**Introduction**

Viruses are considered to be the most ubiquitous and abundant organisms in the world, with an estimated 1031 viruses across the globe (Breitbart and Rohwer 2005). In fact, mathematical models have predicted that the diversity of viruses found in 1 kilogram of marine surface sediment is larger than the diversity of all reptiles on the planet (Breitbart et al. 2004). Not only do they serve as a reservoir for the greatest genetic diversity on Earth, they are important agents of mortality and are central in global geochemical cycles (Suttle 2005). Considering viral abundance, diversity, and ecological importance, surprisingly little is known about their biogeographical distributions, community structure, and ecological dynamics (Breitbart and Rohwer 2005).

 The genus *Ranavirus* encompasses a group of large, double-stranded DNA viruses (family Iridoviridae) and provides an example of the ubiquitous, yet relatively unknown, nature of viruses. These viruses infect a wide range of species, are transmitted through multiple routes, and have large, varying effects between species and populations, making them more general than most viral species, which provides an interesting study system. Ranaviruses are found in 32 countries on 6 continents and possess an extremely large host range, with the capability of infecting multiple species across classes (Chinchar and Waltzek 2014), specifically bony fish (Actinopterygii**)**, reptiles (Reptilia), and amphibians (Amphibia). Amphibians (Lissamphibia) are a diverse and abundant group of organisms that serve as indicators of environmental health and are a vital link in the food web (Wake 1991). However, amphibian populations worldwide have been in decline, and studies point to emerging infectious diseases as one of the major contributors (Gray and Chinchar 2015; Harp and Petranka 2006). Specifically, they have been shown to be particularly susceptible to ranaviral disease: one study reported that the most common cause of amphibian mortality events was infection by ranaviruses (Green et al. 2002). Other studies have reported that ranaviruses are resulting in population declines (Petranka et al. 2003; Teacher et al. 2010; Price et al. 2014; Wheelwright et al. 2014) and have the potential to cause local extinctions (Earl and Gray 2014).

Ranavirus infected wood frog larvae

There are 3 official *Ranavirus* species known to infect amphibians: *Frog virus 3* (FV3), *Ambystoma tigrinum virus* (ATV), and *Bohle iridovirus* (BIV). They have been reported in at least 105 amphibian species in 18 families (Duffus et al. 2015). Transmission of the virus can occur through several routes, including through water and substrate, direct contact, and ingestion of infected individuals. Although the symptoms vary between host and pathogen species, host life stage and transmission route (REF), the viruses generally cause lethargy, internal and external hemorrhaging, swelling of the body and legs, and erratic swimming, with fatal cases involving necrosis in the liver, kidney, and spleen (REF). Additionally, the amphibian ranaviruses have a general trend in the timing of outbreaks, with most die-offs occurring rapidly in the mid to late-summer months (REF). Although these patterns can be observed, there are notable exceptions: the timing of outbreaks in certain species, like Bullfrogs, is much later in the summer (REF) and individuals can be asymptomatic (REF). Unsurprisingly, the mortality rate of infected individuals is also inconsistent.

The outcomes of these outbreaks can vary between species, populations and location, ranging from no apparent mortality to mass die-offs. Factors, such as host life stage, temperature, and anthropogenic influences, have been implicated in this variation. For example, ranavirus outbreaks frequently occur in the mid to late summer months, which coincide with both high temperatures and often the metamorphosis of amphibian larvae. However, studies that tested these influences found conflicting results. Arial et al. (2009) found that multiple amphibian ranavirus species replicated faster with increasing temperature up to a certain optimum, usually between 24°C and 28°C. Contrastingly, another study showed that salamander larvae reared at 10°C or 18°C experienced higher mortality after exposure to ATV compared to larvae reared at 26°C (Rojas et al. 2005), and proposed that the immune system could be suppressed in colder temperatures. Additionally, the process of larval metamorphosis involves natural immunosuppression (Rollins-Smith 1998; Carey et al. 1999); thus, it has been hypothesized that some of the variation in ranavirus outbreak timing could be explained by host life stage. However, although one study found a 1.7-fold increase in mortality of wood frog tadpoles exposed to ranavirus with increasing Gosner (1960) development stages (Warne et al. 2011; Figure 1), another found that metamorphosis was not always the most susceptible stage (Haislip et al. 2011). Finally, further environmental and anthropogenic factors may contribute to the variance in disease prevalence and host mortality, as well. Studies have shown increased probability of outbreaks in areas with cattle access (Gray et al. 2007; Greer and Collins 2008; Hoverman et al. 2012), low elevation (Gray et al. 2009b; Sutton et al. 2014), high elevation (Gahl and Calhoun 2010), and pesticides (Forson and Storfer 2006b; Kerby and Storfer 2009; Kerby et al. 2011).

**Figure 1** shows that the probability of death by ranavirus infection increases with increasing Gosner developmental stage (Warne et al. 2011)

Although there is strong evidence that ranavirus replication and the outcome of infection depend on the host and virus species, as well as other confounding environmental and anthropogenic factors (Speare and Smith 1992; Grant et al. 2003; Rojas et al. 2005; Ariel et al. 2009b), it is clear that ranaviruses have the potential to impact ectothermic vertebrate populations and can often trigger significant morbidity and mortality. Investigating the drivers of outbreak variation would not only further our understanding of generalist viruses and infectious disease ecology in general, but could inform amphibian conservation efforts, as well. Preliminary results from my current research indicate that ranaviruses are indeed present in the state of Vermont. My proposed dissertation research will focus on the ecological and anthropogenic variables associated with disease presence, the effects of these viruses on amphibian communities, how transmission is occurring between ponds and individuals, and the characteristics of both host and pathogen that influence host mortality (Figure 2).

