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Ranavirus infection dynamics and shedding in American bullfrogs: consequences for spread and detection in trade

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ABSTRACT: American bullfrogs *Lithobates catesbeianus* are thought to be important in the global spread of ranaviruses — often lethal viruses of cold-blooded vertebrates — because they are commonly farmed, dominate international trade, and may be 'carriers' of ranavirus infections. However, whether American bullfrogs are easily infected and maintain long-lasting ranavirus infections, or are refractory to or rapidly clear infections, remains unknown. We tracked the dynamics of ranavirus in American bullfrogs through time and with temperature in multiple types of samples and also screened shipments from commercial suppliers to determine whether we could detect subclinical infections. Collectively, we found that tadpoles and juveniles were commonly infected at moderate doses, and while some died, others controlled and appeared to clear their infections. Some individuals, however, harbored subclinical infections for up to 49 d, suggesting that American bullfrogs may be important carriers. Indeed, tadpoles and metamorphosed frogs from 2 of 5 commercial suppliers harbored subclinicial infections. Juveniles at warmer temperatures had less intense but still persistent infections. Because diagnostic performance was strongly related to infection intensity, non-lethal samples (i.e. tail or toe clips, swabs, and environmental DNA) had only a moderate chance of detecting subclinical infections. Even internal tissues may fail to detect subclinical infections. However, viral shedding was correlated with the intensity of infection, so while subclinically infected tadpoles shed virus for 35–49 d, the low levels might lead to little transmission. We suggest that a quantitative focus on virus dynamics within hosts can provide a more nuanced view of ranavirus infections and the risk presented by American bullfrogs in trade.

KEY WORDS: Amphibian disease · Aquaculture · Biosecurity · Carrier state · Detection · Diagnostic performance · Intensity · Non-lethal sampling · *Ranavirus* · Sub-clinical infection

1. INTRODUCTION

Ranaviruses are large, double-stranded DNA viruses in the genus *Ranavirus* (Family *Iridoviridae*) that infect at least 175 species of fish, amphibians, and reptiles around the world (Duffus et al. 2015). They can cause massive die-offs in many taxa in the wild (Whittington et al. 2010, Miller et al. 2011, Duffus et al. 2015) and even community-wide declines (Price et al. 2014), but are also frequently detected in the absence of any apparent mortality or disease (Duffus et al. 2015). Ranaviruses have also been found in a variety of captive settings, from private collections and zoos to aquaculture and ranaculture (Weng et al. 2002, Galli et al. 2006, Majji et al. 2006, Miller et al. 2007, Driskell et al. 2009, Mazzoni et al.

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2009, Cheng et al. 2014, George et al. 2015, Hausmann et al. 2015, Jerrett et al. 2015, Pirarat et al. 2016) and in international trade (Picco & Collins 2008, Schloegel et al. 2009, Duffus et al. 2015). Indeed, the regional and international trade of live animals is thought to be an important pathway by which ranaviruses are introduced into wild and captive amphibian populations (Picco & Collins 2008, Schloegel et al. 2009, Picco et al. 2010, Price et al. 2016). For instance, tiger salamander *Ambystoma mavortium* larvae sold as fishing bait in the southwestern USA are frequently infected with the ranavirus *Ambystoma tigrinum* virus (ATV; Picco & Collins 2008) and may be responsible for the long-distance introductions of novel ATV genotypes (Jancovich et al. 2005). However, American bullfrogs *Lithobates catesbeianus*, which are both highly invasive and are farmed for food around the world (Li et al. 2006, Ficetola et al. 2007, Global Invasive Species Database 2015), appear to play a much larger role in the global spread and spillover of ranaviruses into novel hosts (Mazzoni et al. 2009, Schloegel et al. 2009, Une et al. 2009, Sharifian-Fard et al. 2011, Price et al. 2016).

American bullfrog farms and ranaculture facilities occasionally experience ranavirus outbreaks and die-offs (Majji et al. 2006, Mazzoni et al. 2009, Une et al. 2009, Sharifian-Fard et al. 2011, Saucedo et al. 2019). Outbreaks amplify ranavirus infections, which can then spill over into native species. Ranaculture facilities may also enable recombination events that can produce more virulent strains of ranavirus (Miller et al. 2007, Hoverman et al. 2011, Claytor et al. 2017) as well as other important pathogens such as *Batrachochytrium dendrobatidis* (Schloegel et al. 2012, Yap et al. 2018). American bullfrogs dominate the international trade of live amphibians shipped around the world for food, research, and as pets (Schlaepfer et al. 2005, Schloegel et al. 2009, Altmann & Kolby 2017). Schloegel et al. (2009) estimated that 8.5% of American bullfrogs imported into the USA were ranavirus-infected. Moreover, apparently healthy American bullfrogs can harbor subclinical ranavirus infections (Wolf et al. 1969), thus serving as inapparently infected carriers. Altogether, American bullfrogs appear to be a uniquely important agent of 'pathogen pollution' for ranaviruses and other amphibian pathogens (Daszak et al. 2003).

Several key questions, however, need be addressed to better understand the threat that American bullfrogs represent for ranavirus spread, as well as how we might best detect and prevent this spread. First, it is not yet clear whether American bullfrogs are easily infected or killed by ranaviruses. While ranavirus-related die-offs occur (e.g. Mazzoni et al. 2009, Une et al. 2009), ranavirus can also be common in the apparent absence of mortality (Gray et al. 2007, Hoverman et al. 2012, Tornabene et al. 2018). Given inherent biases in detecting mortality events, it is difficult to draw clear conclusions from sporadic reports from the field. In laboratory exposures, American bullfrog tadpoles appear largely refractory to moderate doses of ranavirus (10^3 plaque-forming units [pfu] ml^{-1}; Hoverman et al. 2011). At higher doses (10^6 pfu ml^{-1}), infections are common, but prevalence declines after the first week post exposure, suggesting infections are cleared (Gray et al. 2012). Together, these results suggest that American bullfrogs are largely resistant to ranavirus and so infections will be rare (i.e. found at low prevalence). The risk of ranavirus movement and spillover would thus seem to be relatively moderate and transient; sufficient quarantine periods could reduce this risk towards zero. However, it is also possible that ranavirus infections in American bullfrogs are not cleared, but reduced to low levels — consistent with the notion of persistent subclinical infections in tadpoles (Hoverman et al. 2019) and adults (Wolf et al. 1969) — which would be difficult to detect, especially in non-lethal samples (Gray et al. 2012). Estimates of prevalence and the duration of infections, and thus the risk of spillover, may therefore be overly conservative. However, quantifying this risk depends a great deal on whether animals with such low-level infections continue to shed virus, and thus remain transmissible, or can recrudesce, as has been observed in salamanders (Brunner et al. 2004) and African clawed frogs *Xenopus laevis* (Robert et al. 2014).

We conducted 2 experiments with American bullfrogs to describe the infection intensity and shedding of ranavirus, firstly, through time as a function of dose, and secondly, across temperatures. We then assessed the correlation between intensity of infection and viral shedding and then estimated the performance of non-lethal methods of ranavirus surveillance (i.e. tail or toe clips, swabs, and environmental DNA [eDNA]). Lastly, we screened shipments of American bullfrogs sent from several suppliers for ranavirus DNA to determine whether we could detect subclinical infections with non-lethal samples. Overall, our results suggest that American bullfrogs may be important carriers of inapparent, yet transmissible, ranavirus infections for long periods. However, non-lethal methods of sampling shipments have a reasonable chance of detecting carriers.

2. MATERIALS AND METHODS

2.1. Expt 1: ranavirus infections through time in tadpoles

American bullfrog eggs were collected from a pond near Lyle, Washington (USA), at which the owner was attempting to eradicate American bullfrogs. The eggs, which had been collected from multiple locations in the pond and likely from many females, were transported to Washington State University's (WSU) Pullman campus in a cooler and housed in 20 gallon (~75 l) aquaria filled with spring water. Upon hatching, the tadpoles were fed pressed algae discs ad libitum (Hikari Sales). To remove organic wastes and excess nitrogen, water in each tank was filtered through activated charcoal and biotic filters (AquaClear 20 Power Filters, Rolf C Hagen) and changed every week (~50% water change). The animal rooms were maintained at ~20°C at densities <100 ind. aquarium^{-1}.

