

NOTE

# Widespread occurrence of the antifungal cutaneous bacterium *Janthinobacterium lividum* on Andean water frogs threatened by fungal disease

Andrew O. Rubio<sup>1,\*</sup>, Sarah J. Kupferberg<sup>2</sup>, Victor Vargas García<sup>3</sup>, Alex Ttito<sup>4</sup>,  
Alexander Shepack<sup>5</sup>, Alessandro Catenazzi<sup>5</sup>

<sup>1</sup>Southern Illinois University, Department of Zoology, Carbondale, IL 62901, USA

<sup>2</sup>Questa Engineering, Pt. Richmond, CA 94807, USA

<sup>3</sup>Asociación Pro Fauna Silvestre Ayacucho, Peru

<sup>4</sup>Departamento de Ecología, Pontificia Universidad Católica de Chile, Santiago, Chile

<sup>5</sup>Florida International University, Department of Biological Sciences, Miami, FL 33199, USA

**ABSTRACT:** Amphibian diversity has declined due to the infectious disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Coexistence between amphibian hosts and this pathogen in some locations is attributed to the presence of the cutaneous bacterium *Janthinobacterium lividum* (*Jliv*). This microbe inhibits the growth of *Bd* on the host, reduces morbidity, and improves survival. Andean water frogs in the genus *Telmatobius* seem to be particularly vulnerable to the disease yet populations of *T. intermedius* and *T. marmoratus* persist in southern and central Peru. We investigated the presence of *Jliv* on these 2 frog species and assessed the relationship of *Jliv* presence with prevalence and intensity of *Bd* infection. By sampling 125 frogs from 7 streams (3323–3950 m a.s.l.) and 27 from a city market, we found spatial variation in the mutualism among populations (range 0–40% proportion of *Jliv*-positives). Overall, 57% of frogs were infected with *Bd*, 12.5% of frogs hosted both *Jliv* and *Bd*, while 7.2% hosted just *Jliv*. We found that the probability of an individual being infected with *Bd* was independent of the presence of *Jliv*; however, we did detect a protective effect of *Jliv* with respect to intensity of infection. The extent of *Jliv* distribution in the high Andes stands in stark contrast to the rarity of *Jliv* on frogs in lower elevation cloud forest biomes.

**KEY WORDS:** Amphibian conservation · Chytridiomycosis · *Batrachochytrium dendrobatidis* · Host defenses · Microbial symbiosis · Peru · *Telmatobius* · Telmatobiidae

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## INTRODUCTION

Amphibians are facing a worldwide threat to their existence: the emerging infectious disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998, Skerratt et al. 2007). After *Bd* zoospores penetrate amphibian skin, they grow and reproduce, thickening the epidermal

layer (hyperkeratosis), causing erosions and ulcers. Colonization by thousands of zoospores leads to osmotic imbalance and can induce cardiac arrest (Voyles et al. 2011). Vulnerability to infection and susceptibility to lethal symptoms vary considerably among species and populations (Voyles et al. 2011) and in relation to environmental factors (Adams et al. 2017).

Some amphibians that co-exist with *Bd* infection may be able to avert the disease via mutualistic relationships with a community of antifungal microbes living on their skin (Harris et al. 2006, Bletz et al. 2017). These antifungal microbes, their metabolites, as well as compounds secreted from the amphibians' skin can alter the outcome of chytridiomycosis (Woodhams et al. 2007, Harris et al. 2009). Populations in which most individuals have anti-*Bd* cutaneous microbes appear to persist in the continued presence of *Bd* (Woodhams et al. 2007, Burkart et al. 2017). One such protective microbe isolated from a diverse set of salamanders and frogs is the betaproteobacterium *Janthinobacterium lividum*, hereafter *Jliv* (Harris et al. 2009). It has been found in many temperate amphibian communities (Harris et al. 2006, Brucker et al. 2008, Becker et al. 2009), but appears to be uncommon in tropical regions (Catenazzi et al. 2018).

