

NOTE

# Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs

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**ABSTRACT:** Amphibian populations are globally threatened by emerging infectious diseases, and 2 pathogens in particular are recognized as major threats: the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses. Here, we evaluated the prevalence of infection by *Bd* and ranavirus in an assemblage of frogs from a lowland wet forest in Costa Rica. We found an overall prevalence of 21.3% for *Bd* and 16.6% for ranavirus, and detected both pathogens widely among our 20 sampled species. We found a positive association between ranavirus and *Bd* infection in one of our 4 most commonly sampled species. We also found a positive but non-significant association between infection by ranavirus and infection by *Bd* among species overall. Our study is among the first detailed evaluations of ranavirus prevalence in the American tropics, and to our knowledge is the first to detect a positive association between *Bd* and ranavirus in any species. Considerable research attention has focused on the ecology of *Bd* in tropical regions, yet we argue that greater research focus is necessary to understand the ecology and conservation impact of ranaviruses on amphibian populations already decimated by the emergence of *Bd*.

**KEY WORDS:** *Batrachochytrium dendrobatidis* · Frog virus 3 · Amphibian decline · Emerging infectious diseases · *Oophaga pumilio* · *Craugastor bransfordii* · *Craugastor fitzingeri*

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## INTRODUCTION

Rapid losses of amphibian populations on several continents have been linked to emerging infectious diseases (James et al. 2009). Two major pathogens have been identified as large-scale threats to amphibians: the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) and ranaviruses (Schloegel et al. 2010). Both *Bd* and ranaviruses have broad host ranges among amphibians (Bancroft et al. 2011, Miller et al. 2011) and broad geographic ranges (James et al. 2009, Miller et al. 2011). While both *Bd* and ranaviruses have been associated with mass mortality events in amphibians (Green et al. 2002, Fox et al. 2006, Lips et al. 2006), both have also been

reported to occur in infections in wild frogs not associated with clinical disease (Miller et al. 2011, Whitfield et al. 2012b).

The amphibian chytrid fungus is currently widespread and enzootic in Central America (Lips et al. 2008, Whitfield et al. in press), most likely as a recent introduction through anthropogenic transport (James et al. 2009). While the geographic origin of *Bd* remains contentious, recent studies have indicated that a 'global pandemic lineage' or 'BdGPL' has been emerging on several continents, and appears largely responsible for *Bd*-associated population declines (Schloegel et al. 2012). The first report of *Bd* from the area of our study is from 1986 (Puschendorf et al. 2006), and 2 *Bd* isolates from our study site are consistent with BdGPL

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(T. James unpubl. data). *Bd* affects the keratinized tissues of amphibians (mouthparts in larvae or skin in adults), and amphibians appear to show little immune response to infection by *Bd* (Rosenblum et al. 2012). In susceptible species, *Bd* may disrupt osmotic balance via the skin and eventually lead to death through cardiac arrest (Voyles et al. 2009).

Ranaviruses are a globally-distributed group of iridoviruses known to affect amphibians, reptiles, and fish (Lesbarrères et al. 2012). At least 2 strains of ranaviruses, *Amyxstoma tigrinum* virus and Frog Virus 3 (hereafter FV3), have been associated with mass mortality events in amphibians (Green et al. 2002, Miller et al. 2011). Depending on the virulence of the strain and the susceptibility of the host species, death resulting from organ failure may occur in less than 3 wk after initial exposure to the virus (Hoverman et al. 2010).

Here we tested for *Bd* and ranavirus in a diverse assemblage of tropical amphibians from lowland wet forests of Costa Rica. Our goals were to describe patterns of infection and co-infection with *Bd* and ranavirus for an assemblage of tropical amphibians, and to determine whether there are any positive or negative associations between these 2 pathogens among species or within species among hosts.

## MATERIALS AND METHODS

### Field site and field sampling

We sampled amphibians at La Selva Biological Station in northeastern Costa Rica (10.43° N, 83.98° W), a predominantly old-growth tropical lowland wet forest (McDade & Hartshorn 1994), between January and March 2011. The amphibian fauna of La Selva includes at least 52 species (Guyer & Donnelly 2005), and amphibian population densities have declined dramatically since 1970 (Whitfield et al. 2007), although there has been little to no species loss as is typical of montane amphibian declines (Lips et al. 2006, Whitfield et al. in press). We conducted all field sampling during the cooler months of the year, during the nominal dry season, because *Bd* infection is apparently limited by high temperatures in warmer months (Whitfield et al. 2012b).

