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NOTE

First report of ranavirus mortality in a common snapping turtle *Chelydra serpentina*

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ABSTRACT: An adult male snapping turtle with marked palpebral edema and multifocal skin ulceration was found alive in a marsh in southern Ontario in summer 2017. The turtle was transported to a rehabilitation facility and died 4 d after arrival. The carcass was submitted to the Canadian Wildlife Health Cooperative for post-mortem examination. Gross lesions included ulcerative conjunctivitis, necrotizing stomatitis, and splenomegaly. Microscopically, this corresponded to multisystemic fibrinonecrotizing vasculitis and severe fibrinous splenic necrosis. Liver tissue tested positive for frog virus 3-like ranavirus and negative for herpesvirus via polymerase chain reaction. The gross and microscopic lesions were consistent with previous reports of ranavirus infection in turtles and were severe enough to have been the cause of death in this case. This is the first report of morbidity and mortality in a common snapping turtle with a ranavirus infection, and the first reported case of ranavirus infection in a reptile in Canada. Ranaviruses are considered to be an emerging infectious disease in chelonians as they are increasing in distribution, prevalence, and host range.

KEY WORDS: *Chelydra serpentina* · FV3 · Ranavirus · Reptile · Snapping turtle

1. INTRODUCTION

Ranaviruses belong to the family *Iridoviridae*, a group of large, icosahedral, enveloped DNA viruses (Gray & Chinchar 2015). *Frog virus 3* (FV3) is the type species of the genus *Ranavirus* and has been associated with mortality events in amphibians, bony fish, and reptiles (Mao et al. 1997, De Voe et al. 2004, Johnson et al. 2008, Price et al. 2017). In the past 25 yr it has been identified as a cause of mass mortalities of amphibians in North America, South America, Europe, and Asia and has been found in over 175 species (Gray et al. 2009, Gray & Chinchar 2015). Although it primarily affects amphibians, there are periodic spillovers into other classes of ectothermic vertebrates, including chelonians (Price et al. 2017).

Reports of FV3 infection in chelonians are less common than in amphibians, but are increasing in number (De Voe et al. 2004, Johnson et al. 2008). The first case of iridovirus infection in a chelonian was in a Mediterranean land tortoise *Testudo hermanni* in 1982 (Heldstab & Bestetti 1982). Since then it has been reported in soft shelled turtles *Trionyx sinensis*, Burmese star tortoises *Geochelone platynota*, gopher tortoises *Gopherus polyphemus*, Florida box turtles *Terrapene carolina bauri*, red eared sliders *Trache-*

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mys scripta elegans and eastern box turtles *Terrapene carolina carolina* with varying degrees of morbidity and mortality (Chen et al. 1999, Marschang et al. 1999, Johnson et al. 2008). In the past decade, FV3 has been associated with multiple mass mortality events in eastern box turtles in the United States, leading to increased awareness of the disease in chelonians (Johnson et al. 2007, 2008, Adamovicz et al. 2018).

In chelonians, commonly reported gross lesions include oral ulceration and abscessation, nasal and ocular discharge, cutaneous abscessation, palpebral edema, and conjunctivitis (De Voe et al. 2004, Johnson et al. 2007, Allender et al. 2013). These lesions correspond microscopically to multisystem fibrinoid vasculitis targeting the spleen and dermis, multifocal hepatic necrosis, and multicentric thrombi (De Voe et al. 2004, Johnson et al. 2007, Allender et al. 2013).

The disease can be transmitted between ectotherm vertebrate classes with the virus shed into the water by infected individuals, as well as through direct contact and predation (Brenes et al. 2014). It has been suggested that larval amphibians act as amplification hosts and that fish and chelonians could be reservoirs for FV3, as some species may act as asymptomatic carriers, which would allow the disease to persist in the environment from year to year (Johnson et al. 2007, Brenes et al. 2014). In many adult and juvenile chelonians, ranavirus infection is often acute and fatal (Johnson et al. 2007, Allender et al. 2011, Allender et al. 2018). Researchers have detected low levels of seroprevalence and PCR prevalence in adult turtles in the few exposed populations that have been examined, which may indicate that most adult turtles die of infection and so are unlikely to be reservoirs for FV3 (Johnson et al. 2007, Allender et al. 2011).

