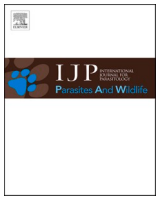
International Journal for Parasitology: Parasites and Wildlife 23 (2024) 100924

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw



Ranavirus and helminth parasite co-infection in invasive American bullfrogs in the Atlantic forest, Brazil

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ARTICLE INFO

Keywords:
Ranavirus
Helminth
Co-infection
Lithobates catesbeianus
Aquarana catesbeiana
Invasive species

ABSTRACT

Emerging infectious diseases threaten amphibian species across the globe. In Brazil, the American bullfrog (*Aquarana catesbeiana*) is a highly invasive species that can potentially transmit parasites and pathogens to native amphibians. This is the first assessment of co-infection of *Ranavirus* and helminth macroparasites in invasive populations of bullfrogs in South America. We collected, measured, and euthanized 65 specimens of *A. catesbeiana* sampled from 9 sites across three states of Brazil in the Atlantic Forest biome. We collected and identified helminth macroparasites and sampled host liver tissue to test for the presence and load of *Ranavirus* with quantitative PCR. We documented patterns of prevalence, parasite load, and co-infection with generalized linear mixed models, generalized logistic regressions, and randomization tests. Most individual bullfrogs did not exhibit clinical signs of infection, but the overall *Ranavirus* prevalence was 27% (95% confidence interval, [CI 17–38]). Bullfrogs were infected with helminth macroparasites from 5 taxa. Co-infection of helminth macroparasites and *Ranavirus* was also common (21% CI [12–31]). Bullfrog size was positively correlated with total macroparasite abundance and richness, and the best-fitting model included a significant interaction between bullfrog size and *Ranavirus* infection status. We observed a negative correlation between *Ranavirus* viral load and nematode abundance (slope = −0.22, P = 0.03). Invasive bullfrogs (*A. catesbeiana*) in Brazil were frequently infected with both *Ranavirus* and helminth macroparasites, so adult bullfrogs could serve as reservoir hosts for both pathogens and parasites. However, many macroparasites collected were encysted and not developing. Coinfection patterns suggest a potential interaction between *Ranavirus* and macroparasites because helminth abundance increased with bullfrog size but was lower in *Ranavirus* infected individuals. Future studies of bullfrogs in the Atlantic Forest should investigate their potential role in pathogen and parasite transmission to native anurans.

1. Introduction

Emerging infectious diseases have been drivers of amphibian population declines around the globe (Daszak et al., 1999). When individuals are co-infected, or simultaneously infected by multiple parasites and pathogens, these concomitant infections have the potential to influence disease dynamics in multiple systems (Herczeg et al., 2021). Although co-infections are ubiquitous in wildlife study systems, most research focuses on one host - one parasite dynamics (Herczeg et al., 2021; Risco et al., 2014; Stutz et al., 2018). Interactions between parasites can lead to patterns of aggregation that may support facilitative interactions (i.e. through immune system suppression), while there might be patterns of segregation that may contribute to inhibitory interactions, such cross-immunity or direct or indirect competition (Herczeg et al., 2021; Ramsay and Rohr, 2021; Risco et al., 2014; Wuerthner et al., 2017). Moreover, infection intensities can vary due to environmental factors, as well as order and timing of exposures (Ramsay and Rohr, 2021; Wuerthner et al., 2017).

The American bullfrog (*Aquarana catesbeiana*) is one of the most successful and harmful invasive species in the world (Nori et al., 2011) and serves as an ideal study system for co-infection dynamics. Bullfrogs can host at least 159 helminth taxa including digeneans, monogeneans, cestodes, acanthocephalans, and nematodes across native and non-native sites (Mata-López et al., 2010). After *A. catesbeiana* was introduced to Brazil in the 1930s and farmed for consumption, many escapes from bullfrog farms led to the invasion in more than 130

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https://doi.org/10.1016/j.ijppaw.2024.100924
Received 13 October 2023; Received in revised form 10 March 2024; Accepted 10 March 2024
Available online 21 March 2024

Brazilian cities and towns in and near the Atlantic Forest region (Both et al., 2011; Cunha and Delariva, 2009), which contains 60% of native Brazilian amphibian species (Jordani et al., 2017). The impacts caused by American bullfrogs are severe, including predation of native anurans (Oda et al., 2019), as well as serving as a reservoir of numerous pathogens (Borzée et al., 2017; Brooks and Hoberg, 2007; Mazzoni et al., 2003; Miaud et al., 2016; Rödder et al., 2013; Ruggeri et al., 2019, 2023).

