The wood frog (*Rana sylvatica*): An emerging comparative model for anuran immunity and host-ranavirus interactions

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Abstract

The wood frog (*Rana sylvatica*) is widely distributed across North America and is the only amphibian found north of the Arctic Circle due to its remarkable ability to tolerate whole-body freezing. Recent mass mortalities attributable to *Ranavirus* spp. (family *Iridoviridae*) in wild juvenile wood frogs, coupled with the apparent high susceptibility of wood frogs to experimental infection with frog virus 3 (FV3), the type species of the Ranavirus genus, or FV3-like isolates underscore the serious threat ranaviruses poses to wood frog populations. Despite the ecological relevance and unique life history of wood frogs, our understanding of the wood frog immune system and antiviral response to ranaviral infections is in its infancy. Here we aim to (1) synthesize the limited knowledge of wood frog immune defences, (2) review recent progress in establishing the wood frog as a study system for ranavirus infection, and (3) highlight the future use of wood frogs as a model anuran to provide insight into the evolution of anuran immune systems and antiviral responses.

Keywords: Antimicrobial peptides; Frogs; Frog virus 3; Immune; Microbiome

1. Introduction

The wood frog (*Rana sylvatica*, LeConte 1825) is widely distributed across Canada and the United States, ranging from Alaska to Labrador with southern limits in the eastern United States and northern limits above the Arctic Circle [see detailed distribution maps provided on (AmphibiaWeb, 2022a; IUCN, 2022)]. Wood frogs (**Fig. 1A)** have a unique life history and are notably one of a few terrestrially hibernating frogs capable of tolerating multiple bouts of whole-body freezing during frigid winters [(Sinclair et al., 2013); and reviewed in (Storey, 1990)]. Wood frogs are one of the first amphibians to arrive at temporary vernal pools (**Fig. 1B**) after thawing from their "frog-sicle" state. As explosive breeders, adult wood frogs only remain in vernal pools for a few days to breed before returning to their terrestrial habitat in the surrounding upland temperate and boreal forests and migrating upwards of one kilometer away from their breeding pools (Berven and Grudzien, 1990). Fertilized eggs hatch and exist as larvae for 3 – 16 weeks before completing metamorphosis (Berven and Gill, 1983), with the length of the larval period influenced by environmental stimuli such as temperature (Berven and Gill, 1983), desiccation signals (Lent and Babbitt, 2020; Thompson et al., 2022), population density (Berven and Gill, 1983), parasite presence (Orlofske et al., 2017) and/or predator presence (Relyea, 2001; Groner et al., 2013). Upon completion of metamorphosis [*i.e.* reaching Gosner Stage 46; (Gosner, 1960)], metamorphs leave vernal pools and emerge onto the terrestrial forest floor as young-of-the-year. Males and females reach sexual maturity within 1 – 2 years or 2 – 3 years of metamorphosis, respectively, and display sexual dimorphism in colour and size (Martof, 1970). For more detailed information on the fascinating life history of wood frogs, readers are referred to information available on AmphibiaWeb (AmphibiaWeb, 2022a). The varied habitats, temperatures and metabolic states experienced by wood frogs on a yearly basis suggest robust adaptations of the wood frog immune system to sustain animal health.

There are over 8,500 known amphibian species (AmphibiaWeb, 2022b) with varied geographical ranges, life histories, and histories of co-evolution with distinct sets of pathogens, suggesting there is likely diversity in amphibian immune systems. Yet, much of our understanding of the immune system of anurans, by far the largest amphibian order, is limited to studies in the African clawed frog (*Xenopus laevis*) model organism [reviewed by (Robert and Ohta, 2009; Rollins-Smith et al., 2009; Chen and Robert, 2011)] and supporting genomic resources (Fortriede et al., 2020). The wood frog (family *Ranidae*) and the African clawed frog (family *Pipidae*) are

separated by over 200 million years of independent evolution (San Mauro et al., 2005; Pyron, 2011), and despite the tetraploidy of the African clawed frog (C-value = 3.09 pg, range 3.00 – 3.85 pg), the nuclear DNA content of the wood frog (C-value = 5.83 pg, range 5.49 – 6.50 pg) is nearly double that of the clawed frog [summarized in the Animal Genome Size Database (Gregory, 2022) and references therein]. Given the wood frog's unique life history, evolutionary distance, and relatively large genome in comparison to the current model anuran (*i.e.*, *X. laevis*), it is tempting to ponder the lessons to be learned from wood frogs. The availability of a much-anticipated annotated wood frog genome is likely to yield evolutionary insight into adaptations of the wood frog immune system shaped by their freeze-tolerant life history and to provide an important point of comparison for the general evolution and diversity of anuran immune genes.

Efforts to explore the diversity of amphibian immune systems are at odds with the global decline of amphibian populations, with over one-third of all amphibians threatened (Baillie et al., 2004). Infectious diseases such as chytridiomycosis, caused by *Batrachochytrium dendrobatidis* and *B. salamandrivorans,* and ranavirosis, caused by ranaviruses such as frog virus 3 (FV3, type species of the genus *Ranavirus,* family *Iridoviridae*), have emerged as contributors to amphibian declines [reviewed by (Chinchar, 2002; Collins, 2010; Chinchar et al., 2017)]. All three of these pathogens are listed diseases reportable to the World Organisation for Animal Health (WOAH, 2021). Although wood frogs are susceptible to both *B. dendrobatidis* (Searle et al., 2011) and FV3 (Forzán et al., 2017), recent reports of *Ranavirus* sp. related mass mortalities in wild wood frog juvenile stages (Wheelwright et al., 2014; Forzán et al., 2019) coupled with the risk of local extinction of isolated populations from ranaviral infection due to their high breeding pond site fidelity (Earl and Gray, 2014) underscores the serious threat ranaviruses pose to wood frog populations. Due to their high susceptibility to ranaviruses and their widespread distribution across North America, the wood frog was highlighted by the members of the Global Ranavirus Consortium as one of two candidate model species for ranavirus challenge experiments in North American studies (Lesbarrères et al., 2012). At the time of writing, the status of the wood frog on the IUCN red list is of "least concern" (IUCN, 2022). However, the lack of historical monitoring of wood frog populations across their extensive geographical range hinders accurate assessment of long-term population stability. While wood frog populations currently appear stable, ongoing rapid changes in the environment resulting from anthropogenic stressors (*i.e.*, climate change, contaminants) can alter host-pathogen dynamics and lead to increasing numbers of die-off and

