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POTENTIAL FITNESS COST OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN *ELEUTHERODACTYLUS COQUI*, AND COMMENTS ON ENVIRONMENT-RELATED RISK OF INFECTION

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Abstract: Body size (SVL) of adult *Eleutherodactylus coqui* frogs infected versus non-infected with *Batrachochytrium dendrobatidis (Bd)* were compared to determine a potential fitness cost of chytridiomycosis in persisting populations. Studies were conducted at two different elevations, in a Cloud Forest (650 m) and an Elfin Forest (850 m), in El Yunque, Puerto Rico. Results showed that *Bd*-infected males were significantly smaller than non-infected males in the Cloud Forest, but not in the Elfin Forest. Although infected females also tend to be smaller than non-infected ones, this effect was not significant. *Bd* prevalence and probability of infection by this fungus was significantly greater in the Cloud Forest than in the Elfin Forest, regardless of sex. We report on differences of environmental factors in these forest types in Puerto Rico, and discuss their implications on the growth of *Bd* and the vulnerability of frogs to infection by this pathogen.

Key Words: Body size, *Eleutherodactylus*, *Batrachochytrium dendrobatidis*, amphibian declines, fitness cost, direct development.

Resumen: P.A. Burrowes, A.V. Longo y C.A Rodríguez. "Costo potencial en aptitud evolutiva de *Batrachochytrium dendrobatidis* en *Eleutherodactylus coqui*, y comentarios sobre el riesgo de infección relacionado con el ambiente". El tamaño corporal (longitud hocico a cloaca) en adultos de *Eleutherodactylus coqui* infectados y no infectados con *Batrachochytrium dendrobatidis* (*Bd*) fue comparado para determinar el costo potencial en aptitud evolutiva de quitridiomicosis en poblaciones resistentes. Los estudios fueron realizados en dos tipos de bosque a diferentes elevaciones, Bosque Nublado (650 m) y Bosque Nublado Enano (850 m), en El Yunque, Puerto Rico. Nuestros resultados demuestran que los machos infectados con *Bd* son significativamente más pequeños que los no infectados en el Bosque Nuboso, sin embargo no sucede lo mismo en el Bosque Nublado Enano. Aunque las hembras que están infectadas por *Bd* también son más pequeñas que las no infectadas, este efecto no es estadísticamente significativo. La prevalencia de *Bd* y la probabilidad de infección por este hongo son significativamente más altas en el Bosque Nublado que en el Bosque Nublado Enano para ambos sexos. Reportamos las diferencias en factores ambientales en estos dos tipos de bosque en Puerto Rico y discutimos las implicaciones en el crecimiento de *Bd* y la vulnerabilidad de las ranas a la infección por este patógeno.

Palabras Clave: Tamaño corporal, *Eleutherodactylus*, *Batrachochytrium dendrobatidis*, declinaciones de anfibios, aptitud evolutiva, desarrollo directo.

INTRODUCTION

It is well documented that many amphibian species die from skin infections by a pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (Berger *et al.* 1998; Longcore *et al.* 1999; Lips 1998, 1999). In Central America, entire amphibian communities have been wiped out by this fungal infection (Lips *et al.* 2006). However, some amphibian species seem to persist with low-level *Batrachochytrium dendrobatidis* (*Bd* hereafter) infections (Beard and O'Neill 2005; Burrowes *et al.* 2004; Puschendorf *et al.* 2006; Oliveira de Queiroz *et al.* 2006). In Puerto Rico, we have reported declining amphibian populations since the 1980's, and these have been linked to a potential synergistic effect between *Bd* infections and climate change (Burrowes *et al.* 2004). The earliest record of *Bd* infection in Puerto Rico is 1976 and at present ten native species (nine *Eleutherodactylus,* and *Leptodactylus albilabris*) have been diagnosed positive (Burrowes *et al.* 2008). One of these species is potentially extinct (*E. karlschmidti*), and six others are declining in mountain areas and are considered endangered or critically endangered (IUCN 2006). At this point, *Bd* infections are endemic and occurring in most amphibians above 500 m in mountain ranges all over the island (Burrowes *et al.* 2008). *Eleutherodactylus coqui* populations show a variation of abundance through time at the two forest elevations, Cloud Forest (650 m) and Elfin Forest (850 m), in the eastern mountains of El Yunque, Puerto Rico (Burrowes *et al*. 2004). In the lower Cloud Forest, this species is declining since the late 1980's, but in the Elfin

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Forest it has recuperated from a decline in the early 1990's and appears to be stable (Burrowes and Joglar 2005). In this paper we investigate these population effects by estimating *Bd* infection risk at these forest types, and discuss the environmental factors that may be associated with differences in *Bd* prevalence.