Some diseases, such as white nose syndrome and chytridiomycosis, can impact wildlife at a population level, and ranaviruses are a particular threat to species that are isolated or in low abundance (Gray & Chinchar 2015). The life history traits of chelonians, including low fecundity, low juvenile survival, and long adult life spans, make them especially prone to population level impacts as a consequence of infection by pathogens like FV3, which cause mortality in adults (Heppell 1998, Gray & Chinchar 2015).

2. CASE REPORT

A 13 kg adult male common snapping turtle was found in Cootes Paradise Marsh at the western end of Lake Ontario, Canada, in June 2017 (Fig. 1). The turtle was noted to be lethargic with facial swelling and skin ulcerations (Fig. 2). It was brought to a wildlife rehabilitation centre, where it received treatment and died 4 d after admittance. All fieldwork was carried out under approved animal use protocols from McMaster University (no. 17-01-05) and site-specific permits (Hamilton Conservation Authority land access, Wildlife Scientific Collector's Authorization no. 1084392 and Royal Botanical Gardens no. 2016-07). The body was submitted to the Ontario/Nunavut Regional Center of the Canadian Wildlife Health Cooperative for diagnostic investigation.

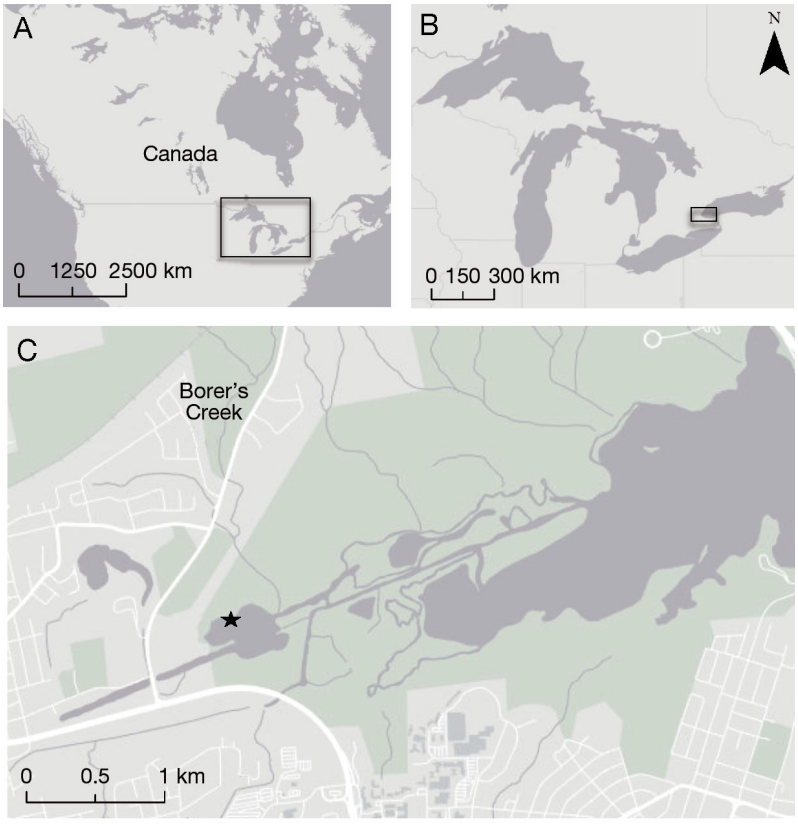

Fig. 1. Location of (A) the Laurentian Great Lakes in Canada and (B) Cootes Paradise Marsh, at the extreme western end of Lake Ontario; (C) the snapping turtle infected with ranavirus was found in West Pond within Cootes Paradise Marsh (43.2713° N, 79.9306° W; star)

