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Host-pathogen relationships between the chytrid fungus *Batrachochytrium dendrobatidis* and tadpoles of five South American anuran species

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The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is one of the most important contributors for the decline of amphibian populations worldwide. Evidence indicates that the harmfulness of *Bd* infection depends on the species and life stage, the fungus strain, the season and environmental factors. In the present paper, we experimentally investigated (i) the susceptibility and sensitivity of five South American tadpole species (*Rhinella fernandezae, Scinax squalirostris*, *Hypsiboas pulchellus*, *Leptodactylus latrans* and *Physalaemus fernandezae*) to a foreign *Bd* strain (JEL423), (ii) the response of two populations of *P. fernandezae* to a native *Bd* strain (MLA1), and (iii) the virulence of native and foreign *Bd* isolates on tadpoles of the same species. We also evaluated the relationship between *Bd* infection and the loss of keratinised mouthparts in *P. fernandezae*. We found that all species except *L. latrans* were susceptible to *Bd* infection with lethal consequences, with *R. fernandezae* being the most sensitive species. In *P. fernandezae,* sensitivity to infection depended on population as well as *Bd* strain, although no relationship was found between fungal infection and the loss of keratinised mouthparts. This is the first experimental study on mortality rates of South American tadpoles exposed to *Bd*.

Key words: Batrachochytrium dendrobatidis, fungal infection, South America, tadpoles

INTRODUCTION

The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), the etiological agent of chytridiomycosis (Longcore et al., 1999), is recognised as a proximate driver of many severe declines of amphibian populations worldwide (Lips et al., 2006). *Bd* infects keratinising tissue such as mouthparts of larvae and the skin of adults (Berger et al., 1998; Altig, 2007). The complexity of host-pathogen interaction in the *Bd*-amphibian system has been studied extensively among different amphibian species, *Bd* strains, populations and environmental conditions (Searle et al., 2011; Gervasi et al., 2013; Ortiz-Santaliestra et al., 2013; Langhammer et al., 2014; Spitzen-Van Der Sluijs et al., 2014). Whereas some species carry constant infections in nature with little or no evidence of disease outbreaks (Kielgast et al., 2009; Reeder et al., 2012), others suffer significant declines (Ryan et al., 2008; Vredenburg et al., 2010). Sensitivity can also vary among amphibian life stages, and tadpoles of most species often show low sensitivity to *Bd* infection until metamorphosis (Rachowicz & Briggs, 2007; Symonds et al., 2007; Narayan et al., 2014), probably because infections only occur on the keratinised mouthparts (Berger et al., 1998). As a consequence, they can act as *Bd* reservoirs for the pathogen to persist (Blaustein et al., 2005; Mitchell et al., 2008), despite reports of reduced survival due to infection in a range of species (Blaustein et al., 2005; Garner et al., 2009; Gahl et al., 2012; Paetow et al., 2013; Hanlon & Parris, 2014).

Most studies on *Bd* and its relationship with the anuran host are from the northern hemisphere and Australia (see Voyles et al., 2011). We believe it is essential to generate knowledge about infection in South American amphibians, in order to carry out future conservation programs and to identify key species to prioritise. Therefore, we used a comparative experimental approach to examine host responses to *Bd* infection (susceptibility, sensitivity and loss of keratinised mouthparts) in tadpoles of five South American anuran species: the burrowing toad (*Rhinella fernandezae*), the white-banded treefrog (*Hypsiboas pulchellus*),

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the striped snouted treefrog (*Scinax squalirostris*), the creole frog (*Leptodactylus latrans*), and the whistling dwarf frog (*Physalaemus fernandezae*). Moreover, we investigated the susceptibility of *P. fernandezae* to *Bd* depending on strain and populations. To our knowledge, this study represents the first experimental bioassays on *Bd* infection of native South American tadpoles.

