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LOW DETECTION OF RANAVIRUS DNA IN WILD POSTMETAMORPHIC GREEN FROGS, *RANA* (*LITHOBATES*) *CLAMITANS*, DESPITE PREVIOUS OR CONCURRENT TADPOLE MORTALITY

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ABSTRACT: Ranavirus (Iridoviridae) infection is a significant cause of mortality in amphibians. Detection of infected individuals, particularly carriers, is necessary to prevent and control outbreaks. Recently, the use of toe clips to detect ranavirus infection through PCR was proposed as an alternative to the more frequently used lethal liver sampling in green frogs (*Rana* [*Lithobates*] *clamitans*). We attempted reevaluate the use of toe clips, evaluate the potential use of blood onto filter paper and hepatic fine needle aspirates (FNAs) as further alternatives, and explore the adequacy of using green frogs as a target-sampling species when searching for ranavirus infection in the wild. Samples were obtained from 190 postmetamorphic (≥1-yr-old) green frogs from five ponds on Prince Edward Island (PEI), Canada. Three of the ponds had contemporary or recent tadpole mortalities due to Frog Virus 3 (FV3) ranavirus. PCR testing for ranavirus DNA was performed on 190 toe clips, 188 blood samples, 72 hepatic FNAs, and 72 liver tissue samples. Only two frogs were ranavirus-positive: liver and toe clip were positive in one, liver only was positive in the other; all blood and FNAs, including those from the two positive frogs, were negative. Results did not yield a definitive answer on the efficacy of testing each type of sample, but resemble what is found in salamanders infected with *Ambystoma tigrinum* (rana)virus. Findings indicate a low prevalence of FV3 in postmetamorphic green frogs on PEI (≤2.78%) and suggest that green frogs are poor reservoirs (carriers) for the virus.

Key words: Blood, fine-needle aspirate, FV3, liver, PCR, *Rana clamitans*, ranavirus, toe clips.

INTRODUCTION

Amphibian populations worldwide have suffered severe recent declines, partly because of the spread of diseases, including those caused by ranavirus infection (Daszak et al., 1999). Ranaviruses (Iridoviridae) can infect a variety of species of amphibians, fish, and reptiles, but there is some host-dependent susceptibility: Frog Virus 3 (FV3) usually, but not exclusively, infects frogs and toads (Chinchar, 2002) wheras *Ambystoma tigrinum* virus (ATV) tends to cause infection in salamanders (Jancovich et al., 1997). Infection causes acute mortality of tadpoles (98–100%), and can result in severe dermal and visceral lesions and death of adults (Greer and Collins, 2007; Cunningham et al., 2008). Dissemination of ranavirus infection has been enhanced by introduction of foreign amphibian species into naïve populations (Harp and Petranka, 2006).

Prevention of the spread of disease, in the wild or in captivity, requires accurate diagnosis of the infection status of representative individuals in a population. Detection of ranavirus infection can be achieved by PCR testing on liver tissue (adult and tadpole), whole-body samples (usually tadpoles), or tail clips from salamanders and tadpoles (Brunner et al., 2004; Gray et al., 2009). Lethal methods of detection are adverse to threatened or endangered populations and preclude long-term studies on individuals. Recently, toe clips were proposed as nonlethal samples to detect ranavirus infection in wild frogs through PCR (St-Amour and Lesbarreres, 2007). Although a welcomed

alternative, toe-clipping may cause inflammation, necrosis, or decreased survival, so its use is often discouraged (Canadian Council on Animal Care, 2004). Other potentially suitable samples include blood and hepatic fine-needle aspirate (FNA): ranavirus has been detected in circulating lymphocytes (Cunningham et al., 2008) and FNA is an invasive but nonlethal procedure. In the wild, more than one species of amphibian are likely to be present, and thus targeting a particular species may increase or decrease the chances of finding a particular pathogen, depending on how likely that species is to be infected. We evaluate postmetamorphic green frogs (*Rana* [*Lithobates*] *clamitans*) as a target species for the detection of ranavirus in a natural environment, and whether hepatic FNA or blood collection onto specialized filter paper (Smith and Burgoyne, 2004) could replace toe-clipping or liver sampling in detecting ranavirus infection using PCR.