local extinction events. Thus, our aims are to (1) synthesize the limited knowledge of wood frog immune defences, (2) review recent progress in establishing wood frogs as a study system for ranavirus infection, and (3) highlight the future use of wood frogs as a model anuran to provide insight into the evolution of anuran immune systems and antiviral responses (**Fig. 2**). To maintain the focus of this short review on the above aims, we limit our discussion to studies of *R. sylvatica*. Aspects of anuran immune functions which have yet to be explored in *R. sylvatica* are not discussed but references to several insightful reviews of these topics are provided.

2. Immune defences of *R. sylvatica*

2.1. Brief outline of anuran innate immune components

The anuran innate immune system is the first line of defence against infection and encompasses a wide variety of physical (e.g., epithelial barriers), chemical (e.g., antimicrobial peptides, and plasma proteins such as complement, lysozyme, and cytokines), cellular (e.g., granulocytes, macrophages), and commensal microbial barriers to infection. Much of our present knowledge of anuran innate immunity has been skillfully reviewed in recent publications, covering skin as an anatomical barrier [reviewed in (Varga et al., 2019)], chemical defences such as antimicrobial peptides [reviewed in (Rollins-Smith, 2023)], the complement system [reviewed in (Rodriguez and Voyles, 2020)] and the interferon system [reviewed in (Adeyemi et al., 2023)]. Similarly, cellular defences of hematopoietic origin, such as granulocyte development [reviewed in (Hauser et al., 2023), and microbial defences, such as the adaptive microbiome hypothesis [reviewed in (Woodhams et al., 2023)], have also been expertly reviewed elsewhere. A vital element of the anuran innate immune system that requires more investigation are the germline encoded pattern recognition receptors (PRRs). PRRs allow for targeted, cell-mediated responses through the recognition of pathogen- and damage-associated molecular patterns (PAMPs, DAMPs). Studies of PRRs in anurans have largely focused on the membrane-spanning Toll-like receptors, which presumably detect PAMPs and DAMPs within intracellular vesicles and in the extracellular space (Ishii et al., 2007), although some more recent studies have considered other PRR families [(Zhao et al., 2014); reviewed in (Jiang et al., 2021)]. Presently, the only PRRs which have been identified in *R. sylvatica* are Class A scavenger receptors, but this puts them among a very small group of anurans for which attempts to characterize scavenger receptors have been made. While the ongoing research into anuran innate immunity provides a growing base for future

comparative studies, studies of innate immune function in *R. sylvatica* have thus far been limited to antimicrobial peptides, blood leukocytes, scavenger receptors, and host-associated microbial communities, which we explore in the following section.

2.2. Innate immune defences of R. sylvatica

2.2.1. Antimicrobial peptides

Antimicrobial peptides (AMPs) are short (12 – 50 amino acids), cationic, amphipathic peptides with broad-spectrum antimicrobial activity, and some AMPs have been shown to function as key regulators of the innate immune response in human and mouse models [reviewed in (Lai and Gallo, 2009; Hancock et al., 2016)]. Over 1162 diverse antimicrobial peptides have been identified from anurans and are listed in the Antimicrobial Peptide Database (Wang et al., 2016). Most of these AMPs are synthesized as prepropeptides and secreted from dermal glands onto anuran skin as mature peptides following cleavage of the active peptide from the preproregion (Reilly et al., 1994). *X. laevis* is known to produce at least 12 antimicrobial peptides, belonging to the magainin, caerulein precursor fragment (CPF), peptide glycine-leucine-amide (PGLa), xenopsin precursor fragments (XPF), and *Xenopus laevis* antibacterial peptide (XlAsp) families (Zasloff, 1987; Soravia et al., 1988; Moore et al., 1991; Hou et al., 2011; Li et al., 2016; Zhang et al., 2017). While many of these are among the best characterized AMPs, the species is by no means the richest source of AMPs identified. The diversity of skin-secreted AMPs is particularly high in Ranid frogs, which often express many AMPs belonging to several distinct families including brevinins, esculentins, japonicins, nigrocins, palustrins, ranacyclins, ranatuerins, and temporins [reviewed in (Conlon et al., 2009)]. For example, North American ranids possess a wide arsenal of skin-secreted AMPs, ranging from three in *Rana muscosa* to twenty-three in *Rana palustris* (Wang et al., 2016). Skin-secreted AMPs of anuran origin have been of particular interest in recent years as several have been demonstrated to inhibit or inactivate *Bd* and FV3 [reviewed in (Rollins-Smith, 2023)].