METHODS

Tadpoles were collected from natural breeding sites in temporary ponds located at three sites in Buenos Aires province, Argentina. *R. fernandezae* (stages 29–34 [Gosner, 1960]), *H. pulchellus* (30–34), *S. squalirostris* (34–37), and *L. latrans* (33–36) larvae were collected in the outskirts of La Plata [35° S, 57° W] and used for Bioassay 1. *P. fernandezae* larvae (stages 33–37) were collected from La Balandra [34° S, 57° W] and Pinamar (25–35) [37° S, 56° W], and used for Bioassays 2 and 3. Data from individual *P. fernandezae* from La Balandra were also used in Bioassay 1. Upon arrival at the laboratory, all individuals were held at 37°C for 16 h to eliminate any *Bd* (Woodhams et al., 2003); tadpoles were subsequently acclimated for five days at 17°C, and a photoperiod of 16:8 hours of light:dark. After acclimation, tadpoles were placed individually into 500 ml cylindric polypropylene containers with perforated plastic lids and 56 ml of dechlorinated water.

Zoospores we collected from two different *Bd* strains: JEL423 isolated from an adult *Agalychnis lemur* from Panama, and MLA1 isolated from larvae of *Hypsiboas cordobae* from Argentina. Zoospore collection was done by washing three-day-old 1% tryptone agar plates (grown at 23°C) for 1 hour with 4 ml of distilled water over three consecutive days (the same procedure was performed with *Bd*-free agar plates for control groups), obtaining a final suspension of 4×10^6 zoospore ml^{-1}. A Neubauer chamber was used for zoospore counts.

For *Bd* exposure treatments, we inoculated containers containing 56 ml of dechlorinated water with 4 ml of daily harvested zoospore suspension (*Bd*-free suspension for the control group) for three consecutive days (exposure time), obtaining a final concentration of 6×10^4 zoospore ml^{-1} in each container. After this period, the water in experimental containers was replaced with fungus-free water every day. Tadpoles were fed liquefied lettuce *ad libitum* and checked daily for mortality counts. Bioassays were ended when mortality was recorded in all exposed individuals, or in 10% of the individuals of the control group. A solution of the anesthetic MS222 (tricaine methane sulfonate) was used to humanely euthanise tadpoles, which were then fixed in 10% formalin. To determine the presence of abnormalities in keratinised mouthparts, we extracted the oral disc for inspection with a stereomicroscope (Wild M3 Heerbrugg). *Bd* presence was identified through direct and histological examination of oral structures following Berger et al. (1999; hematoxylin and eosin staining using a compound optical microscope Hund Wetzlar H600).

Results were assessed considering three criteria: (1) susceptibility, defined as the ability to become infected with *Bd* (a species was considered susceptible to *Bd* when at least one individual was infected with the pathogen); (2) sensitivity, defined as survival time after *Bd* exposure; (3) mouthpart deformity, as partial or total absence of keratinised mouthparts on the oral disc. To investigate whether anurans species are susceptible to infection by *Bd* and to assess their sensitivity, Bioassay 1 consisted in exposing tadpoles of *R. fernandezae*, *H. pulchellus*, *S. squalirostris*, *L. latrans* and *P. fernandezae* to *Bd* strain JEL423. Results from Bioassay 3 (using Bd strain JEL423) performed on *P. fernandezae* were also included in the data analysis. With Bioassay 2, we tested whether different populations of a single species were differentially affected by *Bd*. We used the same experimental design as in Bioassay 1 and compared two *P. fernandezae* populations (La Balandra and Pinamar) to a locally isolated *Bd* strain (MLA1). In Bioassay 3 we exposed tadpoles of *P. fernandezae* from the La Balandra population to MLA1 or to JEL423. Fungal exposures for each trial were conducted simultaneously, and we used 10 exposed and 10 control larvae for each species, population and *Bd* strain.

