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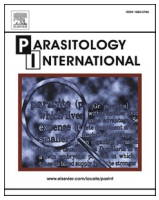

Short communication

High prevalence and concomitant infection of *Ranavirus* and *Eustrongylides sp.* in the invasive American Bullfrog in Brazil

Karla Magalhães Campião [a,*], Julia Anselmo da Luz Rico [a], Gabriel de Souza Monteiro [a], Lauren V. Ash [b], Cauê Pinheiro Teixeira [a], Nicholas J. Gotelli [b]

[a] *Biological interactions Lab, Department of Zoology Universidade Federal do Paraná, Curitiba, Brazil*
[b] *University of Vermont, Department of Biology, 109 Carrigan Drive, Burlington, VT 05403, USA*

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ABSTRACT

American Bullfrogs, *Aquarana catesbeiana,* are invasive anuran species distributed worldwide. One of the adverse impacts that this species causes in native communities is as a reservoir host for pathogens and parasites. Here, we report the coinfection of two pathogenic organisms in A. *catesbeiana*: *Ranavirus* and the nematode *Eustrongylides*. Bullfrogs were collected in the wild in a pond close to the urban area of São Paulo, Brazil. The prevalence of both pathogens was high: 77% were infected with ranavirus with a mean viral load of 1010.3 viral copies, and 100% of the bullfrogs were infected by *Eustrongylides* sp. with a mean intensity of infection of 13.4 specimens per host. Four host specimens (31%) presented pathological signs that seemed to be related to the *Eustrongylides* sp. infection, such as internal organs adhered to each other due to high intensity and large size of the nematodes, ulcers, and raw flesh wounds caused by the nematode. The pathogenic and concomitant infections have potential zoonotic implications and raise concerns about human infection risks for *Eustrongylides* infection. Moreover, such infections may represent an additional level of threat to native communities through the potential shifts in patterns of parasite and pathogen transmission. Future research involving the native anuran community is essential to ascertain whether invasive bullfrogs are attenuating or exacerbating diseases such as ranavirosis and eustrongylidiosis.

The American Bullfrog, *Aquarana catesbeiana*, naturally inhabits the eastern regions of the United States and Canada, yet it has been intentionally introduced to multiple countries for commercial purposes [1]. Notably, in Brazil, this species was brought in during the 1930s for farming dedicated to human consumption. Brazil's bullfrog production has flourished within captive environments, positioning Brazil among the world's prominent producers [2]. However, the abandonment of several farms in the early 1990s prompted bullfrog populations to escape into native ecosystems, and the species is now documented in at least 130 municipalities across Brazil [3]. Bullfrogs cause diverse negative impacts in native ecosystems, such as the spread of infectious diseases, due to their role as hosts for microparasites and macroparasites. In this paper, we concentrate on two common and potentially pathogenic parasites, Ranavirus and *Eustrongyloides* sp. [4–6].

Ranavirus are extensive double-stranded DNA viruses of the Iridoviridae family. These viral pathogens can infect reptiles, fish, and farmed and wild amphibians [7]. Ranavirus species are known to induce a variety of symptoms in amphibians, which encompass cutaneous ulcerations, edema, and internal hemorrhages. Additionally, infected amphibians exhibit signs of lethargy, reduced appetite, respiratory difficulties, and abnormalities in internal organs [7,8]. The transmission of ranavirus occurs through water and moist substrate contamination, direct contact with infected individuals, and ingestion of contaminated tissues, including necrophagy or cannibalism [8]. Ranavirus species can demonstrate a broad geographical distribution, encompassing South America, North America, Asia, Europe, and Oceania [8]. While studies on ranavirus have predominantly focused on North America and Europe, it is important to note that most research on ranavirus cases in amphibians also centers around species belonging to the Ranidae family [8]. Cases of infected bullfrog tadpoles have been documented globally, including two reports in Brazil [6,9], and the virus has been linked to significant mortality events in nature and on frog farms [7,9]. Adult bullfrogs are generally tolerant to the virus, resulting in low mortality [5,7]. However, frogs can still exhibit subclinical effects, and spread the

* Corresponding author.
E-mail address: karla.campiao@ufpr.br (K.M. Campião).

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virus [5].

