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Sublethal effects of wild-type and a vIF-2α-knockout *Frog virus 3* on post-metamorphic wood frogs (*Rana sylvatica*)

Joe-Felix Bienentreu[1], Leon Grayfer[2], Danna M. Schock[3], Matthew Guerreiro[4], Stephanie J. DeWitte-Orr[4], Jacques Robert[5], Craig R. Brunetti[6] and David Lesbarrères[1*]

[1] Department of Biology, Laurentian University, Sudbury ON P3E 2C6, Canada; fbienentreu@laurentian.ca, dlesbarreres@laurentian.ca
[2] Department of Biological Sciences, George Washington University, Washington, DC 20052, USA; leon_grayfer@email.gwu.edu
[3] University Studies and Environmental Technology, Keyano College, Fort McMurray, AB T9H 2H7, Canada; Danna.Schock@keyano.ca
[4] Department of Health Sciences, Wilfrid Laurier University, Waterloo, ON N2L 3C5, Canada; guer5930@mylaurier.ca, sdewitteorr@wlu.ca
[5] Department of Microbiology and Immunology, University of Rochester, Rochester, NY 14642, USA; Jacques_Robert@urmc.rochester.edu
[6] Department of Biology, Trent University, Peterborough, ON K9J 7B8, Canada; craigbrunetti@trentu.ca
* Correspondence: dlesbarreres@laurentian.ca

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Abstract: Ranaviruses have been associated with rising numbers of mass die-offs in amphibian populations globally. With life-stages occupying different environments and presenting distinct physiologies, amphibian of different ages are likely to play an important role in pathogen persistence. To assess the potential role of post-metamorphic amphibians as a *Ranavirus* reservoir, we performed a bath-exposure study on wood frogs using environmentally relevant doses (~10^3 and ~10^4 PFU/mL) of wild-type (WT) and knockout *Frog virus 3* (FV3), deficient for the vIF-2α immune-evasion gene, the effects of which have never been addressed in post-metamorphic anurans. We observed 42% infection prevalence and low mortality (10%) across the virus challenges, with half of the mortalities attributable to ranavirosis. Prevalence and viral loads followed a dose-dependent pattern. Notably, when exposed to the vIF-2α knockout (ΔvIF-2α) FV3, individuals exhibited significantly decreased growth and increased lethargy in comparison to WT FV3 treatments. Although 85% of individuals in the virus treatments exhibited stereotypic signs of ranavirosis throughout the experiment, at termination (40 days post exposure) most individuals were clear of signs of infection. Overall, this study provides evidence that even a single short time exposure to environmentally relevant doses of *Ranavirus* may cause sublethal infections in post-metamorphic amphibians, thus indicating their possible role as a reservoir for this pathogen.

Keywords: *Ranavirus*; FV3, vIF-2α knockout mutant; bath exposure; wood frog; sublethal effects; reservoir host

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1. Introduction

Viruses belonging to the genus *Ranavirus* (family Iridoviridae) are believed to be responsible for a number of mass mortality events in wild and captive amphibians, fish, and reptiles by causing a systemic disease involving necrosis of liver, nephretic and gastrointestinal tissues [1-5]. Four species of *Ranavirus* are known to infect a wide range of amphibian hosts: *Ambystoma tigrinum virus*, *Bohle iridovirus*, *Common midwife toad virus*, and *Frog virus 3* [6]. *Frog virus 3* (FV3), the type species of the genus, is known to have a significant impact on amphibians worldwide [7], especially in North America [8,9] and is the best described member of the genus. Full genomes of several FV3 isolates have been sequenced, the elemental features of viral replication are known, and 98 open reading frames (ORFs) have been identified [10]. Several conserved protein-coding regions are likely involved in virulence, host tropism, and immune evasion strategies [11], but the specific functional role is presently only known for a few FV3 ORFs [12]. By creating knockout mutants, and conducting subsequent experimental challenge of *Xenopus laevis* tadpoles, Chen et al. [10] identified the gene vIF-2α, which plays a crucial role in viral virulence and is essential to the FV3 evasion of the host antiviral response [11]. However, the *in-vivo* effects of the vIF-2α knockout FV3 (ΔvIF-2α FV3) have not been studied in any post-metamorphic anuran.

