Development of a Sin Nombre Virus Transmission Model in Deer Mice and its use for Assessing Oral Bait Vaccine Candidates

By

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Abstract

Hantaviruses are zoonotic viruses in the Order *Bunyavirales*, and cause two distinct diseases for which there are no approved vaccines or therapeutics. In the Americas, hantavirus infection causes hantavirus cardiopulmonary syndrome (HCPS), a severe respiratory disease with a fatality rate of 35%. In North America, the primary agent responsible for HCPS is Sin Nombre virus (SNV) carried by *Peromyscus maniculatus,* or deer mice, and humans contract disease through exposure to the virus in contaminated rodent excreta and/or secreta. One major issue of working with certain hantaviruses is the need to propagate viral stocks *in vivo* within the virus's natural host. Here we refined an infection protocol for developing viral stocks *in vivo* by utilizing intraperitoneal infection route as opposed to the classical intramuscular route. We also show that age and sex of deer mice has little effect on viral replication and shedding and that these factors are not an issue for viral stock production. We also developed a reliable experimental model of SNV transmission in deer mice. Transmission of SNV between deer mice is not well understood and the few studies that have attempted to examine transmission have either not been successful or have failed to shed light on how the virus is transmitted between animals. We show that direct contact is the main driver of SNV transmission rather than exposure to the virus in the environment, which is the case for human infection. Additionally, we use our developed transmission model to show that heat shock responses and changes in testosterone levels in SNV infected deer mice do not influence viral replication, shedding, or transmission. Finally, we used our transmission model to test the efficacy of vaccine platforms for protecting deer mice against acquiring SNV. Vaccination with rVSVΔG/SNVGPC was able to significantly reduce the risk of becoming infected with SNV in our model. Overall, we were able to develop a reliable SNV

transmission model in deer mice and use this model to test various hypotheses on SNV ecology and to examine the protective efficacy of vaccines developed against SNV.

Publications Arising from this Work

1. **Warner, B. M.**, Stein, D. R., Griffin, B. D., Tierney, K., Leung, A., Sloan, A., ... & Safronetz, D. (2019). Development and Characterization of a Sin Nombre Virus Transmission Model in Peromyscus maniculatus. *Viruses*, *11*(2), 183.
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Chapter 1 – Introduction

1.1 Hantavirus History

1.1.1 Old World Hantaviruses

Historically there has been a dichotomous classification of hantaviruses into so called "Old World" and "New World" hantaviruses. "Old World" hantaviruses are the causative agent of hemorrhagic fever with renal syndrome (HRFS) or nephropathia endemica (NE) seen predominantly throughout Europe and Asia (Jonsson et al. 2010). Originally described as Korean hemorrhagic fever (KHF) following an outbreak of the disease during the Korean War in the early 1950s, HFRS had been known by several different names depending on the region (Lee et al. 1978). The clinical manifestations of HFRS were described in 1954, more than twenty years before discovery of the etiologic agent(s) responsible for the disease (Sheedy et al. 1954). After speculation for many years that the infectious agent responsible for HFRS was rodent borne, in 1978 Lee et al were able to show that HFRS was indeed caused by a virus carried by *Apodemus agrarius*, or striped field mouse (Lee et al. 1978). Tissues from these mice immunofluoresced when treated with serum from convalescent KHF patients and the agent was successfully passaged through these mice, but interestingly not in various tissue cultures or in normal laboratory animals (Lee et al. 1978). The agent discovered by Lee was named Hantaan virus (HTNV) and its finding led the realization that KHF and other hemorrhagic diseases such as HFRS and NE in Europe were similar. This resulted in the discovery of several other similar agents throughout Asia as well as Europe such as Seoul virus (SEOV) and Dobrava virus (DOBV) among others in the 1980s and 1990s (Avsic-Zupanc et al. 1992; Lee et al. 1982; Chan et al. 1987). NE, a milder form of HFRS that was described much earlier was discovered to also be caused by a rodent-borne virus found in Finland named Puumala virus (PUUV) after the region in which it was discovered (Brummer-

Korvenkontio et al. 1980). In 1985, following the antigenic and genetic characterization of several different HFRS-causing agents and their classification into what was then the *Bunyaviridae* family, the *Hantavirus* genus name was proposed by Schmaljohn et al in reference to the prototypical member HNTV (1985).

1.1.2 New World Hantaviruses

The prior discovery of several hantaviruses that can cause HFRS made the identification of New World hantaviruses easier than the initial discovery of Hantaan virus. In 1993, there was a cluster of cases of acute respiratory distress syndrome (ARDS) resulting in the deaths of 75% of cases during an outbreak in the southwestern Four Corners region of the United States (Nichol et al. 1993). Initial serologic surveys of patients from the outbreak identified antibodies that were cross-reactive with hantavirus antigens, an unexpected finding as hantaviruses had previously not been associated with disease in North America. RT-PCR using primer sets from known hantaviruses resulted in identification of sequences that were similar to those hantaviruses, but represented a novel virus that most closely resembled Prospect Hill virus (PHV), the only known North American hantavirus at the time (Nichol et al. 1993). New genetic analysis revealed that all the ARDS patients tested were positive for the newly identified hantavirus that was originally named the Four Corners virus, but eventually renamed Sin Nombre virus (SNV). The cardiac and pulmonary involvement during infection with newly discovered hantaviruses in the Americas led to the designation of the disease caused by these “New World” hantaviruses as hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS) (Schmaljohn and Hjelle 1997). Following the initial outbreak, rodent trapping around the homes of those infected with SNV were able to identify the deer mouse (*Peromyscus maniculatus)* as the most likely reservoir for the virus. Deer mice were the most prevalent species caught during trapping and around one third of deer mice caught were seropositive for antibodies reactive against various

hantaviruses (Nichol et al. 1993; Childs et al. 1994). While other species were found to also be seropositive, deer mice were identified as the primary reservoir for SNV in United States and eventually also in Canada (Drebot et al. 2000). Following the identification and characterization of SNV, viruses that can cause HCPS were soon identified in several countries in North and South America including Canada, Brazil, Chile, Argentina, Paraguay, and Bolivia among others (Jonsson et al. 2010).

1.2 Taxonomy

For over thirty years following the first designation of Hantaviruses, all known hantaviruses made up a genus within the family *Bunyaviridae* (ICTV). In 2016, the International Committee on the Taxonomy of Viruses (ICTV) reclassified the bunyaviruses from a family to an order, thereby altering the taxonomy of all the viruses that were a part of the previous family (ICTV 2016). *Bunyaviridae* become the Order *Bunyavirales,* and the *Hantavirus* genus became the Family *Hantaviridae.* As of the 2018b release and an update to the taxonomy in 2019 by ICTV, the Family *Hantaviridae* now consists of four subfamilies and seven genera made up of 47 distinct viruses (ICTV 2018b; Abudurexiti et al. 2019). The type species HTNV, officially *Hantaan orthohantavirus,* falls under the *Orthohantavirus* genus, the prototypical genus that includes all of the rodent-borne hantaviruses and falls under the *Mammantavirinae* subfamily, which distinguishes it from the three non mammal-borne subfamilies. The genus includes 36 of the 47 species that make up the family and all hantaviruses that are known to cause human disease. The species that make up the *Hantaviridae* family are distinguished by criteria set forth by ICTV and are so named *Sin Nombre orthohantavirus*, *Puumala orthohantavirus*, etcetera officially. These include having a unique rodent reservoir, having a greater than 7% difference at the amino acid level of the complete nucleocapsid (N) and glycoprotein precursor (GPC) proteins, having a

greater than four-fold difference in cross-neutralization, and having not undergone a re-assortment event (Schlegel et al. 2014; Plyusnin et al. 2011). Fulfilling these criteria can be challenging, and would include characterization of viruses that typically has not occurred and require cell culture isolates of each virus. This has led to confusion in the naming and classification of different viruses, with some previously acknowledged hantaviruses being removed as official species and reclassified as strains under others (ICTV 2016). The lack of meeting all criteria led to a proposal to change the classification requirements to having greater than 10% disparity at the amino acid level in the N protein and 12% in the GPC and there have been discussions on the ability to classify given viruses based solely on genetic data (ICTV 2016; Maes et al. 2009).

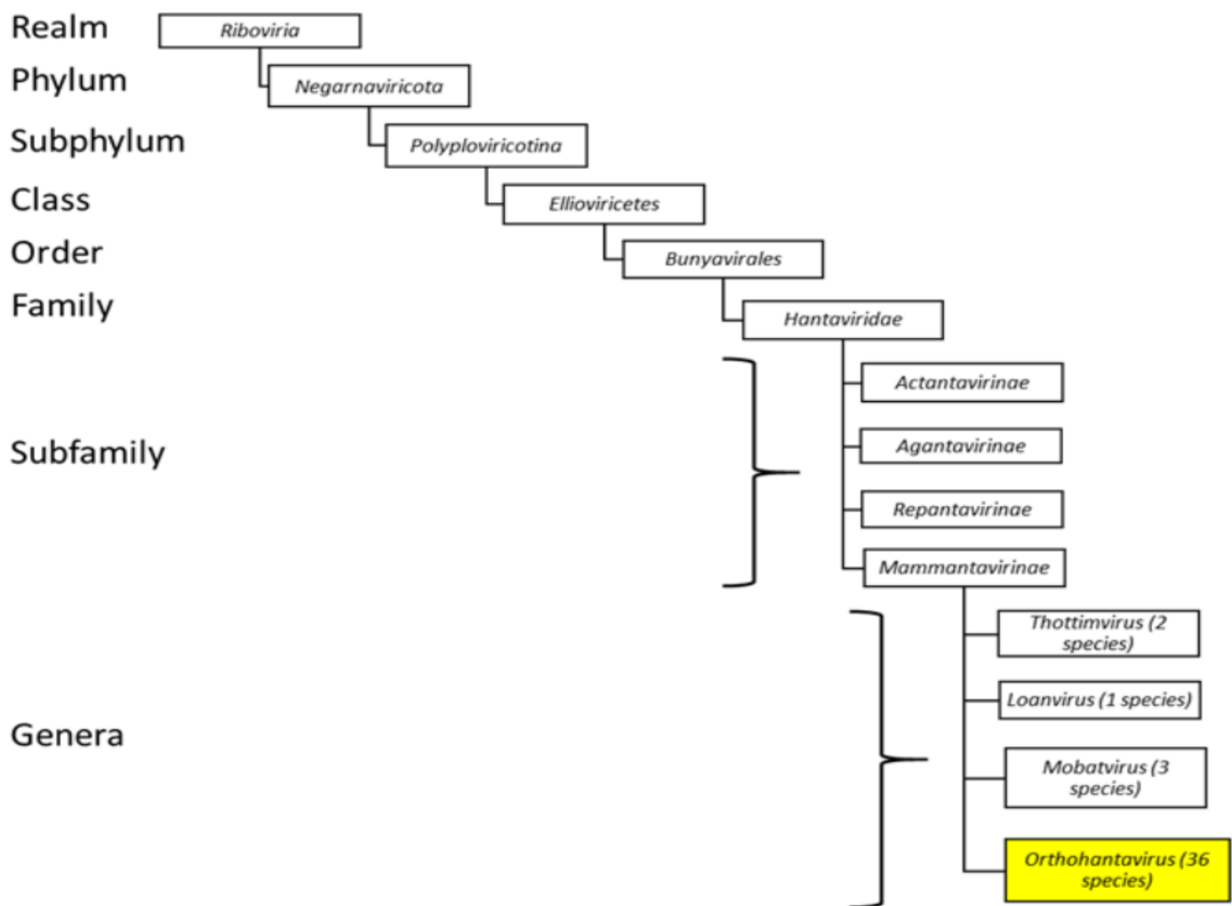

Figure 1. Taxonomy of hantaviruses. Toxonomic classification of hantaviruses. Hantaviruses that have been discovered and can cause human disease are part of the *Orthohantavirus* genus, consisting of 36 separately described species (highlighted).

1.3 Distribution

Hantaviruses have a global distribution, with human cases and/or the presence of hantaviruses in rodent or mammal populations on every continent aside from Antarctica. Our knowledge of hantavirus distribution comes from both human and field surveillance data. Globally, it is estimated that there are tens of thousands of hantavirus infections each year, with the vast majority of these HFRS or NE cases occurring in Europe and Asia (Jonsson et al. 2010). HCPS incidence in the Americas is far lower, however the number of both Old and New World hantavirus infections is likely underreported due to subclinical infection, for which there is little data available. There is also little data regarding hantaviruses in Africa and Australia. There are reports of seroprevalence in humans in certain areas of Africa as well as reports of molecular evidence of rodent and shrew-borne hantaviruses (Schlegel et al. 2014; Klempa et al. 2006; Klempa et al. 2007; Klempa et al. 2010). In Australia, there have been reports of seropositive rodents, but no humans cases have been reported (Leduc et al. 1986; Bi et al. 2005). Due to the proximity and the trade between Australia and Asian countries in which HFRS is prevalent, monitoring and surveillance of the presence of hantavirus could be warranted (Bi et al. 2005).

The presence of human cases and outbreak situations typically lead to investigation into the presence of viruses in the rodent populations in the area of the outbreak. The distribution of hantaviruses relies mainly on the habitat range of each virus's rodent host(s). The classical dichotomy of hantaviruses into "Old World" and "New World" viruses is based on their infection of species found in the "Old World" and "New World", respectively. This affects the range of humans cases seen that are caused by a given virus, which is generally limited to the habitat range

of its host. Therefore, the incidence of human infection caused by certain hantaviruses seen in different parts of the world is due to a combination of viral evolution alongside its chosen host species, prevalence within the host population, ability to transmit amongst that population, and its ability to spillover into humans. The exception to the limited geographical range of hantaviruses seems to be the HFRS-causing SEOV, carried by the brown or Norway rat, which has been shown to have a global distribution (Jonsson et al. 2010). Questions remain regarding the transmission and reach of hantaviruses within rodent populations, but in general the presence of a given host species in a geographical area indicates that human cases can occur.

1.4 Hantavirus Biology

1.4.1 Genome

Hantavirus virions are spherical, enveloped, and can be anywhere between 70-210 nm in diameter (Schlegel et al. 2014). Their genomes are comprised of three segments of negative sense RNA, termed the small (S) segment, medium (M) segment, and large (L) segment, respectively, based on their relative sizes (Jonsson et al. 2010). The sequence identity between different hantavirus species ranges anywhere from between 50-75% at the nucleotide level and 42-85% at the amino acid (aa) level (Schlegel et al. 2014). The most and least conserved sequences between species are in the M segment and the L segment, respectively.

The S segment is approximately 1500 base pairs (bp), but its size can vary greatly between different viruses based on the length of not only the open reading frame (ORF), but also the 5' and 3' non-coding regions (NCR) (Schlegel et al. 2014). The S segment encodes the viral nucleocapsid protein, a 428-433 aa residue protein that binds to each genome segment to form ribonucleoprotein complexes (RNPs) within the virion. Additionally, some hantavirus species encode a non-structural protein (NSs) within a second ORF in a +1 position on the S segment (Schlegel et al.

2014). The size of the NSs is highly variable depending on the virus, and multiple functions for this protein have been proposed, including adaptation to rodent hosts and interferon antagonism (Ulrich et al. 2002; Jaaskelainen et al. 2007; Jaaskelainen et al. 2008).

The M segment is approximately 3500-3800 bp and encodes the glycoprotein precursor (GPC) (Schlegel et al. 2014). During translation, GPC is cleaved into two separate proteins at a conserved WAASA site within the protein sequence. The cleaved N terminal half of the protein makes up the approximately 510 aa protein Gn, and the C terminal half consists of a 480 aa protein, Gc. Gn and Gc are both transmembrane proteins made of up globular heads and have cytoplasmic C terminal regions that may interact with the nucleocapsid protein or have roles in virus assembly (Cifuentes-Munoz et al. 2014; Muyangwa et al. 2015; Shi et al. 2004). These cytoplasmic tails have also been suggested to contain motifs important for cellular trafficking and endoplasmic reticulum (ER) retention (Spiropoulou 2001). Together Gn and Gc form heterodimers on the surface of the virion made up of four subunits each of Gn and Gc (Cifuentes-Munoz et al. 2014; Muyangwa et al. 2015). The structure of this glycoprotein dimer allows for interaction with cellular receptors leading to binding and entry, discussed in the following section. Both glycoproteins are also glycosylated, and bound by high-mannose type oligosaccharides and may be important for protein folding and trafficking (Shi et al. 2004).

The L segment is approximately 6500 bp and encodes the 2100 aa RNA-dependent RNA polymerase (RdRp) (Schlegel et al 2014). RdRp facilitates the transcription of mRNA and cRNA from the genome as well as replication of the genomic vRNA from cRNA required for viral packaging (Jonsson et al. 2010). There are structurally conserved motifs found in all RdRps, and L proteins of hantaviruses contain the motifs preA, A, B, C, D, and E typically found in RdRps of viruses containing negative sense, segmented genomes (Kukkonen et al. 2005). Because of the

relatively limited expression of the L protein and lack of a developed reverse genetics system for studying hantaviruses, much of the knowledge of the hantaviral L protein comes from the study of other members of the *Bunyavirales* order.

1.4.2 Life Cycle

The hantavirus life cycle begins with viral entry via receptor mediated endocytosis and clathrin-coated vesicles (Hepojoki et al. 2012; Jin et al. 2002). Hantaviruses are able to infect multiple different cell types including endothelial and epithelial cells, macrophages, follicular dendritic cells, and lymphocytes (Mackow and Gavrilovskaya 2001a; Markotic et al. 2007; Raftery et al. 2002). To facilitate entry, viral Gn protein interacts with one of a number of potential receptors, initiating endocytosis. The most well known receptors for hantavirus entry are β integrins, which bind to various α integrin subunits to form heterodimers on the surface of cells and are involved in platelet aggregation and binding to the extracellular matrix (Bennet 2005). Specifically, pathogenic hantaviruses have been shown to preferentially bind integrins containing β_3 subunits such as α_{IIa}/β_3 and α_v/β_3, while non-pathogenic hantaviruses bind to integrins consisting of β_1 subunits (Gavrilovskaya et al. 1998; Gavrilovskaya et al. 1999a). Other receptors for hantavirus entry have been suggested, and there is some *in vitro* evidence for their role in viral entry including CD55 or decay-accelerating factor (DAF), and also the β_2 integrins complement receptor 3 (CR3) and complement receptor 4 (CR4) (Hepojoki et al. 2012; Raftery et al. 2014). Recently, the cadherin-superfamily receptor protocadherin-1 was found to be a critical receptor for the entry of New World hantaviruses (Jangra et al. 2018).

Following receptor-mediated entry into endosomes, fusion of the viral and endosomal membranes occur through the viral Gc protein following endosomal pH reduction (Hepojoki et al. 2012). Uncoating of the virion releases the S, M, and L RNP complexes into the cytoplasm, where

transcription, translation, and replication can then occur. The first step to occur is transcription of S, M, and L mRNA by RdRp within the cytoplasm. Viral mRNA does not contain poly A tails, but the viral L protein is capable of “snatching” 5’ caps from host transcripts which are then bound by N and stored for use as primers for transcription of mRNA (Cheng and Mir 2012). S and L mRNA is translated on free ribosomes in the cytoplasm, while M mRNA is translated at the rough ER (Jonsson et al. 2010; Schlegel et al. 2014). This transcription and translation allows for the accumulation of viral proteins and to allow for RdRp to switch its role from transcription of mRNA to replication of vRNA through cRNA.

Exact mechanisms of viral replication are not clear, but it has been suggested that the panhandle structure of the vRNA formed via complementary 3’ and 5’ NCRs allows for priming of replication by RdRp (Schlegel et al. 2014). Once sufficient vRNA levels have been transcribed, N protein then binds vRNA to form RNPs which are shuttled to the ER-Golgi-intermediate compartment via dynein (Ramanathan et al. 2007). Recruitment of RNPs by the cytoplasmic tail of viral glycoproteins embedded in the Golgi results in assembly and envelopment of RNPs and RdRp followed by budding at the Golgi (Spiropoulou 2001). There is also evidence that some “New World” hantaviruses assemble and bud at the cell surface rather than the Golgi (Ravkov et al. 1997). While evidence supports likely budding and assembly at the golgi for most or all hantaviruses, there may be late budding at the plasma membrane for some “New World” viruses. The segmented genome of hantaviruses also allows for potential re-assortment of genomic segments during co-infection with multiple hantavirus strains or species. This may allow, in the absence of well-developed reverse genetics systems for hantavirus, the development of re-assortant viruses for studies of pathogenesis, immune system evasion, replication, or transmission.

1.5 Hantavirus Ecology

1.5.1 Infection of Reservoir Hosts

The worldwide distribution of hantaviruses is due to their zoonotic nature and co-evolution alongside their reservoir hosts. Most commonly, hantaviruses are found within rodent species, but recently characterized hantaviruses have been found in bats, shrews, and moles (Klempa et al. 2007; Weiss et al. 2012; Arai et al. 2007; Kang et al. 2011). The main human-disease causing hantaviruses are carried by *Crecitid* and *Murid* members of the Order *Rodentia* (Schlegel et al. 2014). Generally, it is thought that hantaviral infection of host species does not cause any overt disease. However it has been shown that the growth rates of rats infected with SEOV and of deer mice infected with SNV may be altered (Childs et al. 1989; Douglass et al. 2007). There is also evidence that infection with PUUV results in impaired over-winter survival of infected bank voles, likely due to a need to invest resources in dealing with persistent infection (Kallio et al. 2007). Because hantaviruses can be contracted via inhalational of viral particles, and the high morbidity and mortality associated with infection, their handling requires CL-3 containment for *in vitro* study or CL-4 for *in vivo* experiments. This requirement, along with the need for specific host species has prevented the study of experimental infection of host species for most hantaviruses. Therefore the knowledge we have gained on infection in reservoir species comes from various experimental and field studies examining various ecological aspects of infection such as maintenance, transmission, prevalence, distribution, spillover into humans and other animals, and viral evolution (Jonsson et al. 2010). A number of studies of Old and New World hantaviruses have been performed. Here, I will briefly go over some of the main hantaviruses of consequence for human disease and what has been revealed about infection of their reservoir hosts.

The three most common host species for hantaviruses that cause HFRS or NE are part of the *Apodemus, Rattus,* and *Myodes* genera (Jonsson et al. 2010). The genus *Apodemus* consists of

several species that harbor hantaviruses, the two most prevalent being *Apodemus agragius* carrying HTNV in Asia and *Apodemus flavicollis* carrying DOBV in Europe (Lee et al. 1978; Avsic-Zupanc et al. 1992). There are multiple rat-borne hantaviruses, but SEOV carried by *Rattus norvegicus,* or Norway rats, is the most common and is the only hantaviruses that is found worldwide, including being identified in Canada (Kerins et al. 2018). *Myodes glareolus,* or bank vole is the most widely distributed reservoir in Europe is the primary reservoir for PUUV. In terms of human disease burden, these pathogens are responsible for most hantavirus infections in the Old World.