We sampled amphibians using a strategy to maximize the number of sampled amphibian species, families, and life histories. We collected post-metamorphic amphibians from a variety of habitats (forest, ponds, streams) and targeted both diurnal and nocturnal species. Amphibians were caught opportunistically,

as climatic conditions and natural history allowed. We sampled terrestrial habitats in all 3 months, and searched along existing trails in primary and secondary forests, as well as highly disturbed terrestrial habitats near laboratory buildings. Pond habitats were only visited during our January sampling period, because the seasonal ponds at this site normally do not hold water after early January. We sampled a single stream in secondary forest, and only during the February and March sampling periods, because high stream flow during heavy rains prohibits effective field collection in streams.

We collected all amphibians using clean, unused plastic bags to prevent cross-contamination among individuals or contamination by observers. We sampled for *Bd* by swabbing the body surfaces with Fisher brand cotton-tipped wooden swabs. We swabbed the ventral surface of each amphibian 10 times, each side of the abdomen 5 times, and each hand and foot 2 times. We stored swabs dry in 2 ml centrifuge tubes that were frozen until laboratory analysis. To sample for ranavirus, we took small tissue samples (a single toe clip from the outermost toe of the right hind foot) from each sampled animal, and held toe clips in 70% ethanol until laboratory analysis. We always swabbed amphibians for *Bd* before sampling for ranavirus. We are confident that no individuals used in this study were sampled twice because none showed evidence of previous toe clipping.

### Pathogen detection

To test for presence of *Bd* and ranavirus, we used qPCR following protocols described previously (Boyle et al. 2004, Brunner et al. 2004, Forson & Storfer 2006, Hyatt et al. 2007, Kerby & Storfer 2009, Whitfield et al. 2012a, Kerby et al. 2013). For both *Bd* and ranavirus, we extracted nucleic acids from swabs using Qiagen DNeasy Blood and Tissue spin column kits following the manufacturer's protocols. For *Bd*, we used 10 µl reactions with 3 µl extracted template DNA, while for ranavirus we used 13 µl reactions containing 2 µl of DNA template. qPCR reactions for *Bd* included 900 nmol forward primer, 900 nmol reverse primer, 250 nmol probe, and 2× Taqman Fast Universal Master Mix. We ran plates for 1 cycle of 95°C (20 s), and 50 cycles of 95°C (3 s) and 60°C (20 s). We ran ranavirus reactions using 300 nmol forward primer, 900 nmol reverse primer, 250 nmol probe, and Taqman 2× Universal PCR master mix. We ran plates for an activating cycle at 95°C (10 min), and then 40 cycles at 95°C (20 s), 54°C (20 s), and 72°C (30 s). Samples for both

*Bd* and ranavirus were run on a StepOne Plus qPCR machine (Applied Biosystems), and each plate contained negative controls and standards as internal positive controls. All samples were run in triplicate. We designated samples that were positive in 2 wells as positive; we reran samples that were positive in only 1 well and from the rerun we designated these samples positive if 2 wells were positive and negative if no wells were positive.

### Statistical analysis

To evaluate trends in infection prevalence for *Bd* and ranavirus, we used generalized linear models with infection status as a binomial response variable, species and sampling period (month) as predictor variables, and binomial error distributions. To evaluate differences among species in *Bd* and ranavirus loads, we used linear models with log-transformed *Bd* load and ranavirus load as response variables and species and month as predictor variables. To test for patterns of co-infection at the species level, we used a linear regression of prevalence of *Bd* infection and ranavirus infection by species, and used the number of individuals sampled for each species as weights. To test for patterns in co-infection at the individual level, we analyzed 2-way contingency tables with infection by ranavirus and *Bd* using Fisher's exact test, first for individuals of all species pooled, and subsequently for each species represented by >20 samples and infection by both pathogens. We conducted all statistical analyses in R (R Development Core Team 2012).