Some of the pathogens found in American Bullfrogs have been drivers of amphibian population declines across multiple continents, including ranaviruses (Gray and Chinchar, 2015). *Ranavirus* outbreaks can lead to mass-mortality events, especially in immunologically naïve populations (Gray and Chinchar, 2015). American Bullfrogs have shown resistance to *Ranavirus* infection (Hoverman et al., 2011) and the ability to harbor subclinical levels of virus (Brunner et al., 2019), indicating they could influence community transmission dynamics as reservoir species. Recent studies have shown a widespread prevalence of *Ranavirus* in bullfrog tadpoles across Brazil (Ruggeri et al., 2019, 2023), yet little is known about the role of adult bullfrogs in native amphibian communities. As adults, bullfrogs can also host a variety of endo- and ecto-macroparasites, however research on the dynamics of viral pathogens and macroparasites is generally sparse (Herczeg et al., 2021). Previous studies have indicated the abundance of nematode and trematode species can have effects on *Ranavirus* viral levels in laboratory settings (Ramsay and Rohr, 2021; Wuerthner et al., 2017), yet there are few field studies investigating natural co-infection prevalences (Stutz et al., 2018); and to our knowledge, there are no field studies that have investigated co-infections of helminth parasites and *Ranavirus* in amphibians of South America, where biodiversity is highest.

Here, we investigated whether invasive adult American bullfrogs in the Brazilian Atlantic Forest 1) harbor *Ranavirus*, potentially serving as reservoirs in native anuran communities; 2) host helminth parasites; and 3) exhibit nonrandom patterns of co-infection between *Ranavirus* and helminth parasites at population and community scales.

2. Materials and methods

2.1. Field sites and sampling

We sampled adult *A. catesbeiana* at nine different sites in the Brazilian Atlantic Forest in the states of Santa Catarina (SC), Paraná (PR) and São Paulo (SP) (Table 1). The most widely separated sites are approximately 550 km apart. Anurans were collected during the breeding season between October 2018 and April 2019. We used a visual search method to detect anuran specimens (Crump and Scott Jr., 1994). We spent a minimum of 3 days at each site and sampled for at least 5 hours each night, collecting a total of 65 bullfrogs throughout the survey. We euthanized collected bullfrogs with the application of an overdose of lidocaine 4% (as approved by the Ethics Committee of the Universidade Federal do Paraná and in accordance with Ordinance CFBio 148/2012).

2.2. Parasite and pathogen detection

After bullfrogs were euthanized, we measured the snout-vent length (SVL), and dissected the specimens, storing ~1g of liver tissue in 70% ethanol for later *Ranavirus* testing. We checked for the presence of clinical signs of *Ranavirus*, such as organ lesions or hemorrhaging and redness or swelling near the legs (Gray et al., 2009; Miller et al., 2011). We searched for macroparasite infection by examining the lungs, intestines, stomach, kidneys, gonads, bladder, and liver under a stereomicroscope. We fixed macroparasites in a 70% alcohol solution and visually identified them to phylum or class under a 40X stereomicroscope. We classified each individual parasite as an adult with a direct life cycle, or a larva (with an indirect life cycle), according to their morphological development, i.e. if a parasite was found encysted and did not present developed reproductive organs, it was classified as larva. Necropsied bullfrogs were fixed in a 10% formaldehyde solution, stored in 70% ethanol.

We extracted DNA from adult bullfrog liver samples using the Omega Bio-tek E.Z.N.A. Tissue DNA kit following the manufacturer's protocols. To test for the presence and viral load of *Ranavirus*, we used quantitative PCR (qPCR) to amplify a portion of the major capsid protein using previously described protocols (Stilwell et al., 2018). We ran 10 μL Taqman qPCR reactions with 0.9 μM of each primer and 0.2 μM of probe, 5 μL of Taqman Universal PCR mix, 2.5 μL of nuclease-free water, and 2 μL of DNA template. We ran plates on a CFX Connect Real-Time PCR System (Bio-Rad) for an activating cycle at 95 °C (2 min), and then 45 cycles at 95 °C (20 s), 54 °C (20 s), and 72 °C (30 s). All plates were run with an extraction negative, non-template control (nuclease-free water), and gBlock (IDT DNA) standards as internal positive controls. Each sample was run in duplicate, with a positive assigned if both wells amplified. If only one well amplified, the sample was rerun and assigned positive if at least two wells amplified. An 18S assay (Applied Biosystems Assay ID Hs99999901_s1) was used for the positive samples to quantify DNA and to normalize viral load (viral copies per ng DNA).

2.3. Statistical analysis

We conducted all statistical analyses using R and Rstudio (R Core Team, 2023; RStudio Team, 2023). For the entire collection, as well as for individual sites, we used a beta distribution to estimate helminth and *Ranavirus* prevalence and 95% confidence intervals (CIs). Prevalence (%) was estimated as the expectation of the beta distribution where *shape1* and *shape2* are the parameters (*shape1* = the number of infected samples + 1; *shape2* = the number of uninfected samples + 1). The beta distribution accounts for uncertainty due to sample size and generates asymmetric 95% CIs. To test for differences in *Ranavirus* and helminth

Table 1
Collection locations, site numbers, *Ranavirus* infection status, and viral load of American bullfrogs (*Aquarana catesbeiana*) in 9 sample sites within the Brazilian Atlantic Forest. Site numbers were assigned to the corresponding Brazilian state and town where samples were collected; Sample size = number of collected bullfrogs; Ranavirus + N = number of *Ranavirus* positive bullfrogs; RV Prevalence = *Ranavirus* prevalence estimated from the beta distribution and given with the 95% confidence interval in parentheses (95% CI); Mean viral load = average estimates include zeroes, are in units of viral copies per ng of DNA, and given with standard error in parentheses (SE).