Much of the ecological knowledge of these hantaviruses and their hosts is gleaned from field work and retrospective studies of HFRS outbreak and epidemics that can be correlated with multiple factors including climate patterns such as temperature changes and precipitation, rodent abundance, and anthropogenic alteration of rodent habitats. The clearest association is between rodent abundance and infection incidence. HFRS outbreaks are correlated with rodent abundance (Jonsson et al. 2010). Changes in the rodent population can be brought about by several factors including mast years in Europe, which occur when a high abundance of tree nuts accumulate and are available to provide nutrients for the rodent population (Schwarz et al. 2009; Tersago et al. 2009). Mast years have typically been predicted to fall two years following high summer temperatures and one year following high autumn temperatures, thus regional temperature patterns can help predict rodent abundance and possible HFRS outbreaks (Schwarz et al. 2009; Tersago et al. 2009). Another factor affecting reservoir abundance is predation of these species, where in places such as northern Europe harsh conditions may favor predation of certain rodent species leading to cycles of low and high rodent populations (Heyman et al. 2008; Heyman et al. 2009). Climate change may also present a risk of higher incidence of hantavirus infection. Sudden snow melts that subsequently lead to freezing temperatures with a lack of normal snow cover can push

bank vole populations into buildings, thereby increasing the potential for contact with humans (Olsson et al. 2009). In China, low rainfall and a lack of flooding has resulted in increases in *A. agrarius* and associated HFRS cases (Bi et al. 1998). The much higher incidence of HFRS in Europe and Asia have made studying the association of climatic factors and their effect on the rodent population with HFRS easier than the same types of studies in the New World, particularly in North America, where the number of cases is relatively low.

In North and South America, hantaviruses most commonly associated with human disease are SNV, Andes virus (ANDV), and Araraquara virus (ARAV) all carried by rodents of the *Cricetidae* family. These include *Peromyscus maniculatus,* or deer mouse, as the primary reservoir for SNV, *Oligoryzomys longicaudatus*, or long-tailed pygmy rice rat, the primary reservoir for ANDV, and *Necromys lasiurus*, the hairy-tailed bolo mouse, the reservoir for ARAV (Jonsson et al. 2010). While these three viruses cause the majority of HCPS cases, there are several others that have been associated with human disease in North, Latin, and South America. Others have been discovered and classified, but have not been reported to cause human disease.

Similar to "Old World" hantaviruses, there are several potential factors that may be altering the rodent population such that the incidence of HCPS could begin to rise. In Brazil, where ARAV is responsible for most fatal HCPS cases, its rodent reservoir has experienced drastic habitat change due to deforestation and farming (Figueiredo et al. 2009). High temperatures due to climate change as well as changes in land use from native to cultivatable land such as for sugar cane, have led to changes in the abundance of rodent species that harbor hantaviruses in Brazil (Prist et al. 2017). Through modelling, it has been suggested that the increase in sugar cane cultivation may increase HCPS risk by 20% and rising temperatures may increase risk by as high as 34% (Prist et al. 2017). In North America, studies examining the ecology and biology of SNV

in deer mice have provided most of our knowledge of hantavirus ecology. Fluctuations in the abundance of deer mice can be used to predict SNV outbreak situations. Based on the basic reproductive number of SNV and the number of deer mice present in a given year due to favorable weather conditions, a specific threshold can be predicted whereby SNV circulation in the deer mouse population will occur leading to potential exposures (Luis et al. 2015). The number of animals falling below this threshold, estimated at approximately 17 mice/hectare, may lead to disappearance of the virus within populations are certain sites. Via this model, it has been suggested that SNV within deer mice follows delayed density dependent prevalence, however others studies have suggested that this may be the case only on a larger, regional scale and that the host-pathogen interaction for SNV in deer mice in local sites does not necessarily follow this pattern (Carver et al. 2015).

Based on the evidence that deer mouse prevalence can be a predictor of SNV exposure, insight into what may cause increases in deer mouse abundance can lead to better prediction and preparedness for avoiding SNV infection. Climate, precipitation, rodent density, habitat, behaviour, movement of the host population, species diversity, and predation among other factors have all been examined for their role in affecting deer mouse populations and thus SNV prevalence (Khalil et al. 2014). For HCPS caused by SNV, disease cases have tended be found more generally in arid regions that are susceptible to rare climatic events such as El Nino, which has been hypothesized to have contributed to multiple HCPS outbreaks (Carver et al. 2015). Following the El Nino Southern Oscillation, which is a weather phenomenon during which parts of North America experience warmer conditions and sometimes increased levels of precipitation during autumn and winter, increased incidence of SNV infection has occurred (Carver et al. 2015; Hjelle and Glass 2000; Rasmussen and Wallace 1983; Glass et al. 2006). The increase in precipitation

leads to an increase in not just deer mouse density, but also in the density of other rodent species. However, the increase in deer mouse abundance leads to increased SNV transmission and exposure risk leading to outbreaks. Therefore, it is possible that climatic patterns can be used to predict changes in deer mouse abundance.

Related to deer mouse abundance in a given habitat is the density of deer mice within the entire ecosystem of that habitat. The dilution effect describes the phenomenon in which increased diversity of species within an area leads to lower SNV infection rates in the deer mouse population (Dizney and Ruedas 2009). The effect works by reducing the number of competent vector hosts for the pathogen by increasing the number of non-competent hosts (Dizney and Ruedas 2009). For pathogens like SNV that are likely transmitted directly between deer mice, the increase in non-competent host species decreases deer mouse density and also the number of encounters between deer mice that would lead to transmission (Khalil et al. 2014; Dizney and Ruedas 2009). Experiments aimed at determining how diversity may decrease SNV prevalence in deer mice have shown that it is likely due to a reduced number of interactions between deer mice rather than reduced interaction time (Clay et al. 2009a). How environmental, anthropogenic, and climatic factors influence species diversity and density could be critical to monitor going forward as these may affect deer mouse populations and SNV prevalence. Increased rates of infection within deer mouse populations could have important implications for risk of human exposure and infection. There a multitude of factors that can contribute in some way to hantavirus infection and prevalence within a population of a reservoir species. Field and environmental studies can shed light on how many of these factors correlate with infection rates and human exposure risk, but due to the complexity of the ecology of a specific area, its climate, human activity, and species richness, it is often difficult to determine what factors are critical for hantavirus infection and transmission

occurring. Despite the difficulties of working with hantaviruses due to the need for high containment laboratories and specific reservoir hosts, experimental infection models have been developed to help shed light on some of these issues.

While some hantavirus infection models have focused on pathogenesis, there have been some experimental systems developed for studying hantavirus infection of reservoir hosts. The first experimental hantavirus infection with Old World viruses was of *A. agrarius* with HTNV (Lee et al. 1981a; Lee et al. 1981b). Infection of these mice with HTNV by multiple routes showed that intramuscular (IM), intraperitoneal (IP), subcutaneous (SC), oral, intrapulmonary, and intranasal (IN) all resulted in productive infection. Intrapulmonary and IM were the most efficient routes of infection seen, and further experiments used IM as the primary route due to difficulties with intrapulmonary infections. During experimental infection, viremia appeared on day 7 post-infection (dpi), but was negligible at 15 dpi (Lee et al. 1981a). Viral loads in the tissues, salivary glands, and excreta indicated that virus was present in these animals up to one year following infection and potentially secreted in the urine and feces for as long as one year. These indicated that hantaviruses can cause persistent infection of their rodent hosts. Similar findings were made for PUUV infection of laboratory bred bank voles (Yanagihara et al. 1985). Transient viremia beginning after 7 dpi is seen in IM infection followed by viral loads in the tissues occurring at 14 dpi and lasting as long as 270 dpi in the lungs and liver (Yanagihara et al. 1985). Infectious virus in the excreta and secreta of experimentally infected bank voles was present as early as 14 dpi and was detectable at 103 dpi (Yanagihara et al. 1985). Interestingly, during persistent PUUV infection despite the presence of virus for as long as 270 days, sporadic shedding of virus in urine, feces, and saliva is seen (Yanagihara et al. 1985; Hardestam et al. 2008). In another experimental system, despite bank voles being positive for PUUV RNA at 133 dpi, viral RNA could only be detected

on day 44 for excreta and day 84 in saliva (Hardestam et al. 2008). This sporadic replication and shedding has been found to be common among different hantavirus species. For SEOV, initial experimental infections were done in Wistar rats, where newborn and adult rats were permissive to infection (Kariwa et al. 1996; Kariwa et al. 1998). Experimental infections of the natural rodent reservoir for SEOV, the Norway rat have been used consistently for at least the last twenty years, as these animals are available commercially. Experimental infection of these rats with SEOV has led to important insights into the differences between infection of male and female animals, immune responses generated in reservoir hosts, and how sex hormones can alter these immune responses (Hannah et al. 2008; Easterbrook and Klein 2008b; Klein et al. 2000; Klein et al. 2001). In general, infection of male rats with SEOV leads to higher levels of virus in the lung and viral shedding compared with females, and this can be reversed by gonadectomy either in the neonatal period of life or during adulthood (Hannah et al. 2008). This data and other studies indicate the importance of sex hormones on the immune response in the context of hantavirus infection. The role of sex hormones in SNV infection in deer mice has not been studied previously, although data showing that regulatory T cells play an important role in infection suggest that alteration of sex hormones may affect the course of infection. However, the overall generalizability of the results of these experiments for infection of wild hosts and for other hantaviruses is difficult to determine due to the inbred nature of the animals used for these experiments. Genetic diversity of the host population may have an important impact on factors such as the immune response, where differences in the MHC affect the nature of the immune response generated (Jeltsch et al. 2014). Nevertheless experimental infection of Norway rats with SEOV has provided a suitable model for the study of the interaction of SEOV with its rodent host.

For New World hantaviruses, fewer models of hantavirus infection within their rodent hosts exist. Experimental infection of cotton rats with Black Creek Canal virus (BCCV) revealed a persistent infection with peak viral titers at 14 dpi followed by reduced levels and sporadic appearances of live virus in the blood thereafter, similar to what was seen in experimental Old World hantavirus infections (Hutchinson et al. 1998). These rats were also positive for virus in nearly all tissues tested as late as 150 dpi. This infection model also allowed for the further characterization of transmission of BCCV, which will be discussed in detail later (Hutchinson et al. 2000). Cano Delgadito virus infection of its rodent host, the Alston's cotton rat, showed acute and persistent infections occur in this host (Fulhorst et al. 2002). Both of these infection models were able to show the presence of virus in the salivary glands, saliva, urine and/or feces of infected animals, which may be critical for virus transmission. Development of other hantavirus-host infection models have been limited due to the need for high containment facilities and lack of access to specific host species.

A number of studies of SNV in deer mice have been performed following the first experimental infection that was described in 2000 by Brian Hjelle's group (Botten et al. 2000). Initially, they were able to isolate a new strain of SNV, 77734, from the tissues of an SNV-infected deer mouse that was then used for all subsequent passages and infections. Experimental infection of deer mice revealed the presence of viral RNA in most animals as early as 7 dpi and in all animals on day 14 pi (Botten et al. 2000). These findings reported that the lungs and heart of deer mice were the tissues that had the highest levels of viral RNA present, followed by the spleen and brown adipose tissue (BAT). Persistent infection also occurred, as they were able to detect viral RNA in the tissues and N protein via immunohistochemistry (IHC) on 90 dpi. The persistence of SNV was followed up by the same group, wherein they showed that during experimental infection, SNV

RNA was present in the heart at day 270 pi (Botten et al. 2003). The authors were able to determine sites of persistent and active replication of SNV through the expression of positive strand SNV RNA revealing the heart and BAT as likely reservoirs for the virus throughout persistence. Positive strand RNA levels also correlated with the amount of N protein detected via IHC and with live virus isolated from the tissues. There is not as much data on presence of virus in excreta and/or secreta in experimental infections, though viral RNA is readily detectable following spiking of these samples with RNA. Therefore, the shedding of virus in these sample may be limited, resulting in the limited detection of viral RNA in these samples following infection. Data on the level of virus in excreta and secreta is an important component of examining SNV transmission between deer mice. These results highlighted the potential importance of these sites of SNV replication during persistent infection and how they might play a role in viral shedding and transmission. Further experiments using this experimental SNV infection model in deer mice have shed light other virus-host interactions as well as immune responses of deer mice during SNV infection. The immune response generated against hantaviruses in their rodent hosts plays an important role in how these viruses persist within the rodent population without causing detrimental overt disease. What is known about hantavirus-specific immune responses in host species is covered in the next section.

In addition to experimental systems that describe the interaction between various hantaviruses and their hosts, reports have been made on infectious challenge of deer mice with hantaviruses other than SNV. Maporal virus (MAPV), a South American hantavirus carried by *Oligoryzomys delicates*, which is not known to cause disease in humans, but does cause disease in Syrian hamsters, was used to infect deer mice in an attempt to develop a surrogate model of SNV infection that does not require BSL-4 containment (McGuire et al. 2016). Deer mice infected with

MAPV developed a persistent infection similar to that seen with SNV, with development of virus specific antibodies and the presence of viral RNA and N protein in the tissues as late as 56 dpi. The expression profile of a set of immune related genes was also similar to what is seen during SNV infection in deer mice. In contrast to SNV infection, MAPV infection resulted in mild pathology upon histological analysis of tissues, however there were no overt sign of disease in the mice throughout the course of infection. Interestingly, experimental infection of deer mice with ANDV, a known pathogen of consequence in humans, differed significantly from infection with either SNV or MAPV (Spengler et al. 2013; Schountz et al. 2014). Deer mice were permissive to ANDV infection, with deer mice developing anti-ANDV IgG and neutralizing antibody responses and viral RNA detectable in tissues at 21 dpi. However, deer mice were able to clear the infection by day 56 and did not develop a persistent infection as seen with SNV or MAPV (Spengler et al. 2013). Another study comparing the immune response to both SNV and ANDV revealed that the antibody response against ANDV occurred faster and that expression of immune genes in cervical lymph node cells was upregulated during ANDV infection compared with SNV infection (Schountz et al. 2014). The resulting immunity that differs during SNV and ANDV infection could be the driver of viral clearance seen in experimental ANDV infection of deer mice, and will be discussed in the following section.

One of the difficulties of working with hantaviruses and trying to develop experimental models of host-hantavirus relationships is that for some viruses, cell culture adaption of the virus results in an inability to infect its host or other animals. For example, a comparison of wild type, bank vole only passaged PUUV with a VeroE6-adapted PUUV revealed mutations within the non-coding region of the S segment, specifically at positions 26 and 1577 in one experiment and in position 1580 in another, that reduced the ability of the virus to infect bank voles (Lundkvist et al.

1997). Additionally, the cell culture adapted virus induced much lower levels of neutralizing antibodies 10 weeks after infection. Wild type virus induced reciprocal neutralizing titers of greater than 1280 while VeroE6-adapted virus induced titers of only 20 to 80, suggesting a possibly lower ability of this virus in replicate *in vivo* (Lundkvist et al. 1997). A further analysis of the mutations responsible for this reduced infectivity revealed that cell culture adaptation resulted in one amino acid change, found in the RdRp, specifically a S to F mutation at position 2053 of the protein (Nemirov et al. 2003). This mutation along with the previously found mutations in the S segment non-coding regions reduce the ability of VeroE6-adapted virus to infected both the virus's natural rodent host as well as other species. For SNV, a similar phenomenon has been shown in which VeroE6 cell adaptation of SNV results in virus that is no longer capable of infecting deer mice (Warner et al. 2019a). In addition to no longer being infective in deer mice, cell culture adapted SNV is not pathogenic in non-human primates, but deer mouse only passaged SNV causes severe pathology and HCPS-like disease (Safronetz et al. 2014). Only two nucleotide mutations occurred following VeroE6 culturing of the virus, both in the RdRp, one at position 166 resulting in a K to E amino acid change, and another which was silent (Safronetz et al. 2014). These examples illustrate the close evolutionary relationship between hantaviruses and their hosts, and that consideration of the passage history of the virus should be taken into account when attempting to develop experimental models of hantavirus-host infections or even HFRS and HCPS models of disease.

A final consideration when studying host-hantavirus interactions is the possibility of genetic differences between geographically distinct or separated rodent host populations or the viruses that are found within these populations. Development of the original experimental SNV model in deer mice was done in colony bred animals originating in the same area from which the

virus was also isolated, minimizing any genetic differences altering the kinetics of infection or the ability of the virus to infect those animals (Botten et al. 2002). There is evidence that genetic compatibility between host and virus can impact the ability of a given strain of hantavirus to infect rodents of a certain population. This is an important factor to be taken into consideration when doing experimental hantavirus infections of rodent hosts and developing models of hantavirus infection.

1.5.2 Immune Responses of Reservoir Hosts

The sections of this thesis that involve immunological details of hantavirus infections are written such that those with cursory knowledge of immunology will hopefully be able to gain from what is described. Several descriptions of hantavirus-host interactions have been described so far. The experimental infection models that have furthered our understanding of the immunological mechanisms mediating hantavirus persistence in reservoir hosts the most are models of SEOV infection in rats and SNV infection in deer mice. Over the last twenty or so years, there have been indications that during human infection, inflammatory responses mediated by mononuclear cell infiltration and CD8 T cells may be responsible for disease symptoms. In contrast it has been shown that a reduction of the inflammatory response and a shift toward a regulatory response mediated by regulatory T cells may allow for viral persistence in rodent hosts, possibly at the expense of clearing the virus, but also avoiding overt disease (Easterbrook and Klein 2008a). The inflammatory response induced by infection in humans and how this leads to disease will be covered in a later section. Here I will discuss what has been uncovered regarding the immune response in rodent hosts of hantaviruses, mainly SEOV and SNV, and how these responses have been hypothesized to lead to persistent infection.

It has been hypothesized that during the co-evolutionary history of each hantavirus and its host, viruses and the host have evolved mechanisms that suppress the immune response against the virus, allowing for the virus to remain within the host while not causing disease. These mechanisms may include immune evasion by the virus, suppression of immune function by the virus, alteration of host responses, delayed or suppressed innate immune responses, and development of regulatory rather than deleterious inflammatory responses by the host (Easterbrook and Klein 2008a).

Expression of innate and adaptive immune response genes such as IFN-β, IL-1β, TNF-α, Mx2, and IFN-γ are reduced in the lungs of infected rats, the main target organ for the virus (Easterbrook and Klein 2008b). Interestingly, the expression of many of the same genes are elevated in the spleen, where peripheral immune cells are likely residing. This suggests that rats that are infected are not fully immunosuppressed, but mechanisms have resulted in reduced inflammation and antiviral responses in the main target tissues of the virus (Easterbrook and Klein 2008b). Although the expression of some pro-inflammatory and innate genes is elevated during SEOV infection in the spleen, there is also an expansion of regulatory T cells (Treg) found in the spleen during infection as well as in the lungs (Easterbrook et al. 2007b). It appears that the development of Treg cells during infection allows for persistence of the virus. Elimination of or reduction of the Treg population decreases the expression of TGF-β, which is thought to be the driver of the regulatory response leading to persistence (Easterbrook et al. 2007). Reduction of the Treg population also leads to increased TNF-α expression and a significant decrease in SEOV RNA in the lung of infected animals, potentially due to immune clearance of the virus (Easterbrook et al. 2007b).

It has been shown that in wild rodent populations, males are typically infected at a higher rate than females due to various reasons such as habitat range and aggressive behaviour. Much of the work done on the immune response of rats to SEOV has also examined the effect of sex differences

on SEOV infection including how sex hormones and glucocorticoids can influence infection. During experimental SEOV infection of Norway rats, it has been shown that the expression of certain pattern recognition receptors increases depending on the sex of the infected animal (Hannah et al. 2008). In female rats, TLR7 and RIG-I expression increase immediately following infection and remain stable, while in male rats, TLR3 expression increases while the others do not (Hannah et al. 2008). During experimental SEOV infection, there are higher levels of viral RNA and viral protein in the tissues of male rats than females, however gonadectomy of either sex reverses this effect (Hannah et al. 2008). The lower viral loads in females correspond to higher expression levels of pro-inflammatory and antiviral genes in females compared to males, and this may be in part due to sex hormones such as testosterone and estradiol, as gonadectomy reverses the effect. Levels of corticosterone, an immunosuppressive hormone, have also been shown to be reduced in SEOV infection in male rats correlating with development of a regulatory T cell response (Easterbrook and Klein 2008c). These immunological differences between male and females hosts may play an important role along with other factors such as behaviour, habitat range, and mating in the ecology of hantaviruses and their transmission.

SNV infection of deer mice has received the most attention when it comes to studying the immune response of rodent hosts infected with hantaviruses. Similar to what has been seen in rats infected with SEOV, deer mice that are persistently infected with SNV have a predominant regulatory T cell response to SNV antigens (Schountz et al. 2007). Interestingly, during the acute phase of infection, T cells from deer mice do express genes for Th1 and Th2 type cytokines such as IFN-γ, IL-4, and IL-5 while also having moderate expression levels of TGF-β and IL-10, Treg promoting cytokines. Detectable expression of transcription factors T-bet and GATA-3 in these cells also indicate a productive T cell response before transition to Treg-like phenotypes. However,

once SNV infection progresses toward persistence, the T cell response becomes dominated by expression of TGF-β. In addition, despite the pro-inflammatory phenotype of some T cells present during acute infection, these cells show a weak proliferative response when presented with antigen in a recall assay (Schountz et al. 2007). This weak recall response to antigen is similar to what has been shown in wild caught deer mice, where SNV infected individuals had a weaker response when challenged with phytohemagglutinin (PHA) indicating general immunosuppression, which could be due to the predominance of Treg cells present in these animals, although this was not antigen specific (Lehmer et al. 2007). Additional analysis of the kinetics of the immune response in SNV infected deer mice has been performed and showed that CCL2, CCL3, CCL5 expression is increased significantly in the spleen and moderately in the lungs of infected mice (Schountz et al. 2012). Cytokine expression in the lungs and spleen is limited during infection, with only a limited number of cytokines such as IL-12 and IL-21 significantly upregulated, while TGF-β expression is high early, drops, and increases again as infection enters persistence (Schountz et al. 2012). This provided further evidence that while some immunity is generated against SNV, this does not lead to viral clearance. However, this experiment compared overall expression of immune genes in the tissues examined rather than in purified lymph node cells cultures and T cell populations as in other publications. Therefore, it is difficult to speculate on how representative the expression profiles seen are in terms of the phenotypes of responding T cells. Further experiments have examined the immune responses of deer mice during ANDV and MAPV infection to attempt to gain insight into mechanisms behind persistence vs clearance of viruses. During ANDV infection, no hematological changes such as levels of various lymphocytes or leukocytes are noticeable compared with SNV infection (Schountz et al. 2014). However, deer mice infected with ANDV generate immune responses that differ substantially from those infected

with SNV. During ANDV infection there is a significant increase compared with SNV infection in genes for cytokines such as IFN-γ, IL-4, and IL-13 as well as a number of chemokines and transcriptions factors promoting pro-inflammatory responses (Schountz et al. 2014). Clustering of the genes that are upregulated during ANDV infection indicate a Th2 bias during infection that results in clearance of ANDV by at least 56 dpi (Schountz et al. 2014; Spengler et al. 2013). During MAPV infection of deer mice, which results in persistent infection, the immune profile in the lungs and spleen is more representative of SNV infection than ANDV infection (McGuire et al. 2016). Expression of CCL2, CCL3, and TGF-β in the spleen of MAPV infected deer mice resembled the expression of the same genes in SNV infected animals while IL-23 expression was closer to what was seen in ANDV infected deer mice (McGuire et al. 2016). In the lungs, CCL2 and TGF-β again had similar expression profiles to SNV infection, but Ccl3 resembled what is seen during ANDV infection. Overall, the immune response during MAPV, a virus that is able to cause persistent infection, resembled the response seen during SNV infection. The data suggests that the regulatory type response generated in host animals prevents disease and allows for persistence at the cost of being unable to clear the virus.

