggests a competent immune response that may not characterize juvenile frogs (Rollins-Smith 1998, Rollins-Smith et al. 2011).

In this study, adult *E. coqui* frogs were exposed to repeated high doses of Bd, were maintained at temperatures favorable to Bd (Piotrowski et al. 2004), and had opportunities for indirect re-infection via zoospores shed onto terraria substrate (see Chapter 3). Research is underway by P.A. Burrowes to study potential eco-immunological mechanisms of *E. coqui*, including symbiotic anti-Bd bacteria on the skin (Harris et al. 2006), which could provide insight into how *E. coqui* adults resist and clear infection. It is unknown when the immune system of direct developing frogs matures.

This study represents a first step toward understanding the ontogenetic susceptibility of direct-developing frogs to Bd, and the results have important ecological and conservation implications. If juveniles are susceptible to chytridiomycosis in the field, or if there is a fitness cost to Bd infection (Burrowes et al. 2008b), then the decline of *E. coqui* populations reported by Burrowes et al. (2004) could be a function of reduced recruitment of juveniles despite adult frogs showing resistance (Longo and Burrowes 2010). This possibility is particularly worrying for the eight species of *Eleutherodactylus* occurring in Puerto Rico that are globally threatened with extinction (IUCN 2012), assuming they share a similar ontogenetic response to the pathogen.

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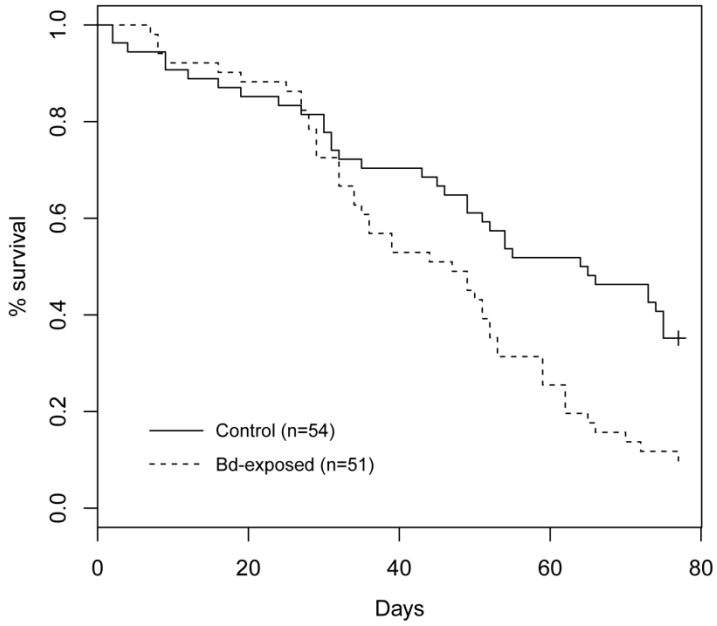
Fig. 1.1 Survival for *Eleutherodactylus coqui* (a) juveniles (N=105) and (b) adults (N=43) exposed to either Bd strain JEL427 or a sham solution (control).

(a)



(b)

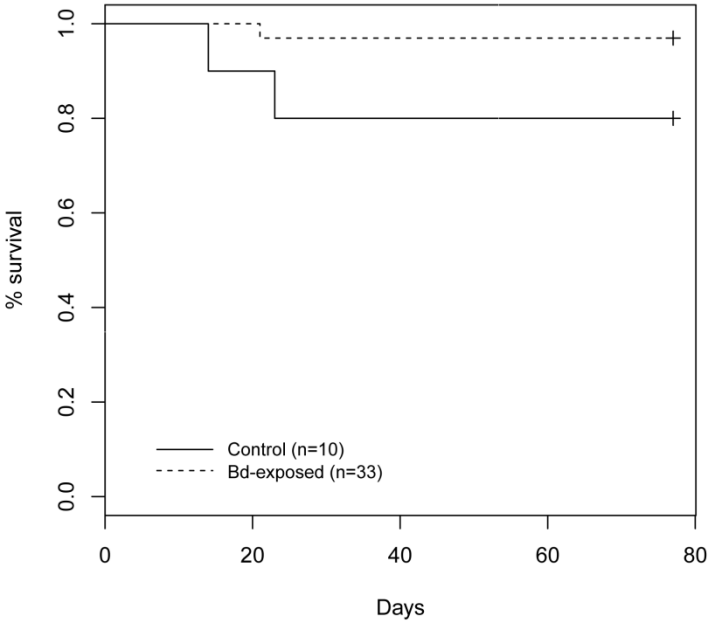
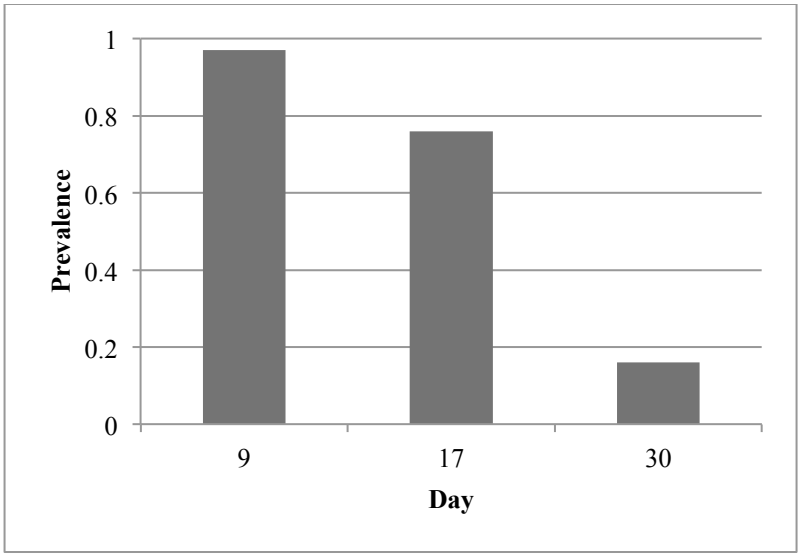
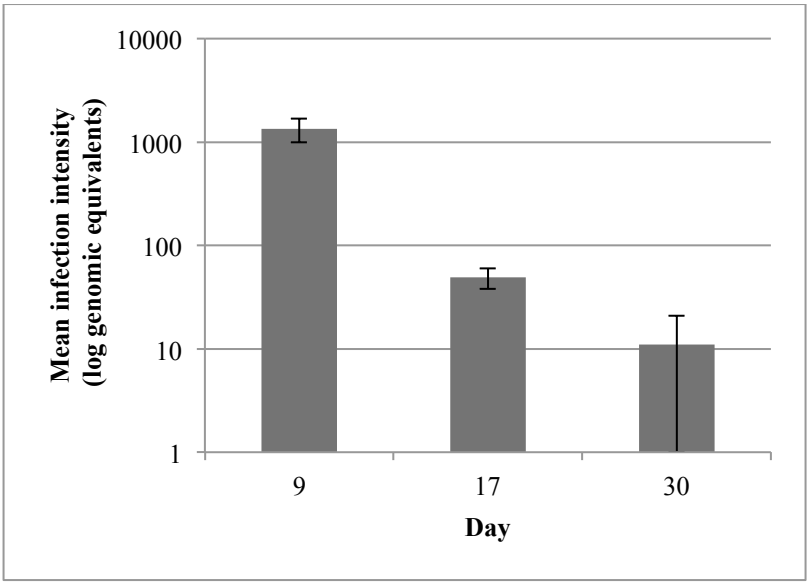


Fig. 1.2 (a) Prevalence and (b) mean infection intensity over time of adult *Eleutherodactylus coqui* frogs exposed Bd strain JEL427. Data for the three groups exposed to Bd are combined. Error bars represent the standard error.

(a)



(b)



Chapter 2

TRANSMISSION OF THE FUNGAL PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* IN A TERRESTRIAL DIRECT-DEVELOPING FROG

ABSTRACT

Batrachochytrium dendrobatidis (Bd), a fungal pathogen of amphibians that causes chytridiomycosis, has received considerable attention due to its role in the decline and extinction of frogs and salamanders worldwide. Infectious disease theory predicts host extinction from a pathogen under a limited set of conditions, most notably when pathogen transmission is independent of host density. There are two likely pathways to extinction in the Bd system. Amphibian species that tolerate the pathogen can carry and transmit it to susceptible species even when the latter's population density declines below a transmission threshold. Alternatively, a non-amphibian pathogen reservoir can allow for indirect disease transmission even at low host population densities. Previous experimental work on Bd transmission comes exclusively from aquatic systems. This study tested experimentally whether terrestrial, direct-developing *Eleutherodactylus coqui* frogs can become infected with Bd indirectly from contact with zoospores shed onto vegetation by an infected animal, and directly from exposure to an infected frog. Both types of transmission were observed, and the proportion of frogs infected indirectly and directly was equal (~30%). Indirect transmission occurred on both moss and bromeliad substrates. New infected frogs cleared or reduced their infections relatively quickly, suggesting that low-level recurring infections may typify infection dynamics where Bd is enzootic. This is the first known demonstration that amphibians can become

infected indirectly in non-aquatic habitats and to quantify Bd transmission in a terrestrial species. The findings lay the groundwork for future studies on Bd transmission, which are essential for predicting population-level impacts of epizootic and enzootic disease.

INTRODUCTION

The fungal pathogen *Batrachochytrium dendrobatidis* (Bd), the etiological agent of amphibian chytridiomycosis, is one cause of the decline of amphibian species worldwide (Skerratt et al. 2007, Collins and Crump 2009). Bd appears to be among a small number of infectious diseases capable of causing species extirpations and extinctions (McCallum 2012), because most diseases have a host density threshold below which a pathogen cannot persist in a host population (Anderson and May 1979). However, extinction can occur when transmission is density-independent, as occurs in many vector-borne and sexually transmitted diseases or through the existence of abiotic or biotic disease reservoirs (De Castro and Bolker 2004).

Despite the rapidly expanding body of Bd research, little is known about the mode and frequency of its transmission. Bd infection occurs via aquatic zoospores that infect amphibian skin (Longcore et al. 1999). Theoretical work shows that if Bd can persist outside of its host in an environmental reservoir, such as a pond, the probability of host extinction greatly increases (Mitchell et al. 2008). Experimental work on Bd transmission to date has exclusively focused on aquatic rather than terrestrial systems, even though Bd is known to infect frogs in a range of habitats. Researchers studying mountain yellow-legged frogs (*Rana muscosa*) demonstrated that Bd transmission occurs both from direct contact between animals and indirectly through contact with Bd-contaminated water

(Rachowicz and Briggs 2007). Others researchers have shown that bullfrogs (*Lithobates catesbeianus*) can transmit Bd to wood frogs (*L. sylvaticus*) (Greenspan et al. 2012), that infected adult Fowler's toads (*Bufo fowleri*) and gray treefrogs (*Hyla chrysoscelis*) can infect tadpoles of the same species in aquatic enclosures or tanks (Parris and Cornelius 2004), and that crayfish infected with Bd can transmit to southern leopard frog tadpoles (*L. sphenocephalus*) (McMahon et al. 2013).

Little is known about Bd transmission in terrestrial direct-developing amphibians, which lack an aquatic larval stage and do not rely upon water bodies for their reproduction. In the Neotropics alone, at least 31% of anuran species (881/2816) have direct development of terrestrial eggs (Hedges et al. 2008, IUCN 2012), and this mode of reproduction characterizes many genera in Old World tropical forests as well (Wells 2007). There is low Bd prevalence in direct-developing microhylid frogs in Australia (Hauselberger and Alford 2012), while in Central America, the abundances of direct-developers have declined significantly in some areas (Lips et al. 2006), even if these species initially fare better than aquatic breeders in a Bd epidemic (Crawford et al. 2010). Lower prevalence and higher initial survival may be due to reduced susceptibility or fewer opportunities for transmission and re-infection in terrestrial habitats. Bd occurs in bromeliad axils in Panama (Cossel and Lindquist 2009), and researchers suspect that indirect transmission occurs for terrestrial species (Burrowes et al. 2008b), although it has never been demonstrated.

This study investigated if terrestrial frogs can become infected with Bd indirectly by making contact with zoospores shed onto vegetation or soil by an infected animal. The proportion of frogs infected via the environment was compared to the proportion of frogs

infected when directly exposed to an infected frog. The common coqui (*Eleutherodactylus coqui*) was chosen as the study species, because extensive existing field and laboratory research provided useful background information, and unlike other congeners, is still relatively abundant. This terrestrial, direct-developing frog is native to Puerto Rico and was introduced to Hawaii, Florida, and neighboring Caribbean islands. *E. coqui* currently persists with enzootic Bd in Puerto Rico although mid-elevation populations are declining, presumably due to population-level effects of chytridiomycosis (Longo and Burrowes 2010). In Puerto Rico, clumping of *E. coqui* in the humid retreats of drought-exposed experimental terraria correlated positively with increased frog mortality, suggesting that drought or increased Bd transmission acting individually or together could drive population declines during environmental stress (Longo et al. 2010).

METHODS

This study follows three earlier transmission experiments involving 55 *E. coqui* frogs in which Bd transmission was possibly observed in only one frog. It has since been discovered that the Bd culture used for experimental inoculation had attenuated in pathogenicity after a long history of artificial maintenance (Langhammer et al. 2013, Chapter 3). The experiment described in this chapter was designed in ways that address this limitation. The research followed guidelines set by the Institutional Animal Care and Use Committees of Arizona State University and the University of Maryland.

Frog collection and husbandry

A total of 35 *E. coqui* frogs were collected from El Yunque, Puerto Rico (n=18) and from an introduced population near Hilo, Hawaii (n=17), for this experiment. Frogs

were captured at night using individual plastic bags inverted and worn as gloves to prevent Bd contamination. In the laboratory, the frogs were assessed for mass, length and sex, and each animal was toe-clipped for individual identification. A new pair of powder-free nitrile gloves was used every time a different frog was handled.

Frogs were housed individually in 5.7 liter Sterlite plastic storage boxes lined with one of two vegetation types: sphagnum moss or a small bromeliad in potting soil. Terraria were misted daily; water accumulated in bromeliad axils, and both moss and soil remained damp. The laboratory was maintained at 21-22°C on a 12-hour day-night cycle, and frogs were fed vitamin-dusted crickets twice a week.

Bd diagnostics

Infection status was determined using sterile fine-tipped cotton swabs (Medical Wire & Equipment #113) and standard protocol for swabbing frog skin (Hyatt et al. 2007). DNA was extracted from swabs using PrepMan Ultra and zoospore genomic equivalents were quantified on skin swabs using a real-time quantitative polymerase chain reaction (qPCR) assay (Boyle et al. 2004) modified by Hyman and Collins (2012).

All animals had been in captivity for one year at the beginning of this study following use in a previous experiment by PFL. All frogs were confirmed to be Bd-negative for at least 9 months prior to this study. The prior infection of some of the study animals was not a concern because this species is known to lose and regain infections readily in the lab. For example, of 83 *E. coqui* frogs collected from El Yunque, Puerto Rico, for another study, 18% were naturally infected with Bd upon arrival at the laboratory. All but two frogs cleared their infections within 5 weeks, and after testing Bd-negative for several weeks, tested Bd positive following experimental Bd inoculation

(PFL unpublished data). Researchers suspect that similar dynamics occur in the field, where frogs can gain, lose, and regain Bd infections (P.A. Burrowes, pers. comm.).

Experimental set-up

Frogs were randomly assigned to one of three treatments: (a) inoculation with Bd zoospores (n=12), (b) indirect transmission experiment (n=12), or (c) direct transmission experiment (n=11). For the Bd inoculation, a cryopreserved sample of Bd strain JEL427 was obtained. This strain was originally isolated from an infected *E. coqui* frog in El Yunque, Puerto Rico, and several passages were made in 1% tryptone broth. The Bd was grown on 1% tryptone agar plates for 5-7 days, plates were flooded with 1% tryptone broth, and zoospore concentration was estimated using both wells of a hemacytometer and averaging the counts. The zoospore solution was filtered to remove sporangia and diluted with purified water to obtain a concentration of 10^6 zoospores/ml. During inoculation, frogs were placed in 236 ml plastic deli cups with lids containing 10 ml of the Bd inoculate for 10 hours on two consecutive days. Each of 12 inoculated frogs was placed in individual terraria to develop their infections for 9 days, at which point all frogs were swabbed to determine infection status.

The direct and indirect transmission experiments were conducted simultaneously (Fig. 2.1). For the direct transmission experiment, on day 9 each infected frog was randomly placed into a terrarium containing an uninfected frog on a moss substrate. There were not enough females to have all male-female pairings, so 4 of 11 pairings were male-male. After frogs were housed together for 2 weeks, they were placed into individual clean terraria and infection intensity was monitored over time.

For the indirect transmission experiment, 12 additional uninfected frogs were randomly assigned on day 9 to each of the 12 empty terraria previously occupied by the inoculated animals, where they had presumably shed zoospores. Four of these terraria contained a bromeliad substrate, and the remaining 8 contained damp sphagnum moss. Both substrate types were included because water accumulated in bromeliad axils should support aquatic Bd zoospores. It was important to know if water was necessary for indirect transmission, and bromeliads are a common habitat for *E. coqui* in the wild. The frogs were kept in the Bd-contaminated terraria for 2 weeks before they were moved to new, clean terraria.

Direct transmission was defined as that occurring after close contact between individuals, since the frogs were not forced into physical contact and immediately separated during the experiment. A similar assumption was made in a study of *Rana muscosa*, where tadpoles were kept in small (20 x 30 cm) mesh cages suspended in a lake to assess the impact of tadpole density on Bd transmission (Rachowicz and Briggs 2007). It is recognized that both direct and indirect transmission is possible in terraria containing an infected and an uninfected frog.

All frogs were swabbed at the following intervals: day 0, 9, 16, 23, and 37. Day 1 of the experiment was the first inoculation day. Frogs were monitored daily to assess behavior and condition. At the conclusion of the experiment frogs were euthanized by immersion in 300 mg/l of tricaine mesylate (MS-222).

RESULTS

The average infection intensity of the 12 inoculated *E. coqui* frogs on day 9 of the experiment was 1,220 ± 613 zoospore genomic equivalents (Fig. 2.2). Two frogs developed clinical signs of chytridiomycosis and died 2 weeks after inoculation; a third frog was consumed by its cage mate in the direct transmission treatment. The remaining inoculated frogs cleared their infections or reduced them to very low levels (6.3 ± 4.6 genomic equivalents) by day 37, the end of the study.

Transmission was detected in both treatments (Fig. 2.3). The proportion of frogs that became newly infected with Bd during the experiment did not differ between the two treatments: 3/10 (30%) in the direct transmission treatment and 3/11 (27%) in the indirect transmission treatment (Fisher's exact test, p=1.0). Indirect transmission occurred in two moss and one bromeliad terraria, indicating that zoospores can survive outside the host long enough on these substrates to infect a new host. The average infection intensity of the newly infected frogs on day 23, marking the end of the two-week transmission treatments, was low (direct = 31.4 ± 18.9 Bd zoospore genomic equivalents, indirect = 16.8 ± 16.8 Bd zoospore genomic equivalents, and the infections were reduced or lost by the following sample date (Fig. 2.1).