Tadpoles that attained Gosner (1960) stages 25–28 were randomly assigned to 1 of 3 virus exposure treatments: 150 to a low-dose (10^3 pfu ml^{-1}, diluted in aged tap water), 150 to a high-dose (10^5 pfu ml^{-1}), and 90 to a mock exposure (with an equivalent amount of cell culture media). The ranavirus used in this and the following experiment was the second passage of AEC37, a frog virus 3 (FV3)-like ranavirus isolated from wood frogs in New York State (Brunner et al. 2011) and grown and titered by plaque assay in fathead minnow cells. The tadpoles were placed in individual 900 ml plastic containers filled with 400 ml of water with the appropriate concentration of ranavirus and fitted with lids with holes drilled through them for air circulation. After 24 h, during which animals were not fed, each tadpole was removed with a piece of window screen, briefly rinsed with aged tap water, and transferred to a new container with clean, aged tap water and ~1/4 of an algae disc (effectively fed ad libitum). Animals were moved to new containers with clean water and food every 7 d. Screens and gloves were changed between individuals to prevent cross-contamination. All containers and equipment were disinfected in 10% commercial bleach solution for >30 min, rinsed, and dried between uses.

At the start of the experiment, individuals were randomly assigned to 1 of 10 sampling times (2, 4, 6, 8, 14, 21, 28, 35, 42, and 49 d post exposure) with 15 low-dose, 15 high-dose, and 9 control tadpoles per time point. The day prior to sampling, each tadpole was placed in a new container with clean water (but without food to avoid clogging eDNA filters) to collect virus shed just in the prior 24 h. Animals that died before their assigned sampling day were removed from their container and samples collected in a similar fashion on the day of their death.

2.2. Expt 2: ranavirus shedding and detection across temperatures in juveniles

Juvenile American bullfrogs (n = 21, mean snout–vent length [SVL] ~60 mm, mean mass ~20 g) were obtained from a biological supplier (Rana Ranch Bullfrogs) for a herpetology class experiment, and an additional 5 were screened and tested negative for ranavirus DNA (see Section 2.3 and Biological Supplier B in Table 1). The animals were individually housed in 10 gallon (~38 l) glass aquaria filled with 10 cm (12.9 l) of aged tap water treated with Amquel (Kordon) and a plastic haul-out consisting of an inverted paint bucket liner. The class experiment involved measuring feeding rates, escape speeds, and time to exhaustion in animals acclimated to 1 of 6 water temperatures (10–35°C in 5° increments). Three frogs were randomly assigned to each experimental temperature, and an additional 3 frogs were held in aquaria at room temperature (~18°C) and used for demonstration purposes only.

Water temperature was elevated to each level with a variety of heaters: 20°C (Eheim Jäger 25 W adjustable), 25°C (Aqueon 50 W preset to 25.5°; Aqueon Products), 30°C (ViaAqua 50 W adjustable; Commodity Axis), and 35°C (ViaAqua 150 or 200 W adjustable). Water temperatures were decreased in the 10° and 15°C treatments by running cold water in a closed loop from an ice-filled cooler through silicon aquarium tubing looped through the 10°C and then 15°C aquaria, returning to the cooler. Ice was changed daily. Each frog was fed 5 crickets per week following a ~50% water change.

When the class experiment was finished, 15 d after arrival, the animals in their aquaria were transferred to an environmental chamber in a research laboratory and the temperature conditions and feeding regime continued. After a total of 31 d held at these temperatures, each frog was intraperitoneally injected with 100 µl of ranavirus diluted 1:10 in molecular grade water for a total of >10^4 pfu, except for the 3 frogs held at ambient temperatures, which were injected with water only and served as negative controls. At 24 d post exposure, each frog was housed for 24 h in a 900 ml plastic container with lid filled with 500 ml of aged, treated tap water, which had been placed in the container the previous day to allow the

temperature to equilibrate. In this way, the eDNA sample collected (see Section 2.4) included virus shed over a 24 h period. The American bullfrogs were then euthanized and sampled the next day.

2.3. Observational Study: ranavirus infections in shipments from commercial suppliers

From January through May 2016, 5 shipments of American bullfrogs from commercial suppliers were received at WSU (Table 1). Upon arrival, tadpoles were immediately euthanized and sampled. Metamorphosed frogs, which were sent in moist sphagnum moss, were first group-housed by shipment in a glass aquarium with ~2 l of aged tap water for 24 h, from which eDNA could be filtered, before being euthanized and sampled. Note that 1 shipment included individuals that were involved in Expt 2, which were separated before the other 5 were placed in a single aquarium for 24 h (Biological Supplier B, Table 1).

2.4. Sampling methods

Tadpoles were euthanized by immersion in ~200 ml of 250 mg l^{-1} buffered benzocaine HCl solution in clean containers for 10 min beyond cessation of righting behavior (Leary et al. 2013). Metamorphosed frogs were euthanized with a topical benzocaine gel (20% w v^{-1}; Orajel, Church and Dwight) applied to the head. Then a swab was turned for 5 rotations in the oral cavity and surrounding skin of tadpoles (Expt 1) or 5 times around the cloaca of terrestrial morphs (Expt 2 and Observational Study). After swabbing, a small, 2–4 mm distal section of the tail (for tadpoles) or toe (metamorphosed frogs) was cut off. Lastly, a small (~4–8 mm^3) amount of liver and nephritic tissues, which we call kidneys for simplicity, was dissected out and combined in a single tube. In between samples, the dissecting scissors or scalpel and forceps were wiped clean and then flame sterilized with an ethanol lamp or lighter for >15 s.

Samples of eDNA were collected by vacuum filtering water in which the animal was housed through a 0.22 µm cellulose nitrate disposable filter (Nalgene 145-2020, ThermoFisher). The volume of water filtered was reduced from 250 ml in Expt 1 and 125 ml in Expt 2 and the Observational Study to reduce issues with filters clogging (note that the quantities of ranavirus detected in eDNA filters is reported as the number per 125 ml) Sloughed skin, bacteria, etc. built up quickly in the water of dead tadpoles, so filters often clogged. If unfiltered water remained after being vacuum-filtered overnight, the filter disc was collected and the amount filtered noted. In the Observational Study, 3 eDNA filter samples were collected from the water from each shipment in addition to a filter negative sample (spring water purchased from a local grocery store). For all samples, the forceps used to transfer the filter disc to a storage tube were disinfected between uses with 50% commercial bleach for >90 s and then rinsed in fresh water. Gloves were changed between samples, and any equipment that might have been contaminated was disinfected with bleach before re-use. All filtering was done in the room with the animals or the surrounding laboratory, which is free of PCR products. All samples were stored in 1.5 ml snap-cap microcentrifuge tubes and frozen at –80°C.