While much of the work on immune responses in the SEOV and SNV models focuses on T cell responses, particularly Treg cells, antibodies against hantaviruses are generated in reservoir hosts. Generally persistence occurs in spite of antibody generation in the host animal, including neutralizing antibody (Easterbrook and Klein 2008a). Around 14 dpi, antibodies begin to be detectable in the serum of infected hosts, however the antibodies generated are unable to help eliminate the infection. There is evidence that the presence of maternal antibodies are protective in pups of mothers infected with hantavirus, suggesting some protective efficacy (Zhang et al. 1988; Kallio et al. 2006a; Borucki et al. 2000; Botten et al. 2002). Because of the limited reagents

available for a number of difference reservoir hosts, there is little data available on the isotypes of antibody generated during persistent hantavirus infection. Development of reagents or suitable models in animals for which there are reagents could further develop our understanding of what types of immune responses lead to persistent infections.

1.6 Hantavirus Transmission

1.6.1 Transmission Between Rodents

Similar to hantavirus ecology, what is known about transmission of hantaviruses within their rodent host populations comes from studies of specific host-virus interactions, including PUUV in bank voles, SEOV in Norway rats, and SNV in deer mice, while transmission of mole, shrew, and bat-borne hantaviruses has not been well characterized to this point. There have been field studies done to attempt to characterize transmission between rodents as well as attempts at defining transmission in experimental systems. Transmission of hantaviruses is generally thought to be horizontal, with vertical transmission rarely being reported, perhaps due to the an inability of the virus to cross the placenta to cause neonatal infection or due to the presence of maternal antibodies that can protect rodent pups (Borucki et al. 2000). Horizontal transmission can occur by two mechanisms, either through direct contact between an infected animal and an uninfected animal or through exposure of uninfected animals with virus-contaminated excreta or secreta left by infected animals (Jonsson et al. 2010; Schlegel et al. 2014; Forbes et al. 2018). Direct contact can include transmission of virus in the saliva of infected animals via biting and wounding during aggressive behaviour or through grooming (Forbes et al. 2018). As male rodents have been shown to be disproportionately infected with hantaviruses it is thought that their tendency to act aggressively and compete for habitats and food may lead to transmission. To this end, wounding has been correlated with higher seroprevalence, viral titers, and viral shedding in SEOV infected

rats and SNV infected deer mice (Hinson et al. 2004; Bagamian et al. 2012). While this may be a primary route of exposure for SEOV, it has been difficult to demonstrate what directly leads to infection following direct contact of rodents. Indirect infection of rodents through inhalation of the virus in the environment is also able to contribute significantly to transmission. Some hantaviruses have been shown to be shed in the urine and feces as well as persist in the environment for long periods, leading to exposure (Kallio et al. 2006b). There are likely differences in the main mechanism(s) of transmission for different hantaviruses, but studies have begun to shed light on how some of these viruses are able to transmit amongst their reservoir hosts.

For Old World hantaviruses some experimental work has shown that transmission through direct contact or through exposure to virus in the environment can occur. HTNV was shown to be shed in the urine, saliva, and feces of *A. agrarius* following experimental infection and direct contact between caged animals led to transmission (Lee et al. 1981b). Virus shedding began at 10 dpi and virus was detectable in the urine as late as 360 dpi (Lee et al. 1981b). The majority of transmission to uninfected cage mates occurred during the acute phase of infection, from 10-35 dpi, which saw 24 out of 29 exposed animal become infected despite only being exposed for 5 days to infected animals. Early during infection and following day 35, during the persistent phase, HTNV transmission did not occur as readily, due to reduced viral titers and shedding. Following day 35, only four of 66 animals became infected, showing that the most likely period of transmission is during acute infection, at least experimentally.

During PUUV infection of bank voles, similar kinetics are seen in terms of peak viral titers around 10-20 dpi followed by viral shedding (Kallio et al. 2006b; Gavrilovskaya et al. 1990). For PUUV however, there is evidence that the virus's ability to survive in the environment could drive transmission. Exposure of uninfected bank voles to PUUV-contaminated bedding led to over 20%

of exposed animals becoming infected, showing that PUUV found in the bedding remained infectious for up to 15 days (Kallio et al. 2006b). Infected donor voles were removed from their cages on day 17 pi, during the peak of acute infection. Similarly, SEOV is detectable in the urine of Wistar rats and exposure of rats to contaminated urine led to SEOV infection (Kariwa et al. 1998). However this study was done in newborn Wistar rats, so they relevance to natural infection in Norway rats is not clear. As mentioned above, there is evidence that direct contact between rats is the main driver of SEOV transmission, as wounding and body size are correlated with SEOV infection in male rats (Hinson et al. 2004). Data on wounding and transmission comes from wild caught animals, and no laboratory studies have been done to determine the necessity of wounds or aggressive behaviour for transmission of SEOV.

Some experimental studies on the transmission of New World hantaviruses have been performed. A study of transmission of ANDV between wild caught long-tailed pygmy rice rats showed that between 12 and 17% of directly exposed animals became infected with ANDV (110). However, unlike Old World hantaviruses in which indirect transmission seems likely to occur, no rats that were separated from infected rats by wire mesh became infected, nor did any that were exposed to contaminated bedding (Padula et al. 2004). Additionally, it was mentioned by the authors that biting was not a prerequisite for infection occurring during direct contact, in contrast to the data for SEOV suggesting wounding as a major mechanism of transmission. A small study of BCCV infection in cottons rats showed 100% of directly exposed uninfected cage mates became infected with BCCV regardless of sex (Hutchinson et al. 2000). In that study, aggressive behaviour and fighting may have been a driver of transmission as all animals showed evidence of fighting, with some animals even dying due to injuries sustained during caging with other cotton rats (Hutchinson et al. 2000). The same study also provided evidence that vertical transmission can

occur in rodent hosts, as infectious virus or viral RNA was detected in both fetuses and newborn pups born to infected females (Hutchinson et al. 2000). The aggressive nature of the animals and artificial setting for the BCCV experiments may have led to the high transmission rate seen, but the articles described demonstrate the discrepancies in the modes and likelihood of transmission between different hantavirus species.

SNV transmission is the most well-studied of the New World hantaviruses. Field studies have indicated that male deer mice are more likely to be infected with SNV than females and perhaps they are responsible for the majority of SNV transmission (Carver et al. 2015). Males typically will display more aggressive behaviour which may lead to transmission and also may have a larger habitat range, resulting in more contacts with other deer mice (Calisher et al. 2007; Forbes et al. 2018). However, factors such as wounding, sex of deer mice, age and size of deer mice, and density of the rodent population that have been suggested to play a role in SNV prevalence and transmission only sometimes can be correlated with transmission rates, and there are differences between rodent populations at different sites (Calisher et al. 2007). There is also evidence that SNV within the deer mouse population follows the so-called "20/80 rule" whereby a small percentage of the deer mouse population accounts for a large percentage of SNV transmission (Clay et al. 2009b). This could be due to individual differences in deer mouse susceptibility and exposure. Clay et al reported that in a deer mouse population, the majority of contacts between mice happen between a small number of individuals, with 25% of deer mice responsible for 100% of contacts between mice (2009b). In addition to a small number of animals being responsible for all contact between deer mice, a small number of individuals had long contact duration, providing more potential for transmission. A combination of the number of contacts and their duration correlated with SNV prevalence along with body mass, which was predictive of

higher numbers of and longer contacts with other deer mice (Clay et al. 2009b). Interestingly, sex and breeding condition did not have an effect in their analysis of SNV prevalence (Clay et al. 2009b). Field studies have shed some light on transmission dynamics within deer mouse populations, but there have been very few laboratory studies examining the mechanism(s) of SNV transmission. While some Old World hantaviruses are responsible for thousands of human cases per year, New World hantaviruses cases are relatively rare, suggesting either limited transmission amongst rodent populations or limited transmission to humans. Few studies have attempted to study experimental transmission of SNV.

Botten et al used their deer mouse colony and housed the animals in an outdoor enclosure setting to attempt to observe SNV transmission (2002). Groups of deer mice were caged with one or two SNV-infected deer mice for 14 days at a time throughout the first 60 days of infection (Botten et al. 2002). In infected deer mice, the authors were only able to sparsely detect SNV RNA in the saliva, while no urine or feces samples had any SNV RNA or live virus. Through the first 60 days of infection and up to 213 dpi, only 1 naïve deer mouse, exposed from day 29-42, became infected following direct exposure to infected deer mice (Botten et al. 2002). The authors also looked at wounding throughout the experiment, and showed a low number of wounds overall that did not correlate with transmission. Vertical transmission from dams to pups also did not occur, with mothers passively transferring antibody to the pups that appeared to be protective. Another study by Bagamian et al sought to determine whether SNV transmission could occur in an experimental setting. Once again they used outdoor enclosures, however this time using SNV infected wild-caught deer mice as donors during direct contact experiments (Bagamian et al. 2012). During this study, a higher number of transmission events occurred, with one set of experiments resulting in 26% of naïve deer mice becoming infected. The mechanism of transmission was not

studied, however it was shown that the number of new wounds was significantly increased in animals that became infected compared with those that did not, while overall wounding did not differ between infected and uninfected mice (Bagamian et al. 2012). These studies of SNV in a natural setting provide evidence that studying SNV transmission can be accomplished, but also that the frequency of SNV transmission in deer mouse populations is low, likely due to low replication of the virus, little shedding, and instability of the virus in the environment, resulting in a requirement of direct contact for transmission. The main mechanisms behind SNV transmission remain to be uncovered, and a proper model for experimental study of SNV transmission is deer mice would provide a valuable tool for studying those mechanisms.

One variable that has been studied for certain hantaviruses, but overall had not been well characterized is the stability of hantaviruses in the environment and how the ability of the virus to survive under various conditions leads to transmission not only to other rodents, but to humans as well. As mentioned above, PUUV has been shown to be relatively stable and infectious as long as 15 days following shedding of the virus (Kallio et al. 2006b). For other Old World hantaviruses, data is lacking on how well the virus can persist in the environment. One study showed that the stability of HTNV was reliant upon temperature in wet conditions, as virus remained infectious after just 8 days at 37°C, but for 9 days at 20°C and for 96 days at 4°C (Hardestam et al. 2007). After allowing the virus to dry, only 6-14% of the original infectious titer remained following just 90 minutes, while no infectious virus was detectable 24 hours after drying (Hardestam et al. 2007). There is a lack of data on the *ex vivo* stability of other hantaviruses, including New World hantaviruses. Experiments on how well those viruses can remain infectious outside the host as well as the effects of various environmental conditions on virus survival may shed light on why there

are low prevalence rates within rodent populations as well as low incidence of human cases of HCPS.

While hantaviruses are thought to be spread directly between rodents and to humans and are not thought to be vector-borne, there is the possibility that insect vectors, such as mosquitos and ticks could play some role in transmitting virus amongst rodent populations. Other members of the *Bunyavirales* Order are carried by some form of arthropod vector, and this form of transmission could account for certain aspects of hantavirus transmission that are not well understood. This includes the difficulty to show SNV transmission in experimental settings, the low rate of infection seen in humans when virus prevalence is high, and the persistence of virus amongst rodent populations at high prevalence despite apparent limited interactions between animals. This is an area of hantavirus research that has not been well studied.

1.6.2 Transmission to Humans

Transmission of hantaviruses to humans is a spillover infection that almost always represents a dead end host for the virus, resulting either in death or clearance of the infection. Transmission to humans is thought to occur through exposure to virus found in rodent excreta or secreta, though this can be difficult to show definitively to be the cause of infection. Rarely, bites by infected rodents are thought to contribute to human disease (Schlegel et al. 2014). Human-to-human transmission of ANDV either in the household or nosocomially can occur, but these few cases are the only known instance of non-rodent borne hantavirus disease occurring (Martinez et al. 2005; Martinez-Valdebenito et al. 2014). Detection of virus in the saliva and salivary glands of patients has been reported, highlighting the potential for future human-to-human transmission, particularly for ANDV which appears to be more resistant to anti-viral factors in human saliva (Hardestam et al. 2009). Risk factors for contraction of hantaviruses include certain occupations that increase the

likelihood of exposure including farming and forestry or activities such as cleaning sheds, barns, or cabins (Forbes et al. 2018). Handling and cutting firewood has been shown to increase risk of human infection as well as activities like cleaning animal storage areas and sheds, seeding, and plowing (Vapalahti et al. 2010; Van Loock et al. 1999; Zeitz et al. 1995). Any buildings that may potentially be infested with rodents and are unused or do not have good air circulation could pose a hazard for aerosolization of virus. Increased risk of exposure has been linked to rodent sightings in the home or sighting of rodent droppings, use of rodent traps, and living within a close radius of a forest (Figueiredo et al 2014; Vapalahti et al. 2010; Van Loock et al. 1999; Watson et al. 2014). One of the main factors in whether humans become infected is the ability of hantaviruses to remain infectious outside their hosts, which as stated earlier can vary significantly between different viruses. Overall, the risk of hantavirus infection in humans involves multiple factors such as rodent abundance, climate, rainfall, occupation, and human activity.

1.7 Pathogenesis and Human Disease

1.7.1 HFRS and NE

The clinical course of HFRS begins with an incubation period that lasts from 10 to 42 days (Jonsson et al. 2010). The severity of infection depends on the causative agent, with HTNV and DOBV infections typically being more severe, while SEOV slightly less so, and PUUV infection being relatively mild. Once symptoms begin to appear, five stages of disease occur: in order they include febrile, hypotensive, oliguric, polyuric, and convalescent phases (Jonsson et al. 2010). While HFRS is characterized by vascular leakage and renal dysfunction, pulmonary and neural involvement have been known to occur (Alexeyev and Morozov 1995; Linderholm et al. 1997). Nearly all infections lead to fever following incubation that includes generalized symptoms such as myalgia, malaise, pain, chills, enanthema, exanthema, and bradycardia (Muranyi et al. 2005).

At this stage, hemorrhaging may begin to occur as well as renal involvement leading to hematuria, proteinuria (Jonsson et al. 2010). Anywhere from 10 to 40% of febrile patients develop hypotension along with leukocytosis, thrombocytopenia, and infiltration of mononuclear cells into the lungs (Jonsson et al. 2010; Muranyi et al. 2005; Scholz et al. 2017). During the hypotensive stage, vascular leakage, abdominal pain, and tachycardia occur while renal conditions tend to manifest such as tubulointerstitial nephritis, glomerulonephritis, and IgA nephropathy (Muranyi et al 2005). One-third of patients at this stage develop shock and mental confusion (Chun et al. 1989). About half of febrile patients advance to the oliguric stage of infection, characterized by a urine output of less than 400ml per day. This stage represents the peak of illness responsible for half of deaths caused by HFRS and can last up to 16 days (Jonsson et al. 2010). Renal insufficiency can lead to the need for dialysis and patients often suffer hypertension and hemorrhaging. Hemorrhage, shock, and renal insufficiencies are the main cause of death from HFRS, in which the fatality rate can range from as low as less than 1% for SEOV and PUUV to as high as 12% for DOBV and 15% for HTNV (Jonsson et al. 2010). If the infection is controlled, the polyuria and convalescent phases follow, and can last as long as six months (Muranyi et al. 2005). Long term sequelae in HFRS patients is thought to be rare, but can include renal failure, hypotension, myopia, myocarditis, visual impairment, gastrointestinal hemorrhages, and CNS manifestations (Muranyi et al. 2005). Despite a classical dichotomy of disease caused by Old and New World hantaviruses, HFRS patients can develop cardiopulmonary manifestations of disease. Pulmonary infiltrates and edema can occur in HFRS patients (Linderholm et al. 1997). HFRS patients have also been shown to have changes in electrocardiogram during acute infection (Makela et al. 2009; Markotic et al. 2002b). This cardiopulmonary involvement may lead to hemorrhaging and shock in severe cases.

1.7.2 HCPS

While HFRS cases range from mild to severe and target mainly the kidneys, HCPS tends to be a more severe disease targeting the heart and lungs. Kidney involvement has been reported in HCPS, specifically in one patient with renal involvement accounting for the initial hospitalization, before respiratory illness led to death. Kidney involvement has also been reported in patients infected with New World hantaviruses (Passaro et al. 2001; Campos et al. 2009). Overall, however, HCPS primarily affects the lungs and heart of infected patients. The onset of HCPS symptoms typically occurs following an incubation period that has a range of 9 to 33 days and a mean time of 14 to 17 days following exposure (Young et al. 2000). The disease course is similar to HFRS in that it commonly follows distinct stages that can vary in severity depending on the causative pathogen. Following the incubation period, a prodrome phase begins that includes fever, myalgia, malaise, headache, and thrombocytopenia (Jonsson et al. 2010; Borges et al. 2006). About one-quarter of patients also have gastrointestinal involvement including nausea, vomiting, and diarrhea. Disease then progresses to cardiopulmonary symptoms that include dyspnea, cough, tachycardia, and low blood pressure between four and six days following symptom onset (Jonsson et al. 2010; Borges et al. 2006). Additionally, respiratory distress and failure leads to cyanosis in one-fifth of patients. The most severe phase of illness involves bilateral lung infiltrates, pulmonary edema, hypotension, and cardiogenic shock resulting in need for mechanical ventilation and intensive care treatment. In HCPS, renal failure, as assessed by increased creatinine levels, and some forms of hemorrhaging such as hematuria, intestinal bleeding, and vomiting blood, can occur in about half of patients (Jonsson et al. 2010). The majority of patients suffer from thrombocytopenia, metabolic acidosis, reduced oxygen saturation, leukocytosis, and increased hemoconcentration. HCPS disease course is rapid, with most hospital admissions occurring 3 to 6

days following symptom onset and fatal outcomes occurring within 2 days of hospital admission (Jonsson et al. 2010).

1.7.3 Epidemiology

As I have already discussed some of the factors that may play a role in transmission of hantaviruses to humans, here I will only briefly discuss the disease burden seen in particular areas of the world with respect to HFRS and HCPS. The most common hantaviruses responsible for human disease and their geographic locations are depicted in figure 2. In terms of the number of global cases, HFRS and NE cases outnumber HCPS cases by thousands. The greatest number of cases occurs in Asia, with China and South Korea experiencing the majority of cases among Asian countries (Watson et al. 2014). Cases in other Asian countries are either sporadic or lacking sufficient data. China experiences by far the most cases globally, with tens of thousands of HFRS cases reported each year mostly caused by HTNV and SEOV (Jonsson et al. 2010; Watson et al. 2014). South Korea has reported hundreds of cases HFRS caused by Hantaan virus per year, but a lack of reporting, knowledge, and diagnostic testing could account for the few cases seen in other Asian countries. HFRS and NE cases in Russia are primarily caused by PUUV and number in the thousands per year, however the majority of these cases occur in European Russia (Watson et al. 2014). Among the rest of Europe, several countries report high numbers of HFRS and NE cases each year with PUUV accounting the majority of those cases. Finland and Sweden each report thousands of NE cases each year, while other countries such as Norway, France, Belgium, The UK, and Germany experience fewer cases, ranging from zero to hundreds per year (Jonsson et al. 2010; Watson et al. 2014). More severe HFRS cases caused by DOBV in the Mediterranean region of Europe can range from zero to 100 cases in countries such as Greece, Bulgaria, Bosnia, and

Slovenia. Data from certain European countries is lacking due to their non-participation in the European hantavirus registry (Watson et al. 2014).

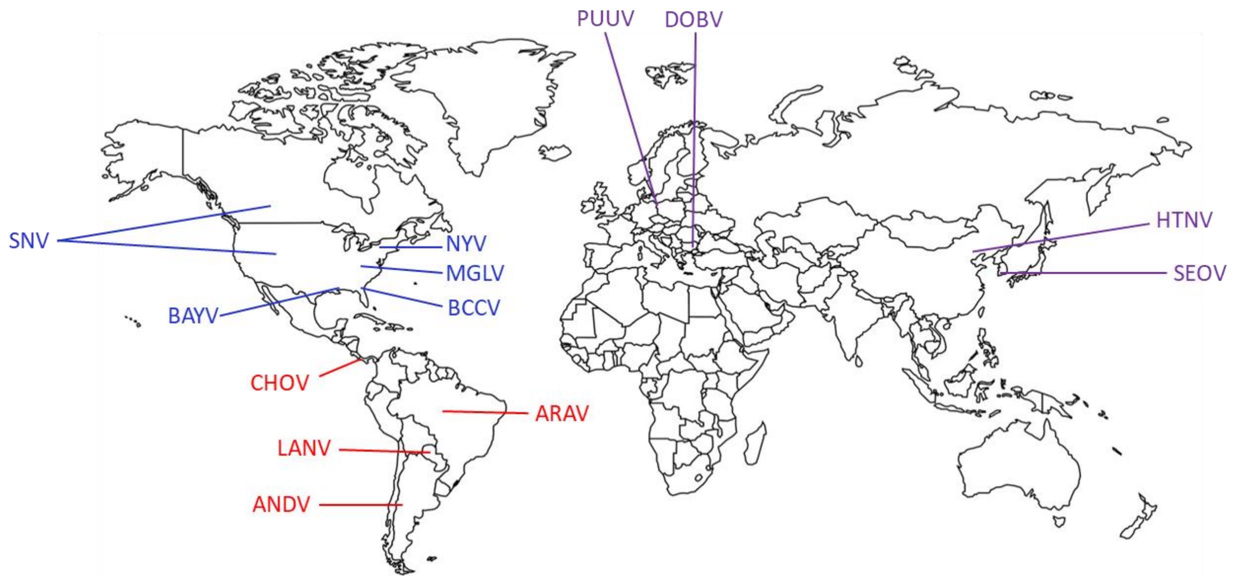

Figure 2. Global distribution of disease causing hantaviruses. Hantaviruses found in North and South America are typically associated with HCPS, while those in Europe and Asia cause HFRS. Seoul virus (SEOV) has a worldwide distribution. Abbreviations: SNV = Sin Nombre virus, NYV = New York virus, MGLV = Monongahela virus, BCCV = Black Creek Canal virus, BAYV = Bayou virus, CHOV = Choclo virus, ARAV= Araraquara virus, LANV = Laguna Negra virus, ANDV = Andes virus, PUUV = Puumala virus, DOBV = Dobrava-Belgrade virus, HTNV = Hantaan virus, SEOV = Seoul virus.