DISCUSSION

In tropical forest sites where declines in amphibian biodiversity follow Bd epidemics, few species occur in water most of the time. Even frogs with aquatic larvae spend much of their lives resting or foraging on leaf litter, soil, rocky substrate, and vegetation (Lips et al. 2006). Understanding the likelihood of Bd transmission, both

direct and indirect, in terrestrial habitats is critical for monitoring, and perhaps eventually managing, amphibian populations declining due to Bd. These results show experimentally, for the first time, that amphibians can become infected with Bd in non-aquatic habitats via an environmental pathogen reservoir. Bd DNA has been detected in the environment by a number of researchers (Brem and Lips 2008, Cossel and Lindquist 2009, Hyman and Collins 2012), and the results presented here show that some of the Bd detected is likely to represent viable zoospores.

Given the low sample size, a possible relationship between infection intensity and likelihood of transmitting Bd could not be determined. The frog with the highest pathogen load, by an order of magnitude, was the only frog that transmitted Bd in both treatments, suggesting that infection intensity is an important driver of Bd transmission in terrestrial species as predicted for aquatic species (Briggs et al. 2010). *E. coqui* can die from chytridiomycosis with varying loads of Bd (see Chapter 3), and the many opportunities for indirect re-infection from humid substrates in tropical forests may explain how Bd can survive enzootically among frogs that can clear infection.

A larger study may have permitted investigation of the importance of habitat type in indirect transmission. Indirect transmission was observed in 1 of 3 terraria containing bromeliads and 2 of 8 terraria containing moss. Future indirect transmission experiments should include more replicates of each habitat type and also include a leaf litter treatment, which is a preferred habitat for many terrestrial direct-developing frog species (Wells 2007). In an ongoing study to assess the relationship between microhabitat use and Bd infection in eastern Puerto Rico, *E. coqui* with the highest infection levels are found in bromeliads (Burrowes, et al. manuscript in preparation). The relative importance of

different habitat types in supporting an environmental reservoir of Bd for terrestrial amphibians warrants further investigation.

The transmission rate was expected to be higher in the direct transmission treatment, because the inoculated frogs were exposed to a very high dose of Bd and animals were confined together in a small space for two weeks. *E. coqui* are territorial and it is likely that physical contact between the frogs was avoided, except in one case where an uninfected frog consumed its infected cage-mate (but it did not become infected). Direct Bd transmission between frogs that are amplexing, sharing retreat sites, or providing parental care may be higher (Longo et al. 2010).

None of the frogs in the direct transmission treatment sustained their new Bd infections through the end of the experiment. Although the frogs were not swabbed after 37 days, it is unlikely that the frogs in the indirect transmission treatment, or even the inoculated animals, would have sustained their infections much longer than 37 days. Adult coqui frogs have been readily observed to clear natural and induced Bd infections in the laboratory within a matter of weeks under normal climate conditions (Chapters 1-3). As a result, it is recommended that future experiments with terrestrial amphibians consider re-inoculating every 3-5 days to ensure infections are sustained. Similar infection dynamics are hypothesized to occur for *E. coqui* in its native range, where Bd is enzootic: frogs become infected via another frog or non-amphibian pathogen reservoir, clear infection if conditions are favorable, and re-gain infection readily at some point in the future. How infection dynamics change when conditions are unfavorable for frogs, such as prolonged dry spells, is an area ripe for investigation.

This study is a first step in understanding Bd transmission in terrestrial frogs, which is essential for predicting population-level impacts of epizootic and enzootic disease. The three species of *Eleutherodactylus* that disappeared in recent decades, possibly as a result of Bd, had greater affinity to water compared with other species (Burrowes et al. 2004). High levels of indirect transmission and continual re-infection via ambient zoospores may explain how terrestrial frogs can decline, even to extinction, in the face of endemic chytridiomycosis.

ACKNOWLEDGEMENTS

P. Burrowes, K. Lips, and J. Collins made significant contributions to this study. Many students in the Burrowes/Joglar laboratories and R. McGuire assisted with collection of frogs. C. Palmer, A. Lewis, C. Arlotta, and A. Khaldar worked diligently in the lab. J. Longcore provided Bd isolate JEL427 and D. Boyle kindly sent Bd standards. M. Langhammer created illustrations for the methods figure. A. Smith provided helpful comments on the chapter. Collection and export permits were granted by Hawaii DNLR (permit EX10-08) and Puerto Rico DRNA (permits 2009-IC-015, 2009-IC-014). Funding for this study was provided by an EPA STAR Fellowship and a P.E.O. Scholar Award.

Fig. 2.1. Experimental design for direct and indirect transmission treatments. Mean infection intensity during the 2-week transmission window is given for each inoculated frog. All frogs in the direct transmission treatment were housed on moss substrate.

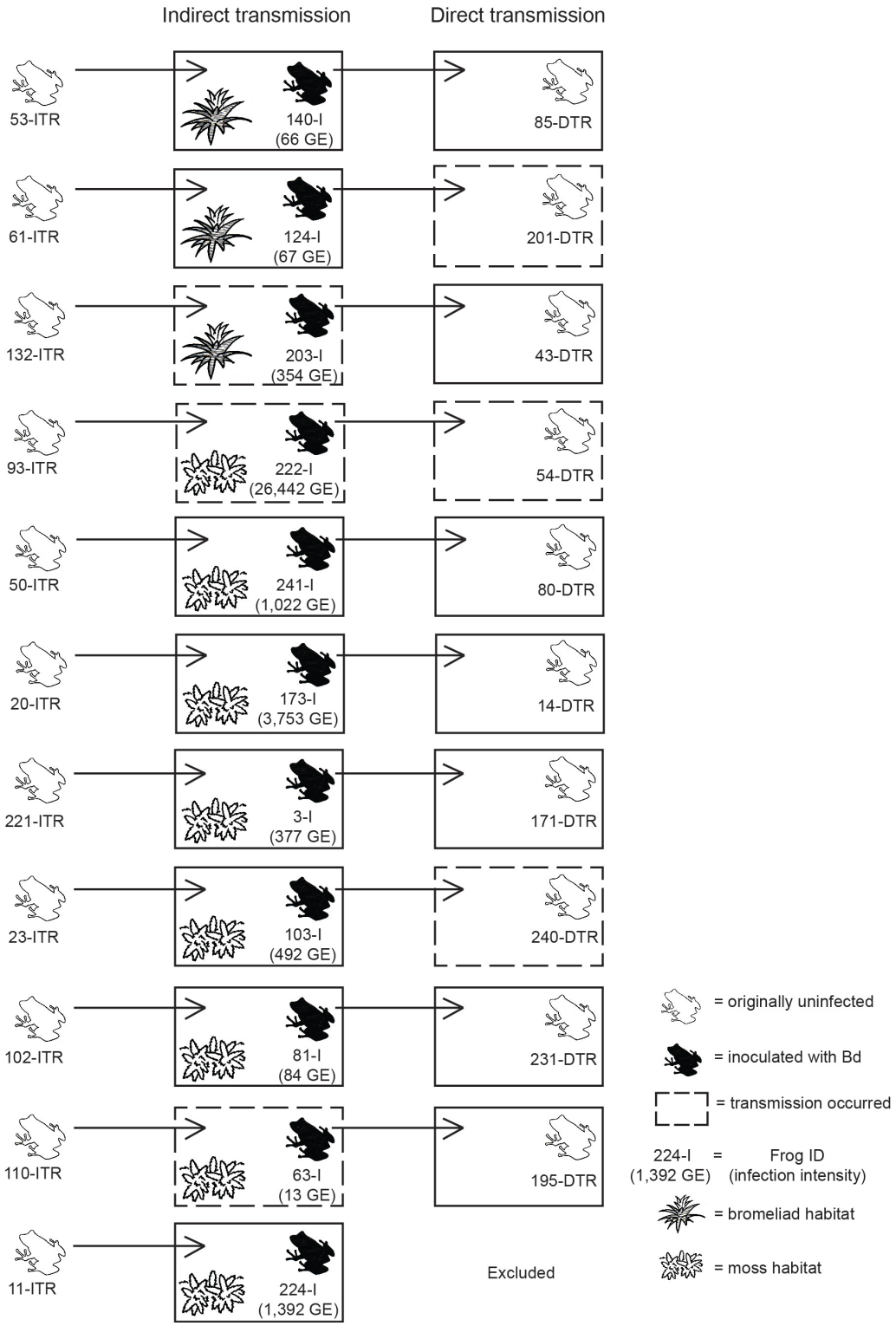


Fig. 2.2. Mean infection intensity over time (log Bd zoospore genomic equivalents) of inoculated and newly infected frogs in the direct and indirect transmission treatments. Inoculated frogs were exposed to Bd on day 1, the transmission treatments began on day 9, and frogs were separated and/or moved to clean terraria on day 23. Error bars represent standard error.

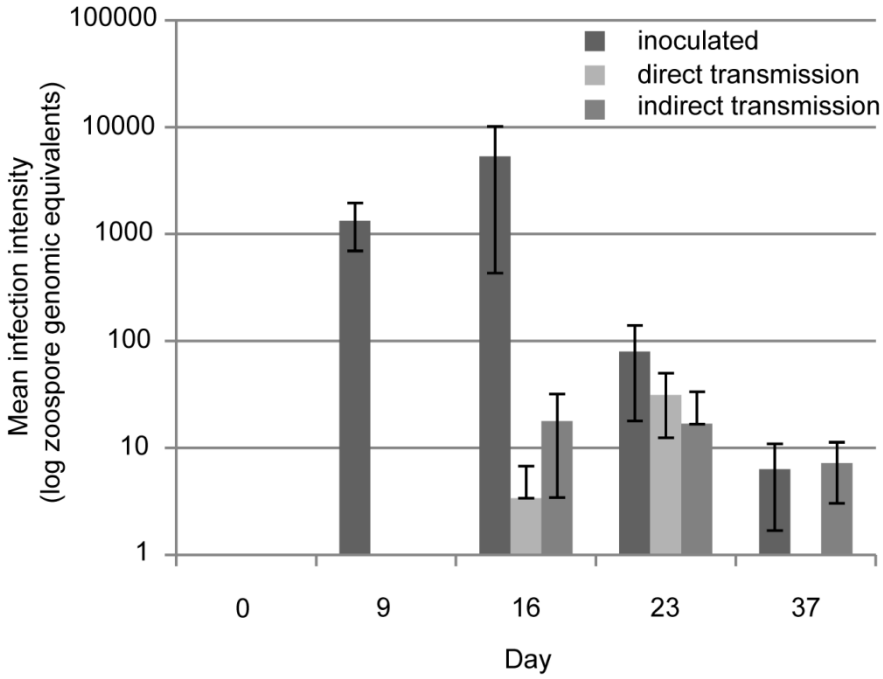
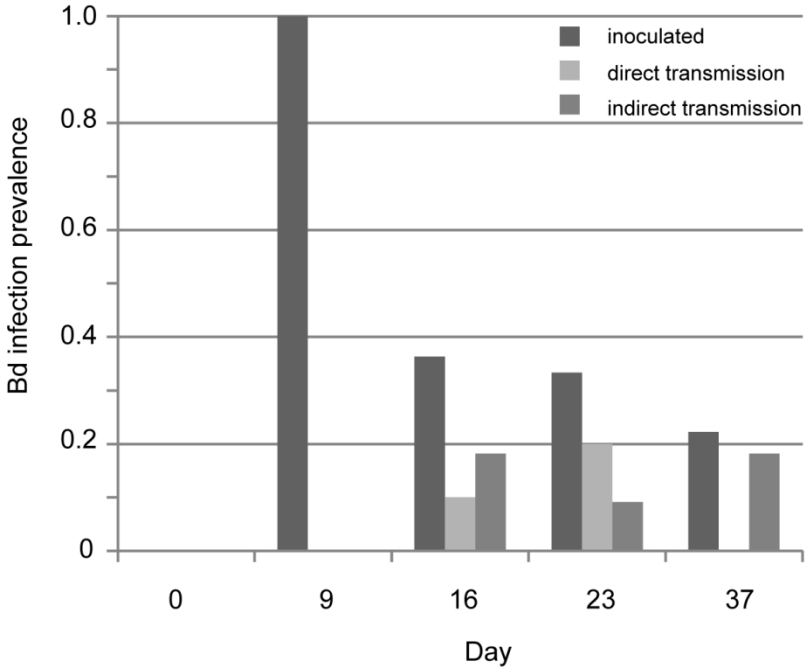


Fig. 2.3. Bd infection prevalence over time of inoculated and newly exposed frogs in the direct and indirect transmission treatments. Day 16 represents the first sampling date after exposure to either an infected frog or habitat.



Chapter 3

A FUNGAL PATHOGEN OF AMPHIBIANS, *BATRACHOCHYTRIUM DENDROBATIDIS*, ATTENUATES IN PATHOGENICITY WITH *IN VITRO* PASSAGES[1]

ABSTRACT

Laboratory investigations into the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), have accelerated recently, given the pathogen's role in causing the global decline and extinction of amphibians. Studies in which host animals were exposed to Bd have largely assumed that lab-maintained pathogen cultures retained the infective and pathogenic properties of wild isolates. Attenuated pathogenicity is common in artificially maintained cultures of other pathogenic fungi, but to date, it is unknown whether, and to what degree, Bd might change in culture. In this study, zoospore production over time was compared in two samples of a single Bd isolate having different passage histories: one maintained in artificial media for more than six years (JEL427-P39), and one recently thawed from cryopreserved stock (JEL427-P9). In a common garden experiment, two different amphibian species, *Eleutherodactylus coqui* and *Atelopus zeteki*, were exposed to both cultures to test whether Bd attenuates in pathogenicity with *in vitro* passages. The culture with the shorter passage history, JEL427-P9, had significantly greater zoospore densities over time compared to JEL427-P39. This difference in zoospore production was associated with a difference in

[1] This chapter was previously published: Langhammer PF, Lips KR, Burrowes PA, Tunstall T, Palmer CM, Collins JP. 2013. A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with *in vitro* passages. PLoS One 8: e77630.

pathogenicity for a susceptible amphibian species, indicating that fecundity may be an important virulence factor for Bd. In the 130-day experiment, *Atelopus zeteki* frogs exposed to the JEL427-P9 culture experienced higher average infection intensity and 100% mortality, compared with 60% mortality for frogs exposed to JEL427-P39. This effect was not observed with *Eleutherodactylus coqui*, which was able to clear infection. The differences in phenotypic performance observed with *Atelopus zeteki* are hypothesized to be rooted in changes of the Bd genome. Future investigations enabled by this study will focus on the underlying mechanisms of Bd pathogenicity.

INTRODUCTION

The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) causes the skin disease chytridiomycosis in susceptible amphibians (Berger et al. 1998) and has contributed to the decline and extinction of amphibian species worldwide (Skerratt et al. 2007). As a result, studies on this pathogen and many host species have increased greatly since Bd was described in 1998 (Collins and Crump 2009). Laboratory experiments in particular have provided insights into the differential susceptibility of host species to chytridiomycosis (Bishop et al. 2009, Searle et al. 2011), the role of environmental factors in disease dynamics (Bustamante et al. 2010, Murphy et al. 2011), methods of disease transmission (Rachowicz and Briggs 2007, Greenspan et al. 2012), pathogenicity of different Bd strains (Berger et al. 2005b, Retallick and Miera 2007, Farrer et al. 2011), and the mechanism by which Bd causes amphibian mortality (Voyles et al. 2009).

As with other pathogens, conducting laboratory experiments with Bd requires maintaining the pathogen in artificial media, because isolating a new strain for each

experiment is often not feasible or desirable. Studies in which host amphibians are intentionally infected with Bd often assume that *in vitro* cultures retain the properties of wild isolates, particularly the ability to infect hosts and cause disease (Brem et al. 2013). Several studies have shown that Bd isolates differ in pathogenicity for amphibian hosts in controlled laboratory exposure experiments (Berger et al. 2005b, Retallick and Miera 2007, Fisher et al. 2009, Farrer et al. 2011), and others have demonstrated that Bd isolates differ in phenotypic characters possibly linked to virulence (Fisher et al. 2009, Voyles 2011). However, it is not clear whether these differences are a result of variable environmental conditions faced by the isolates in nature or of different *in vitro* management and time since isolation (Berger et al. 2005b). Recent work has shown that the *in vitro* passage history of Bd strains can affect phenotypic traits potentially linked to pathogenicity (Voyles 2009).

Attenuated pathogenicity is common in artificially maintained cultures of fungi pathogenic to insects, plants, and humans (Butt et al. 2006, Safavi 2012), yet some fungal species remain pathogenic even after dozens of passages (Butt et al. 2006). The question of whether Bd attenuates in culture has received very little attention until recently, and it has been unclear whether, and to what degree, Bd attenuates in culture (Voyles et al. 2012). Brem et al. (2013) showed that an artificially maintained Bd isolate became more pathogenic in subsequent exposures after it was passed through an amphibian host, suggesting that Bd virulence attenuates in culture. However, these authors used a Bd strain originating from *Lithobates pipiens* to infect and then re-isolate Bd from *Scaphiopus holbrooki*, introducing a variable (i.e., the *Scaphiopus* epidermis) unrelated to *in vitro* versus *in vivo* maintenance that could have accounted for greater pathogenicity in

S. holbrooki in subsequent exposures. The conservative interpretation is that Brem et al. demonstrate phenotypic plasticity in Bd performance, which is a notable result.

The present study was initiated after several transmission experiments yielded unexpected results. *Eleutherodactylus coqui* experimental frogs expected to be susceptible to infection following high doses of Bd either did not become infected or cleared their infections rapidly. It was hypothesized that the Bd isolate used had attenuated in pathogenicity during 6 years of *in vitro* maintenance. Virulence is an emergent property of host, pathogen, and environment (Casadevall et al. 2011), and the design of this study was constructed to tease apart this integrated triangle of cause and effect. To that end, the approach differs from Brem et al. (2013) in several important ways. First, two samples of the same Bd strain (JEL427) were used, one that was cryopreserved upon isolation and one maintained as active culture since 2005. Next, variation in a phenotypic trait, zoospore production, was examined between these two cultures of the same Bd isolate with different passage histories (Voyles 2011). Zoospore production has been a focus of other studies comparing Bd isolates (Woodhams et al. 2008, Fisher et al. 2009, Voyles 2011) and the effects of passage history on a single isolate (Voyles 2009). This trait is also important because there is an intuitive, mechanistic link with pathogenicity: more zoospores should increase opportunities for transmission between hosts and re-infection of an individual host (Briggs et al. 2010).

Finally, the two cultures were exposed to the amphibian species from which JEL427 was isolated, *Eleutherodactylus coqui*, and a second species, *Atelopus zeteki*, under identical environmental conditions. *E. coqui*, a terrestrial direct-developing frog native to Puerto Rico, typically carries low to moderate Bd-infections where this strain is

enzootic (Longo et al. 2013), succumbs to chytridiomycosis in the laboratory (Longo et al. 2010), and is thought to be experiencing disease-related declines at mid-elevations in eastern Puerto Rico (Burrowes et al. 2004). *Atelopus zeteki* is a stream-associated frog with larval development endemic to Panama. Bd-related declines in the wild (La Marca et al. 2005, Lips et al. 2006) are common in this species and it is also highly susceptible to chytridiomycosis in the laboratory (Bustamante et al. 2010, Becker et al. 2011). Including *A. zeteki* in the study design yields a common garden experiment, a framework for testing the performance of two cultures of the same Bd isolate with different evolutionary histories under conditions in which the treatment environment and host were held constant (a "common garden"). This control over two elements of the virulence triangle allowed more confidence that differences in Bd performance resulted from variation in the third element of the triangle—genetics—and explore the possibility that variation in Bd's performance between two cultures of the same strain with different histories reflected genetic changes in Bd rather than phenotypic plasticity.