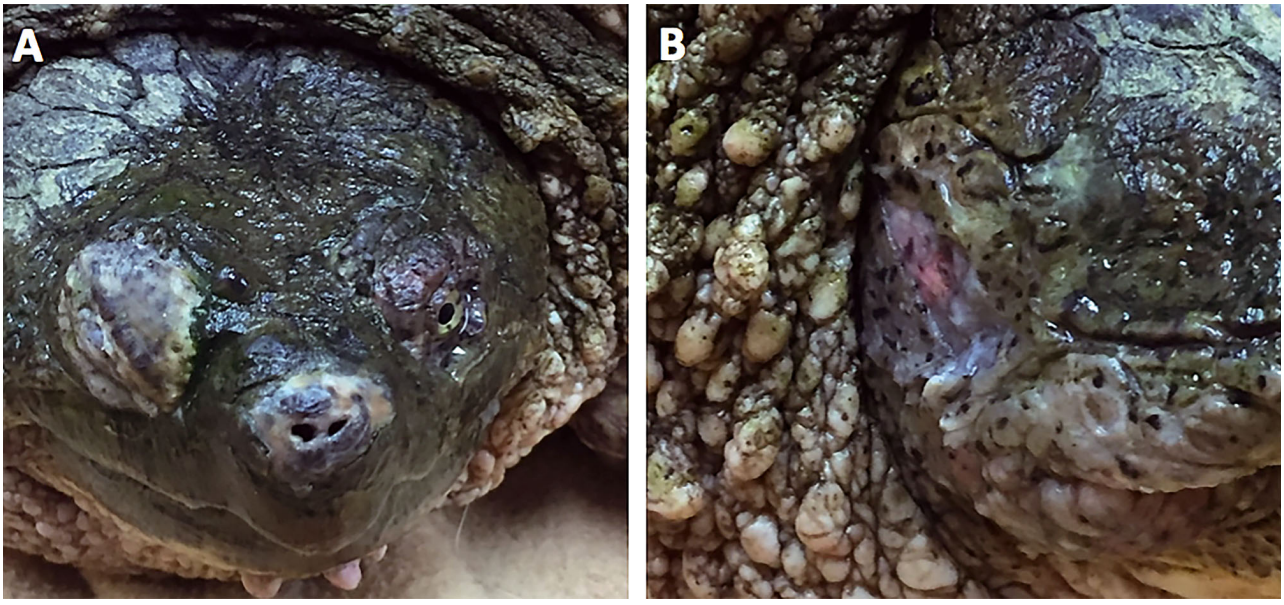

Fig. 2. Snapping turtle showing (A) severe palpebral and rostral swelling and (B) epidermal ulceration on the lateral neck. These lesions are consistent with ranavirus infection

A post-mortem examination was performed; a standard set of tissues were prepared routinely for histologic examination and stained with hematoxylin and eosin. Additional stains included Masson's trichrome and Martius scarlet blue (MSB) trichrome on select tissues. Swabs of the oral lesions were taken for bacterial culture.

Necropsy revealed marked bilateral palpebral swelling that effectively occluded vision, with associated conjunctival ulceration and yellow-tan crusting. A 1.5 cm diameter soft tissue swelling was present at the right oral commissure. There was a focal 1.5 × 1.0 cm area of ulcerative necrosis in the dorsal soft palate, just caudal to the bill, and there was a rim of necrotic tissue lining the inner aspect of the upper beak. The oral cavity contained a small amount of red-tinged opaque fluid. The spleen appeared to be enlarged to approximately 5.0 × 3.0 cm and the liver was diffusely pale yellow-brown. Lungs were moderately diffusely congested. The turtle had moderate amounts of subcutaneous and perivisceral fat, although the stomach and intestinal tract were empty, with only a few hard fecal pellets present in the rectum.