Virus	Abbreviation	Geographic Location	Disease	Annual # of Cases
Puumala virus	PUUV	Europe	HFRS/NE	>1,000
Dobrava-Belgrade virus	DOBV	Europe	HFRS	Hundreds
Hantaan virus	HTNV	China, Korea, Russia	HFRS	10,000-100,000
Seoul virus	SEOV	Worldwide	HFRS	Thousands
Sin Nombre virus	SNV	North America	HCPS	20-50
Andes virus	ANDV	South America (Chile, Argentina)	HCPS	~75-100
Araraquara virus	ARAV	South America (Brazil)	HCPS	75-100

Table 1. List of described hantaviruses. Listed are the most common hantaviruses responsible for causing human disease, including their location and their annual incidence.

Following the discovery of New World hantaviruses in the early 1990s, several countries in both North and South America have reported cases of HCPS. In South America, Venezuela, French Guiana, Peru, Bolivia, Brazil, Paraguay, Uruguay, Chile, and Argentina have reported cases (Figueiredo et al. 2014). In Brazil, the causative agent of most cases of HCPS is ARAV, while in Chile and Argentina, ANDV causes most cases. HCPS cases occur less frequently than do HFRS and NE cases, with only several thousand cases occurring since the disease was characterized. In the South American countries listed, the number of cases each year ranges from single digits to as high as 100, but comprehensive reporting on HCPS occurrence in South America is lacking. In North America, the majority of HCPS cases are caused by SNV, and since the initial outbreak in 1993, greater than 700 cases in the United States and 120 cases in Canada have been diagnosed, for an average of around 30 and five cases per year, respectively (Centers for Disease Control and Prevention; Public Health Agency of Canada). The far fewer number of HCPS cases may be due to a combination of the ability of the virus to transmit, its stability in the environment and the rodent population, and a lack of strong data on subclinical infection and exposures.

Seroprevalence studies have suggested that there could be higher rates of exposure that previously thought (Castillo et al. 2012). Individuals have been shown to be IgM and IgG positive against ARAV and ANDV despite no diagnosed HCPS (Castillo et al. 2012; Pereira et al. 2012). Currently our knowledge of HCPS prevalence relies upon up to date and proficient reporting and laboratory confirmation of cases.

1.7.4 Immune Responses to Hantavirus Infection

1.7.4.1 HFRS

During hantavirus infection, the vascular endothelium is the primary target of infection. Endothelial cells become infected and vascular permeability leads to the classical symptoms of infection described above, but infected cells do not show signs of cytopathic effect (CPE). Due to the lack of CPE and cell death during hantavirus disease, it has been suggested that pathology may be mediated by the immune response to the virus. Following viral entry, recognition of viral RNA by pattern recognition receptors triggers immune signaling and the production of innate interferons (IFN) (Vaheri et al. 2013). During HFRS, innate cytokines IFN-α, IFN-β do become elevated and IFN-λ has been shown to be decreased during HTNV infection, but there is evidence that some hantaviruses are able to evade or antagonize the innate immune response, at least *in vitro* (Krakauer et al. 1994; Stoltz et al. 2007; Jaaskelainen et al. 2008; Krakauer et al. 1995). Established hantavirus infection can also inhibit the phosphorylation of STAT1 and STAT2, major inducers of immune signaling pathways (Stoltz et al. 2007). Other cytokines that play a major role in antiviral immune responses that are generated early during infection are tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6. These cytokines play a significant role in fever, systemic inflammation, and antiviral immune responses in cells. TNF-α is detectable in the serum of HFRS and NE patients, which likely contributes to the flu-like symptoms that occur following incubation

(Krakauer et al. 1995; Linderholm et al. 1996). IL-1β and IL-6 are also increased during this acute stage of infection (Linderholm et al. 1996; Takala et al. 2000). IL-6 has been implicated in severe HFRS disease, with IL-6 levels identified as a strong predictor of disease severity (Outinen et al. 2010). Additionally, a number of chemokines that aid in leukocyte trafficking and adhesion are upregulated during the acute phase of hantavirus infection, including CXCL8, CXCL10, ICAM, VCAM, and E-selectin (Takala et al. 2000; Klingstrom et al. 2008; Krakauer et al. 1995). These proteins are produced by endothelial cells in response to innate and adaptive cytokines and they function in chemo-attraction and adhesion of leukocytes and lymphocytes. Infected endothelial cells also express viral glycoproteins on their surface and are able to recruit neutrophils, leading to the release of neutrophil extracellular traps (Hepojoki et al. 2014; Raftery et al. 2014; Schonrich et al. 2015). Neutrophil extracellular traps can enhance inflammation and activation of platelets that can lead to immunothrombosis (Hepojoki et al. 2014; Schonrich et al. 2015). Nitric oxide produced mainly by macrophages during the innate immune response may have anti-viral effects and have been detected during acute HFRS (Outinen et al. 2010). The inflammatory milieu resulting from the innate immune response to infection likely drives the immunopathogenesis seen during the HFRS disease course.

The cell types that are involved in mediating the immune response to hantavirus infection have mostly been investigated in the context of human infection due to the lack of suitable animal models of HFRS. During acute infection, innate immune cells such as macrophages and mononuclear phagocytes (MNPs) are recruited to the lungs of HFRS and NE patients (Scholz et al. 2017; Linderholm et al. 1993). While MNPs were detected at high levels in the lungs of patients, they were significantly decreased in the peripheral blood, suggesting strong recruitment of these cells to the site of infection (Scholz et al. 2017). A similar phenomenon is seen with NK cells

during acute infection, with an expansion of the NK cell population occurring in the blood and lungs (Bjorkstrom et al. 2011; Braun et al. 2014; Linderholm et al. 1993). One report showed that NK cell numbers slightly decreased during acute PUUV infection, but this is in conflict with other studies showing rapid expansion and activation of NK cells in the peripheral blood of patients (Bjorkstrom et al. 2011; Braun et al. 2014; Linderholm et al. 1993). Nevertheless, activated NK cells are able to kill endothelial cells and contribute to immune mediated pathology (Braun et al. 2014). Interestingly, hantaviruses may be able to prevent endothelial cell killing through the upregulation of MHC class I in infected cells (Braun et al. 2014). A study of T cell numbers in the lungs of HFRS patients showed that there are increased numbers of CD4 and CD8 T cells expressing activation markers during infection along with an increase in NK cells (Rasmuson et al. 2011). Another examination of NE patients infected with PUUV showed a significant increase in cytotoxic CD8 T cells present in the lungs, while CD4 T cell numbers remained steady (Linderholm et al. 1993). This contrast may be dependent upon the severity of infection or be dependent on host-specific factors and the causative virus. One case report of a Croatian infected with PUUV showed that the patient had an increase in CD4 T cells, but no increase in the number of CD8 T cells in the peripheral blood compared to baseline, in contrast with what is generally seen during HFRS (Markotic et al. 2002a). A study of Finnish PUUV patients showed that cytotoxic T cell immunity generated during infection is long lived, with PUUV N protein-specific CD8 T cells that produce interferon-γ (IFN-γ) present years following infection (Van Epps et al. 2002). This in line with studies showing that IFN-γ is typically detected in the serum of HFRS patients and may play a key role in disease. While IL-10 has also been shown to be upregulated, there is little data on the presence of regulatory T cells during infection such as with reservoir hosts. The activation of CD8 T cells and their interaction with infected endothelial cells has been

hypothesized to be the driving factors in pathogenesis during HFRS (Terajima et al. 2007). This hypothesis along with the data collected during human HFRS disease are in contrast to what is seen in persistent infection of reservoir hosts, as described above. Studies to this end, examining the differential pathology seen during PUUV infection and compared to DOBV infection have attempted to shed light on what leads to development of severe HFRS. IgM and IgG responses are generated during acute infection, and the robustness of the antibody response during infection has been shown to be correlated with infection outcome, independent of viral load (Pettersson et al. 2014). Patients with severe disease had lower levels of IgG and higher leukocyte numbers in the blood compared to those with mild disease, suggesting perhaps a more predominant cell-mediated response to infection. This type of response may lead to an increase in CD8 T cell and NK cell mediated pathology, while a strong Th2 type response may lead to protection and avoidance of excess inflammation. A comparison of PUUV and DOBV infection revealed that DOBV infection, which typically causes more severe disease, not only results in longer lasting and higher viremia, but also a weaker IgG response and a longer lasting inflammatory cytokine response (Korva et al. 2013). These studies provide evidence that developing a strong antibody response, while avoiding deleterious inflammatory cytotoxic responses may be key to reducing pathology mediated by the immune response. While differences in infection depending on the causative virus likely play a role in disease severity, immune system mediated pathology appears to play an important role in the development of HFRS. An understanding of the key underlying mechanism(s) that drives persistent versus pathogenic infection outcomes could provide a means for controlling hantavirus infection in humans and limiting human disease.

1.7.4.2 HCPS

More experimental work on the role of the immune system in the disease course of HCPS has been performed, thanks to the development of suitable animal models of the disease in addition to *in vitro* and human clinical studies. Similar to HFRS, following entry immune activation occurs, and it is thought that an excessive inflammatory response contributes to disease severity (Gavrilovskaya et al. 1999b). Contributions to pathogenesis response are likely to be from both the innate and adaptive immune response to infection. *In vitro,* type I IFN responses occur following SNV infection, and innate responses are not dependent upon replicating virus (Borges et al. 2006; Prescott et al. 2005). As in HFRS, innate cytokines such as TNF-α, IL-1α, and IL-6 are elevated during human HCPS cases, with high IL-6 levels and low transforming growth factor-β (TGF-β) levels correlated strongly with disease severity and lethality (Borges et al. 2008; Morzunov et al. 2015). A number of other upregulated cytokines and chemokines have been identified in HCPS patients including INF-α, TNF-β, VEGF, and CXCL10 while the increased levels of others such as IL-2RA, IL-18, CXCL1, CXCL9, hepatocyte growth factor, macrophage colony-stimulating factor, macrophage migration inhibitory factor, and stem cell factor have been correlated with fatality in HCPS (Morzunov et al. 2015; Khaiboullina et al. 2017). A direct comparison of the serum of HFRS and HCPS patients showed that while several of the same cytokines and chemokines are found during infection, HCPS patients generally undergo stronger inflammatory and Th1 type immune responses with earlier and stronger activation of innate immune responses (Khaiboullina et al. 2017). While several innate and pro-inflammatory cytokines with a Th1 bias may have a role in HCPS disease, a mixed Th1/Th2 immune profile has also been documented with an increase in IL-5 and antibody isotypes IgG1 and IgG3, indicative of a Th2 type response (Bostik et al. 2000; Bharadwaj et al. 2000). During HCPS, infected endothelial cells produce chemokines such as IP-10 and RANTES, which promote macrophage

infiltration into the lungs (Sundstrom et al. 2001). Activated, lung infiltrating macrophages produce pro-inflammatory cytokines that may exacerbate pathology. Additionally, the infiltration of cells into the lungs during the inflammatory response includes activated CD4 and CD8 T cells (Borges et al. 2006). High numbers of activated T cells are present during HCPS, particularly activated CD8 T cells in the blood (Kilpatrick et al. 2004; Ennis et al. 1997). Significantly higher levels of circulating, SNV-specific CD8 T cells were found in severe cases of HCPS that required ventilation, and particular HLA haplotypes presenting specific epitopes were found to be major drivers of disease severity (Kilpatrick et al. 2004). It is possible that the presence of higher numbers of lymphocytes seen during certain cases is due to less controlled viral replication early during infection, leading to higher antigen levels and activation of these cells. This may represent a correlation between illness and the presence of T cells, but may not necessarily implicate T cells as drivers of disease. Contrastingly, certain HLA class I and II haplotypes may not correlate with disease severity in HCPS caused by ARAV (Borges et al. 2006). While activated CD4 and CD8 T cells have been implicated in disease during hantavirus infection, cell killing mediated by cytotoxic T lymphocytes is abolished during hantavirus infections through interactions of the nucleocapsid protein with caspases that aid in apoptosis and via the downregulation of death receptors on infected cells (Sola-Riera et al. 2019a; Sola-Riera et al. 2019b; Gupta et al. 2013). The inability to kill virally-infected cells may drive a continued inflammatory response that exacerbates disease in affects tissues. Further examination into how different haplotypes presenting hantavirus antigens corresponds to HCPS pathology could provide further evidence that disease is mediated by deleterious inflammatory responses.

While immunopathogenesis is thought to play a key role in HCPS disease progression, some evidence from animal models suggests it may not be necessary for disease. Animal models

of HCPS have been valuable in examining different aspects of the immunopathology of HCPS. Studies utilizing the Syrian hamster model of HCPS have mirrored human case studies and revealed roles for certain cytokines in HCPS pathogenesis such as IL-6, IFN-γ, TNF-α, and IL-4 (Safronetz et al. 2012; Safronetz et al. 2011). However, other studies using this model have also shown that CD4 and CD8 T cells are not required for pathogenesis in the ANDV model of HCPS, and immunosuppression leads to lethal SNV infection in Syrian hamsters (Hammerbeck et al. 2011; Prescott et al. 2013). These studies oppose the idea that a strong adaptive immune response to the causative virus is needed for disease pathogenesis. An NHP model of HCPS has shown that during disease there is an increase in the presence of CD8 T cells as well as cytokines such as IL-1, IL-6, GM-CSF, IFN-γ, and TNF-α compared with animals with no disease, indicating again that these cytokines and others could mediate HCPS disease (Safronetz et al. 2014). The differences seen in animal models and what is hypothesized to occur during human infection could be due model specific differences in hantavirus infections. Further examination of the role of the immune response in HCPS pathogenesis is warranted.

1.7.5 Pathogenic vs Non-pathogenic Hantavirus Infection

Insights into the pathogenic mechanisms hantaviruses use to cause disease can be gathered from comparisons of pathogenic viruses to those which do no cause overt disease in humans such as Tula virus (TULV) and Prospect Hill virus (PHV). Both pathogenic and non-pathogenic viruses infect the same cell types, neither cause cytopathic effects, and both are able to inhibit apoptosis of infected cells, likely through interaction with caspases (Klingstrom and Ahlm 2011; Sola-Riera et al. 2019b). Vascular leakage that occurs during hantavirus infection of endothelial cells is likely to occur through a few different mechanisms in the absence of apoptosis or cell death. ANDV N protein has been shown to directly interact with tuberous sclerosis complex (TSC) protein that is

responsible for regulation of the pathways involved in cell growth such as RheB-mTOR-pS6K, and in regulation of adherens junctions between endothelial cells (Gorbunova et al. 2016). Interaction of hantaviruses with their receptor $\alpha_v\beta_3$ integrins results in its inhibition and subsequent RhoA activation leading to increased cell permeability (Gorbunova et al. 2016). N protein interaction intracellularly with TSC prevents its repression of RheB GTPase mediated signalling leading to cell growth and permeability of the endothelial layer. TSC can also control the activity of RhoA, therefore this interaction could be directly involved in increased endothelial permeability. Pathogenic, but not non-pathogenic hantavirus infections have also been shown to increase endothelial cell permeability through sensitizing cells to vascular endothelial growth factor (VEGF), and VEGF production during infection leads to increased vascular permeability (Hepojoki et al. 2014; D'ignazio et al. 2016; Kurihara et al. 2014). VEGF, which is important for angiogenesis, may play an important role in permeability through promoting its own production. TNF-α produced early during hantavirus infection as well as hypoxia that is experienced during inflammation in the lungs, induce the upregulation of NF-κB in cells such as macrophages (D'ignazio et al. 2016). NF-κB expression leads to increase in inflammatory responses as well as expression of hypoxia inducible factors (HIF), which are transcription factors that regulate expression of genes for VEGF and nitric oxide synthases, which produce NO that plays a role in hantavirus immunopathology (D'ignazio et al. 2016; Kurihara et al. 2014). VEGF also promotes upregulation of HIF, in a feedback loop that continues to promote angiogenesis, but also leads to increased permeability within the inflammatory environment during hantavirus infection. While VEGF has been postulated to play an important role in disease through *in vitro* and human studies, targeting of VEGF *in vivo* in the Syrian hamster model of suggests that its role may not be critical for severe or lethal infection (Bird et al. 2016). These mechanisms at play that lead to increased

vascular permeability and immune-mediated pathology in hantavirus infection differ between pathogenic and non-pathogenic viruses. Since non-pathogenic viruses utilize β_1 integrins for entry rather than β_3, it is possible that there are different interactions between these integrins and their extracellular matrix ligands. Endothelial cell migration is inhibited by pathogenic viruses that bind to β_3 integrins, but not by non-pathogenic viruses binding to β_1 integrins (Gavrilovskaya et al. 2002). While pathogenic and non-pathogenic viruses may share properties such as the inhibition of caspase mediated apoptosis, a detail as small as their receptor for entry leads to extreme differences in infection outcome in humans. Further study of these differences could provide insight into pathways to target during treatment of hantavirus infections and provide valuable information on the pathogenesis of diseases which involve excessive vascular leakage.

1.7.6 Vaccines and Therapeutics

The high lethality rate of HCPS and the overall burden of HFRS in European and Asian countries underscores the need for effective preventative and therapeutic countermeasures against hantavirus infections. Currently there are no FDA approved vaccines or therapeutics for use in humans against New or Old World hantaviruses. The major focus of vaccine development for hantaviruses has been for prevention of HFRS. An HTNV infected suckling rodent brain-derived inactivated vaccine, Hantavax, has been used extensively in Asia, however questions remain regarding its immunogenicity and efficacy (Lee et al. 2009; Song et al. 2016; Jung et al. 2018). Two initial doses followed by a booster after one year are required to achieve seropositivity, and less than 50% of those vaccinated develop neutralizing antibodies (Song et al. 2016). A tissue culture-derived bivalent inactivated vaccine against HTNV and SEOV has been tested and used in China as well, and is able to induce anti-HTNV neutralizing antibodies and seropositivity in a high percentage of vaccinated individuals (Li et al. 2016; Brocato and Hooper 2019). This bivalent

vaccine has been administered to millions of people throughout China (Brocato and Hooper 2019). A vaccinia virus (VV) vectored vaccine against HTNV was tested in phase I and II clinical trials and was able to induce humoral responses, including neutralizing antibody, however issues with pre-existing immunity to vaccinia limited its effectiveness in this case (McClain et al. 2000). A phase I trial of DNA vaccines against PUUV and HTNV has also been completed, albeit with a very small number of participants (Hooper et al. 2014). Three doses of PUUV, HTNV, or a combined vaccine was able to elicit neutralizing antibodies in five, seven, and seven out of nine participants, respectively (Hooper et al. 2014). Aside from Hantavax and the inactivated HTNV vaccine, which are given annually to millions of people in Asia, no other vaccines for HFRS have progressed fully through human trials. Four nucleic acid based vaccines against HFRS and HCPS are currently undergoing clinical trials, however none are currently approved in Europe or the Americas (Schmaljon 2012; Brocato and Hooper 2019). For HCPS, no vaccines have progressed into human trials aside from DNA vaccines, while multiple platforms for both HFRS and HCPS have been tested in pre-clinical studies. DNA vaccines have made up the majority of vaccines that have been tested for immunogenicity and protective efficacy in animal models. A DNA vaccine against SEOV was shown to be immunogenic in mice and also able to protect hamsters from challenge with SEOV and HTNV (Hooper et al. 1999; Kamrud et al. 1999). A HTNV DNA vaccine was also able to provide homologous and heterologous protection against SEOV and DOBV in hamsters and elicit high titer neutralizing antibodies in NHPs (Hooper et al. 2001). DNA vaccines that express Gn/Gc of SNV have been shown to be immunogenic in mice and rabbits as well as be able to protect hamsters and deer mice against SNV infection and lethal ANDV infection (Bharadwaj et al. 1999; Bharadwaj et al. 2002; Hooper et al. 2013). The utility of these vaccines in the context of lethal SNV infection is difficult to determine due to the lack of suitable animal

models of lethal SNV infection other than the NHP model. For ANDV, a number of different platforms for vaccination have been developed and tested for immunogenicity and protective efficacy using the Syrian hamsters model of HCPS. A DNA vaccine expressing ANDV M segment is immunogenic in Rhesus macaques, and passive transfer of immune sera from vaccinated macaques into Syrian hamsters protected the hamsters from lethal ANDV infection (Custer et al. 2003). Viral vectored vaccines expressing ANDV glycoproteins have also been developed and tested in the Syrian hamster model of HCPS. Adenovirus vectors expressing ANDV Gn, Gc, or N were protective against ANDV both on their own and in combination (Safronetz et al. 2009). Recombinant vesicular stomatitis virus expressing ANDV GPC was also able to full protect hamsters against lethal ANDV challenge, including challenge at six months and one year post-vaccination (Brown et al. 2011; Prescott et al. 2014). While several candidates have shown efficacy in animal models, progression toward clinical trials has been limited. For HCPS, due to the rare occurrence of the disease, its sporadic nature, and the logistical issues that would arise with testing the vaccine, it is likely not practical to attempt to develop a vaccine for common use. However, for HFRS, the burden of disease remains high, and development of an effective vaccine could prevent tens of thousands of cases per year in Europe and Asia. Therefore the most likely advancement toward an HCPS vaccine might be for development of a vaccine for HFRS in combination with one targeting HCPS causing agents. Some of the DNA vaccines mentioned above were able to provide cross-protection against other hantavirus species. The DNA vaccine against ANDV elicits antibodies against SNV and BCCV (Custer et al. 2003). While some of these vaccines may be immunogenic against multiple viruses, vaccination of hamsters with a SNV DNA vaccine did not fully protect against lethal ANDV infection (Hooper et al. 2013). The type of immunity elicited by vaccines candidates could play an important role in protection in the context

of protection against multiple different hantaviruses, as passive transfer of immune sera from SNV-vaccinated rabbits was able to protect against homologous SNV challenge, but not against heterologous challenge with lethal ANDV (Kwilas et al. 2014). This suggests that cross-protection could be conferred in part by cell-mediated immunity. Dual HFRS/HCPS vaccines have been tested in pre-clinical models, with DNA vaccines combined to produce a cross-clade vaccine. An HNTV/ANDV DNA vaccine has been shown to elicit high antibody titers against both viruses (Hooper et al. 2006). Additionally, a mix of SNV, ANDV, HTNV, and PUUV DNA vaccines has been administered to rabbits and was able to elicit a strong humoral response (Hooper et al. 2013). However, the utility of this approach has not been assessed in infectious challenge models.