2.5. Ranavirus detection

DNA was extracted from the liver+kidney samples and tail clips with Qiagen DNeasy Blood and Tissue kits following the manufacturer's protocol. eDNA was extracted from half of each filter sample using the QIAshredder/DNeasy Blood and Tissue DNA extraction kit method described by Goldberg et al. (2011). DNA was extracted from swabs collected in Expt 1 using PrepMan Ultra (Applied Biosystems), following the manufacturer's protocol. However, PCR

Table 1. Number of positive/samples tested from American bullfrogs in 5 shipments. Biological suppliers were companies that regularly provide animals for research and education. Internet sources sold directly to consumers via eBay.com. SVL: snout–vent length

Supplier	Live stage (average Gosner stage)	Average SVL (mm)	Liver+ kidney	Swab	Tail/ toe clip	eDNA filter
Internet source A	Larva (35.8)	31.3	0/9	0/9	0/9	0/3
Internet source B	Larva (33.1)	30.8	5/13	2/13	4/13	3/3
Biological supplier A	Larva (35.6)	32.7	0/7	0/7	0/7	0/3
Biological supplier B	Metamorphosed	53.7	0/5	1/5[a]	0/5	0/3
Biological supplier C	Metamorphosed	140	3/5	5/5	3/5	3/3

[a]The one positive swab from Biological supplier B was, by all indications, a false positive

inhibition was common in these samples (detected using an Exogenous Internal Positive Control [Applied Biosystems, ThermoFisher] in the third well of each sample) unless the extracted template DNA was diluted 1:10 in water. Therefore, DNA was extracted from swabs collected in Expt 2 and the Observational Study with DNeasy kits. All steps in the sample extraction processes involving open tubes or pipetting took place in a PCR cabinet (Esco) with positive pressure from HEPA-filtered air in a room free of PCR products, except the eDNA filters in Expt 2, which were extracted in a laboratory dedicated to low-quantity DNA samples, with additional requirements for entry (see Hall et al. 2016 for details). The hood was disinfected between batches of samples with a 50% bleach solution followed by a germicidal UV light for 15 min. We included samples of liver from American bullfrogs known to be free of ranavirus infection (provided by M. Gray, University of Tennessee, Knoxville) or chicken as negative extraction controls.

Ranavirus DNA was detected and quantified using a quantitative Taqman real-time PCR (qPCR) reaction that amplifies a 96 bp region of the major capsid protein gene of all known members of the genus *Ranavirus* except for the Santee-Cooper ranavirus lineage of fish and does not amplify members of other genera in the family *Iridoviridae*, including *Lymphocystivirus* and *Megalocytivirus* (Stilwell et al. 2018). Each sample was run in triplicate 20 µl reactions using the TaqMan Gene Expression Master Mix (Applied Biosystems) on 96-well plates with 5 µl of DNA template and a final concentration of 1.8 µM of the forward and reverse primers and 900 nM of the probe, run for 10 min at 95°C followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Serial dilutions of gBlock synthetic oligonucleotide sequences containing the target sequence (Integrated DNA Technologies), diluted to a final concentration of 5×10^1 to 5×10^6 copies per reaction, served as the standard against which unknown samples were quantified. Samples with clear amplification in 2 or 3 wells were scored as positive. Those without amplification in any of the wells were scored as negative. Ambiguous samples were re-run, and if at least 1 well showed amplification, the sample was scored as positive. Viral quantities for positive samples are reported as the mean across all wells of the sample, including zeros. We included zeros in the calculations because the analytical limit of detection of this assay is quite low (>90% probability of detecting ~10 copies; Stilwell et al. 2018), detection probabilities are very much concentration dependent (Wilcox et al. 2018), and zeros provide information on the actual concentration of target DNA in a sample. Samples that were dropped or lost were excluded from the relevant analysis, so sample sizes vary slightly among analyses.

2.6. Statistical analyses

Differences in survival in Expt 1 were compared with a log-rank test on Kaplan-Meier survival estimates, accounting for censoring of tadpoles euthanized for sampling. Changes in prevalence through time were analyzed with logistic regressions. Changes in quantities of the viral MCP gene ($\log_{10}$[copy number + 1]) through time, centered on Day 7 to aid fitting (Expt 1), and across temperatures (Expt 2) were described with natural splines regression with 3 degrees of freedom using the 'ns' function in the 'splines' package in R version 3.5.2 (R Core Team 2016). The overall effects of time and treatment in these regressions were assessed with ANOVAs. The relationship between virus titers in non-lethal samples and liver+kidney samples, both log-transformed as above, were analyzed with linear regressions including the main effects of time (again centered on Day 7) and treatment and their interactions with the titer in liver+kidney samples.

Diagnostic test sensitivity and specificity and confidence intervals for each non-lethal sample type were evaluated relative to the 'gold standard' of internal tissues (liver+kidney) using the 'epi.tests' function in the package 'epiR' (Stevenson et al. 2016). The probability of virus detection in samples as a function of time or intensity of infections ($\log_{10}$[copy number + 1] in liver+kidney samples) was analyzed with logistic regression using the bias-reduction method of Firth (1993) as implemented in the 'brglm' function (Kosmidis 2013) to avoid issues with quasi-separation in some analyses.

3. RESULTS

3.1. Expt 1: ranavirus infections through time in tadpoles

Survival varied among treatments ($\chi^2 = 88.047$, $p < 0.001$; Table 2, Fig. 1A,B) — 38.5% of tadpoles died in the high-dose treatment compared with 7.3% in the low-dose treatment — but the difference in mortality between the low-dose and 'mock' exposure treatments (3.3%) was negligible ($\chi^2 = 1.731$, $p < 0.188$). Most tadpoles became infected (Table 2, Fig. 1C,D).

Table 2. Prevalence of infection and numbers of American bullfrog tadpoles positive by 2 criteria: a positive liver+kidney sample only or a positive liver+kidney or 2 or more positive non-lethal samples (tail clip, oral swab, or eDNA filter) in Expt 1. Tadpoles were exposed to a high (10^5 pfu ml^{-1}), low (10^3 pfu ml^{-1}), or 'mock' (low, but unknown; see Sections 2.1 and 3.1 for details) dose of an FV3-like ranavirus. Note that the numbers tested vary because some samples were lost during the experiment or processing

Treatment	Prevalence (positive/tested)		
	Liver+kidney only	Liver+kidney or ≥2 non-lethal samples	Mortality (died/total)
'Mock'	0.516 (32/62)	0.556 (50/90)	0.033 (3/90)
Low	0.623 (71/114)	0.667 (100/150)	0.073 (11/150)
High	0.946 (105/111)	0.959 (142/148)	0.385 (57/148)

Considering only the liver+kidney samples, prevalence of infection increased with dose, from 62.3% in the low-dose (10^3 pfu ml^{-1}) to 94.6% in the high-dose (10^5 pfu ml^{-1}) treatments. If we assume that tadpoles were infected if either the liver+kidney sample or at least 2 non-lethal samples were positive, these values increase slightly to 66.7 and 95.9%, respectively. Ranavirus DNA was detected in internal tissues of all tadpoles that died, including the 'mock'-exposed controls. Unfortunately, over half of the 'mock'-exposed tadpoles tested positive for ranavirus. This was not statistically different from the low-dose treatment (χ^2 = 1.469, p = 0.226), nor were there differences in survival rates (Fig. 1A,B). It is unlikely that half the tadpoles were naturally infected (e.g. as hatchlings), as none of 5 tadpoles that were not used in this experiment were infected when subsequently tested (probability of 0 of 5 infected with prevalence of 50% ≈ $(1-0.5)^5$ = 3%). Contamination in the process of screening animals (e.g. DNA extraction, qPCR) also seems unlikely, as negative extraction controls and