Microscopically, there was multifocal full thickness necrosis of the conjunctival epithelium with fibrin, edema and aggregates of poorly preserved leukocytes expanding the underlying connective tissues (Fig. 3A). Fibrinoid change was apparent in many blood vessels within the underlying connective tissue and was accompanied by moderate numbers of leukocytes within the wall and in the perivascular connective tissue (Fig. 3B). Superficially, there were colonies of coccoid bacteria within the necrotic tissue.

The spleen was severely affected by fibrinonecrotizing vasculitis with fibrin, necrosis, hemorrhage, and a mixed population of leukocytes effacing over 95% of the parenchyma (Fig. 3C). There was diffuse lymphoid depletion and no appreciable remaining follicular structures. Fibrinoid change was present in the vast majority of blood vessels, characterized by amorphous eosinophilic material segmentally expanding the walls, along with frequent granulocytic cells, which was highlighted with MSB stain (Fig. 3D). These blood vessels were surrounded by concentric, fibrillar, hypereosinophilic areas in which there was loss of differential staining and scattered karyorrhectic debris (fibrin and coagulation necrosis) accompanied by variable numbers of poorly preserved monocytes and granulocytes. Numerous embolized trematode eggs measuring 100–150 µm in length were found within the spleen. There was evidence of vascular fibrinoid change in the liver, kidney, and lungs, but not to the extent observed in the spleen or conjunctiva, with no necrosis or inflammation of the adjacent tissues.

Within the kidney, there was extensive tubular epithelial necrosis with sloughing of cells into the lumen. Glomeruli frequently contained marked seg-