**Question II: What are the effects of ranaviruses in amphibian communities of Vermont?**

**Objective II: I aim to determine a) whether there is a relationship between species diversity and disease presence and b) if disease severity varies across sites.**

**Hypotheses and Predictions:**

H: Ranavirus reduces species and genetic diversity

P: Ranavirus will cause mortality in susceptible species, which will reduce species richness, evenness, and genetic diversity in ranavirus present sites after periods of high disease prevalence.

H: Host community and genetic structure causes variation in disease severity between amphibian populations and communities

P: Communities that have reservoir species will have increased ranavirus severity.

P: Communities and populations with low genetic diversity, richness, and evenness will have increased ranavirus severity.

Background:

- community stability/equilibrium

- species diversity metrics in general/ in system

- genetic diversity in general? In system?

- mortality and viral load are correlated (find reference)

- reservoir species (in general, and in ranavirus system)

- Not too many community analyses in this system (make sure)

- Variance in virulence

- Most susceptible species (wood frogs)?

Approach: measure disease severity with viral load

- Add graph that has species richness over time in each site; probably doesn’t change much; abundance changes (add graph) but we do not know prevalence or load data yet

- viral load with qPCR to determine how virulent; estimate host abundance and host community structure; estimate differences in these measurements between present sites and absent sites; look at abiotic factor comparison between present and absent sites

*Viral load quantification*

Viral load will first be estimated for all samples by comparing the cycle threshold (the crossing point of the amplification curve with the preset threshold of fluorescence detection) of the sample to the standard curve. Using Nanodrop, the amount of DNA in each sample was estimated and then diluted to the same concentration of 5 ng/uL. A dilution factor was applied to samples that had a concentration of less than 5 ng/uL. Using this method, a rough estimate of viral copy number for each positive sample is calculated, and the resulting estimates can then be compared. In the future, to obtain more precise calculations of viral copy number, the positive samples will be run again in duplicate with an internal control in the form of an amphibian gene (yet to be determined).

Expected/alternative results:

If no change in species richness, evenness, or abundance is observed, perhaps ranavirus is not causing severe mortality (further studies of fitness effects?)

Not too many community analyses in this system (make sure)

**Question III: How are ranaviruses being transmitted among individuals and between sites?**

H1: Transmission between communities varies due to the environment, human influence, and disease prevalence

P1: Transmission between communities will increase with increasing human visitation, increasing virions in the environment, and increasing statewide and within-community disease prevalence.

H2: Transmission within communities varies due to different species interaction strengths and contact rates.

P2: Individuals and species will interact with each other non-randomly, which will influence disease transmission

P2: Increased density of conspecifics and heterospecifics will increase transmission; increased predation by amphibians on amphibians will increase transmission; increased mating will increase transmission

H3: The number of hosts influences pathogen fitness and transmission.

P3: Pathogen fitness will decrease with increasing number of hosts, so transmission mode will expand.

How does pathogen host switching influence transmission (come up with probabilities of ranavirus infection across species due to interactions: does ranavirus have the same efficiency across species?) – use bacteriophages as model; “efficiency” = fitness?

expose bacteriophage to multiple hosts vs single host – trade offs? Does primary transmission mode shift? “Evolutionary Reversals During Viral Adaptation to Alternating Hosts” - W. D. Crill, H. A. Wichman and J. J. Bull

Background:

- how is it transmitted between individuals? (more detail; which has bigger dose)

- how do humans influence transmission (other ways to go between sites)?

- Previous eDNA research

- Host-switching behavior (general/ranavirus)

- Experimental evolution: viruses dominate our planet and their evolution is a broad and applied field that can be studied in the real time

Approach 1: **use transmission network analyses to make SIR models - how different interactions (and hypotheses of these interactions) influence disease prevalence**

Approach 2: Predict the probability of being transmitted to another site using environmental data (eDNA), anthropogenic influences (visitation estimates), and ranavirus prevalence

Approach 3: experimental evolution

Expected results:

First transmission network/SIR model in system

**Question IV: Are there host and/or pathogen characteristics that increase or decrease pathogen fitness? Are there environmental stressors that increase susceptibility, resilience, or tolerance?**

* Does mortality/fitness change with multiple stressors and at different life stages?
* If there are any that are asymptomatic – are they differentially expressing genes?
* Are the survivors differentially expressing genes?
* There are adaptive alleles: allelic shifts as summer passes/infection prevalence increases?
* Characteristics of the host that increase transmission? [Genetic structure] (QII?)
* Characteristics of pathogen [connected with how is it getting around/how sick is it making them] (QIII)
* gene flow (migration) – landscape genetics (not disease related?); estimate migration; high migration could be introducing the disease at a higher rate but could also add to genetic diversity which could buffer effects of disease (QII)
* Co-infection with chytrid?

H: Multiple stressors increase host susceptibility to ranavirus and the susceptibility to these stressors varies between life stages.

P:

H: There are adaptive alleles and differences in gene expression in asymptomatic and surviving individuals, which signal increased host tolerance

P:

Background

- Multiple stressors

- Define host susceptibility, tolerance, and resistance

- has differential gene expression been done in this system?

- what is known about ranavirus genomics

Approach:

Genomics

Expected Results:

- the only expression studies deal with immune response (check); not asymptomatic or surviving individuals

- multiple stressors have not taken life stage into consideration or exposed individuals to pesticide before the disease

ideas

adding timing of pesticide to experiment? (pesticide first then disease, disease first then pesticide)