Only two AMPs – brevinin-1SY and temporin-1SY – have been reported from the skin of *R. sylvatica* at different developmental stages. Brevinin-1SY, was the first AMP from *R. sylvatica* to be identified (Matutte et al., 2000). Like other brevinin-1 family members, brevinin-1SY is 24 amino acids in length, retains the four conserved residues Pro^{3}, Ala^{9}, Cys^{18}, Cys^{24} and the C-terminal cystine-bridged heptapeptide ring (Matutte et al., 2000), and is predicted to have a +3

charge and adopt an amphipathic alpha helical secondary structure (Katzenback et al., 2014). Transcripts for brevinin-1SY were detected as early as Gosner stages 14 – 20, with levels increasing towards the end of metamorphosis (Gosner stages 36 – 45) and were detectable in dorsal and ventral skin tissues, as well as other tissues, of adult male wood frogs captured from spring breeding ponds (Katzenback et al., 2014). Brevinin-1SY was later detected in the skin secretions of recently metamorphosed *R. sylvatica* (Groner et al., 2013), indicating that brevinin-1SY peptides are translated as early as post-metamorphosis. A second AMP of 14 amino acids in length, temporin-1SY, was identified in the skin secretions of recently metamorphosed wood frogs (Groner et al., 2013). Temporin-1SY has a low similarity to the temporin consensus sequence, but is predicted to have a +2 charge, high hydrophobicity and an amidated C-terminal residue, typical of this peptide family. Aside from brevinin-1SY and temporin-1SY, Groner et al. (2013) also detected three other unidentifiable peptides suggesting that *R. sylvatica* metamorphs may express additional skin-secreted AMPs. We recently presented preliminary findings at the 2022 North American Comparative Immunology Workshop that indicate the transcription of additional AMP families in the skin of *R. sylvatica* (Douglas and Katzenback, unpublished data), and better aligns the AMP repertoire of wood frogs with that of other North American ranids. Abiotic factors such as temperature (Matutte et al., 2000), dehydration, anoxia, freezing (Katzenback et al., 2014), contaminant exposure (Groner et al., 2013), and anthropogenic noise levels (Tennessen et al., 2018) have been demonstrated to alter AMP expression (transcript and/or protein levels) in wood frogs. Similarly, predator presence, such as a larval dragonfly (*Anax junius*), has been observed to lower total skin peptide yield in wood frog metamorphs (Groner et al., 2013). This suggests that expression of brevinin-1SY, and possibly other wood frog AMPs, is differentially regulated in response to stressors, potentially as a compensatory or preparatory mechanism. Further study is needed to determine the extent of AMP diversity in *R. sylvatica*, the regulatory mechanisms that control AMP expression, and how AMPs might contribute to defence against pathogens of anuran concern through potential direct antimicrobial properties or through indirect immunomodulation of host responses.

2.2.2. Blood leukocytes

Anuran leukocytes are generally divided into neutrophils, eosinophils, basophils, monocytes and lymphocytes, although terminology varies, as it often remains unclear how closely

the function of these cell types aligns with the mammalian leukocytes they resemble (Campbell, 2015). Hematologic reference intervals for adult *X. laevis* have long been available and serve as a key comparison for studies in other anurans (Hadji-Azimi et al., 1987), but standard intervals do not appear to be reported for *X. laevis* larvae. During metamorphosis *X. laevis* experience a dramatic remodelling of blood leukocyte populations and a temporary downregulation of immune function [reviewed in (Robert and Ohta, 2009)]. This shift in leukocyte development appears to also occur in ranid species based on hematological observations (Davis, 2009), but has not been directly examined in wood frogs. Anuran leukocyte function has been studied in *X. laevis*, and the functional characterization of leukocytes has been supported and complemented by the characterization of an increasing number of *Xenopus* cytokines [e.g. (Koubourli et al., 2018; Yaparla et al., 2019; Fukui and Matsunami, 2022)]. Unfortunately, functional investigation of leukocytes has rarely been performed in other anurans and any observations of leukocyte responses are assumed in comparison to *Xenopus* or mammalian models.

Several studies have examined *R. sylvatica* blood cell profiles, primarily of larvae and metamorphs. In larval, recently metamorphosed, and adult wood frogs, blood leukocytes are comprised of lymphocytes, neutrophils, eosinophils, basophils, and monocytes [(Gervasi and Foufopoulos, 2008; Forzán et al., 2016; Szuroczki et al., 2019), summarized in **Table 1]**. While the proportions of eosinophils and monocytes observed in adult *R. sylvatica* agree with values from *X. laevis*, *R. sylvatica* has a comparatively higher percentage of lymphocytes, with fewer neutrophils and basophils. Leukocyte proportions in tadpoles and metamorphs largely fall within the ranges observed for adult wood frogs, except for a lower percentage of lymphocytes in tadpoles and higher percentage of lymphocytes in metamorphs. However, there appears to be observational variation in the presence of blood cell types in recently metamorphosed frogs, such as a lack of basophils or monocytes (Gavel et al., 2019), or proportions of blood cell types such as a low level of monocytes (Szuroczki et al., 2019). These observed hematological ranges provide some insight into hematologic trends across development, but all the studies relied on animals held in a controlled, captive environment for an extended period, so any comparisons to observations made from individuals maintained under different conditions, or directly captured from the wild, should be made with caution.

Leukocyte profiles are often used to assess stress levels in animals, as levels of glucocorticoid stress hormones influence the proportion of circulating cells from the various

leukocytic lineages. The neutrophil to lymphocyte (N : L) ratio is a common metric, as high N : L ratios reliably indicate high levels of glucocorticoid hormones (Davis et al., 2008). A few studies have applied this method to wood frogs, examining the effects of diet (Szuroczki et al., 2019), pesticides (Gavel et al., 2019; Szuroczki et al., 2019), proximity to agriculture (Ruso et al., 2021), parasites (Szuroczki et al., 2019), and ranavirus infection (Forzán et al., 2016). Notably, the study considering the effects of agricultural stress on wood frogs observed that Gosner stage of the individual was the only factor considered that influenced N : L ratios, which were stable until metamorphic climax then suddenly dropped (Ruso et al., 2021), likely reflecting turn-over of larval lymphocytes. While leukocyte profiles may be useful for assessing stress in *R. sylvatica*, additional study is needed to better understand the effects of development, season, and other factors.