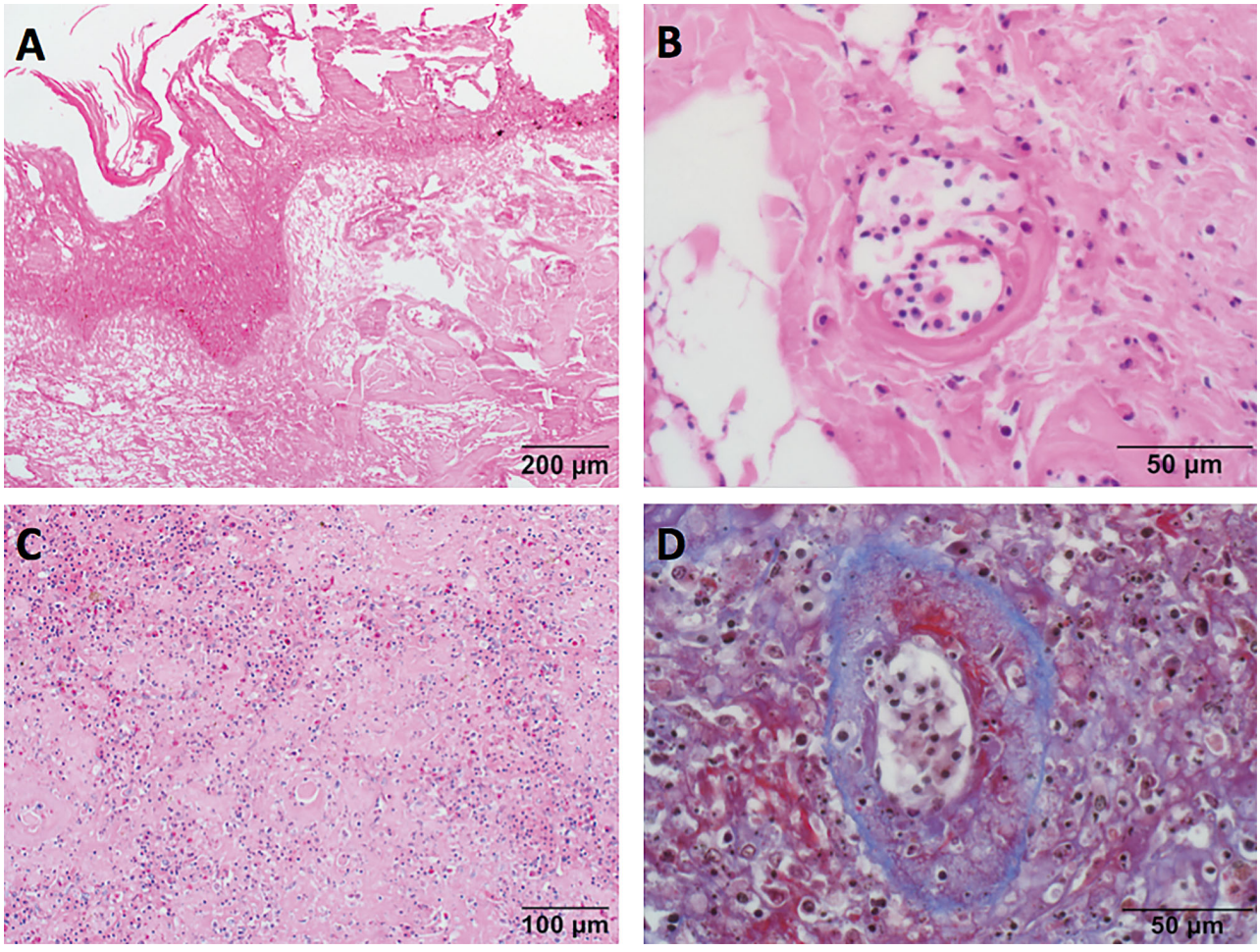

Fig. 3. Photomicrographs of histologic lesions in a snapping turtle with a ranavirus infection. (A) Necrotic and edematous conjunctiva (hematoxylin and eosin stain). (B) A palpebral blood vessel with fibrinoid necrosis characterized by hyalinized hypereosinophilic matrix segmentally effacing the wall and karyorrhectic debris (hematoxylin and eosin stain). (C) The spleen has been entirely effaced by necrosis, fibrin and hemorrhage (hematoxylin and eosin stain). (D) A vessel within the spleen displaying fibrin (red) within and surrounding the vessel wall, characteristic of fibrinoid necrosis (Martius scarlet blue trichrome stain)

mental thickening of capillary loops. Embolized trematode eggs, similar to those noted in the spleen, were embedded in the mucosa of the stomach and sometimes associated with adjacent necrosis and heterophilic inflammation. Lungs were markedly congested. Testes were inactive.

Using electron microscopy (EM; courtesy of BC Animal Health Centre), iridovirus particles were visualized in the liver, displaying their characteristic icosahedral shape (Fig. 4). Microscopically, all hepatocytes had variable cytoplasmic vacuolation, consistent with glycogen or lipid, and there were aggregates of melanomacrophages (considered to be within normal limits; Allender et al. 2013). Hepatic sinusoids were mildly enlarged, but no thrombi were noted.

Swabs of the oral lesions produced moderate growth (3+) of *Acinetobacter calcoaceticus*, *Providencia rettgeri*, *Aeromonas veronii*, *A. eucrenophila*, *Klebsiella oxytoca*, *Citrobacter braakii*, and *Pseudomonas rhodesia*. No *Mycoplasma* spp. were isolated from the mouth swab. These results are interpreted as bacterial overgrowth given the distribution of bacteria visible histologically.