We used Kaplan-Meier analyses (XLSTAT software version 2013.5.04; Addinsoft) to generate survival curves for species (Bioassay 1), populations (Bioassay 2), and groups exposed to *Bd* strains (Bioassay 3), comparing them using a Log-Rank Test. A contingency table analysis was performed on infection data (*Bd*/no *Bd*) with characteristics of mouthparts (normal/deformed), to determine whether the presence of *Bd* was related to oral disc deformation in *P. fernandezae* (individuals of Bioassays 2 and 3). We considered an oral disc to be deformed when keratinised mouthparts (labial teeth and jaw sheath) were absent (Altig, 2007).

RESULTS

Bioassay 1

Nine out of ten *R. fernandezae* individuals, and five out of ten *S. squalirostris* individuals treated with *Bd* died within 24 h of inoculation, and the remainder died on day 2. The other species survived longer, with *P. fernandezae* having the longest survival time (Fig. 1A). No animals in the control groups died except for one tadpole of *L. latrans* on day 5. Treatment and control groups differed significantly for all species, with the following Mean Survival Times (MST) in the treatment groups: *R. fernandezae* ($p<0.001$) 1.1 d, *S. squalirostris* ($p<0.001$) 1.5 d, *L. latrans* ($p=0.004$) 3.4 d, *H. pulchellus* ($p<0.001$) 3.9 d, and *P. fernandezae* ($p<0.001$) 6.5 d. We also observed significant differences in survival among all species ($p<0.001$).

We found no evident oral disc deformations except in *P. fernandezae* (nine out of ten individuals, see Bioassay 2). Four out of the five species tested positive for the presence of *Bd* (direct examination of fresh oral disc surface at 400 × without stain; Fig. 2): *H. pulchellus* (4/10 inoculated individuals), *R. fernandezae* (5/10), *S. squalirostris* (4/10), and *P. fernandezae* (2/10); *L. latrans* tested negative (0/10). The histological analysis of sectioned and stained mouthparts of larvae of all species revealed no evidence of *Bd*.