Eustrongylides species are pathogenic nematodes that naturally occur in wildlife. These nematodes, characterized by being large and red, infect aquatic birds and fish-eating mammals [10,11]. Their life cycles involve multiple hosts and stages. Piscivorous birds are the definitive hosts, where the parasites mature, reproduce and release eggs that are excreted in the bird's feces into water bodies like lakes, rivers, or ponds. These eggs are ingested by the first intermediate host that is often a small aquatic invertebrate. Inside the invertebrate host, the eggs hatch, and the larvae develop into the second and third stage larvae, and the second intermediate host is usually a small fish or other vertebrate that consumes the infected invertebrates containing second-stage larvae. Once inside the second intermediate host, the larvae develop into fourth-stage larvae [10]. At this stage, the larvae of *Eustrongylides* species may cause pathogenic effects in the intermediate host and the infection is considered a zoonotic disease, mainly, in fish [11,12]. Infection in fish is a zoonotic problem because these parasites can be transmitted to humans through ingestion of raw or undercooked fish [11], as documented in cases where humans became infected after consuming fish infested with fourth-stage larvae [10–12]. In addition to fish, amphibians and reptiles have also been reported as intermediate or paratenic hosts [10,13,14]. Farmed bullfrogs have been reported infected by third-stage larvae of *Eustrongylides* in Mexico [4], as well as wild bullfrogs collected in California [15].

Here, we report for the first time the co-occurrence of these highly pathogenic parasites in a population of the invasive American Bullfrog in Brazil.

Thirteen specimens of bullfrogs were collected from the wild in São Paulo, Brazil (23°37′52.7″S 46°49′14.5″W). We checked bullfrogs for the presence of clinical pathological signs, such as organ lesions or hemorrhaging and redness. We then carried out the necropsy, we extracted ~1 g of liver tissue and stored it in 70% ethanol for later Ranavirus testing. We searched for macroparasite infection by examining all organs under a stereomicroscope. We fixed macroparasites in a 70% alcohol solution and observed the specimens in a temporary slide mounted with lactophenol for morphological identification. We extracted DNA from adult bullfrog liver samples and used quantitative PCR (qPCR) to amplify a portion of the major capsid protein. The qPCR reactions 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 run for the positive samples to quantify DNA and to normalize viral load (viral copies per ng DNA). In a subset of Ranavirus qPCR positive samples, we amplified a 498 bp portion of the major capsid protein using PCR protocols and primers. Prevalence was calculated as the proportion of infected individuals in the entire sample, and binomial confidence intervals for prevalence were calculated in R using the *binom.confint* function of the *binom* package. All procedures were approved by the Brazilian Institute of Environment and Natural Resources (63620–2) and the Ethics Committee of Universidade Federal do Paraná (23,075.008635/2019–03).

We found a 76.9% prevalence of ranavirus infection (estimate confidence intervals: 49.74% - 91.82%) and a 100% prevalence of infection by the nematode larvae of *Eustrongylides* sp. (estimate confidence intervals: 77.19% - 100%). Viral load was successfully evaluated for 6 specimens (Table 1), with an average of 1010.3 copies/ng (SD + - 614.7), and *Eustrongylides* sp mean intensity of infection was 13.4 (SD + - 14.6). *Eustrongylides* sp. larvae were found in the abdominal cavity and accessory organs, and all gastrointestinal tract. Four host specimens (30.7%) presented pathological signs that seemed to be related to the *Eustrongylides* sp. pathology, such as internal organs adhesion due to the high intensity and large size of the nematodes, ulcers, and raw flesh wounds caused by the nematode (Fig. 1) Three of these specimens were co-infected and all four had a high intensity of infection by *Eustrongylides* sp. (Table 1).

Table 1
Infection by ranavirus and *Eustongylides* sp. in invasive specimens of the American Bullfrog (A*quarana catesbeiana)* in Southeast Brazil. We present the status of ranavirus infection, viral load (viral copies/ng), number of *Eustongylides* specimens, and the occurrence of pathology for each bullfrog analyzed.

Host	Ranavirus infection	Viral load	N *Eustongylides* sp	Pathology
1	Positive	427	20	yes
2	Negative	0	11	yes
3	Positive	398	18	no
4	Positive	564	3	no
5	Negative	0	19	no
6	Positive	1408	7	yes
7	Positive	1486	20	yes
8	Positive	na	7	no
9	Positive	na	1	no
10	Positive	na	1	no
11	Negative	0	56	no
12	Positive	na	6	no
13	Positive	1779	5	no

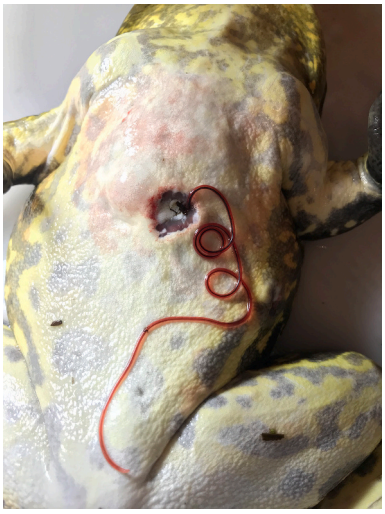

Fig. 1. Lesion caused by a larva of *Eustrongylides* sp. in a naturalized specimen of the invasive Bullfrog (*Aquarana catesbeiana*) in Brazil.