METHODS

The research herein followed guidelines set by the Institutional Animal Care and Use Committees of Arizona State University, the University of Maryland, and the Maryland Zoo in Baltimore. Collection and export permits were obtained from Hawaii DNLR (permit EX10-08) and Puerto Rico DRNA (permits 2009-IC-015, 2009-IC-014).

Strain phenotype experiment

Bd produces motile zoospores that infect amphibian skin. Zoospores encyst on the skin surface and develop into thalli within the skin. The mature zoosporangia then cleave

into new zoospores after 3-5 days (Berger et al. 2005a). To test if passage history yielded measurable phenotypic differences in Bd isolate JEL427, zoospore production over time was compared in two different samples of this single isolate (Voyles 2009). JEL427 was isolated in 2005 from an infected *Eleutherodactylus coqui* frog collected in the El Yunque forest of eastern Puerto Rico. Some of this original culture was cryopreserved (Boyle et al. 2003), and some of the culture was transferred into new solutions of 1% tryptone broth every 3-4 months and maintained at 4°C at the University of Maine by J.E. Longcore. In 2010, some of the JEL427 maintained as active culture since 2005 was obtained, because the cryopreserved sample was initially unavailable. The culture was subsequently maintained at 18°C for one year and passed into 1% tryptone broth monthly. This culture, which experienced a total of at least 39 passages between the two laboratories, will be hereafter referred to as "JEL427-P39".

In 2011, a sample of the cryopreserved JEL427 held at the University of Puerto Rico was revived by P.A. Burrowes for this experiment and transferred into 1% tryptone broth. It was then passed into new 1% tryptone broth monthly and maintained at 18°C. This culture, which experienced roughly 9 total passes, will be hereafter referred to as "JEL427-P9". Thus, these two cultures are the same age, originating in 2005 from the same source isolate, but have different passage histories. The selection pressure on the JEL427-P9 culture was effectively halted during cryopreservation.

Although the temperature at which Bd isolates are maintained in artificial media can influence zoospore production (Woodhams et al. 2008, Voyles et al. 2012), the prior maintenance of JEL427-P39 at a colder temperature was not considered to be a concern for the study, because the culture was maintained at 18°C for at least 15 months before

using it in the strain-phenotype and frog-exposure experiments. This procedure should have provided enough time for the JEL427-P39 culture to acclimate to the warmer temperature.

For the strain-phenotype experiment, the JEL427-P39 and JEL427-P9 cultures were grown on 1% tryptone agar plates using week-old broth culture. Plates were sealed and incubated at 18°C for 5-6 days, and on inoculation day, were flooded with 2 ml of sterile 1% tryptone broth and allowed to sit for 30 minutes while the zoosporangia released zoospores into solution. The solution was then collected from each plate using a sterile pipette and allowed to drain through a sterile #4 cone-style coffee filter lining a glass funnel, which allows zoospores to pass through but not zoosporangia (Myers et al. 2012). Separate filters and funnels were used for each culture to prevent cross-contamination, and the zoospore filtrate for each culture was collected in separate 50 ml sterile tubes.

The zoospore concentration of each filtrate was quantified using a hemacytometer (Hausser Scientific ® Bright-Line). Two concentration counts of total zoospores for each culture were made and averaged. The stock solutions were diluted to an identical starting concentration of 10^5 zoospores/ml. One milliliter of stock solution for each culture was transferred into 10 ml of 1% tryptone broth in sterile BD Falcon™ 25 cm^2 tissue culture flasks yielding 5 replicate flasks of the JEL427-P39 culture and 5 replicate flasks of the JEL427-P9 culture. Flasks were maintained at 18°C.

Zoospore density was quantified daily for 12 consecutive days for each replicate flask. Two concentration counts were made for each replicate per day using both chambers of the hemacytometer and averaging them. The same researcher (PFL) made

concentration counts each day to be consistent, but the order of flasks to be counted was randomized and kept unknown to the counter to avoid bias. The experiment was replicated a month later to ensure repeatability of results, and this time both motile and total zoospores were counted for each replicate daily. Zoospore density over time was compared using repeated-measures ANOVA in JMP® 10 software.

Eleutherodactylus coqui *exposure experiment*

Although the passage history of Bd strains is thought to cause measurable phenotypic changes in culture (Woodhams et al. 2008, Voyles 2009), it is unknown if these changes correspond to a difference in pathogenicity for an amphibian host. To answer this question, 36 adult common coqui frogs (*Eleutherodactylus coqui,* mean snout-vent length [SVL]=41.2 mm) were collected from El Yunque, Puerto Rico and near Hilo, Hawaii to use in an exposure experiment (18 frogs per site). Frogs were captured at night using plastic bags worn as gloves to prevent cross-contamination with Bd zoospores. In the laboratory, frogs were swabbed using sterile fine-tipped cotton swabs (Medical Wire & Equipment #113) to determine Bd infection status (Hyatt et al. 2007), and each animal was weighed, measured, and sexed.

The frogs in this study served as uninfected controls in a previous experiment, so all animals had been in captivity for approximately one year at the beginning of the experiment. The laboratory was maintained at 21°C on a 12-hour day-night cycle. Frogs were housed individually in 5.7 liter Sterlite plastic storage boxes lined with damp sphagnum moss. Each container had a water dish to maintain humidity and *Cecropia* leaves for refugia. Frogs were transferred to new terraria with clean sphagnum moss bi-weekly. Terraria were sterilized with a 10% bleach solution and rinsed three times before

re-use. Moss was autoclaved and rinsed thoroughly for re-use a maximum of three times, at which point it was discarded and new moss was used. Frogs were fed vitamin-dusted crickets *ad libitum* twice a week and terraria were misted daily.

All frogs were confirmed to be Bd-negative at the beginning of the study. DNA was extracted from skin swabs using PrepMan Ultra, and samples were analyzed using the standard real-time quantitative polymerase chain reaction (qPCR) assay (Boyle et al. 2004) modified by Hyman and Collins (2012). Frogs were given unique identification numbers and randomly assigned to three treatment groups: exposure to JEL427-P39 (n=12), exposure to JEL427-P9 (n=12), and exposure to a sham solution (control, n=12).

For the Bd exposure, both the JEL427-P9 and JEL427-P39 cultures were grown on 1% tryptone agar plates for 5-7 days, flooded with 1% tryptone broth, and filtered to obtain a pure zoospore stock solution, as described above. Zoospore concentration was determined by counting motile zoospores in both chambers of the hemacytometer and averaging the counts. The stock solutions were diluted with purified water to obtain a concentration of $1x10^5$ zoospores/ml for both cultures. The sham solution for the control group consisted of an equivalent amount of 1% tryptone broth (without zoospores) diluted with purified water. During inoculation, frogs were placed in 236 ml plastic cups with lids containing 10 ml of the JEL427-P9 culture, the JEL427-P39 culture, or the sham solution for 10 hours on two consecutive days. A new pair of nitrile gloves was used whenever a different frog was handled to prevent cross-contamination.

Frogs were swabbed and weighed every ~15 days, and morbidity and mortality were monitored daily for 80 days. To minimize suffering, moribund frogs were euthanized by immersion in 300 mg/l tricane mesylate (MS-222) when their righting

reflex was lost. For both *E. coqui* and *A. zeteki*, loss of righting reflex was the only reliable indicator of imminent death. Because the most important dependent variable in our study was survival time, euthanizing frogs sooner as they began exhibiting milder clinical symptoms of chytridiomycosis (e.g. darkening pigmentation) may have biased our experimental results. At the end of the experiment, inoculated frogs were euthanized with an overdose of Tricaine mesylate (MS-222) and uninfected control frogs were donated to the National Aquarium in Baltimore.

To compare the average infection intensity over time between frogs exposed to JEL427-P9 and JEL427-P39, a mixed effects model was designed with first order autocorrelation using the package 'nlme' in R (R Core Development Team 2008, Pinheiro et al. 2013). Infection intensity data were log-transformed prior to analysis. Time and passage history were treated as fixed effects and individual frog ID as a random effect. A second model was analyzed that included an interaction between time and treatment in the model.

Atelopus zeteki *exposure experiment*

A second exposure experiment was conducted using Panamanian golden frogs (*Atelopus zeteki*, mean SVL=46.5 mm), a species known to be highly susceptible to chytridiomycosis in the lab (Bustamante et al. 2010, Becker et al. 2011) and the wild (Lips et al. 2006). Seventy captive-bred *Atelopus zeteki* frogs, 15 months post-metamorphosis, were obtained from the Maryland Zoo in Baltimore with support of Project Golden Frog. Although this species is Critically Endangered (IUCN 2012), captive-bred surplus animals were approved for scientific research.

Frogs were allowed to acclimate to laboratory conditions for one month. Animal husbandry was identical to that described for *E. coqui*, with the following exceptions: frogs were fed fruit flies or small crickets every other day, terraria were changed weekly, and plastic refuges were used instead of leaves.

Frogs were randomly assigned to three treatment groups as in the previous experiment: exposure to JEL427-P39 (n=30), exposure to JEL427-P9 (n=30), and exposure to a sham solution (control, n=10). This experiment was undertaken in two phases, 5 weeks apart, with the treatment groups subdivided evenly, e.g. JEL427-P39 (phase 1: n=15, phase 2: n =15). The experiment was conducted twice to ensure repeatability of results. Since *A. zeteki* is highly susceptible to chytridiomycosis, frogs in the exposure groups were given a lower dose of Bd than in the *E. coqui* experiment (10^2 zoospores/ml in both phases) (Becker et al. 2011). As before, frogs were exposed to Bd zoospores in 236 ml plastic cups for 10 hours on two consecutive days. Frogs were swabbed and weighed every ~15 days, and morbidity and mortality were monitored daily for 130 days. At the end of the experiment, surviving frogs were reserved for use in a subsequent experiment by researchers at the University of Maryland.

RESULTS

Strain phenotype experiment

Data for both strain phenotype experiments were combined, since the only difference between them was a six-week difference in start date. The JEL427-P9 culture showed significantly greater zoospore density over time (repeated measures ANOVA, $p < 0.0001$; Fig. 3.1) compared to the JEL427-P39 culture. The trends were nearly identical

when counts were made using motile zoospores instead of total zoospores (data not shown).

Eleutherodactylus coqui *exposure experiment*

Given the results of the strain phenotype experiment, it was hypothesized that the higher zoospore output in the culture with shorter passage history would yield greater pathogenicity in susceptible amphibians. Specifically, it was predicted that JEL427-P9 would lead to higher Bd infection prevalence, infection intensity, and frog mortality than JEL427-P39.

On day 15 post-exposure, infection prevalence among *E. coqui* frogs was 67% in the JEL427-P9 group and 75% in the JEL427-P39 group, indicating that not all frogs became infected during exposure (Fig. 3.2a). Prevalence after day 15 dropped sharply, as frogs started clearing infection despite the large exposure dose. The results of the mixed effects model indicate no significant difference in mean infection intensity over time between the two groups (-0.937 log genomic equivalents, p=0.15). Finally, there was no significant difference in mortality between the three treatment groups, including the controls (Log-rank test, p=0.37). Only 1 frog died during the experiment, from the JEL427-P39 group. The frog may have succumbed to chytridiomycosis given its relatively high pathogen load (12,816 zoospore genomic equivalents), but all other Bd-exposed frogs cleared infection within 80 days.

Atelopus zeteki *exposure experiment*

The results with *E. coqui* motivated a second experiment with a more susceptible species, *Atelopus zeteki*, which was predicted to more readily become infected upon initial exposure to Bd. Given the much lower Bd dose used in the *A. zeteki* experiment, it

took longer for infections to build to detectable levels. Most *A. zeteki* frogs yielded Bd-negative swabs at day 15 but were positive on day 30, indicating a large proportion of false negatives up through day 15 (Fig. 3.2b). This suggests that researchers may be significantly under-sampling early or low-level Bd infections in the lab and field. Eventually, all but one Bd-exposed *Atelopus*, in the JEL427-P39 group, tested positive during the course of the experiment, while none of the control frogs became infected. By day 86, Bd prevalence among surviving frogs in the JEL427-P9 group reached 100%.

The mixed effects model in which time and passage history were treated as fixed effects revealed that *A. zeteki* frogs exposed to JEL427-P9 experienced higher average infection intensity over time compared to the JEL427-P39 group (3.22 log genomic equivalents, $p<0.01$). Including the treatment*time interaction decreased the model's AIC by 1, indicating only slight improvement.

Survival rate differed markedly between JEL427-P9 and JEL427-P39 groups. Most Bd-exposed *A. zeteki* eventually showed clinical signs of chytridiomycosis including skin sloughing, darkening pigmentation, weight loss, abnormal posture, lethargy, and ultimately loss of righting reflex, at which point frogs were euthanized. None of the *A. zeteki* frogs exposed to the JEL427-P9 culture survived (0/30), while 40% of the frogs exposed to the JEL427-P39 culture survived (12/30) the 130 day experiment (Fig. 3.4). However, all but one of the frogs exposed to JEL427-P39 were Bd-positive at the end of the experiment and eventually died while waiting to be used in a subsequent experiment. The JEL427-P9 frogs experienced shorter survival times following inoculation than JEL427-P39 frogs (Log-rank test, $p < 0.0001$), and all control frogs (10/10) survived the full 130 days.

DISCUSSION

Concern about the possible degeneration of artificially-maintained Bd isolates has surfaced relatively recently (Fisher et al. 2009, Voyles 2009, Brem et al. 2013), as researchers noticed that some Bd cultures no longer infected or caused disease in amphibian subjects (Brem et al. 2013). Attenuation is well known in fungi pathogenic to insects, plants, and humans, but other species of fungi show no loss of pathogenicity after dozens of passages (Butt et al. 2006). This study combined strain phenotype and amphibian exposure experiments using a single Bd isolate to demonstrate that an experimental Bd isolate attenuated in pathogenicity when maintained in artificial media. *A. zeteki* frogs exposed to a sample of JEL427 cryopreserved upon isolation experienced greater infection intensity over time, died sooner, and had 100% mortality in the 130 day experiment, compared with frogs exposed to a sample of the same isolate with a longer *in vitro* passage history. This effect was not apparent in *E. coqui*, a species that is currently persisting with low to moderate Bd-infections where this strain is endemic (Longo et al. 2013), as all but one frog survived and cleared infection across both treatments.

This difference in Bd pathogenicity for *A. zeteki* was associated with phenotypic differences between the two cultures, specifically zoospore production over time. A previous study (Voyles 2009) demonstrated that passage history can affect the zoospore production of a single Bd isolate, and it was hypothesized that this might translate into variation in pathogenicity based on a culture's history. Other fungal pathogens exhibit reduced sporulation when maintained in artificial media (Lord and Roberts 1986, Butt et al. 2006, Safavi 2012). This study predicted that the JEL427 culture with the shorter

passage history would have higher fecundity for two reasons. First, evolution of JEL427-P9 was effectively halted during cryopreservation, making it more likely that this culture retained the infective and pathogenic capabilities of wild type Bd. Second, cultures were transferred into new media monthly, when zoospore production was low due to diminishing food supply. In contrast, Voyles (2009) transferred cultures into new media weekly when zoospore density was high, likely selecting for greater zoospore output over time. The *in vitro* strain phenotype experiments showed that the JEL427-P9 culture indeed produced significantly more zoospores/ml over time than the JEL427-P39 culture.

Data showing that average infection intensity in *A. zeteki* frogs exposed to JEL427-P9 was significantly greater for the JEL427-P39 group further suggests that zoospore production is an important component of Bd pathogenicity. In studies with other susceptible species, individual amphibians with the greatest pathogen loads are more likely to succumb to chytridiomycosis (Voyles et al. 2009, Vredenburg et al. 2010). However, factors that co-vary with increased zoospore production may have led to the greater pathogenicity of the JEL427-P9 culture. Sporangium size, rather than zoospore production, was associated with pathogenicity of Bd isolates in a previous study (Fisher et al. 2009). A recent study of the entomopathogenic fungus *Beauveria bassiana* showed that attenuation in artificial media was characterized by both reduced sporulation and a decline in the activity of a spore-bound alkaline serine protease, which is known to be important in penetration of insects and subsequent pathogenicity (Safavi 2012). Genomic analyses have uncovered expansions in protease gene families in Bd relative to other fungi, leading researchers to hypothesize that proteases play an important role in Bd

pathogenicity (Rosenblum et al. 2008, Joneson et al. 2011). More work is needed to resolve the relationship between zoospore production and Bd pathogenicity.

Because it is not clear how long it takes for Bd to attenuate in culture, or even which strains will or will not attenuate, freshly isolated Bd or cryopreserved stock should be used in amphibian exposure experiments (Brem et al. 2013), especially those designed to test for susceptibility to Bd. The experiments with *A. zeteki* and *E. coqui* showed that artificially maintained isolates may still kill frogs, but only if the species is highly susceptible.

This work further highlights the importance of choosing an appropriate amphibian host species in Bd challenge experiments. The first frog exposure experiment, with *E. coqui*, shed no light on the affect of passage history on Bd pathogenicity. All but one *E. coqui* frog cleared infection within 60 days after exposure to a high dose of Bd, despite being the original source species for JEL427. This finding suggests that species surviving an epidemic can develop resistance or tolerance to the original source of an infection, but may still be vulnerable if a new strain is introduced or environmental conditions change. Conversely, in the exposure experiment with *A. zeteki*, a difference in pathogenicity between the cultures with different passage histories was evident. This species shows high susceptibility to Panamanian Bd strains in the field (Lips et al. 2006) and lab (Becker et al. 2011), and it proved highly susceptible to JEL427 from Puerto Rico.

Most importantly, this research lays the groundwork for future investigations into the mechanisms of Bd virulence. It is hypothesized that the differences in phenotypic performance observed in this study are rooted in changes of the genome. Maintenance in artificial media may impose intense selection pressure on genes regulating production of

zoospores or spore-bound proteases. Whole genome sequencing will enable comparisons between a pathogenic and an attenuated version of these isolates (Rosenblum et al. 2008, Joneson et al. 2011), potentially providing insights into the genetic determinants of pathogenicity and help explain the genetic variation and performance among Bd lineages (Farrer et al. 2011, Rosenblum et al. 2013). Better understanding of the mechanisms of Bd pathogenicity will strengthen our ability to manage a disease having widespread and often devastating impacts on amphibian species (Schloegel et al. 2006, Vredenburg et al. 2010) and communities (Crawford et al. 2010).

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Fig. 3.1. Mean zoospore densities over 12 days for two cultures of the same Bd strain with different passage histories: JEL427-P9 (~9 passes) and JEL427-P39 (~39 passes). Error bars represent standard error.