### RESULTS

We sampled 253 amphibians from 20 species and 8 families (Table 1). Of these, 54 individuals of 11 species tested positive for *Bd* (overall prevalence 21.3%), and 42 amphibians from 9 species tested positive for ranavirus (overall prevalence 16.6%, Table 1). Fourteen individuals from 4 species (*Craugastor fitzingeri*, *C. bransfordii*, *Oophaga pumilio*, and *Rhaebo haematiticus*) tested positive for both *Bd* and ranavirus. We detected infections both by *Bd* and by ranavirus in each of the 3 major habitats (terrestrial, ponds, and streams), and no patterns in habitat association were obvious for either pathogen.

We found strong differences in prevalence of infection by *Bd* among months (deviance = 18.846,  $df = 2$ ,  $p < 0.0001$ , Fig. 1) and species (deviance = 59.471,  $df = 20$ ,  $p < 0.0001$ , Fig. 2). Prevalence of infection by *Bd* was highest in January and decreased in February and March. We also found differences in *Bd* load among species ( $F_{9,36} = 2.535$ ,  $p = 0.0229$ ), but not among months ( $F_{2,36} = 0.889$ ,  $p = 0.4196$ ).

We found differences in prevalence of infection by ranavirus among months (deviance = 9.1394,  $df = 2$ ,  $p = 0.0104$ ), but no difference in infection prevalence

Table 1. Prevalence of infection by ranavirus and *Bd* in 20 species of amphibians from lowland wet forests of Costa Rica. 'Habitat' indicates where a given species was sampled (T: terrestrial; P: ponds; S: streams). Individuals that tested positive for both ranavirus and *Bd* are listed and in the columns for 'Ranavirus' and '*Bd*', and are also included in the columns for 'Ranavirus + *Bd*'; consequently the sum of the columns 'Ranavirus,' '*Bd*,' 'Ranav. + *Bd*,' and 'No infect.' may sum to greater than the column 'Total sampled'

Taxon	Habitat	Ranavirus	<i>Bd</i>	Ranav. + <i>Bd</i>	No infect.	Total sampled
<b>Bufonidae</b>						
<i>Rhaebo haematiticus</i>	T, S	4	4	1	6	<b>13</b>
<i>Rhinella marina</i>	T	4	0	0	16	<b>20</b>
<b>Hylidae</b>						
<i>Agalychnis callidryas</i>	T, P	0	0	0	4	<b>4</b>
<i>Agalychnis</i> spp. <sup>a</sup>	P	0	0	0	2	<b>2</b>
<i>Dendropsophus ebraccatus</i>	P	0	0	0	1	<b>1</b>
<i>Hypsiboas rufitelus</i>	P	0	0	0	2	<b>2</b>
<i>Scinax boulengeri</i>	T	0	0	0	1	<b>1</b>
<i>S. elaeochroa</i>	T, P	5	2	0	27	<b>34</b>
<i>Smilisca boudinii</i>	T	2	0	0	0	<b>2</b>
<b>Centrolenidae</b>						
<i>Espadarana prosoblepon</i>	S	0	1	0	0	<b>1</b>
<i>Teratohyla spinosa</i>	T, S	1	3	0	6	<b>10</b>
<i>Hyalinobatrachium fleishmanii</i>	T	0	0	0	1	<b>1</b>
<b>Leptodactylidae</b>						
<i>Leptodactylus savagei</i>	T	0	1	0	1	<b>2</b>
<b>Ranidae</b>						
<i>Lithobates warszewitschii</i>	T	0	0	0	1	<b>1</b>
<b>Craugastoridae</b>						
<i>Craugastor bransfordii</i>	T	10	8	2	54	<b>70</b>
<i>C. fitzingeri</i>	T, P, S	9	15	8	11	<b>27</b>
<i>C. megacephalus</i>	T	1	2	0	4	<b>7</b>
<i>C. mimus</i>	T	0	1	0	1	<b>2</b>
<b>Strabomantidae</b>						
<i>Pristimantis cerasinus</i>	T	0	0	0	2	<b>2</b>
<i>P. ridens</i>	T	0	1	0	10	<b>11</b>
<b>Dendrobatidae</b>						
<i>Oophaga pumilio</i>	T	6	16	3	21	<b>40</b>
<b>Grand total</b>		<b>42</b>	<b>54</b>	<b>14</b>	<b>171</b>	<b>253</b>

