Experimental *Frog Virus 3* infection using Brazilian strain: amphibians susceptibility

Infecção experimental utilizando estirpe brasileira de Frog Virus 3: suceptibilidade de anfíbios

Sthefany Rosa Alfaia[1] ; Marcelo Cândido[2] ; Ricardo Luiz Moro de Sousa[2] ; Ricardo Harakava[3];
Luara Lucena Cassiano[3]; Ana Maria Cristina Rebelo Pinto da Fonseca Martins[3]; Cláudia Maris Ferreira[1]

[1] Instituto de Pesca, Agência Paulista de Tecnologia dos Agronegócios, Secretaria de Agricultura e Abastecimento do Estado de São Paulo - SP, Brazil
[2] Universidade de São Paulo, Faculdade de Zootecnia e Engenharia de Alimentos, Pirassununga – SP, Brazil
[3] Instituto Biológico, Agência Paulista de Tecnologia dos Agronegócios, Secretaria de Agricultura e Abastecimento do Estado de São Paulo - SP, Brazil

ABSTRACT

An alarming number of global warnings concerning amphibian mortality outbreaks have been released in recent years. Emerging diseases stand out as the main potential causes. Ranavirus is a worldwide-spread highly infectious disease capable of affecting even other ectothermic animals such as fish and reptiles. One major issue regarding this pathology is the lack of clinical signs before it leads up to death. Aiming at having a better understanding of anurans susceptibility, this study analyzed bullfrog (*Lithobates catesbeianus*) survival rate, when challenged with three doses of a Brazilian strain of *Frog Virus 3* (FV3). The qPCR analysis indicated a low infectivity rate in these animals both as larvae and as adults. To elucidate the results, the following hypothesis was performed: 1) The amount of inoculum used on the frogs was insufficient to trigger an infection; 2) For the FV3 to produce clinical signs in this species, there is the need for a cofactor; 3) The animals did undergo FV3 infection but recovered in the course of the experiment, and 4) The inoculum utilized might have been low-virulence. Finally, the presence of actual clinical signs of ranavirus is discussed, with the more likely hypothesis.

Keywords: *Lithobates catesbeianus*. Ranaviruses. Ranavirus. Iridovirus. Emergent disease, Amphibians

RESUMO

Um número alarmante de notificações globais sobre surtos de mortalidade de anfíbios tem sido realizado nos últimos anos. As doenças emergentes destacam-se como as principais causas potenciais. O ranavírus é uma doença altamente infecciosa disseminada em todo o mundo, capaz de afetar até outros animais ectotérmicos como peixes e répteis. Uma questão importante em relação a essa patologia é a falta de sinais clínicos antes de levar à morte. Com o objetivo de compreender melhor a suscetibilidade dos anuros, o presente trabalho analisou a taxa de sobrevivência de rãs-touro (*Lithobates catesbeianus*), desafiadas com três doses de uma estirpe brasileira do *Frog virus 3* (FV3). A análise de qPCR indicou baixa taxa de infectividade nesses animais, tanto como larvas quanto como adultos. Procurando esclarecer os resultados, foram formuladas as seguintes hipóteses: 1) A quantidade de inóculo aplicada nas rãs foi insuficiente para desencadear uma infecção; 2) Para que o FV3 dê sinais clínicos nesta espécie, é necessário um cofator; 3) Os animais sofreram infecção por FV3, mas se recuperaram no decorrer do experimento, e 4) O inóculo utilizado pode ter sido de baixa virulência. Finalmente, foi discutida a presença de sinais clínicos reais de ranavírus e levantada a hipótese mais provável

Palavras-chave: *Lithobates catesbeianus*. Ranavirose. Ranavírus. Iridovírus. Doenças emergentes. Anfíbios.

Correspondence to:
Cláudia Maris Ferreira
Instituto de Pesca, Agência Paulista de Tecnologia dos
Agronegócios, Secretaria de Agricultura e Abastecimento do
Estado de São Paulo
Av. Francisco Matarazzo, 455
CEP: 05001-900, São Paulo – SP, Brazil
e-mail: cmferreira@sp.gov.br

Introduction

Over the last few decades, an increasing number of notifications has tackled the global decline of amphibian diversity. Potential causes may be associated with pollution, loss, and fragmentation of natural habitats, climatic changes, increasing UV-B radiation, the arrival of predators and exotic competitors, urbanization, and the advent of emerging diseases (Berger et al., 1999; Chinchar et al. 2011, 2017; Earl et al., 2016; Hamer & McDonnell, 2008; World Organisation for Animal Health, 2017). Two pathogens were predominantly analyzed as agents of emerging outbreaks: the *Batrachochytrium dendrobatidis* (Bd) fungus and ranaviruses. Both diseases caused by these particular agents are vastly spread and can affect a wide spectrum of hosts (Daszak et al., 2000; Duffus et al., 2015; Miller et al., 2011; Soto-Azat et al., 2016).

Ranavirus is currently classified as the second most common infectious agent in amphibians, both in the wild and in captivity (Grayfer et al., 2012). Belonging to the Iridoviridae family, it is characterized for being an icosahedral, large (120-300nm), double-stranded-DNA virus. As previously mentioned, ranavirus is highly infectious and usually affects both young and adult frogs. The Iridoviridae family is divided into five genera (Megalocytivirus, Lymphocystivirus, Chloridovirus, Ranavirus, and Iridovirus). However, only the Ranavirus genus, especially the *Frog virus 3* (FV3) species, has enough plasticity to infect amphibians, fish, and reptiles (Chinchar et al., 2011; Lesbarrères et al., 2012; Robert & Jancovich, 2016). Due to dissemination risks and the lack of control and cautionary measures, in 2012 the World Organization for Animal Health members declared the disease to require mandatory notification (World Organisation for Animal Health, 2017).

Bullfrogs (*Lithobates castebeianus*), formerly classified as *Rana castebeiana*, originate from North America. The species has been kept at high stocking densities for human consumption on the grounds of economic interests in quite a few of its traits, among which are size, fast growth, abundance, and the various nutritional qualities of its meat. The first records of bullfrogs found in Brazil date back to 1935, and breeding farm activities started in the 70s (Dias et al., 2010; Schloegel et al., 2010). Today, countries such as Brazil, China, Indonesia, Taiwan, and Mexico employ breeding methods inside intensive confinements, a practice which, although more profitable in terms of productivity, also contributes to the rise in diseases resulting from the high densities (Altherr et al., 2011; Freitas et al., 2017).

Previous studies identified FV3 strains in frog culture coming from various regions in Brazil (Alencar, 2016; Mazzoni et al., 2009; Neves et al., 2016; Oliveira et al., 2020). In time, the first isolated ranavirus genome in Brazil was recently published (Cândido et al., 2019). However, identifying the presence of Ranaviruses relying solely on visual observation proves to be a tough challenge, since, as far as clinical signs go, the only often observed signs are either characteristics also perceived in several other pathological processes or the ultimate death of the animal (Haislip et al., 2011; Miller et al., 2011). Ranaviruses, when apparent, manifest in the form of systemic infections featuring petechiae and ulcerations along the skin and limbs, ascites, weight loss, hemorrhage, and lethargy. Internal injuries are mainly found on the spleen, liver, kidney, and gastrointestinal wall (Hoverman et al., 2011; Mazzoni et al., 2009; Miller et al., 2011; Oliveira et al., 2020; Robert et al., 2005).