Different *Ranavirus* species and their respective isolates possess a high variability in their virulence, commonly measured as the time to death [13,14]. Such variation typically depends on the viral strain [14], the susceptibility of the host species present and their interaction [13]. Amphibians exhibit a high interspecific variation in susceptibility to *Ranavirus* infections in general [15,16], but intraspecific variation in susceptibility and infection severity among life stages also plays an important role in pathogen dynamics [13,17]. Many larval amphibians show a high probability of developing lethal ranavirosis [e.g. 16,18,19], which coincides with observations of die-offs in late stage tadpoles or newly metamorphosed individuals [1,20]. However, *Ranavirus* likely relies upon reservoirs such as sublethally infected individuals to persist in amphibian communities [8,17,18]. To gain greater insight into the routes of *Ranavirus* transmission, it is essential to conduct studies using distinct life-stages of model species, environmentally relevant exposure routes and doses to determine the role that particular hosts and specific life-stages play in the epidemiology of a pathogen [21,22]. Accordingly, to evaluate the role of sublethally infected post-metamorphic amphibians as reservoirs for *Ranavirus*, we exposed terrestrial wood frogs (*Rana sylvatica*) via bath-exposure to environmentally relevant doses [18] of two strains of *Ranavirus*; wild-type (WT) FV3 and a ΔvIF-2α FV3. Bath-exposure can be effective for amphibian species with semi-aquatic life as it mimics natural routes of infection, as suggested by Gray et al. [18] and Bayley et al. [23]. To date, there have been only a few studies that have assessed the susceptibility of post-metamorphic anurans to *Ranavirus* infection via bath exposure [23-25], and only a single study used a North American species [*R. sevosus*, 26]. Mortality events in wild anuran populations in North America are often associated with communities containing wood frogs [e.g. 1,27,28], which could indicate their roles as reservoirs for the pathogen in such communities.

2. Materials and Methods

Ranavirus isolates and anuran larvae

We used the *Frog virus 3* wild-type isolate ATCC VR-567, which was originally isolated from *Rana pipiens* in the early 1960s [29]. The ΔvIF-2α FV3 was constructed by replacing the vIF-2α gene of the WT FV3 ATCC VR-567 isolate with a puromycin resistance gene (18Kprom-Puro-EGFP) through homologous recombination [10]. The viral stocks were each propagated in 5 plugged T75 flasks utilizing epithelioma papulosum cyprini cells at room temperature (18C–20°C) in Roswell Park Memorial Institute medium (RPMI), supplemented with 2% fetal bovine serum and 1% antibiotic PenStrep (Invitrogen, Burlington, ON). This cell line is known to consistently produce high *in vitro* amounts of *Ranavirus* [30]. Flasks were allowed to reach approx. 80% confluence before cells were infected with FV3 at a multiplicity of infection (MOI) of 0.01. Flasks were rocked for 1hr at room temperature to allow for even distribution of the virus particles, and then incubated for 5 days at

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30°C to allow for viral proliferation. The resulting media and cell/virus mixture of each virus strain were pooled, subjected to 3 freeze-thaw cycles to lyse any remaining cells, and then passed through a 0.2 μm filter to remove cellular debris. Finally, the virus stocks were aliquoted and frozen at -70°C for later use

Wood frogs have been suggested as a model species for challenge experiments involving North American strains of *Ranavirus* due to their widespread distribution, sympatry with numerous other amphibian species, and relatively high *Ranavirus* prevalence levels [3,31]. We capitalized upon the robust literature available for wood frog-*Ranavirus* dynamics and used this species in our study. Six clutches of wood frog eggs were collected from a natural, semi-permanent wetland in Wood Buffalo National Park near Fort Smith, Northwest Territories, Canada, on 14 May 2018. The wood frog population at this wetland had low *Ranavirus* prevalence during the year previous to the egg collection (5% in terrestrials and 0% in tadpoles [32], identified as FV3-like strain; see [33]) and the wetland had pH and conductivity values within usual ranges for wood frog habitats in the area [34]. Each egg clutch was individually packed in a new 3.7 L Ziploc® bag filled with pond water and then placed into a thermo-insulated cooler filled halfway with water and ice packs. The temperature inside the coolers was maintained at approximately 4°C until arrival at Laurentian University animal care facility ~48 hours later. This low temperature decreases egg development rates significantly, yet is well within the normal environmental range for this species [35]. Upon arrival, the clutches were carefully removed from their respective bags, washed three times in aged, de-chlorinated water to remove any external debris, and then transferred into individual 45 L plastic tubs (food grade) and monitored daily. Two clutches, that started to hatch while being transported, were not used in the experiment because we could not exclude the possibility that the tadpoles had come into contact with waterborne parasites and pathogens, nor could we exclude the possibility that any developmental issues that might arise during the experiment were due to hatching under suboptimal conditions. The remaining four clutches of eggs were hatched under controlled conditions as stated below. To minimize the spread of pathogens among containers from the reception of the clutches and all across the experiment we used new gloves when handling each individual and all equipment was disinfected with 10% bleach solution, 95% isopropyl, and subsequently rinsed with tap water.