<sup>a</sup>Metamorphs of *Agalychnis callidryas* and *A. saltator* cannot be readily distinguished, and were therefore lumped into the grouping '*Agalychnis* spp.'

among species (deviance = 24.317,  $df = 20$ ,  $p = 0.2288$ ). Prevalence of infection by ranavirus was higher in January and March than in February (Fig. 1). We found no difference in ranavirus load among species ( $F_{8,24} = 0.7033$ ,  $p = 0.6857$ ) or months ( $F_{2,24} = 1.5432$ ,  $p = 0.2342$ ).

We found a positive but non-significant linear relationship between the prevalence of *Bd* and the prevalence of ranavirus among species ( $F_{1,18} = 1.7461$ ,  $r^2 =$

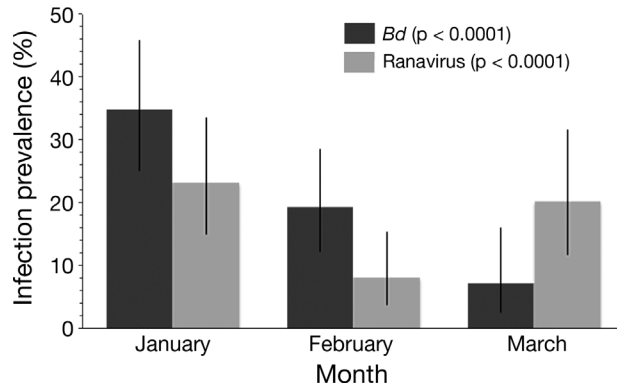


Fig. 1. Monthly variation in infection prevalence ( $\pm 95\%$  CI) of *Bd* and ranavirus among amphibians from 3 sampling events in 2011. Prevalence of infection by *Bd* was highest in January, the coolest month. Prevalence of infection by ranavirus was lower in February than in January or March

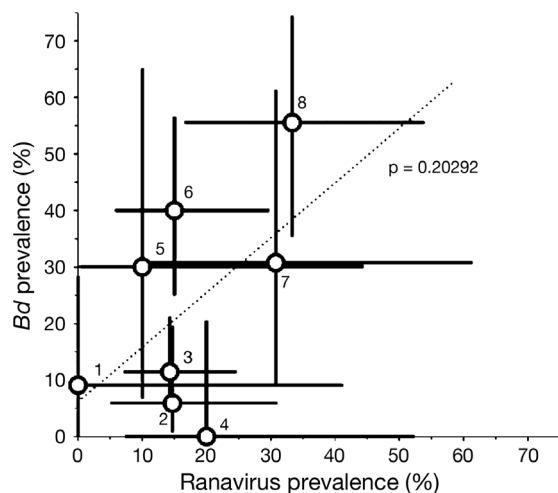


Fig. 2. Interspecific variation in infection prevalence ( $\pm 95\%$  CI) of *Bd* and ranavirus in 8 species of amphibians for which we had a sample of  $>10$  individuals. Among species, the prevalence of infection by *Bd* is positively but non-significantly related to the prevalence of infection by ranavirus. Numbers indicate species as follows: 1: *Pristimantis ridens*; 2: *Scinax eleaochroa*; 3: *Craugastor bransfordii*; 4: *Rhinella marina*; 5: *Teratohyla spinosa*; 6: *Oophaga pumilio*; 7: *Rhaebo haematiticus*; 8: *C. fitzingeri*. The p-value in the figure refers to the regression between prevalence of infection by *Bd* and prevalence of infection by ranavirus among species

0.08843  $p = 0.20292$ , Fig. 2). When all species were pooled, there was a non-significant but apparent trend in the association between infection by *Bd* and infection by ranavirus within individual hosts (odds ratio = 2.13, 95% CI: 0.94–4.64,  $p = 0.061$ , Fig. 3). We found no association between ranavirus infection and *Bd* infection for *Craugastor bransfordii* (odds ratio = 2.21, 95% CI: 0.18–15.64,  $p = 0.320$ , Fig. 3), *Oophaga pumilio* (odds ratio = 1.60, 95% CI: 0.19–13.80,  $p = 0.668$ , Fig. 3), or *Scinax elaeochroa* (odds ratio = 0, 95% CI: 0–33.19,  $p = 1.0$ , Fig. 3). However, we found a significant positive association between infection by *Bd* and infection by ranavirus in *C. fitzingeri* (odds ratio = 11.43, 95% CI: 1.12–604.86,  $p = 0.019$ , Fig. 3).