2.2.3. Pattern recognition receptors - Class A scavenger receptors

Scavenger receptors (SRs) encompass twelve classes of structurally diverse PRRs which are expressed on the surface of a variety of leukocytes and barrier cell types, such as epithelial and endothelial cells. SRs are known to bind a variety of ligands, including PAMPs and DAMPs, and participate in their internalization via endocytosis [reviewed in (Taban et al., 2022)]. Class A scavenger receptor (SR-A) members include macrophage scavenger receptor 1 (MSR1) also known as SR-AI/II, SCARA1), scavenger receptor class A member 3 (SCARA3), SCARA4, SCARA5 and macrophage receptor with collagenous structure (MARCO) and have been implicated in host-virus interactions in human, mouse, and fish systems, as well as one anuran (Vo et al., 2019a); they bind and traffic dsRNA, a viral PAMP, to endosomal Toll-like receptor 3 to initiate a type I interferon response (DeWitte-Orr et al., 2010) and are used by some viruses in receptor-mediated entry of host cells (Haisma et al., 2009; MacLeod et al., 2013, 2015).

The expression and partial binding profiles of SR-As have been investigated in several anuran cell lines, including lines derived from *R. sylvatica* [reviewed in (Douglas et al., 2023)]. Cell lines derived from metamorphic *R. sylvatica* tadpole hind leg bone (WoodTad-bone), cephalic region (WoodTad-HE1) or retinal tissue (WoodTad-rpe) expressed detectable levels of a core set of SR-A transcripts, including *scara3*, *scara4* and *scara5* (Vo et al., 2019c). In addition, WoodTad-bone and WoodTad-HE1 expressed transcripts for *srai/ii* and WoodTad-bone expressed transcripts for *marco*. In agreement with observations from mammals and fish (Suzuki et al., 1997; Kraal et al., 2000; Jiang et al., 2006; Fukuda et al., 2011), only wood frog cell lines positive for

srai/ii or *marco* transcripts (*i.e.* WoodTad-bone and WoodTad-HE1) were able to bind acetylated low-density lipoprotein (acLDL, a common SR-A ligand) (Vo et al., 2019c). These studies confirm that *R. sylvatica* cells transcribe various SR-A transcripts and possess the ability to bind typical SR-AI and MARCO ligands. While the immune functions associated with these receptors have yet to be evaluated in *R. sylvatica*, they present the only PRRs yet identified in the species.

2.2.4. Mucosal microbiomes

A growing body of research has highlighted the complex interactions between microbiota and the immune system. Microbial communities are not only regulated by host immune mechanisms but can support host immunity [reviewed in (Thaiss et al., 2016; Pandiyan et al., 2019)]. This framework has been applied to studies of anuran mucosal microbiomes and has resulted in a wealth of information on anuran skin microbiomes, and to a lesser extent gut microbiomes [reviewed in (Jiménez and Sommer, 2017)]. Recent reviews have outlined the role of skin bacteria in defense against *B. dendrobatidis* [reviewed in (Rebollar et al., 2020)], and the complex interplay between commensal microbes and the host immune system (Woodhams et al., 2023). Exploration of the *R. sylvatica* microbiome has only begun, but the few published studies on the skin and gut microbiome point to dynamic microbial communities. The varied environmental conditions faced by wood frogs throughout their life history contrast sharply with the consistent aquatic environment of captive *X. laevis*, making them a preferable system for studying how the environment can influence the anuran microbiome and any resulting effects on disease susceptibility.

The skin-associated bacterial microbiome of adult *R. sylvatica* is mainly composed of members of Proteobacteria, Bacteroidetes and Actinobacteria, with relatively few core genera or families shared by all individuals (Douglas et al., 2021). While the average diversity within skin-associated communities remained mostly consistent between seasons, we observed a large difference in microbial community structure between individuals sampled from spring breeding ponds and individuals sampled from the forest floor in summer and fall (Ontario, Canada), with the latter having an increased abundance of microbial phyla which are often associated with soil (Douglas et al., 2021). The effect of season was considerably larger than for any other factor considered. A small, but significant difference was observed between frogs captured from different breeding ponds, and sex of the frog did not appear to have a significant effect. While our initial

study did not explore the immune relevance of these changes, the findings suggest that the skin microbiome of adult *R. sylvatica* is a complex and highly variable community. Additional studies are needed to determine if skin-associated microbial communities differ geographically across the wood frog's broad range, the role of host skin-associated molecules in regulating the skin microbiome and *vice versa*, and how they might be affected by abiotic factors implicated in anthropogenic change and biotic factors such as pathogen challenge.

Experiments on *R. sylvatica* tadpoles have demonstrated the importance of early life gut microbiome colonization on host mortality to FV3 challenge (Warne et al., 2019). Sterilized *R. sylvatica* egg clutches inoculated with either sterile water, a homogenate from unmanipulated *R. sylvatica* eggs from their original clutch, or a homogenate of *R. catesbeiana* gut tissue, initially developed distinct gut microbial communities which then became similar as the tadpoles developed (Warne et al., 2019). While this suggests that host-selection or later larval diet was more influential to gut microbiota composition, the initial inoculation did appear to have some lasting effects. When exposed to FV3, uninoculated larvae experienced a higher overall mortality rate and died more rapidly than inoculated larvae (Warne et al., 2019). It remains unclear whether this difference might arise from direct contribution of gut microbiota to defence against FV3, or an indirect benefit resulting from improved tadpole health.