Husbandry conditions

For each clutch, water was maintained at 21°C, and a pH of 7.5. All water conditions were established in accordance to the parameters recorded in the wild across three seasons of field-work. The photoperiod was set to 14:10 h light-dark hours using full spectrum 4000 k LED light bars (1 per 10 experimental units). A 75% water change with de-chlorinated aged water (21°C) was conducted on a six-day basis, and two air diffusers were added to each tub for oxygenation. Tadpoles were fed standard dried tadpole micropellets (ZooMed®, Zoo Med Laboratories Inc., San Luis Obispo, CA) on a 2 day basis: 30 mg/tadpole for week 1, 60 mg/tadpole for week 2, and 120 mg/tadpole for week 3 and until metamorphosis. This amount of food corresponded to an intermediate level of resources, appropriate for not over-feeding the larvae [36]. Lose coconut fibres were provided as environmental enrichment. Dead tadpoles were immediately removed and examined for gross signs of ranaviral disease. Upon tadpoles reaching Gosner stage 32 [37], we randomly chose 10 tadpoles per clutch, euthanized them via immersion in 6% MS222 solution. We pooled tissues per clutch and screened for *Ranavirus* using qPCR. All samples tested negative. Subsequently, 30 tadpoles from each of the four clutches were separated into 120 individual plastic tubs (2.1 L food grade), filled with 1.6 L de-chlorinated aged water, and monitored daily. We randomly redistributed the plastic tubs on our shelving units every 3 days to eliminate any effects due to specific placement in the setup (e.g., light intensity and micro-temperature differences). After reaching Gosner stage 40, each tadpole was provided with a floating cork platform to allow for resting and to prevent drowning. The amount of water in each tub was gradually decreased to 0.2 L as each animal progressed to Gosner stage 45. A perforated lid was added to each tub to prevent the animals from escaping. To prevent fouling of the water, we stopped offering food during metamorphic climax (Gosner stages 42-45), a point in anuran

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development when they temporarily stop eating. All 120 tadpoles completed metamorphosis within the first week of July 2018 (average time hatch to metamorphosis: 49 ± 3 days). All water was then removed from the enclosures. Coconut fibre substrate (Eco Earth®, Zoo Med Laboratories Inc., San Luis Obispo, CA), a small amount forest moss (Exo Terra®, Rolf C. Hagen Inc., Montreal, QC) to maintain moisture, and a 100 mm petri dish filled with de-chlorinated water were added to each tub. Natural cork sheets were used to create a shelter but arranged such that animals could not hide out of sight. Frogs were held at 21°C day and 18°C night temperature and the photoperiod was set to 14:10 h light-dark cycles. Approximately 15 flightless fruit flies (*Drosophila melanogaster*) were provided every 2 days for the first 14 days. We then introduced bigger fruit flies (*D. hydei*), in variation with House- (*Acheta domesticus*) and Banded crickets (*Gryllus sigillatus*), as well as mealworms (*Tenebrio molitor*) as food sources. Individuals were either fed 20 fruit flies, 2 small crickets, or 2 small mealworms on a 2-day basis. We used Exo Terra® Calcium and Multi Vitamin Powder in a 1:1 ratio to dust insects and provide additional nutrient and mineral supply. We used Repashy® Superfly Fruit Fly Culture Medium (Repashy Ventures Inc., Oceanside, CA) as well as Fluker's® Orange Cube Complete Cricket Diet (Fluker's Cricket Farm Inc., Port Allen, LA) to maximize the nutritional value of the insects.

Experimental design

The exposure trials were conducted with wood frogs that were 45 - 50 days post metamorphosis. A total of 100 frogs (25 from each of the 4 clutches) were randomly selected and equally assigned to the 5 treatments (20 frogs per treatment). Within each treatment, frogs had a snout-vent-length (SVL) of 22.5 ± 2.5 mm and a weight of 1.5 ± 0.5 g. To simulate a natural route of infection we conducted a 2 h individual water bath exposure in 200 mL glass jars, containing 15 mL water with either $10^{2.97}$ PFU/mL (low titre) or $10^{3.97}$ PFU/mL (high titre) of the appropriate virus isolate suspended in RPMI medium. Individuals in the negative control treatment were challenged with RPMI medium only, following the same procedure. The media volume used was sufficient to guarantee full coverage of the ventral and dorsal body surfaces of the frogs without the possibility of drowning. Plaque assays and viral quantification using qPCR were conducted to determine exact virus stock concentration in PFU/mL at exposure date, as previously described [38].

All treatments were held under identical husbandry conditions, as described above. All individuals were monitored twice daily for morbidity and gross signs of ranavirosis [e.g. 18,39]. Activity level and feeding behaviours were recorded each time on a 3-level scale (0 = inactive, 1 = passive/sheltered, 2 = active/outside and 0 = no reaction, 1 = interest/approach, 2 = feeding, respectively). All frogs were measured and weighed every 3-days, and thoroughly examined for external signs of ranavirosis (e.g. lesions/haemorrhages and skin ulceration). All dead frogs were removed from their tanks and necropsies were immediately performed: toe-clips and liver sections were removed and stored individually in 1.5 mL microcentrifuge tubes (Eppendorf® filled with 95% ethanol) at -70°C. Carcasses were stored in either 10% buffered formalin or RNAlater for further analysis. The experiment was terminated after 40 days, a time shown to be sufficient for morbidity and mortality to occur [21,24]. All remaining frogs in the virus treatments and the control group were euthanized by immersion in 6% MS222 solution, followed by post-mortem procedures as described above. The experiment was conducted under Laurentian University Animal Care Protocol #6013781.