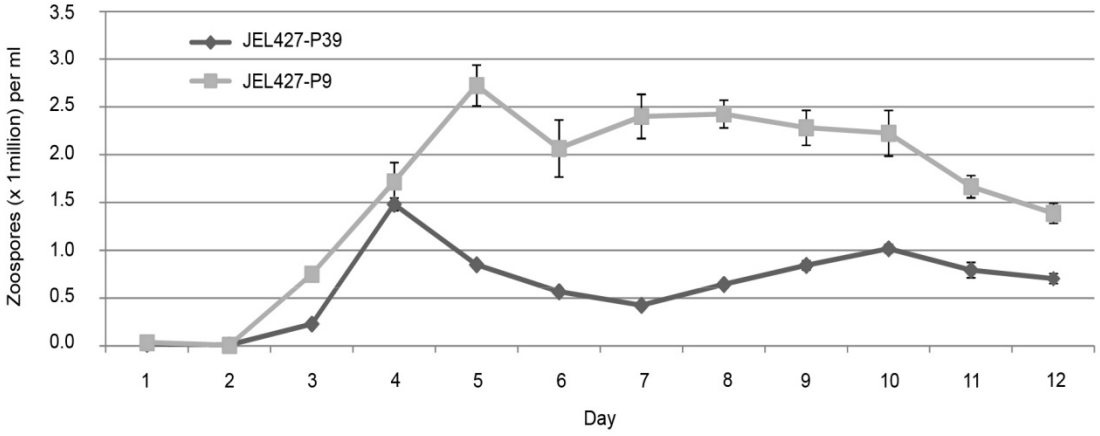


Fig. 3.2. Prevalence over time of Bd infection in (a) *Eleutherodactylus coqui* and (b) *Atelopus zeteki* exposed to either the JEL427-P9 or the JEL427-P39 culture.

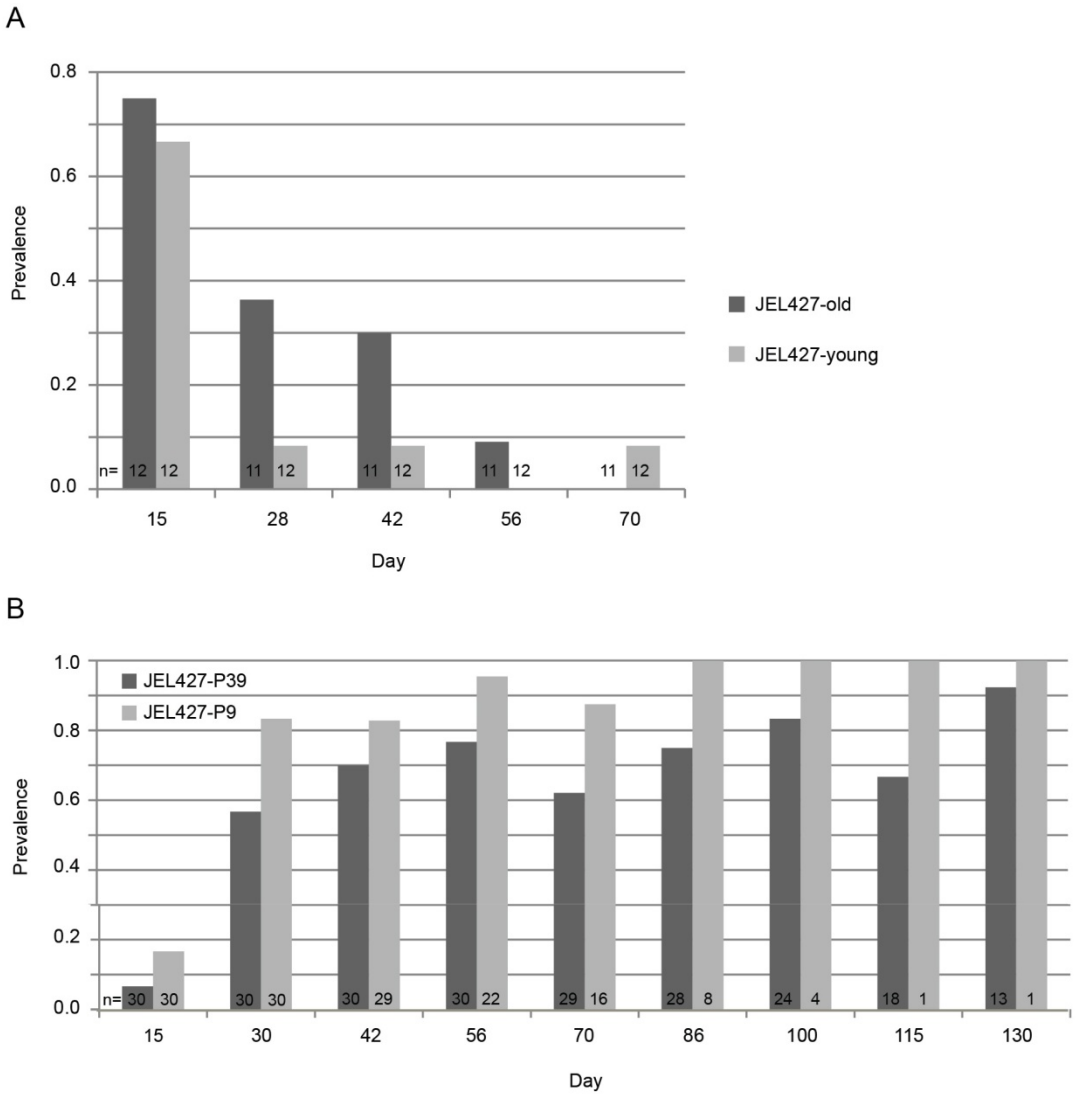


Fig. 3.3. Average infection intensity over time for (a) *Eleutherodactylus coqui* and (b) *Atelopus zeteki* frogs exposed to either the JEL427-P9 culture or the JEL427-P39 culture.

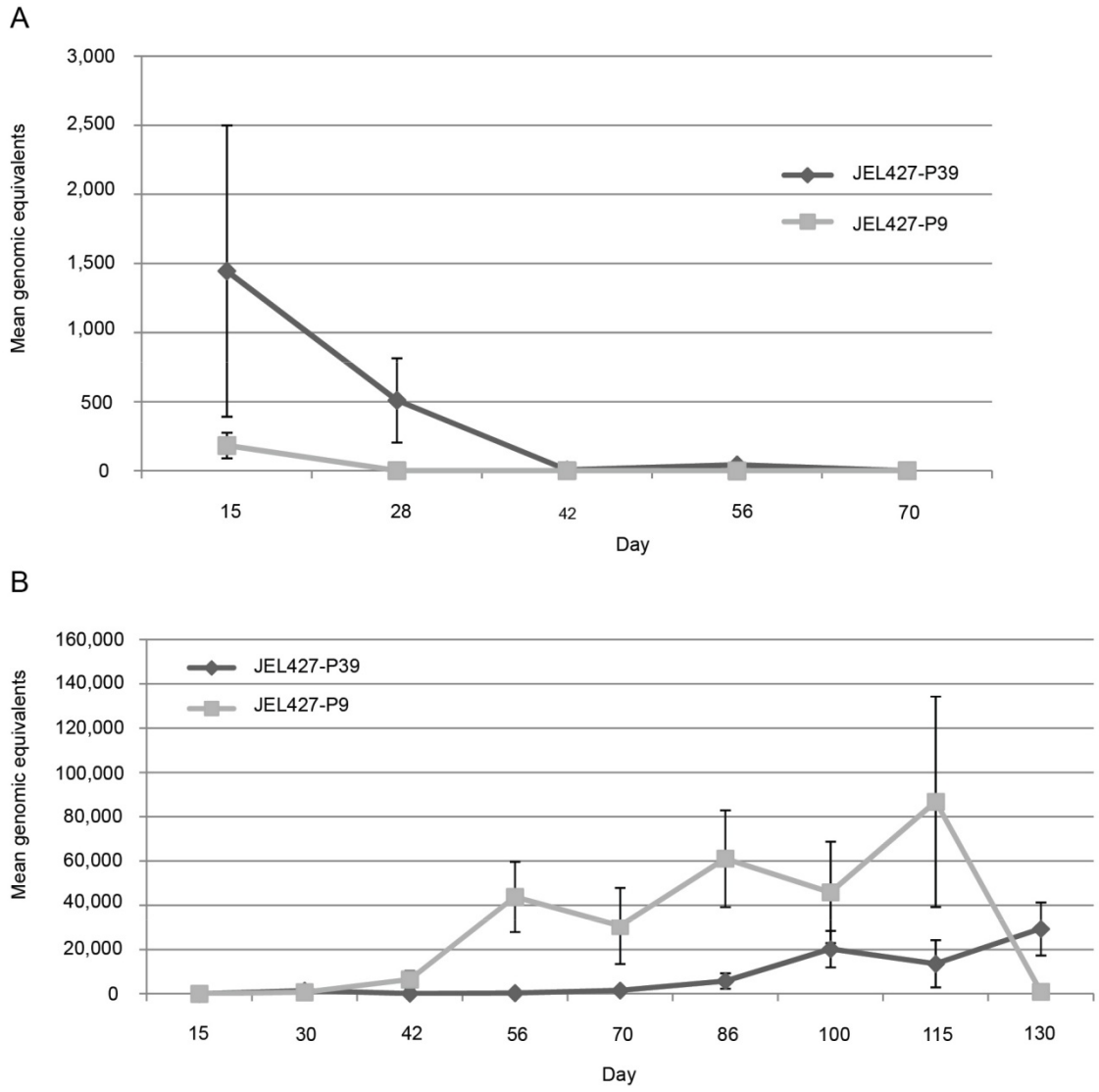
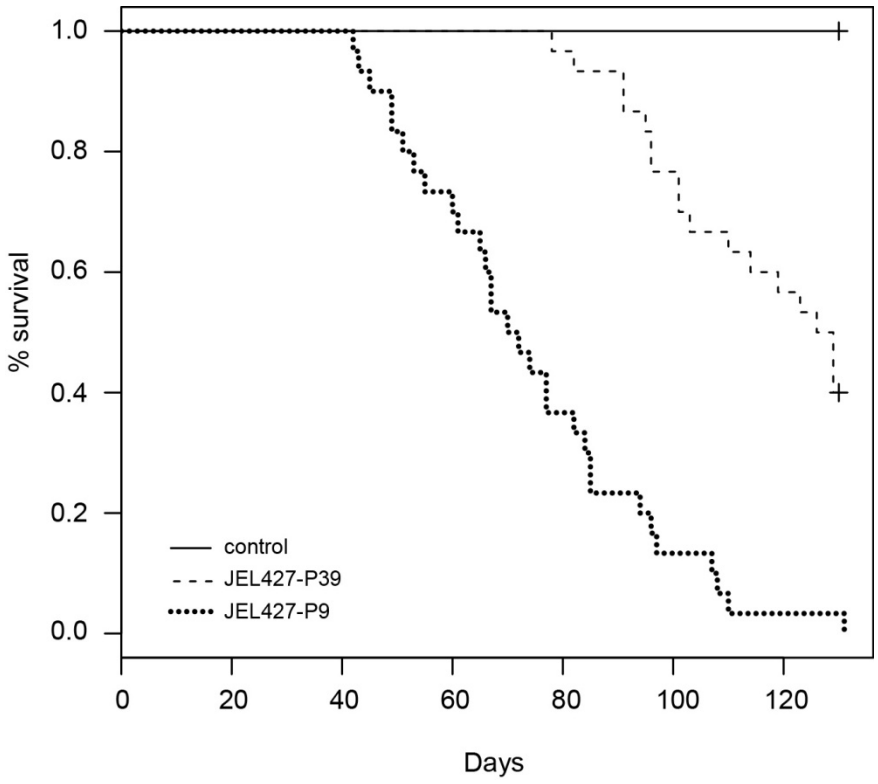


Fig. 3.4. Survival pattern for *Atelopus zeteki* frogs exposed to two versions of the same Bd strain with different passage histories, JEL427-P9 (n=30) and JEL427-P39 (n=30), or to a sham solution (n=10, control).



Chapter 4

POPULATION-LEVEL IMPACTS OF ENDEMIC CHYTRIDIOMYCOSIS ON *ELEUTHERODACTYLUS COQUI*

ABSTRACT

Mathematical models are often used to study emerging infectious diseases of wildlife with the aim to identify key features of host-pathogen dynamics that can inform conservation and management actions. For more than two decades amphibians have declined, even to extinction, from chytridiomycosis, and mathematical models have proven useful for evaluating the impact of infectious disease on amphibian populations and for identifying critical system parameters to target in field and laboratory research. All current models of chytridiomycosis focus on temperate species with seasonal breeding, aquatic larvae, and one mode of pathogen transmission. In this study a continuous-time deterministic model is developed to explore the impact *Batrachochtyrium dendrobatidis* (Bd), the fungal pathogen that causes chytridiomycosis, on a suite of Puerto Rican frogs that breed throughout the year, develop directly with no larval stage, and experience both direct and indirect disease transmission. The model is parameterized with data from lab experiments in Chapters 1 and 2 and long-term field studies of *Eleutherodactylus coqui*. Model analysis revealed that high rates of direct and indirect transmission drive the population to very low levels, but indirect transmission must be accompanied by high zoospore production or long zoospore lifespan to drive populations below an "extinction" threshold. Except under these high rates of indirect transmission and zoospore production (or low rate of zoospore mortality), coexistence of

host and pathogen is possible for all values of the pathogen-induced mortality rates. Because juveniles are the life stage more susceptible to chytridiomycosis, recruitment rates of both susceptible and infected juveniles into the adult class are major determinants of adult population size following pathogen introduction. This work identifies priorities for future field and laboratory investigations on direct-developing frogs persisting with the amphibian chytrid fungus.

INTRODUCTION

For more than a century we have recognized the importance of mathematical modeling for understanding the dynamics of infectious diseases in humans (Anderson and May 1991). Wildlife biologists have also adopted models to study emerging infectious diseases (Hudson et al. 2002), recently recognized as a major threat to biodiversity (Daszak et al. 2000). A case in point is chytridiomycosis, an emerging infectious disease implicated in the global decline and extinction of amphibians (Collins and Crump 2009). Since the discovery of the fungal pathogen that causes chytridiomycosis, *Batrachochytrium dendrobatidis* (Bd), in the late 1990s, the impact of the disease on frog populations has been explored using various mathematical models (Briggs et al. 2005, Emmert and Allen 2006, Mitchell et al. 2008, Briggs et al. 2010, Fisher et al. 2012, Doddington et al. 2013). Most of these models focus on temperate amphibian species with seasonal breeding, aquatic larvae, and one mode of pathogen transmission.

Modeling can be especially powerful when combined with field and laboratory investigations in an iterative manner. Data from lab experiments and field studies can

parameterize models, and model analysis uncovers sensitive parameters that would benefit from additional field or lab work (Plowright et al. 2008). Modeling also often leads to better-targeted research and novel hypotheses. Briggs et al. (2005) first modeled chytridiomycosis. They used an individual-based model to understand how some populations of the mountain yellow-legged frog (*Rana muscosa*) could be driven extinct by Bd while others persisted with the pathogen. They showed that for populations to persist with Bd following an outbreak, some infected individuals must survive through metamorphosis in the wild, an occurrence not observed in the lab where mortality reaches 100%. In a second model, Briggs et al. (2010) tracked the number of zoospores in the environment and the pathogen load on individual mountain yellow-legged frogs (*Rana sierrae*) frogs. The authors found that the long-lived tadpole stage can promote pathogen persistence by acting as a biotic reservoir and that epidemic vs. endemic dynamics alone were sufficient to explain the persistence of some frog populations and not others.

Emmert and Allen (2006) used a discrete-time model to explore the impacts of Bd on an explosive-breeding amphibian species by tracking density of juveniles, adults, and an environmental pool of zoospores. The authors assumed no recovery from infection, that Bd survives and reproduces on the keratin of dead animals, and that amphibians mature sexually after birth. The researchers found that the birth rate and the transmission rate are key to determining infection outcome, and host extinction can occur when disease reduces the host population to such low levels that stochastic factors become important.

Mitchell et al. (2008) explored the effect of a free-living stage of Bd on the European common toad (*Bufo bufo*), a pond-breeding species with aquatic larvae. Bd can

survive for several weeks in sterilized water (Johnson and Speare 2003). Their model revealed that in habitats with free-living zoospores the chances of host population extinction increased greatly. Zoospore mortality and production rate were key parameters controlling the outcome of pathogen introduction in their model.

Extending the Mitchell et al. (2012) model to the Majorcan midwife toad (*Alytes muletensis*), Doddington et al. (2013) showed that local environmental factors such as temperature were more important than genetic differences between host populations or Bd strains in determining the outcome of a pathogen introduction. Finally, in a generic two-species model, Fisher et al. (2012) demonstrated that high densities of a Bd-tolerant species acting as a pathogen reservoir led to extinction of Bd-susceptible species.

Collectively, these models suggest that a biotic or abiotic pathogen reservoir combined with high transmission and high mortality of metamorphs can lead to host extinction. However, aside from the generic model in Fisher et al. (2012), all of these models were developed for temperate species with an obligate aquatic larval stage. The biology of direct-developing amphibians may require a different approach. Bd infects the keratinized tissue of amphibian skin (Longcore et al. 1999), which is restricted to tadpole mouthparts. As a result, tadpoles are generally much less susceptible than metamorphosed frogs to Bd (Berger et al. 1998). Direct-developing frogs lack a tadpole stage, and newly-hatched juveniles, likely newly metamorphosed frogs, have keratin throughout their skin. However, the hatchlings of direct-developers are typically much smaller than metamorphs. Juvenile amphibians are thought to have underdeveloped immune systems compared to adults (Rollins-Smith et al. 2011), making this life stage potentially more vulnerable to Bd (Chapter 1). Finally, most direct-developing frogs

breed year-round and occupy terrestrial habitats where transmission dynamics are likely different than in ponds or streams.

In this chapter I develop a mathematical model as one way to project the impact of chytridiomycosis on direct developing frogs in Puerto Rico, where Bd is endemic (Longo et al. 2010). Three island species— *Eleutherodactylus karlschmidti*, *E. jasperi*, *E. eneidae*—are recently extinct and chytridiomycosis is a suspected cause (Burrowes et al. 2004). Eight other *Eleutherodactylus* species are globally threatened with extinction, matching trends across the Caribbean as a whole (IUCN 2012). My model builds upon previous work but differs in several important ways. First, it explicitly includes direct and indirect disease transmission; laboratory studies demonstrate the importance of both transmission modes (Chapter 2). Second, juvenile and adult stages can die or recover from infection as shown in Chapter 1 and in recent field work (Longo et al. 2010, Longo et al. 2013). Finally, I parameterized the model with data collected on *E. coqui*, making this the first model for direct developing amphibians, a common life history strategy among amphibians in the humid tropics (Duellman and Trueb 1986).

I use the model to assess the conditions most likely to lead to population extirpation from infectious disease, host persistence with an infectious disease, or pathogen-free hosts. I also address the following questions: Over time how does a high Bd-induced mortality rate of juveniles affect frog populations with endemic Bd? What is the impact of simultaneous direct and indirect disease transmission on a population?