2.3. Brief outline of anuran adaptive immune components

The vertebrate adaptive immune system allows for the highly specific recognition and elimination of previously encountered pathogens through a diverse set of rearranged receptors to achieve immunological memory. This involves the presentation of antigens in major histocompatibility complex (MHC) molecules, and the recognition of non-self antigens by rearranged receptors on B-cells or T-cells which mediate pathogen-tailored effector functions. In mammals these adaptive immune cells are known to follow well-defined patterns of maturation and activation, much of which occurs in lymphoid organs such as the thymus and spleen. While studies of *X. laevis* have revealed similarities to the mammalian adaptive immune system, there are also clear differences and readers are referred to reviews on the *X. laevis* immune system (Robert and Ohta, 2009; Flajnik, 2018). Our understanding of the adaptive immune response in *R. sylvatica* is currently in its infancy and is limited to anatomical observations of the thymus and spleen, and preliminary characterization of MHC genes.

2.4. *Adaptive immune defences of R. sylvatica*

2.4.1. *Lymphoid organs*

Reports on the structure of the thymus and spleen of *R. sylvatica* likely represent the earliest immunological work on the species. Early observations focused on developing tadpoles, reporting the absence of an observable effect on the spleen or remaining thymus following partial thymectomy (Hoskins, 1921), and outlining stages of thymic development (Fabrizio and Charipper, 1941). Several studies have observed the effects of FV3 infection on thymus and spleen histologically (Forzán et al., 2015, 2017), and a single study has examined splenocyte proliferation in response to stress and/or FV3 infection (Kirschman et al., 2018). However, studies have not directly examined the function of these lymphoid organs in *R. sylvatica.*

2.4.2. *Major histocompatibility complex*

Major histocompatibility complex (MHC) proteins play an essential role in the adaptive immune responses of vertebrates by presenting self and non-self epitopes for recognition by T-cells [reviewed in (Pishesha et al., 2022)]. MHC Class I proteins are generally expressed by all nucleated cells and usually present epitopes from the proteome of the MHC I expressing cell on the cell surface for recognition by $CD8^+$ T-cells. MHC Class II proteins are typically expressed by professional antigen-presenting cell types, where they bind exogeneous epitopes that have been engulfed by the cell and present them on the cell surface for recognition by $CD4^+$ T-cells. The MHC system has been characterized in *X. laevis* and shown to be similar to that of mammalian models [reviewed in (Flajnik, 2018)], but has been less thoroughly investigated in other anurans, with studies generally observing genetic diversity of MHC genes and/or patterns of expression related to factors such as disease susceptibility [*i.e.*, chytridiomycosis, reviewed in (Fu and Waldman, 2017)].

Studies of MHC diversity in *R. sylvatica* populations have been limited to the MHC Class IIβ gene (Hernández-Gómez et al., 2019; Savage et al., 2019). While some ranid species appear to have experienced MHC Class IIβ gene duplications, *R. sylvatica* appears to have a single MHC Class IIβ locus (Kiemnec-Tyburczy et al., 2010). Studies of *R. sylvatica* populations have relied on amplification of the MHC Class IIβ gene exon 2, which includes the peptide binding region, and have observed a maximum of 2 alleles per individual, with homo- and heterozygous individuals present (Hernández-Gómez et al., 2019; Savage et al., 2019). Studies of wood frogs

from breeding ponds in Maryland and Pennsylvania (USA) have respectively suggested that tadpoles with heterozygous MHC Class IIβ alleles tended to have a lower intensity of FV3 infection (Savage et al., 2019), and that populations with greater MHC Class IIβ gene diversity experienced lower experimental infection loads from a parastitic *Echinoparyphium* sp. (Hernández-Gómez et al., 2019). The latter study also observed that particular MHC Class IIβ alleles tend to be enriched in populations living in close proximity to agriculture beyond what would be predicted based on comparisons of neutral genetic structure, suggesting that differences in MHC allele frequencies were resulting from local selective pressure. These findings highlight the intraspecific immune diversity of *R. sylvatica* and establish that population-level variation is present even within relatively small geographic areas. *R. sylvatica* appear well-suited to studies of this nature and may serve as a model for studies of other MHC loci to determine if similar trends exist for Class I and Class IIα genes.

2.4.3. T-cell mediated immunity

Administration of phytohaemagglutinin (PHA) and assessment of the resulting swelling is a method, although an often problematic one, used to gauge the immunocompetence of vertebrates in ecological studies [reviewed by (Kennedy and Nager, 2006)]. PHA is a toxic lectin derived from red kidney beans which has established activity as a T-cell mitogen in mammals and is traditionally employed to assess the delayed-type hypersensitivity response, an increased T-cell-mediated response to an antigen upon subsequent exposure [reviewed in (Demas et al., 2011)]. Unfortunately, this method is often incorrectly applied (*i.e.*, secondary stimulation with PHA is not performed, making it impossible to determine whether an increased adaptive response occurs) or misinterpreted (*i.e.*, PHA stimulated inflammation is assumed to be the result of lymphocyte infiltration, when it is known to promote an inflammatory response involving multiple cell types *in vivo*). PHA treatment has been applied to *R. sylvatica* to assess the effects of water body desiccation (Gervasi and Foufopoulos, 2008), predator presence (Seiter, 2011), and dietary antioxidants in combination with various environmental stressors (Szuroczki et al., 2019) on immune function. In each case a single injection of PHA was administered and swelling was measured after 24 h without additional analysis. Therefore, it is difficult to interpret the immunological significance of any observed differences in swelling between *R. sylvatica* treatment

groups beyond a potential difference in overall capacity for an inflammatory immune response to PHA.

