



High prevalence of infection in tadpoles increases vulnerability to fungal pathogen in high-Andean amphibians



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ABSTRACT

The amphibian pathogen *Batrachochytrium dendrobatidis* (Bd) is causing population declines and species extirpations worldwide. Montane amphibians in tropical and temperate regions are especially vulnerable to chytridiomycosis. High-elevation amphibian assemblages typically include few species, so that epizootics should be limited once high frog mortality hinders transmission of the pathogen. We hypothesized that tadpoles of a high-elevation frog in the Peruvian Andes, *Telmatobius jelskii*, could function as reservoir for Bd in Andean streams. We postulated that, for tadpoles to function as an efficient reservoir of Bd, they should inhabit streams over extended periods of time, and have high prevalence of Bd. We surveyed streams between 2400 and 4850 m in the wet and dry seasons of 2010, where we captured, swabbed and determined the developmental stage of 458 tadpoles. We found that cohorts of tadpoles overlap continuously in these streams, as a consequence of multiple breeding events throughout the year. Prevalence of Bd among tadpoles averaged 53.1% (95% confidence interval: 49.8–56.3%); 8 out of 13 streams inhabited by *T. jelskii* had a prevalence greater than 50%. Prevalence of Bd was also higher during the dry season and increased with the age of the tadpoles. Our results support the hypothesis that the year-long presence of infected tadpoles in streams makes high-Andean *Telmatobius* frogs especially vulnerable to chytridiomycosis. The genus is already extirpated in Ecuador, and has been observed to decline rapidly in Peru, Bolivia and Argentina. Conservation strategies to mitigate the impact of Bd on populations of *Telmatobius* should consider aquatic life-stages.

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1. Introduction

High elevation amphibians are threatened by chytridiomycosis, an emergent and highly virulent disease caused by the fungus *Batrachochytrium dendrobatidis* (Bd; Berger et al., 1998; Kilpatrick et al., 2010; Vredenburg et al., 2010). Bd epizootic events, which cause population declines and extirpations, have been documented in montane areas in California (Vredenburg et al., 2010), the Andes in Peru (Catenazzi et al., 2011; Seimon et al., 2007), and the Pyrenees and similar regions of the Iberian peninsula (Walker et al., 2010). The devastating impact of chytridiomycosis on montane amphibians is puzzling for a number of reasons. The optimal growth for Bd in culture is reportedly 15–25 °C (Piotrowski et al., 2004; Woodhams et al., 2008). These temperatures seldom occur at high elevation, even in tropical regions. The persistence of Bd in populations of highly vulnerable hosts is especially intriguing, because amphibian species richness at high-elevations is low, which limits the number

of alternative hosts. During a chytridiomycosis outbreak, the high mortality of infected host frogs should quickly reduce transmission, and therefore reduce the risks of future outbreaks and epizootics (Anderson and May, 1979). Therefore, in order to persist Bd requires an alternative host or an environmental reservoir. In contrast to adults, chytridiomycosis is not lethal to tadpoles of most species (Blaustein et al., 2005). Bd infects the keratinized mouthparts of tadpoles (Fellers et al., 2001), and infected tadpoles can be recognized by their abnormal and depigmented oral disks (Knapp and Morgan, 2006; Vredenburg and Summers, 2001).

The larval stages of montane amphibians often spend extended periods of time in water before completing metamorphosis, and frequently over-winter in temperate regions (Bosch and Martinez-Solano, 2006; Bosch et al., 2001; Briggs et al., 2010). Understanding the link between amphibian larval development and Bd epidemiology is crucial for designing strategies to reduce the impact of this disease on potentially susceptible species (Bosch and Martinez-Solano, 2006; Briggs et al., 2010; Conradie et al., 2011). Here we explore the hypothesis that long-lived tadpoles function as reservoirs for Bd in populations of high-Andean frogs. We investigated whether tadpoles of *Telmatobius jelskii* continuously occupy aquatic habitats throughout the year, and denoted their Bd infec-

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tion status. We also quantified Bd infection intensity and prevalence in both tadpoles and post-metamorphic stages of *T. jelskii* in order to evaluate the threat of chytridiomycosis in the Puna ecosystem of the Peruvian central Andes.

The genus *Telmatobius*, consisting of approximately 60 species is mainly distributed in the tropical Andes, with most species distributed in Peru and Bolivia (Lehr, 2005). Species in this genus have declined dramatically over the past three decades (Merino-Viteri et al., 2005). For example, the three *Telmatobius* species known from Ecuador were extirpated in the late 1980s and early 1990s and are now thought to be extinct (Merino-Viteri et al., 2005). The last individuals of *Telmatobius* found in Ecuador showed symptoms of chytridiomycosis (Merino-Viteri et al., 2005). Moreover, population declines of *Telmatobius marmoratus*, *Telmatobius mendelsoni* and *Telmatobius timens* in Peru (Catenazzi et al., 2011; Seimon et al., 2007; von May et al., 2008) and of two species of *Telmatobius* in Argentina (Barrionuevo and Mangione, 2006) have been associated with outbreaks of Bd. Additional threats for species of *Telmatobius* are habitat loss, agricultural expansion, trout farming, contamination from mining and agriculture, and human harvesting and consumption (Angulo, 2008; Catenazzi et al., 2010; De la Riva and Lavilla, 2008; von May et al., 2008).