METHODS

Common coqui life cycle

Eleutherodactylus coqui is a terrestrial breeder with direct development. Lacking an aquatic tadpole stage, juvenile *E. coqui* emerge from the egg as tiny, fully formed juveniles. Frogs reach sexual maturity after approximately one year (Joglar 1998). Breeding occurs throughout the year but decreases in the dry season, as reproductive effort is affected by temperature and rainfall. Male frogs provide parental care to developing eggs. Females lay 4-6 clutches per year with a mean clutch size of 27; hatching success averages 60% (Townsend and Stewart 1994). Roughly 81% of juvenile frogs die within the first year, while the annual mortality rate of adult *E. coqui* frogs is 94% (Stewart and Woolbright 1996).

Model description

To capture the dynamics of this life cycle, a susceptible-infected-susceptible (SIS) compartment model (Kermack and McKendrick 1927) was adapted to the ecology of the Bd-coqui system. I use a continuous time model because frog reproduction and Bd infection occur throughout the year (i.e., cohorts overlap). The model tracks the population density of susceptible and infected individuals of the non-reproductive juvenile class (S_J, I_J), the reproductive adult class (S_A, I_A), and an environmental reservoir of Bd zoospores (Z).

Experimental work in Chapter 1 showed that juveniles and adults can die from Bd or recover from infection, and parameters governing the Bd-induced mortality rates (α_J, α_A) and recovery rates from infection (γ_J, γ_A) are included. Unlike earlier efforts, the Bd-coqui model allows for indirect transmission via an environmental reservoir of zoospores

(at rate ε) and/or direct transmission through animal-animal contact (at rate β), which is supported by experimental results in Chapter 2. Infected frogs shed Bd zoospores at a constant rate (ϕ), and zoospores die at a constant rate (μ). For simplicity, I assumed no direct transmission between life stages because juveniles and adults typically occupy different microhabitats in the forest. However, vertical transmission from attending fathers to newly hatched juveniles is untested and could be incorporated into future versions of the model.

To model the effects of endemic chytridiomycosis on an *E. coqui* population over decades, I included parameters governing population birth rate (b), natural mortality rate of adults and juveniles (d_J, d_A), and recruitment rate of juveniles into the adult class (χ_S, χ_I). The recruitment rates represent the proportion of individuals surviving to sexual maturity within a year. The birth rate is limited in a density-dependent manner by adult carrying capacity (K), based on the field observation that *E. coqui* reproduction is limited by nest site availability (Townsend and Stewart 1994).

The Bd-coqui model (Fig. 4.1) is a system of six equations:

$$dS_J/dt = b\Psi N_A(1-(N_A/K)) - d_J S_J - \varepsilon S_J Z - \beta_J S_J I_J - \chi_S S_J + \gamma_J I_J$$

$$dI_J/dt = \varepsilon S_J Z + \beta_J S_J I_J - d_J I_J - \alpha_J I_J - \chi_I I_J - \gamma_J I_J$$

$$dS_A/dt = \chi_S S_J - \varepsilon S_A Z - \beta_A S_A I_A - d_A S_A + \gamma_A I_A$$

$$dI_A/dt = \chi_I I_J + \varepsilon S_A Z + \beta_A S_A I_A - d_A I_A - \alpha_A I_A - \gamma_A I_A$$

$$dZ/dt = \phi I_A + \phi I_J - \mu Z$$

$$N_A = S_A + I_A$$

Parameter estimation

I used data from long-term published field studies of *E. coqui* to estimate frog life history parameters. Yearly female reproductive output is the product of the mean number of egg-laying events annually (π=5), clutch size (θ=27), and hatching success (ρ=0.6) (Townsend and Stewart 1994). The birth rate is thus calculated as $b=\pi*\theta*\rho=81$ yr^{-1} or 0.22 $dy^{-}1$. The birth rate is multiplied by an additional constant representing survival probability of juveniles in the immediate post-hatching period (Ψ). The value of 0.3 is an estimate based on the mortality rate of very young juveniles in the lab (chapter 1) and predation rates in the field (Stewart and Woolbright 1996). These juveniles, which are <4 weeks old, are likely unaccounted for in published juvenile mortality rates because they are extremely difficult to see and mark. Excluding this parameter leads to an unnaturally high transition rate from eggs to young juveniles. The natural mortality rates for juveniles and adults are from yearly survival data (Stewart and Woolbright 1996) (d_J=0.81 yr^{-1} or 2.2^{-3} dy^{-1}; d_A=0.94 yr^{-1} or 2.5^{-3} dy^{-1}). I set adult carrying capacity (K) at 4000 frogs per hectare, which approximates mean density of adults at one site in northeastern Puerto Rico (Stewart and Woolbright 1996).

The recruitment rate of susceptible juveniles was informed by juvenile survival data (Stewart and Woolbright 1996) and estimates that juveniles reach sexual maturity in about one year (Joglar 1998). As explained above, the survival rate may underestimate mortality of the juveniles in the immediate post-hatching period, and thus in this analysis, the yearly recruitment rate of susceptible individuals is assumed to be χ_S=0.16 yr^{-1} (or 4.4^{-04} dy^{-1}). I assumed that the recruitment rate of infected juveniles was about half that of susceptible juveniles, χ_I=0.07 yr^{-1} (or 2.0^{-04} dy^{-1}) because their survival rate was much

lower in the lab (Chapter 1). Infected juveniles may also take longer to reach sexual maturity. The uncertainty surrounding these parameters necessitated exploring them over a large range of values.

The pathogen-induced mortality rates of adults and juveniles are estimates drawn from experimental data in Chapter 1. Bd-induced mortality over the 77 day experiment in excess of the control group was 25% for juveniles and 0% for adults (α_J=0.25/77=0.003 dy^{-1}; α_A=0/77=0 dy^{-1}). The mortality rate for juveniles estimated by this experiment is likely low due to poor survival of control frogs in the experiment perhaps as a result of poor nutrition (J. Stabile, pers. comm.). For purposes of this analysis, the default rate of juvenile Bd-induced mortality was increased to α_J=.0065 dy^{-1}, but I explored limits of 0-0.01 dy^{-1}. In addition, because adult coqui can die from Bd infections in the lab (Longo et al. 2010) and field (Longo et al. 2013) I explored α_A from 0-0.003 dy^{-1} . The recovery rate from Bd infection was estimated as the proportion of Bd-exposed frogs that cleared infections during the 77 day experiment in Chapter 1 ($\gamma_{J=}$0.0005 dy^{-1}; γ_A=0.01 dy^{-1}).

Chapter 2 experiments demonstrated that direct and indirect transmission is possible in *E. coqui*. The transmission probability in each experiment was 30% over 14 days. However, the transmission rate is a product of the probability of transmission and the contact rate, and there are no field data estimating the contact rate between infected and susceptible frogs or between ambient zoospores and susceptible frogs. For this model I used the range of transmission rates explored in Fisher et al. (2012) (β=1.0^{-6} dy^{-1*Ij}, ε=1.0-7 dy^{-1*Z}) and varied them over three orders of magnitude to accommodate the uncertainty in transmission rate estimation.

Estimates of the lifespan of Bd zoospores vary from a mean of about 24 hours (Piotrowski et al. 2004) to at least 4 weeks in sterilized tap water and 7 weeks in sterilized lake water (Johnson and Speare 2003). In the terrestrial habitats frequented by *E. coqui*, standing water is restricted to sites such as tree holes or the axils of plants. In contrast, temperate ponds provide ample aquatic sites where Bd might persist for long periods. Therefore, I used 24 hours as the estimated value for zoospore mortality rate (μ=1 dy^{-1}), but μ was explored over limits reflecting these extremes (0.04-1 dy^{-1}).

Previous studies estimating zoospore production rate directly by placing an infected frog in a known volume of water for a period of time and then filtering the zoospores have widely varying results (Mitchell et al. 2008, Reeder et al. 2012). Similarly, zoospore loads of infected *E. coqui* (Chapters 1-3) assessed by qPCR analysis of skin swabs taken from naturally- and lab-infected frogs, vary greatly. To accommodate this uncertainty, I used the same zoospore production rate as Fisher et al. (2012) (ϕ=10 dy^{-1}), but varied it over three orders of magnitude. See Table 1 for a description of all parameters I used in the model, their estimated values, and limits.

Model analysis

I conducted model analysis in R language and environment for statistical computing (R Core Development Team 2008). I explored each parameter's sensitivity by simultaneously varying it and the transmission parameter ε across a biologically meaningful range of values while holding all other parameters constant at the values given in Table 1. I solved the system of equations described earlier to estimate host population size at equilibrium for each parameter combination (see Appendix C for analytical code). Initial conditions for all model analyses were: Sj=529, Ij=1, Sa=99,

Ia=1, Z=1, reflecting a realistic initial ratio of juveniles to adults (Stewart and Woolbright 1996). My analyses showed that the model is not sensitive to initial conditions.

I explored parameter combinations that could lead to (a) pathogen free conditions, (b) coexistence of host and pathogen, or (c) host extinction from Bd. For this analysis, I set a threshold number of adult frogs below which the population is effectively extinct due to stochastic factors because in this deterministic model the host population cannot reach zero. My extinction threshold was 50 adult frogs, which is consistent with Mitchell et al. (2008).

RESULTS

Introducing Bd into an *E. coqui* population can cause an epizootic leading to a permanent reduction in host population size (Fig. 4.2). The system has two equilibria: a pathogen-free state in which Bd cannot establish, and coexistence of host and pathogen at reduced host numbers. At low rates of direct or indirect transmission, the pathogen-free equilibrium is stable. As the rate of direct or indirect transmission increases, a bifurcation point is reached where the disease-free equilibrium becomes unstable and the coexistence equilibrium becomes stable (Fig. 4.3). The Bd-free state does not exist if both types of transmission occur in the population for the parameter values explored in this analysis. Varying the initial density of zoospores up to 10^{12} per hectare suggests this terrestrial system does not have a threshold number of zoospores that must be exceeded in order for an epizootic to occur; rather, transmission rates drive the dynamics for *E. coqui*.

In addition to the transmission rates β and ε, the model is highly sensitive to zoospore production rate ϕ, and zoospore mortality rate μ (Fig. 4.4a,b). For a given rate

of transmission, changing the zoospore production rate or the zoospore mortality rate has a large effect on number of adult frogs at equilibrium. High rates of direct and indirect transmission drive the population to very low levels, but only high zoospore production or long zoospore lifespan combined with high indirect transmission drives the population below the "extinction" threshold of <50 mature individuals (Fig. 4.5).

I predicted that disease-induced mortality rates of adults (α_A) and juveniles (α_J) would have a large effect on model outcomes given previous results (Briggs et al. 2005, Doddington et al. 2013), but my model was not particularly sensitive to either of these parameters. For a given transmission rate (ε), varying the Bd-induced mortality rate of adults has a relatively small effect on equilibrium population size, as evidenced by the horizontal striation in Fig. 4.4c. Coexistence of host and pathogen is possible for all values of the pathogen-induced mortality rates, except under extreme values of indirect transmission, zoospore production, and/or zoospore mortality described above. At the highest rates of transmission and juvenile Bd-induced mortality, the model is more sensitive and high juvenile mortality can result in small adult populations (Fig. 4.4d).

If there is no adult mortality from disease, why is the equilibrium population of adults so low at high rates of transmission? The answer is in the low recruitment rate of infected juveniles: if many juveniles become infected when transmission is high, and they have a lower recruitment rate than susceptible individuals, then adult population size declines. The model is highly sensitive to recruitment rate of both susceptible (χ_S) and infected juveniles (χ_S) (Fig. 4.4e, f) and to the population birth rate (b), immediate post-hatching survival (Ψ), and natural adult mortality rate (d_A) (see Appendix B for sensitivity results of other parameters).

DISCUSSION

In tropical montane environments, many direct developing frog populations persist at reduced numbers following Bd outbreaks while populations of other species decline to extinction (Lips et al. 2006). The biology of direct developing amphibians is one explanation for these different outcomes, specifically, the absence of an aquatic larval stage, skin characteristics that may influence infection susceptibility, year-round infection and breeding, and different transmission dynamics in terrestrial relative to aquatic habitats. I designed this study with the goal to better understand the impacts of chytridiomycosis on *E. coqui* and related species on Puerto Rico, which are persisting with Bd (Longo et al. 2010) following introduction of the pathogen in the 1970s or earlier (Burrowes et al. 2004). A number of the *Eleutherodactylus* species on Puerto Rico are globally threatened with extinction and three species are recently extinct, with Bd as a suspected cause (Burrowes et al. 2004). While the most serious outbreaks of chytridiomycosis have likely already occurred, the introduction of new pathogenic Bd strains is a possibility (Farrer et al. 2011) that could further threaten surviving species.

My model addresses the distinctive biology of direct developers. Preliminary model analysis led to specific experiments on ontogenetic susceptibility (Chapter 1) and disease transmission (Chapter 2). I then parameterized the model with data from those lab experiments and long-term field studies (Townsend and Stewart 1994, Stewart and Woolbright 1996) to assess the conditions favoring host-pathogen persistence and possible population extinction from infectious disease, to understand the importance both

direct and indirect transmission, and to evaluate the impact of high Bd-induced mortality of juvenile frogs.

In this model host extinction from disease is possible when transmission rates are high and either zoospore production rate is high or zoospore mortality rate is low. Zoospore mortality and production rates also influenced the outcomes of Bd infection in other models (Mitchell et al. 2008, Doddington et al. 2013). If either direct or indirect transmission is sufficiently high, my model indicates that Bd readily becomes endemic in a population, a conclusion consistent with the observation that Bd is now endemic in Puerto Rico (Longo et al. 2010). Bd's survival in arboreal water sources (Cossel and Lindquist 2009), and in relatively tolerant adult *E. coqui* and potentially other reservoir species, likely affords ample opportunities for both indirect and direct transmission. Estimating transmission rates and zoospore production rates in the field is a high priority for future research.

There are no data indicating whether *E. coqui* frogs are more likely to become infected with Bd through direct contact with another frog or indirectly via zoospores surviving temporarily in the environment. Because *E. coqui* is a territorial species, direct transmission is likely to occur only during amplexus, sharing of humid retreat sites during dry conditions, and during parental care of eggs and hatchlings. Depending on transmission rates, having both types of transmission in the population can lead to lower equilibrium population sizes, but the effect is not dramatic. Indirect transmission is likely key to explaining how some populations may have gone extinct following Bd introduction. The three species gone from Puerto Rico in the past few decades were microhabitat specialists with greater affinity for water (e.g., bromeliad axils) where

indirect transmission is likely higher (Burrowes et al. 2004). Incorporating both direct and indirect transmission into the model allows for future investigation into (a) the impact of vertical transmission through parental care and (b) seasonal differences in the relative importance of direct and indirect transmission.

The high rate of Bd-induced juvenile mortality described in Chapter 1 led to the prediction that this parameter would have a large impact on model outcomes. Instead, the more important parameter was the recruitment rates of both susceptible and infected juveniles into the adult class. Because the recruitment rate incorporates aspects of survival and maturation (i.e., juveniles that become adults survive to reach sexual maturity within a given time frame), recruitment rate is not independent of juvenile Bd-induced mortality. The model requires both parameters because removing the juvenile Bd-induced mortality rate results in an unrealistically large number of individuals getting "stuck" in the infected juvenile class.

What these results may be revealing is that a population can tolerate relatively high mortality of young juveniles due to Bd infection as long as enough infected juveniles survive to reach sexual maturity. This unsurprising result leads to an interesting prediction: increased selection pressure for early age at first reproduction, as suggested by Hyman (2012) for the boreal chorus frog (*Pseudacris maculata*). Any reduction of success for a species with one or few chances to breed can lead to extinction, since there is no capacity in the life history to absorb any variation in opportunities to breed due to pathogen-induced low survival. Tracking the fate of infected juveniles beyond one year is a high priority for future field investigations.

For my model I explored important population parameters over a large range of estimates to understand their sensitivity and impact on infectious disease dynamics. Future laboratory and field investigations can be used to improve estimates of direct and indirect transmission rates, zoospore production and mortality, and recruitment of susceptible and infected juveniles. As we learn more about the mechanisms that contribute to persistence of direct-developing frogs with Bd, these data will reveal the critical points in the life histories of these animals that can guide management and conservation practices.

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Table 4.1. Description and estimated values of model parameters. Parameter estimates not based on my experimental work or the literature are described in the Methods.

Parameter	Value	Range explored	Description	Source
Frog life cycle				
π	5 yr^{-1}	-	number of egg laying events/yr	Townsend & Stewart 1994
θ	27	-	number of eggs laid/pair	Townsend & Stewart 1994
ρ	0.6	-	egg hatching success	Townsend & Stewart 1994
b	0.22 dy^{-1} (81 yr^{-1})	0.11-0.33	birth rate	$\pi^*\theta^*\rho$
Ψ	0.3	0.15-0.45	immediate post-hatching survival	See Methods
K	4000	2000-6000	adult carrying capacity	Stewart & Woolbright 1996
d_J	2.2^{-3} dy^{-1} (0.81 yr^{-1})	$1.1\text{-}3.3^{-3}$	mortality rate of juvenile frogs	Stewart & Woolbright 1996
d_A	2.5^{-3} dy^{-1} (0.94 yr^{-1})	$1.25\text{-}3.75^{-3}$	morality rate of adult frogs	Stewart & Woolbright 1996
χ_S	4.4^{-4} dy^{-1} (0.16 yr^{-1})	$2.2\text{-}6.6^{-4}$	recruitment rate of susceptible juveniles	See Methods
χ_I	2.0^{-4} dy^{-1} (0.07 yr^{-1})	$2.75^{-5}\text{-}4.4^{-4}$	recruitment rate of infected juveniles	See Methods
Bd infection				
γ_J	5.0^{-4} dy^{-1}	$2.5\text{-}7.5^{-4}$	infection clearing rate of juvenile frogs	This study
γ_A	0.01 dy^{-1}	0.005-0.015	infection clearing rate of adult frogs	This study
α_J	0.0065 dy^{-1}	0-0.01	juvenile Bd-induced mortality rate	This study
α_A	0	0-0.003	adult Bd-inducted mortality rate	This study
β	1.0^{-6} dy^{-1*lj}	$1.0^{-7}\text{-}1.0^{-5}$	direct transmission rate	See Methods
ε	1.0^{-7} dy^{-1*Z}	$1.0^{-8}\text{-}1.0^{-6}$	indirect transmission rate	See Methods
μ	1 dy^{-1}	0.04-1	zoospore mortality rate	Piotrowski et al. 2004 Johnson & Speare 2003
ϕ	10 dy^{-1}	10-1000	zoospore shed rate by infected frogs	Fisher et al. 2012

Fig. 4.1. Schema for the Bd-coqui model. See Table 1 for parameter descriptions.