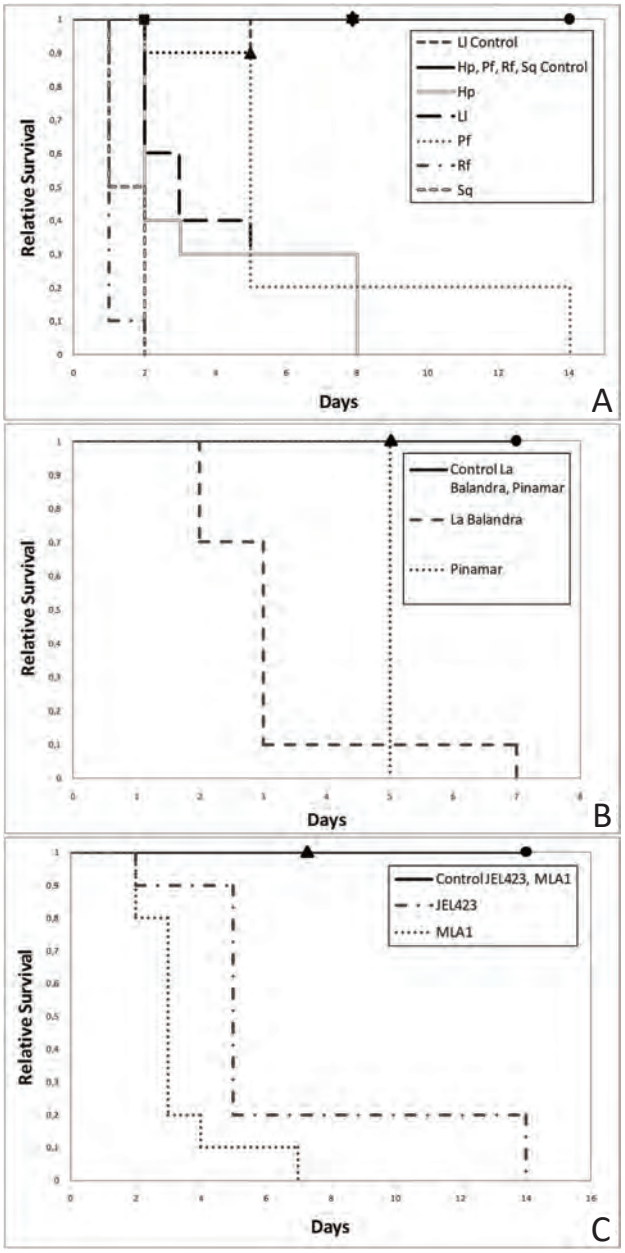

Fig. 1. Relative survival through time. A). Tadpoles of five anuran species exposed to *Batrachochytrium dendrobatidis* (*Bd*). Hp: *Hypsiboas pulchellus*, Ll: *Leptodactylus latrans*, Pf: *Physalaemus fernandezae*, Rf: *Rhinella fernandezae*, Sq: *Scinax squalirostris*. B) *P. fernandezae* tadpoles from La Balandra and Pinamar exposed to *Bd* strain MLA1. C) *P. fernandezae* tadpoles from La Balandra exposed to *Bd* strains MLA1 and JEL423. Symbols in lines of control groups show the end of the bioassay for each species. A) Square: Rf and Sq; triangle: Ll; star: Hp; circle: Pf. (no survival in exposed or control individuals but in 10% of Ll control individuals). B) Circle: La Balandra; triangle: Pinamar (no survival in exposed or control individuals). C) Circle: JEL423; triangle: MLA1 (no survival in exposed or control individuals).

Bioassay 2

We found significant differences in survival between La Balandra and Pinamar populations exposed to *Bd* and their respective controls (p<0.001); MST were 3.1 d and 5.0 d respectively. In specimens from the Pinamar population, survival decreased from 100% on day 4 to 0% on day 5, while for La Balandra the decrement was more gradual (Fig. 1b). All tadpoles in control groups survived throughout the bioassay. The survival analysis showed significant differences between La Balandra and Pinamar (p= 0.001), and neither group had survivors at the end of the bioassay (day 5 for Pinamar and day 7 for La Balandra). Oral discs were deformed in about half of the larvae, including the total loss of keratinised mouth parts in the upper and/or lower jaws (Fig. 3).

We identified *Bd* thalli in mouthparts of individuals from La Balandra (6/10 infected individuals) and Pinamar (3/10), although infection was mild in both (1–10 zoosporangia), and negative in controls. Infection was detected in tadpoles with normal mouthparts and those with some degree of depigmentation. Histological analyses were negative for individuals from Pinamar and controls, whereas *Bd* sporangia were present in individuals from La Balandra (Fig. 4).

Bioassay 3

Survival differed significantly between *P. fernandezae* tadpoles inoculated with isolate JEL423 (MST=6.5 d), isolate MLA1 (MST=3.3 d), and control groups (100% survival; p<0.001), as well as between the treatment groups (Fig. 1C; p=0.003). Between 50 and 90% of treated larvae and controls presented loss of keratinised mouth parts (typified in Bioassay 2). *Bd* thalli were detected in tadpoles exposed to either *Bd* strain in mouthparts either with or without deformation (6/10 and 2/10 infected individuals for MLA1 and JEL423, respectively), but not in control tadpoles. Histological analysis yielded negative *Bd* infection for both treatments and controls. The contingency table with all individuals of *P. fernandezae* showed a dependency between depigmentation/no *Bd* (19/40) and normal/no *Bd* (2/40). Although no formal behavioural analyses were performed, we observed a change in the normal activity of the exposed tadpoles (Bioassays 1, 2 and 3) such as slow reaction to stimuli.