In addition to vaccines, there are also no currently approved therapeutics for HFRS or HCPS. The efficacy of ribavirin, a nucleoside analog and broad spectrum antiviral, has been assessed in HFRS and HCPS clinical trials, with varying results, and its usefulness is not clear (Mertz et al. 2004; Huggins et al. 1991; Malinin et al. 206; Rusnak et al. 2009; Moreli et al. 2014). Due to the excess inflammation underlying disease during infection, steroids have often been employed, but their efficacy is also not clear. A double-blind, placebo controlled trial of intravenous methylprednisolone did not show any clear benefit to HCPS patients (Vial et al. 2013). There are case reports of steroids being administered to patients with HFRS, but the benefits are not clear, and no placebo controlled trials have been performed to assess their efficacy. For HCPS, there is evidence that the use of immune plasma from convalescent HCPS patients may provide some benefit to patients (Vial et al. 2015). Patients administered 5,000 nAb U/kg of immune plasma trended toward a significantly lower case fatality rate, although there were not enough patients to reliably confirm efficacy (Vial et al. 2015). Further trials of immune plasma with high neutralizing antibody titers may confirm the effectiveness of this approach. Due to the rare occurrence of

HCPS, it is difficult to obtain the number of patients required to reliably test the protective effects of medical countermeasures.

1.8 Oral Bait Vaccines

For zoonotic diseases carried by animals such as rodents and larger mammals, one method of preventing spread of these infections within the animal population and to humans is to immunize the wild animal population. The concept of wildlife vaccination involves the dropping of bait that contains vaccines, which subsequently immunize animals following their ingestion (MacInnes et al. 2001). In Ontario, the original implementation of this strategy involved aerial dropping of live attenuated rabies vaccines within a certain area in the south of the province, targeted at reducing the incidence of rabies within the red fox population, the main carrier of the virus in that area (MacInnes et al, 2001). Within four years of bait introduction, rabies was eliminated from the fox population in that area. Since the description of this effective implementation, a number of bait style vaccines have been employed against a variety of pathogens found in wildlife. The development of these types of vaccines for wildlife has certain benefits such as cost and a faster path toward regulatory approvals, and multiple platforms have since been utilized to deliver vaccines to wildlife populations including Adenoviruses and pox viruses (Mendoza et al. 2018). Here I will briefly discuss bait vaccine strategies that have been used for other zoonotic pathogens in hopes of laying a foundation upon which to develop a similar strategy for reducing hantavirus incidence.

1.8.1 Vaccines Against Rabies Virus

Following the employment of oral baits for vaccination of wildlife against Rabies in the late 1980s and early 1990s, the use of live attenuated rabies strains fell out of favor due to a small number of rabies cases developed following vaccination (Knowles et al. 2009). The mutation rate

of the attenuated virus, and the potential for reversion to virulence in some of the strains used for bait vaccines led to the preferred use of recombinant vaccines expressing rabies glycoprotein as vaccine vectors. A recombinant vaccinia virus expressing rabies glycoprotein, V-RG was developed and used extensively in the USA, Canada, and Europe (Kieny et al. 1984; Brochier et al. 1991; Hanlon et al. 1998; Rosatte et al. 2001). A commercially licensed vaccinia-rabies vaccine RABORAL V-RG has been distributed globally since 1987 and has been shown to be effective in foxes, coyotes, skunks, and raccoons (Maki et al. 2017). It has also been shown to be safe, with no adverse events occurring in any off target species, which may inadvertently come across the baits in the wild (Maki et al. 2017). Additionally, human Adenovirus type 5 (hAd5), due to its immunogenicity, ability to grow to high titers, stability, and ability to infected multiple cell types has commonly been used as a vaccine vector (Mendoza et al. 2018; Graham and Prevec 1992). hAd5 vectors expressing rabies glycoprotein have been developed and optimized for a number of species that can harbor rabies virus (Rosatte et al. 2001). A commercially available hAd5, ONRAB has been used in Canada to successfully target foxes, skunks, and raccoons and is generally stable, safe in non-target wildlife, and effective (Knowles et al. 2009; Fry et al. 2013; Brown et al. 2014; Sobey et al. 2013; Slate et al. 2014). A direct comparison of RABORAL and ONRAB suggested that ONRAB is likely more effective due to its relative effectiveness in more species (Fehlner-Gardiner et al. 2012). However, both have been used extensively for decades with high effectiveness and safety profiles, suggesting this approach could be used to target other viral zoonotic pathogens.

1.8.2 Vaccines Against Borrelia burgdorferi

Oral bait vaccines against the causative bacteria of Lyme disease, *Borrelia burgdoferi,* have been developed to limit the transmission of this pathogen within its enzootic cycle. Lyme

disease is carried by ticks from the *Ixodes* genus, and the enzootic transmission cycle for the spirochete involves infected ticks feeding on reservoir hosts such as white-footed mice (*Peromyscus leucopus)*, which are competent reservoirs for the bacteria (Gomes-Solecki 2014). Once mice or other competent reservoirs for Lyme are infected, subsequent blood meals provided to non-infected ticks continues the transmission cyle. Other species such as deer and cattle are essential for tick survival, but these animals are not competent reservoirs for *B. burgdorferi,* therefore prevention of infection in the main reservoir, white-footed mice and other rodents, may lead to the elimination of the spirochete from the mouse and tick populations. Prevention of infection of the vector, or the tick species, through vaccination of the mouse population has been shown to be an effective method to curb transmission of Lyme disease. While the white-footed mouse is the main reservoir for *B. burgdorferi,* several other small mammals such as chipmunks, squirrels, and shrews are competent reservoirs as well (Gomes-Solecki 2014). Two successful platforms have been employed to prevent transmission of Lyme disease through the vaccination of mice that may become infected through tick feeding. Recombinant strains of *E. coli* expressing OspA, an outer surface protein of the spirochete against which protective immune responses can be generated, formulated into baits are capable of inducing anti-OspA antibodies in lab mice and in white-footed mice following oral gavage or feeding of the vaccine (Richer et al. 2011; Voordouw et al. 2013). They are also effective at reducing the burden of spirochetes following infected tick feeding of vaccinated animals (Richer et al. 2011; Voordouw et al. 2013). One limitation of this approach is the reduced immunogenicity and requirement for multiple oral doses of the vaccine. This could limit its effectiveness in a wild setting in which mice may not come across the multiple baits needed for effective vaccination. A second approach utilizes recombinant vaccinia virus, similar to rabies. Vaccinia virus expressing OspA is also able to induce high levels

of anti-OspA antibodies and provide protection to vaccinated mice from infection (Bhattacharya et al. 2011). This vaccination is an effective means of disrupting the transmission cycle of *B. burgdorferi* by reducing the number of infected mice and ticks. Although the life cycle and transmission of *B. burgdorferi* is more complex than that of hantaviruses, these studies provide evidence that this type of approach could be effective when targeting smaller species such as rodents for bait vaccination. Rabies vaccines are typically directed toward animals that are many times greater in size than mice and rats, and therefore may more easily consume baits laid out for their consumption. An important question is the suitability of this approach for small animals like rodents, and these studies show that this method has merit.

1.8.3 Vaccines Against Yersinia pestis

Yersinia pestis, which causes sylvatic plague in prairie dogs and black-footed ferrets as well as pneumonic, septicemic and bubonic plagues in humans has been targeted through vaccination of wildlife reservoirs of the bacteria. Prairie dogs are affected by plague and are also an important transmitter of the bacteria to humans. Once again recombinant pox viruses have been used as vectors expressing plague proteins for induction of immunity against the bacteria. A modified vaccinia virus expressing a truncated version of the V antigen of *Y. pestis* or a fusion protein combination of the F1 capsular protein and the LcrV type III secretion component of the bacteria have been shown to be immunogenic and protective in mouse models (Brewoo et al. 2010; Bhattacharya et al. 2010). A recombinant raccoon pox virus expressing the F1 antigen was also found to be immunogenic and able to provide some protection to prairie dogs against infection in field studies (Sun et al. 2011; Rocke et al. 2017; Rocke et al. 2008). The incorporation of Rhodamine B, a dye that used in ecological and behavioural research, into bait vaccines can also track which animals have been exposed to them in the wild (Suder et al. 2018). This could provide

a means of tracking the efficacy and the uptake of vaccines deployed in a given area. This is another example of the use of pox viruses as live vectored vaccines that are effective in providing protection to wildlife against infectious agents.

1.8.4 Oral Bait Vaccine Platforms

Several different approaches have been used for the development of bait style vaccines for wildlife, some of them listed above. The most common and seemingly most effective approach is to use virally-vectored vaccines expressing an immunogen of interest, which will invoke a protective immune response. Pox viruses such as vaccinia, canary pox, or raccoon pox are vectors which are immunogenic even when administered orally, stable, safe in non-target animals, and safe in immunocompetent humans (Mendoza et al. 2018). One concern with pox viruses as vectors is their safety in immunocompromised individuals that may come into contact with the vaccine. Serious adverse effects could occur when infections with pox viruses occur in these individuals. Proper warning and descriptive labels on bait vaccines containing pox viruses are typically used, however unintentional exposures could occur. Recombinant Adenoviruses offer another suitable option as a platform for bait style vaccines. Being non-enveloped, adenoviruses are stable and immunogenic when given through an oral route (Mendoza et al. 2018). They also are capable of infecting a wide range of host cell types and animals. hAd5 for use in humans can be limited because of pre-existing immunity due to prior exposures, but this is less of an issue when targeting wildlife. Other vectors that have potential as bait vaccines include vesicular stomatitis virus (VSV) and attenuated recombinant rabies virus. VSV as a vaccine platform is immunogenic and has been used extensively in humans and animal models (Mendoza et al. 2018; Suder et al. 2018). Its use as an oral vaccine has not been thoroughly tested, and its enveloped nature may limit its immunogenicity via this route. However in an experiment in which mice were orally vaccinated

with VSV expressing Ebola virus glycoprotein, this platform provided complete protection against lethal Ebola virus challenge (Jones et al. 2007). Attenuated rabies viruses have been used successfully for vaccination of wildlife against rabies, but the use of recombinant rabies virus as a vector expressing other viral antigens has been explored (MacInnes et al. 2001; Gomme et al. 2011; Abreu-Mota et al. 2018). The use of rabies virus as a vaccine vector has the additional benefit that it may provide protection against both rabies and the targeted virus of interest through expression of its antigens (Mendoza et al. 2018). Overall, a number of suitable candidate vaccine platforms for development of bait style vaccines for zoonotic pathogens such as hantaviruses are available. Their development and testing could potentially lead to employment of this strategy to limit the transmission of hantaviruses within their host populations and ultimately reduce the burden of human disease.

1.9 Rationale and Significance

Hantaviruses have a global distribution and are a significant contributor to human disease in Europe, Asia, and the Americas. Despite the identification and characterization of dozens of new hantavirus species since their discovery and classification, research remains to be done in several important areas pertaining to hantaviruses. A viable vaccine candidate for either New or Old World hantaviruses likely remains years away and the advancement of therapeutics for treating either HFRS or HCPS has not occurred. Study of infection with hantaviruses remains difficult due to the need for specific host species to study persistent infections *in vivo* as well as the need for high containment facilities. Some hantavirus' tendencies to mutate following cell culture adaptation limits the applicability of some *in vitro* work in the context of human and rodent infection. Field studies have shed light on important ecological considerations with regard to hantaviruses, but studies of experimental infections and transmission have been lacking due to the

difficulties mentioned. For several aspects of hantavirus biology such as viral entry, fusion, replication, viral structure, and host responses, there is little data for many hantaviruses, and much of the work has focused on certain viruses. There may be differences in many of the aspects between different species of hantaviruses that have not been discovered. The fact that these differences may occur highlights the need for expanded research efforts for many areas of both Old and New World hantavirus biology.

1.9.1 Difficulties of Working with Hantaviruses

As mentioned above, working with particular hantaviruses such as PUUV or SNV in cell culture can provide challenges to researchers hoping to study different aspects of these viruses such as replication *in vitro* and *in vivo,* pathogenesis, and persistent infections. One issue is that hantaviruses do not cause obvious CPE of infected cells, making quantification of live virus difficult. Another is that growing either of these viruses in cell culture such as in VeroE6 cells requires adaptation for growth in these cells, eventually resulting in mutations which render these viruses incapable of infecting their reservoir hosts or other animals (Lundkvist et al. 1997; Nemirov et al. 2003; Safronetz et al. 2014; Warner et al. 2019a). This limitation requires that virus stocks to be used for infection of either host animals for ecological experiments or studies of pathogenesis be passaged only within natural host species. For deer mice, this requires the passaging of SNV within deer mice and the use of viral stocks derived from tissue homogenates for *in vivo* experiments (Safronetz et al. 2014; Warner et al 2019a). Protocols for generating high titer virus stocks have not been optimized for any hantaviruses with this requirement. Determinations of an appropriate dose and route of infection for producing virus stocks *in vivo* have not been performed. It was previously assumed that IM infection of young male deer mice was the most efficient route for viral replication leading to high titer virus from tissue

homogenates, however other routes such as IP may lead to higher levels of virus (Warner et al. 2019a). Testing infection of different ages and both sexes of deer mice infected through multiple routes may provide a more efficient means of production of SNV for its use in infectious models, and this is something that has previously not been examined.

1.9.2 SNV Transmission Model

Experimental transmission of multiple hantavirus types has shown that these viruses transmit readily between the reservoir hosts and can persist within the environment for relatively long periods of time, leading to infection of naïve animals. However for SNV, attempts at examining transmission of the virus between deer mice have had unsatisfying results, including seeing a very low transmission rate in an experimental outdoor setting (Botten et al. 2002). One study was able to show transmission of SNV between deer mice at rates as high as 26% (Bagamian et al. 2012). However this study was unable to determine whether SNV is transmitted primarily through direct contact between mice as has been suggested through field studies, or through persistence in the environment, which occurs for other hantaviruses and is the main mode of transmission to humans. Successful characterization of how SNV is transmitted between deer mice could have important implications for our understanding of hantavirus ecology and the biology of SNV infections in deer mice. Additionally, the development of a suitable, reliable model of SNV transmission would allow for study of what factors, either mouse intrinsic, environmental, or immunological, might lead to increases in transmission. It also allows for the testing of different intervention measures for prevention of SNV transmission within deer mouse populations.

1.9.3 Mechanisms Behind Intermittent SNV Shedding and Transmission

As discussed above, during persistent infection of reservoir host species, hantaviruses replicate at low levels in several tissues with sporadic periods of high viral replication and shedding

that are thought to lead to increased risk of SNV transmission. What is responsible for triggered higher levels of replication within the tissues of infected mice leading to shedding of SNV is currently unknown. Factors such as cold stress, heat shock, and hormone levels may potentially influence SNV levels in infected deer mice. A determination of what factors lead to increased levels of SNV replication, shedding, and transmission would provide valuable information for researchers and public health officials for advising measures to prevent infection. It will provide important information as to why the trends seen in ecological and field studies of SNV infection are occurring. Currently, none of these aspects have been examined experimentally for SNV infection in deer mice.

1.9.4 Use of Bait Vaccines

Due to the rare occurrence of HCPS cases caused by SNV in North America, the development and testing of vaccines for use in humans is likely an infeasible and impractical option for prevention of SNV infections. A cheaper, potentially more useful approach could be the use of wildlife vaccines which would prevent the spread of SNV among the deer mouse population in high risk areas for human exposure. Bait style vaccines for prevention of infection with several pathogens have been utilized successfully in wildlife, including of rabies, Lyme disease, and plague as discussed previously. As most hantaviruses are carried by mammalian reservoir hosts, employment of a similar approach for hantaviruses could prove effective. The use of vaccines in specific areas to reduce the prevalence of hantaviruses within a rodent community could significantly improve the outcome seen in terms of the number of human infections. This approach has not been developed or tested for any hantaviruses to this point, but has been suggested to be an approach that could be effective (Mendoza et al. 2018). In order to develop and test vaccines to this effect, a suitable model of transmission and a strong characterization of the dynamics of

hantavirus transmission are needed. With an appropriate infection model of SNV in deer mice, the testing and developing of bait style vaccines for SNV could provide a strong proof of concept for this approach against hantaviruses.

1.10 Hypothesis and Objectives

1.10.1 Hypotheses

Based upon evidence and previous work done with regard to SNV transmission in deer mice and previous utilization of bait style vaccines for reduction or elimination of pathogens within wildlife populations, we have formulated a two part hypothesis upon which the majority of the work in this thesis is based. The hypotheses are as follows: i) transmission of Sin Nombre virus between deer mice occurs via a direct mechanism, i.e. direct contact between deer mice, rather than an indirect mechanism such as via contaminated excreta and secreta in the environment, as occurs during human contraction of infection, and that stress responses and/or fluctuations in sex hormone levels result in an increase in viral replication and shedding in Sin Nombre virus infected deer mice leading to increased SNV transmission; and ii) transmission of Sin Nombre virus can be prevented via vaccination of naïve mice with vaccines expressing Sin Nombre virus glycoproteins.

1.10.2 Objectives

1.10.2.1 Effect of Age and Sex on SNV Replication

Overall, this thesis includes four objectives. The first is to optimize experimental SNV infections in deer mice, both for further experimental use and for producing SNV viral stocks *in vivo* from tissue homogenates of infected mice. I want to determine whether the sex and age of deer mice has any significant effect on the kinetics of SNV replication and shedding during infection. Similarly I want to determine whether infection of male and female or differently aged mice produce SNV stocks of similar viral titers. Both of these objectives are aimed at increasing

the number of mice available for both virus production and for experimental use, rather than limiting experiments to juvenile or young male deer mice.

1.10.2.2 Evaluation of Direct and Indirect SNV Transmission

The second objective is to determine whether SNV is primarily transmitted directly between deer mice through direct contact, which has been proposed and hypothesized to be the case through evidence from field studies, or if shedding of SNV by deer mice and environmental exposure leads to infection. The second part of this objective is to also develop and characterize a transmission model for SNV in deer mice that can be used to further study various ecological aspects of SNV infection and transmission in deer mice.

1.10.2.3 Effect of Heat Shock Responses and Testosterone Levels During SNV Infection

The third objective involves the use of the established SNV infection and transmission models to test my hypothesis regarding what leads to increased viral replication, shedding, and transmission in persistently infected deer mice. If particular factors lead to an increase in viral replication, shedding, or transmission rates, we should be able to detect this through both experimental infection and transmission experiments.

1.10.2.4 Prevention of SNV Transmission Through Vaccination

Finally, the last objective of this thesis is to show to that the transmission of SNV between deer mice can be prevented via oral vaccination of naïve deer mice. The goal of this final objective to is to test various vaccine platforms expressing SNV glycoproteins (either Gn, Gc, or full GPC) in an established transmission model to provide a proof of concept for the use of bait style vaccines in preventing hantavirus transmission amongst the rodent host population.

Chapter 2 – Materials and Methods

2.1 Animal Ethics Statement

All experiments described were carried out at the National Microbiology Laboratory (NML) of the Public Health Agency of Canada. Animal experiments were approved by the Animal Care and Use Committee at the Canadian Science Centre for Human and Animal Health in accordance with Canadian Council on Animal Care guidelines. All infectious work described was performed under CL-4 conditions at the NML. All animals were given food and water *ad libitum* and monitored daily throughout the course of experiments.

2.2 Cells and Viruses

The SNV used for all deer mouse experiments, strain 77734 is the original genotypically matched virus strain for the subspecies of deer mice in our colony and previously used by our group. The virus was originally isolated from a wild *P. maniculatus rufinus* (deer mice) and used for inoculation of deer mice in the original description of experimental SNV infection in this species (Botten et al. 2000). Chilean ANDV strain 9717869 propagated on VeroE6 cells was used for lethal ANDV challenge. Hamster-adapted Sin Nombre virus (HA-SNV) used for infectious inoculations was developed previously through 20 serial passages through Syrian golden hamsters. Virus obtained from tissues homogenates of HA-SNV infected hamsters were subsequently used for hamster challenges. Recombinant Vesicular Stomatitis virus expressing SNV glycoprotein, rVSVΔG/SNVGPC and rVSVΔG/SNVGPC expressing green fluorescent protein, rVSVΔG/SNVGPC-GFP, were sent to our lab by Dr. Kartik Chandran from Albert Einstein College of Medicine. Recombinant Vesicular Stomatitis virus expressing ANDV glycoprotein, rVSVΔG/ANDVGPC and rVSVΔG/ANDVGPC expressing green fluorescent protein,

rVSVΔG/ANDVGPC-GFP were created and characterized in our lab previously by Dr. Kyle Brown (Brown et al. 2011). Recombinant Adenoviruses expressing either SNV Gn or Gc (Ad-Gn and Ad-Gc) were designed and purchased from Applied Biological Materials, Incorporated (ABM, Vancouver, Canada).

VeroE6 (African green monkey kidney) cells and human embryonic kidney cells (HEK 293T) were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) containing 2-10% fetal growth serum + penicillin-streptomycin (1000U) (Hyclone).

2.3 Animals and Infections

2.3.1 Deer Mice

Peromyscus maniculatus rufinus were provided by a breeding colony housed at the University of Manitoba Bannatyne campus. This breeding colony was established with deer mice brought in from a colony at Rocky Mountain Laboratories in Hamilton, Montana, USA, that was established from the originally established colony at the University of New Mexico (Botten et al. 2001). All deer mice used from the colony were seronegative and Sin Nombre virus free. All deer mice brought to the NML from the university were acclimated for at least one week prior to experimental procedures. Animals were housed within veterinary technical services (VTS) at the NML under CL-2 conditions until just prior to infectious challenge. For all experiments, deer mice were anaesthetized with inhalational isoflurane infected with 2 x 10^5 genome copies of SNV (strain 77734). For SNV model refinement experiments and virus stock preparation, animals were infected either intramuscularly (IM) or intraperitoneally (IP). For all subsequent replication and transmission experiments, deer mice were infected IM.

2.3.2 Hamsters

All Syrian golden hamsters (*Mesocricetus auratus)* were purchased from Charles River Laboratories and acclimated for at least one week prior to experimental procedures. For vaccination studies, hamsters were 4-6 weeks old at the onset of the experiments. Hamsters were housed within VTS until being moved into CL-4 just prior to infectious challenge. For experiments involving hamsters, animals were anaesthetized with inhalational isoflurane and were given an intraperitoneal injection of 200 focus forming units (FFU) of ANDV 9717869 or 2 x 10^5 genome copies of HA-SNV. For ANDV challenge studies, animals were monitored daily for signs of disease including lethargy, hunched posture, and labored breathing. Animals requiring euthanasia due to clinical score were exsanguinated via cardiac puncture after induction of deep anasethesia.

2.4 SNV Stock Preparation

For SNV stock preparation, groups of deer mice were infected with SNV and their lungs were harvested at 10 days post infection, pooled, and homogenized via manual tissue homogenizers (VWR). Pooled lung homogenates were then clarified by low speed centrifugation twice for 15 minutes at 525 x *g*. Lung homogenates were prepared in 1 mL of Dulbecco's modified eagle medium (DMEM) per set of lungs and pooled homogenates aliquoted into 500 µL aliquots and stored at -80°C for future infections. 140 µL was taken from each viral stock for RNA extraction and determination of viral RNA copy number by RT-qPCR.