2.5. R. sylvatica immune omics analysis

Genomic, transcriptomic, and proteomic resources support deeper immunological understanding by providing a wealth of gene sequence and gene expression data which can be directly compared to similar datasets from other organisms. Excitingly, during the writing of this manuscript, a whole genome assembly for *R. sylvatica* was released (GenBank Accession: JAQSEC000000000.1) but remains unpublished and unannotated. While some transcriptomic analysis has been performed on *R. sylvatica* previously (Tompsett et al., 2013), only one publication has investigated the *R. sylvatica* transcriptome from an immunological perspective (Eskew et al., 2018). The study isolated RNA from the ventral skin of *R. sylvatica* exposed to *B. dendrobatidis*, as well as untreated controls, and identified differentially expressed genes at all time points, including a considerable number of genes annotated with immune related gene ontology terms. While this study did not assemble a *R. sylvatica* reference transcriptome, raw RNA sequencing data is available through the NCBI sequencing read archive, and supporting data and code are publicly available. A single proteomic study on *R. sylvatica* liver tissue did not indicate any findings of immune relevance (Kiss et al., 2011). While there are few resources at present, the availability of genomic and transcriptomic and resources will undoubtedly support future studies, and we look forward to the deeper understanding of wood frog immunity that they might provide.

3. **Frog virus 3 infection of *R. sylvatica***

FV3 is a large double-stranded DNA virus which has been used to study the anuran antiviral response, particularly in *X. laevis* which was established as a model anuran to study host-FV3 interactions (Gantress et al., 2003). While larvae tend to be highly susceptible, adult *X. laevis* are generally tolerant of FV3-infection, experiencing transitory pathology and viral replication that is largely limited to the kidneys [reviewed in (Chen and Robert, 2011)]. Adult *X. laevis* can mount a successful immune response despite the immunoevasive properties of FV3, which encodes viral homologs of host genes that act as pseudosubstrates to prevent the activation of a cellular antiviral response [reviewed in (Grayfer et al., 2012)]. In this section, we focus on the recent advances in establishing *R. sylvatica* as a susceptible host to investigate host-FV3 interactions.

Documented die-off events of larval wood frogs in the wild (Wheelwright et al., 2014; Forzán et al., 2019) in combination with *in vitro* and *in vivo* experiments have demonstrated *R. sylvatica* to be highly susceptible to FV3. *In vitro* challenge of anuran cell lines originating from different species demonstrated differing susceptibilities to FV3, with the wood frog embryonic kidney cell line, KERS, particularly permissive to FV3 (Rafferty, 1965). Similarly, *in vivo* challenge experiments have demonstrated that all wood frog life stages post hatch (hatchlings, larvae, pro-metamorphs, metamorphs, adults) are highly susceptible to experimental infection with FV3 or FV3-like isolates, although hatchlings and larvae appear particularly susceptible (Haislip et al., 2011; Forzán et al., 2015, 2019). Wood frog susceptibility to FV3 and host mortality also appears to be dependent on the viral strain (Echaubard et al., 2014), level of initial viral infection (Forzán et al., 2015), developmental stage at the time of infection (Warne et al., 2011) and possibly host population density depending on host life stage (Reeve et al., 2013; Crespi et al., 2015). External environmental conditions and stimuli have mixed effects on wood frog susceptibility to FV3 infection, viral load and/or mortality. For example, temperature (Brand et al., 2016; Echaubard et al., 2014; Hall et al., 2018), and chronic host stress responses (Kirschman et al., 2018) alter wood frog susceptibility to FV3 and/or mortality outcomes while others, such as predator cues (Haislip et al., 2012; Reeve et al., 2013), do not.

Several studies have explored the progression of ranavirus infection of *R. sylvatica* across their various life stages to better understand how infection is established and the resulting ranavirosis pathology. Oral infection studies in adult wood frogs have determined a viral lethal dose where 50% of the animals die (LD_{50}) of just $10^{2.93}$ plaque forming units (pfu) and doses of $\geq 10^{4.43}$ pfu are observed to be uniformly fatal. While individuals administered an FV3 dose below the LD_{50} tended to avoid systemic infection and survive, FV3 was still detected in several tissues suggesting that there is potential for infection and recovery or ongoing sub-lethal infection. Infection with a fatal dose results in initial superstructural changes to mucosal epithelia and bone marrow which progress to a systemic infection characterized by inflammation and necrosis of a wide range of tissues, resulting in mortality 10 – 14 days post-infection (Forzán et al., 2015, 2017). The observation of an apparent tropism for hematopoietic tissues lead to a hematological study, which noted an increase in neutrophil levels and reduction in basophil levels throughout infection, while lymphocyte levels were initially reduced but elevated in the later stages of infection (Forzán et al., 2016). FV3 infection also affected the morphology of several leukocytes including the

apparent activation of monocytes and nuclear deterioration in neutrophils. Several studies have relied on bath infection methods to investigate larval wood frog susceptibility to FV3 and FV3-like viruses (Haislip et al., 2011; Reeve et al., 2013; Echaubard et al., 2014; Kirschman et al., 2018). Dose response experiments have suggested that mortality increases with the dose of inoculum (Warne et al., 2011), but methods vary with baths often ranging from 10^3 - 10^4 pfu/mL and lasting from 12 – 72 h. Results have consistently indicated a higher susceptibility of *R. sylvatica* to FV3 than other *Rana* species (Haislip et al., 2011; Echaubard et al., 2014). Larvae experience 30 - 100% mortality, with fatally infected individuals surviving anywhere from days to weeks (Haislip et al., 2011; Reeve et al., 2013; Echaubard et al., 2014; Kirschman et al., 2018). Despite the numerous studies demonstrating susceptibility of *R. sylvatica* to FV3 we currently have a very limited understanding of the host-virus interactions leading to these health outcomes, or whether *R. sylvatica* that recover from infection develop immunological memory to FV3.