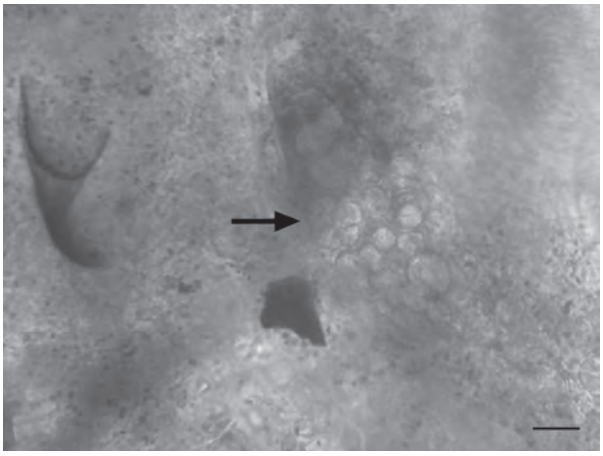

Fig. 2. *Rhinella fernandezae* fresh oral disc surface (without stain), infected with *Bd* (400X). Note mature and empty zoosporangium in the *stratum corneum* (arrow). Scale bar=10 µm.

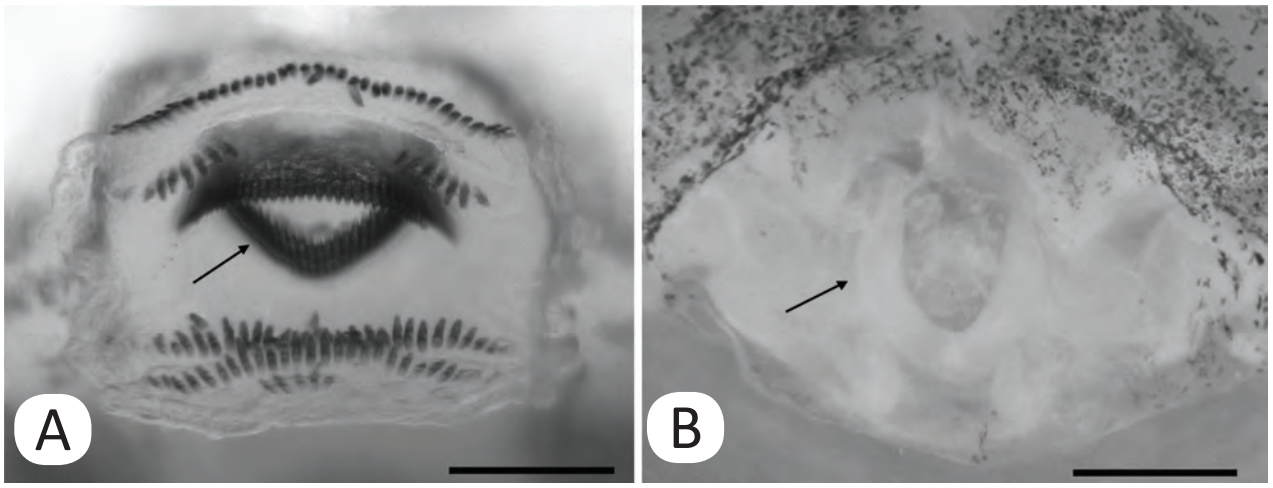

Fig. 3. Normal (A; 25 stage; Pinamar population) and abnormal (B; 38 stage; La Balandra population) oral discs in *Physalaemus fernandezae* tadpoles (Gosner,1960). Arrows indicate lower sheath jaw (A) and lower jaw (B; showing lack of keratinised structures). Scale bars=200 µm (A); 500 µm (B).

DISCUSSION

To our knowledge, our results represent the first report of disease susceptibility in native South American tadpoles experimentally exposed to *Bd*.

Bioassay 1

All the species we tested died after exposure to *Bd* but varied widely in their sensitivity; whereas all individuals of *R. fernandezae* and *S. squalirostris* died on day 2, other species survived between 8 and 14 days. This low larval survival, recorded also in other species (Blaustein et al., 2005; Garner et al., 2009; Paetow et al., 2013; Hanlon & Parris, 2014), shows that amphibian larvae not only act as carriers and reservoirs of *Bd* (Narayan et al., 2014) but also experience mortality. The high mortality and low infection degree registered suggests that the study species are sensitive to exposure of *Bd*. That *Bd*-exposed tadpoles could invest a high amount of energy to prevent infection, perhaps through mechanisms that inhibit zoospore attachment to host cells, could ultimately lead to larval mortality before metamorphosis (Garner et al., 2009). High concentration of *Bd* zoospores also produce harmful chemicals which might have contributed to the rapid mortality observed in our experiments (McMahon et al., 2012).