MATERIALS AND METHODS

Postmetamorphic (≥1-yr-old juveniles and adults) green frogs ($n=190$) were collected from five permanent ponds on Prince Edward Island (PEI), Canada (46°12′N, 63°12′W), during August 2010 and July–August 2011. Sampling in 2010 was restricted to a toe clip and blood sample ($n=118$ and 116, respectively), sampling in 2011 included toe-clip, blood, liver, and hepatic FNA samples ($n=72$ of each sample). The ponds were selected based on their abundant green frog population (all ponds) and on the diagnosis of tadpole mortalities due to a ranavirus (ponds BUE, DWM, and EW2; Fig. 1) the previous or contemporary summer (Canadian Cooperative Wildlife Health Centre, unpubl. data). Frogs were captured using dip nets, placed in individual solid plastic containers with breathing holes and enough water to keep them moist, and kept in a shady area until capture was completed and sampling began. All equipment was disinfected between captures using a spray of 5% bleach (sodium hypochlorite) solution and rinsed after at least 30 sec of contact (The Australian Treatment Abatement Plan, 2006).

Each frog was similarly sampled to collect blood and a toe clip in 2010. A few drops of 2% lidocaine (Lurocaine, Vétoquinol N. A. Inc., Quebec, Canada) were applied with a syringe onto the first digit of the left caudal limb (Fig. 2). While the lidocaine numbed the area, blood was collected by puncture of the facial (maxillary) vein with a 25-gauge needle and recovered onto a heparinized capillary tube (Microhematocrit Capillary Tubes, Fisher Scientific, Pittsburgh, Pennsylvania, USA; Fig. 3; Forzán et al., 2012). Blood was immediately transferred to an FTA™ Minicard (Whatman-GE Healthcare UK Limited, Buckinghamshire, UK; Fig. 3). Once dry, the FTA card was placed in a multi-barrier pouch with a desiccant (Whatman-GE Healthcare), which was instantly sealed. The first digit, along with a small amount of webbing, was then amputated, holding it gently with tissue forceps and cutting swiftly with Stevens curved tenotomy scissors (Fig. 2); the toe clip was placed in a 2-mL screw-capped microcentrifuge tube with 70% ethanol. The amputation site was sprayed with Bactine antiseptic (Bayer Inc., Ontario, Canada); the frog was placed back in the plastic container and, after a few minutes of demonstrating no adverse effects from the procedures, released back into the pond. In only two cases were we unable to extract blood from a frog.

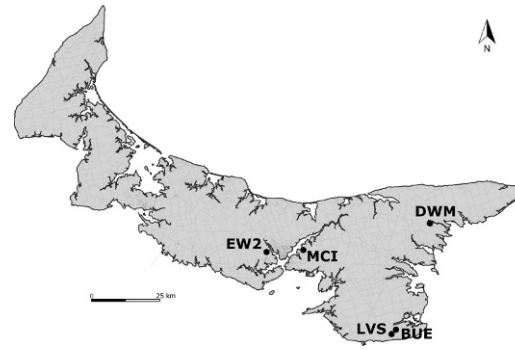

FIGURE 1. Map of Prince Edward Island, Canada, showing ponds were green frogs (*Rana* [*Lithobates*] *clamitans*) were sampled and tested for ranaviruses in 2010 and 2011: MCI ($n=30$ frogs), BUE ($n=57$), LVS ($n=35$), DWM ($n=59$), and EW2 ($n=9$). Scale bar=25 km.