A pressing question in the field is the potential reservoir(s) of FV3. Interestingly, surveys of adult male wood frogs migrating to, or present at, vernal breeding pools revealed individuals from 25 of 27 independent sites in Eastern Canada and the United States tested positive for ranaviral infection, with an average prevalence of ~39% among individuals as determined by detection of viral DNA and mRNA (Crespi et al., 2015). Together with the noted absence of external signs of ranavirosis, the authors concluded that these individuals were experiencing asymptomatic, low-level, active ranaviral infections. We propose that wood frogs that recover from ranavirosis may serve as a reservoir and that immunosuppressive events, such as multiple bouts of freezing coupled with depleted energy reserves, may permit reactivation of infection, like what has been observed in FV3-recovered *X. laevis* that have been irradiated to induce immunosuppression (Robert et al., 2007). However, this is a theory that needs to be tested.

Very little is currently known about the cellular mechanisms of FV3 infection in *R. sylvatica*. One *in vitro* study of SR-A function has indicated that FV3 may be bound by SR-As on the cell surface to initiate receptor-mediated entry in the wood frog larval retinal tissue cell line WoodTad-rpe (Vo et al., 2019b). As WoodTad-rpe does not appear to express SR-AI or MARCO, this suggests that SCARA3, SCARA4, SCARA5 and/or additional unknown SR family members are able to act as receptors for FV3 entry. An additional study comparing infection with wildtype FV3 or a gene-knockout mutant deficient for a viral homologue of eukaryotic translation initiation factor 2 subunit α known as vIF-2α, a putative immune-suppression gene, observed reduced

activity and growth in juvenile *R. sylvatica* infected with the mutant (Bienentreu et al., 2020). While the authors hypothesized that this may be due to a more energetically expensive immune response because of the failed immune evasion, viral load and mortality did not differ significantly from wood frogs infected with wild-type FV3, so it remains unclear to what extent the vIF-2α gene supports infection in wood frogs (Forzán et al., 2019). While studies have demonstrated that host genotype and virus strain interactions influence susceptibility (Echaubard et al., 2014), no studies have been published examining the *R. sylvatica* immune response to FV3, so it remains unclear how the virus is cleared in cases of sub-lethal infection, what immune responses are evaded, or potentially what immunological differences exist in wood frog antiviral responses to result in systemic infection and mortality of this highly susceptible anuran.

4. Concluding Remarks

Wood frogs have an intriguing life history. Their development relies on healthy aquatic and terrestrial ecosystems, and they have an impressive capacity to withstand whole-body freezing. Yet, we have a limited and fragmented understanding of the wood frog immune system and know virtually nothing of how their immune system has evolved to support host defence throughout their life history. We believe wood frogs present an exciting opportunity as an emerging anuran study system for comparison with the *X. laevis* model organism, and vertebrates in general, to identify points of conservation and divergence in the evolution of the immune system. Furthermore, the high susceptibility of wood frogs to FV3 further supports their use as a comparator anuran to the relatively resistant *X. laevis* model to elucidate the host- and/or viral-mediated factors that underlie host susceptibility to FV3. Advancing research on the wood frog immune system will require key resources – an annotated genome, transcriptomes, proteomes, an expanded invitrome (*i.e.*, cell lines) and reagents (*e.g.*, antibodies, recombinant cytokines). As more resources begin to become available, we look forward to the immunological lessons that may be learned from this fascinating and far-reaching species.

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6. Conflicting Interests

The authors declare no conflicts of interest.