qPCR-based *Ranavirus* quantification

Tissue samples were assessed for *Ranavirus* following the protocol for quantitative PCR (qPCR) described by Leung et al. [40], and viral loads were quantified as described by Hoverman et al. [14]. DNA extraction from liver sections was performed using Qiagen DNEasy® Blood and Tissue kits according to manufacturer specifications (QIAGEN® Inc., Valencia, CA, USA). For quantification of genomic DNA a Synergy H1 Hybrid Multi-Mode Reader (BioTek, Winooski, VT, USA) and a Quant-iT™ dsDNA BR Assay Kit (Invitrogen Corp., Carlsbad, CA, USA) were used. For qPCR we used a Mx3005P qPCR System (Agilent Technologies, Santa Clara, California). The qPCR mixture contained:

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250ng of template DNA, 10 µL TaqMan Universal PCR Master Mix 2X (Thermofisher Scientific), 1 µL forward primer MCPRV_F-5GTCCTTTAACACGGCATACCT3 (10µM), 1µL reverse primer MCPRV_R-5ATCGCTGGTGTTGCCTATC3 (10 µM), and 0.05µL TaqMan probe MCP_NFQ-5TTATAGTAGCCTRTGCGCTTGGCC3 (100 µM), as well as PCR grade water in the appropriate amount to reach the final reaction volume of 20 µl. Samples were run in duplicate at 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 sec, and 60°C for 30 sec. Individuals were considered positive if both duplicates showed a clear amplification (e.g. surpassing the respective cycle threshold). If only one of the two runs showed amplification, a third run was conducted, to either confirm or dismiss previous results. Each 96-well plate included a no-template control (DNA grade water), and a serial dilution of a known quantity of cultured *Frog virus 3* (10^6 - 10^1 PFU/mL) to create standard curves with precise fit (R^2 = > 0.95). The primers used target a consensus sequence within Major Capsid Protein (MCP) that is shared among the majority of amphibian associated Ranaviruses (FV3, GenBank No. AY548484 [12]; TFV, AF389451 [41]; CMTV, JQ231222 [42]; EHNV, FJ433873 [43]), and allows for a high analytical sensitivity when used in combination with a TaqMan probe [40]. Subsequently, following Yuan et al. [44], the standard curves were used to calculate viral load in copies/250ng gDNA, as recommended by Gray et al. [9]. Due to large standard deviations viral loads are reported as log (copies/250ng gDNA).

Statistical analyses

Statistical analyses were conducted using R v3.3.3 [45], in R Studio V3.4.1 [46]. We used Chi^2 tests to compare survival among all treatments, whereas infection rates and exhibition of pathological signs were only compared between the virus treatments. We conducted a one-way ANOVA to test for differences in the response variables among all individuals in the treatment groups: percentage growth (SVL and weight), activity level, and feeding behaviour. Clutch identity was treated as random factor. Since the control group had no infected individuals, resulting in a mean and SD = 0, the treatment was removed from the ANOVAs for viral loads. We used Tukey's HSD test to detect differences between means for ANOVAs where the overall null was rejected.

3. Results

No frogs from the control treatment died, tested positive for via qPCR, or exhibited any gross pathology associated with ranavirosis (Table 1). Overall, we observed 5% *Ranavirus* related mortality and 42% infection prevalence in post-metamorphic wood frogs exposed in the different virus treatments (Table 1). We did not observe any significant differences in survival rates, infection prevalence, mortality due to ranavirosis, or gross signs of infection. Infection prevalence followed a dose-dependent pattern in both the WT and ΔvIF-2α FV3 challenges although the trends were not statistically significant (Table 1). Two individuals in the high dose WT FV3 group died between 8-10 days post-exposure, and one individual died in each ΔvIF-2α FV3 treatment, 8 and 11 days post-exposure. We observed severe haemorrhaging in these animals 1 to 2 days before death, consistent with gross pathology associated with ranavirosis in other studies [e.g. 18,39]. Four other individuals died within the last 10 days of the experiment, 1 frog in each virus treatment. All 4 of these frogs tested negative for *Ranavirus* but two of the four individuals had shown signs of infection prior to death (WT FV3 low and ΔvIF-2α FV3 high treatment). Starting approximately at day 7 post exposure, individuals in the virus treatments started to show gross signs of ranavirosis, such as diffusely congested blood vessels and erythema, primarily on the extremities, toes and fingers, as well as on the anterior ventral body surface (Figure 1). Such signs were present in 79% and 94% of the individuals exposed to the respective low and high dose of WT FV3, as well as 72% and 83% in the low and high dose of ΔvIF-2α FV3, respectively. At the end of the study, 87% and 81% of individuals previously exhibiting lesions in the low and high WT FV3 infection groups, and 85% and 73% of the low and high ΔvIF-2α FV3, groups, no longer had any observable signs of disease; Table 1.