State	Site	Town	Latitude	Longitude	Sample size	*Ranavirus* + N	RV Prevalence (95% CI)	Mean viral load (±SE)
São Paulo	1	Embu das Artes	−23.6313	−46.8207	13	10	53% (29–77)	680 (±195)
	2	Piedade	−23.7111	−47.3684	10	0	–	–
	3	Iporanga	−24.5853	−48.5991	4	0	–	–
Paraná	4	Quatro Barras	−25.3213	−49.0169	10	4	50% (23–77)	17801 (±14201)
	5	Piraquara	−25.5247	−49.0855	2	0	–	–
	6	São José dos Pinhais	−25.6997	−49.0798	4	0	–	–
Santa Catarina	7	Blumenau	−27.0291	−49.0940	9	1	18% (3–45)	11403 (±0)
	8	Urubici	−28.0801	−49.5413	10	0	–	–
	9	Chapecó	−26.9670	−52.7159	3	1	40% (7–81)	30 (±0)

prevalence between locations, we analyzed 2-way contingency tables using Fisher's exact tests for each site represented by 5 or more samples. We compared helminth taxa diversity across bullfrogs by calculating total abundance, total taxa richness, and evenness. We calculated evenness using Hurlbert's (1971) probability of interspecific encounter (PIE), which ranges between 0 and 1, with maximal evenness closer to 1. We used analysis of variance (ANOVA) to test whether there were significant differences in *Ranavirus* viral load (number of viral copies per ng of DNA in infected individuals) and in the abundance and richness of helminth taxa in individuals among sites. We used a $\log_{10}(x+1)$ transformation of count data for ANOVAs. To test for the fit of the data to a Poisson distribution, we regressed the variance of the counts of parasites per taxon against the mean abundance per host. Because the data were over-dispersed (slope $\gg$ 1), we used a negative binomial distribution (Lindén and Mäntyniemi, 2011) as the link function in generalized linear and mixed effects models.

To detect nonrandom co-infection patterns of *Ranavirus* with macroparasites, we used a randomization test in the *EcoSimR* package (Gotelli et al., 2015). We first created a 6 × 65 occurrence matrix in which each row is a taxon (*Ranavirus* + 5 macroparasite taxa) and each column is an individual bullfrog. We randomized the occurrence of each taxon among frogs, with probabilities of occurrence assigned proportionally to frog body size (SVL), using the sim10 algorithm. It is reasonable to assign occurrence probabilities proportional to bullfrog body size because many other studies have shown a strong positive relationship between parasite abundance, parasite taxa richness, and host body size (Campião et al., 2015; Gregory et al., 1996; Kamiya et al., 2014). We then calculated the matrix-wide C score (Stone and Roberts, 1990) as an overall index of segregation or aggregation. We created 1000 null matrices in this way and used them to estimate the tail probability for the C-score of the observed matrix. To test community co-occurrence at the site level, we first created a 6 × 9 occurrence matrix (6 taxa × 9 sites). In this analysis, we assumed sites were equiprobable, but we fixed row totals to preserve differences among taxa in their occurrence frequencies (sim2 algorithm). Additionally, we conducted three separate analyses using the sim9 algorithm where row totals were fixed and sites were weighted by the number of bullfrogs collected, the average bullfrog length, and the sum of bullfrog lengths.

We used generalized linear regression models (GLM) and generalized linear mixed effects models (GLMM) to test the relationships at different spatial scales among *Ranavirus* prevalence and viral load, helminth taxa abundance, helminth taxa richness, and bullfrog length (SVL mean centered and scaled). We tested the effects of parasite abundance, richness, and bullfrog SVL on *Ranavirus* infection status by fitting a GLM with the logit-link function. We restricted these analyses to individuals from locations with documented *Ranavirus* infections (N = 35 individuals) to ensure a relevant context for assessing infection drivers and to guarantee exposure potential was present. To test for statistically significant predictors of total helminth abundance, we first estimated the theta parameters for the negative binomial distributions of total helminth abundance using the *fitdistr* function in the *MASS* package (Venables and Ripley, 2002). We fit a Poisson GLM to test the effect of bullfrog size and *Ranavirus* infection on helminth taxa richness. The theta parameters for abundance were used for the negative binomial linear mixed effects models with the *glmer* function in the *lme4* package (Bates et al., 2015). Collection location was included as a random effect in all the mixed effects models. We also tested pairwise relationships among taxa using a mixed effects model with a negative binomial link function. For these pairwise tests, we used the Benjamini-Hochberg (Benjamini and Hochberg, 1995) procedure to adjust p-values for multiple testing. Model comparison and selection were performed using likelihood ratio (LR) tests. We used the R packages *tidyverse* (Wickham et al., 2019) and *interactions* (Long, 2019) to visualize the relationships as generalized linear regressions.