Collection of samples in 2011 varied slightly: it included euthanasia and additional collection of liver tissue and hepatic FNA. Frogs were collected and bled as per description above; after bleeding, each frog was euthanized by applying 20% benzocaine (Orajel PM, Church & Dwight Co., Ontario, Canada) gel (Altig, 1980) to its ventral abdomen and inner thighs using a wooden tongue depressor. Once the frog became nonresponsive, toe-clipping was performed, and the frog placed in

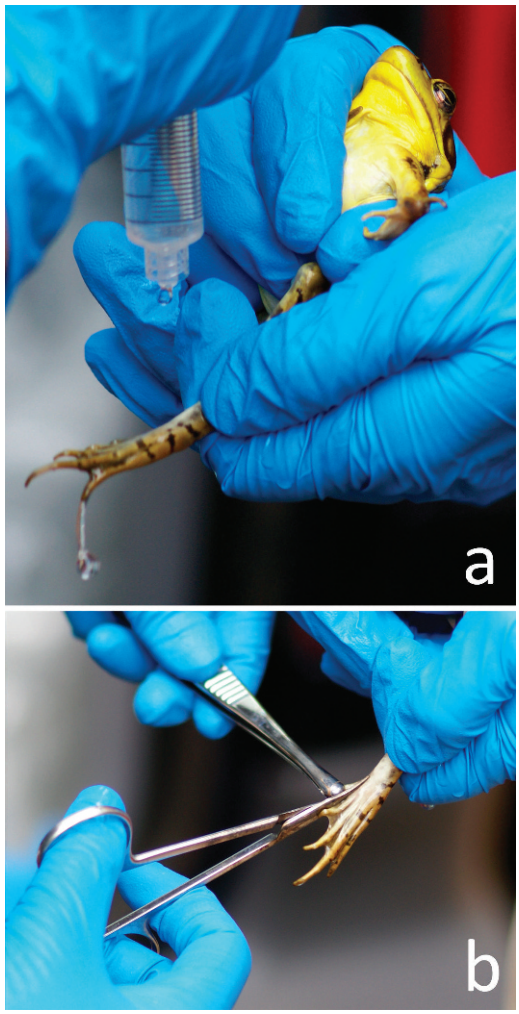

FIGURE 2. Toe-clipping of a green frog (*Rana* [*Lithobates*] *clamitans*) for sampling and testing for ranaviruses. (a) Lidocaine is applied to the left foot of a green frog. (b) The first digit is amputated with tissue forceps and Stevens curved tenotomy scissors.

a sealable plastic bag until death. Dead frogs were kept in a Styrofoam cooler with ice packs and transported to the necropsy room (Atlantic Veterinary College, Charlottetown, Canada) where weighing, FNA, and tissue collection were performed. The FNA was obtained using a 5-cc syringe with a 22-gauge needle; collected material was expelled from the syringe onto an FTA card which was dried and subsequently placed in a sealed pack (Whatman-GE Healthcare). The coelomic cavity was incised with a new scalpel blade, and a small portion of liver was collected, using forceps and that same blade, and placed in a microcentrifuge tube with 70% ethanol. Samples of spleen and kidney were also obtained and preserved in microcentrifuge tubes at −80 C. Nine kidney samples were later thawed to obtain a subsample that was placed in 70% ethanol for PCR testing. The rest of the carcass was placed in 10% buffered formalin. Unlike in 2010, when blood was not obtained from two sampled frogs, in 2011 all samples (blood, toe clip, hepatic FNA, liver and kidney tissues) were collected from each of the 72 frogs collected.

The surgical instruments used for toe-clipping in the field (2010 and 2011) were sterilized between each frog with a dip in 70% alcohol immediately followed by exposure to the open flame of a wickless metal alcohol burner (model 97-5320, C&A Scientific, Manassas, Virginia, USA) or a candle, and kept on a clean stainless steel tray while not in use. A new tongue depressor was used to apply the euthanizing benzocaine gel to each frog. The forceps used to collect the liver, kidney, and spleen samples were sterilized with a micro-incinerator (Bacti-cinerator, McCormick Scientific LLC, St. Louis, Missouri, USA) and a new scalpel blade was used for each frog. Both in the field and in the necropsy room, specific people restrained the frogs, performed the sampling, or handled the samples to avoid