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Average viral loads at the end of the experiment differed in a dose dependent pattern, with the high-dose treatments showing 51% higher loads on average and relative to the low dose treatments although the trend was not statistically significant (Table 2). There were no significant differences among treatments with respect to feeding behaviour or relative weight gain among the treatments. We observed significant variation in relative length gain among treatments ($F_{4,87}$ = 7.73, p < 0.001; Table 2). Post hoc tests revealed that the length gain in the ΔvIF-2α FV3 high treatment was significantly lower than the WT FV3 and the control treatments, with the largest difference relative to the high dose WT FV3 group (p < 0.001). Overall, the two ΔvIF-2α FV3 infection groups exhibited a 11% lower relative length increase compared to the control group (Table 2; Figure 2), whereas the WT FV3 treatments showed a 2% higher length gain than the control animals. Activity level of individuals showed significant variation among treatments ($F_{4,87}$ = 7.32, p < 0.001; Table 2). Individuals in the WT FV3 high-dose treatment were significantly more active than the WT FV3 low treatment (p < 0.01), and the control group (p < 0.001). The two ΔvIF-2α FV3 treatments showed generally lower activity levels relative to the WT FV3 groups, but only the comparisons to the WT FV3 high dose treatment were significant (p < 0.01 for the low dose, and p = 0.09 for the high dose ΔvIF-2α FV3).

Table 1. Survival at 40 days post exposure, proportion qPCR positive individuals, frequency of haemorrhages, and cleared haemorrhages in post-metamorphic wood frogs after bath exposure to WT FV3 and a ΔvIF-2α FV3. Respective Chi² and p-values included. Viral titres: $10^{2.97}$ PFU/mL (low) or $10^{3.97}$ PFU/mL (high).

	WT FV3 low	WT FV3 high	ΔvIF-2α FV3 low	ΔvIF-2α FV3 high	control	df	χ^2	p
survival	19/20 (95%)	17/20 (85%)	18/20 (90%)	18/20 (90%)	20/20 (100%)	4	0.11	0.991
positive alive	7/19 (37%)	9/17 (53%)	4/18 (22%)	10/18 (56%)	0/20 (0%)	3	2.80	0.424
positive dead	0/1 (0%)	2/3 (67%)	1/2 (50%)	1/2 (50%)	n/a	3	2.01	0.572
hemorrhages alive	15/19 (79%)	16/17 (94%)	13/18 (72%)	15/18 (83%)	0/20 (0%)	3	0.32	0.956
hemorrhages cleared alive	13/15 (87%)	13/16 (81%)	11/13 (85%)	11/15 (73%)	n/a	3	0.33	0.954
hemorrhages dead	1/1 (100%)	2/3 (67%)	1/2 (50%)	2/2 (100%)	n/a	3	0.67	0.881
hemorrhages cleared dead	1/1 (100%)	0/2 (0%)	0/1 (0%)	1/2 (50%)	n/a	3	n/a	n/a

Table 2. Relative growth (length and weight gain), average viral loads, and activity level and feeding behaviour for post-metamorphic wood frogs after bath exposure to WT FV3 and ΔvIF-2α FV3, 40 days post-exposure. Average viral loads are shown as log (copies/250 ng gDNA). For each variable, respective df, F and p included. Viral titres: $10^{2.97}$ PFU/mL (low) or $10^{3.97}$ PFU/mL (high). AVL = average viral load.

	WT FV3 low	WT FV3 high	ΔvIF-2α FV3 low	ΔvIF-2α FV3 high	control	df	F	p
length gain %	19.11 ± 6.16	22.47 ± 5.93	14.5 ± 6.1	12.83 ± 5.95	18.75 ± 5.05	4, 87	7.73	< 0.001
weight gain %	57.16 ± 27.01	65.65 ± 29.64	52.22 ± 22.73	43.78 ± 20.95	59.1 ± 24.62	4, 87	1.87	0.129
AVL all individuals	0.86 ± 1.18	1.3 ± 1.29	0.5 ± 0.97	1.54 ± 1.52	n/a	3, 68	2.44	0.072
AVL positive individuals	2.34 ± 0.32	2.45 ± 0.35	2.26 ± 0.09	2.78 ± 0.75	n/a	3, 26	1.52	0.232
AVL dead individuals	n/a	8.37 ± 0.47	5.72 ± 0	8.84 ± 0	n/a	2, 1	1.52	0.497
activity level	1.24 ± 0.13	1.41 ± 0.14	1.23 ± 0.09	1.3 ± 0.16	1.19 ± 0.15	4, 87	7.32	< 0.001
feeding behaviour	1.38 ± 0.20	1.42 ± 0.24	1.37 ± 0.18	1.44 ± 0.17	1.33 ± 0.22	4, 87	0.83	0.518