Few studies in South America have discussed the presence of the virus in aquaculture, its prevalence, variation possibility of the strains, and its deleterious effects on aquaculture, specifically on frog farming. To better understand anurans' susceptibility to this disease, this study analyzed bullfrog (*Lithobates catesbeianus*) survival rate, both in its larval and adult forms, when challenged with three doses of a Brazilian strain, as well as closely followed the evolution of a potential symptomatic clinical case.

Materials and Methods

Collection of samples

The animals were acquired from a commercial facility located in Pindamonhangaba city (22º 50' 31" S/45º 36' 31" W),

São Paulo, Brazil. The individuals had no history of previous diseases and approximately 20% of the livestock acquired (35 tadpoles and 25 frogs) were previously examined through molecular diagnosis (PCR) for FV3 (Oliveira et al., 2020). By the time the tadpoles were collected, they had grown up to 31-36 Gosner (1960) stages and weighed an average of 4.7g +1.2 (n=144), whereas the adult frogs weighed 60g + 10 (n=112) on average. In the laboratory, acclimatization and the experimentation were set up along with controlled photoperiod (12:12) and temperature (22 °C + 0,8), each stage lasting one week. The subjects were fed once a day with the same ration fed to the frog farm, (Laguna® 32% rough protein, 5% ethereal extract, 10% rough fiber, 2,5% calcium, 1% phosphorus, 14% ash, 21% carbohydrates, Vitamin C 300mg, rough energy 4180 Kcal/kg), the amount of feed being equivalent to 3% of the group's live weight both for tadpoles and adult frogs. The biomass proportion was accordingly adjusted in each stage.

Infection and experimental routine

Aliquots of an isolated sample were used for the experimental infection, a remnant from an outbreak that took over a commercial farm in 2012 in São Paulo, southeast Brazil (GenBank access MH351268) (Cândido et al., 2019). Viruses were isolated in BF-2 cells (bluegill fry ATCC® CCL-91®) that had been kept and sub cultivated in MEM (minimum essential medium - Gibco®, Life Technologies, USA), having received fetal bovine serum supplement 10% (SFB), L-Glutamine 1%, penicillin 100 UI/mL, streptomycin 100µg/mL (PenStrep - Gibco®, Life Technologies, USA) incubated at 25 °C and 5% CO_2 atmosphere. As for the virus, subsequent passages were executed, from which the sixth passage of cells was utilized in this study, whose total volume was 106.8 PFU/mL.

Under a deficit of studies on the examined species, we based in experiments of Forzan et al. (2015) when determining how much inoculum should be administered. Both adults (nº=112) and tadpoles (nº=144) were inoculated by oral route and the treatments (T) were divided into Control group (only 50 MEM µL), T1: 102.6 PFU/mL, T2: 104.6 PFU/mL, and T3: 106.8 PFU/mL, with each group bring replicated four times. The experiment was carried out for 21 days.

The inoculation protocol, as well as the sanitary and analysis protocols, followed the routine from smallest to largest viral presence to avoid cross-contamination. Tadpoles were kept in 13 L aquariums, while adult animals were placed in modified cages with wells whose deepest end held 4 L of water and the shallow end was 45 cm deep.

Two samplings were performed to determine what was likely an evolution of the disease and its effects, both consisting of internal organs (liver, spleen, and kidney). The first sampling, in which two random individuals from each replica (nº=32) were euthanized, was performed 14 days p.i. (post-inoculation). The second sampling, in which two animals were once again taken from each replica) and euthanized (nº=32d, was conducted 21 days p.i. In summary, a total of 64 samples were collected per experimental group (tadpoles and adults).

For the euthanasia, after all the animals had been anesthetized with ice, tadpoles were submitted to Eugenol protocols (7mL/L), while frogs were submitted to benzocaine hydrochloride (4g/L). Liver, spleen, and kidney samples were collected following the protocol recommended by World Organisation for Animal Health (2017) to verify the effectiveness of the infection. When switching between treatments, utensils, and materials utilized were sterilized with 70% alcohol and Virkon®, the counters cleansed and gloves changed. All procedures had previously been submitted to and approved by São Paulo/Brazil's Fisheries Institute (No.04/2016).

Analysis

Molecular analyses were conducted first. DNA extraction was performed with gene pools of the collected organs (spleen, liver, and kidney) following the protocol recommended by the Wizard SV Genomic DNA Purification System kit (Promega®, Brazil). As for reactions, the GoTaq Colorless Mastermix 2X kit (Promega®, USA) was used along with two primer pairs recommended by World Organisation for Animal Health (2017). The fragments were obtained from the conserved MCP (major capsid protein) gene and were denominated MCP1 (321 pb) (M151 - 5'-AACCCGGCTTTCGGGCAGCA-3' and M152 - 3'-CGGGGCGGGGTTGATGAGAT-5'), and MCP2 (625pb) (M153 - 5'- ATGACCGTCGCCCTCATCAC-3' and M154 - 3' CCATCGAGCCGTTCATGATG-5'). Analyses by enzymatic digestions also followed the World Organisation for Animal Health (2017) recommendations.

Real-time quantitative PCR protocol (qPCR) was used to determine the viral load in the infected animals for comparison purposes among the different doses administered in each treatment. Following the standard Allender et al. (2013) TaqMan-MGB (Invitrogen®, USA) system and primers, whose target is the major capsid protein (5'-AACGCCGACCGAAAACTG-3'), (3'-GCTGCCAAGATGTCGGGTAA-5'), as well as the probe(CCGGCTTTCGGGC), it was used TaqMan Platinum

PCR SuperMix-UDG 12.5μL with ROX 2X (Invitrogen®, USA), 1.25μL TaqMan probe, 2.5μL of tissue pool dilution and water to make for a final concentration of 25μL. The thermocycling protocol followed: 95° for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and a final extension of 72 °C for 10 min. The standard curves were obtained from the dilutions' threshold values by using positive control plasmid.

Samples that came back positive were sequenced to guarantee the veracity of the results. The PCR products utilized were purified using PEG 6000 and sequenced using the Sanger method. Bioedit was used to compare the obtained results to other sequences of its kind coming from GenBank.

Following standard protocol for histology, the samples were fixed in 10% formalin and dehydrated in ascending concentrations of ethanol followed by diaphanization with 3 xylol baths. After embedding the tissues in paraffin, the blocks were sliced into 5 μm pieces using an HRAZ M 55 Zeiss® microtome and hematoxylin-eosin stain. The viewing and capturing of images were performed in light microscopy (CARL-ZeissAxioScope.a1®) using ZEN® image capture software.