2.5 Blood, Swab, Excreta, and Tissue Collection

For blood, tissue, and swab collections animals were anaesthetized under inhalation of isoflurane followed by swabbing and exsanguination via cardiac puncture. Euthanasia was then completed by cervical dislocation. Whole blood was collected in K2-EDTA tubes and serum was collected in serum separator tubes (BD Biosciences and SAI Infusion Technologies). Serum was

collected via centrifugation at 21,000 x *g* for 1 min. Oral and rectal swabs were taken using fine tip rayon swabs (MWE) and placed in 500 µL DMEM. Pieces of tissue (lung, heart, spleen, liver) were removed and placed into 1 mL RNALater for future viral RNA detection. For urine collection, animals were housed singly overnight in sterile foil lined cages with grated bottoms to allow for excreta to fall through. Any urine and feces were collected the following day. For feces, collection was performed as for urine stated above, or during euthanasia, fresh feces was removed directly from the rectum. Feces was stored in RNALater for future detection of viral RNA.

2.6 Detection and Quantitation of Viral RNA

For tissue, excreta, swab, and blood RNA, samples were removed from CL-4 as per the CL-4 standard operating procedure (SOP). Tissues and feces were removed from RNALater at least 24 hours following collection and homogenized in 600 µL RLT lysis buffer (Qiagen, Germany). Homogenates were clarified by centrifugation at 1500 x *g* for 10 minutes, diluted to 30 mg equivalents in RLT lysis buffer, and mixed with 600 µL of 70% ethanol. RNA was extracted using an RNeasy mini kit (Qiagen) according the manufacturer's instructions. For blood, swabs, and urine, 140 µL of sample was mixed with buffer AVL for 10 minutes before being briefly spun and mixed with 95% ethanol for inactivation. RNA was extracted using viral RNA mini kits (Qiagen) according to manufacturer's instructions. RT-qPCR for detection of SNV or ANDV S segment genes was performed on a QuantStudio 3 instrument (Applied Biosystems) in triplicate with a one-step protocol using a Quantitect Probe RT-PCR kit (Qiagen) according to manufacturer's instructions. RT-PCR was carried out in 3 stages: reverse transcription (50 °C for 30 min), Taq activation (95 °C for 15 min), and amplification (40 cycles of 94 °C for 15 s and 60 °C for 60 s). Data acquisition occurred at the end of the annealing/extension stage (60 °C for 60 s) of each amplification cycle. All primer and probe concentrations were 200nM. A standard curve

of *in vitro* transcribed RNA ranging from 5 x 10^7 to 5 copies per mL or mg of tissue was used to calculate genome copy numbers via interpolation. A Ct value of 35 was used a cut-off point for identification of positive RNA samples, as this corresponded to a copy number of less than one.

2.6.1 Primers and Probe for Detection of SNV RNA

Forward – GCAGACGGGCAGCTGTG

Reverse – AGATCAGCCAGTTCCCGCT

Probe – 5’FAM-TGCATTGGAGACCAAACTCGGAGAACTC-3’IAbkFQ

2.6.2 Primers and Probe for Detection of ANDV RNA

Forward – AAGGCAGTGGAGGTGGAC

Reverse – CCCTGTTGGATCAACTGGTT

Probe – 5’FAM-ACGGGCAGCTGTGTCTACATTGGA-3’TAMRA

2.7 Transmission Experiments

2.7.1 Direct Transmission

During direct, intra-cage transmission experiments, one SNV-infected deer mouse was housed with 2-4 naïve, uninfected sex-matched deer mice for 6 weeks. Two-three weeks following the onset of each experiment, infected seeder mice were bled retro-orbitally using heparinized capillary tubes to test for SNV RNA via RT-qPCR to confirm infection. At six weeks post-infection, all animals were euthanized and whole blood, serum, and lungs were collected to determine the presence or absence of SNV and/or seroconversion. To assess the effect of heat

shock responses on SNV transmission, identical experiments were carried out except that each SNV-infected deer mouse was given paeoniflorin (50 mg/kg) via oral gavage every 2 days for 3 weeks following infection. To assess the effect of different levels of testosterone on SNV transmission, again identical experiments were carried out, except infected seeder deer mice were first castrated and then implanted with an osmotic pump delivering either testosterone enanthate (200 µg/day for 28 days) or vehicle as negative control. To determine the protective efficacy of oral vaccination against SNV, identical transmission experiments were carried out with the exception that all uninfected deer mice were vaccinated against SNV no less than 28 days before the onset of exposure to infected animals.

2.7.2 Indirect Transmission

For indirect transmission experiments, infected deer mice were housed independently for 3 weeks. Contaminated, dirty cages housing the deer mice were not changed during the second and third week of infection. Three weeks post-infection, infected deer mice were removed from the cages and three naïve, uninfected deer mice were housed in the cages for 1 week. Following one week of exposure to the dirty caging, the naïve deer mice were separated and housed independently to avoid any potential intra-cage transmission between these animals. Three weeks post-exposure, all animals were euthanized and blood, serum, and lung samples taken for detection of SNV and/seroconversion as above.

2.8 Determination of Seroconversion by ELISA

For non-vaccination experiments, Black Creek Canal virus (BCCV) cellular lysate was used as antigen for ELISA detection of anti-SNV antibodies confirming seroconversion as antibodies specific for SNV cross-react with BCCV. For seroconversion confirmation from

vaccination experiments, purified SNV nucleocapsid protein was used as antigen. For each, 96-well, half-area, high-binding polystyrene plates (Corning) were incubated with either 50 ng per well of BCCV infected cell lysate or 30 ng per well of purified SNV N protein in PBS and incubated overnight at 4°C. The next day, plates were washed three times with PBS-T (PBS + 0.1% Tween 20) and blocked with 5% skim milk PBS-T for one hour at 37°C. Following the blocking step, plates were washed again three times with PBS-T. Deer mouse serum samples were diluted 1:100 in PBS + 5% skim milk and were added to the plate in triplicate overnight at 4°C. The following day, plates were washed three times with PBS-T and secondary HRP-conjugated anti-Peromyscus leucopus antibody (KPL; 1:1000) was added to the plates for 1 h at 37 °C. Plates were then washed again three times with PBS-T and one-step ABTS substrate (Thermofisher) added for 30 minutes at room temperature for development. OD values were then read at 405nm. Positive samples were those that had an OD greater than the mean OD plus 3 standard deviations seen in the negative control wells.

2.9 Induction of Heat Shock Responses

Deer mice were given either 500 mg/kg geranylgeranylacetone (GGA) or 50 mg/kg paeoniflorin (PFL) in 100 µL via oral gavage using plastic feeding needles. Eight, 12, or 24 hours following oral gavage, the deer mice were euthanized and their blood and tissues were collected in RNALater for determination of *hsp70* mRNA expression. For longer term assessment of *hsp70* mRNA expression, deer mice were given PFL via oral gavage daily for one week. Groups of deer mice were euthanized four, eight, and 12 days following the onset of treatment for tissue collection.

2.10 Detection of Heat Shock Protein mRNA Expression

Tissues were removed from RNAlater, and RNA extracted using an RNeasy plus mini kit (Qiagen) following the manufacturer's instructions. Two-step RT-qPCR was performed in triplicate on a StepOne Plus instrument (Applied Biosystems). For two-step RT-qPCR, 100 ng of tissue or blood extracted RNA was reverse transcribed using a superscript III RT first strand synthesis kit (Invitrogen) followed by qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems). For reverse transcription, 100 ng of template RNA was mixed with random hexamers as primers in a 20 µL reaction for five min at 65 °C followed by 10 min at 25 °C and 50 min at 50 °C. For subsequent qPCR, two µL of cDNA was used in 20 µL reactions. The cycle parameters for qPCR using the SYBR Green Master Mix were 2 min at 50 °C and 2 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Oligonucleotide concentrations were 2 µM. Fold change in gene expression for *hsp70* was calculated by the ΔΔCt method using *GAPDH* as a reference gene.

2.10.1 Primers for *hsp70* mRNA Expression

HSP70 Forward – GCGGGTGGCGTGATGA

HSP70 Reverse – GAAGATCTGCGTCTGCTTGGT

GAPDH Forward – TCCGTCGTGGATCTGACATG

GAPDH Reverse – ACGCCTGCTTCACCACCTT

2.11 Induction of Thermogenesis

The administration of CL316,243 to deer mice for the induction of thermogenesis was done through osmotic pumps (model 1004, Alzet) which were filled with either PBS or CL316,243 and implanted into deer mice subcutaneously as per manufacturer's instructions. Briefly, deer mice

were anesthetized and the right side of their abdomen shaved. A small incision in the flank of the body of each mouse was made and pumps were implanted between the skin and peritoneum. Incisions were then held together with surgical staples that were removed 7-8 days following pump implantation. Animals were administered meloxicam (2 mg/kg subcutaneous) following pump implantation and monitored daily for any adverse complications due to implantation or to determine if the staples became removed.

2.12 Castration of Male Deer Mice

For the castration of male deer mice to eliminate exogenous testosterone, a protocol by Valkenburg et al (2016) was modified as per recommendations by our veterinary staff. First, male deer mice were anaesthetized via inhalation isoflurane and then shaved and cleaned using chlorhexidine and 70% ethanol. The mice were maintained under isoflurane anaesthetization and secured to a heating pad for the duration of the surgical procedure. Before surgery, mice were given a 2mg/kg subcutaneous dose of Meloxicam as an analgesic. With a sterile scalpel, a small (less than 1 cm) incision was made to the skin and peritoneum adjacent to the rectum. Using sterile forceps the peritoneum was opened and the testicular fat pad and testes were pulled out of the peritoneal opening with a second pair of sterile forceps. The fat pad, testes, and epididymis were clamped off using hemostats, and the testes removed. The remaining vasculature was allowed to clot and then placed back into the opening in the peritoneum and skin. Incisions were glued shut using veterinary glue. The same procedure was then repeated on the other testes and deer mice were recovered in their own cages until fully alert. The animals were monitored daily for any signs of stress or opening of surgical incisions. Animals were also administered meloxicam (2 mg/kg subcutaneous) daily for 3 days following the surgical procedure as an analgesic.

2.13 Testosterone ELISA

Levels of serum testosterone were quantified using a commercial testosterone ELISA kit (ENZO Life Sciences) according to the manufacturer's instructions. Briefly, deer mouse serum was collected and diluted 1:40 in sample assay buffer and incubated with anti-testosterone antibody for 1 hour at room temperature (RT) in 96 well assay plates. Wells were then incubated with an alkaline phosphatase-testosterone conjugate for 1 hour at RT before washing and adding a pNpp substrate for an additional hour. A stop solution was added and absorbance readings were taken at 405nm. Absorbance levels were inversely proportional to the testosterone concentration in each well. Serum testosterone levels were determined using a standard curve of known testosterone concentration.

2.14 Implantation of Osmotic Pumps

The administration of exogenous testosterone to castrated male deer mice was done through osmotic pumps as above for CL316,243 and described in section 2.11 (model 1004, Alzet). Pumps were filled with either propylene glycol or testosterone enanthate and implanted into deer mice subcutaneously as per manufacturer's instructions. Briefly, castrated male deer mice were anesthetized and the right side of their abdomen shaved. A small incision in the flank of the body of each mouse was made and pumps were implanted between the skin and peritoneum. Incisions were then held together with surgical staples that were removed 7-8 days following pump implantation. Animals were administered meloxicam (2 mg/kg subcutaneous) following pump implantation and monitored daily for any adverse complications due to implantation or to determine if the staples became removed. All experiments using testosterone enanthate, a

controlled substance in Canada, were conducted with approval and with a controlled substance exemption by the Public Health Agency of Canada.

2.15 Characterization of Recombinant Adenoviruses

For determination of SNV glycoprotein expression by recombinant Adenoviruses, Ad-Gn and Ad-Gc we used to infect 70% confluent monolayers of HEK293T cells at a multiplicity of infection (MOI) of 1. Following 48 hours, cells were washed once with PBS and harvested in 1x RIPA buffer. Cells were then placed on ice for 30 minutes with periodic agitation to lyse the cells. The cells were then spun at 13,000 x g for 10 minutes. Supernatants were collected and protein concentrations were determined. 15 µg of protein from cell lysates was prepared for loading onto SDS-PAGE gels. 15 µg of protein was diluted 1:1 with tricine/SDS buffer and heated to 95°C for 10 minutes to denature the proteins. Samples were then loaded into pre-cast 4-12% Bis-Tris protein gels. Gels were run for 40 minutes at 140V. Proteins were dry-transferred to nitrocellulose membranes using an iBlot 2 as per manufacturer's instructions. Membranes were then dried and blocked in Odyssey blocking buffer for one hour at RT. Membranes were washed, incubated in primary antibody (convalescent hamster serum) against SNV/ANDV glycoprotein diluted 1:1000 in blocking buffer (1:1 PBS-t:Odyssey blocking buffer) overnight at 4°C. The next day, membranes were washed four times in PBS-T for five minutes each. Membranes were then incubated in secondary peroxidase labelled anti-hamster IgG antibody diluted 1:1000 in blocking buffer for one hour at RT. For development, membranes were once again washed four times in PBS-T and then incubated in DAB substrate for 10 minutes until color development.

2.16 Vaccination of Animals

2.16.1 Deer Mice

Deer mice were first anaesthetized with inhalational isoflurane and either vaccinated IM or via oral gavage using plastic feeding tubes (20ga x 30mm, Instech laboratories). For vaccination with recombinant Adenoviruses, deer mice were vaccinated with 10^8 PFU in 100 µL PBS. rVSVΔG/SNVGPC was given to deer mice at a dose of 2 x 10^4 PFU in plain DMEM.

2.16.2 Hamsters

Syrian golden hamsters were anaesthetized with inhalational isoflurane and were given an IP injection of 10^5 PFU of either rVSVΔG/SNVGPC, rVSVΔG/ANDVGPC, or rVSVΔG/LASVGPC as a negative control. Hamsters were monitored for 28 days and then challenged inside CL-4 with either ANDV or HA-SNV.

2.17 Determination of Anti-SNV or Anti-ANDV IgG Responses by ELISA

Detection of anti-SNV and anti-ANDV antibody responses was done by ELISA. Following immunizations, 96 well half-area plates (Corning) were coated with 500 ng/well SNV or ANDV virus-like particles overnight at 4°C in the hamster immunization experiments. For deer mouse vaccination experiments, plates were coated with 500 ng/well of purified, concentrated rVSVΔG/SNVGPC particles. The following day, plates were washed three times with PBS-T and coated for 1 hour with 5% skim milk + 0.01% tween 20. Following blocking, plates were washed three times with PBS-T and serum diluted in blocking buffer was added to plates in triplicate and incubated at 4°C overnight. The next day, the plates were washed three times with PBS-T and secondary peroxidase labelled anti-hamster IgG was added to the plates (1:1000) for 1 hour at 37°C. Following three washes with PBS-T, 75 µL/well of one-step ABTS substrate (Thermofisher) was added to the plates for 30 minutes at room temperature. Plates were then read at 405nm and analyzed using SoftMax Pro software.

2.18 Determination of Neutralizing Antibody Responses

rVSVΔG/SNVGPC-GFP and rVSVΔG/ANDVGPC-GFP we incubated with dilutions of serum for one hour at room temperature (RT) and then the viruses were used to infect monolayers of VeroE6 cells in 96 well plates. Cells were scored for infection at 14 hours post-infection via GFP expression levels using an automated counter, CellInsight fluorescence microscope. A percentage of relative infection was determined compared with the infection with the same virus in the absence of serum. Neutralizing titer 80, or NT_{80} was determined by the Reed-Meunch method as the titer of serum that was able to provide an 80% or 50% reduction in relative infection. Alternatively, the number of cells expressing GFP were enumerated manually under fluorescence microscopy. Neutralizing antibody titers were calculated as the dilution of serum which was able to reduce the number of cells expressing GFP by a given percentage as compared to virus incubated with negative control serum.

2.19 Assessment of Protective Efficacy of SNV Vaccines

2.19.1 SNV Challenge

Following vaccination, deer mice were challenged with SNV 28 days post-vaccination via IM route. Animals were euthanized on day 14 post-infection during the peak of acute infection to test for the presence of SNV RNA in the blood and tissues.

2.19.2 SNV Transmission Model

For testing whether vaccination prevented acquisition of SNV during transmission experiments, deer mice were vaccinated and then 28 days post-vaccination exposed to infected deer mice for 6 weeks. Following six weeks of intra-cage exposure to infected deer mice,

vaccinated animals were euthanized and tested for either SNV anti-nucleocapsid antibodies or SNV RNA in the lungs.

2.19.3 Lethal ANDV Challenge

Twenty-eight days following vaccination, hamsters were challenged IP with 200 focus-forming units (FFU) of ANDV as described above. Animals were monitored for clinical signs of disease including hunched posture, labored breathing, and lethargy daily according to an approved scoring sheet. Animals requiring euthanasia due to clinical score or from a pre-determined experimental time point were exsanguinated via cardiac puncture after induction of deep anesthesia.

2.20 Statistical Analysis

Statistical analysis was performed using Prism 5 software (GraphPad). Statistical significance between groups was determined using Mann-Whitney test, one-way analysis of variance (ANOVA), two-way ANOVA, Kaplan-Meier analysis with log-rank test, or Fisher's exact test where appropriate. For analyses that yielded statistically significant differences between groups, i.e. $p<0.05$, p values or * have been included within figures.

Chapter 3 - Refinement of SNV Infections in Deer Mice

3.1 Introduction

Studying SNV infection and pathogenesis can be difficult due to a lack of suitable small animal models mimicking human disease, and the need to passage the virus in its rodent host to maintain virulence (Safronetz et al. 2014; Warner et al. 2019a). Currently the only lethal animal models of SNV infection include an immunocompromised Syrian hamster model and a non-human primate model requiring SNV obtained directly from infected deer mouse tissue homogenates (Safronetz et al. 2014; Brocato et al. 2014). Additionally, the need for viruses taken directly from infected rodent hosts appears to be a common trend for at least two different hantaviruses, as cell culture adaptation of Puumala virus (PUUV) has been shown to result in mutations that alter its ability to infect its rodent host (Lundkvist et al. 1997; Nemirov et al. 2003). Passaging SNV in vitro also results in mutations in the RNA dependent RNA polymerase leading to a loss of virulence and infectivity of deer mice (Safronetz et al. 2014; Warner et al. 2019a).

SNV infections have classically been done IM, as this route is thought to most closely mimic natural infection (Botten et al. 2000; Bagamian et al. 2012). Additionally, because SNV is thought to be transmitted primarily by male mice, these mice have been used exclusively for virus propagation. Initial experimental infections of deer mice with SNV used the 77734 strain passaged in juvenile deer mice, therefore young male deer mice have since been used for virus passaging. The ability of SNV to replicate to high titers following other routes of infection or in the tissues of female and/or older deer mice has not been determined. Because propagation of viruses within specific rodent hosts rather than in cell culture requires a consistent and reliable protocol to generate high titer virus while using as few animals as possible, we sought to determine whether IM infection results in higher levels of SNV titers than IP infection in experimentally infected deer

mice. Additionally we also sought to identify any age and/or sex related differences that may have a significant effect on viral replication and subsequent SNV production. These experiments had important implications for all subsequent experimental SNV infections in deer mice. The requirement for a certain age or sex would severly limit the number of mice available to us at any given time, therefore determining our ability to use a wider range of host animals was an important consideration.

3.2 Results

3.2.1 IP infection route results in equivalent or higher SNV levels as IM infection

Before beginning experiments for optimization of route of infection, we first confirmed that a VeroE6-adapted SNV (77734) is unable to cause productive infection in deer mice, requiring the need for SNV stocks produced in deer mice. As can be seen in Figure 3, our VeroE6 cell culture adapted SNV was not able to infect deer mice, with no viral RNA detectable in the blood or tissues of infected animals during what is normally the acute stage of SNV infection, day 10. Next, since IM has been the route in infection chosen for most experimental SNV infections in deer mice, including for passaging of SNV *in vivo,* we wanted to determine whether this route was the most effective for generation of high titer SNV stocks (Botten et al. 2000; Botten et al. 2002). To determine whether IM infection results in higher levels of SNV than IP infection, groups of deer mice (IM: n = 10, IP: n = 12) were infected with SNV. Infected deer mice were euthanized on day 10 post-infection, which is during the acute phase of infection and was previously determined to be an optimal time for tissue collection and virus preparation (Botten et al. 2000; Botten et al. 2003). Pooled lung homogenate from IM infected animals had a viral copy number that trended lower than that seen in homogenates produced following IP infection (Figure 4A). While the homogenates produced from three groups of IP infected deer mice tended to be higher than that

from IM infected mice, viral RNA levels were not significantly higher, as IP viral copy numbers were only compared to one stock of virus produced from IM infection preventing appropriate statistical analysis by t test. Those infected IM were less likely to have detectable viral RNA in their blood compared with IP infected mice, with only 50% of IM infected mice having detectable SNV RNA in their blood versus 100% of IP infected mice at day 10 (Figure 4B; $p = 0.0107$). Other than in the blood, no tissues or swabs had significantly different levels of SNV RNA between IM and IP infected groups. When we examined viral RNA levels in the lungs, heart, spleen, or swabs of infected deer mice, there were no differences seen between infection routes (Figure 4B). The IM group appeared to have lower levels of RNA in the lungs, although this was not statistically significant (Figure 4B). Therefore IP infection appears to provide a more reliable method of productive infection as assessed by the presence of viral RNA in the blood, but overall levels of viral RNA in the tissues did not differ.

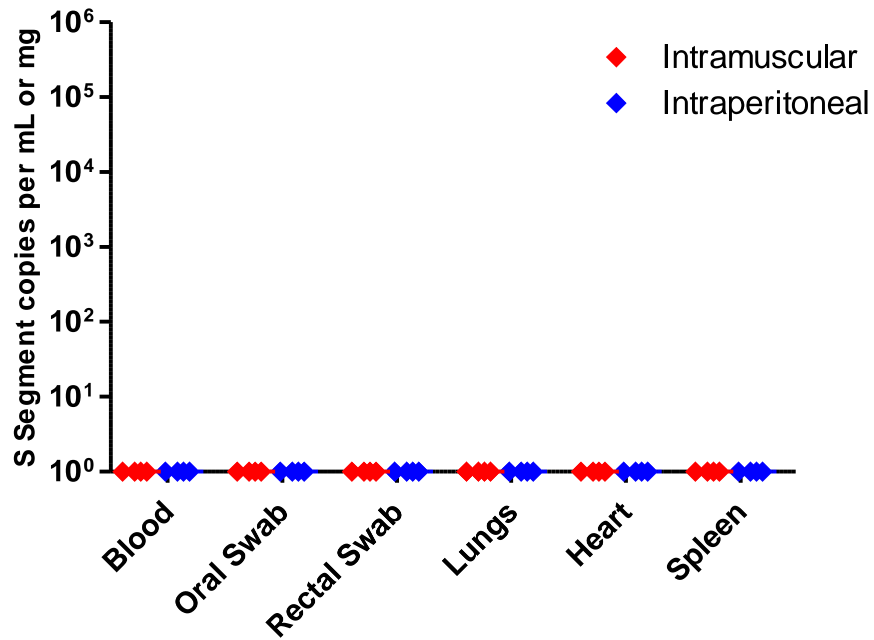

Figure 3. Infection of Deer Mice with VeroE6-adapted SNV. Groups of 1-2 month old deer mice were inocoluated with a VeroE6-adapted SNV and viral copy numbers in the listed tissues were assessed at 10 dpi. n=4. Figure adapted from Warner et al. 2019a.