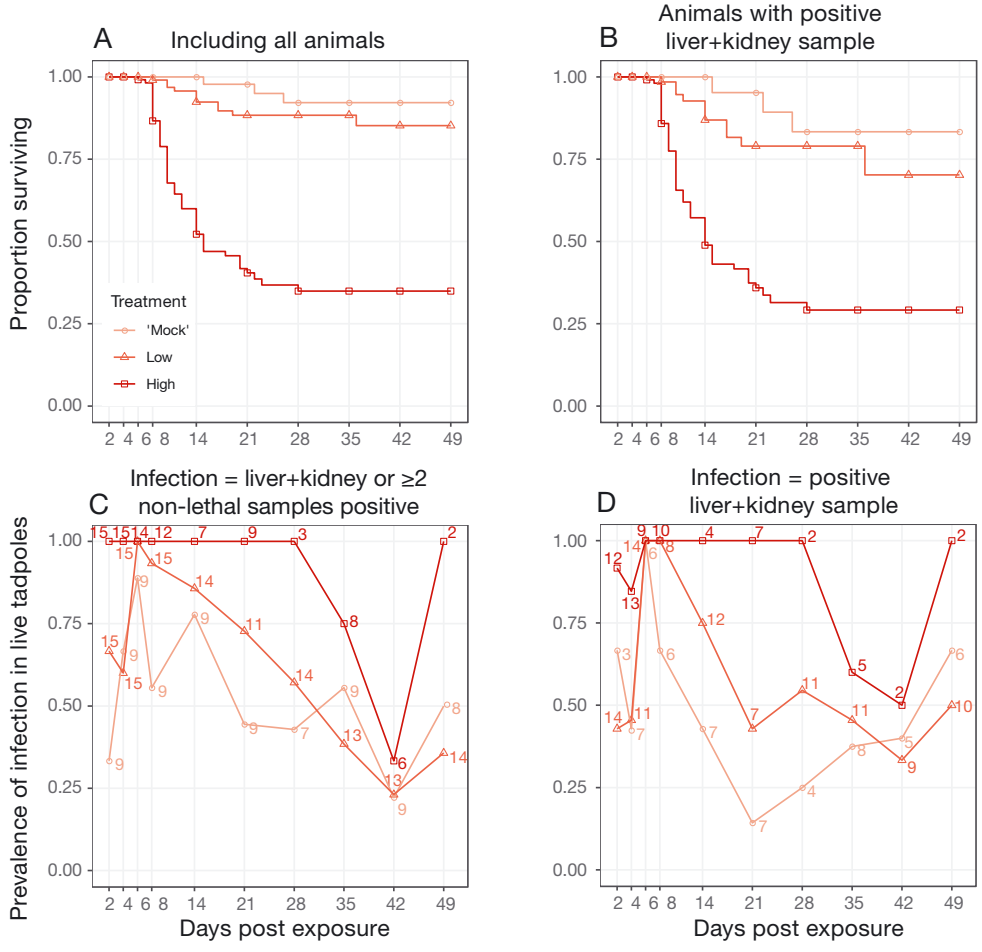

Fig. 1. (A,B) Survival and (C,D) prevalence of infection in American bullfrog tadpoles through time after experimental exposure to a high (10^5 pfu ml^{-1}), low (10^3 pfu ml^{-1}), or 'mock' (low, but unknown) dose of an FV3-like ranavirus. Note that animals that died prior to sampling were excluded from the estimates of prevalence; the numbers of live animals tested are indicated next to each point

no-template controls (water in place of template DNA in qPCR reactions) were all negative in this and other studies. These observations, and the fact that subsequent testing found ranavirus DNA in several aliquots of cell culture media used contemporaneously with this experiment, strongly suggest that our 'mock' exposure was actually an exposure to an unknown, but low, dose of ranavirus, and we treat it as such hereafter.

The odds of infection in live animals (i.e. those euthanized during sampling) tended to decline after ranavirus exposure in the high-dose ($\beta = -0.161 \pm 0.057$, $z = -2.83$, $p = 0.005$) and low-dose ($\beta = -0.055 \pm 0.012$, $z = -4.483$, $p < 0.001$) treatments, but not in the 'mock' treatment ($\beta = -0.022 \pm 0.014$, $z = -1.609$, $p = 0.108$; Fig. 1C). However, infections were still detected for well over 1 mo: 10 of 16, 13 of 40, and 11 of 26 tadpoles sampled after the first month in the high, low, and 'mock' exposures, respectively, had a positive liver+kidney sample or at least 2 positive nonlethal samples (Fig. 1C). If we include only those with a positive liver+kidney sample, the numbers are 6 of 9, 13 of 30, and 9 of 19 tadpoles, respectively (Fig. 1D). These were low-level infections (Fig. 2), but of the 28 tadpoles with positive liver+kidney samples on Days 35, 42, or 49 post exposure, 10 (35.7%) had detectable virus DNA in the water or on oral swabs, suggesting they were still shedding rana-

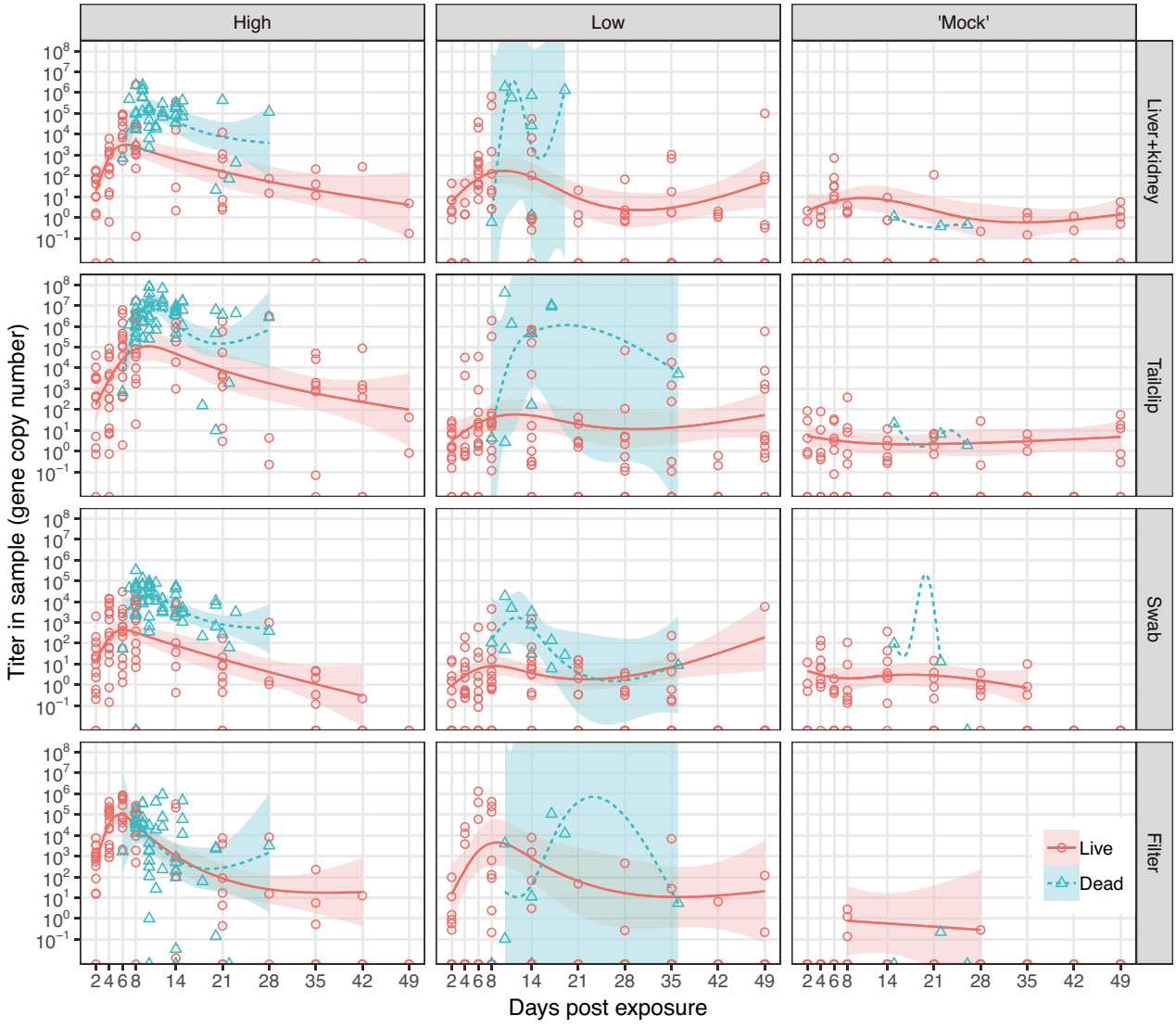

Fig. 2. Titers of ranavirus DNA as measured by qPCR in 4 types of samples collected through time since exposure in American bullfrog tadpoles exposed to a high (10^5 pfu ml^{-1}), low (10^3 pfu ml^{-1}), or 'mock' (low, but unknown) dose of an FV3-like ranavirus. Points along the abscissa represent samples with no ranavirus DNA detected. Lines are natural splines regression fit with 3 df, and the shaded area is the 95% confidence envelope

virus. Note that the apparent increase in prevalence at the last time point was likely an artifact of small sample sizes (Fig. 1); there was no statistical support for a second-order function of time (not shown).