The absence of oral disc deformation in all species except *P. fernandezae* may be linked to a short time of exposure. As evidenced by the examination of unstained mouthparts, *Bd* was present in all species except *L. latrans* (see Peterson et al., 2007 for a similar example on another species). Although tadpoles of *L. latrans* may be resistant, the presence of *Bd* was confirmed in adults and juveniles of wild populations (Herrera et al., 2005; Ghirardi et al., 2009). Given the localised nature of an early stage of infection (Berger et al., 1999), false negatives could arise from histological analyses. This can be related to the absence of mouthpart deformation,

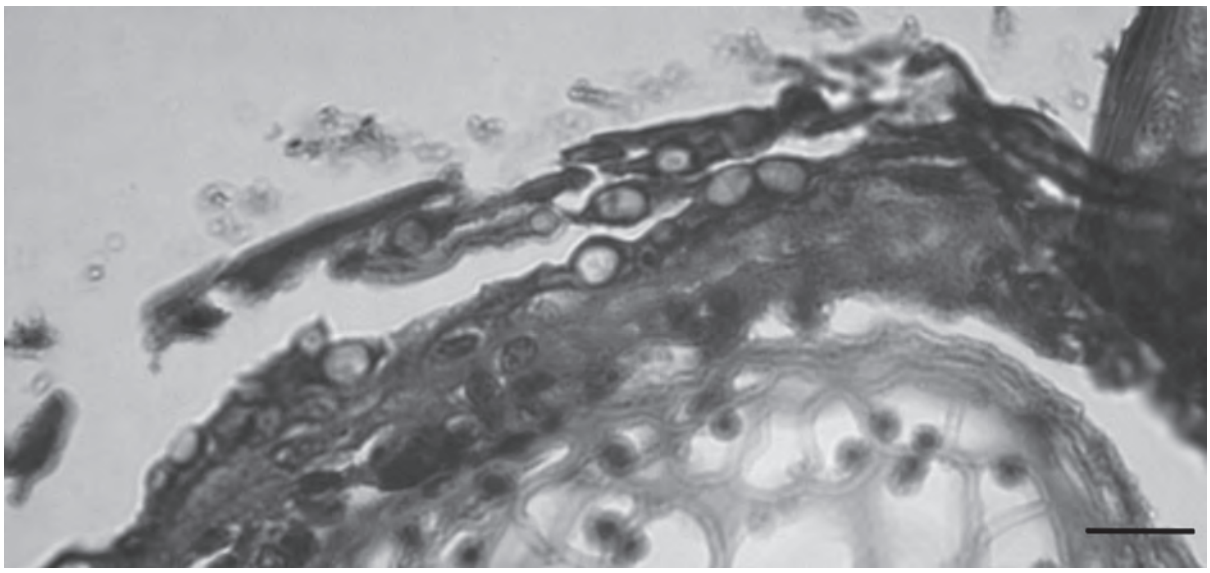

Fig. 4. Oral disc sections of *Physalaemus fernandezae* tadpoles from La Balandra population showing empty *Batrachochytrium dendrobatidis* zoosporangia (arrow). Scale bar=14 µm.

provided there exists a strong association between oral disc depigmentation and histological confirmation of *Bd* (Rachowicz & Vredenburg, 2004; Knapp & Morgan, 2006). The presence of deformations on the oral disc of *P. fernandezae* is noteworthy, and is discussed below.