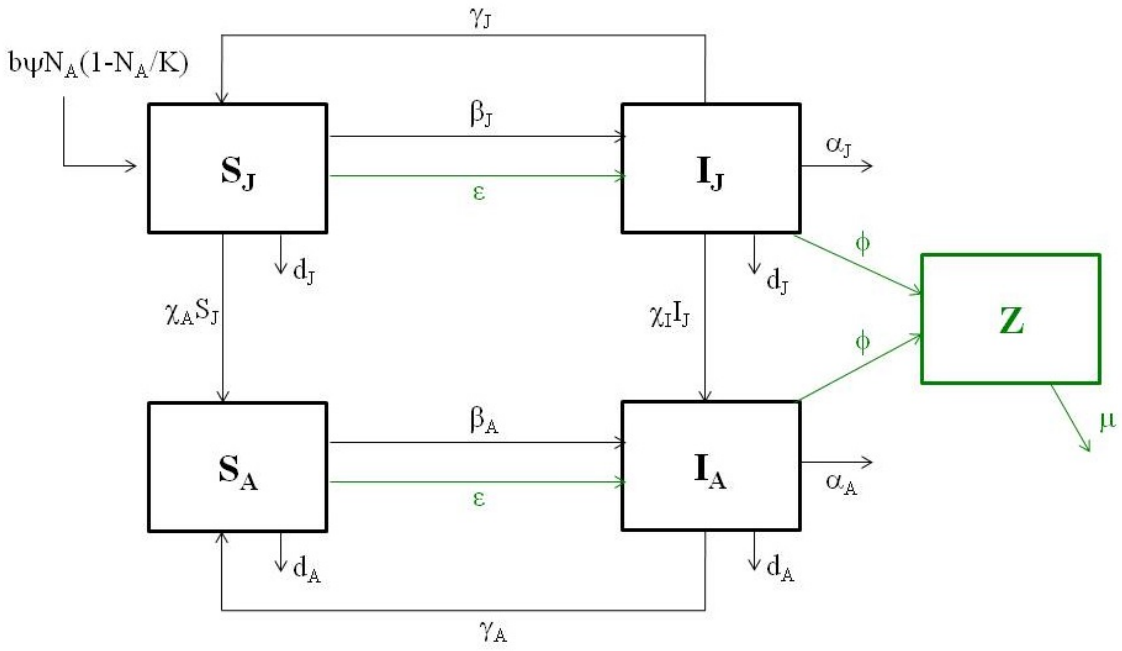
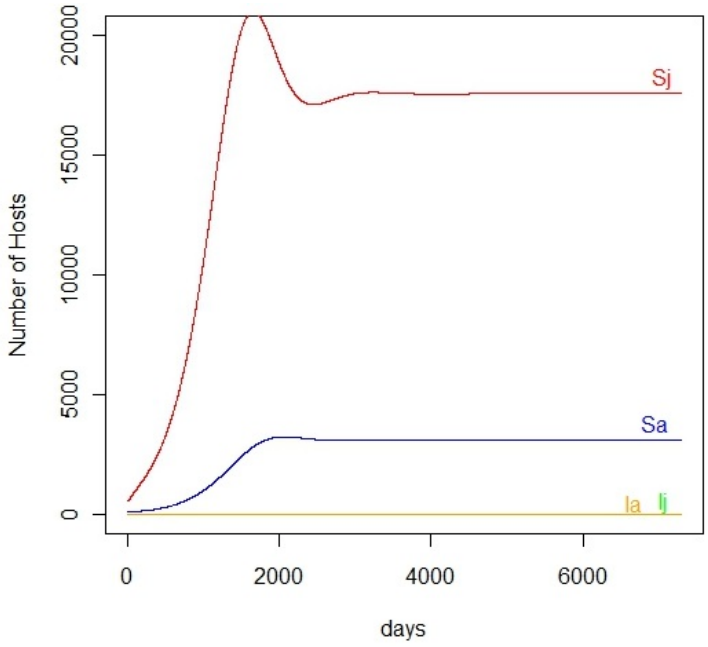


Figure 4.2. Number of susceptible and infected adult and juvenile *E. coqui* over time (a) in absence of Bd and (b) following introduction of Bd. See Table 1 for estimated parameter values; both direct and indirect transmission is assumed; initial conditions: Sj=529, Ij=1, Sa=99, Ia=1, Z=1, where Sj=susceptible juveniles, Ij=infected juveniles, Sa=susceptible adults, Ia=infected adults, and Z=zoospores.

(a)



(b)

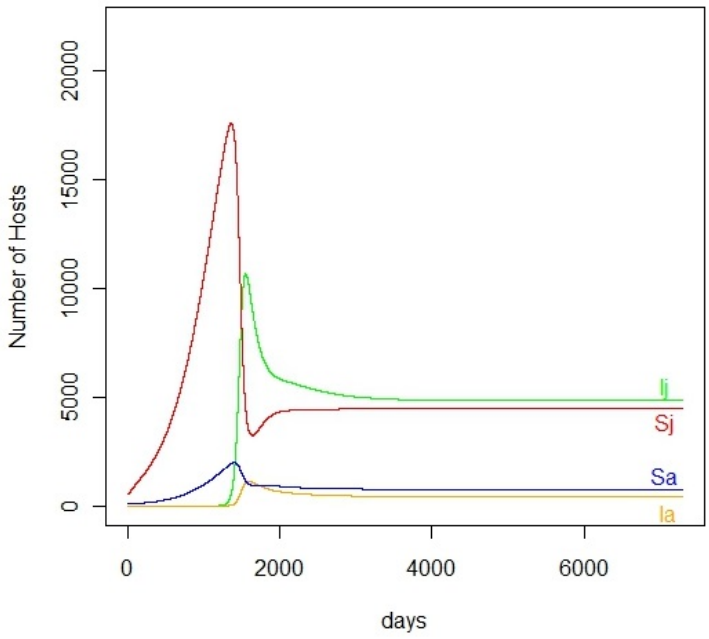
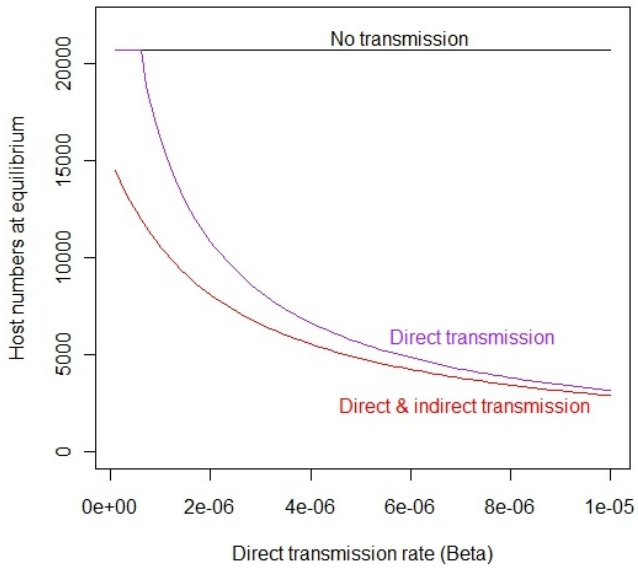


Figure 4.3. Impact of transmission parameters (β and ε) on infectious disease dynamics. As the rate of (a) direct or (b) indirect transmission increases, size of the *E. coqui* population at equilibrium decreases. Both transmission rates show a bifurcation at $\varepsilon > 5e^{-08}$ or $\beta > 6e^{-07}$ (for parameter values shown in Table 1), where the pathogen-free equilibrium becomes unstable and the coexistence equilibrium is stable. Note that values on the abscissa shift by an order of magnitude between (a) and (b).

(a)



(b)

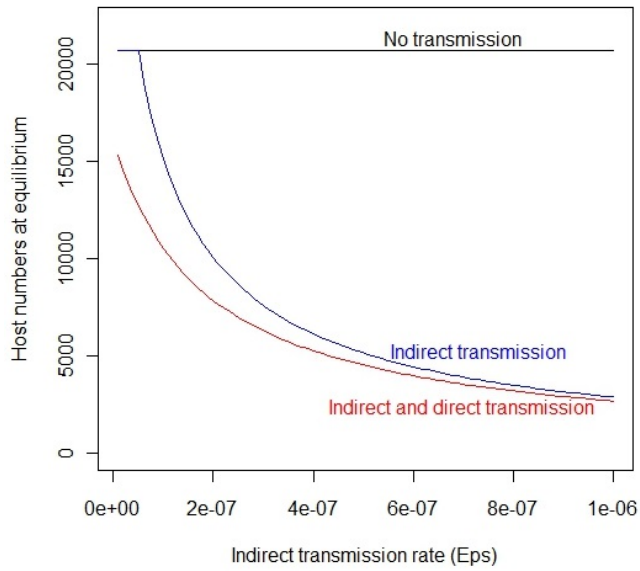


Fig. 4.4. Sensitivity of key model parameters: number of adult *E. coqui* frogs at equilibrium for different values of the indirect transmission rate ε and rates of (a) zoospore production, (b) zoospore mortality, (c, d) Bd-induced mortality, and (e, f) juvenile recruitment. Other parameters held constant at values in Table 1.

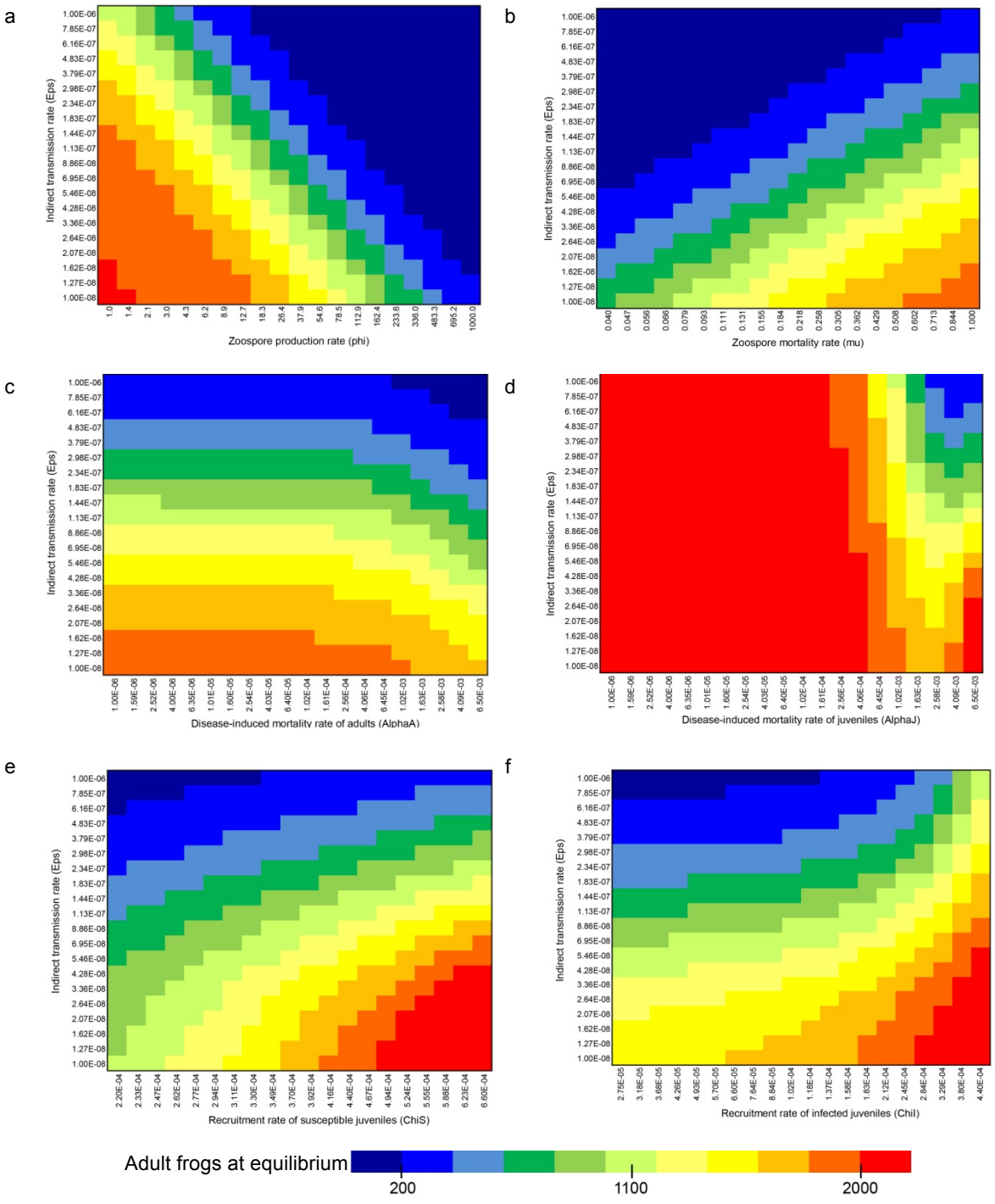
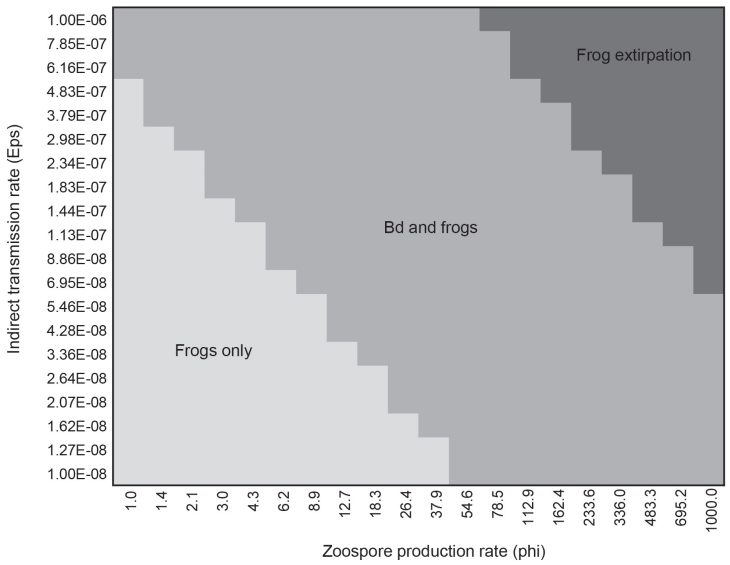
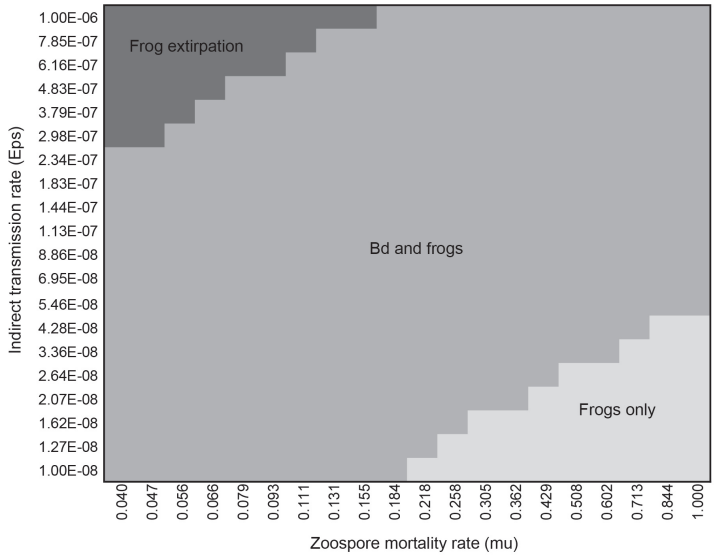


Fig. 4.5. Importance of (a) zoospore production rate ϕ and (b) zoospore mortality rate μ. For parameter estimates in this model only ϕ and μ can reduce the number of adult *E. coqui* frogs below the "extinction" threshold of 50 individuals at certain values of the transmission parameter ε. Other parameters held constant at the values in Table 1, except β=0 to show the disease-free state.

(a)



(b)



CONCLUSION

Amphibians are the world's most threatened class of vertebrates (Vié et al. 2009) and face a current extinction rate more than 200 times the background rate (McCallum 2007). The scientific community has called for concerted, scaled-up action to tackle the most serious threats to amphibian species (Mendelson et al. 2006). The Amphibian Conservation Action Plan (ACAP) highlights priorities for conservation and research to address threats of habitat loss, over-exploitation, contaminants, climate change, and infectious disease (Gascon et al. 2007).

We have learned much about chytridiomycosis since the ACAP was published, in particular we have a better understanding of how this emerging infectious disease causes amphibian extinction (Skerratt et al. 2007, Collins and Crump 2009). The role of a pathogen in causing extinction of its host, not typically predicted by theory (Anderson and May 1979), motivated my research into the impact of chytridiomycosis on Caribbean frogs. Bd has caused the near-disappearance of *Leptodactylus fallax* on Dominica (IUCN 2012) and is implicated in the extinction of *Eleutherodactylus karlschmidti*, *E. jasperi*, *E. eneidae* from Puerto Rico (Burrowes et al. 2004). In particular, I wanted to understand how this aquatic pathogen impacts terrestrial frogs that do not depend on water bodies for breeding.

In studying the ontogenetic susceptibility of *E. coqui* to Bd, I discovered that juvenile frogs suffer high pathogen-induced mortality even as adults can resist or clear infection. This finding has several important implications. First, a species may still be negatively affected by chytridiomycosis if adults are healthy or uninfected. Second, there is a need to investigate the susceptibility of older juveniles. The fate of infected juveniles

should be tracked to determine whether they reproduce and endure fitness costs as a result of Bd infection.

By investigating Bd transmission in *E. coqui*, I determined that indirect transmission of an aquatic pathogen can occur in frogs occupying humid terrestrial substrates. Indirect transmission gives a possible pathway to extinction (De Castro and Bolker 2004). It is a high priority to study transmission dynamics in the field, to determine how transmission varies seasonally, and whether vertical transmission occurs via frogs providing parental care to offspring.

An unanticipated line of research into the attenuation of artificially-maintained Bd strains has important implications for Bd-amphibian lab investigations. Amphibian exposure experiments need to consider the passage history of Bd strains when infecting frogs and use freshly isolated or cryopreserved Bd whenever possible. Genomic comparisons between attenuated and non-attenuated strains may uncover the genetic mechanisms of Bd pathogenicity, a line of research currently underway as a result of this study.

My mathematical model showed that Bd can cause decline of direct-developing frogs if transmission remains high and infection reduces recruitment of juveniles into the reproductive adult class. Extinction can occur under high rates of indirect transmission and zoospore production (or low zoospore mortality), as may be common in epidemics. Model analysis helped pinpoint priorities for future research; namely, tracking the survival and reproduction of infected juveniles and studying pathogen transmission and zoospore shedding rates in the field.

Once Bd is introduced into a region, it is extremely difficult, if not impossible, to eliminate (Woodhams et al. 2011). The pathogen is maintained either by amphibian species that tolerate infection (Reeder et al. 2012) or by other biotic and abiotic pathogen reservoirs (Kilburn et al. 2011, Garmyn et al. 2012, McMahon et al. 2013). Treating individual amphibians with probiotics or anti-fungal agents may be possible for highly threatened species where resources permit (Woodhams et al. 2011), but these approaches are not yet practical for speciose amphibian communities where many of the most severe declines are occurring.

My research points to the importance of preventing introduction of new pathogenic Bd strains into Puerto Rico, monitoring the infection status and population trends of all *Eleutherodactylus* species on the island, and ensuring that key sites are safeguarded to minimize other threats to amphibian biodiversity. Indeed, these actions are necessary for most amphibian communities impacted by chytridiomycosis. An additional priority is to investigate the mechanisms by which some adult *E. coqui* frogs resist or clear Bd infection, given recent evidence that Bd can inhibit the amphibian immune system (Fites et al. 2013). Studying the populations that survive Bd outbreaks, such as *E. coqui* and congeners on Puerto Rico, is key to understanding the mechanisms of pathogen resistance and ultimately to understanding how amphibians persist with chytridiomycosis.

