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Local phenotypic variation in amphibian-killing fungus predicts infection dynamics

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ABSTRACT

Environmental factors can limit the distribution of organisms if they are not able to respond through phenotypic plasticity or local adaptation. *Batrachochytrium dendrobatidis* (*Bd*) is a broadly distributed pathogen, which shows spatially patterned genotypic and phenotypic variation; however, information on the functional consequences of this variation on disease dynamics in natural hosts is limited. We genotyped and quantified variation in *Bd* phenotypes across an elevational gradient and quantified host infection dynamics at each site. All *Bd* strains were members of the global panzootic lineage yet differed in phenotype. We hypothesize that this phenotypic variance results from adaptive processes due to the interaction between pathogen, hosts, and environment. We detected a correlation between zoospore and zoosporangia sizes and a positive association between zoosporangia size and *Bd* prevalence. Given that *Bd* phenotype predicted disease status in our wild populations, we developed an index to identify critical environments where the fungus could be more deleterious.

1. Introduction

Organisms that have broad distributions must contend with highly variable environmental factors that affect their fitness (Thoday, 1953; Hall et al., 1992; Travis, 1994). Depending on their degree of tolerance, many environmental factors such as temperature, seasonality, precipitation, humidity, and elevation can limit organismal distributions or directly affect their performance and probability of persistence (Ignoffo, 1992; Inglis and Sagripanti, 2006). Organisms use a number of mechanisms to respond to these environmental challenges that fall along a continuum from phenotypic plasticity to microevolution (Reed et al., 2010). Independent of the mechanism, the result is phenotypic or genetic variation that has functional consequences not only for species persistence, but also for interactions with other species in the community (Agrawal, 2001; Smits et al., 2006). Functionally significant phenotypic changes will be especially important in interactions between hosts and pathogens, because they can change patterns of disease dynamics in populations. For example, the pathogenic fungus *Cryptococcus neoformans* is capable of producing significantly larger cells when infecting particular hosts as a means of evading host immune responses (Zaragoza et al., 2010), thus altering infection rates in host populations. While we know that pathogens often vary in phenotype (Brown and Barker, 1999; Drenkard and Ausubel, 2002; Matz et al., 2005; Smits et al., 2006), we know less about how those phenotypes translate into patterns of disease in natural populations.

The amphibian-killing fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), causes chytridiomycosis, which is a main factor contributing to amphibian population declines and extinctions worldwide (Wake and Vredenburg, 2008; Fisher et al., 2012). *Bd* is

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broadly distributed, infects a wide range of hosts (Olson et al., 2013; Valencia-Aguilar et al., 2015), and persists across a broad temperature range (Piotrowski et al., 2004; Woodhams et al., 2008; Becker and Zamudio, 2011). These characteristics increase its success as a host-generalist pathogen. Distribution models based on climatic and topographic variables have been used to predict the current and future (Ron, 2005; Rödder et al., 2008, 2009, 2010; Puschendorf et al., 2009; Murray et al., 2011; Rohr et al., 2011; Liu et al., 2013; James et al., 2015) ranges of this pathogen. These models infer the potential occurrence of *Bd* at continental and global scales but only indirectly predict the likelihood of disease outcomes. Additionally, we know that phenotypic variation in *Bd* can be explained by life history trade-offs (Woodhams et al., 2008) and potential changes in gene expression conferred by aneuploidy or other genomic features (Rosenblum et al., 2013), but rarely are we able to link these changes in phenotype to community level disease epidemiology. These interactions between environmental and genetic control are evident in the many field surveys of pathogen prevalence in wild populations (Burdon, 1977; Vercelli, 2004; Hunter, 2005). For example, models predict that *Bd* has a higher probability of occurrence at high elevations, where mean temperatures and cloud cover provide better (or ideal) conditions for its growth (Piotrowski et al., 2004; Pounds et al., 2006). However, not all high elevation sites appear to respond equally to *Bd* infection (Lips, 1998; La Marca et al., 2005; Brem and Lips, 2008; Kriger and Hero, 2008; Gründler et al., 2012).

Bd strains can be genetically subdivided into enzootic and panzootic lineages (Farrer et al., 2011; Schloegel et al., 2012; Rosenblum et al., 2013) that differ in chromosomal copy number and virulence (Farrer et al., 2013; Rosenblum et al., 2013). *Bd* strains also vary in phenotypes related to transmission. For example, among strains, zoospore sizes vary from 2 to 6 μm (Longcore et al., 1999; Fisher et al., 2009; Schloegel et al., 2012), and zoosporangium sizes vary from 15 to 68 μm in diameter (Fisher et al., 2009; Farrer et al., 2011; Flechas et al., 2013). Further, zoosporangium size is correlated to virulence (Fisher et al., 2009), and zoospore size is correlated to DNA content (Schloegel et al., 2012), suggesting that phenotype and virulence may also be related.