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Figures

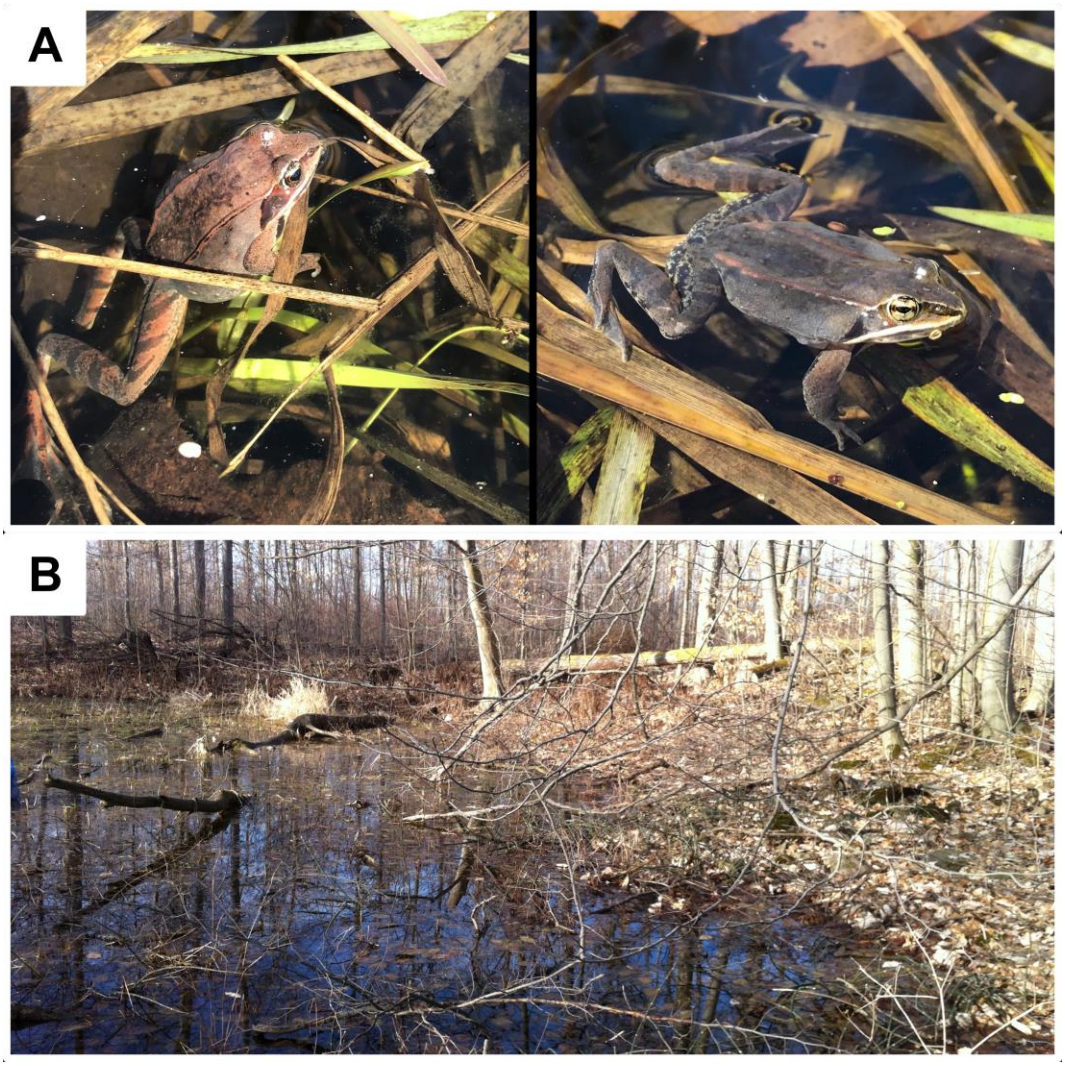
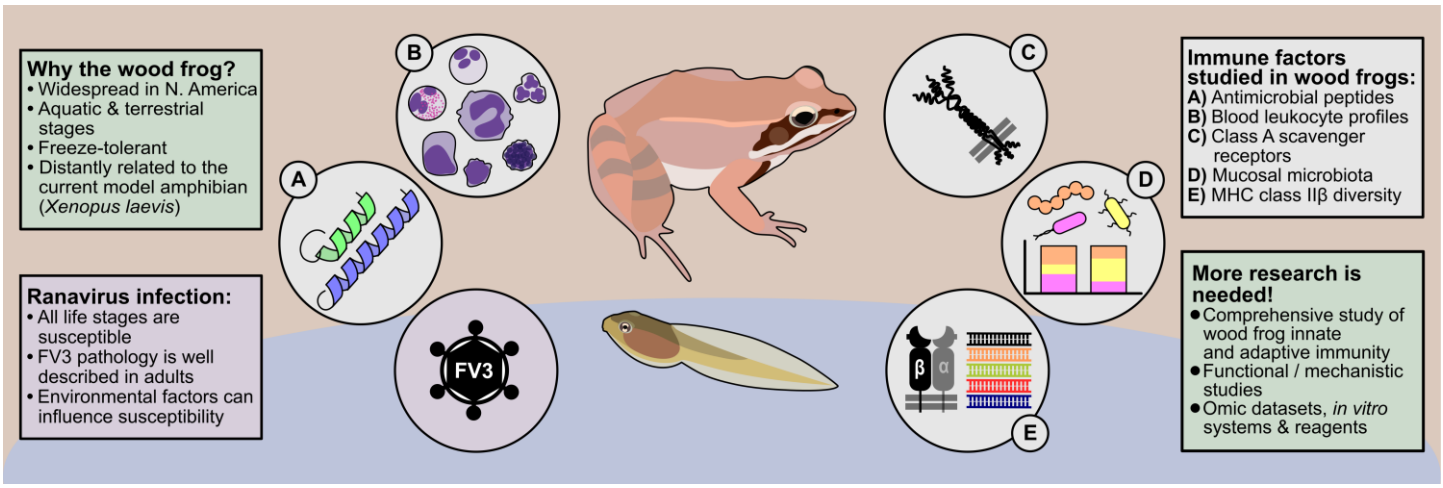


Fig. 1. Wood frogs in spring vernal pools. Photographs of (A) Female (left) and male (right) wood frogs (*Rana sylvatica*) amongst vegetation in spring vernal pools in Southern Ontario, Canada; and (B) typical topography of spring vernal pools characterized by gradually sloping edges, shallow water containing floating vegetation and shrubs, and fallen trees and/or branches. Photographs taken by B. Katzenback.



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821 **Fig 2.** A graphical summary of immune-relevant studies performed in *R. sylvatica*. Interesting
822 aspects of *R. sylvatica* biology are highlighted, including their susceptibility to Frog Virus 3.
823 Elements of innate and adaptive immunity which have been investigated in *R. sylvatica* are
824 depicted, and recommendations for further research are made.

Tables

Table 1: Observed wood frog blood cell proportions

Husbandry information	Treatment Group	Life Stage	Blood sample site	Percent leukocyte type (%)					Reference
				L	N	Eo	B	M	
Wild-collected eggs masses reared in a laboratory environment at 19°C. Held for 10+ weeks.	Control, All Diets	Tadpole	Heart	20 - 60	3 - 12	1 - 5	2 - 10	N/R	(Szuroczki et al., 2019)
Wild-collected eggs masses reared in a laboratory environment at 21 - 23°C until metamorphosis.	Control and desiccation signals; 72 h after PHA-injection	Metamorph	Heart; brachial vein	N/R	N/R	N/R	N/R	N/R	(Gervasi and Foufopoulos, 2008)
Adult frogs captured in April and bred in captivity. Tadpoles reared in outdoor mesocosms for 5+ weeks. Metamorphs housed in a laboratory environment at 22°C for 21 days.	Control	Metamorph	Heart	88 - 100	0 - 12	0 - 1	N/O	N/O	(Gavel et al., 2019)
Adult frogs captured from breeding pools in May and held in a laboratory environment at 20 - 22°C for 6 months.	Control	Adult	Maxillary Vein	63.7 - 90	0.4 - 13.3	0 - 4.5	5.9 - 14.8	0 - 3.4	(Forzán et al., 2016)

L, lymphocyte; N, neutrophil; Eo, eosinophil; B, basophil; M, monocytes; N/R, leukocytes observed but numbers were not reported; N/O, Leukocytes not observed.