Stressors such as competition, predation pressure or disease are known to affect growth rates in amphibians (Richards 1962; Beebee and Wong 1992; Kiesecker *et al.* 1999 Parris and Cornelius 2004). *Bufo fowleri* and *Hyla chrysoscelis* tadpoles were found to metamorphose at lower body masses when infected with *Bd* (Parris and Cornelius 2004). In addition, Kriger *et al.* (2006, 2007) found that smaller frogs of *Mixophyes iteratus* and the *Litoria leuseri* complex in Australia, had higher prevalence, and intensity of *Bd* infections, and suggested developmental stress in infected frogs as one possible explanation for these results. The purpose of this work was to investigate a potential decrease in fitness in *Eleutherodactylus coqui* that are infected with *Bd* but persist in the rainforest.

We used snout-to-vent length (SVL) of adults as an indicator of fitness, because size is highly correlated with anuran female fecundity (Crump 1974, Lauck 2005) and particularly, with *E. coqui* clutch size (Townsend and Stewart 1984, Joglar 1998). Although reproductive success of male *E. coqui* has been correlated to calling activity and not directly to body size (Woolbright and Stewart 1987), larger males have higher call intensities, and thus a potential advantage towards getting a mate (Narins and Hurley 1982). Thus, we hypothesize that if infection by *Bd* induces a developmental constraint that results in smaller body size in coqui frogs, it would represent a fitness cost to individuals, and in case of an epidemic, to entire populations resulting in observable declines. The existence of such a response to *Bd* infection would have critical implications for understanding patterns of disease dynamics in amphibian communities that are rich in direct-development Eleutherodactyline frogs.

MATERIALS AND METHODS

Study Site

Our study took place at El Yunque National Forest, a tropical rainforest reserve in eastern Puerto Rico where we have been conducting studies on the effect of *Bd* infection on frog populations for the past six years. We report on two frog populations, one in Palo Colorado Forest (18º18'2.14''N, 65º47'7.4''W) at 650 m, and the other in Bosque Enano Forest (18º18'6.72''N, 65º47'40.498''W) at 850 m. These forests differ in structure mostly as a response to elevation. The Palo Colorado Forest at El Yunque is part of the subtropical wet forest association (Ewel and Whitmore 1973), and its name is related to the abundance of the native tree *Cyrilla racemiflora.* Bosque Enano is an Elfin Forest formation classified as lower montane wet forest in the Holdridge system (Ewel and Whitmore 1973), and characterized by permanent cloud cover and nocturnal fog. For the purposes of comparability, hereafter we will refer to the commonly known *Palo Colorado* forest as Cloud Forest, and to *Bosque Enano* as Elfin Forest (Lugo 2005).

Field Methods

In both forest types we walked a 100 X 3 m linear transect in order to monitor frog relative abundance and prevalence of *Bd*. Frogs were captured in individual plastic bags worn as gloves and inverted to prevent cross contamination. SVL was measured using digital calipers (Digimatic Plastic Caliper; Mitutoyo Corporation) within the deflated plastic bag. Molding the plastic around the frog helped keep individuals still and in a flat position to minimize measurement errors. Frogs were examined for physical condition and sampled for *Bd* by swabbing their ventrum, feet and toes as described by Kriger *et al.* (2007). In addition, frogs were toe-clipped for marking purposes following a modification of Twitty (1966); toe tissue was preserved in 70% ethanol and stored at room temperature until *Bd* diagnostics. A fresh pair of powder-free nitrile gloves was worn every time a different frog was manipulated.

Bd diagnostics

Bd detection was done by Polymerase Chain Reaction (PCR) performed by Pisces Molecular (Boulder, Colorado, U.S.A.), following Annis *et al*. (2004); and Taqman quantitative PCR according to Boyle *et al.* (2004) at Zoological Society of London and University of Puerto Rico facilities. Quantitative results on zoospore number provided by the qPCR method were not taken into account for scoring infection status in this paper, only presence or absence of the pathogen was considered. Toe clippings were preferred over swabs because when comparing results from swabs and toes from the same frog, we found that swabs did not detect low-level infection (Longo and Burrowes, *unpublished data*).

Analysis

SVL data of all frogs sampled for *Bd* were divided by sex, and forest type and analyzed using Minitab (Release 14). Mann Whitney tests for equality of medians were performed to determine differences in SVL between *Bd*-infected and non-infected frogs by sex and forest type, and Chi square tests of independence were performed to determine potential associations between prevalence of *Bd* with sex or forest type. We used a statistical package for epidemiology (Epi-Info) to calculate the odds ratio (OR), in order to compare the probability of having *Bd* in two populations from different forest types.