Results

There was no occurrence of mortality during the acclimation on tadpoles or intercurrences during the inoculation. A low mortality rate was observed among the inoculated specimens, indicating that none of the three doses used on the animals were lethal. However, a few clinical signs were detected that were divided, in general, if present in all treatments except control group, and specific, if present in only some particular individuals. General signs included loss of floatability and appetite, reduced movement, and lordosis on the tail. On the other hand, petechiae, abdominal swelling, weight loss, and epithelial desquamation were only observed in three specific specimens. By the end of 21 days, 75% of the animals had gone into metamorphosis, most of whose tails had already shrunk or disappeared. It was not possible to estimate the median lethal time of the infection after inoculation due to the elevated survival rate.

Conventional PCR analyses detected the presence of the virus in two animals (12.5%) in treatment 102.6 PFU/mL, the first one (g1) having been collected 14 days p.i. and the second one (g17) having been collected 21 days p.i. The qPCR analyses also detected positive samples (g1: 1.79×10^3 viral copies/ng DNA, and g17: 1.09×10^4 viral copies/ng DNA).

No morphologic alterations or other significant signs were found in the collected organs (liver, spleen, and kidney). In all treatments, the histological analyses verified the presence of melanomacrophages in the liver, as well as rarefaction of hepatocytes on a large scale, the control group included. This was attributed to a mineral protein deficiency in the ration fed to the animals back in the breeding farm (Seixas et al., 2017).

PCR detected some positive tadpole samples in which the kidney glands had damaged cellular organization, leading to glomerular hypoplasia and, consequently, to an increase in Bowman's capsular space, tubulo-nephrosis, dystrophic calcification in some areas, and presence of hyaline material in the tubular light. A significant presence of eosinophils was also noticed in the kidney parenchyma (Figure 1). Lastly, signs of monolymphatic and eosinophilic hepatitis were found in the liver and inclusion corpuscles in the cytoplasmic region of hepatic cells.
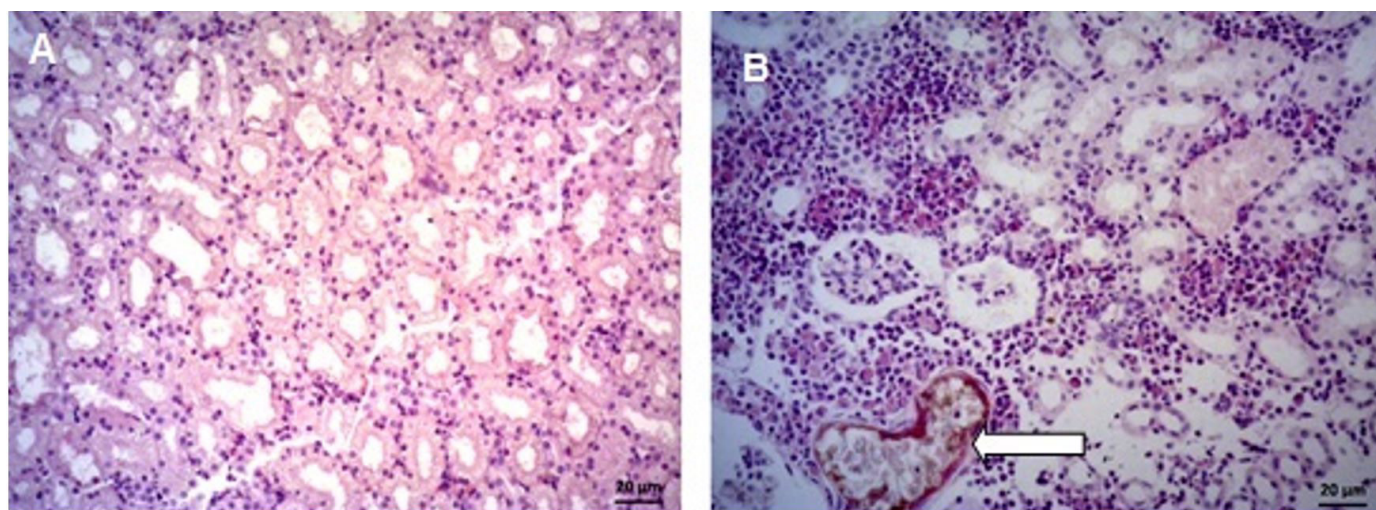

Figure 1 – Adult bullfrog kidneys (*Lithobates catesbeianus*) comparative photomicrograph. (A) Control group without the presence of the virus, 200 x; (B) Variation in the kidney architecture resulting from an increase in Bowman's capsular space along with glomerular hypoplasia, dystrophic calcification zone (white arrow), and sheets of eosinophils, 200 x. H&E stain.

Figure 2 – Adult bullfrogs (*Lithobates catesbeianus*) experimentally infected with Brazilian strain Frog virus 3. (A) Comparison between two individuals submitted to the same treatment (104.6 PFU/mL) showing signs of weight loss; (B) In the same treatment alteration in head position was detected along with alterations in both posture and swimming ability, resulting in circle swimming and loss of leaping capacity.
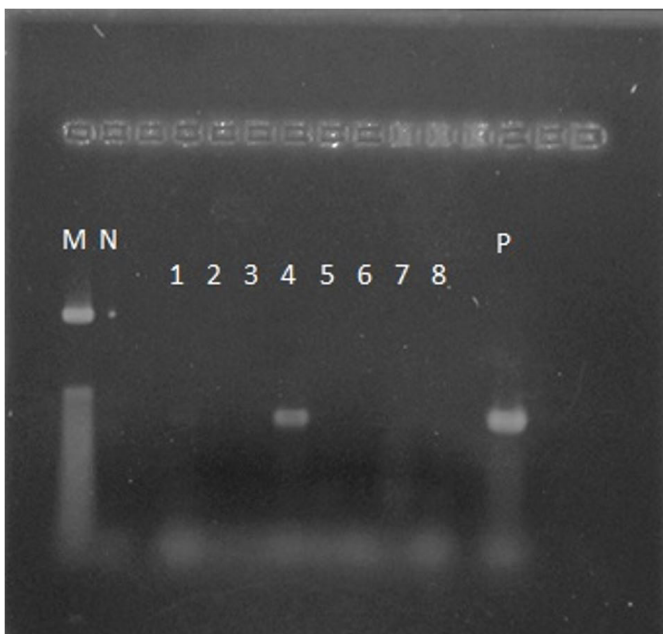

Figure 3 – Photomicrograph of a 1.0% agarose gel stained with SYBR® Gold, observed in ultraviolet light (UV), illustrating the results of the collection of adult *Lithobates catesbeianus* infected with FV3 performed 14 p.i. (being individuals submitted to treatment 1 (102.6 PFU/mL). Out of the eight specimens examined, individual 4 was the only positive one. The photograph shows the molecular-weight marker (M), negative control (N), analyzed samples (1-8), and positive control (P).