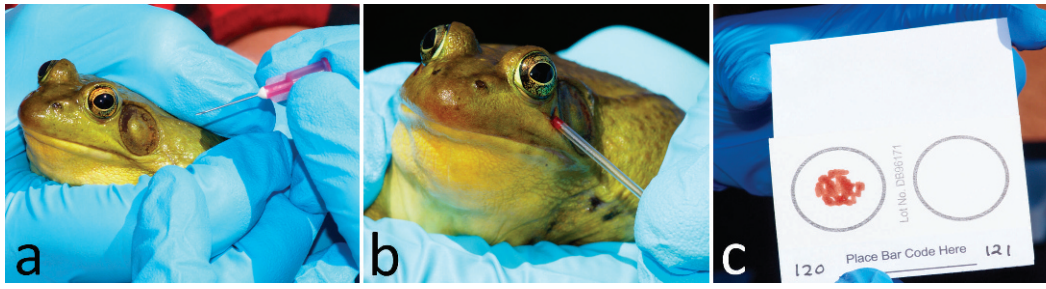

FIGURE 3. Blood sampling of a green frog (*Rana* [*Lithobates*] *clamitans*) for testing for ranaviruses.(a) Bleeding is elicited by puncturing the facial vein with a needle. (b) Blood is collected into a heparinized capillary tube. (c) Blood is immediately transferred onto filter paper.

cross-contamination; nitrile gloves were worn by all and discarded after each frog was sampled or euthanized.

Opportunistically, three dead and agonizing green frog tadpoles were collected from pond EW2 either 2 wk prior to sampling (2010) or on the day of sampling (2011). Dead green frog tadpoles had been collected from BUE and DWM the previous summer (2009) when up to eight carcasses were found on a single day in ponds that measured <25 m across; none were collected in the sampling years as the only dead tadpole found (BUE, 3 wk after sampling) was too autolyzed to test. Livers from all collected tadpoles were placed in 70% ethanol to be tested by PCR and, in the case of the EW2 tadpoles found in 2010, the rest of the carcasses were fixed in 10% buffered formalin and processed routinely for histopathologic examination following the routine procedure for wildlife mortality investigation of the Canadian Cooperative Wildlife Health Centre, Atlantic region.

In the laboratory (Pisces Molecular, Boulder, Colorado, USA), the skin and soft tissue removed from each toe, a cut-out piece of each FTA card containing blood or hepatic FNA material, and a portion of each liver and kidney sampled (including livers of moribund tadpoles from pond EW2) were individually transferred into tissue lysis buffer. Total DNA was extracted from all samples using a spin-column DNA purification procedure (Qiagen DNeasy 96, Qiagen, Valencia, California, USA). The blood, toe clips, hepatic FNA, and liver and kidney tissue samples were tested for the ranavirus major capsid protein gene with single-round PCR amplification (Mao et al., 1997), using primers covering the same region of the MCP gene as the MCP1 assay recommended by the Aquatic Animal Health Code (World Organisation for Animal Health, 2012).

To confirm the quality and quantity of DNA in the samples from 2010, six randomly selected toe clips and eight FTA cards were selected and tested by PCR for DNA from the amphibian mitochondrial gene cytochrome oxidase I (CO1), using primers LepF1 and LepR1 (Smith et al., 2008).

A subset of the positive PCR reactions was sequenced: livers from moribund tadpoles from EW2 (2010 and 2011), DWM, and BUE (2009), and kidney from an adult frog from DWM (2011). The PCR products were run through an ExoSAP-IT™ PCR clean-up reaction (GE Healthcare UK Limited, Buckinghamshire, UK) and used as template in dideoxy-cycle sequencing reactions (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Carlsbad, California, USA) for both forward and reverse strand sequence determination. Both sets of reactions were run on a capillary electrophoresis DNA sequencer (Genetic Analyzer 3130, 36-cm array, POP7; Applied Biosystems) and base-called with Sequence Analysis v5.2 software (Applied Biosystems). For each sample, the resulting forward- and reverse-strand DNA sequence files were assembled into a consensus (forward+reverse) sequence using Vector NTI/Contig Express software (Life Technologies, Grand Island, New York, USA) and aligned for homology with published sequences for various ranaviruses (VectorNTI/AlignX software; Life Technologies). Northern leopard frogs, *Rana* (*Lithobates*) *pipiens*, (two in 2010 and two in 2011) were also opportunistically sampled in one of the ponds (EW2) and tested.