We observed a characteristic pattern to viral titers in live animals through time, generally rising rapidly over the first week post exposure, after which point tadpoles began to die in large numbers (Fig. 1A,B), and then declining more slowly to lower levels (Fig. 2; $F_{3,226}$ = 26.412, p < 0.001). This pattern was most pronounced in the high-dose group, principally because tadpoles in this treatment attained a higher peak in titers than those in other treatments ($F_{2,226}$ = 41.489, p < 0.001). Titers in live animals in the high- and low-dose treatments declined to similar levels by 35–49 d post exposure (mean ± SE = 1.23 ± 1.19 for the high dose, 1.24 ± 1.63 for the low dose on the $\log_{10}$ scale), but animals in the mock exposure treatment had somewhat lower titers (–0.04 ± 0.48, β = –1.266 ± 0.673, t = –1.881, p = 0.072). Individuals that died during the experiment tended to have higher titers than those sampled alive ($F_{1,280}$ = 52.94, p < 0.001), although this was not apparent in the 'mock' treatment (Fig. 2).

There was a strong positive relationship between viral titers in the internal tissues (liver+kidney samples) of live animals and the copy numbers found in filters, swabs, and tail samples (Fig. 3), which suggests that the titers in these non-lethal samples largely reflect the intensity of infection within the animal. Note, however, that while the slopes of the regression between copy numbers in filters and liver+kidney samples (β = 0.948 ± 0.079, t = 12.041, p < 0.001) and tail and liver+kidney samples (β = 0.801 ± 0.083, t = 9.601, p < 0.001) were close to 1, overall, the slope between copy numbers found in swab and liver+kidney samples was much lower (β = 0.528 ± 0.059, t = 8.873, p < 0.001; Fig. 3). There were also changes to the overall pattern in live animals with time since exposure and with treatment. For instance, the slope of filter samples tended to flatten with time since exposure (interaction between liver+

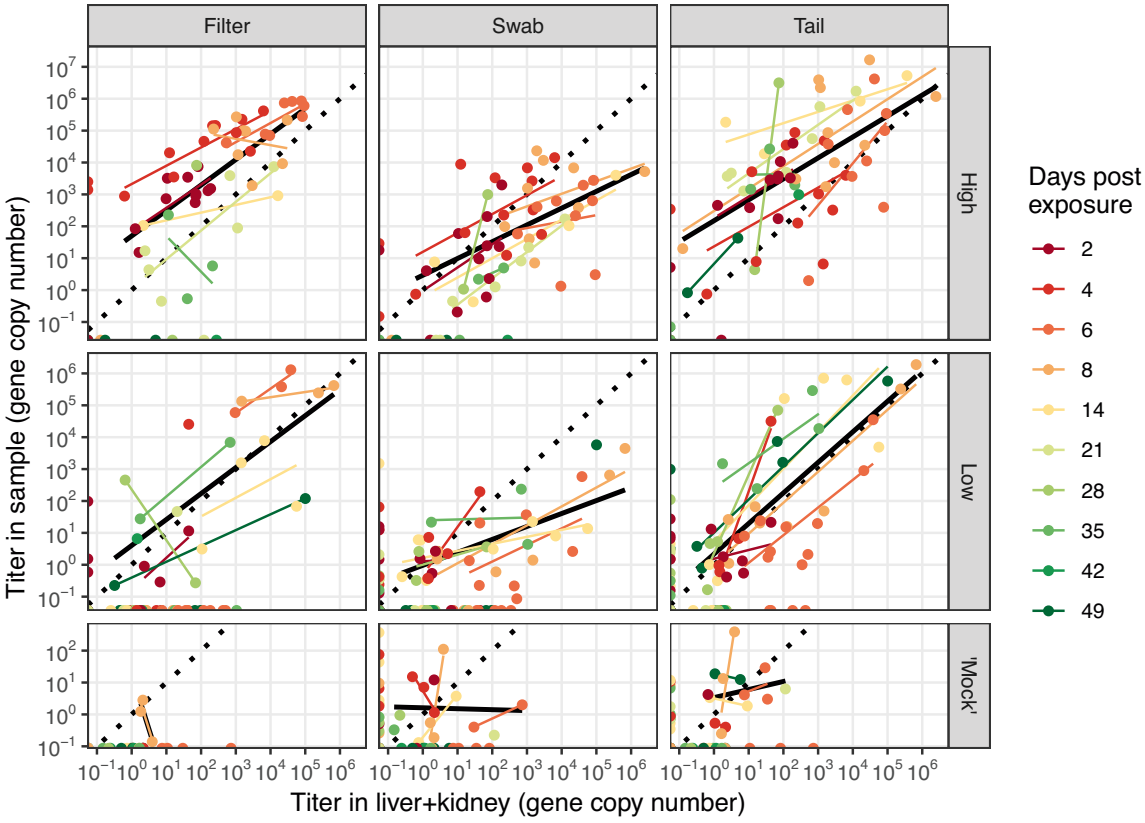

Fig. 3. Titers of ranavirus DNA measured in non-lethal samples (y-axis) from American bullfrog tadpoles exposed to 1 of 3 doses of virus (rows) as a function of titers in the liver+kidney samples collected at the same time (x-axis). Note that individuals that died before sampling are excluded. Colors correspond to the date of sampling. Black solid lines are the best-fit regression line across all sampling dates. Black dotted lines are 1:1 lines for context. Points along the abscissa and ordinate represent samples with no ranavirus DNA detected in the non-lethal sample or liver+kidney sample, respectively

kidney and days post exposure; β = −0.02 ± 0.004, t = −5.589, p < 0.001), and its intercept decreased at the low and 'mock' exposure doses (β = −1.285 ± 0.233, t = −5.516, p < 0.001 and β = −1.107 ± 0.254, t = −4.36, p < 0.001, respectively), all of which implies that virus shedding in water is reduced more rapidly than internal titers. Similarly, the intercept of the line between swab samples and liver+kidney samples tended to decline with time since exposure (β = −0.014 ± 0.004, t = −3.532, p = 0.001), although the slope did not change (interaction: β = 0.003 ± 0.003, t = 1.122, p = 0.263), nor were there main effects of treatment (both p > 0.4). Overall, while the relationship between titers found in non-lethal samples and internal titers varied among treatments and with time in sometimes idiosyncratic ways, they were usually positive (Fig. 3). Moreover, when we excluded negative samples, we found that the relationships were always positive and the interaction terms (i.e. changing slopes) were no longer significant, except in the case of swabs in the 'mock' treatment (not shown). Thus, while more intense infections tended to yield more virus copies in non-lethal samples, there is evidence that this relationship was more complex than a simple, constant ratio.

3.2. Expt 2: ranavirus shedding and detection across temperatures in juveniles

Three American bullfrogs in the temperature experiment died before sampling: 2 held at 35°C and 1 at 25°C. These individuals were excluded from analyses because samples were not collected suitably close to the time of death. All 15 surviving ranavirus-exposed American bullfrogs were infected, regardless of the criterion, and all 3 control American bullfrogs were negative. All individuals appeared to be healthy, maintained normal behaviors, and continued to eat until death or the end of the experiment.

The intensity of infections (copies in the liver+kidney samples) in these experimentally infected juveniles appeared to decline with increasing environmental temperature, which was mirrored to a degree by changing quantities in toe clips (Fig. 4; β = 0.833 ± 0.167, t = 4.985, p < 0.001). The quantity of ranavirus DNA found in filter samples, however, was not significantly related to those in liver+kidney samples (β = −0.364 ± 0.235, t = −1.551, p = 0.145). Only 3 swab samples detected ranavirus DNA, and these titers were very low.