A few deaths occurred among the adult animals throughout the first days of acclimation, which led us to have them examined not only for FV3 (the result was negative) but also for bacteria, since the only bacterial presence found in the examined samples was that of Streptococcus sp. In terms of infection, the same pattern observed on the larvae appeared to apply to the adults, meaning the doses proved not to be lethal, culminating in a mortality rate no higher than 6.25% among the animals in experimentation. Differently from tadpoles, there was no occurrence of general signs in frogs. The observed signs were attributed to specific individuals: apathy, loss of appetite, edema, redness in the ventral region, loss of posture and swimming ability, and alteration in head position (Figure 2). Signs appeared eight days p.i., since the evolution of both losses of posture and alteration in head position happened at an impressively fast pace, becoming more aggravated each day.

The majority of the animals ate regularly regardless of being infected. Weight loss was only noticed in specific individuals, which was frequently associated with loss of posture, swimming ability, or alteration in head position.

On the other hand, the infectivity rate verified through conventional PCR was as low as 1.56%, occurring in one of the individuals from treatment 1 (Figure 3). The qPCR also detected positive results, indicating 4.13 x 103 viral copies/ng DNA.

Similar histological alterations were observed in tadpoles and adults: monolymphatic hepatitis with the presence of melanomacrophages in the liver, the control group included. Rarefaction of hepatocytes was observed, possibly as a result of poor nutritional quality of the ration formerly fed to the animals, which culminated in mineral protein deficiency. While bullfrogs feed off invertebrates and small vertebrates in the wild, at breeding farms these

animals are fed high rough-protein-rated rations (about 40% PB). Some studies suggest such a diet may lead to tissue damage and negatively affect animal performance. (Seixas et al., 2017). Although there was a decrease in the presence of eosinophils in the kidney parenchyma of PCR-positive adults as compared to the tadpoles, we found glomerular alterations, the presence of hyaline material inside the tubules, alteration in Bowman's capsule walls, and tubular-nephrosis in some areas (Figure 4).

It was not possible to establish correlations between the administered viral load and the viral load detected in the infected animals, given that very few positive results were found. It is necessary to seek information concerning the actual viral load to which these animals are exposed in real conditions, either in the wild or breeding environments, to recreate an outbreak setting under laboratory conditions.

Sequencing indicates with 99.9% accuracy that the samples belonged to the Ranavirus genus, frog virus 3 species.

Discussion

This study aimed at analyzing bullfrog survival rate when challenged with a Brazilian strain of FV3 through experimental infection, despite species such as *Xenopus laevis* and *Rana sylvaticus* being classified as experimental models on account of their higher susceptibility to ranaviruses infection (Earl & Gray, 2014; Forzán et al., 2017; Grayfer et al., 2012; Robert et al., 2007). The *L. catesbeianus* species require special care given its distinct profile, as it is both inserted in breeding environments and geographically spread in various communities as an exotic animal, therefore remaining vulnerable to constant relocation, either as breeding sources or pets (Schloegel et al., 2010, 2012). High bullfrog mortality outbreaks caused by FV3 have been reported both in the wild and in commercial environments (Landsberg et al., 2013; Majji et al., 2006; Miller et al., 2007; Oliveira et al., 2020). Some authors argue that captivity can potentialize the virulence of FV3 (Hoverman et al., 2011; Majji et al., 2006).
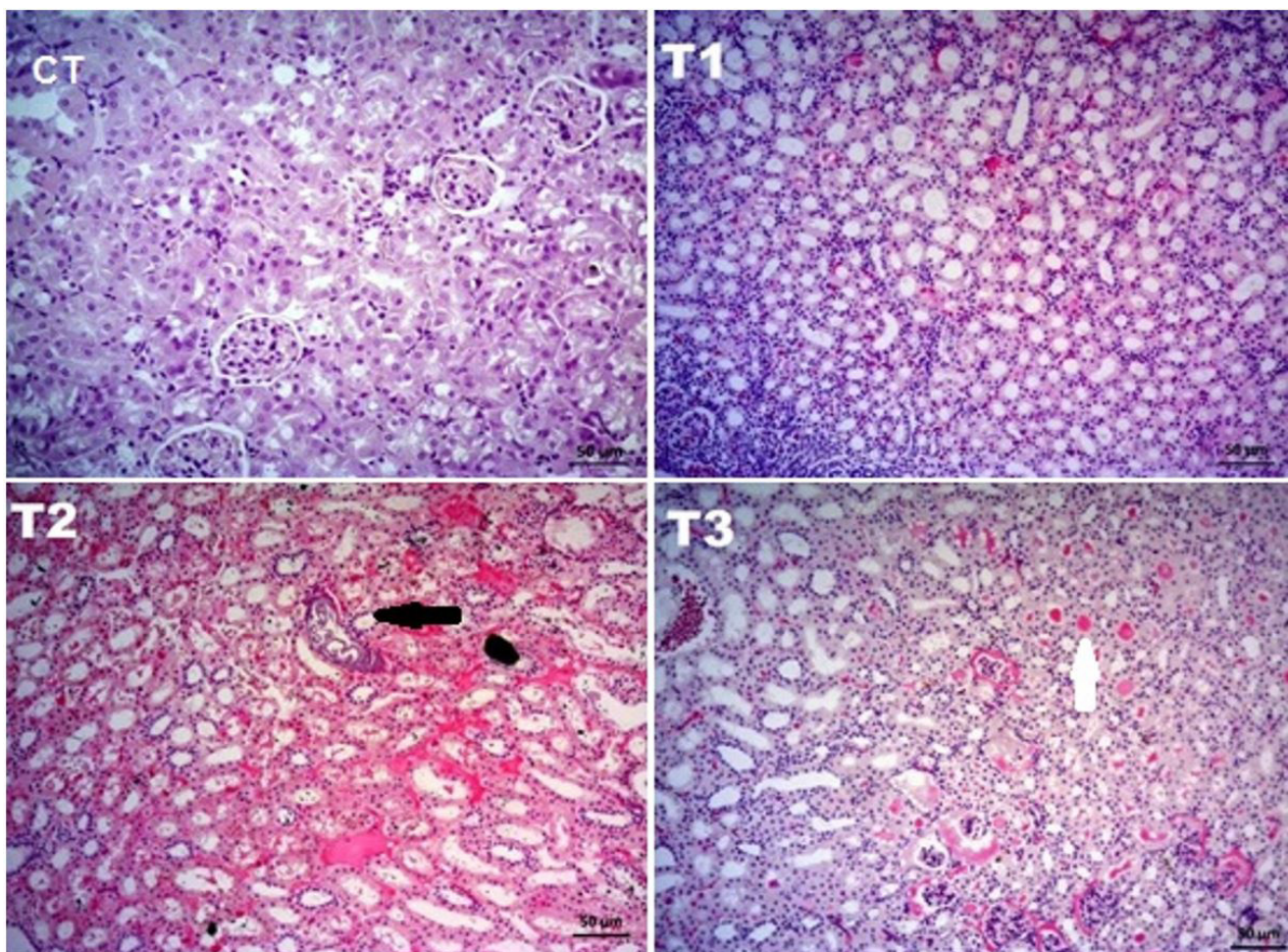

Figure 4 – Comparative photomicrograph of an adult bullfrog kidney (*Lithobates catesbeianus*) experimentally infected with Frog virus 3 and its monitoring (CT). The picture compares all four treatments, demonstrating a variation in cell architecture as the viral concentration increased. Calcification zones were present in Treatment 2 (104.6 PFU/mL) (black arrow), while a huge amount of hyaline material (white arrow) can be seen inside the tubules in treatment 3 (106.8 PFU/mL). 200 x. H&E stain. T1 – 102.6 PFU/mL; T2 -104.6 PFU/mL; T3 - 106.8 PFU/mL.