A sample of frozen liver was tested by herpesvirus consensus PCR (VanDevanter et al. 1996) and FV3 PCR using primers FV3-MCP-1-F (5′-GCA GGC CGC CCC AGT CCA-3′) and FV3-MCP-2-R (5′-GGG CGG TGG TGT ACC CAG AGT TGT-3′) (Dr. Greg Appleyard pers. comm.) targeting the major capsid protein gene. The same sample tested positive for FV3 and negative for herpesvirus. Positive FV3

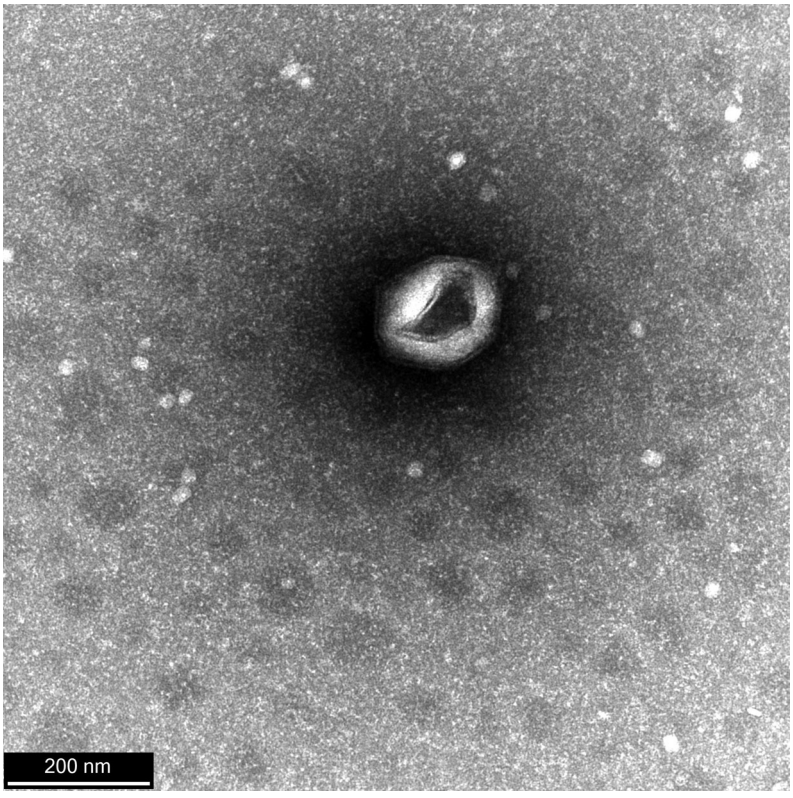

Fig. 4. Electron micrograph of the icosahedral iridovirus particle from the liver of a snapping turtle (courtesy of the Animal Health Centre, BC Ministry of Agriculture)

PCR results were confirmed by direct sequencing of the 482 base pairs (bp) amplicon using a commercial DNA sequencing kit and ABI 3500 genetic analyzer (ThermoFisher Scientific) according to the manufacturer's instructions. DNA sequences were assembled to a 440 bp fragment and analyzed using DNASTAR Lasergene 14 and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST search showed 100% match with *Lacerta monticola* ranavirus (GenBank accession number KM516719.1) and FV3 major capsid protein gene sequences (GenBank accession numbers FJ459783.1 and DQ897669.1).

3. DISCUSSION

In this case, the diagnosis of an FV3-like ranavirus infection is supported by systemic gross and microscopic lesions consistent with viral infection, visualization of viral particles by EM from hepatic tissue and genetic confirmation via PCR. Prior to this report, snapping turtles were not known to be susceptible to ranavirus infection. This is also the first reported case of ranavirus induced mortality in a reptile in Canada.

The gross and microscopic findings in this case are consistent with previous reports of FV3 infection in turtles and the lesions are severe enough to have been the cause of death. In chelonians, FV3 infection presents clinically with ocular and nasal discharge, respiratory distress, palpebral edema, anorexia and lethargy (De Voe et al. 2004, Johnson et al. 2007). Gross lesions include fibrinonecrotic oral ulceration, conjunctivitis, cellulitis, splenomegaly, and subcutaneous edema (Heldstab & Bestetti 1982, Marschang et al. 1999, De Voe et al. 2004, Johnson et al. 2007, 2008, Allender et al. 2013).