Bioassay 2

Although the survival time of tadpoles from both *P. fernandezae* populations was similar, individuals from Pinamar died in large numbers a single day, a difference which may have been caused by genetic differences between the two groups. Natale (2006) found differences in the sensitivity of tadpoles to a Chromium (VI) solution in two populations of *P. fernandezae*, but studies on mortality variation among different tadpole populations exposed to *Bd* are largely lacking (for examples on adults see Tobler & Schmidt, 2010; Bradley et al., 2015; Piovia-Scott et al., 2015). Although no individuals from either population survived longer than 7 days, survival in La Balandra tadpoles began to decrease 3 days earlier than for Pinamar. Individuals from Pinamar population were on average at earlier developmental stages compared to La Balandra when exposed to *Bd*, which might lead to a higher sensitivity to infection (*Bd*: Hanlon & Parris, 2014; see also Bunn et al., 2001; Johnson et al., 2011).

The pattern of mouthpart deformation in both the treatment and control groups was more consistent with that shown by tadpoles exposed to low temperatures (6°C) than with the pattern of 'gaps' that characterises *Bd* infection (Rachowicz & Vredenburg, 2004). We also identified *Bd* sporangia in both normal and abnormal mouthparts, suggesting loss of keratinised mouthparts in the absence of *Bd*, as well as unaffected mouthparts in the presence of *Bd* (see also Blaustein et al., 2005; Padgett-Flohr & Goble, 2007; Smith & Weldon, 2007). It is worth considering that *P. fernandezae* tadpoles generally have abnormalities in oral disc structures and in the pattern of ossification when reared under laboratory conditions (Barrasso, unpublished).

Bioassay 3

Survival of groups inoculated with the Argentina (MLA1) and Panama (JEL423) *Bd* strains differed markedly. While the survival of both groups declined 48 hours after exposure, all individuals exposed to the Argentina strain died twice as fast than individuals exposed to the Panama strain. Experiments comparing the effects of different *Bd* strains revealed differences among host species which may be associated with environmental factors (Berger et al., 2005; Retallick & Miera, 2007; Gahl et al., 2012). JEL423 was isolated 6 years before MLA1 from an adult of *A. lemur* from Panama, and has produced symptoms and mortality in different species (Becker & Harris, 2010; Brannelly et al., 2012), whereas MLA1 was isolated from larvae of *H. cordobae* from a mountain stream in San Luis province (Argentina), and these are the first bioassays performed with this strain. Differences in the *in vitro* handling of the strains as well as in the time since their isolation may cause changes in their virulence (Berger et al., 2005; Brem et al., 2013). MLA1 has larger sporangia than JEL423 (Arellano et al., 2010), supporting its higher virulence (see also Fisher et al., 2009). Genomic studies have revealed deep phylogenetic diversity, cryptic recombination and the existence of *Bd*-specific genes with possible pathogenicity factors (Joneson et al., 2011; Farrer et al., 2013; Rosenblum et al., 2013). The two strains used in our experiment are included in a global panzootic lineage (GPL) that contains the most infectious *Bd* isolates (Lips et al., 2006; Becker & Harris, 2010; Brannelly et al., 2012; Gahl et al., 2012; Rosenblum et al., 2013). Different effects on the survival of tadpoles can also be attributed to immunotoxicity (Piovia-Scott et al., 2015).

The finding that prevails in all experiments was high mortality of tadpoles, although reports of mass mortalities in the wild are lacking (but see Barrionuevo & Magione, 2006; Ghirardi et al., 2014). Tadpoles were exposed to concentrations of *Bd* zoospores ($6\times10^4 ml^{-1}$) which are likely higher than concentration in nature where *Bd* diffuses and becomes reduced through predators that forage on *Bd* zoospores (Searle et al., 2013; Schmeller et al., 2014; Groner & Relyea, 2015), and where chemical agents can have a fungicidal effect (Gahl et al., 2011; Hanlon & Parris, 2014; Rumschlag et al., 2014). Native tadpoles also might only experience *Bd*-caused declines when subjected to more virulent or allopatric strains (James et al., 2009).

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