3.3. Observational Study

Two of 5 suppliers sent infected American bullfrogs to WSU: 3 of 5 metamorphosed frogs from one supplier and 5 of 13 tadpoles from a separate supplier, for a total of 8 (19%) of the 42 animals shipped to our lab (Table 1). None showed any outward signs of disease or gross lesions when dissected. The intensities of infection in the liver+kidney samples ranged from $10^{0.691}$–$10^{2.64}$ in the tissues for metamorphosed frogs and from $10^{0.124}$–$10^{2.35}$ copies for tadpoles.

Tail and toe clips detected ranavirus in most of the infected tadpoles (4 of 5) and metamorphosed individuals (3 of 3) shipped from suppliers. Swab samples were less congruent with the results of internal tissues, detecting infection in just 2 of the 5 infected tadpoles, but scoring as positive all 5 metamorphosed frogs from the shipment in which 3 were infected, according to the liver+kidney samples. In addition,

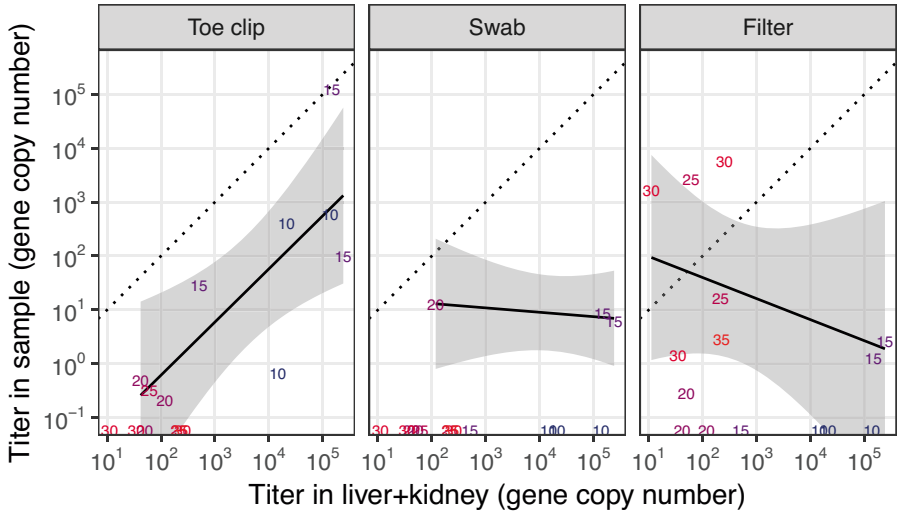

Fig. 4. Titers of ranavirus DNA measured in non-lethal samples (y-axis) from juvenile American bullfrogs as a function of the titer in liver+kidney samples collected at the same time (x-axis). Numerals represent the environmental holding temperature (°C) of each individual. Solid black lines are best-fit regression lines across all temperatures and the shaded areas represent their 95% confidence envelopes. Black dotted lines are 1:1 lines for context. Numbers along the abscissa represent samples with no ranavirus DNA detected in the non-lethal sample

1 of 5 swabs from another supplier in which no other samples were positive produced a false positive with consistent, low-level amplification. It is unclear where this contamination may have come from, but there were no false positives in the eDNA filters, and all 3 filters in both infected shipments detected ranavirus DNA.

3.4. Diagnostic performance

Including data from both experiments and the observational study and using the liver+kidney samples as a gold standard, we found that tail/toe clips had an overall diagnostic sensitivity of 0.831 (95% CI: 0.776–0.878; 187 of 225 tail/toe clips from infected individuals correctly tested positive) and a specificity of 0.714 (95% CI: 0.621–0.796; 80 of 112 tail/toe clips from uninfected individuals correctly tested negative; see Fig. A1 in the Appendix. Swabs were less sensitive than tail clips (0.714, 95% CI: 0.651–0.772), but similarly specific (0.664, 95% CI: 0.566–0.752; Fig. A1). (It is worth noting that we did not attempt to standardize the amount of starting material in our samples, or the amount of DNA in the qPCR reactions, between sample types. These samples may have similar diagnostic performance when provided the same amount of material collected.) While filter samples were less sensitive than the other 2 non-lethal samples (0.61, 95% CI: 0.539–0.678), they were more specific (0.936, 95% CI: 0.857–0.979). Note that there was no evidence that diagnostic performance differed between larval and metamorphosed animals, although with only 28 metamorphosed animals across studies, our ability to detect differences was minimal.

Restricting ourselves to the 266 samples with complete data, we found that 19 animals had at least 2 of the 3 non-lethal samples test positive, but negative liver+kidney samples. Treating the collection of non-lethal samples as the gold standard, liver+kidney samples would thus have a diagnostic sensitivity of 0.879 (95% CI: 0.817–0.926), suggesting that even internal tissues samples are not necessarily perfectly sensitive. If we define a positive as having all 3 non-lethal samples positive, we still found 2 apparent false negative liver+kidney samples and a diagnostic sensitivity of 0.98 (95% CI: 0.931–0.998). The lack of concordance between internal tissues and non-lethal samples tended to occur when viral copy numbers were relatively low.

The probability of detecting infections (sensitivity) in non-lethal samples increased with the intensity of infection in the host (Fig. 5). The odds of detection in tail/toe clips increased 3.49-fold with every order of magnitude increase in the quantity of ranavirus in liver+kidney samples ($\beta = 1.25 \pm 0.18$, $z = 6.956$, $p < 0.001$), and 2.08- and 2.59-fold in swabs and eDNA filters, respectively (swabs: $\beta = 0.731 \pm 0.1$, $z = 7.338$, $p < 0.001$; eDNA: $\beta = 0.951 \pm 0.113$, $z = 8.403$, $p < 0.001$). However, the precise relationship between sensitivity and the intensity of infections varied with exposure dose; sensitivity was greater in tadpoles exposed to a high dose of ranavirus, even if the titer found in the liver+kidney sample was low (Fig. 5, top row of facets). Moreover, there was no clear relationship between sensitivity and the intensity of the infection in the juveniles in Expt 2 or the tadpoles and adults shipped to us (Fig. 5, bottom 2 rows of facets), although sample sizes were low.

Diagnostic sensitivity also decreased with time since exposure, while specificity increased (Fig. S1), in large part, but not entirely, due to changes in the intensity of infection. When the intensity of infection (titers in liver+kidney samples) was included in logistic regressions of virus detection in the non-lethal samples, the effect of time since exposure was small, but still significant, for tail clips ($\beta = -0.027 \pm 0.011$, $z = -2.506$, $p = 0.012$), somewhat larger for filters ($\beta = -0.037 \pm 0.014$, $z = -2.557$, $p = 0.011$), and fairly large for swabs ($\beta = -1.22 \pm 0.465$, $z = -2.626$, $p = 0.009$), whereby the odds of detecting infection in swabs declined 2.03-fold every 10 d, even after accounting for infection intensity (Fig. S1).