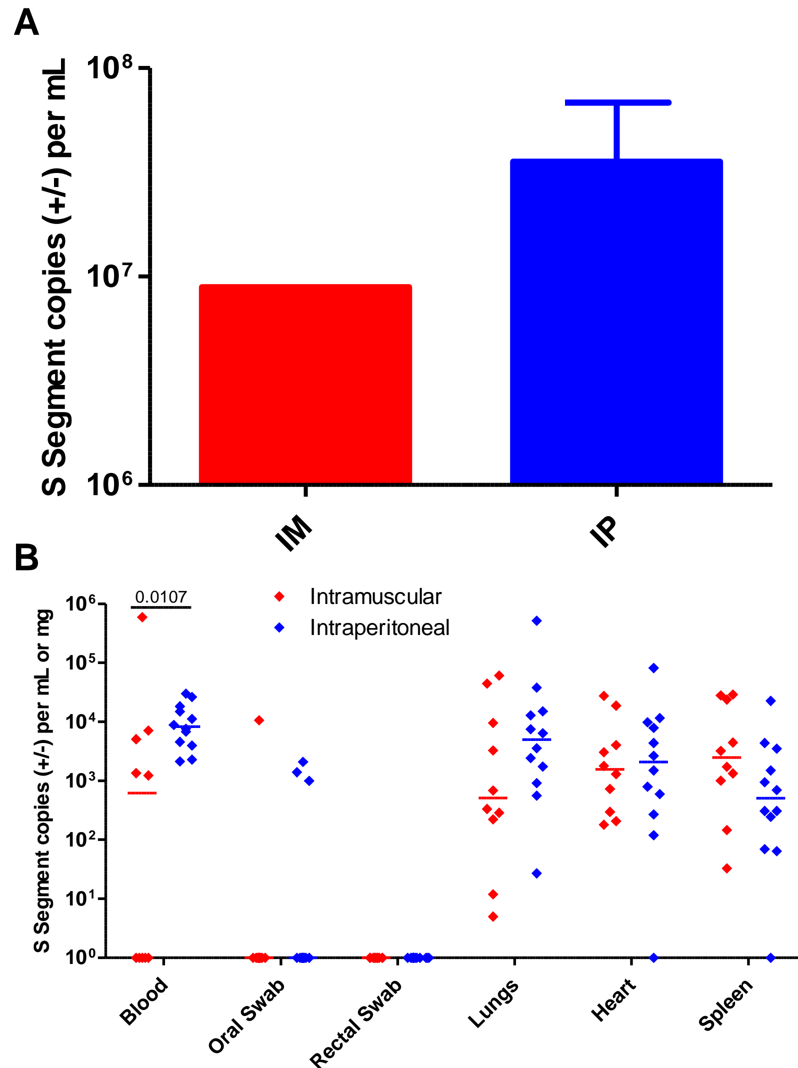

Figure 4. Production of SNV Stocks *in vivo* by IM and IP infection. Groups of 10 (IM) or 12 (IP) deer mice were infected with SNV and S segment copies were detected in (A) pooled lung homogenates from each group for producing viral stocks or (B) each individual mouse to determine amounts of viral replication and shedding 10 dpi. For (A) 1 IM group was tested and 3 IP groups were tested. Data shown are the mean + SEM (A) and medians (B) for each group. Numbers indicate the p value as assessed by Mann-Whitney test. Figure adapted from Warner et al. 2019a.

3.2.2 Effect of Age and Sex on SNV Infection in Deer Mice

We sought to determine whether the age and sex of infected deer mice has an influence in the kinetics of SNV infection, and whether these factors might impact the SNV levels of stocks produced *in vivo.* We infected groups of juvenile (1-2 months old maximum) and adult (5-6 months old minimum) deer mice with SNV via the IP route. There were no differences in SNV RNA levels in the blood and spleen of male and female deer mice, but interestingly, females had higher levels of SNV RNA in the lungs and heart compared to males (Figure 5A). Median viral RNA copy number in the lungs and heart of female deer mice were 10^4 and 1.3 x 10^3 per mg of tissues compared with 1.3 x 10^3 and 10^2 per mg for males, respectively (p values = 0.0043 for lungs and 0.0194 for heart, Figure 5A). Therefore there may be slightly higher levels of SNV RNA seen in the lungs and heart, the primary tissues targeted by the virus in female deer mice compared with males.

These differences in viral RNA in the lungs and heart between sexes appears to be due to differences in younger mice. Younger females had significantly higher SNV RNA levels than did younger males in the lungs and heart, but there were no differences seen in the tissues of older males and females. A comparison of younger and older deer mice regardless of sex showed significantly higher SNV copy numbers in the blood and hearts of younger mice (p values = 0.0194 for blood and 0.0120 for heart, respectively) (Figure 5B). When controlling for age and sex of infected deer mice however, we found no significant differences in SNV copy number in the blood, tissues or swabs tested. In terms of viral shedding, which may play a role in future experiments, we were only able to detect SNV RNA in oral swabs from 3 male deer mice, and in no rectal swabs tested (Figure 5). These data indicated there may be subtle differences in viral levels between groups based on the age and sex of deer mice infected with SNV.

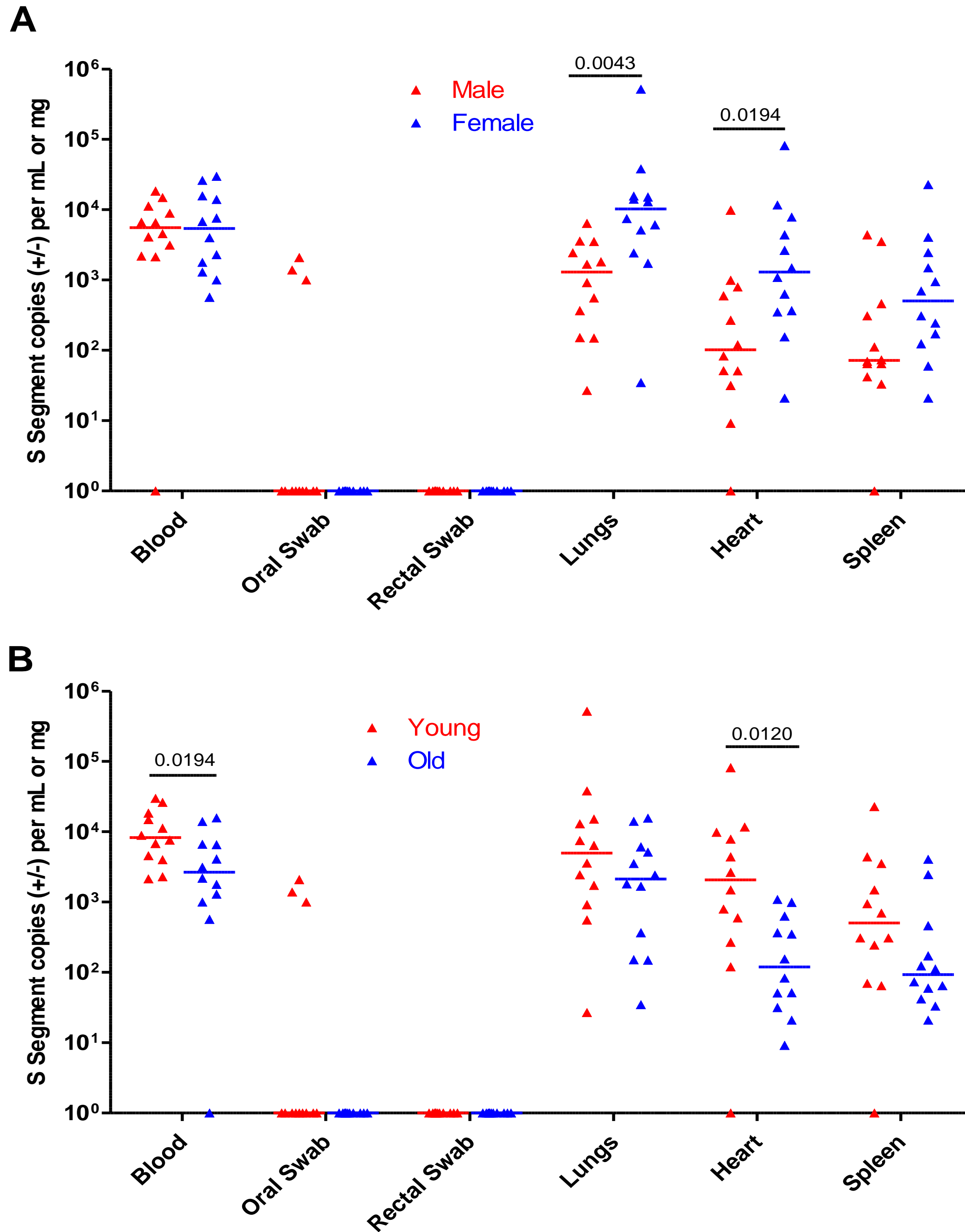

Figure 5. SNV replication and shedding in male and female deer mice of different ages. Comparison of replication and shedding seen in (A) male vs female deer mice 10 days postinfection or (B) 1-2 month old (young) vs 5-6 month old (old) deer mice 10 days post-infection. Data shown are medians. Numbers indicate the p value as assessed by Mann-Whitney test. Figure adapted from Warner et al. 2019a.

3.3 Discussion

Infection of juvenile deer mice with SNV via the IM route has been performed for passaging of infectious virus stocks since the first description of experimental SNV infections in deer mice (Botten et al. 2000). While there has been anecdotal evidence that once SNV becomes adapted to cell culture it is no longer capable of productively infecting deer mice, no data had previously been published confirming this, although VeroE6 passaged SNV does not cause disease in Rhesus macaques (Safronetz et al. 2014). Here we have shown that a VeroE6 passaged strain of SNV (77734) can no longer productively infect deer mice, highlighting the need for optimized protocols for producing viral stocks through *in vivo* passaging.

There are certain characteristics of some hantaviruses such as SNV and PUUV, which make them difficult to study experimentally including the need for passage within its natural rodent host. Also, the nature of the evolutionary relationship between each virus and its host means that each particular host species is needed for laboratory-based ecological studies, and maximizing the use of reproducible infection models is needed. On the issue of developing a more reliable method of producing viral stocks in vivo, our goal was to refine SNV infections to maximize SNV collection for viral stocks made from tissues of infected deer mice. We showed that IP infection produces viral stocks of roughly equivalent RNA copy number and appears to be a more reliable infection route compared with IM. Overall, it seems as though IP infection results in SNV titers at least as high as those seen after IM infection, with more detectable and consistent viremia. Therefore it is likely a more practical and reliable method of infection for production of SNV stocks considering the technical aspects of performing IP and IM infections within CL-4. IM infections involve shaving of mice and infection of musculature that is smaller and less accessible,

potentially leading to injections that do not end up in the muscle. IP are generally considered to be easier to perform under the constraints of sharps work inside CL-4.

While there were subtle differences in the levels of SNV RNA in the blood and tissues between certain groups of mice, these differences could be accounted for when controlling for the sex or age of the mice. These differences will likely have little effect on the final titer of viral stocks produced from these mice or on the outcome of future experimental infections assessing SNV infection outcome in deer mice. Overall, similar levels of SNV replication and production are seen regardless of the age and sex of infected deer mice although we showed statistically significantly higher levels of viral RNA in the lungs and heart of female deer mice and in the blood and heart of younger deer mice. Because we do not see these differences when controlling for age and sex, we believe that any difference seen will have negligible effects in future experiments and in producing viral stocks from different ages and both sexes of deer mice. We have further provided evidence that stocks of high viral RNA copy number can be achieved through infection of either sex and at different ages. This has implications not only for our SNV infection model and virus preparation, but also for other hantaviruses that need to be passaged in their natural rodent host to maintain virulence. In addition, showing that SNV infection of both sexes of deer mice at different ages does not result in significantly different SNV levels during acute infection, we were able to expand our use of animals we are able to use in future infection and transmission studies to include all those that are available to us at a given time.

Chapter 4 - Development of a Sin Nombre virus Transmission Model

4.1 – Introduction

Through field studies, a lot of hypotheses and data have been gathered regarding the transmission of SNV between deer mice, including that transmission is driven by infected males and is mediated through direct contact. However, there have been few studies examining the dynamics of SNV transmission within deer mouse populations experimentally, using controlled, defined conditions. This is partly due to the requirement of biosafety level 4 facilities to work with hantaviruses *in vivo* and the need for a source of deer mice such as a breeding colony. Previous reports on SNV transmission in deer mice utilized outdoor enclosures and either colony bred or wild caught deer mice (Botten et al. 2002; Bagamian et al. 2012). While these experiments most likely reflect what is occurring in a natural setting, it is difficult to control and determine what mechanism(s) of transmission might be the primary mode of transmission. Interestingly, in the first attempt to show SNV transmission, following up to 60 days of exposure to SNV infected deer mice, no uninfected mice except for one, became infected with SNV (Botten et al. 2002). Following exposed mice long term, no mice became infected between days 60-213 of exposure to an infected deer mouse. The lack of transmission events seen could have been due to several factors including little to no shedding of virus in urine, feces, or saliva, which was shown to be minimal in SNV infected deer mice. Additionally, there is very little data on the stability of SNV in the environment, therefore even virus that is shed by infected mice may not remain infectious for periods long enough to lead to exposure resulting in infection. The mice in that study also did not show significant wounding, which has been hypothesized and shown to lead to hantavirus transmission in the case of SEOV (Hinson et al. 2004). Thus the temperament of the mice in those experiments carried out may not have resulted in the best chance to see transmission events.

Bagamian et al (2014) reported on successful transmission events using wild caught deer mice housed within outdoor enclosures. Using already infected mice caught in the wild resulted in the use of animals in various stages of infection, with highly variable immune responses against SNV, and highly variable levels of SNV RNA in their blood. Those that were able to transmit SNV more readily typically had higher viral loads, and deer mice which became infected throughout these experiments gained a significant number of wounds, potentially revealing an important mechanism of transmission of SNV. While this report provided the first instance of SNV transmission in an experimental setting, many questions remain regarding how SNV is primarily transmitted among deer mice.

We sought to determine whether the main mechanism of transmission of SNV is through direct contact between deer mice as we hypothesized, or if indirect transmission, through contact with virus found in excreta or secreta occurs, as it does in most human infections. Because we have access to laboratory bred deer mice and a reproducible and characterized SNV infection model we are in a position to best study the ecology of SNV in an experimental setting. In addition to studying how SNV is transmitted between deer mice, we also wanted to develop an experimental transmission model system to better study SNV infections and transmission moving forward, including determing what might drive SNV transmission in persistently infected host animals and testing preventative measures such as vaccines using this model.

4.2 – Results

4.2.1 – Direct, Intra-cage Transmission

Firstly, we wanted to determine if and how readily, SNV will be transmitted between deer mice in an experimental setting within our caging set up within the level 4 lab. Based on field

seropositivity data and previous experimental attempts, we expected a relatively low transmission rate even given the housing conditions inside our high containment cages. For our experimental system, we housed 3 naïve, uninfected deer mice with one infected "seeder" deer mouse for six weeks, changing the cages weekly. To provide the greatest chance at seeing transmission events, we exposed deer mice directly following infection of the seeder mouse to allow for exposure throughout the acute stage of infection, approximately between 10-21 dpi. For our initial direct, intracage transmission attempts, 33 uninfected deer mice were exposed to 11 SNV infected deer mice. Following six weeks of exposure, eight deer mice became infected with SNV, as determined by either the presence of SNV RNA in the blood or lungs, or the presence of antibodies against SNV detected by ELISA, or 24% of exposed mice (Table 2). This transmission rate was similar to the highest rate seen in the outdoor enclosure studies done by Bagamian and colleagues, providing evidence that this system may result in a significantly higher number of transmission events, providing us with a valuable tool for studying SNV transmission. We were able to demonstrate a model that can produce a high rate of experimental SNV transmission that has not been achieved previously in a controlled laboratory setting. It is also possible that there was an underestimation of the number of transmission events as deer mice that were exposed to SNV may have been euthanized before seroconverting or developing a productive infection. This newly developed model gave us the foundation upon which to perform all of our subsequent studies of SNV transmission.

4.2.2 – Indirect Transmission

Once we were able to demonstrate transmission between co-housed deer mice, we wanted to determine whether indirect transmission through exposure of deer mice to virus found in the environment is a signifincant cause of SNV infection of deer mice. To allow ourselves the best

chance of detecting indirect transmission events, we designed our initial experiment such that uninfected deer mice would be exposed to contamined cages following their housing of infected deer mice, which would presumably be shedding SNV during the peak of acute infection. In our experimental SNV infection model, following IM infection, deer mice have peak viral copy numbers in the blood and tissues around day 14, following by a gradual drop off through until day 28. Therefore we used this as a guideline for our housing of infected deer mice before exposure of uninfected mice. We housed infected deer mice for 21 days and did not change the soiled cages between 7-21 dpi. On day 21, SNV infected deer mice were removed from the cages and three uninfected deer mice were placed in each soiled cage for one week. Following one week of exposure these deer mice were separated to prevent any potential intracage, direct contact transmission. Three weeks following exposure, deer mice were then euthanized and the presence of either viral RNA in the blood or lungs, or anti-SNV antibodies was determined. Following housing in the contaminated cages and exposure to contaminated bedding, none of the 30 deer mice across 10 cages became infected with SNV (Table 2). While we did not see any transmission events occurring through this mechanism, it is difficult to determine whether this type of exposure could result in transmission. Given our attempt at optimizing the timing of exposure and the contained nature of the experimental setting, our results suggest that indirect transmission in this manner is unlikely to be a major contributor to SNV transmission between deer mice.

Table 2. Direct and indirect transmission of SNV in deer mice

Group	Exposed, Naïve Mice	Transmission Events*	% of Naïve Infected	Risk Ratio (95% CI)	P Value (Fisher's exact test)
Direct Transmission	33	8	24	1.320 (1.088 to 1.601)	0.0051
Indirect Transmission	30	0	0		

*** Seropositive or qRT-PCR positive**

Table adapted from Warner et al. 2019a

4.3 – Discussion

Since there have been very few attempts at studying SNV transmission experimentally, our goal was to determine how SNV is primarily transmitted between deer mice and to develop a reliable transmission model for future use. Following direct, intra-cage exposure to SNV infected deer mice, we saw a transmission rate of 24% after six weeks of exposure. This result was in line with the highest number of transmission events seen by Bagamian et al in their outdoor enclosure experiments. This also reflects the relatively poor transmissibility of SNV in comparison to other hantaviruses, which transmit far more readily in similar experimental settings. Alternatively, when we exposed deer mice to contaminated caging that had previously housed infected deer mice for three weeks, we did not see any transmission events. This is in contrast to other hantaviruses such as PUUV, which has been shown to be stable in the environment and transmissible after as long as two weeks outside its host (Hardestam et al. 2008; Kallio et al. 2006a). There is also evidence that BCCV, ANDV, and SEOV are shed in the urine, feces, and saliva of infected host animals, and can also be readily transmitted through an indirect, horizontal manner (Hutchinson et al. 2000; Padula et al. 2004; Kariwa et al. 1998). Experimental transmission of other hantaviruses occurs at much higher frequencies compared to what we detected in our experiments with SNV. Interestingly, indirect transmission through contact with contaminated rodent excreta and/or secreta is thought to be the main mechanism of transmission to humans. Because the acute phase of infection is when deer mice experience the highest levels of viremia, viral loads in the tissues, and levels of viral shedding, our experimental design used here, was optimized to attain the highest possible number of transmission events. Our transmission and infection data demonstrate that SNV is shed at low levels, even during the acute stage of infection, thus shedding and subsequent exposure events are likely quite rare. Another issue that has not been examined, but of future

interest is the environmental stability of SNV and other New World hantaviruses. Some data on the stability of HTNV has been collected, showing that viral particles are relatively stable at 4°C, but do not remain infectious longer than one week at room temperature or warmer, and are not resistant to drying (Hardestam et al. 2007). The ability of SNV to persist in the environment and remain infectious may provide key information about the transmissibility of the virus through this mechanism. Overall, the direct intracage, and indirect, exposure transmission data accumulated, we feel reflects the relatively rare seropositivity rate seen in wild deer mouse populations and well as epidemiological data with regard to human SNV cases in North America. We cannot rule out the possibility of indirect transmission occurring, however we feel that we have demonstrated that direct contact between deer mice is the main driver of SNV transmission. Due to the difficulties of assaying for live hantaviruses, it is difficult to determine when the highest levels of viral shedding occur. There is also some evidence that experimental infection of deer mice with SNV may result in different infection kinetics and shedding patterns than those seen in wild, infected deer mice. Therefore the optimal timing of shedding and exposure in our experimental design could differ from what has been done. An experimental system employing co-housed deer mice separated by a barrier that restricts direct contact, but allows the flow of viral particles could better shed some light on the likelihood of indirect transmission occurring. Another aspect of SNV transmission that was not examined here was wounding seen in deer mice that were co-housed. This is thought to potentially play a role in direct transmission through biting and aggressive behaviour, therefore this is one thing that may be important to monitor and test moving forward.

The second major goal of the transmission experiments was to develop a reliable system to perform future studies on the mechanisms of SNV replication, shedding, and transmission. Here we were able to show that a high enough number of transmission events can occur within a

reasonable time frame so as to perform future transmission experiments to test differences in the transmission rate between two experimental groups. The development of this system, on top of determining the mechanism of SNV transmission, laid the foundation for the rest of the experimental work outlined in this thesis. The remaining results chapters would not have been possible without first performing these initial studies.

Chapter 5 – Effects of Heat Shock, Thermogenesis, and Testosterone Levels on SNV Infection and Transmission

5.1 – Introduction

During infection of deer mice, SNV is able to cause a persistent infection, residing in several tissues for what is presumed to be the lifetime of the mouse. To maintain persistence, the virus must be able to reside within cells while avoiding causing a cytopathic effect, evading immune clearance, and replicating its genome at a low level. SNV infection does not cause any apparent morphological or pathological changes in infected cells throughout acute or persistent infection (Botten et al. 2000; Botten et al. 2003). The co-evolutionary relationship of SNV and deer mice has resulted in a situation in which lifelong infection occurs, with low levels of viral replication in the tissues of infected animals coupled with recurrent, sporadic events during which SNV is shed. Aside from potential transmission events that might occur during the acute stage of SNV infection, periodic episodes of recrudescence may play an important role in viral shedding and transmission not only to other deer mice, but to humans as well. In general, following peak viral loads during acute infection around 21 dpi, SNV infectious titers, antigen expression, and RNA levels are thought to drop continuously until low levels of SNV are detectable in the tissues of infected animals. While this appears to be the case for viral RNA, antigen expression in the tissues appears to be consistent until around three months post-infection (Botten et al. 2003). Regardless of when persistence occurs, the cyclical nature of SNV replication and shedding is likely critical for driving SNV transmission. Insights into what leads to transitions from low levels to high levels of SNV in infected deer mice could help inform preventative strategies and when periods of exposure to SNV are most likely.