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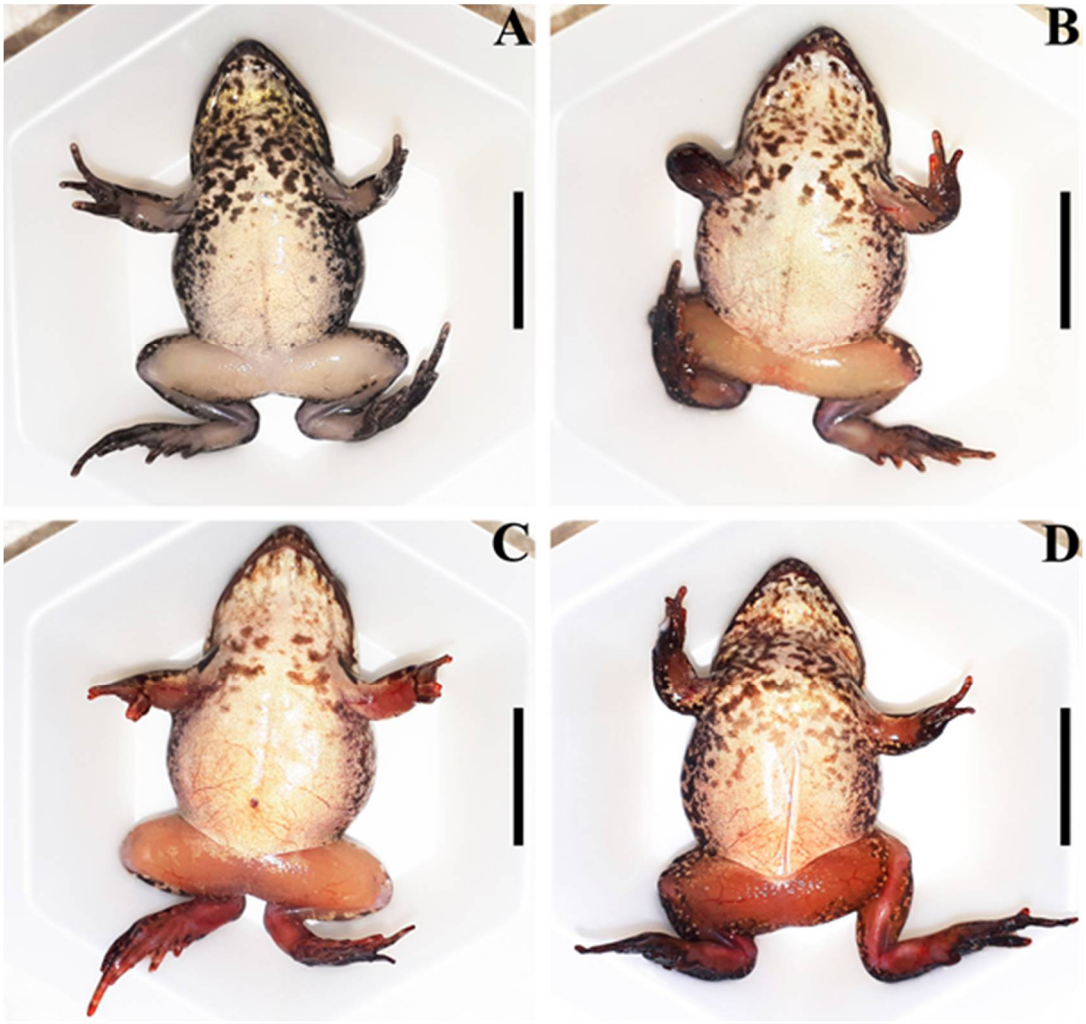

Figure 1. Gross lesions of varying intensity observed in post-metamorphic wood frogs (*Rana sylvatica*) experimentally infected with *Ranavirus*, 40 days post exposure: (A) no obvious signs (WT FV3 low); (B) Slight haemorrhages in fingers, and minor erythema on the anterior ventral body surface (WT FV3 high); (C) Moderate erythema in extremities, as well as haemorrhages in fingers and toes (ΔvIF-2α FV3 high); (D) Severe erythema in extremities and anterior ventral body surface, diffusely congested blood vessels, as well as haemorrhages in fingers and toes (WT FV3 high); scale bar = 10 mm.