4. DISCUSSION

American bullfrogs are thought to be key players in the spread and spillover of ranaviruses, and other pathogens around the world (Mazzoni et al. 2009, Schloegel et al. 2009, Une et al. 2009, Sharifian-Fard et al. 2011, Price et al. 2016, Saucedo et al. 2019). Yet for all their importance, few key details about their epidemiology are well established. For instance, while American bullfrogs are often described as 'carriers,' both tadpoles and adults are largely refractory to experimental exposures to ranaviruses (Clark et al. 1968, Hoverman et al. 2011) and even when infected are unlikely to die (Gray et al. 2012). However, ranavirus infections are not uncommon in at least some American bullfrog populations (Gray et al. 2007, Hoverman et al. 2012, but see Sharifian-Fard et al. 2011, Martel et al. 2013, Sekowska et al. 2014), and die-offs involving American bullfrogs have been noted around the world (e.g. Mazzoni et

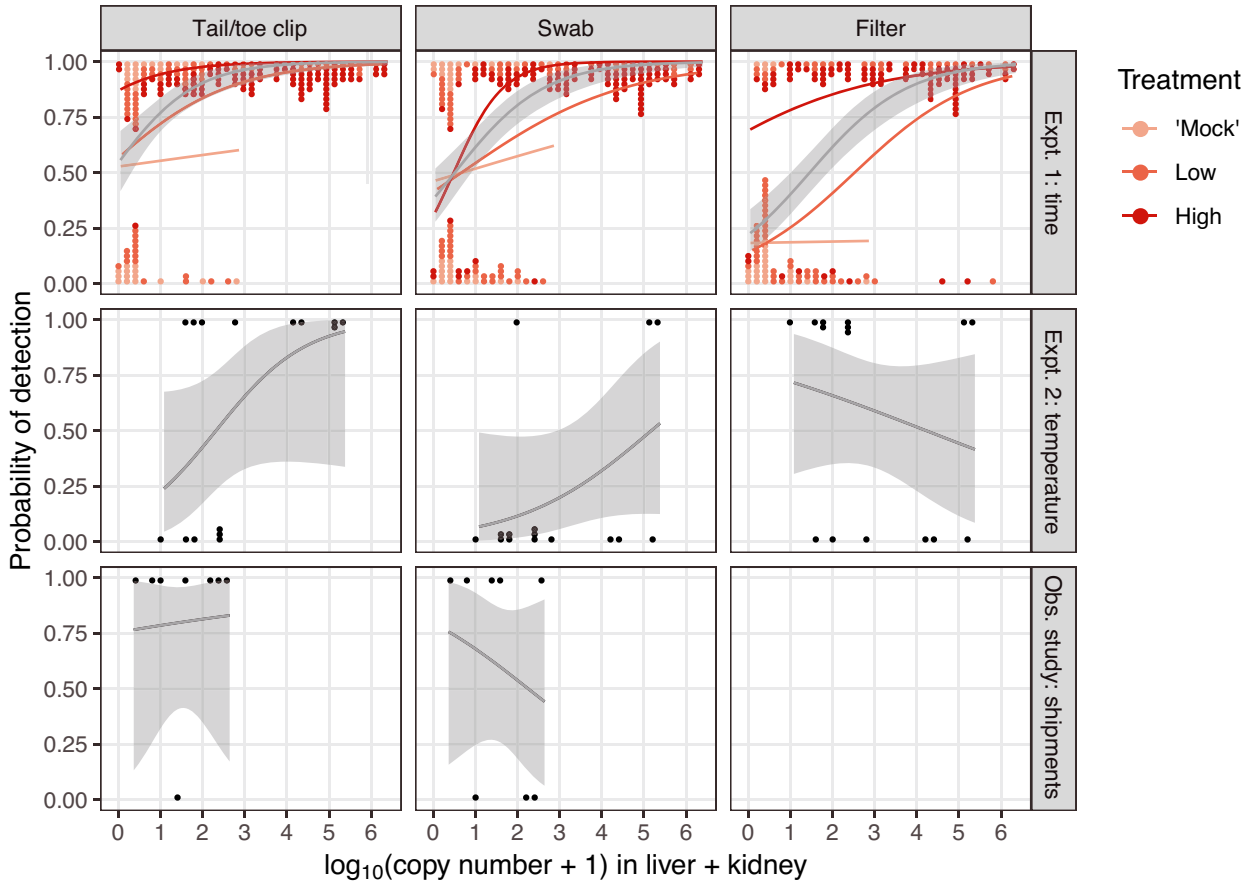

Fig. 5. Relationship between ranavirus detection in non-lethal samples (columns) and the intensity of infection (titer in liver+kidney) in American bullfrog tadpoles (Expt 1), juveniles (Expt 2), or tadpoles and adults (Observational Study). Best-fit logistic regression lines are in black with the 95% confidence envelope in gray. In the first experiment, the lines are also fit to each dose treatment separately (colored lines, including the contaminated 'mock'-exposure). Marginal histograms (stacked dots) are included to show the distribution of intensities of infection in individuals with positive (top margin) and negative (bottom margin) non-lethal samples. Note that only individuals in which virus was detected in the kidney are included

al. 2009, Une et al. 2009). So even the basic question of whether American bullfrogs are easily infected or killed by ranavirus infections remains open. Our experimental results collectively suggest that (1) American bullfrogs may be important carriers of inapparent, yet transmissible ranavirus infections for long periods, (2) the intensities of infections are sensitive to temperature, and (3) detecting low-level, persistent infections with non-lethal samples may prove challenging. They also highlight the importance of infection intensities to the outcome and transmissibility of ranavirus infections.

We found that American bullfrogs were more susceptible to infection than in prior experiments (Clark et al. 1968, Hoverman et al. 2011). A majority of the tadpoles were infected by exposure to even a moderate dose of virus (10^3 pfu ml^{-1}; Table 2), in contrast to 0 or 10% in a similar waterbath exposure (Hoverman et al. 2011). Gray et al. (2012) found a maximum of 69% of tadpoles exposed to 10^6 pfu orally became infected, which is much lower than the ~95% we observed at a similar dose, albeit by waterbath. These differences might be attributable to differences in methods (e.g. room temperature of 20°C here vs. 23°C in the other studies), virus strains, or the details of virus detection. Gray et al. (2012), for instance, used a much more conservative cycle threshold (Ct) for scoring positives of 30 compared with Ct ≤ 40 in the present study, which corresponds to a difference of roughly 3 orders of magnitude in the minimum titers that would be scored as positive under ideal conditions. While our study is not designed to sort out ideal qPCR settings, we believe our more liberal standard is reasonable given the general concordance among multiple samples and would, by definition, be more sensitive. In contrast, overly conserva-

tive diagnostic criteria could lead to the potentially false impression that American bullfrogs, or other host species, are especially resistant to ranavirus infections. In any case, the level of mortality we observed in the low dose was similar to that observed by Hoverman et al. (2011) at a similar dose; most American bullfrog tadpoles do not die from ranavirus infections unless exposed to much higher doses.

Similar to prior work, we found that the proportion of tadpoles infected declined with time since exposure (Gray et al. 2012, Hoverman et al. 2019), suggesting that infections were cleared. While titers in internal tissues initially increased rapidly, presumably due to viral replication, they declined in the following weeks by 2 to 3 orders of magnitude, on average (Fig. 2), presumably due to viral clearance by the immune system (see also Mihaljevic et al. 2019). It also seems that infections beginning from higher doses of inoculum were more difficult for the host's immune system to control — peak viral titers were higher in the high-dose treatment than the low-dose and 'mock' treatments, and prevalence of infections declined more slowly. Perhaps constitutive levels of immune molecules (e.g. interferon; Grayfer et al. 2014, 2015) are able to control viral growth when it begins from low virus densities, but are overwhelmed by higher doses of inoculum before additional immune responses can be mounted. Mihaljevic et al. (2019) fit mathematical models to the time series data in Expt 1, and provide further discussion of the dynamics between viral growth and the host's immune system.

Both the growth rate of the virus as well as the host's immune response are temperature dependent (reviewed by Brunner et al. 2015), so environmental temperature can have strong, but idiosyncratic, influences on the outcome of ranavirus infections (Rojas et al. 2005, Brand et al. 2016). Here we found that infections were less intense in juvenile American bullfrogs held at warmer temperatures (Fig. 4), suggesting that the immune system responds more strongly to temperature than the virus, although none cleared their infection within 24 d. In contrast to our expectations based on the correlation between infection intensity and shedding (Fig. 3) and the strong temperature dependence of virus degradation (Brunner & Yarber 2018), we found more virus in water at warmer temperatures (Fig. 4). We suspect that the frogs might have simply been excreting more given the higher metabolic rates at warmer temperatures — American bullfrog eDNA in the water increased with temperature (data not shown) — but this needs further research.