3. Results

3.1. Ranavirus prevalence and viral load

There was an overall *Ranavirus* prevalence of 27% (95% CI [17–38]) across the 65 *A. catesbeiana* individuals (Table 1). However, we did not observe clear *Ranavirus* clinical signs among collected bullfrogs. Four out of 9 sites had individuals that tested positive for *Ranavirus* (Table 1, Fig. 1). Most of the infected individuals came from Site 1, but we also detected *Ranavirus* infection in Sites 4, 7, and 9. Sites that tested positive did not differ significantly in *Ranavirus* prevalence (P = 0.06; *simulate.p.value* option in Fisher test). Viral loads ranged from 90 to 143,358 viral copies ng^{-1} per individual with a median of 986 copies ng^{-1}, and Site 4 had the highest average viral load (Table 1). Only one bullfrog out of 9 collected in Site 7 was infected, but the viral load was 102,631 copies ng^{-1}. When that sample was removed, there were no statistically significant differences among sites in average viral load (Fig. 1).

3.2. Helminth macroparasite infection

Bullfrogs hosted nematodes, acanthocephalans, cestodes, trematodes, and pentastomids. Nematodes were the most prevalent (72% [62–83]) and abundant (N = 1848), and most nematode individuals were found encysted (62.8%). Acanthocephalans also exhibited high prevalence (33% [22–44]), while cestodes, trematodes, and pentastomids were found in lower prevalence and abundance (Table 2). Acanthocephalans also had a high number of cysts, and the majority of cestodes and pentastomids were larvae. Total helminth abundance and prevalence did not vary significantly among sites (P > 0.05), although there were differences in helminth taxa richness (P < 0.001) and evenness (P < 0.001, Fig. 1). We observed a high prevalence of macroparasites in most collection sites (Table 1). All sites had at least one helminth taxon represented, and only 11 out of the 65 bullfrogs had no infections.

3.3. Co-infection patterns

Twenty-one percent (prevalence with 95% CI [12–31]) of bullfrogs were coinfected by *Ranavirus* and 1 or more helminth taxa. There was a strong positive relationship between average abundance and variance of helminth taxa in individual hosts ($R^2 = 0.979$), and it was stronger when including both helminth taxa abundance and *Ranavirus* viral load ($R^2 = 0.992$). The fitted negative binomial distributions indicated all parasite taxa were aggregated (i.e. high abundance in only a few hosts).

In the community occurrence matrix (6 helminth taxa × 65 bullfrogs), helminth taxa exhibited aggregated associations (C score index smaller than expected by chance; P = 0.015, Fig. 2), so we continued with pairwise mixed models. Acanthocephalans and cestodes co-occurred more frequently than expected, as did nematodes with platyhelminthes (cestodes and trematodes; corrected P < 0.001, Fig. 2). Across individual bullfrogs, there was a significant positive correlation between acanthocephalan and platyhelminth abundances (slope = 0.80, corrected P < 0.0001) and a negative correlation between *Ranavirus* viral load and nematode abundance (slope = −0.22, corrected P = 0.03, Fig. 3a). There were no significant parasite taxa associations among collection sites (Fig. S1).

Macroparasite total abundance had a positive relationship with bullfrog length (slope = 1.94, P < 0.0001). At both the individual and site scale, *Ranavirus* infection status and prevalence alone were not significantly related to bullfrog SVL, helminth richness, or helminth taxa abundance. However, the best fitting model of macroparasite abundance included an interaction between bullfrog size (SVL) and *Ranavirus* infection status (P < 0.0001; Table S1): *Ranavirus*-negative individuals exhibited a steeper positive relationship (slope = 2.07, P < 0.0001) between SVL and macroparasite abundance than did *Ranavirus*-positive individuals (slope = 1.82, P < 0.05; Fig. 3b). Additionally, in the final