Results

Eleutherodactylus coqui males measured 34.9 ± 0.39 mm (mean ±SE) SVL and females 43.7 ± 0.39 mm SVL. Because

subadults measure less than 25 mm in the highlands of Puerto Rico (Joglar 1998), we are confident that only adults were considered in this study. Sexual dimorphism by size, where females are approximately 22% larger than males is characteristic of *E. coqui* (Joglar 1998); thus, all analyses were run separately for males and females. We found that prevalence of *Bd* was independent from sex, suggesting that males and females of *E. coqui* are equally likely to get infected by this fungus (X^2 = 0.528, *DF* = 1, *P* = 0.465). In both sexes, *Bd*-infected frogs had smaller SVL than non-infected individuals (Fig. 1). However, a significant difference in body size between *Bd* infected and non-infected adults (regardless of forest type) was found only for males (*W* = 14908.5, *P* < 0.0003, *N* = 208).

Bd infection rate and the relationship between *Bd* infection and male body size varied between forest types. Males of *Eleutherodactylus coqui* infected with *Bd* were significantly smaller than non-infected males (*W* = 10349.5, *P* < 0.0008, *N* = 179), only in the Cloud Forest (Fig. 2). Also, prevalence of *Bd* was significantly different in the Cloud Forest than in the Elfin Forest (X^2 = 22.86, *DF* = 1, *P* = 0.000), suggesting that vulnerability to *Bd* is dependent of forest type (see Table 1 for sample sizes). Odds ratio analysis was significant for forest types (*OR* = 3.02, *P* < 0.001, 95% C.I. = 1.81, 5.01), suggesting that adult *E. coqui* frogs are about three times more likely to get infected by *Bd* in the Cloud Forest than in the Elfin Forest. When divided by sex, the association between *Bd* prevalence and forest type was significant for both males (*OR* = 3.92, *P* < 0.001, 95% C.I. = 1.94, 8.04) and females (*OR* = 2.13, *P* < 0.03, 95% C.I. = 0.98, 4.65) of *E. coqui* (Table 1).

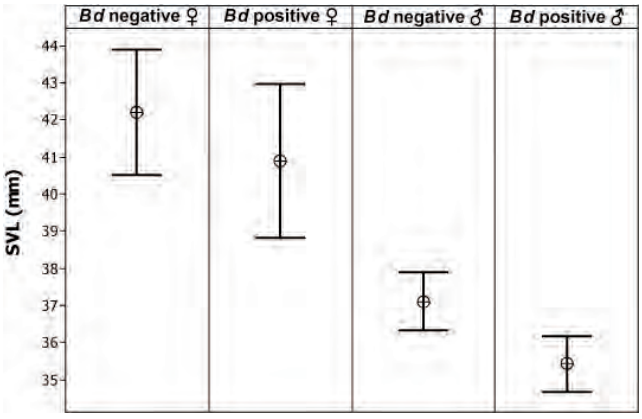

FIG. 1. Mean and 95% confident intervals of SVL's of adult males and females of *Eleutherodactylus coqui* that are infected versus non-infected with *Batrachochytrium dendrobatidis* at both study sites. Differences in size are significant only for males (*W* = 14908.5, *P* = 0.0003, *N* = 208).

Media e intervalos de confianza 95% de LCR de machos y hembras adultas de Eleutherodactylus coqui *que están infectados versus no-infectados con* Batrachochytrium dendrobatidis *en ambos sitios de estudio. Diferencias en tamaño son sólo significativas para machos (W = 14908.5, P = 0.0003, N = 208).*

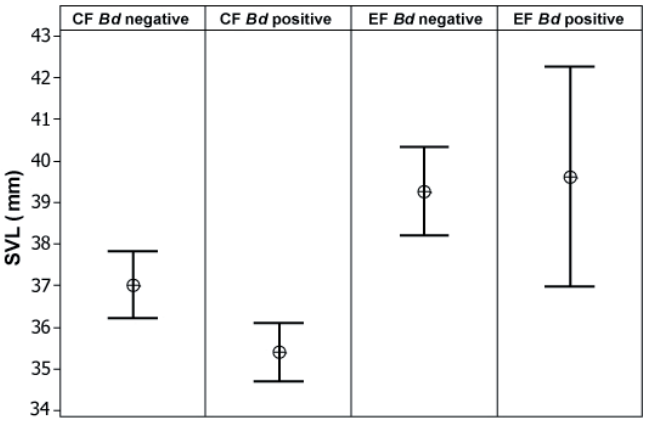

FIG. 2. Mean and 95% confident intervals of SVL's of adult males of *Eleutherodactylus coqui* that are infected versus non-infected with *Batrachochytrium dendrobatidis* in two forest types, Cloud Forest (CF) at 650 m, and Elfin Forest (EF) at 850 m. Differences are significant only in the Cloud Forest (*W* = 10349.5, *P* = 0.0008, *N* = 179).