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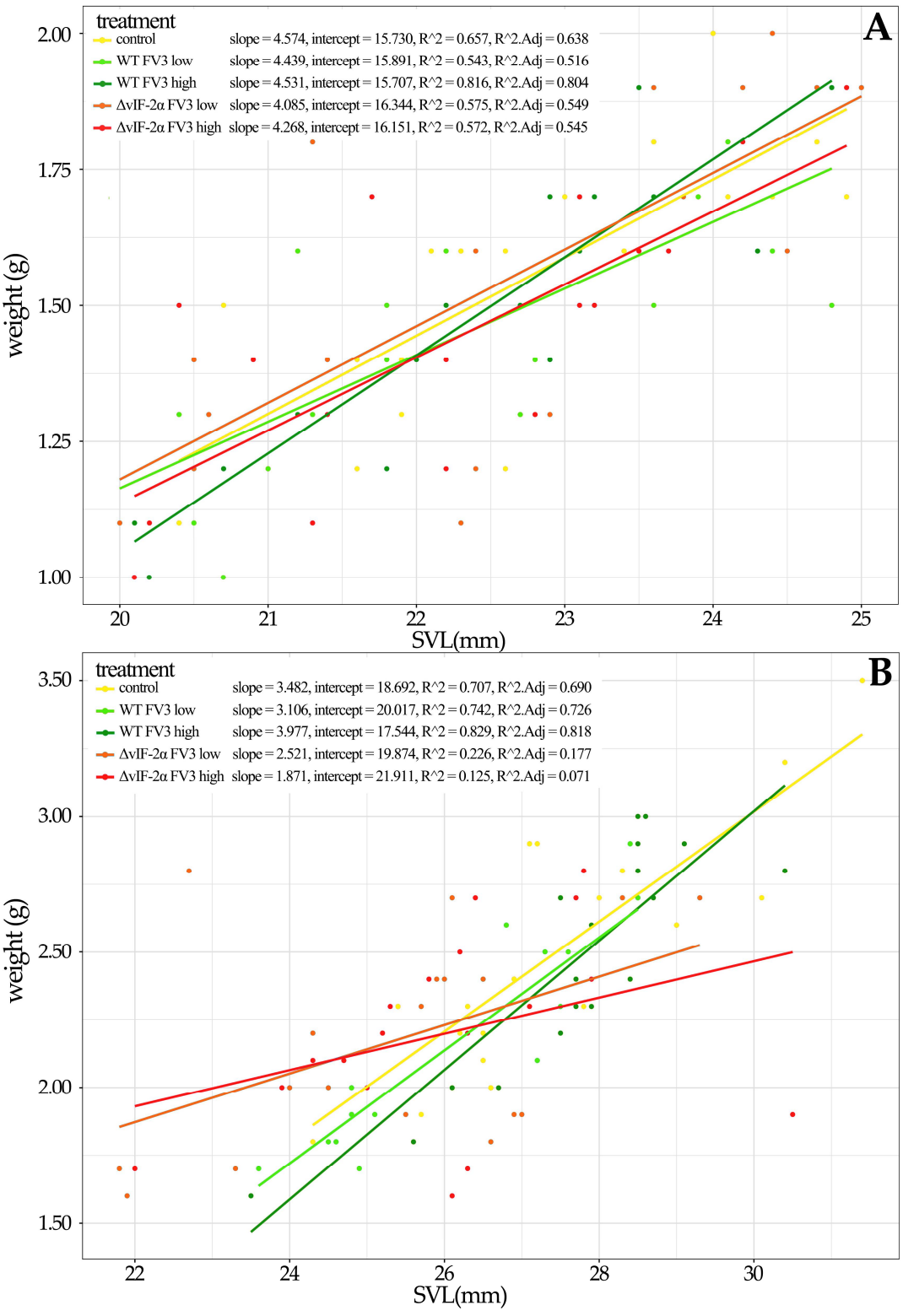

Figure 2. Comparison of body condition (regression of length~weight) of post-metamorphic wood frogs (*Rana sylvatica*) water-bath exposed to ecologically relevant doses of wild type FV3 and ΔvIF-2α FV3, at the start (A) and end (B) of the experiment (including respective slope, intercept, R^2, and adjusted R^2). SVL = snout-vent length.

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4. Discussion

Mortality was very low in post-metamorphic wood frogs when exposed via water bath to environmentally relevant doses of *Ranavirus*. Although there was abundant gross pathological evidence of infection within 2 weeks of exposure, only 42% of the frogs across the treatments tested positive for *Ranavirus* at the end of the 40 day experiment. Moreover, we did not observe any mortality due to ranavirosis in the WT FV3 low treatment, and even frogs exposed to the higher dose experienced low mortality due to ranavirosis (5%), with most individuals only showing minor signs of infection (Figure 1 B, D). The low infection prevalence may be a consequence of selection towards more resilient genetic lineages within the source population, induced by repeated exposure to the pathogen [47]. The genetic compositions of distinct population may have significant impact on the outcomes of a *Ranavirus* emergence [48], and a co-evolutionary history of the host immune system with the pathogen can lead to decreased susceptibility to re-introduced pathogens [or strains; 47]. All wood frogs used in our study were raised from four egg clutches collected from a natural population within Wood Buffalo National Park which showed low *Ranavirus* prevalence and low viral loads in 2017 [32]. However, *Ranavirus* has been commonly observed in amphibians in the area [32,34], with two confirmed die-off events at wetlands 7 and 29 km away in 2017 [28], and mortality occurring among wood frog tadpoles within 3.5 km in 2009 [49]. Additionally, sequencing led to the identification of two different FV3-like viruses in amphibian populations in the research area [33,50]. These observations, in combination with our experimental results, could indicate that the source population evolved partial immunity against FV3-like ranaviruses due to repeated exposures to the pathogen. By contrast, other studies using FV3/ FV3-like viruses with similar doses and bath exposure observed high mortality (97% and 100% for *R. temporaria* [23] and *R. servosus* [26] respectively) and post-metamorphic wood frogs orally inoculated with similar doses of FV3 as our study showed 80-100% mortality [31]. These contrasting results indicate that mortality rates are route-specific, even within the same host species. In post-metamorphic individuals, infection through ingestion of infected tissue is less likely to occur than in larva and tadpoles [see 51,52]. For semi-aquatic species like wood frogs, direct transmission between individuals through contact with infected individuals [as shown in 22,24] probably only occurs during breeding aggregations in the Spring. For the rest of the active season, short time exposure to water containing virus particles shed by other (possibly asymptomatic) infected individuals is the most likely route of infection, since post-metamorphic individuals only infrequently visit wetlands [53].