The majority of reported FV3 mortality outbreaks indicated that larvae and juvenile forms have a higher susceptibility to the virus (i.e. tadpoles and pre-metamorphosis) (Forzán & Wood, 2013; Galli et al., 2006; Jesús Andino et al., 2016; Miller et al., 2011; Warne et al., 2011). Therefore, it is reasonable to presume such susceptibility could be directly associated with two factors: first, energy expenditure, stress and hormonal alterations resulting from metamorphosis, especially due to an increase in corticosteroids, which may lead to suppression of the immune system (Gervasi & Foufopoulos, 2008), and second, the amphibian immune system itself (Rollins-Smith, 1998). Juvenile forms are less receptive to the virus. However, their immune system has an inferior amount of recognition mechanisms as well as a deficit in the major histocompatibility complex molecules (MHC) expression, as they have yet to go through metamorphosis. As a consequence, antibody responses in juveniles is rather less diverse, as opposed to adult individuals (Du Pasquier, 2001; Morales et al., 2010; Rollins-Smith, 2017). Adults, on the other hand, have a more competent immune response due to their innate (macrophages and neutrophils) and adaptive (CD8T cells and antibodies) immune system (Chinchar et al., 2004; Morales & Robert, 2007; Warne et al., 2011).

In the present investigation, no differences were observed regarding the infection rate between bullfrog tadpoles and adults. Although there were a few cases of animals infected with FV3, the survival rate observed throughout the experiment was high. As a consequence, it was not possible to evaluate the disease evolution and a potential clinical case to the desired extent. However, we did observe the presence of clinical signs historically associated by various authors to ranaviruses (Cunningham et al., 1996; Mazzoni et al., 2009; Miaud et al., 2016; Miller et al., 2011). Chen & Robert (2011) highlight the importance of cautious observation when looking for ranavirus clinical signs. These may or may not manifest when the animal is suffering from the disease, as well as may not always necessarily result in the animal's death, being only "transitory" at times. A suggested approach to classify ranavirus signs, according to some authors (Forzán et al., 2017; Majji et al., 2006; Miller et al., 2009), is categorizing them into "mild" (non-lethal) and "severe" (lethal). In the current study, "mild" clinical signs (lethargy, appetite reduction, and floatability alterations) were observed in most treatments featured in the experiment, unlike "severe" signs (erythema, hemorrhage, petechiae, and edema), which were only observed in a few individuals. However, considering the small number of infected animals 21 days p.i., none of the observed signs could be associated exclusively with ranavirus, because they are common to various *L. catesbeianus* pathologies, as stated by Densmore & Green (2007).

To explain the obtained results, we formulated the following hypothesis:

1) The amount of inoculum used on the frogs was insufficient to trigger an infection. As stated before, due to the lack of studies involving experimental FV3 infection in *L. castebeianus*, there is no knowledge about the amount of inoculum to be administered in the animals for reproducing the disease in experimental conditions. The conditions applied were following experiments performed by Majji et al. (2006) and Forzán et al. (2015) and when the experiment started, it was assumed that the chosen amount of inoculum would be enough to promote the disease. Even knowing that bullfrogs are stricken by ranaviruses, as reported by Galli et al. (2006); Mazzoni et al. (2009); Alencar (2016); Neves et al. (2016) and Oliveira et al. (2020), and that ranaviruses viral virulence is even higher in captivity (Chinchar et al., 2017; Claytor et al., 2017), the amount of inoculum administered must be species-specific. It is important to highlight that different population of the same species respond differently to offending agents. Ideally, we would conduct in vivo tests on "healthy disease-free" *L. castebeianus* populations. Unfortunately, there are still no "healthy and disease-free" populations in *L. catesbeianus*;

2) A cofactor is necessary for FV3 to produce clinical signs in this species since a virus strain alone is not responsible for triggering an infection. Much like other diseases, ranavirus virulence is connected to a variety of elements, among which are environmental causes, stressors, dose, temperature, intrinsic factors, host history, and susceptibility of the species (Altizer et al., 2013; Brand et al., 2016; Brunner et al., 2005; Echaubard et al., 2010). The current study was carried out in controlled environments where an attempt was made to minimize some of the external factors mentioned above. No significant water temperature variations were verified and certain measures were adopted to minimize stress inflicted upon the animal (Teixeira et al., 2015), such as calm environments, appropriate density, and minimum necessary manipulation. According to Echaubard et al. (2010), in a setting where there are environmental alterations, the host and its genotype become the main point of analysis. So it must be reinforced that temperature stress is important and a mandatory factor in many outbreaks involving bullfrogs in Brazilian frog farms (Brand et al., 2016; Mazzoni et al., 2009; Neves et al., 2016; Oliveira et al., 2020);

3) The animals did undergo FV3 infection but recovered in the course of the experiment. It is known that animals can live normally while hosting pathogenic agents as long as these don't multiply over the threshold. Only then will they become pathogenic by the excessive amount of pathogens (i.e., quantity). The manifestation of symptomatic or asymptomatic diseases may be determined by the viral integrity of the virus and/or host barriers (Ariel & Jensen, 2009; Brunner et al., 2005; Lancaster & Pfeiffer, 2012; Miller et al., 2009). Asymptomatic manifestations, silently persistent, are a problematic issue, making the host a strong spreading source (Jesús Andino et al., 2016; Miller et al., 2011). Therefore, considering that bullfrogs have a low susceptibility to FV3 together with the animal well-being ideal conditions under which the experiments were conducted, it is reasonable to suggest most animals did get infected with FV3 but managed to recover, preventing the disease manifestation;