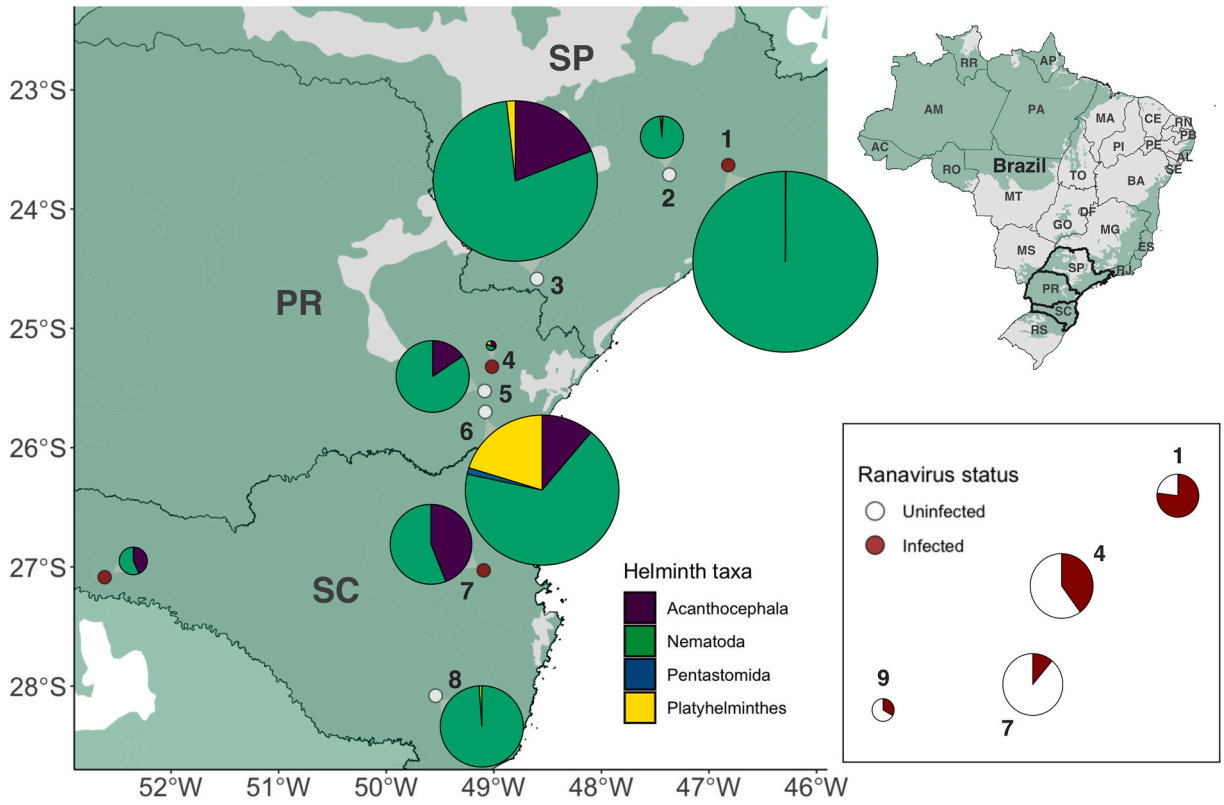

Fig. 1. Distribution of helminth macroparasite taxa and *Ranavirus* infection status and prevalence across sampling locations. The thick gray outline shown in the inset map of Brazil highlights the states where American bullfrog (*Aquarana catesbeiana*) sampling took place (SP = São Paulo, PR = Paraná, SC = Santa Catarina). Green shading represents tropical and subtropical forest biomes. Sample locations included Embu das Artes (Site 1, N = 13), Piedade (Site 2, N = 10), and Iporanga (Site 3, N = 4) in São Paulo; Quatro Barras (Site 4, N = 10), Piraquara (5, N = 2), and São José dos Pinhais (Site 6, N = 4) in Paraná; and Blumenau (Site 7, N = 9), Urubici (Site 8, N = 10), and Chapecó (Site 9, N = 3) in Santa Catarina. Helminth pie chart size corresponds to average helminth abundance in bullfrogs. Differences in helminth taxa richness ($P < 0.001$) and evenness (PIE; $P < 0.001$) were detected; however, helminth abundance did not differ among sites between collection sites. Sites where *Ranavirus* was detected (N = 4) have a corresponding pie chart, in which the size corresponds to average viral load (copies^{-ng}) per individual at a site. Estimated *Ranavirus* prevalence among the positive sites did not differ significantly ($P = 0.06$). Viral load of positive individuals differed among sites ($P < 0.05$), but differences were driven by the high load found in the single Blumenau (Site 7) *Ranavirus*-positive bullfrog. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Collection locations, site numbers, and helminth endoparasite abundance and prevalence in American bullfrogs (*Aquarana catesbeiana*) in 9 sample sites within the Brazilian Atlantic Forest. Site numbers were assigned to the corresponding Brazilian state and town where samples were collected. Total abundance of each endoparasite taxon is shown with the prevalence of bullfrogs infected with that taxon (the number of infected bullfrogs over the total number collected) in parentheses.

State	Site	Town				Platyhelminthes	
			Acanthocephala	Nematoda	Pentastomida	Cestoda	Trematoda
São Paulo	1	Embu das Artes	1 (1/13)	852 (12/13)	0	0	0
	2	Piedade	1 (1/10)	150 (9/10)	0	0	2 (2/10)
	3	Iporanga	44 (1/4)	185 (4/4)	0	0	4 (2/4)
Paraná	4	Quatro Barras	12 (3/10)	15 (4/10)	1 (1/10)	7 (3/10)	0
	5	Piraquara	8 (2/2)	44 (2/2)	0	0	0
	6	São José dos Pinhais	24 (4/4)	147 (3/4)	3 (2/4)	30 (1/4)	14 (2/4)
Santa Catarina	7	Blumenau	115 (8/9)	146 (3/9)	0	0	0
	8	Urubici	0	292 (7/10)	0	0	3 (1/10)
	9	Chapecó	13 (1/3)	17 (3/3)	0	0	0

model, *Ranavirus* load had a slight negative effect on helminth abundance (slope = −0.17, $P < 0.05$, Table S1). Helminth taxa richness was also positively correlated with bullfrog SVL and had an infection status interaction term (Fig. S2). *Ranavirus*-negative individuals exhibited a larger exponentiated coefficient (estimate = 0.73, $P < 0.05$) compared to infected individuals (estimate = 0.48, $P < 0.1$).