An alternative explanation for the low prevalence observed in our experiment is the immune status of the post-metamorphic individuals. Experimental studies conducted with different life-stages of *Xenopus laevis* identified significant differences in the antiviral responses mounted by tadpoles and adult frogs to FV3, presumably reflecting the morphological and immunological differences between pre- and post-metamorphic anurans [54]. Gene expression studies showed that tadpoles exhibit considerably less robust and delayed anti-FV3 inflammatory gene responses relative to adults [55], although they have rather timely antiviral (type III interferon) responses to this pathogen [56]. Post-metamorphic *X. laevis* are capable of clearing FV3 infection within one month after exposure [57]. However, infected adult *X. laevis* have been shown to hold considerably higher viral loads than tadpoles [56], which typically succumb to ranavirosis [58-60]. Therefore, our results may be an underestimate of the actual prevalence and viral loads among the animals studied here. Perhaps, a considerably greater proportion of the frogs exposed in our study was infected with FV3, and subsequently eliminated the majority of their FV3 inocula to a level beyond detection by the methods employed here. This is further supported by the observation that most animals that exhibited gross signs of infection in the first two weeks post-exposure were apparently healthy by the end of the experiment. Moreover, animals that persistently exhibited gross signs of infection until the end of the study, had low viral loads.

Notably, wood frogs exposed to the ΔvIF-2α FV3 showed lower activity, decreased growth, and in the high dose treatment, slightly higher average viral loads than wood frogs exposed to the WT

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FV3. These effects are likely due to energetically costly specific immune responses. Vertebrate antiviral defenses are highly dependent on interferon (IFN) cytokine-mediated immunity. Recent *in vitro* studies suggest that the FV3 vIF-2α gene product is crucial to counteracting the host interferon-induced antiviral states [56]. Andino et al. [11] showed that the ΔvIF-2α FV3 exhibited reduced replication *in vitro* in the immuno-competent *X. laevis* A6 kidney epithelial cell line and elicited less pronounced type I and III IFN responses in A6 cultures, as compared to the wild type FV3. However, when the A6 cell, IFN responses elicited by the ΔvIF-2α FV3 were reassessed as a function of the viral loads in those cultures, the ΔvIF-2α FV3 actually elicited proportionately greater IFN responses than the WT FV3 [61], in turn confirming that the FV3 vIF-2α gene product is crucial to dampening the frog host IFN responses [56]. We postulate that the lower activity and the decreased growth we observed in wood frogs infected with the ΔvIF-2α FV3 reflect their more pronounced, and thus presumably more energetically costly cytokine responses relative to animals infected with WT FV3.

The long-term persistence of *Ranavirus* in a host community is linked to competent amphibian reservoirs [18]. Field studies, as well as experimental exposures to *Ranavirus* have shown that individuals may sustain sublethal infections [17,62-65], and can shed sufficient amounts of *Ranavirus* virions to infect other individuals (10^3 to 10^4 PFU/mL) [18,66,67]. Overwintering tadpoles and paedomorphic salamanders can act as reservoir in aquatic habitats [18,73] and several studies have identified sublethally infected post-metamorphic amphibians, with only a minority exhibiting pathological signs (Australia and Europe: [22,25,74,75]; North America: [17,34,73]. Our findings, as well as reports of re-occurring mass mortality events in other amphibian populations inhabiting semi-permanent and ephemeral wetlands [68-70] further support a specific life-history stage reservoir theory. In such environments, suitable amphibian life-history stages are the most likely reservoirs for *Ranavirus* persistence because virions will not remain active and viable for extensive period of times outside their host without a moist, or even aquatic environment [71,72]. Therefore, both larval and post-metamorphic amphibians can contribute to the persistence of the pathogen in the environment [8].

There is still a lack of knowledge on how ranaviruses persist in amphibian communities. In particular, it is important to identify reservoir species which may allow the pathogen to persist in the environment and facilitate its spread in the amphibian communities. Wood frogs are the most widely distributed amphibian species in North America [76], and due to their sympatry with numerous other species, they are an ideal model for viral challenge experiments [3]. In particular, post-metamorphic wood frogs have been suggested as model to study host response and pathogenesis of ranavirosis in North American amphibians [31]. Here, we document important *in vivo* effects of infection of WT FV3 and ΔvIF-2α FV3 in post-metamorphic wood frogs, contributing to the overall knowledge on infection in adult anurans, and highlighting the function of the vIF-2α gene in *Ranavirus* pathogenesis. Low viral loads in the infected individuals underline the importance of high-sensitivity pathogen screening [77]. Finally, we provide evidence that even short duration exposures to environmentally relevant doses of *Ranavirus* may cause sublethal infections in post-metamorphic wood frogs, indicating the role of this species as a plausible reservoir for FV3, and possibly for *Ranavirus* in general.

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