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APPENDIX A

SUPPLEMENTARY TABLES

S1. *Eleutherodactylus coqui* frogs wild-collected from Puerto Rico (PR) and Hawaii (HI).

Frog ID	Date of collection	Location of collection	Sex	SVL (mm)	Infection intensity upon collection
1	09/13/10	PR	M	42.4	0
2	09/13/10	PR	M	39.3	0
3	09/13/10	PR	M	31.4	123
4	09/13/10	PR	F	40.4	0
5	09/13/10	PR	M	37.3	241
10	09/13/10	PR	F	49.1	0
11	09/13/10	PR	F	48.9	0
12	09/13/10	PR	F	35.4	0
13	09/13/10	PR	M	40.8	0
14	09/13/10	PR	F	37.3	0
15	09/13/10	PR	F	50.2	0
20	09/13/10	PR	F	34.7	2955
21	09/13/10	PR	F	46.9	0
22	09/13/10	PR	F	50.8	0
23	09/13/10	PR	F	39.9	0
24	09/13/10	PR	M	39.5	0
25	09/13/10	PR	M	38.6	0
30	09/13/10	PR	M	34.1	19
31	09/13/10	PR	F	33.9	113
32	09/13/10	PR	F	29.9	0
33	09/13/10	PR	M	40.1	0
34	09/13/10	PR	F	55.0	0
35	09/13/10	PR	F	35.3	0
40	09/13/10	PR	M	38.5	0
41	09/13/10	PR	F	37.9	0
42	09/13/10	PR	M	35.3	0
43	09/13/10	PR	M	40.9	0
44	09/13/10	PR	F	39.2	0
45	09/13/10	PR	M	40.3	0
50	09/13/10	PR	M	41.0	0
51	09/13/10	PR	M	41.0	0
52	09/13/10	PR	M	38.1	0
53	09/13/10	PR	F	55.2	268
54	09/13/10	PR	M	40.2	51
55	09/13/10	PR	F	41.8	0
60	09/13/10	PR	M	39.2	0
61	09/13/10	PR	M	37.3	0
62	09/13/10	PR	M	36.9	0
63	09/13/10	PR	M	35.5	0
64	09/13/10	PR	M	49.2	0
65	09/13/10	PR	M	39.7	0
70	09/13/10	PR	F	51.8	0
71	09/13/10	PR	F	39.9	8001
72	09/13/10	PR	F	34.6	0
73	09/13/10	PR	M	35.4	549
74	09/13/10	PR	F	50.2	0
75	09/13/10	PR	M	36.7	0
80	09/13/10	PR	M	41.1	0

81	09/13/10	PR	M	33.4	0
82	09/13/10	PR	M	37.1	1340
83	09/13/10	PR	M	39.6	0
84	09/13/10	PR	M	38.8	0
85	09/13/10	PR	F	37.8	62
90	09/13/10	PR	M	35.5	0
91	09/13/10	PR	M	39.6	0
92	09/13/10	PR	M	39.0	0
93	09/13/10	PR	F	37.2	0
94	09/13/10	PR	F	50.6	0
95	09/13/10	PR	F	38.4	0
100	09/13/10	PR	F	31.7	0
101	09/13/10	PR	M	37.7	0
102	09/13/10	PR	F	35.5	0
103	09/13/10	PR	M	43.3	41
104	09/13/10	PR	F	40.2	0
105	09/13/10	PR	F	29.7	0
110	10/20/10	HI	F	36.3	0
111	10/20/10	HI	M	32.3	0
112	10/20/10	HI	F	30.5	0
113	10/20/10	HI	M	32.2	0
114	10/20/10	HI	M	34.9	0
115	10/20/10	HI	M	34.2	0
120	10/20/10	HI	M	30.2	694
121	10/20/10	HI	M	32.7	0
122	10/20/10	HI	M	31.5	0
123	10/20/10	HI	M	32.5	0
124	10/20/10	HI	F	40.3	0
125	10/20/10	HI	M	29.3	0
130	10/20/10	HI	M	30.1	301
131	10/20/10	HI	M	28.1	0
132	10/20/10	HI	F	33.9	0
133	10/20/10	HI	F	31.4	15
134	10/20/10	HI	M	34.4	0
135	10/20/10	HI	M	32.1	0
140	10/20/10	HI	M	32.9	0
141	10/20/10	HI	M	34.2	0
142	10/20/10	HI	M	37.6	78
143	10/20/10	HI	M	30.7	613
144	10/20/10	HI	M	32.1	0
145	10/20/10	HI	M	32.2	0
150	10/20/10	HI	M	32.1	0
151	10/20/10	HI	F	27.7	5816
152	10/20/10	HI	M	31.2	415
153	10/20/10	HI	M	34.1	0
154	10/20/10	HI	F	31.8	122
155	10/20/10	HI	M	33.1	0
160	10/20/10	HI	M	29.1	0
161	10/20/10	HI	M	34.7	0
162	10/20/10	HI	M	34.1	0
163	10/20/10	HI	M	29.1	0
164	10/20/10	HI	F	38.1	49
165	10/20/10	HI	M	34.5	0

170	10/20/10	HI	?	31.1	0
171	10/20/10	HI	M	29.1	0
172	10/20/10	HI	?	NA	1381
173	10/20/10	HI	M	28.0	406
174	10/20/10	HI	F	32.2	0
175	10/20/10	HI	M	29.3	164
180	10/20/10	HI	F	38.5	0
181	10/20/10	HI	M	33.4	2964
182	10/20/10	HI	M	33.1	0
183	10/20/10	HI	M	32.9	0
184	10/20/10	HI	F	38.1	0
185	10/20/10	HI	M	29.6	0
190	10/20/10	HI	M	33.2	0
191	10/20/10	HI	F	41.4	0
192	10/20/10	HI	M	36.8	496
193	10/20/10	HI	M	31.5	0
194	10/20/10	HI	M	33.6	0
195	10/20/10	HI	?	41.5	0
200	10/20/10	HI	M	30.1	199
201	10/20/10	HI	M	28.5	557
202	10/20/10	HI	F	29.9	0
203	10/20/10	HI	M	32.7	0
204	10/20/10	HI	F	31.2	0
205	10/20/10	HI	M	28.7	0
210	10/20/10	HI	F	36.8	0
211	10/20/10	HI	M	26.8	39
212	10/20/10	HI	M	29.7	0
213	10/20/10	HI	?	33.6	0
214	10/20/10	HI	M	29.5	26
215	10/20/10	HI	M	28.9	0
220	10/20/10	HI	M	31.9	2286
221	10/20/10	HI	M	29.9	0
222	10/20/10	HI	M	28.0	4854
223	10/20/10	HI	M	30.0	0
224	10/20/10	HI	M	32.3	0
225	10/20/10	HI	M	NA	105
230	10/20/10	HI	?	25.7	0
231	10/20/10	HI	M	29.7	8412
232	10/20/10	HI	M	25.0	0
233	10/20/10	HI	M	27.7	0
234	10/20/10	HI	M	28.7	0
235	10/20/10	HI	M	31.5	0
240	10/20/10	HI	F	38.4	0
241	10/20/10	HI	F	34.5	0
242	10/20/10	HI	M	31.7	0
243	10/20/10	HI	M	28.6	0
244	10/20/10	HI	F	34.8	0

Infection intensity = zoospore genomic equivalents

S2. Adult *E. coqui* frogs used in ontogenetic susceptibility experiment (Chapter 1).

Frog ID	Experimental treatment	Days survived	Censor	Infection intensity		
				Expt start	Post-inoc	Expt end
1	control	77	Y	0	0	0
4	control	77	Y	0	0	0
10	control	77	Y	0	0	0
11	control	77	Y	0	0	0
13	control	14	N	0	0	0
15	control	23	N	0	0	0
63	control	77	Y	0	0	0
64	control	77	Y	0	0	0
83	control	77	Y	0	0	0
105	control	77	Y	0	0	0
2	Bd-exposed 10^6	77	Y	0	20	0
14	Bd-exposed 10^6	77	Y	0	1314	0
22	Bd-exposed 10^6	77	Y	0	131	0
23	Bd-exposed 10^6	77	Y	0	5208	0
25	Bd-exposed 10^6	77	Y	0	1536	6
43	Bd-exposed 10^6	77	Y	0	1812	0
50	Bd-exposed 10^6	77	Y	0	1452	0
52	Bd-exposed 10^6	77	Y	0	798	24
61	Bd-exposed 10^6	77	Y	0	217	0
80	Bd-exposed 10^6	77	Y	0	244	0
93	Bd-exposed 10^6	77	Y	0	9774	0
101	Bd-exposed 10^6	77	Y	0	5294	0
102	Bd-exposed 10^6	77	Y	0	722	0
20	Bd-exposed 10^5 (PI)	77	Y	0	1669	0
31	Bd-exposed 10^5 (PI)	21	N	0	1042	0
53	Bd-exposed 10^5 (PI)	77	Y	0	10	0
82	Bd-exposed 10^5 (PI)	77	Y	0	177	0
85	Bd-exposed 10^5 (PI)	77	Y	5	1092	0
103	Bd-exposed 10^5 (PI)	77	Y	0	2088	0
130	Bd-exposed 10^5 (PI)	77	Y	0	0	0
133	Bd-exposed 10^5 (PI)	77	Y	1392	1362	0
173	Bd-exposed 10^5 (PI)	77	Y	0	0	0
200	Bd-exposed 10^5 (PI)	77	Y	398	702	4
211	Bd-exposed 10^5 (PI)	77	Y	165	124	318
214	Bd-exposed 10^5 (PI)	77	Y	79	143	0
225	Bd-exposed 10^5 (PI)	77	Y	74	1740	13
115	Bd-exposed 10^5	77	Y	0	646	0
125	Bd-exposed 10^5	77	Y	0	1553	0
163	Bd-exposed 10^5	77	Y	0	1458	0
193	Bd-exposed 10^5	77	Y	0	444	0
224	Bd-exposed 10^5	77	Y	50	104	0
240	Bd-exposed 10^5	77	Y	51	216	0
241	Bd-exposed 10^5	77	Y	24	203	0

PI = previously infected. 10^6 or 10^5 indicates Bd dose in zoospores/ml. Censor = Y frogs survived to the end of the experiment. Infection intensity = zoospore genomic equivalents.

S3. Juvenile *E. coqui* frogs used in ontogenetic susceptibility experiment (Chapter 1).

Frog ID	Experimental treatment	Days survived	Censor	SVL (mm)	Age (wks)	Infection intensity at death or end of expt
405	control	9	N	7.2	8.5	0
406	control	4	N	7.6	8.5	0
408	control	31	N	8.6	8.0	0
411	control	77	Y	9.4	8.0	0
415	control	54	N	9.2	8.0	0
424	control	77	Y	11.8	6.0	0
426	control	77	Y	12.0	6.0	0
431	control	73	N	NA	6.0	0
432	control	73	N	11.4	6.0	0
435	control	49	N	11.4	6.0	0
438	control	74	N	10.9	6.0	0
445	control	77	Y	11.6	7.0	0
447	control	49	N	11.4	7.0	0
448	control	77	Y	11.9	7.0	0
451	control	77	Y	12.1	7.0	0
457	control	77	Y	12.4	7.0	0
459	control	77	Y	12.3	7.0	0
467	control	77	Y	11.9	7.0	0
468	control	77	Y	11.7	7.0	0
472	control	77	Y	13.2	7.0	0
473	control	32	N	8.5	8.5	0
474	control	24	N	7.6	8.5	0
480	control	12	N	8.3	8.5	0
484	control	66	N	9.9	8.5	0
495	control	77	Y	10.4	9.0	0
499	control	77	Y	9.6	9.0	0
502	control	52	N	9.1	9.0	0
503	control	77	Y	9.7	9.0	0
505	control	77	Y	10.5	9.0	0
600	control	2	N	10.8	9.0	0
601	control	30	N	12.5	9.0	0
602	control	2	N	12.8	9.0	0
609	control	51	N	13.8	9.0	0
610	control	64	N	11.1	9.0	0
616	control	43	N	12.8	9.0	0
618	control	77	Y	13.9	9.0	0
620	control	77	Y	12.0	9.0	0
624	control	27	N	11.9	9.0	0
626	control	54	N	11.2	9.0	0
633	control	45	N	12.3	9.0	0
635	control	77	Y	11.9	10.0	0
636	control	77	Y	11.1	10.0	0
637	control	75	N	12.1	10.0	0
640	control	75	N	11.4	10.0	0
641	control	75	N	12.2	10.0	0
646	control	31	N	9.8	6.5	0
650	control	46	N	12.7	21.0	0
651	control	30	N	13.5	21.0	0
655	control	9	N	13.3	21.0	0
658	control	65	N	13.2	21.0	0
659	control	16	N	13.2	21.0	0

661	control	35	N	11.6	21.0	0
663	control	19	N	13.4	21.0	0
665	control	55	N	13.1	18.0	0
402	Bd-exposed	29	N	7.4	8.5	363
403	Bd-exposed	29	N	6.9	8.5	5
412	Bd-exposed	32	N	8.0	8.0	5
414	Bd-exposed	62	N	8.5	8.0	12
417	Bd-exposed	34	N	7.4	8.0	42
419	Bd-exposed	51	N	9.5	8.0	5
420	Bd-exposed	25	N	7.5	8.0	21
422	Bd-exposed	59	N	11.1	6.0	511
423	Bd-exposed	59	N	10.0	6.0	1198
437	Bd-exposed	27	N	10.7	6.0	10
441	Bd-exposed	36	N	10.8	6.0	2
449	Bd-exposed	77	Y	11.7	7.0	0
452	Bd-exposed	77	Y	11.5	7.0	1416
453	Bd-exposed	62	N	11.0	7.0	0
454	Bd-exposed	72	N	10.9	7.0	0
461	Bd-exposed	47	N	11.8	7.0	6
463	Bd-exposed	62	N	10.8	7.0	21
464	Bd-exposed	44	N	10.3	7.0	5
465	Bd-exposed	65	N	10.4	7.0	1152
466	Bd-exposed	50	N	12.0	7.0	0
469	Bd-exposed	77	Y	12.7	7.0	1801
477	Bd-exposed	53	N	7.5	8.5	8281
479	Bd-exposed	29	N	8.6	8.5	8
483	Bd-exposed	39	N	8.4	8.5	3160
488	Bd-exposed	39	N	7.9	8.5	141
494	Bd-exposed	49	N	8.8	9.0	5
496	Bd-exposed	59	N	9.4	9.0	31
498	Bd-exposed	35	N	8.5	9.0	19
604	Bd-exposed	53	N	13.5	9.0	22064
606	Bd-exposed	77	N	13.0	9.0	0
608	Bd-exposed	32	N	13.2	9.0	223488
611	Bd-exposed	32	N	12.3	9.0	5724
613	Bd-exposed	34	N	13.5	9.0	45156
615	Bd-exposed	66	N	12.7	9.0	0
623	Bd-exposed	27	N	11.7	9.0	3
627	Bd-exposed	70	N	11.4	9.0	227
628	Bd-exposed	28	N	12.0	9.0	43860
629	Bd-exposed	52	N	13.8	9.0	32208
630	Bd-exposed	49	N	12.7	9.0	0
631	Bd-exposed	51	N	12.8	9.0	2988
632	Bd-exposed	36	N	12.0	9.0	6004
634	Bd-exposed	77	Y	11.4	9.0	0
639	Bd-exposed	7	N	11.5	10.0	764
642	Bd-exposed	19	N	10.1	6.5	644
643	Bd-exposed	16	N	9.9	6.5	277
647	Bd-exposed	28	N	10.4	6.5	12096
649	Bd-exposed	8	N	11.6	21.0	14136
652	Bd-exposed	8	N	13.2	21.0	14832
657	Bd-exposed	9	N	13.0	21.0	622
662	Bd-exposed	77	Y	13.3	21.0	60
664	Bd-exposed	52	N	13.3	18.0	62

Censor = Y frogs survived to the end of the experiment. Infection intensity = zoospore genomic equivalents.

S4. *E. coqui* frogs used in direct and indirect transmission experiments (Chapter 2).

Bd inoculated frogs

Frog ID	Expt	Infection intensity				Transmitted Bd
		Day 9	Day 16	Day 23	Day 37	
140	both	132.6	0.0	0.0	0.0	no
222	both	1536.0	51348.0	dead	dead	to 54 (direct), 93 (indirect)
224	indirect	2610.0	174.6	14.4	0.0	no
124	both	134.4	0.0	60.0	39.0	to 201 (direct)
241	both	436.2	1608.0	594.0	dead	no
203	both	354.0	dead	dead	dead	to 132 (indirect)
173	both	7506.0	0.0	46.2	11.4	no
3	both	754.2	0.0	0.0	0.0	no
103	both	984.6	0.0	0.0	0.0	to 240 (direct)
81	both	167.4	0.0	0.0	0.0	no
63	both	25.2	1.2	0.0	0.0	to 110 (indirect)
101	excluded*	0.0	0.0	0.0	0.0	NA

Originally uninfected frogs

Frog ID	Expt	Habitat	Infection intensity			Experimental set-up
			Day 16	Day 23	Day 37	
152	excluded*	moss	0	0	0	shared cage with 101
231	direct	moss	0	0	0	shared cage with 81
85	direct	moss	0	0	0	shared cage with 140
171	direct	moss	0	0	0	shared cage with 3
14	direct	moss	0	0	0	shared cage with 173
240	direct	moss	10.2	0	0	shared cage with 103
80	direct	moss	0	0	0	shared cage with 241
54	direct	moss	0	28.8	0	shared cage with 222
195	direct	moss	0	0	0	shared cage with 63
201	direct	moss	0	65.4	0	shared cage with 124
43	direct	moss	0	0	0	shared cage with 203
11	indirect	moss	0	0	0	used cage of 224
221	indirect	moss	0	0	0	used cage of 3
110	indirect	moss	0	50.4	7.2	used cage of 63
20	indirect	moss	0	0	0	used cage of 173
50	indirect	moss	0	0	0	used cage of 241
61	indirect	bromeliad	0	0	0	used cage of 124
23	indirect	moss	0	0	0	used cage of 103
132	indirect	bromeliad	46.2	0	0	used cage of 203
93	indirect	moss	7.2	0	14.4	used cage of 222
102	indirect	moss	0	0	0	used cage of 81
53	indirect	bromeliad	0	0	0	used cage of 140
192	excluded*	bromeliad	0	0	0	used cage of 101

* Excluded from analysis because frog 101 did not become infected following Bd exposure. Infection intensity = zoospore genomic equivalents.

S5. *E. coqui* frogs used in Bd strain attenuation experiment (Chapter 3). Frogs were exposed to 10^5 zoospores/ml of JEL427-39 (39 passes) or JEL427-P9 (9 passes).