4. Discussion

This is the first assessment of concomitant infection of the American bullfrog with *Ranavirus* and macroparasites in South America. Although *Ranavirus* infections were common across disjunct localities in three Brazilian states (Fig. 1), most infected adult bullfrogs were asymptomatic, which to our knowledge, has not been reported previously. Macroparasite infections were also common, and taxa were highly aggregated (Fig. 3). Nematodes and acanthocephalans were common at most sites, suggesting they are prevalent in the environment (Goater et al., 2013) and in the prey of bullfrogs (Leivas et al., 2012). As in other studies, we found a positive, linear relationship between host body size and macroparasite abundance (Fig. 3) (Poulin, 2007), possibly

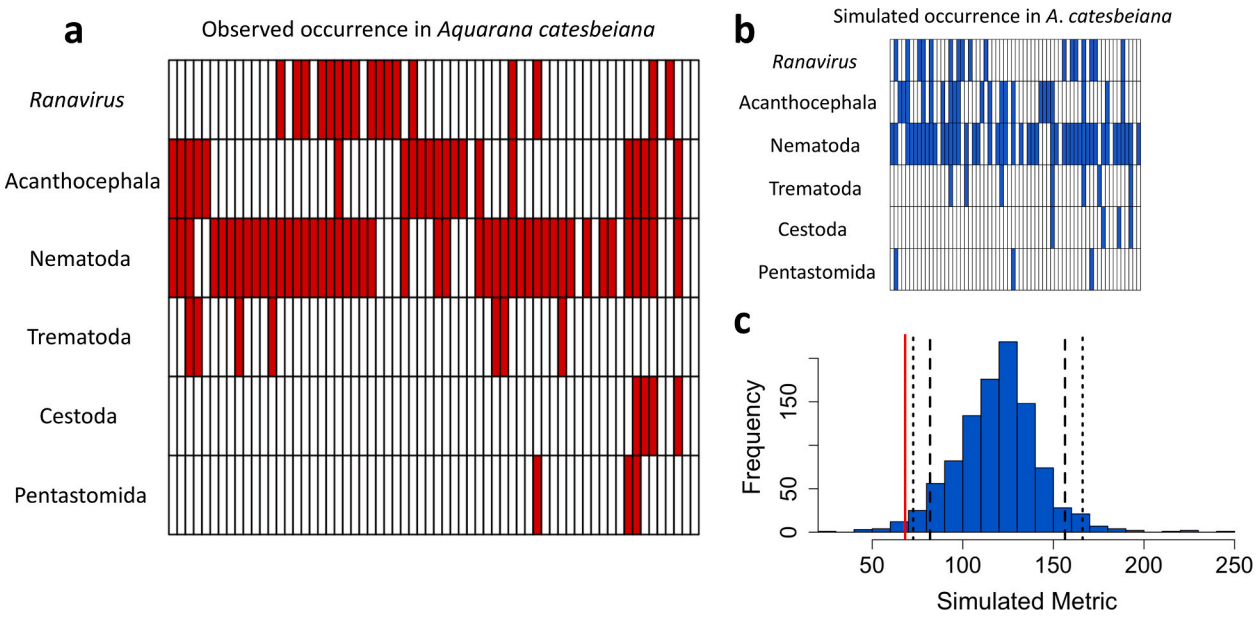

Fig. 2. (a) Observed occurrence matrix of presence (red cells) and absence (white cells) of *Ranavirus* and helminth taxa (6 rows) infection in individual bullfrogs (*Aquarana catesbeiana*; 65 columns). (b) Simulated occurrence matrix (65 columns, one null matrix out of 1000 simulations). (c) Distribution of simulated co-occurrence metric (blue histogram bars; 1000 null matrices). Vertical red line = observed co-occurrence metric. Dashed vertical lines = 95% and 99% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