RESULTS

All blood (n=188) and FNA samples (n=72) were negative for ranavirus DNA (Table 1). Two liver samples (n=72) and one toe clip (n=118), both sampled in 2011, were weakly positive, and represented only two positive frogs: an adult frog from the EW2 site (positive liver and toe clip) and an adult from the DWM site (positive liver only). The nine kidney samples tested corresponded to the two positive frogs and seven others, randomly selected from samples from the positive ponds (DWM and EW2). The kidney of the frog with positive signals from the liver and toe clip was negative; the kidney of the frog with positive signal from the liver only was strongly positive. The other seven kidneys were negative.

Prevalence of infection in postmetamorphic green frogs on PEI, based on the number of samples with a positive PCR signal for ranavirus DNA, could be calculated as 3% (95% CI=0.86–9.55%, n=2/72) and 0.5% (95% CI=0.13–2.88%, n=1/190), based on liver and toe clips, respectively. Using results from the second sampling year only, prevalence by toe-clip samples would be 1% (95% CI=0.33–7.40%, n=1/72). Prevalence per pond, if calculated for those sites with one positive frog, would vary highly: DWM, 1/37=3% (95% CI=0.64–13.81%); EW2, 1/7=14% (95% CI=3.19–52.65%). The small numbers of positive samples preclude a proper

TABLE 1. Samples collected from green frogs, *Rana* (*Lithobates*) *clamitans*, during 2010 and 2011 on Prince Edward Island, Canada, and results of corresponding PCR tests for presence of ranaviruses (ponds shown in Fig. 1). Dashes indicate pond not sampled.

	Samples collected per pond								PCR results		
	MCI	BUE		LVS	DWM		EW2		Ranavirus DNA (+/−)		
Sample	2010	2010	2011	2010	2010	2011	2010	2011	2010	2011	Total
Blood	29	28	28	35	22	37	2	7	0/116	0/72	0/188
FNA[a]	—	—	28	—	—	37	—	7	—	0/72	0/72
Toe clip	30	29	28	35	22	37	2	7	0/118	1/72	1/190
Liver	—	—	28	—	—	37	—	7	—	2/72	2/72
Kidney	—	—	0	—	—	4	—	5	—	1/9	1/9
Positive frogs	0	0	0	0	0	1[b]	0	1[c]	0/118	2/72	2/190

[a] FNA = fine needle aspirate.

[b] Positive liver sample only.

[c] Positive liver and toe clip samples.

statistical analysis to compare the efficacy of testing each type of sample.

The dead green frog tadpoles from ponds EW2 (2010) and BUE (2009) had microscopic lesions consistent with lethal ranavirus infection: hepatic and renal (glomerular and hematopoietic) necrosis (Fig. 4), with occasional intracytoplasmic inclusions in hepatocytes (Green et al., 2002; Une et al., 2009). The livers of moribund and dead tadpoles (EW2, BUE, and DWM) all were strongly positive for ranavirus DNA via PCR.

All 14 samples (six toe clips and eight blood samples) from 2010 tested for the CO1 gene showed very strong amplified bands, confirming the presence of significant amounts of nondegraded amphibian DNA in the extracts from these samples. The 495–base-pair sequence of the ranavirus DNA from dead tadpoles (EW2 and DWM) and one of the positive frogs (EW2) matched FV3 (GenBank accession FJ601916) with 100% identity. The similarly sized sequence of tadpoles found dead in BUE matched the sequence title "Chinese giant salamander virus strain CGSV-L" (GenBank accession HQ684746) exactly. Of the five northern leopard frogs sampled opportunistically, the toe clip from one juvenile frog from EW2 sampled in 2011 was positive for ranavirus DNA, identified through sequencing as FV3. The kidney of this positive frog was also weakly positive for ranavirus DNA. All liver, blood, FNA, and other toe-clip samples were negative.