Water frogs of the genus *Telmatobius*, native to the Andean highlands of South America (Lavilla & De la Riva 2005), are especially vulnerable to chytridiomycosis (Catenazzi & von May 2014). Infected individuals exhibit the pathological conditions associated with chytridiomycosis and quickly die (Catenazzi et al. 2018). The genus has been extirpated from Ecuador, where 3 species were previously reported (Merino-Viteri et al. 2005). Many other species have been locally extirpated throughout the Andes (Barrionuevo & Ponssa 2008, Catenazzi & von May 2014, De la Riva & Reichle 2014). In our study area of high elevation (>3200 m) grasslands in central and southern Peru, the focal species are the marbled water frog *T. marmoratus* (which also occurs in Bolivia and northern Chile) and the endemic Allipaca water frog *T. intermedius*. The 2 species are classified, respectively, as Vulnerable (IUCN SSC Amphibian Specialist Group 2015) and Data Deficient (Icochea et al. 2004). In contrast many *Telmatobius* species have disappeared from cloud forest sites on the eastern slopes of the Andes, where *Bd* has caused the collapse of entire frog communities (Catenazzi et al. 2011). Furthermore, *Jliv* is extremely rare in these cloud forests, having been found only once at the treeline transition (Catenazzi et al. 2018).

Our goal here was to explore the distribution of *Jliv* in high-Andean grassland streams (well above the treeline transition) with extant populations of *Telmatobius*. A wide distribution of *Jliv* in these high elevation streams might help to explain why many forest species of *Telmatobius* have disappeared while highland populations persisted following the epizootics of chytridiomycosis across their range (Catenazzi et al. 2010). Specifically, we asked (1) Where is *Jliv* pres-

ent, and is there a relationship with *Bd* prevalence and (2) Do *Bd*-infected frogs that host *Jliv* have lower concentrations of zoospores on their skin compared to frogs lacking *Jliv*?

## MATERIALS AND METHODS

We compiled data from 7 streams in the southern Peruvian Andes and a market in Cusco that sold live frogs, presumably collected locally (see Fig. 1, Table 1). We grouped sites within a 50 km radius into a single region. We searched in creeks, captured frogs with nets or by hand, and marked frogs with visible implant elastomer to prevent repeated sampling.

We collected skin swabs to detect *Bd* and *Jliv* from 125 stream-caught frogs, and 27 individuals from the market. We stroked each frog 30 times (5× on each side of abdominal midlines, hind-leg inner thighs, and feet webbing) with a dry, sterile, rayon-tipped swab (Catenazzi et al. 2010). We dry-stored swabs individually in sterile 1.5 ml centrifuge tubes before extracting DNA using Prepman Ultra® (Life Technologies). For each swab, we performed one quantitative PCR (qPCR) with a probe-based Taqman assay for *Bd* (Boyle et al. 2004) and one SYBR assay (Harris et al. 2009) for *Jliv* on a StepOnePlus™ (Life Technologies). We compared the samples to positive controls with DNA amounts equivalent to 1, 10, 100, and 1000 zoospores for *Bd* and cells for *Jliv*; this provides genomic equivalence for the number of *Bd* zoospores (henceforth  $Z_e$ ) and *Jliv* cell genomes ( $C_e$ ). We converted genomic equivalence values to account for dilution during DNA extraction. We used gBlocks synthetic DNA (IDT) of the internal transcribed spacer (ITS) gene (Genbank no. AY598034) for *Bd* standards. For standards of *Jliv*, we cultured the strain CCOS 423 (courtesy of D. Woodhams' laboratory) and aliquoted DNA extracts from samples of known cell quantities.

We defined infection status as *Bd*-positive when  $Z_e > 0$ . For *Jliv*, we inspected the melting curve of the qPCR reaction to confirm *Jliv* presence for samples with amplification curves. We considered a frog positive for *Jliv* when the amplification curve and  $C_e > 0$ , and at least one peak in the melting curve corresponded to the temperature producing the largest (single) peak in the qPCR reactions for the serial dilutions of our standards.

Because relatively few individuals were positive for both *Bd* and *Jliv*, we used Fisher's exact test of independence to determine if there was an association between *Bd* infection status and *Jliv* status

within stream-sampled frogs of each species. We used a Mann-Whitney *U*-test to compare median  $\log(Z_e)$  of *Bd*-positive *Telmatobius intermedius* that were *Jliv*-positive and negative. For *T. marmoratus*, we used a Kruskal-Wallis 1-way ANOVA to compare  $\log(Z_e)$  among frogs from the market and stream-sampled frogs that either hosted or lacked *Jliv*.