Microscopic lesions often involve systemic fibrinonecrotizing vasculitis characterized by hyalinized vessel walls (De Voe et al. 2004, Johnson et al. 2007). This can be especially prominent in the spleen, leading to effacement of splenic architecture by hemorrhage and fibrin with depletion of lymphoid follicles (Johnson et al. 2007, Allender et al. 2013). It has been hypothesized that the severe damage to splenic ellipsoids observed with chelonian FV3 infections could be the result of their filtering function leading to antigen trapping and a subsequent immune response (Johnson et al. 2007). Other common microscopic lesions include fibrin thrombi, necrotizing hepatitis and ulceration with fibrinonecrotic plaques on mucous membranes (Heldstab & Bestetti 1982, Marschang et al. 1999, De Voe et al. 2004, Johnson et al. 2007, 2008, Allender et al. 2013, Adamovicz et al. 2018). The damage to mucous membranes, most notably the oral mucosa and conjunctiva, could be the result of viral replication in epithelial cells or secondary to thrombus formation and ensuing infarction (Johnson et al. 2007).

Large, basophilic, intracytoplasmic inclusion bodies are often visible in epithelial cells in amphibian and fish iridovirus infections but, in chelonians, viral inclusion bodies are unpredictable and their presence varies based on the species (Marschang et al. 1999, Johnson et al. 2008, Allender et al. 2013). In this case, inclusion bodies were not observed microscopically. However, the characteristic icosahedral viral particles of iridoviruses were visualized using EM of the liver (Heldstab & Bestetti 1982).

The major differential diagnosis for the observed stomatitis and conjunctivitis is a herpesviral infection, which was ruled out via PCR of liver tissue (Divers & Mader 2005). A differential for upper respiratory disease in chelonians is mycoplasma infection, which was ruled out through bacterial culture (Feldman et al. 2006). Systemic fibrinoid vasculitis can also be caused by herpesvirus, as well as bacterial septicemia, rickettsial disease and systemic fungal infections — of which there was no microscopic evidence (De Voe et al. 2004).

Ranavirus has been present in southern Ontario in amphibians since at least 1999 (Greer et al. 2005). It has been found in species such as the spring peeper *Pseudacris crucifer*, green frog *Lithobates clamitans*, northern leopard frog *Lithobates pipiens* and eastern newt *Notophthalmus viridescens* (Gray & Chinchar 2015), which are species known to populate Cootes Paradise Marsh (M. L. Piczak unpubl. data). Given that the same or similar strains of FV3 have been found in neighboring amphibian populations in other FV3-related chelonian mortality events, it is possible that this snapping turtle contracted the virus from amphibians sharing the same environment (Johnson et al. 2008, Brenes et al. 2014).

In amphibians, anthropogenic stressors such as agricultural activity, housing, and road traffic can increase the prevalence of ranavirus infections (Forson & Storfer 2006, Gray et al. 2007, Miller et al. 2011). Unfortunately, Cootes Paradise Marsh has been heavily impacted by urbanization, habitat conversion, and discharge of sewage effluent (Mudroch & Capobianco 1979, Thomasen & Chow-Fraser 2012). Chronic stress can lead to immunosuppression and make individuals more susceptible to disease (Martin 2009). As ranavirus has been present in southern Ontario for some time, it is possible that this disease has emerged in snapping turtles as a new host species due to a change in environmental stressors (Greer et al. 2005).

At this point, the prevalence of an FV3-like ranavirus in Cootes Paradise Marsh is unknown, as is the impact it could have on species living there. Based on the literature, it is clear that ranavirus is a significant pathogen of chelonians, and it was the cause of death in this snapping turtle (Johnson et al. 2007, Allender et al. 2013). Long-term population studies with disease monitoring in amphibian, fish, and chelonian populations will be required for a better understanding of the complex ecology of ranaviruses (Mao et al. 1997, Gray et al. 2009).

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