DISCUSSION

Our original purpose in 2010 was to compare blood sampling to toe-clipping in the detection of ranavirus infection via PCR testing of wild green frogs. Toe-clipping had been proposed as an alternative to the lethal, and more commonly employed, liver sampling in green frogs (St-Amour and Lesbarreres, 2007); we intended to explore an even less invasive technique based on reported detection of ranavirus in circulating lymphocytes of infected individuals (Cunningham et al., 2008). The lack of positive samples in 2010 (0/116 and 0/118 toe clips and blood samples, respectively) was unexpected. It left the question of whether blood sampling could replace toe-clipping unanswered and raised further concerns regarding the validity of sampling apparently healthy green frogs to detected ranavirus in a population.

A more targeted and thorough sampling followed in 2011: only ponds with confirmed tadpole mortalities due to ranavirus, either contemporary or recent, were sampled, reducing the number of ponds studied from five to three. The number of

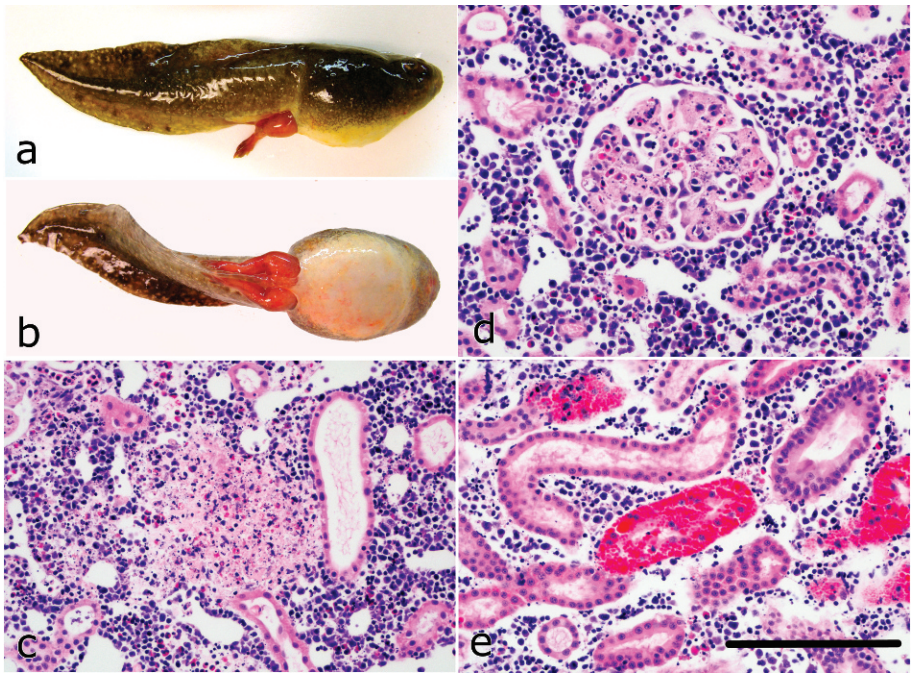

FIGURE 4. Ranavirus lesions in green frog (*Rana* [*Lithobates*] *clamitans*) tadpoles collected at pond EW2 on Prince Edward Island, Canada, in 2010. Gross lesions: (a) hind-limb hyperemia, (b) ventral epidermal petecchia. Microscopic renal lesions: (c) hematopoietic necrosis, (d) glomerular necrosis, (e) hyaline droplets in tubular epithelium. Bar=200 μm.

frogs sampled was reduced ($n=72$) but the number of samples obtained from each frog was increased to include hepatic FNA, liver tissue, and, for a subset of animals, kidney tissue. Positive samples were sequenced and determined to belong, with the notable exception of tadpoles from pond BUE, to the FV3 genus. To ensure that the negative results, particularly in 2010, were not due to poor quality or quantity of DNA in the samples, or to problems during DNA extraction, a subset was tested for the amphibian CO1 gene. The presence of nondegraded amphibian DNA of good quality and sufficient quantity was confirmed in all 14 samples tested. As all of the samples were collected and processed in a similar manner, it may be assumed that most (or all) other DNA samples were of equal quality and that the negative PCR results accurately reflect the absence of virus in the samples, or its presence in such minute amounts that even an extremely sensitive test such as PCR could not detect it.