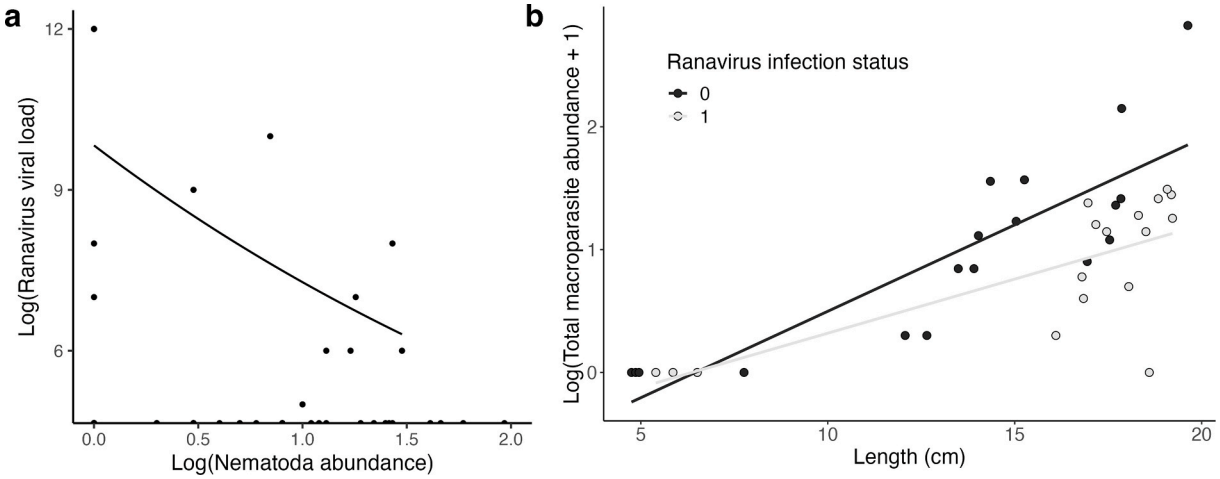

Fig. 3. (a) The negative relationship between log-transformed Nematoda abundance and *Ranavirus* load (copies^{-ng}; $P < 0.05$). Points represent individuals that tested positive for *Ranavirus* infection with viral load assay data (N = 12). The nematode abundances in *Ranavirus*-negative individuals are shown on the x-axis. (b) The relationship between log-transformed total macroparasite abundance and bullfrog snout-vent length (cm) and *Ranavirus* infection status with fitted regression lines ($P < 0.001$). Data represent individuals from sites that had at least one *Ranavirus* infection (4 sites, N = 35).

indicating infections accumulate through aging (Hudson et al., 2006; Hudson and Dobson, 1995; Raffel et al., 2009). However, a negative interaction with *Ranavirus* infection status was also observed (Fig. 3), which could suggest synergistic mechanisms.

Ranavirus is a prevalent and virulent pathogen group that has been previously documented in other South American countries (Candido et al., 2019). *Ranavirus* infections are usually severe in larval and metamorphic stages (Miller et al., 2011), so infected adult bullfrogs may either be the survivors of a prior *Ranavirus* infection and now host subclinical infections (Driskell et al., 2009; Gray et al., 2009) or they were exposed to the virus as adults (Robert et al., 2007). We do not know whether these infected adults are shedding viral particles into the environment, but previous studies have shown a correlation between viral shedding rates and viral copies within liver and kidney tissue (Brunner et al., 2019). The impact of the viral shedding of asymptomatic infections is poorly understood in this system (Brunner et al., 2019; Gray et al., 2009; Robert et al., 2007), as is the relationship between viral plaque-forming units (pfu) and genome copies (Leung et al., 2017). Viral shedding by adults into the water could affect community disease dynamics (Price et al., 2017) by amplifying transmission or, at lower shedding rates, by 'priming' native anuran immune systems (Nelson et al., 2015). Because half of the collected adults had relatively high viral loads ($>10^3$ copies/ng; Table 1, Fig. 1), American bullfrogs could be important reservoirs for *Ranavirus* in the Brazilian Atlantic Forest.

In addition to *Ranavirus* infection, we found an abundant helminth assemblage in the internal organs of adult bullfrogs (Fig. 1). All the main helminth classes have been found in bullfrogs in their native and invaded sites (Mata-López et al., 2010) and are frequently found in native Brazilian anurans (Campião et al., 2014). This finding is consistent with a previous review (Mata-López et al., 2010) but has not been

reported in South America where previous surveys on farmed (Antonucci et al., 2012) and wild-caught specimens (González et al., 2014) recorded low infection prevalence and abundance of parasites. For North America, where the invasive bullfrogs are closer to their native range, Dare and Forbes (2013) found that bullfrogs had lower helminth species richness than their endemic counterparts, but demonstrated high levels of infection for some parasites known to be harmful. Thus, our results, in addition to previous studies, indicate that invasive bullfrogs are not free from helminth parasites in their non-native range, with some studies reporting high levels of infection (Lemke et al., 2008) while others report low levels (González et al., 2014). This suggests that helminth prevalence, richness, and intensity of infection found in these bullfrogs vary by location, which are most likely determined by interactions between biotic and abiotic factors (Anderson and Sukhdeo, 2010). The presence of these parasites in non-native bullfrog populations may have implications for the health of both the bullfrogs and the ecosystems they inhabit (Pulis et al., 2011).

The high aggregation in parasite communities that we observed (Fig. 2) has been reported for many other systems (Poulin, 2007; Shaw et al., 1998). Site-level aggregation may reflect heterogeneity in exposure or susceptibility to parasites among populations and most likely contributes to the observed difference in helminth richness and evenness among sites. Similarly, the positive relationship between metrics of parasite infection and body size has been consistently documented in parasitological literature [refer to Kamiya et al. (2014) for a comprehensive review, and Campião et al. (2015)]. Beyond body size, foraging behavior emerges as another influential factor likely to impact parasite acquisition. This may explain the aggregated co-occurrence (nematodes-platyhelminthes and cestodes-acanthocephalans) and a positive correlation of the abundances of acanthocephalans and cestodes that we observed. Bullfrogs are voracious generalist predators of potentially infected intermediate hosts (Goater et al., 2013; Leivas et al., 2012), and this behavior may contribute significantly to the acquisition of diverse parasites across different taxa, explaining the observed relationships in our study.