Media e intervalos de confianza 95% de LCR de machos adultos de Eleutherodactylus coqui *que están infectados versus no-infectados con* Batrachochytrium dendrobatidis *en dos tipos de bosques, Bosque Nublado (CF) a 650 m, Bosque Nublado Enano (EF) a 850 m. Diferencias son significativas sólo en Bosque Nublado (W = 10349.5, P = 0.0008, N = 179).*

Discussion

Results of this work provide further evidence that *Bd*-infection is associated with a decrease in body size in populations affected by this disease. Several explanations have been proposed to explain this relationship, including the possibility that (1) larger/older frogs may have more resistance to *Bd* (Kriger *et al.* 2006), (2)*Bd* infection may cause a developmental constraint leading to small body sizes (Parris and Cornelius 2004; Kriger *et al.* 2007), (3) infected frogs die before they reach large body sizes (Kriger *et al.* 2006), and (4) smaller frogs occupy different microhabitats where the risk of infection with *Bd* is higher (Beard and O'Neill 2005). Long-term *Bd* diagnosis of *E. coqui* at our study sites at El Yunque, have revealed that prevalence of *Bd* in juveniles is 20% higher than adults, and Beard and O'Neill (2005) found greater infection rates in subadults than adults of this species in Hawaii. Juveniles and subadults of *E. coqui* are active in the lower vegetation (d < 50 cm) where humidity levels are higher, and hence, the chances of getting infected with *Bd* may be greater (Beard and O'Neill 2005). In addition, *E. coqui* eggs have diagnosed positive for *Bd* suggesting that male parental care can serve as a means to transmit the infection to newborns in direct-developing frogs (Longo and Burrowes,*unpublished data*). Thus, we favor the developmental constraint hypothesis for *E. coqui* because newborns are vulnerable to contract *Bd* directly from infected parents at the nest, and juveniles and subadults are more likely to spend more time in moist *Bd*-favorable microhabitat. We suggest that once a newborn or

TABLE 1. Prevalence (number of infected individuals/ number of frogs sampled X 100) of *Batrachochytrium dendrobatidis* (*Bd*) in *Eleutherodactylus coqui*. Odd ratio statistic is significant in all cases, and indicates that the probability of frogs being infected by *Bd* is about 3 times higher in the Cloud forest than in the Elfin Forest.

TABLA 1. Prevalencia (número de individuos infectados / número de ranas muestradas X 100) de Batrachochytrium dendrobatidis *(Bd) en* Eleutherodactylus coqui. *La estadística de odd ratio es significativa en todos los casos, e indica que la probabilidad de las ranas de ser infectadas por Bd es unas 3 veces más alta en el Bosque Nublado que en el Bosque Nublado Enano.*

Forest Type	Prevalence of *Bd*	Total number of frogs	Statistic (Odds Ratio, Probability, 95 % Confidence Interval)
All adult frogs:			
Cloud Forest	44.1%	299	*OR* = 3.02,*P* < 0.001, C.I. = 1.81, 5.01
Elfin Forest	20.7%	130	
Males:			
Cloud Forest	42.5%	179	*OR* = 3.92, *P* < 0.001, C.I. = 1.94, 8.04
Elfin Forest	15.9%	82	
Females:			
Cloud Forest	46.7%	120	*OR* = 2.13,*P* < 0.03, C.I. = 0.98, 4.65
Elfin Forest	29.2%	48	

juvenile of *E. coqui* contracts *Bd*, it will die when infection intensity is very high, but it may survive by carrying a low infection at a fitness cost of smaller body size. Calling intensity of males is influenced by body size (Narins and Hurley 1982), such that smaller *Bd*-survivors may have a reduced chance of getting a mate. As a result *Bd* infection, even when carried at low levels, would represent a fitness cost to individuals, and in case of an epidemic, to entire populations resulting in observable declines.

We found that males and females of *E. coqui* are equally likely to be infected by *Bd*. The lack of a significance of an effect of *Bd* infection on female SVL, does not mean that females are not negatively affected by this pathogen. Our results may be an artifact of smaller female sample sizes in our data,or that body size is not the best estimate of fitness in direct-developing female frogs.