Bullfrogs are important drivers in the global spread of ranaviruses, especially through commercial trade [5,8]. These anurans can be frequently infected at moderate levels of the virus, and their responses to infection vary significantly. Certain individuals succumb to the infection, while others can effectively manage and actively eliminate infections. In contrast, some individuals can maintain subclinical infections for extended periods, acting as virus reservoirs without displaying signs of disease [5]. As disease carriers, bullfrogs can perpetuate ranavirus circulation within both captive and wild environments, potentially affecting native amphibian populations and overall ecosystem health. In our study, we observed pathological signs that can be attributed as result of ranavirus infection [7,8], as well as to *Eustrongylides* sp. [11,12].

The pathological condition induced by *Eustrongylides* species is commonly reported for both fish and birds, eliciting a host inflammatory response [10,11,16,17]. Although scarcely documented in amphibians, the pathogenicity of these parasites is linked to their larval stages, in which the larvae can infiltrate the tissues of anurans, establishing residence in sites such as muscles and internal organs. This infestation process can lead to visible tissue lesions as those observed in this study (Fig. 1), often presenting as nodules or protrusions on the skin and within muscles. These lesions can induce localized inflammation and are frequently accompanied by hemorrhagic events, characterized by red

discolorations in the affected tissues [4,10], multifocal peritonitis and multiple granulomas in the liver, compressive atrophy of host organs, and chronic inflammatory response [10]. The presence of these lesions and hemorrhages can provoke discomfort and impact the mobility of host species. Moreover, muscle damage can result in impaired movement, thereby reducing the organisms' ability to feed. This, in turn, compromises their overall health, rendering them more susceptible to secondary infections and diseases [4]. Infections can lead to alterations in behavior, increased susceptibility to predation, and a diminished ability to cope with stress in the intermediate/paratenic host. When parasites infect a host, it triggers a range of host responses aimed at neutralizing or expelling the intruders. These responses encompass both cellular and humoral reactions, which can span from acute or persistent inflammation to extensive tissue necrosis [12]. *Eustrongylides* sp. larvae can cause perforation of the internal organs [18], and ultimately hosts' death [19].

Eustrongylides larvae are acquired by bullfrogs through the consumption of the first or second intermediate host [10]. The invasive bullfrogs are recognized as voracious predators [18,19], which may have played a significant role in the elevated prevalence and intensity of *Eustrongylides* infection. Indeed, Lezama and Sarabia [2001] propose that bullfrogs could potentially contract these parasites by ingesting infected fish. The potential correlation between nematode-induced pathology and the presence of open wounds raises the possibility of increased susceptibility to ranavirus infection due to the higher viral copy release into the water [19]. Furthermore, the high intensity of *Eustrongylides* infection and associated larval pathology might compromise the host's immune response, rendering them more susceptible to ranavirus infection—an essential factor driving ranavirus propagation [19,20]. Simultaneously, the virus could reciprocally suppress the host's immune response, further exacerbating the pathological effects induced by the nematodes.

Frog farming is an economic activity established over a few decades, which has mainly encompassed the exportation of frog meat to other regions. Currently, Brazil holds a significant position as one of the world's major suppliers of bullfrog meat for consumption [2]. In parallel with the economic benefits, frog farming has raised concerns due to its potential environmental repercussions. Bullfrogs are susceptible to ranavirus which underscores the potential risk posed by amphibian translocation in the global distribution of pathogens through this activity [9]. Furthermore, within the scope of bullfrog commercialization, the lesions and hemorrhages induced by *Eustrongylides* sp. can carry significant economic implications. The existence of tissue lesions can substantially erode the commercial quality of these animals when designated for human consumption. The adverse visual aspect stemming from evident lesions has the potential to detrimentally influence the product's reception within the market [4]. Additionally, infections raise concerns about the plausible risks of human infection, considering the prevalent practice among inhabitants of neighboring local communities to hunt these amphibians for consumption in areas where bullfrogs have become naturalized [KMC personal observation]. Moreover, the Atlantic Forest harbors a remarkable diversity of amphibians, the majority of which are endemic, and many of them facing the threat of extinction. The presence of bullfrogs already imposes an additional level of threat to native communities [2], and the potential pathogens spillover poses an even greater risk. Future studies within the native anuran community are necessary to determine whether the invasive bullfrogs are diluting or amplifying diseases such as ranavirosis and eustrongylidiosis.

CRediT authorship contribution statement

Karla Magalhães Campião: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Julia Anselmo da Luz Rico:** Writing – review & editing, Writing – original draft, Data curation. **Gabriel de Souza Monteiro:** Writing – original draft, Data curation. **Lauren V. Ash:** Writing – review & editing, Writing – original draft, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cauê Pinheiro Teixeira:** Writing – original draft, Data curation, Conceptualization. **Nicholas J. Gotelli:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

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