Frog ID	Experimental treatment	Days survived post-exposure	Censored	Infection intensity	
				day 15	death / end of expt
14	control	80	Y	0	0
80	control	80	Y	0	0
54	control	80	Y	0	0
171	control	80	Y	0	0
201	control	80	Y	0	0
240	control	80	Y	0	0
22	control	80	Y	0	0
85	control	80	Y	0	0
43	control	80	Y	0	0
195	control	80	Y	0	0
231	control	80	Y	0	0
152	control	80	Y	0	0
155	JEL427-P39	80	Y	2130	0
174	JEL427-P39	80	Y	145	0
123	JEL427-P39	80	Y	2	0
90	JEL427-P39	80	Y	0	0
42	JEL427-P39	80	Y	2	0
33	JEL427-P39	80	Y	4	0
185	JEL427-P39	80	Y	1506	0
150	JEL427-P39	80	Y	0	0
145	JEL427-P39	12	N	NA	12816
65	JEL427-P39	80	Y	0	0
51	JEL427-P39	80	Y	706	0
84	JEL427-P39	80	Y	34	0
162	JEL427-P9	80	Y	35	0
210	JEL427-P9	80	Y	10	20
233	JEL427-P9	80	Y	9	0
64	JEL427-P9	80	Y	0	0
10	JEL427-P9	80	Y	0	0
1	JEL427-P9	80	Y	0	0
182	JEL427-P9	80	Y	586	0
191	JEL427-P9	80	Y	670	0
114	JEL427-P9	80	Y	3	0
105	JEL427-P9	80	Y	0	0
4	JEL427-P9	80	Y	857	0
55	JEL427-P9	80	Y	30	0

Censor = Y frogs survived to the end of the experiment. Infection intensity = zoospore genomic equivalents.

S6. *Atelopus zeteki* frogs used in Bd strain attenuation experiment (Chapter 3). Frogs were exposed to 10^2 zoospores/ml of JEL427-39 (39 passes) or JEL427-P9 (9 passes). Frogs were obtained in June 2012 from the Maryland Zoo in Baltimore.

Frog ID	SVL (mm)	Mass (g)	Experimental treatment	Days survived post-exposure	Censored	Infection intensity day 30	Infection intensity death / end of expt
701	51.3	8.28	control	130	Y	0	0
706	46.4	7.40	control	130	Y	0	0
709	51.0	8.11	control	130	Y	0	0
720	43.2	6.92	control	130	Y	0	0
721	51.1	9.11	control	130	Y	0	0
724	50.9	7.04	control	130	Y	0	0
740	52.6	7.59	control	130	Y	0	0
744	41.2	6.12	control	130	Y	0	0
765	35.9	3.83	control	130	Y	0	0
770	51.0	9.73	control	130	Y	0	0
703	41.0	4.71	JEL427-P39	130	Y	0	373
704	41.4	6.31	JEL427-P39	119	N	0	152064
705	51.2	8.92	JEL427-P39	114	N	1	13298
707	52.9	9.91	JEL427-P39	130	Y	19	121
708	52.5	10.10	JEL427-P39	96	N	0	756
710	42.8	5.86	JEL427-P39	130	Y	43	362
711	51.0	8.88	JEL427-P39	95	N	0	114516
712	40.1	5.25	JEL427-P39	130	Y	17	84
714	46.4	6.76	JEL427-P39	129	N	2587	28620
719	49.5	8.32	JEL427-P39	130	Y	464	10140
722	51.2	9.68	JEL427-P39	130	Y	4	799
725	42.9	7.64	JEL427-P39	129	N	5355	10044
732	40.9	5.92	JEL427-P39	91	N	0	152400
734	48.3	7.17	JEL427-P39	110	N	0	235986
735	42.8	5.67	JEL427-P39	130	Y	0	0
736	50.8	8.96	JEL427-P39	78	N	6	43182
738	50.9	7.86	JEL427-P39	101	N	367	128640
741	44.5	7.02	JEL427-P39	130	Y	4952	2892
745	46.2	6.56	JEL427-P39	91	N	2	78300
747	43.3	5.36	JEL427-P39	82	N	6	34
748	50.5	7.14	JEL427-P39	126	N	0	52020
752	47.8	6.28	JEL427-P39	130	Y	0	33
753	47.8	9.25	JEL427-P39	130	Y	4	13920
754	48.2	7.64	JEL427-P39	103	N	2	27678
755	42.9	5.43	JEL427-P39	101	N	0	12300
760	49.5	8.63	JEL427-P39	96	N	0	3300
761	50.8	8.80	JEL427-P39	130	Y	66	309
763	47.9	7.45	JEL427-P39	130	Y	384	648
766	43.3	7.03	JEL427-P39	129	N	0	123960
768	50.7	7.35	JEL427-P39	123	N	28724	131130
702	48.1	8.19	JEL427-P9	84	N	6338	153510
713	46.0	6.74	JEL427-P9	77	N	3498	263550
715	46.4	6.65	JEL427-P9	97	N	166	66018
716	50.5	6.90	JEL427-P9	45	N	25	223536
717	48.1	8.44	JEL427-P9	49	N	27	273132

718	53.2	9.08	JEL427-P9	70	N	85	109500
723	43.1	6.53	JEL427-P9	74	N	412	1120
726	42.7	5.74	JEL427-P9	121	N	6	83040
727	51.0	9.42	JEL427-P9	61	N	1	109458
728	48.2	8.72	JEL427-P9	107	N	0	218994
729	42.6	6.30	JEL427-P9	72	N	2756	123948
730	38.4	4.32	JEL427-P9	42	N	3	149046
731	43.3	6.61	JEL427-P9	49	N	70	110940
733	43.2	5.54	JEL427-P9	110	N	187	40788
737	43.0	6.96	JEL427-P9	96	N	706	154890
739	52.9	10.03	JEL427-P9	43	N	4	12570
742	44.6	6.61	JEL427-P9	85	N	53	49692
743	43.3	7.56	JEL427-P9	65	N	0	473
746	41.5	7.49	JEL427-P9	55	N	1	104832
749	40.9	6.30	JEL427-P9	67	N	2901	70560
750	48.2	7.17	JEL427-P9	51	N	851	88308
751	47.8	7.36	JEL427-P9	67	N	924	121500
756	41.5	6.52	JEL427-P9	60	N	1	NA
757	41.3	5.75	JEL427-P9	77	N	0	221322
758	46.5	6.75	JEL427-P9	66	N	0	335358
759	44.4	5.56	JEL427-P9	108	N	650	87282
762	46.3	8.03	JEL427-P9	82	N	0	2110
764	51.4	9.55	JEL427-P9	53	N	0	313368
767	43.2	7.88	JEL427-P9	94	N	846	51294
769	47.5	8.26	JEL427-P9	85	N	21	91158

Censor = Y frogs survived to the end of the experiment. Infection intensity = zoospore genomic equivalents.

S7. *Dendrobates tinctorius* frogs used in trial experiment testing the infectivity and pathogenicity of Bd strain JEL427. Exposure dose was 10^5 zoospores/ml. Frogs were obtained from a captive breeder in Sept 2011 and were Bd-negative upon arrival.

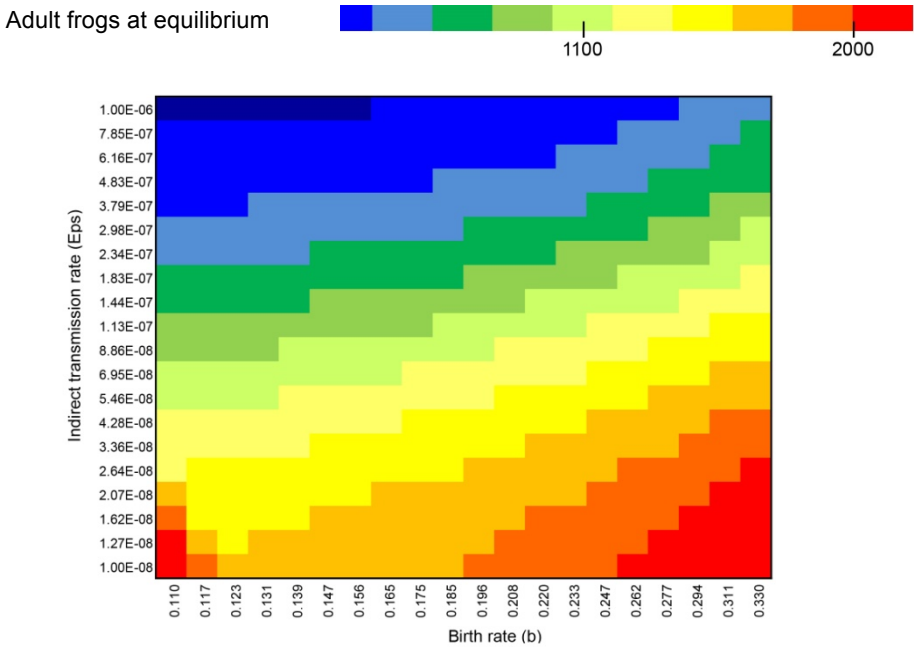
Frog ID	SVL (mm)	Mass (g)	Experimental treatment	Days survived post-exposure	Censored	Infection intensity at death
900	32.78	3.08	Control	250	Y	0
901	30.91	2.31	Control	162	N	0
902	33.15	3.51	control	250	Y	0
903	35.94	3.68	control	250	Y	0
904	32.82	2.86	Bd-exposed	86	N	29052
905	33.33	3.08	Bd-exposed	185	N	2918
906	25.14	1.34	Bd-exposed	50	N	7470
907	32.97	2.86	Bd-exposed	98	N	80100

Censor = Y frogs survived to the end of the experiment. Infection intensity = zoospore genomic equivalents.

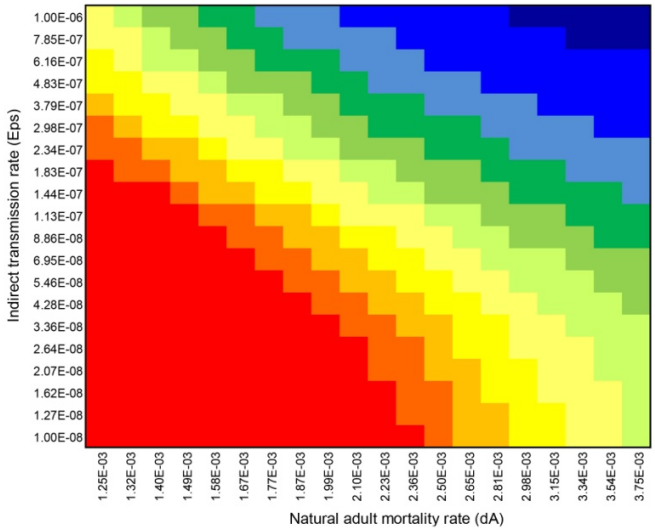
APPENDIX B

SUPPLEMENTARY FIGURES

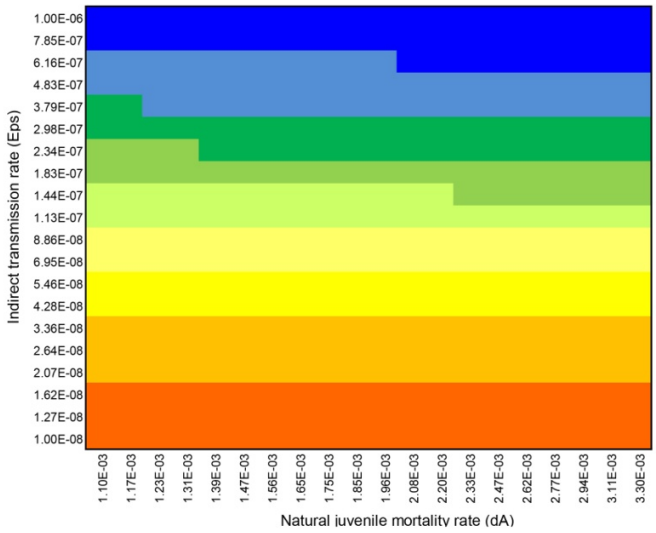
S1. Sensitivity of natural history parameters: number of adult *E. coqui* frogs at equilibrium for different values of the indirect transmission rate ε and (a) birth rate, (b) immediate post-hatching survival rate, (c) natural mortality rate of adults, (d) natural mortality rate of juveniles, and (e) carrying capacity.



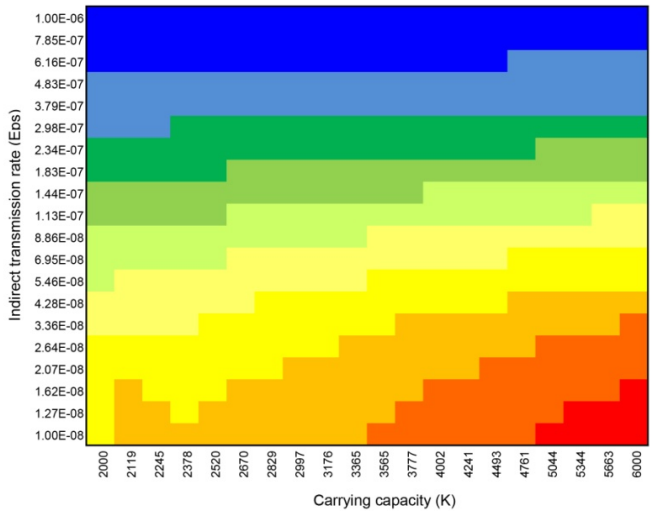
(c)


Indirect transmission rate (Eps)
1.00E-06
7.85E-07
6.16E-07
4.83E-07
3.79E-07
2.98E-07
2.34E-07
1.83E-07
1.44E-07
1.13E-07
8.86E-08
6.95E-08
5.46E-08
4.28E-08
3.36E-08
2.64E-08
2.07E-08
1.62E-08
1.27E-08
1.00E-08
1.25E-03
1.32E-03
1.40E-03
1.49E-03
1.58E-03
1.67E-03
1.77E-03
1.87E-03
1.99E-03
2.10E-03
2.23E-03
2.36E-03
2.50E-03
2.65E-03
2.81E-03
2.98E-03
3.15E-03
3.34E-03
3.54E-03
3.75E-03
Natural adult mortality rate (dA)

(d)


Indirect transmission rate (Eps)
1.00E-06
7.85E-07
6.16E-07
4.83E-07
3.79E-07
2.98E-07
2.34E-07
1.83E-07
1.44E-07
1.13E-07
8.86E-08
6.95E-08
5.46E-08
4.28E-08
3.36E-08
2.64E-08
2.07E-08
1.62E-08
1.27E-08
1.00E-08
1.10E-03
1.17E-03
1.23E-03
1.31E-03
1.39E-03
1.47E-03
1.56E-03
1.65E-03
1.75E-03
1.85E-03
1.96E-03
2.08E-03
2.20E-03
2.33E-03
2.47E-03
2.62E-03
2.77E-03
2.94E-03
3.11E-03
3.30E-03
Natural juvenile mortality rate (dA)

(e)


Indirect transmission rate (Eps)
1.00E-06
7.85E-07
6.16E-07
4.83E-07
3.79E-07
2.98E-07
2.34E-07
1.83E-07
1.44E-07
1.13E-07
8.86E-08
6.95E-08
5.46E-08
4.28E-08
3.36E-08
2.64E-08
2.07E-08
1.62E-08
1.27E-08
1.00E-08
2000
2119
2245
2378
2520
2670
2829
2997
3176
3365
3565
3777
4002
4241
4493
4761
5044
5344
5663
6000
Carrying capacity (K)

APPENDIX C

ANALYTICAL CODE

R code used to create mixed-effects models for analyzing mean infection intensity over time between treatment groups in chapter 3 (credit: Tate Tunstall, University of Maryland)

```
require(nlme)

expdata <- read.table(file="C:/Users/Penny/Atelopusintensity04-04-
13.txt", head=TRUE, sep="\t")

expdata$logint <- log(expdata$intensity+.01)

mod1 <- lme(logint ~ time.numeric + treatment,random=~1|frogID,
correlation=corAR1(form=~time.numeric|frogID, fixed =FALSE),
data=expdata, na.action=na.omit)

summary(mod1)

mod2 <- lme(logint ~ time.numeric + treatment+
time.numeric:treatment,random=~1|frogID,
correlation=corAR1(form=~time.numeric|frogID, fixed =FALSE),
data=expdata, na.action=na.exclude)
```

Example R code used to analyze Bd-coqui mathematic model in chapter 4 (credit: Grace DiRenzo, University of Maryland)

```
library(emdbook)
require(deSolve)
require(rootSolve)

ZSI2<- function(t, y, p)
{
      {
            Sj <- y[1]
            Ij <- y[2]
            Z <- y[3]
            Sa <- y[4]
            Ia <- y[5]
      }
      with(as.list(p),
                  {

dSj.dt <- (b * psi * (Sa + Ia)*(1 - (Sa + Ia)/K)) - (dj * Sj) - (E * Sj
* Z) - (B * Sj * Ij) - (chiS * Sj) + (Gj * Ij)

dIj.dt <- (E * Sj * Z) + (B * Sj * Ij) - (dj * Ij) - (alphaj * Ij) -
(chiI * Ij) - (Gj * Ij)

dZ.dt  <- (p * Ia) + (p * Ij) - (mu * Z)

dSa.dt <- (chiS * Sj) - (E * Sa * Z) - (B * Sa * Ia) - (da * Sa) + (Ga
* Ia)

dIa.dt <- (chiI * Ij) + (E * Sa * Z) + (B * Sa * Ia) - (alphaa * Ia) -
(da * Ia) - (Ga * Ia)
            return(list(c(dSj.dt, dIj.dt, dZ.dt, dSa.dt, dIa.dt)))
            })
}

par_spac <- lseq(0.00000001, 0.000001, 20) #vary parameter E
par_spac2 <- lseq(.000001, 0.01, 20) #vary parameter alphaj
all <- expand.grid(par_spac, par_spac2) #combinations of two params
ZSI2.mat <- matrix(0, nrow = nrow(all), ncol= 5)
colnames(ZSI2.mat) <- c("E", "Aj", "Z", "Nj", "Na")

for (i in 1:nrow(all)){
      ZSI2.mat[i,1] <- all[i,1]
      ZSI2.mat[i,2] <- all[i,2]

      Z <- 1
      Na <- 100
      Ia    <- 1
      Sa <- Na - Ia
      Nj <- 530
      Ij <- 1
      Sj <- Nj - Ij
```

```
      para <- c(b = 0.066, K=4000, dj = 0.0022, B = 0.000001, E =
all[i,1], Gj = 0.0005, alphaj = all[i,2], chiS = 0.00044, chiI=0.0002,
da = 0.0025, Ga = 0.01, alphaa = 0, p = 10, mu = 1)

      x <- runsteady(y = c(Sj, Ij, Z, Sa, Ia), time = c(0, 7300), func
= ZSI2, parms = para)

      ZSI2.mat[i,3] <-  x$y[3]
            # Zoospores
      ZSI2.mat[i,4] <-  x$y[1] + x$y[2]
            # Nj
      ZSI2.mat[i,5] <-  x$y[4] + x$y[5]
            # Na
}

write.csv(ZSI2.mat, file="C:/Users/Penny/File.csv")

col.l <- colorRampPalette(c('blue', 'green', 'yellow', 'red'))(30)
col.m <- colorRampPalette(c('blue', 'red'))(30)
levelplot(ZSI2.mat[,5]~ ZSI2.mat[,2] * ZSI2.mat[,1], cuts = 20, region
= T, scales=list(x=list(log=10), y=list(log=10)),col.regions=col.l,
      main = "Number of adults at equilibrium", ylab = "Indirect
transmission rate (E)", xlab = "Juvenile disease-induced mortality rate
(Aj)", xlim= c(0.000001, 0.01), ylim= c(0.00000001, 0.000001))
```