4) The inoculum utilized might have been low-virulence. Since dosage and lineage virulence are both key elements for triggering outbreaks, they must be taken into consideration. This study used the sixth passage of cells taken from an isolated FV3 sample coming from a frog farm in southeast Brazil. Several studies

have found that performing multiple cell passages in cellular cultivation may result in virulence attenuation (Badgett et al., 2002; Day & Proulx, 2004), since repetitive cell passaging might trigger off mutations or virulence-reducing mechanisms, such as limitations in how effectively the disease would take over the host; a decrease in vivo replication rate; alteration in the plasmatic membrane of the host cells (structure and properties), which make the membrane vulnerable to penetration, besides deletion of viral genes (Brunner et al., 2005; Lee & Lobigs, 2002; Majji et al., 2006). Studies have demonstrated that the dissemination of the virus to new regions is mostly due to the importation and exportation of bullfrogs (Schloegel et al., 2010). Brazil holds a rich diversity of amphibians spread out in various ecosystems (Sasso et al., 2017; Vences & Köhler, 2008). However, a few ranavirus outbreaks have been reported around the country, all associated with the L. castebeianus species (Galli et al., 2006; Mazzoni et al., 2009; Neves et al., 2016; Oliveira et al., 2020). Up to the present, there have been no records of free-living and native species mortality caused by ranavirus. As much as such statistics may be a consequence of the incipiency of studies on the subject, it might also be a result of a genotypic variation spreading in the country, as once verified with the *Batrachochytrium dendrobatidis* fungus (Bd) (Schloegel et al., 2012). Studies have proven the existence of a vast diversity of lineages around the country, Bd-Brazil, and Bd-GLP, and indicated variations in virulence, as well as the adaptation of some native species to one of these lineages (Becker et al., 2017; Carvalho et al., 2017). Following this line of thought, it is reasonable to assume the same may be happening to the FV3 clade spread around the country (Oliveira et al., 2020), where strains presenting distinct virulence circulate through the territory.

Finally, the obtained conclusion was that a large spectrum of factors may influence the manifestations of amphibians infected by ranaviruses both in terms of form and intensity and that species-specific susceptibility consists of only one of such factors.

Conflict of Interest

The authors declare they have no conflicts of interest.

Ethics Statement

The authors declare that the contribution is original and unpublished, the referred manuscript is not being evaluated for publication in another journal, and that the text does not fit in the situations described in editorial policy on plagiarism.

References

Alencar ALF. Isolamento e caracterização de estirpe de *Frog virus 3*-símile detectada em rãs-touro gigante (*Lithobates catesbeianus*) no Estado de São Paulo. Brazil [dissertation]. São Paulo: Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo; 2016. http://dx.doi.org/10.11606/D.74.2016.tde-15082016-141111.

Allender MC, Bunick D, Mitchell MA. Development and validation of TaqMan quantitative PCR for detection of frog virus 3-like virus in eastern box turtles (*Terrapene carolina carolina*). J Virol Methods. 2013;188(1-2):121-5. http://dx.doi.org/10.1016/j.jviromet.2012.12.012. PMid:23274753.

Altherr S, Goyenechea A, Schubert DJ. Canapés to extinction. In: The International Trade in Frog's Legs and its Ecological Impact, editor. A report by Pro Wildlife, Defenders of Wildlife and Animal Welfare Institute. Munich: Pro Wildlife; 2011.

Altizer S, Ostfeld RS, Johnson PTJ, Kutz S, Harvell CD. Climate change and infectious diseases: from evidence to a predictive framework. Science. 2013;341(6145):514-9. http://dx.doi.org/10.1126/science.1239401. PMid:23908230.

Ariel E, Jensen BB. Challenge studies of European stocks of redfin perch, *Perca fluviatilis* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), with epizootic haematopoietic necrosis virus. J Fish Dis. 2009;32(12):1017-25. http://dx.doi.org/10.1111/j.1365-2761.2009.01088.x. PMid:19702625.

Badgett MR, Auer A, Carmichael LE, Parrish CR, Bull JJ. Evolutionary dynamics of viral attenuation evolutionary dynamics of viral attenuation. Science. 2002;76(20):10524-9. PMid:12239331.

Becker CG, Greenspan SE, Tracy KE, Dash JA, Lambertini C, Jenkinson TS, Leite DS, Toledo LF, Longcore JE, James TY, Zamudio KR. Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. Fungal Ecol. 2017;26:45-50. http://dx.doi.org/10.1016/j.funeco.2016.11.007.

Berger L, Speare R, Hyatt AD. Chytrid fungi and amphibian declines: overview, implications and future directions. In: Campbell A, editor. Declines and disappearances of Australian frogs. Canberra: Environment Australia; 1999. p. 23-33.

Brand MD, Hill RD, Brenes R, Chaney JC, Wilkes RP, Grayfer L, Miller DL, Gray MJ. Water temperature affects susceptibility to Ranavirus. EcoHealth. 2016;13(2):350-9. http://dx.doi.org/10.1007/s10393-016-1120-1. PMid:27283058.

Brunner JL, Richards K, Collins JP. Dose and host characteristics influence virulence of ranavirus infections. Oecologia. 2005;144(3):399-406. http://dx.doi.org/10.1007/s00442-005-0093-5. PMid:15891818.

Candido M, Tavares L, Alencar A, Ferreira CM, Queiroz S, Fernandes A, Sousa RLM. Genome analysis of *Ranavirus frog virus 3* isolated from American Bullfrog (*Lithobates catesbeianus*) in South America. Sci Rep. 2019;9(1):17135. http://dx.doi.org/10.1038/s41598-019-53626-z. PMid:31748669.

Carvalho T, Becker C, Toledo LF. Historical amphibian declines and extinctions in Brazil linked to chytridiomycosis. Proc Biol Sci. 2017;284(1848):20162254. http://dx.doi.org/10.1098/rspb.2016.2254. PMid:28179514.

Chen G, Robert J. Antiviral immunity in amphibians. Viruses. 2011;3(11):2065-86. http://dx.doi.org/10.3390/v3112065. PMid:22163335.

Chinchar VG, Bryan L, Silphadaung U, Noga E, Wade D, Rollins-Smith L. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. Virology. 2004;323(2):268-75. http://dx.doi.org/10.1016/j.virol.2004.02.029. PMid:15193922.

Chinchar VG, Waltzek TB, Subramaniam K. Ranaviruses and other members of the family Iridoviridae: their place in the virosphere. Virology. 2017;511:259-71. http://dx.doi.org/10.1016/j.virol.2017.06.007. PMid:28648249.

Chinchar VG, Yu KH, Jancovich JK. The molecular biology of Frog virus 3 and other iridoviruses infecting cold-blooded vertebrates. Viruses. 2011;3(10):1959-85. http://dx.doi.org/10.3390/v3101959. PMid:22069524.

Claytor SC, Subramaniam K, Landrau-Giovannetti N, Chinchar VG, Gray MJ, Miller DL, Mavian C, Salemi M, Wisely S, Waltzek TB. Ranavirus phylogenomics: signatures of recombination and inversions among bullfrog ranaculture isolates. Virology. 2017;511:330-43. http://dx.doi.org/10.1016/j.virol.2017.07.028. PMid:28803676.

Cunningham AA, Langton TE, Bennett PM, Lewin JF, Drury SE, Gough RE, MacGregor SK. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philos Trans R Soc Lond B Biol Sci. 1996;351(1347):1539-57. http://dx.doi.org/10.1098/rstb.1996.0140. PMid:8962441.

Daszak P, Cunningham AA, Hyatt AD. Emerging infectious diseases of wildlife: threats to biodiversity and human health. Science. 2000;287(5452):443-9. http://dx.doi.org/10.1126/science.287.5452.443. PMid:10642539.