## DISCUSSION

Here, we demonstrate widespread infection by *Bd* and ranavirus in wild frogs from lowland wet forests of Costa Rica. Further, we show that in one species (*Craugastor fitzingeri*), occurrence of these 2 pathogens is correlated within hosts. The presence of *Bd* is well-established in Central America, yet robust reports of ranavirus in the region are restricted to a single species, *C. bransfordii* (Whitfield et al. 2012a). Thus, our study provides evidence that not only *Bd*, but also ranavirus may be at least locally prevalent within Neotropical amphibian assemblages.

Several studies have investigated co-infection of multiple pathogens within amphibian hosts, yet we are aware of few studies that have aimed to document co-infections by *Bd* and ranavirus *in situ*. Souza et al. (2012), Fox et al. (2006), and Hoverman et al. (2012) reported co-occurrence of *Bd* and ranavirus within sites (though not within hosts), and Miller et al. (2008) documented concurrent infections by *Bd* and ranavirus in captive amphibians. Positive associations between concurrent infections that we detected in *Craugastor fitzingeri* could be related to temporal or spatial associations in epidemiology of the 2 pathogens (Souza et al. 2012). Alternatively, these positive associations could be related to host immunology such as positive direct (pathogen–pathogen) or indirect (immune-mediated) interactions among pathogens within a host (Pedersen & Babayan 2011). If co-infections are exclusively attributable to spatio-temporal patterns in pathogen distribution, we would expect multiple species to show similar patterns in co-infection, yet they do not. Future field studies should make a more concerted effort to detect *Bd* and ranavirus from the same host amphibians, and experimental trials in controlled conditions may help to un-