Knowledge of phenotypic variation in *Bd* and its functional consequences for disease dynamics is limited; thus, understanding variance in *Bd* phenotypes across varying environments could be key to investigating the evolution of *Bd* virulence at multiple spatial scales (Farrer et al., 2013). Here, we genotyped and quantified phenotypes of *Bd* strains isolated across a 500 m elevational range in the Brazilian Atlantic Forest. First, we predicted that environmental variation across the elevational gradient would explain infection dynamics across populations. Second we hypothesized that *Bd* would vary predictably in phenotypes (such as zoospore and zoosporangium sizes) that are important for disease transmission. Finally, we predicted that those changes in phenotype would affect community disease outcomes. Our study standardized host amphibian species, breeding habitat, latitude, and season, reducing extrinsic factors that might confound the association between the environment, pathogen phenotypes, and infection dynamics in these populations. We discuss the implications of our results using a predictive index that could be applied in disease monitoring of other amphibian communities infected by *Bd*.

2. Methods

2.1. Study site and field sampling

We sampled five Atlantic Coastal Forest streams along an elevational gradient (from 200 to 700 m above sea level [a.s.l.]) (Table 1) at the Parque Estadual da Serra do Mar, Núcleo Itutinga-Pilões. The sampled streams were located in the municipalities of Biritiba-Mirim and Bertioga, São Paulo state, in southeastern Brazil. All streams were located in pristine forest and had approximately the same width, water flow, and canopy cover. We collected 129 adults of our focal host species, *Hylodes phyllodes,* with a minimum of 14 adults per site (Table 1). We also collected up to 10 *H. phyllodes* tadpoles at each elevation for *Bd* isolation. All field collections were approved by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio: 26972-1) and Instituto Florestal (COTEC/SMA: 260106−011.309/2011).

2.2. Bd infection intensity and prevalence

For *Bd* detection and quantification of local prevalence and infection intensity, we collected adult *H. phyllodes* and maintained them individually in plastic bags to avoid cross-contamination. At least five individuals per locality were kept as voucher specimens and deposited at the Museu de Zoologia “prof. Adão José Cardoso” (ZUEC), Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil. At the time of capture, we swabbed all adult *H. phyllodes* following standard field sampling protocols for *Bd* detection (Hyatt et al., 2007). Each swab was placed in a dry and sterile 1.5 ml cryovial in the field and stored at −20 °C in the laboratory until diagnostic analyses were conducted. For detection and quantification of *Bd* zoospores, we first extracted DNA from swabs using 50 μl of PrepMan ULTRA® (Life Technologies) and then quantified infection intensities for each frog using a TaqMan® qPCR Assay (Life Technologies) (Boyle et al., 2004), with modifications described in Kriger et al. (2006) and Lambertini et al. (2013). Briefly, we used strain CLFT 023 as a quantitative standard for the qPCR reactions (Longo et al., 2013). The strain was grown in 1% tryptone in Petri dishes. After growth plates were flooded with distilled water, zoospores were collected and counted with a hemocytometer, before DNA extraction with 100 μl of PrepMan ULTRA® (Boyle et al., 2004; Lambertini et al., 2013). We serially diluted (1:10) the genomic extract to generate the standard curve for our qPCR reactions (10^3 to 10^{-1} genomic equivalents − g.e.). We considered field collected samples positive for *Bd* results when at least one g.e. was detected (Kriger et al., 2007).

2.3. Bd phenotypes and genotypes

We screened up to ten *H. phyllodes* tadpoles from each elevation for possible *Bd* infection by examining their mouthparts (Knapp and Morgan, 2006; Lambertini et al., 2013). We used only tadpoles for *Bd* isolation because it is easier to identify infected individuals in the field by examining mouthpart depigmentation, and also because they typically carry higher *Bd* infection loads than adults (Vieira and Toledo, 2012). Animals showing signs of *Bd* infection were euthanized and oral tissues excised for confirmation of infection by the presence of zoosporangia using compound microscopy (Vieira and Toledo, 2012). The number of euthanized tadpoles varied depending on the number of infected individuals, but we had success in *Bd* isolation with five to ten euthanized tadpoles per locality. Mouthparts containing zoosporangia were dissected and mechanically cleaned by dragging in 1% tryptone culture medium and transferred to sterile plates containing 1% tryptone culture medium with G-penicillin (200 mg l^{-1}) and streptomycin sulfate (350 mg l^{-1}) (Vieira and Toledo, 2012). We grew *Bd* colonies for 1 week and then extracted the DNA from each pure culture for genotyping.

We genotyped five isolated strains, one from each elevation. DNA extraction followed published protocols (James et al., 2008) and we genotyped each individual by sequencing ten SNP markers