Co-infections were common, which is probably due to the ubiquity of infection by helminths and may reflect a pattern where most tend to acquire helminth infection during their lifetimes. *Ranavirus* infection may come first, since it typically occurs earlier in tadpoles and might persist in some adults, whereas helminth infections could be secondary, acquired later in life through foraging or soil contact. The interaction between pathogen and parasites can be harmful for the hosts, as observed in Site 1 (see Campião et al., 2024 for details), and the time between infections may be influential to the course of disease and host condition. Wuerthner et al. (2017) showed that prior infection with a trematode species (*Echinoparyphium* sp.) reduced ranaviral loads, with no reciprocal effect of *Ranavirus* infection on trematode load. Survival rates of hosts infected with the trematode prior to virus exposure were significantly greater compared to hosts only exposed to *Ranavirus*. In this sense, trematode infections appear to benefit hosts that are exposed to *Ranavirus*. Soil-transmitted helminths can also modulate the immune response to other pathogens, with the potential for both positive and negative outcomes (Schlosser-Brandenburg et al., 2023). Ramsay and Rohr (2021), in an experiment where Cuban tree frogs (*Osteopilus septentrionalis*) were exposed to *Ranavirus* and a nematode species (*Aplectana hamatospicula*), showed that co-infected hosts had increased viral loads and decreased nematode loads relative to single infections, regardless of the timing of those infections. They suggested this negative interaction could be mediated by the tradeoff in host T helper 1 (Th1, activated by microparasites) and T helper 2 (Th2, activated by macroparasites) immune response arms, which suppress one another and make it difficult for many vertebrate hosts to mount simultaneous defenses against micro and macroparasites (references within Ramsay and Rohr, 2021). We observed a negative relation between *Ranavirus* infection and total helminth abundance, mediated by bullfrog size. Our results suggest that smaller host individuals that have lower helminth abundances may be more susceptible to pathogen infection, and vice-versa. This could be due to the indirect effects of a trade-off, or possibly, macroparasite infection can reduce microparasite replication rates through cross-reactive immunity (Johnson and Buller, 2011; Wuerthner et al., 2017). However, identifying coinfection patterns in nature is much more complex due to the multiple combinations of various parasite taxa, and further studies are needed for a better understanding of coinfection dynamics in the invasive bullfrogs in Brazil.

Our study presents a comprehensive assessment of both *Ranavirus* and helminth infections within invasive bullfrog populations across a significant range of the Brazilian Atlantic Forest. While our focus in this study was not on providing an exhaustive checklist detailing the precise taxonomy of the helminth parasites, our primary aim was to offer an initial glimpse into the extent of bullfrog infection across various locations. The limited sample size in certain locations also poses a constraint on the ability to extrapolate broader conclusions, yet it also reflects variations in bullfrog abundances in most sites. Furthermore, collecting bullfrogs beyond their native range presents considerable challenges due to the elusive nature of these animals, as they tend to be wary and conceal themselves in remote and inaccessible locations. Thus, although the lack of specific helminth identification and limited sample sizes in some sites constrain the depth of our analyses concerning certain associations, it serves as an essential foundational step in comprehending the broader implications of bullfrog infections. Our findings shed light on the prevalence and co-occurrence of pathogen and parasites, offering valuable insights into their potential interactions within this ecosystem, despite the current limitation in taxonomic detail. This first assessment acts as a crucial preliminary step towards unraveling the impacts of bullfrog infections, signifying the necessity for further detailed investigations in this area.

In summary, this is the first record of widespread *Ranavirus* infection in invasive adult bullfrogs in Brazil. These adult hosts have the potential to act as reservoirs of infection, and our data suggest potential interactions between the virus and macroparasites. Bullfrogs are now widespread invaders of the Atlantic Forest. Future studies should focus on the potential role of bullfrogs as a vector for disease and parasite transmission to native anurans, which are mostly endemic and facing numerous, compounding environmental threats (Anunciação et al., 2021; Vasconcelos et al., 2018).

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

The collection of animals was approved by the Brazilian legislation, with the license ICMBio 63620-2. Manipulation and euthanasia of the studied animals have been approved by The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), which certifies that the procedures using animals in the research project are in consonance with the international guidelines for animal experimentation.

Consent for publication

The work is all original research carried out by the authors. All authors agree with the contents of the manuscript and its submission to the journal. No part of the research has been published in any form elsewhere unless it is fully acknowledged in the manuscript.

Availability of data and materials

All data and analysis will be publicly available here:https://github.com/lvash/Brazil-Bullfrog-Coinfection

Funding

LVA received funding through the University of Vermont (UVM) Department of Biology's John Wheeler award and the UVM Graduate College's Roberto Fabri Fialho Award.

KMC acknowledges the Coordination for the Improvement of Higher Education Personnel and the Federal University of Paraná for the CAPES-PRINT fellowship, and the Council for Scientific and Technological Development (CNPq - proc. 306934/2022-1). Fieldwork was partially funded by the research support call April 2018 of/PRPPG/UFPR, and Fundação Araucária and CNPq in the call to support for young researchers CP 20/2018 PPP.

CPT received a master's scholarship from CAPES.

Acknowledgements

We thank all lab colleagues in UFPR that helped in the field. For laboratory assistance, we thank the undergraduates at UVM: Sarah Clarke, Abbi Smith, Adam Slamin, Raymond Looney, Maddy Degelsmith, Piotr Sowulewski, Conor Kiely, Hannah Lewis, Jessa Houghton, and TJ Mandelkorn.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.100924.

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