Only 2/72 postmetamorphic green frogs thoroughly sampled in 2011 (blood, hepatic FNA, toe clip, and liver) were positive for ranavirus infection through PCR (Mao et al. 1997). The liver was positive in both cases, whereas only one of the frogs had a positive toe-clip (Table 1). Apparent prevalence calculated per pond and based on liver samples would seem different between DWM (1/37, 3%) and EW2 (1/7, 14%), the only two sites where frogs were positive. These numbers are too small for statistical testing, but it is noteworthy that it was only in EW2 where an FV3 outbreak was observed in tadpoles at the time of sampling.

If overall infection prevalences were to be calculated, results would differ depending on the type of sample taken: 3% (2/72) testing liver and 0.53–1.40% (1/190 both years and 1/72 for 2011 only,

respectively) testing toe clips. However, the numbers of positives are too low to convincingly compare the efficacy of these two sample types. Salamanders tested by PCR of tail clips, a sample comparable to a toe clip from a frog, often yield negative results unless they are clinically sick, probably because they do not have sufficient amounts of circulating virus (Greer and Collins, 2007). The prevalence of infection based on PCR testing of tail clips in salamanders leaving a pond after an outbreak of ATV has been reported as 78% (67.4–85.7%, 95% CI), although in those returning 2 yr later the prevalence was only 6.67% (2.0–21.4%, 95% CI; Brunner et al., 2004). Our results seem much lower than those of salamanders immediately after an outbreak, but not too dissimilar to those of salamanders 2 yr later. Tail-clipping in salamanders is thought to underestimate the true prevalence of ranavirus in the wild (Greer and Collins, 2007). Similarly, if a frog is a reservoir but has no active viremia at the time of sampling, PCR tests on toe clips or blood samples could fail to detect infection (false negatives). This, however, does not explain why the liver samples were also, in their majority, negative. The ponds sampled had current or historical outbreaks of ranavirus and were expected to remain infected from one season to the next (Green et al., 2002). Overall negative results from liver tissue suggest that the frogs were truly not infected or had cleared a previous infection, or that the liver is not where the latent infection resides. The low apparent prevalence of infection found in our study suggests that initial detection of infection with FV3 ranavirus in a wild amphibian population, lacking clinical signs and using only PCR, may not be very effective if green frogs are targeted, even when sampling the most likely infected tissue (liver).

When compared to a study on the same species, our results are surprisingly different from those found by testing toe clips and livers in other green frog populations in Canada (St-Amour and Lesbarreres, 2007), where prevalence was much higher: 40/67=60% (47.7–70.6%, 95% CI). These strikingly different results may have been due to an outbreak of ranavirus infection that occurred soon before or at the time of sampling but was not recorded by the authors, a species of ranavirus (not specified in the manuscript) with different disease ecology to that of FV3, or differences in the DNA extraction and PCR methodology.

Our findings suggest that postmetamorphic green frogs are a poor reservoir (carrier) for FV3 ranavirus (low prevalence of latent infection in wild populations of PEI), or that infection levels are so low that conventional PCR testing of preferred (liver) or alternative (toe clips, blood, and hepatic FNA) samples may be an unreliable method to detect subclinical infections in this species. Green frogs may not be the ideal species to sample when determining presence/absence of disease in an area (usually assuming ≥5% prevalence): testing asymptomatic postmetamorphic green frogs could falsely identify an area as free of ranavirus. This could have disastrous consequences in the management of free-ranging and captive populations.

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