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### RESULTS

We found *Bd* on both species (*Telmatobius intermedius*, *n* = 93; *T. marmoratus*, *n* = 59) and at all study sites (Fig. 1, Table 1). We found *Jliv* on *T. intermedius* from 2 out of 3 streams at Pampa Galeras and on *T. marmoratus* from Huaparquilla and Espinar in Cusco Department (Fig. 1, Table 1). Across both spe-

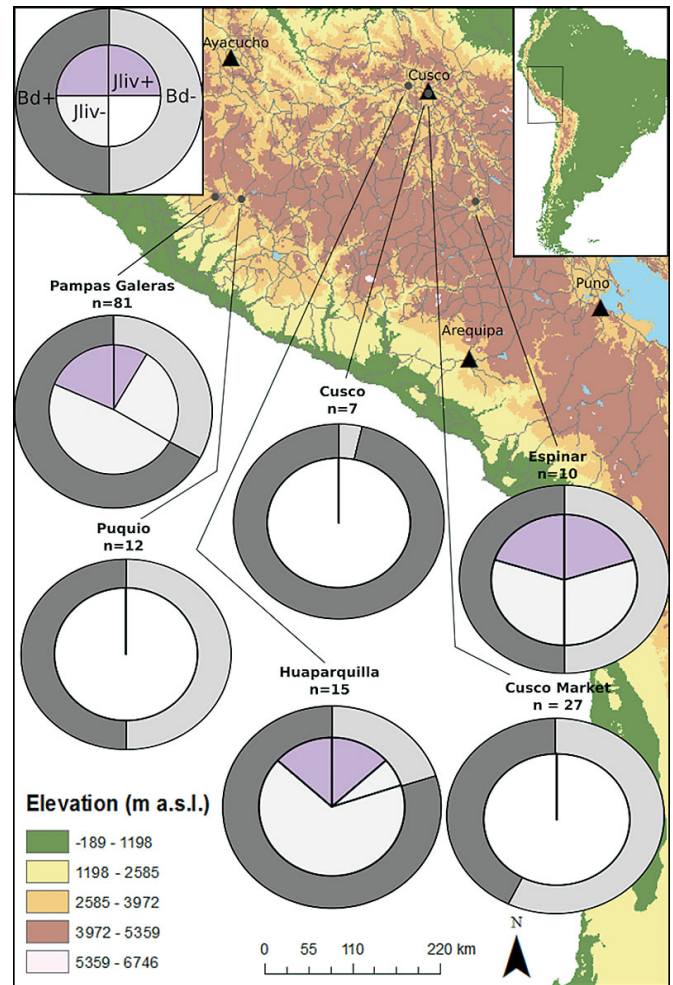


Fig. 1. Locations in Peru of populations of water frogs (*Telmatobius intermedius* and *T. marmoratus*) sampled for the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and the beneficial cutaneous bacterium *Janthinobacterium lividum* (*Jliv*). Outer ring: *Bd* prevalence (dark: infected, light: uninfected); inner circle divisions: proportions of frogs with *Jliv* (violet: presence, white: absence) grouped by *Bd* status. See Table 1 for site details

Table 1. Coordinates (based on WGS84) of sites with *Telmatobius intermedius* (1–4) or *T. marmoratus* (5–8) sampled for presence of *Janthinobacterium lividum* (*Jliv*) and *Batrachochytrium dendrobatidis* (*Bd*). Area name corresponds to localities in Fig. 1. Elev: Elevation above sea level; *n*: number of frogs swabbed; Pr: prevalence;  $C_e$  and  $Z_e$ : median of genome equivalents for bacterial cells or zoospores on *Jliv*- and *Bd*-positive frogs, respectively; Date: year(s) visited