T. jelskii inhabits bogs, streams and rivers with slow-moving waters in the high Andes of central Peru, and are restricted to the regions of Junín, Huancavelica, and Ayacucho (Sinsch, 1986, 1990; Sinsch et al., 1995; Vellard, 1955). Adults of *T. jelskii* reproduce all year long under favorable environmental and habitat conditions (Sinsch, 1990). Tadpoles of *T. jelskii* are thought to spend several months in the cold water of high-elevation streams before completing metamorphosis (>3 months according to Sinsch (1986, 1990). Therefore, if tadpoles carry Bd, they may function as reservoirs for chytridiomycosis over long periods of time. This may keep Bd in the ecosystem when adults are not present or if adults have been extirpated during an outbreak of chytridiomycosis (Briggs et al., 2010, 2005). In this study, we assessed whether tadpoles of *T. jelskii* fulfill two important prerequisites for functioning as reservoir: (1) tadpoles are infected with Bd throughout the year, and (2) tadpoles inhabit aquatic breeding sites all year long.

2. Methods

We studied 22 streams in the Ayacucho region of central Peru (Fig. 1, online kml file), as part of a long-term program that is monitoring the impact of a trans-Andean natural gas pipeline. We sampled 9 streams in both the wet (20 March–6 April 2010) and dry (8–18 July 2010) seasons, 4 streams only in the wet season and 9 streams only in the dry season. These streams were located at elevations between 2400 and 4854 m. Tadpoles and frogs were captured along linear transects of 200 m of stream and with a search effort of six person-hours per transect. We searched for post-metamorphic stages by visually inspecting pools and riparian areas, as well as manually, by feeling for frogs moving under rocks, mud and along the edges of riffles, runs and pools. We determined developmental stage of tadpoles using standard techniques (Gosner, 1960). Tadpoles hatch around Gosner stages 18–20, become active swimmers and algal grazers at stage 25, interrupt feeding and start reabsorbing tail and undergoing metamorphosis at stage 42, and complete metamorphosis at stage 46. At each stream, we measured water temperature in pools occupied by tadpoles between 10:00 and 13:00. We averaged temperature measurements across all occupied pools within a stream.

To measure Bd infection load, each animal was swabbed with a synthetic dry swab (Medical Wire and Equipment Co. Ltd.). In post-metamorphic stages, swabs were stroked across the skin a total of 30 times: 5 strokes on each side of the abdominal midline, 5

strokes on the inner thighs of each hind leg, and 5 strokes on the foot webbing of each hind leg. Tadpoles were swabbed with 10 strokes on the mouthparts. We used a real-time Polymerase Chain Reaction (PCR) assay on material collected on swabs to detect Bd and quantify the level of infection (Boyle et al., 2004). This assay compares the sample to a set of standards and calculates a genomic equivalent for each sample (i.e., the number of copies of DNA of Bd on each swab, or Z_{swab}). We followed DNA extraction and real-time PCR methods of Hyatt et al. (2007) and Boyle et al. (2004), except that we analyzed single-swab extracts once instead of 3 times (Krieger et al., 2006; Vredenburg et al., 2010). The real-time PCR technique for Bd uses DNA extracts from swabs that are diluted 80-fold during extraction and PCR. Thus, to estimate Z_{swab} , we multiplied the genomic equivalent values generated during the real-time PCR by 80. Confidence intervals for prevalence data follow the Wilson procedure (Newcombe, 1998).

We compared Bd prevalence, Z_{swab} and ontogenetic structure of tadpole populations between wet and dry season surveys. These comparisons were made at three levels: (1) within stream comparisons for sites 6, 7 and 13 (Fig. 1); (2) within watershed comparisons, by pooling data from all streams in the Apacheta and Huamanga watersheds (9 streams); and (3) within elevational range comparisons, by pooling data from all streams in the 3800–3899 (2 streams), 3900–3999 (1 stream), and 4000–4099 m ranges (3 streams). We used this approach instead of a general model because we were constrained in our sampling locations. Our permits restricted us to visiting streams near the buried natural gas pipeline where the company had agreements with local communities. As a result, our dataset had important gaps with respect to elevation, number of sites per watershed, and number of streams visited per season.

We additionally evaluated if, when controlling for developmental stage, body size differed between infected and uninfected tadpoles. If Bd infection did not affect tadpole growth and development, curves of body size versus developmental stage would have similar slopes. To test this prediction, we treated developmental stage as a covariate in an analysis of covariance (ANCOVA) comparing the body size of infected and uninfected tadpoles. For this analysis we only considered tadpoles between developmental stages 25 (presence of keratinized mouthparts) and 40 (when tadpoles stop growing in size and enter metamorphosis).

We separated the frequency distribution of tadpole developmental stages into different components using finite mixture distribution models. We assumed that developmental stages followed a gamma distribution corresponding to distinct breeding and egg laying events. We labeled these components as cohorts of tadpoles. The term cohort refers here to tadpoles sharing the same developmental stage at the time of our surveys. We first fitted mixtures of unconstrained gamma distributions, and then produced separate constrained analyses. We chose the unconstrained model only if it was a better fit to the frequency distribution than the constrained model. We used the R package *mixdist* to fit the finite mixture distribution models (<http://cran.r-project.org/web/packages/mixdist/index.html>). This package contains functions for fitting finite mixture distribution models to grouped data and conditional data by the method of maximum likelihood using a combination of a Newton-type algorithm and an expectation-maximization algorithm. The package also reports results of a chi-square test for the fit between an empirical histogram and the histogram calculated from the sum of the mixture distributions.

3. Results

3.1. Prevalence of Bd and infection intensity in tadpoles of *T. jelskii*

Infection with Bd was widespread at the three spatial scales (Figs. 2–4, Table A1). Prevalence of Bd across all tadpoles was

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