Finally, our results show differences in the response of populations of *E. coqui* to *Bd* infection in two different forest types within the same mountain range. Adult frogs in the Cloud Forest are about three times more likely to get infected than in the Elfin Forest, regardless of sex (Table 1). Thus, it is important to determine what environmental factors differ between these two forest types, which might favor *Bd* growth or increase frog's vulnerability to *Bd* infection (Table 2).

Prevalence of *Bd* in natural amphibian populations has been associated to particular climatic envelopes with lower temperatures (< 23 °C) and higher precipitation rates (Berger *et al*. 2004, Retallick *et al*. 2004, Kriger and Hero 2006, Kriger*et al*. 2007). Pounds *et al*. (2006) suggested that global climate change is favoring a temperature optimum for *Bd* growth at mid-elevations in the mountains of Central and South America. In laboratory studies *Bd* grows at a temperature range of 17-25 °C, with optimum results at 23 °C (Piotrowski *et al*. 2004). Table 2 compares the Cloud Forest and the Elfin Forest at El Yunque, Puerto Rico with respect to a variety of environmental factors that may be important for *Bd* growth and dispersal across a mountain range.

Higher moisture and lower temperatures at the Elfin Forest would favor *Bd* growth most of the year (Table 2). However, drier weather at the Cloud Forest may cause frog clumping at humid retreat sites favoring transmission and outbreak of *Bd* (as suggested by Burrowes *et al*. 2004). *Eleutherodactylus coqui* clump at humid retreat sites when deprived of water (Joglar *et al*. 2007), and clumping at humid retreat sites has proven to increase mortality of *Bd*-infected frogs in laboratory studies (Longo and Burrowes, *unpublished data*). Runoff is greater in the Elfin Forest and that water drains into the lower Cloud Forest (Table 2), serving as a source and means for zoospores to spread down to an environment where hosts are more susceptible. In addition, the soil in the Cloud Forest is of sandy texture, and *Bd* zoospores have been shown to survive in moist sand for up to three months (Johnson and Speare 2005).

Thus, the response of frog communities to *Bd* infection may be more difficult to explain than attribution to a localized (mid-elevation) temperature optimum (Pounds *et al.* 2006) or the wave-like spread of zoospores across mountains (Lips *et al.* 2006, 2008). Our data suggests that the response of *E. coqui* to *Bd* infection does not clearly respond to one or the other, but rather to a combination of both in relation to particular environmental factors within a forest range.

The fact that *Bd* is now endemic and widespread in Puerto Rico provides an opportunity to understand how this disease may influence populations that appear to be resistant (Burrowes *et al.* 2008). Herein we show that males and females of *E. coqui* have a potential cost to fitness in spite of underlying

TABLE 2. Comparison of factors that may affect *Bd* prevalence or dispersal in two forest types at El Yunque, Puerto Rico.
TABLA 2. Comparación de factores que pueden afectar la prevalencia o dispersión de Bd en dos tipos de bosque en El Yunque, Puerto Rico.

Attributes Affecting *Bd* Prevalence	Cloud Forest (Palo Colorado)	Elfin Forest (Bosque Enano)	Reference
Mean annual temperature range	17-26 °C	14.5-19 °C	Burrowes *et al.* 2004
Elevation at study sites	650 m	850 m	Joglar, 1998
Mean annual precipitation	4191 mm/yr	4849mm/yr	García Martinó *et al.* 1996.
Mean number of days without rain in one year	77	38	García Martinó *et al.* 1996.
Runoff	3197 mm/yr	3958 mm/yr	García Martinó *et al.* 1996
Evapotranspiration (precipitation – runoff)	994 mm/yr	1292 mm/yr	García Martinó *et al.* 1996.
Cloud cover	Point of cloud formation.	Permanent	Lugo 2005
Soil composition	Granite, sandy texture	Volcanic,rich in organic materials and nutrients	Lugo 2005
Soil O_2 concentration at 10 cm depth	13 % ± 0.21	8 % ± 0.19	Silver *et al.* 1999
Canopy and average tree height	Closed, < 24 m	Closed, < 5 m	Lugo 2005
Average production of green house gases:			
CO_2	15.9×10^{13}	11.2×10^{13}	Keller *et al.* 1986
CH_4	380×10^{10}	6.5×10^{10}	

mechanisms that may contribute to its persistence with chytridiomycosis in the wild. In addition, we show that frogs in the Cloud Forest have greater prevalence and risk of infection of *Bd*, than in the higher Elfin Forest. These findings set the basis for further research to test hypotheses on the effect of climate change on weather patterns that may affect *Bd* growth at different elevations (Pounds *et al.* 2006), and potential venues for *Bd* introductions and spread (Lips *et al.* 2008, Ron 2005).

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