Day T, Proulx S. A general theory for the evolutionary dynamics of virulence. Am Nat. 2004;163(4):E40-63. http://dx.doi.org/10.1086/382548. PMid:15122509.

Densmore CL, Green DE. Diseases of amphibians. ILAR J. 2007;48(3):235-54. http://dx.doi.org/10.1093/ilar.48.3.235. PMid:17592186.

Dias DC, Stéfani MV, Ferreira CM, França FM, Ranzani-Paiva MJT, Santos AA. Haematologic and immunologic parameters of bullfrogs, *Lithobates catesbeianus*, fed probiotics. Aquacult Res. 2010;41:1064-71. http://dx.doi.org/10.1111/j.1365-2109.2009.02390.x.

Du Pasquier L. The immune system of invertebrates and vertebrates. Comp Biochem Physiol B Biochem Mol Biol. 2001;129(1):1-15. http://dx.doi.org/10.1016/S1096-4959(01)00306-2. PMid:11337247.

Duffus ALJ, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, Hick P, Hines MK. Marschang RE Distribution and host range of Ranaviruses. In: Gray MJ, Chinchar VG, editors. Ranaviruses. Cham: Springer International Publishing; 2015. p. 9-57.

Earl JE, Chaney JC, Sutton WB, Lillard CE, Kouba AJ, Langhorne C, Krebs J, Wilkes RP, Hill RD, Miller DL, Gray MJ. Ranavirus could facilitate local extinction of rare amphibian species. Oecologia. 2016;182(2):611-23. http://dx.doi.org/10.1007/s00442-016-3682-6. PMid:27344151.

Earl JE, Gray MJ. Introduction of Ranavirus to isolated wood frog populations could cause local extinction. EcoHealth. 2014;11(4):581-92. http://dx.doi.org/10.1007/s10393-014-0950-y. PMid:24962849.

Echaubard P, Little K, Pauli B, Lesbarréres D. Context-dependent effects of ranaviral infection on northern leopard frog life history traits. PLoS One. 2010;5(10):e13723. http://dx.doi.org/10.1371/journal.pone.0013723. PMid:21060894.

Forzán MJ, Jones KM, Ariel E, Whittington RJ, Wood J, Markham RJF, Daoust PY. Pathogenesis of *Frog virus 3* (*Ranavirus, Iridoviridae*) Infection in Wood Frogs (*Rana sylvatica*). Vet Pathol. 2017;54(3):531-48. http://dx.doi.org/10.1177/0300985816684929. PMid:28060677.

Forzán MJ, Jones KM, Vanderstichel RV, Wood J, Kibenge FSB, Kuiken T, Wirth W, Ariel E, Daoust PY. Clinical signs, pathology and dose-dependent survival of adult wood frogs, *Rana sylvatica*, inoculated orally with *Frog virus 3* (Ranavirus sp., iridoviridae). J Gen Virol. 2015;96(5):1138-49. http://dx.doi.org/10.1099/vir.0.000043. PMid:25593158.

Forzán MJ, Wood J. Low detection of ranavirus DNA in wild post metamorphic green frogs, *Rana* (*Lithobates*) *clamitans*, despite previous or concurrent tadpole mortality. J Wildl

Dis. 2013;49(4):879-86. http://dx.doi.org/10.7589/2013-03-051. PMid:24502715.

Freitas JJG, Viau P, Oliviera CA, Teixeira PC, Tachibana L, Dias DC, Hipolito M, Bordon ICC, Alfaia SR, Ferreira CM. Effect of dietary supplements in American bullfrogs reared in low and high stocking densities. Aquacult Rep. 2017;8:45-8. http://dx.doi.org/10.1016/j.aqrep.2017.09.003.

Galli L, Pereira A, Márquez A, Mazzoni R. Ranavirus detection by PCR in cultured tadpoles (*Rana catesbeiana* Shaw, 1802) from South America. Aquaculture. 2006;257(1-4):78-82. http://dx.doi.org/10.1016/j.aquaculture.2005.06.019.

Gervasi SS, Foufopoulos J. Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. Funct Ecol. 2008;22:100-8. http://dx.doi.org/10.1111/j.1365-2435.2007.01340.x.

Gosner KL. A simplified table for standing anuran embryos and larvae with notes on identification. Herpetologica. 1960;16(3):183-90.

Grayfer L, Andino FDJ, Chen G, Chinchar GV, Robert J. Immune evasion strategies of ranaviruses and innate immune responses to these emerging pathogens. Viruses. 2012;4(7):1075-92. http://dx.doi.org/10.3390/v4071075. PMid:22852041.

Haislip NA, Gray MJ, Hoverman JT, Miller DL. Development and disease: how susceptibility to an emerging pathogen changes through anuran development. PLoS One. 2011;6(7):e22307. http://dx.doi.org/10.1371/journal.pone.0022307. PMid:21799820.

Hamer AJ, McDonnell MJ. Amphibian ecology and conservation in the urbanizing world: a review. Biol Conserv. 2008;141(10):2432-49. http://dx.doi.org/10.1016/j.biocon.2008.07.020.

Hoverman JT, Gray MJ, Haislip NA, Miller DL. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth. 2011;8(3):301-19. http://dx.doi.org/10.1007/s10393-011-0717-7. PMid:22071720.

Jesús Andino F, Jones L, Maggirwar SB, Robert J. Frog virus 3 dissemination in the brain of tadpoles, but not in adult Xenopus, involves blood brain barrier dysfunction. Sci Rep. 2016;6(1):22508. http://dx.doi.org/10.1038/srep22508. PMid:26931458.

Lancaster KZ, Pfeiffer JK. Viral population dynamics and virulence thresholds. Curr Opin Microbiol. 2012;15(4):525-30. http://dx.doi.org/10.1016/j.mib.2012.05.007. PMid:22658738.

Landsberg JH, Kiryu Y, Tabuchi M, Waltzek TB, Enge KM, Reintjes-Tolen S, Preston A, Pessier AP. Co-infection by alveolate parasites and Frog virus 3-like ranavirus during an amphibian larval mortality event in Florida, USA. Dis Aquat Organ. 2013;105(2):89-99. http://dx.doi.org/10.3354/dao02625. PMid:23872853.

Lee E, Lobigs M. Mechanism of virulence attenuation of glycosaminoglycan-binding variants of japanese encephalitis virus and murray valley encephalitis virus. J Virol. 2002;76(10):4901-11. http://dx.doi.org/10.1128/JVI.76.10.4901-4911.2002. PMid:11967307.

Lesbarrères D, Balseiro A, Brunner J, Chinchar VG, Duffus A, Kerby J, Miller DL, Robert J, Schock DM, Waltzek T, Gray MJ. Ranavirus: past, present and future. Biol Lett. 2012;8(4):481-3. http://dx.doi.org/10.1098/rsbl.2011.0951. PMid:22048891.