In any case, while viral titers in internal tissues declined with time, ranavirus DNA remained detectable in 40% (34 of 82) of live tadpoles sampled 35, 42, or 49 d post exposure, none of which had overt signs of disease. While we cannot directly evaluate whether the viral DNA detected represents intact, potentially replicating virus, it seems a reasonable assumption (Morales et al. 2010, Crespi et al. 2015). Moreover, we found ranavirus DNA in swabs and water filters from roughly a third of tadpoles sampled beyond 1 mo post exposure, suggesting that even low-level infections were active and continued to shed virus. Thus, while it appears that American bullfrog tadpoles can usually clear or at least control ranavirus growth and density, our data clearly suggest they can also harbor persistent, subclinical, potentially infectious ranavirus infections.

Subclinical ranavirus infections have been found previously in metamorphosed American bullfrogs (Wolf et al. 1969) and recently in co-housed American bullfrog and green frog *Lithobates clamitans* tadpoles (Hoverman et al. 2019). Long-lived subclinical infections have also been detected in larval and metamorphosed salamanders, which could be made to recrudesce under presumably stressful housing conditions (Brunner et al. 2004), in adult male wood frogs *L. sylvaticus* returning to wetlands to breed (Crespi et al. 2015), and in *Xenopus laevis* purchased from several commercial suppliers (Robert et al. 2007). Robert et al. (2007) found that ranavirus particles persist in peritoneal leukocytes of *X. laevis* juveniles for weeks to months, and these inapparent infections could be made to recrudesce by eliciting an inflammatory immune response (Morales et al. 2010, Robert et al. 2014). Subclinical infections with ranaviruses may thus be more common than previously believed, even among species thought to be largely resistant to infection.

The risk of infection from subclinically infected American bullfrogs may, however, be small in the absence of stressors or immune responses that elicit recrudescence. The amount of virus shed onto the skin or into the water generally correlated with the intensity of infection measured in internal tissues (Fig. 3), and both infection intensities and viral titers in swabs and water filters tended to decline with time since exposure (Fig. 2). Given that probability of infection is generally dose-dependent (Brunner et al. 2005, Duffus et al. 2008, Forzán et al. 2017), the low-level, persistent infections we observed would likely have little chance of infecting naïve hosts. That said, the stress of being housed at high densities (e.g. in ranaculture facilities) or being shipped might lead

otherwise low-level infections to grow to more transmissible levels. Some of the infected animals in shipments to our lab had moderate virus titers, which is consistent with this hypothesis.

The quantitative nature of ranavirus infections is also important for detecting infections. While numerous studies have evaluated the performance of various diagnostic methods of detecting ranaviruses in lethal and non-lethal samples (reviewed by Miller et al. 2015), virtually all treat infection status as binary, lumping together both weakly and intensely infected individuals. However, the intensity of infection varies over several orders of magnitude in time and among individuals (Fig. 2), and so diagnostic sensitivity and specificity vary as well (Figs. 5 & A1; Greer & Collins 2007, Gray et al. 2012). At least in the context of preventing the continued movement and spread of ranavirus in trade and captive settings, we should aim to detect inapparent infections with low-to-moderate intensities, if for no other reason than that individuals with more intense infections are more likely to exhibit obvious signs of disease and die (Brunner et al. 2005, Brunner & Collins 2009). Low-level, persistent infections are likely to persist in breeding colonies, zoos, ranaculture, etc., or be shipped elsewhere (e.g. Robert et al. 2007, Schloegel et al. 2009). Thus, while our estimates of diagnostic performance of swabs and tail/toe clips overall (Fig. A1) are consistent with those of prior studies (Greer & Collins 2007, Gray et al. 2012), we think these estimates of diagnostic sensitivity are overly high in the context of trade.

We found that all 3 non-lethal sampling methods had only moderate chances of detecting low-intensity infections such as those observed several weeks beyond exposure (Figs. 2 & A1), which would make the goal of detecting subclinical, persistent infections very difficult. This may be eased by the fact that our goal is not to detect every infection, but rather to detect at least one. Presumably a single positive test would lead to further investigation, which would better quantify the scale of the problem and reveal false positives. Sampling designs are a topic for other papers (e.g. Gray et al. 2015), but we note that filtering water to collect eDNA may be more efficient than individual-level sampling (i.e. tail/toe clips, swabs). eDNA at least theoretically samples from an entire population (e.g. shipment, tank in captivity), so while individual eDNA samples may have lower sensitivity than tail clips, they avoid the problem of not including what may be rare infections in samples.

Collectively, our results suggest that American bullfrogs are not uniquely resistant to ranavirus infections nor especially likely to be carriers, although they, too, can maintain persistent, potentially transmissible, subclinical infections. Rather, what may make American bullfrogs particularly effective at spreading ranavirus infections around the world is their incredible abundance in trade and status as invaders. By improving conditions in captive settings and trade that might facilitate transmission and viral amplification in hosts and implementing routine surveillance for ranaviruses and other pathogens, we may be able to minimize the threat of pathogen spillover.

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Appendix.

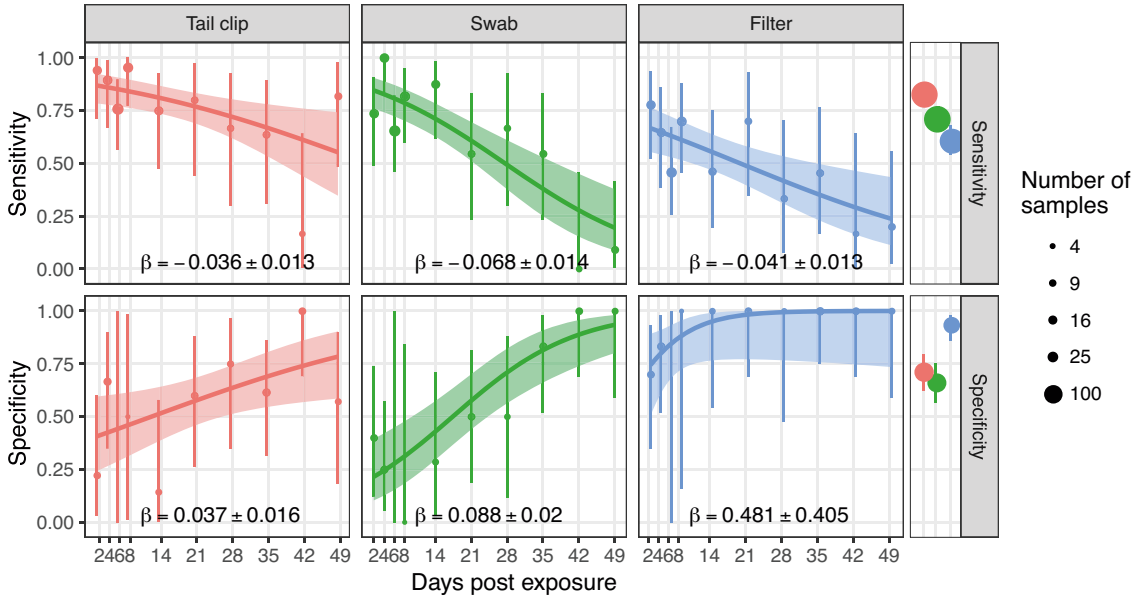

Fig A1. Diagnostic sensitivity and specificity over time since experimental exposure to a frog virus 3 (FV3)-like Ranavirus for each of three sample types collected from American bullfrog tadpoles relative to the gold standard of internal tissues (liver+kidney) in Expt 1. The curves and regression coefficients in each panel come from logistic regressions fit to each metric and sample type and the shading their 95% confidence envelopes. The vertical lines are the 95% confidence interval (Wilson 1927) around the proportions (points) of tests correctly testing positive (sensitivity) or negative (specificity).The right-most panels show sensitivity and specificity across the whole experiment

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