| Site | Area name (stream)        | Latitude (°S), longitude (°W) | Elev. (m) | n  | — <i>Jliv</i> — |                 | — <i>Jliv</i> $C_e$ — |                | — <i>Bd</i> — |               | — <i>Bd</i> $Z_e$ — |            | Date    |
|------|---------------------------|-------------------------------|-----------|----|-----------------|-----------------|-----------------------|----------------|---------------|---------------|---------------------|------------|---------|
|      |                           |                               |           |    | Pr (%)          | n <i>Jliv</i> + | Median                | Range          | Pr (%)        | n <i>Bd</i> + | Median              | Range      |         |
| 1    | Pampa Galeras (Cupitay)   | 14.6635, 74.3995              | 3975      | 63 | 33.3            | 21              | 38389                 | 879–86441710   | 65.1          | 41            | 83.0                | 0.4–9567   | 2016–17 |
| 2    | Pampa Galeras (Lamay)     | 14.6908, 74.3783              | 4000      | 13 | 7.7             | 1               | 3027                  |                | 76.9          | 10            | 57                  | 0.1–759    | 2016–17 |
| 3    | Pampa Galeras (Incahuasi) | 14.7334, 74.3387              | 3940      | 5  | 0               |                 | n/a                   |                | 60.0          | 3             | 8                   | 2–70       | 2016    |
| 4    | Puquio                    | 14.6899, 74.0936              | 3215      | 12 | 0               |                 | n/a                   |                | 50.0          | 6             | 33                  | 0.4–2164   | 2014    |
| 5    | Huaparquilla              | 13.4676, 72.2031              | 3323      | 15 | 26.7            | 4               | 134975                | 1441–26270583  | 80.0          | 12            | 321                 | 60–3927    | 2016    |
| 6    | Cusco (market)            | 13.5215, 71.9824              | 3399      | 27 | 0               |                 | n/a                   |                | 96.3          | 26            | 12580               | 911–473870 | 2013    |
| 7    | Cusco (Rio Chocca)        | 13.5503, 71.9791              | 3399      | 7  | 0               |                 | n/a                   |                | 42.9          | 3             | 60.4                | 6.0–1552.0 | 2014    |
| 8    | Espinar                   | 14.7093, 71.4432              | 3874      | 10 | 40.0            | 4               | 4368                  | 1348–286441710 | 50.0          | 5             | 74                  | 0.1–2077.3 | 2016    |

cies and all sites, *Jliv* was present on 19.7% of all frogs sampled; 12.5% hosted both *Jliv* and *Bd* and 7.2% hosted just *Jliv*. In addition, *Jliv* was absent on all *T. marmoratus* from the market in Cusco (Table 1) and these frogs had the highest prevalence and intensity of infection, above what is considered a lethal level (i.e.  $Z_e > 10000$ ).

Presence of *Jliv* did not affect a frog's probability of being infected with *Bd* (Fig. 2) as indicated by Fisher's exact test (*T. intermedius*,  $p = 0.80$ ; *T. mar-*

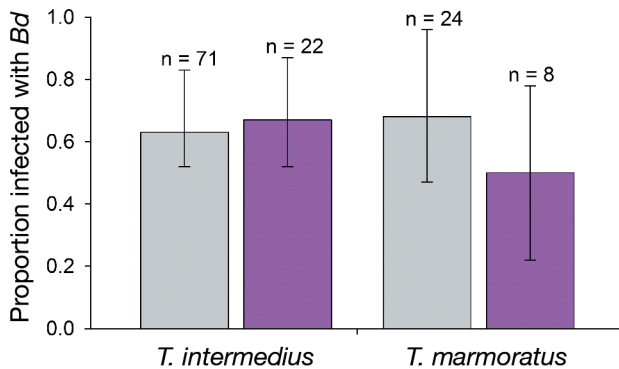


Fig. 2. Proportion of *Telmatobius* individuals infected with *Batrachochytrium dendrobatidis* (*Bd*) grouped across study areas (excluding market frogs). Error bars: 95% Clopper-Pearson binomial confidence intervals; n: number of individuals which hosted (violet) or lacked (grey) the skin symbiont *Janthinobacterium lividum*

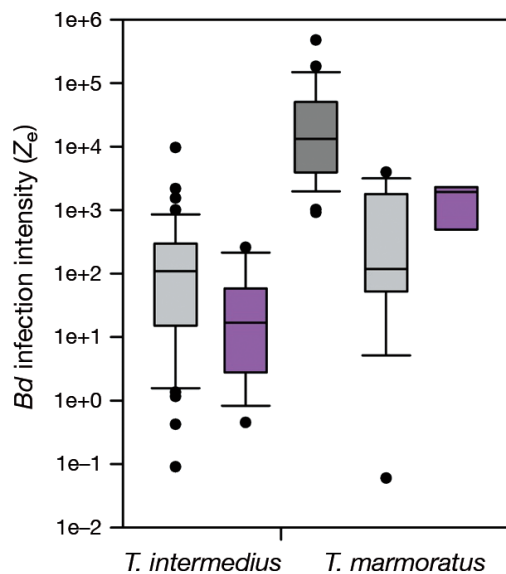


Fig. 3. Median, interquartile, and outlier zoospore equivalent values ( $Z_e$ ) for water frogs in the genus *Telmatobius* infected with *Batrachochytrium dendrobatidis* (*Bd*). Sample sizes: *T. intermedius* individuals which hosted (violet,  $n = 15$ ) or lacked (grey,  $n = 45$ ) *Janthinobacterium lividum*; *T. marmoratus* frogs from Cusco city market (dark grey,  $n = 26$ ), or stream-caught (grey,  $n = 16$ ; violet,  $n = 4$ )