Majji S, LaPatra S, Long SM, Sample R, Bryan L, Sinning A, Chinchar VG. *Rana catesbeiana* virus Z (RCV-Z): a novel pathogenic ranavirus. Dis Aquat Organ. 2006;73(1):1-11. http://dx.doi.org/10.3354/dao073001. PMid:17240747.

Mazzoni R, Mesquita AJ, Fleury LFF, Brito WME, Nunes IA, Robert J, Morales H, Coelho ASG, Barthasson DL, Galli L, Catroxo MHB. Mass mortality associated with a Frog virus 3-like Ranavirus infection in farmed tadpoles Rana catesbeiana from Brazil. Dis Aquat Organ. 2009;86(3):181-91. http://dx.doi.org/10.3354/dao02096. PMid:20066953.

Miaud C, Pozet F, Gaudin NCG, Martel A, Pasmans F, Labrut S. Ranavirus causes mass die-offs of alpine amphibians in the southwestern Alps, France. J Wildl Dis. 2016;52(2):242-52. http://dx.doi.org/10.7589/2015-05-113. PMid:26967128.

Miller D, Gray M, Storfer A. Ecopathology of ranaviruses infecting amphibians. Viruses. 2011;3(11):2351-73. http://dx.doi.org/10.3390/v3112351. PMid:22163349.

Miller DL, Gray MJ, Rajeev S, Schmutzer AC, Burton EC, Merrill A, Baldwin CA. Pathologic findings in larval and juvenile anurans inhabiting farm ponds in Tennessee, USA. J Wildl Dis. 2009;45(2):314-24. http://dx.doi.org/10.7589/0090-3558-45.2.314. PMid:19395741.

Miller DL, Rajeev S, Gray MJ, Baldwin CA. *Frog virus 3* infection, cultured American bullfrogs. Emerg Infect Dis. 2007;13(2):342-3. http://dx.doi.org/10.3201/eid1302.061073. PMid:17479910.

Morales HD, Abramowitz L, Gertz J, Sowa J, Vogel A, Robert J. Innate immune responses and permissiveness to ranavirus infection of peritoneal leukocytes in the frog *Xenopus laevis.* J Virol. 2010;84(10):4912-22. http://dx.doi.org/10.1128/JVI.02486-09. PMid:20200236.

Morales HD, Robert J. Characterization of primary and memory CD8 T-cell responses against ranavirus (FV3) in *Xenopus laevis.* J Virol. 2007;81(5):2240-8. http://dx.doi.org/10.1128/JVI.01104-06. PMid:17182687.

Neves PV, Catroxo MH, Hipolito M, Oliveira CR, Ferreira CM. Uso de anticorpos policlonais, microscopia eletrônica e histologia para detecção de partículas semelhantes ao iridovírus em rãs-touro. Bol Inst Pesca. 2016;42(3):523-31. http://dx.doi.org/10.20950/1678-2305.2016v42n3p523.

Oliveira CR, Alfaia SR, Ikari FL, Tavares LS, Sousa RLM, Harakava R, Ferreira CM. Detection and molecular characterization of *Frog virus 3* in bullfrogs from frog farms in Brazil. Aquaculture. 2020;516:734575. http://dx.doi.org/10.1016/j.aquaculture.2019.734575.

Robert J, Abramowitz L, Gantress J, Morales HD. *Xenopus laevis*: a possible vector of Ranavirus infection? J Wildl Dis. 2007;43(4):645-52. http://dx.doi.org/10.7589/0090-3558-43.4.645. PMid:17984259.

Robert J, Jancovich JK. Recombinant ranaviruses for studying evolution of host–pathogen interactions in ectothermic vertebrates. Viruses. 2016;8(7):187. http://dx.doi.org/10.3390/v8070187. PMid:27399758.

Robert J, Morales H, Buck W, Cohen N, Marr S, Gantress J. Adaptive immunity and histopathology in Frog virus 3-infected Xenopus. Virology. 2005;332(2):667-75. http://dx.doi.org/10.1016/j.virol.2004.12.012. PMid:15680432.

Rollins-Smith LA. Metamorphosis and the amphibian immune system. Immunol Rev. 1998;166(1):221-30. http://dx.doi.org/10.1111/j.1600-065X.1998.tb01265.x. PMid:9914915.

Rollins-Smith LA. Amphibian immunity-stress, disease, and climate change. Dev Comp Immunol. 2017;66:111-9. http://dx.doi.org/10.1016/j.dci.2016.07.002. PMid:27387153.

Sasso T, Lopes CM, Valentini A, Dejean T, Zamudio K, Haddad CFB, Martins M. Environmental DNA characterization of amphibian communities in the Brazilian Atlantic forest: potential application for conservation of a rich and threatened fauna. Biol Conserv. 2017;215:225-32. http://dx.doi.org/10.1016/j.biocon.2017.09.015.

Schloegel LM, Ferreira CM, James TY, Hipolito M, Longcore JE, Hyatt AD, Yabsley M, Martins AMCPF, Mazzoni R, Davies AJ, Daszak P. The North American bullfrog as a reservoir for the spread of *Batrachochytrium dendrobatidis* in Brazil. Anim Conserv. 2010;13:53-61. http://dx.doi.org/10.1111/j.1469-1795.2009.00307.x.

Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, Zhao S, Wangen C, Ferreira CM, Hipolito M, Davies AJ, Cuomo CA, Daszak P, James TY. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Mol Ecol. 2012;21(21):5162-77. http://dx.doi.org/10.1111/j.1365-294X.2012.05710.x. PMid:22857789.

Seixas JT Fo, Camargo C Fo, Pereira MM, Martins AMCRPF, Ribeiro OP Fo, Mello SCRP, Cassiano LL, Hipolito M. Histopathological aspects of the liver of free-living and farmed bullfrogs (*Lithobates catesbeianus*). Braz J Vet Res Anim Sci. 2017;46:275-9. http://dx.doi.org/10.1590/s1806-92902017000400001.

Soto-Azat C, Peñafiel-Ricaurte A, Price SJ, Sallaberry-Pincheira N, García MP, Alvarado-Rybak M, Cunningham AA. *Xenopus laevis* and emerging amphibian pathogens in Chile. EcoHealth. 2016;13(4):775-83. http://dx.doi.org/10.1007/s10393-016-1186-9. PMid:27682604.

Teixeira PC, Vicentini IBF, Ferreira CM, França FM, Bordon IAC, Ranzani-Paiva MJT. Bullfrog hemogram under management stress. Cienc Agrotec. 2015;39(2):154-63. http://dx.doi.org/10.1590/S1413-70542015000200007.

Vences M, Köhler J. Global diversity of amphibians (Amphibia) in freshwater. Hydrobiologia. 2008;595(1):569-80. http://dx.doi.org/10.1007/s10750-007-9032-2.

Warne RW, Crespi EJ, Brunner JL. Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct Ecol. 2011;25(1):139-46. http://dx.doi.org/10.1111/j.1365-2435.2010.01793.x.

World Organisation for Animal Health – OIE. Infection with Ranavirus [Internet]. Paris: OIE; 2017 [cited 2017 Sept 26]. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_ranavirus.pdf.