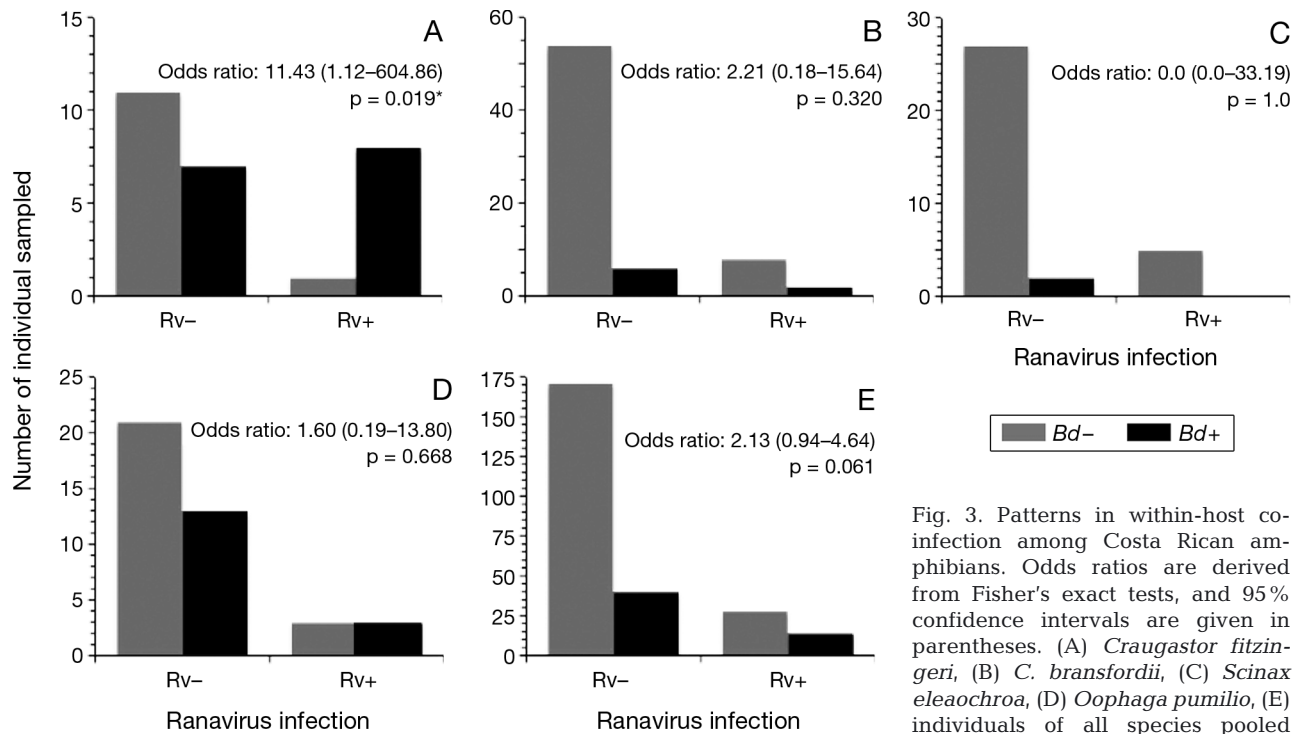


Fig. 3. Patterns in within-host co-infection among Costa Rican amphibians. Odds ratios are derived from Fisher's exact tests, and 95% confidence intervals are given in parentheses. (A) *Craugastor fitzingeri*, (B) *C. bransfordii*, (C) *Scinax eleochoa*, (D) *Oophaga pumilio*, (E) individuals of all species pooled

derstand direct or indirect pathogen interactions in co-infection in amphibian hosts.

Our study considerably expands the known host range of ranavirus in wild amphibian populations. Miller et al. (2011) summarized information on ranavirus infection in amphibians and demonstrated infections in 70 species of amphibians from 14 families. Of the 9 species for which we detected ranavirus infections, only *Rhinella marina* has previously been reported as a potential host (Zupanovic et al. 1998). Our study presents the first report of ranavirus infection in the Centrolenidae, and earlier work from La Selva identified the first infection in the Craugastoridae (Whitfield et al. 2012a). Further, our current study presents the first known infection in the Dendrobatidae in wild populations, although infections in captive dendrobatids have been observed (Miller et al. 2008). Further work with ranavirus in tropical regions is likely to continue expanding the known host range of ranaviruses.

It is unclear whether ranaviruses are endemic or invasive in Central America. Preliminary molecular characterization of ranavirus from La Selva shows 100% concordance to FV3 (Whitfield et al. 2012a), suggesting either that non-native strains of FV3 are present in Central America or that insufficient sequence data are available for comparison among various strains of FV3 and FV3-like viruses. However, an apparent infection by ranavirus in *Rhinella mar-*

*ina* was reported in Costa Rica before 1991 (Speare et al. 1991), indicating that either some strain of ranavirus is endemic to Central America or that an introduction occurred before 1991.

It is also unclear whether ranavirus is associated with mortality events or population declines in the Neotropics. While mass mortality events and population extirpations have been documented widely throughout the American tropics (Lips et al. 2006, 2008), sampling for ranavirus has been conducted at few decline sites—and fewer still during mass mortality events. It is assumed that *Bd* has been exclusively responsible for mass mortality events in the Neotropics, although the presence of ranavirus—which in North America is responsible for more observed mass mortality events than is *Bd* (Green et al. 2002)—in the American tropics may complicate efforts to attribute all known mass mortality events and rapid population declines to a single pathogen.

The Neotropics host extreme amphibian biodiversity, and many species and populations have been dramatically impacted by *Bd*. Resolving the history of ranavirus in the region, the potential impacts of ranavirus to Neotropical amphibians, interactions between *Bd* and ranavirus within amphibian hosts, and the potential for emergence of non-native ranavirus strains should be priorities for research in disease ecology and conservation management for tropical amphibians.

**Acknowledgements.** Funding for this study was provided by an FIU Dissertation Year Fellowship to S.M.W. Costa Rica's MINAET and FIU IACUC provided permits. Genetic samples were run on equipment provided by NSF grant MRI-0923419. C. Brizuela provided assistance in the field.

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