*marmoratus*, excluding market frogs,  $p = 0.43$ ). With respect to *Bd* infection intensity (Table 1), the difference in median  $\log(Z_e)$  was significant for *T. intermedius* ( $U = 2.05$ ,  $p = 0.018$ ); frogs with *Jliv* had almost 10-fold lower magnitude *Bd* loads (Fig. 3). For *T. marmoratus*, there were significant differences among groups ( $H = 29.8$ ,  $df = 2$ ,  $p < 0.001$ ). Dunn's post hoc multiple comparisons indicated that the *Jliv*-negative market frogs had the greatest load, but for stream-caught *T. marmoratus* there was no difference in *Bd* infection intensity when comparing frogs with and without *Jliv* ( $Q = 0.552$ ,  $p > 0.05$ ).

## DISCUSSION

The anti-fungal bacterium *Jliv* was widespread (found on up to 40% of individuals within a site) in grassland populations of *Telmatobius* in the high Andes of Peru. This result stands in contrast to the extreme rarity of *Jliv* on frogs in lower elevation cloud forests that have been sampled extensively (Burkart et al. 2017, Catenazzi et al. 2018). The presence of *Jliv* in high-elevation streams is consistent with the ability of these bacteria to grow at low temperatures (Suman et al. 2015). Yet we found no association between the presence of *Jliv* and decreased probability of *Bd* infection. Probability of infection may be influenced by environmental factors which were not evaluated here, but are important to disease susceptibility in lotic frogs (Adams et al. 2017).

We found that *Jliv*-positive frogs had approximately 10-fold fewer zoospores on their skin than *Jliv*-negative frogs at our most well-sampled site. The natural presence or augmentation of *Jliv* by immersing frogs in probiotic baths reduces peak infection loads and lessens the morbidity and mortality associated with chytridiomycosis (Harris et al. 2009, Becker & Harris 2010), because *Jliv* produces metabolites, including violacein, which inhibit *Bd* growth using *in vitro* bioassays (Brucker et al. 2008). Survival after *Bd* exposure is associated with the concentration of violacein on the skin (Becker et al. 2009), and the presence of anti-*Bd* cutaneous microbes appears to permit populations to successfully co-exist with the fungal pathogen (Woodhams et al. 2007).

We observed high concentrations of *Bd* and no *Jliv* on *T. marmoratus* frogs being sold for human consumption in Cusco. While *Bd* infection prevalence (96%) was similar to previous reports from the same live trade market (Catenazzi et al. 2010, 2017), the median intensity ( $Z_e = 12580$ ) was 6× greater. High prevalence may be attributed to frogs being housed



in confined settings, because constant contact promotes stress and transmission. Frogs could have been infected with *Bd* either before or after being captured, but the result that *Bd* prevalence was lower in the nearby Rio Chocco highlights the risks posed by the live trade (i.e. translocation into the wild and maintenance of a *Bd* reservoir).

Our observations of *Jliv* on the skin of *Telmatobius* frogs suggest that mutualism with this violacein-producing bacterium may be protective for our focal species. Despite the ubiquity of *Bd* in the sampled streams, with infection prevalence reaching 80%, detrimental effects of chytridiomycosis on individual frogs were not immediately obvious. Excluding frogs from the Cusco market, all had loads below the 10 000 *Bd* zoospore lethality threshold, and median intensity of infection ranged from 10s to 100s of zoospores. None appeared moribund when captured. Our finding that 20% of all individuals hosted *Jliv* is likely an underestimate because some sites had small sample sizes and amphibians regularly shed their skin. Both factors decrease the chances of detecting the symbiont. Although we observed that frogs hosting *Jliv* had fewer *Bd* zoospores on their skin, we cannot infer that their survival is simply due to the presence of *Jliv* in their environment. Frog skin harbors diverse bacterial and fungal assemblages, and symbionts other than *Jliv* may protect hosts from *Bd* and other pathogens (Bletz et al. 2017). We cannot rule out the possibility that persisting populations have been negatively affected by chytridiomycosis because we do not know their abundance or demographic distribution prior to the arrival of *Bd*. It is therefore essential to investigate the relationships among species of *Telmatobius*, *Jliv*, and *Bd* suggested by our findings. Because *Telmatobius* are so prone to declines, uncovering the factors that contribute to their survival is vital to their conservation.

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