Previous studies of persistent infection have identified the lungs, heart, and brown adipose tissue (BAT) as primary sites for persistent infection (Botten et al. 2003). These tissues typically harbor SNV nucleocapsid protein and genomic RNA throughout long term infection of deer mice. It is unknown how low level viral replication or harboring of genomic RNA in these tissues subsequently results in production of infectious virus and thus the dissemination of virus within the mouse and eventual viremia and shedding. Viral RNA copy numbers within the tissues of infected deer mice have been shown to outnumber infectious particles by as much as 10-1000 fold, which may indicate the presence of defective viral particles or high numbers of neutralizing antibody bound particles (Botten et al. 2003). Other negative sense RNA viruses persist in tissues in various forms including mainly as vRNA or vcRNA (Chandler et al. 1996; Marschall et al. 1996; Andreoletti et al. 2000). The replicative status of the infected cells or tissues may also play a role in the viral life cycle, and impact its replicative activity, and it has been suggested that infection of BAT by SNV during persistent infection may be influenced by the metabolism of infected cells. In overwintering bank voles, BAT is also a common site of viral persistence, and this tissue may play in important role in recrudescence seen during persistent hantavirus infections (Gavrilovskaya et al. 1983). BAT is responsible for providing heat to mammals through non-shivering thermogenesis (Matz et al. 1995). This is accomplished through expression of uncoupling protein 1 (UCP-1), which is able to uncouple cellular respiration from ATP synthesis in the mitochondria of BAT cells (Matz et al. 1995; Matz et al. 1996). Cellular heat shock proteins (HSP) are critical for shuttling UCP-1 within cells, and their expression is increased during non-shivering thermogenesis (Matz et al. 1996). There is evidence that HSPs are able to interact with several different viruses, and that the presence of different heat shock family proteins can increase viral replication, including HTNV (Santoro et al. 2009; Yu et al. 2009). Cold stress leading to

increased non-shivering thermogenesis and expression of heat shock proteins may play a role in increasing viral mRNA expression and replication leading to recrudescence. We sought to determine whether induction of either heat shock responses or thermogenesis in infected deer mice led to increases in SNV viral replication, shedding, or transmission.

While thermogenesis, heat shock responses, and/or other cellular mechanisms might play a role in regulating SNV replication, immune or behavioural mechanisms may also drive SNV replication and transmission. While immune responses are generated in deer mice against SNV during infection, including neutralizing antibodies, the virus is able to avoid immune clearance through a combination of altering the innate immune response, forming quasi-species, or inducing regulatory T cell responses that result in persistence. The influence of sex hormone levels and hantavirus infection has been studied thoroughly by Sabra Klein's group in the context of SEOV infection in rats. Much of this work has shed light on how the presence of sex hormones influence the immune response to SEOV and affects the kinetics of SEOV infection in male and female rats. For instance, elevated testosterone levels lead to an increased number of wounds and an increase in SEOV titers during infection (Easterbrook et al. 2007a). Gonadectomy in either male or female rats reduces the production of sex hormones, and has opposite effects in males and females infected with SEOV. Gonadectomized male rats see a significant reduction in SEOV levels, while an increase is seen in infected female rats (Hannah et al. 2007). Corticosteroids, which alter the immune function of treated animals, also significantly impacts SEOV infection, as male rats with low corticosterone levels following adrenalectomy had higher levels of SEOV RNA correlated with high number of regulatory T cells (Easterbrook and Klein 2008a). There is also evidence in other infectious models, for example with tick-borne parasites, that high levels of testosterone result in increased susceptibility, likely due to alterations in the immunity generated against

infection (Hughes and Randolph 2001). Sex hormones play a major role in modulating host immune responses in a variety of contexts including during viral infection, and there is interest in the how sex hormones and sex differences may drive hantavirus replication and persistence in reservoir hosts. The role of sex hormones in the context of behaviour is an interesting avenue as well that has not been given much attention, although increases in testosterone can lead to higher levels of wounding in rats which may lead to increased transmission (Easterbrook et al. 2007). Changes in sex hormone levels, due to breeding season or other physiological factors might influence viral infection and transmission through the mechanisms mentioned. We sought to determine how testosterone levels influence replication, shedding, and transmission in male deer mice infected with SNV.

5.2 – Results

5.2.1 – Induction of Heat Shock Responses

The induction of heat shock responses in animals can either be done through cold stress induction, or via chemical means. Two compounds that have been shown to induce heat shock responses are geranylgeranylacetone (GGA) and paeoniflorin (PFL) (Wang et al. 2014; Tokuriki et al. 2017; Hehir and Morrison 2016; Fan et al. 2018; Zhu et al. 2017). We tested these two compounds for their ability to induce the expression of heat shock protein 70 mRNA (Hsp70). Deer mice were given either GGA or PFL via oral gavage and the fold change in expression of Hsp70 mRNA was determined in the blood and lungs of treated mice at eight, 24, and 48 hours post-induction. Treated deer mice had increased levels of Hsp70 mRNA 24 hours following treatment with either drug, but this effect was not seen at 48 hours (Figure 6). Mean fold change in expression at 24 hours was 17 in the GGA group and 7.5 in the PFL group, however PFL induced

expression appeared to be more consistent with the GGA group having a high standard deviation which significantly brought up the mean. Therefore we chose administration of PFL as our choice for heat shock induction experiments moving forward.

PFL induction of heat shock protein mRNA expression also increased Hsp70 mRNA expression 24 hours following oral gavage in the heart and BAT, organs that harbor SNV during persistent infection (Figure 7A). We also wanted to determine whether there are any differences in PFL-induced Hsp70 expression between male and female deer mice. When we analyzed male and female Hsp70 mRNA fold changes, there were no differences in the fold change of Hsp70 mRNA expression levels indicating that sex differences would not impact subsequent SNV infection experiments (Figure 7B).

Initially, we had only given a single dose of PFL for induction of heat shock protein expression and we were planning on using it continuously, including multiple doses over multiple weeks for various experiments during SNV infection. Thus we wanted to confirm that no adverse effects would occur following administration of multiple doses given over a longer period of time. We administered PFL to 12 deer mice once daily for one week, followed by one week of monitoring to determine if continued treatment resulted in any ill effects. Over the course of treatment as well as afterwards, we saw no signs of disease or discomfort in any treated deer mice. We also monitored their weight, and no animals suffered any significant weight loss compared to controls (Figure 8). Therefore we felt comfortable that multiple doses of PFL given over several days would not have any negative impact on the experimental animals. We also wanted to test whether repeated, daily gavage with PFL would result in continued expression of Hsp70 mRNA. Daily doses of PFL appeared to promote a tolerant effect, with expression levels returning to baseline in both the lungs and blood at 4 days after the start of treatment (Figure 9). Following the

recognition of this apparent tolerance when given daily, for future experiments, we administered PFL every other day for induction of heat shock responses. Overall, our data suggested that administration of PFL induces expression of Hsp70 mRNA and a heat shock response appropriate for testing the role of such responses in SNV infection.

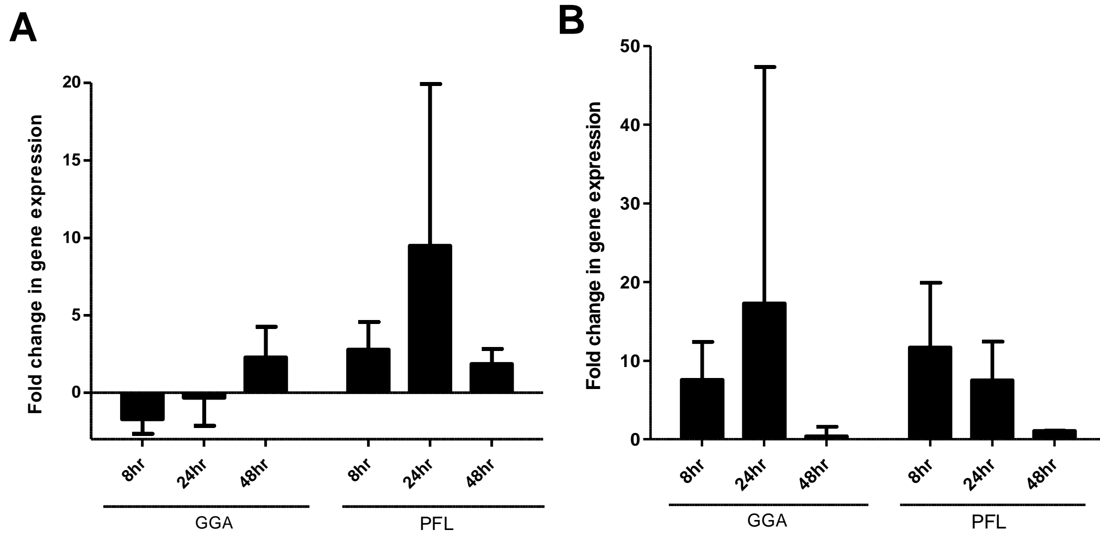

Figure 6. Induction of hsp70 expression in deer mice. Levels of hsp70 expression were assessed in either the (A) lungs, or (B) blood as compared to vehicle treated mice. n=3. Shown are data means + SD. Figure adapted from Warner et al. 2019a.

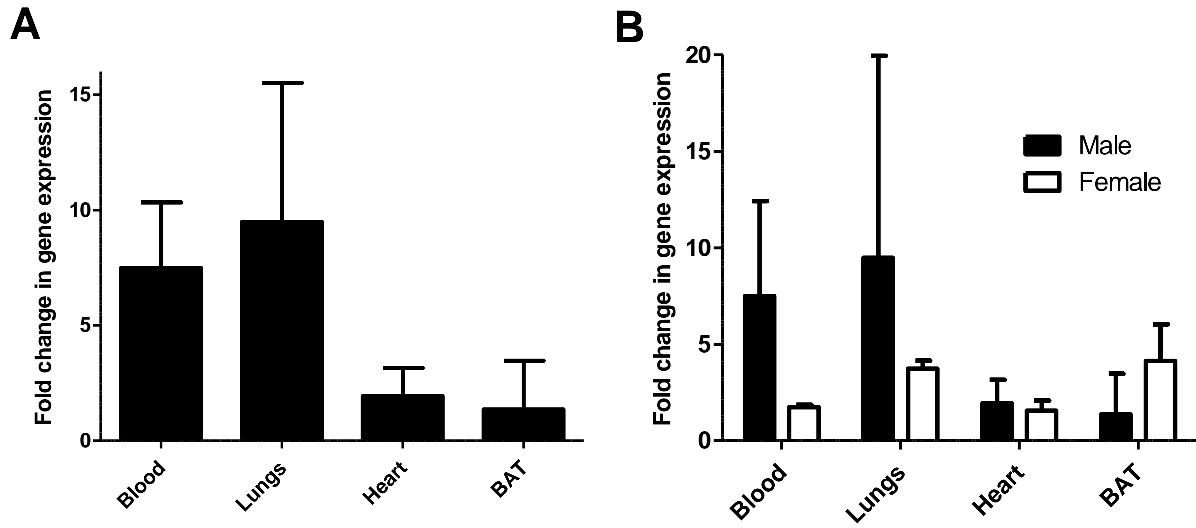

Figure 7. Induction of hsp70 expression in tissues harboring persistent SNV and in male and female deer mice. (A) Mice were given PFL and hsp70 expression was assessed 24 hours later in tissues where SNV persistence has been shown to occur. n=3 (B) hsp70 expression in the tissues of both male and female mice 24 hours following oral gavage of PFL. n=6. Shown are data means + SD. Figure adapted from Warner et al. 2019a.

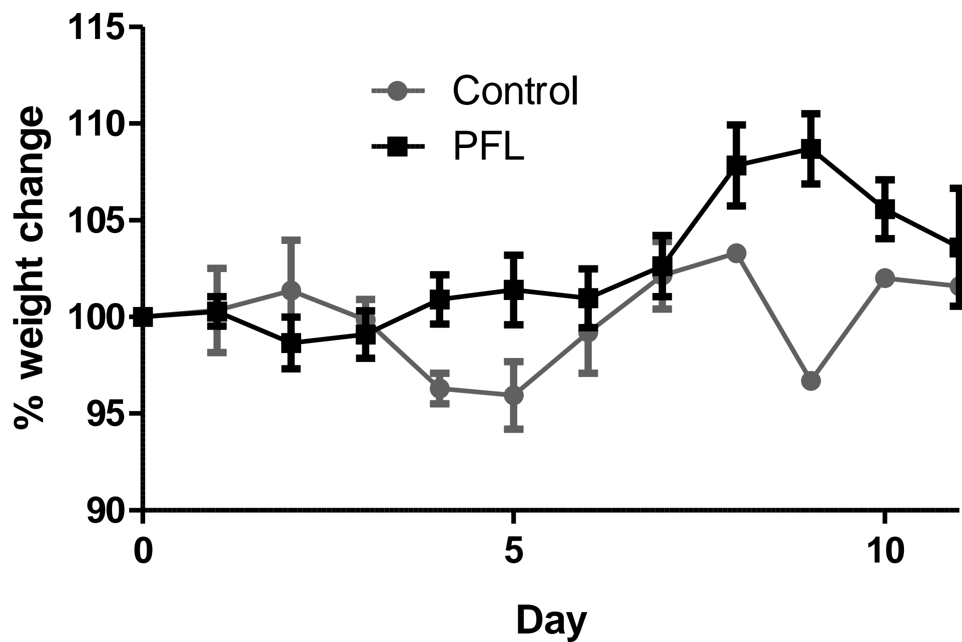

Figure 8. Weight loss during and following continued administration of PFL to deer mice. Deer mice were given daily doses of PFL for 7 days and the change in weight each day was monitored up to 5 days post-administration. n=12. Shown are data means + SD. Figure adapted from Warner et al. 2019a.

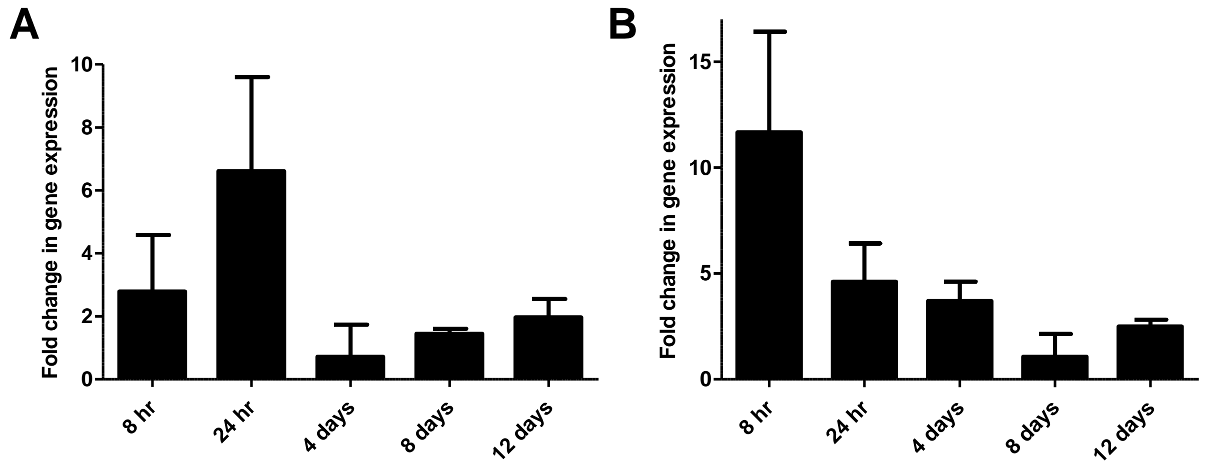

Figure 9. Long term expression levels of Hsp70 following daily doses of PFL. Mice were given PFL once daily for 7 days and hsp70 expression levels at different time points in the (A) lungs and (B) blood of deer mice were determined. n=12. Shown are data means + SD. Figure adapted from Warner et al. 2019a.

5.2.2 – Effect of Heat Shock Responses on SNV Infection and Transmission

Following confirmation that PFL treatment can induce expression of HSP70, we wanted to determine if heat shock induction had any effect on SNV replication, shedding, or transmission. We gave PFL or PBS to groups of deer mice (n=8) via oral gavage every other day for two weeks during acute SNV infection. Contrary to what we had hypothesized, treatment with PFL did not increase the amount of SNV RNA copy numbers in any of the tissues or blood (Figure 10). Interestingly, during the peak of acute infection, there was actually higher viremia seen in the control animals (p=0.0486), but there were no differences in the lung, heart, or spleen (Figure 10). During this experiment we also tested for the presence of SNV RNA in oral swabs, urine, and feces, and were only able to detect SNV RNA in three animals on day 14 post-infection. We did not detect any SNV RNA in the urine of any control animals after day seven, when RNA was detected in two mice early in infection. While there were no significant differences in viral shedding seen in our data, further experiments looking into the effects of PFL treatment or heat shock induction on shedding of SNV in excreta might yield more robust results. From our data it appears that shedding of SNV in urine, feces, and saliva during acute infection is sporadic.

While we were conducting SNV infection and shedding experiments in deer mice treated with PFL, we also examined how treatment might affect the transmission rate using our direct, intra-cage transmission model. Once again, infected deer mice were co-housed with sex-matched mice for six weeks, and then all mice were tested for either the presence of SNV RNA or seroconversion. Based on the previous experiment, when we did not see SNV RNA appear until into the second week of infection, for transmission experiments we continued PFL oral gavage treatments for an extra week, resulting in every other day treatment for 21 days. Following six weeks of exposure, we saw an increased rate of transmission when infected deer mice were given

PFL compared to our group which did not receive any treatment. Sixteen out of 37 deer mice (43%) became infected as compared to 24% in our previous intra-cage experiment (Table 3). While treatment with PFL resulted in an increased transmission rate compared with control animals, this did not reach statistical significance by Fisher's exact test (relative risk = 1.355 (0.9489–1.878), p = 0.1311). One limitation of this experiment is that we were unable to determine which mice were transmitting SNV within one cage following an initial transmission event in that cage. Deer mice which may have became infected following contact with the seeder mouse may then have been responsible for further transmission events within that cage during acute infection. Regardless, the increase in transmission rate provides interesting results, and further experiments need to be done to determine whether PFL treatment or some other means of heat shock or cold stress induction can influence SNV shedding and transmission. Our treatment in the experiments outlined here, did not have a significant effect on viral replication, shedding, or transmission.

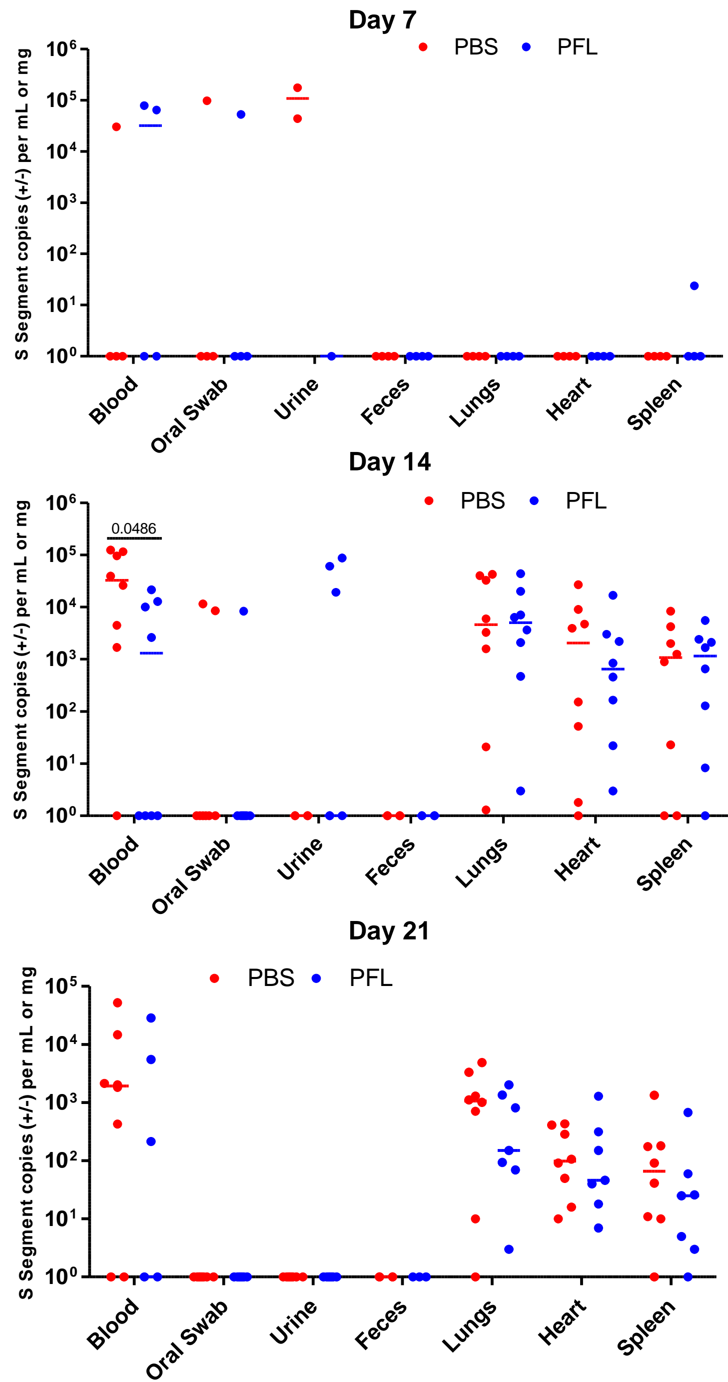

Figure 10. Replication and shedding of SNV in control and PFL treated deer mice. SNV viral copy number was assessed in the tissues and samples listed at the indicated time points post-infection. Shown are data medians. Numbers indicate the p value as assessed by Mann-Whitney test. n=8 per group days 14 and 21, 4/group for day 7. Figure adapted from Warner et al. 2019a.

Table 3. Transmission of SNV among untreated and heat shock induced, infected deer mice

Group	Exposed, Naïve Mice	Transmission Events*	% of Naïve Infected	Risk Ratio (95% CI)	P Value (Fisher's exact test)
Direct Transmission	33	8	24	1.355 (0.9489 to 1.878)	0.1311
PFL Treated$^{\psi}$	37	16	43		

*** Seropositive or qRT-PCR positive**

Ψ Treated with PFL every two days for 3 weeks

Direct transmission data same data from Table 2 as comparison with PFL treated.

Table adapted from Warner et al. 2019a

5.2.3 – Castration of Deer Mice

In order to study the effects of testosterone on SNV infection in male deer mice, we wanted to eliminate all exogenous testosterone to be able to effectively control the amount of the hormone in experimental animals. The amount of testosterone found in the serum of deer mice at any given time is highly variable, thus reduction or elimination of endogenous testosterone followed by supplementation with either testosterone or a control substance was required for proper study of its effects. We modified a protocol published by Valkenburg et al (2016) in which testes are surgically removed from mice, to include two lateral incisions on either side of the scrotum, rather than one single larger incision from which the testes are pulled out. Administration of meloxicam before and for three days following surgery was provided as an analgesic and deer mice were monitored for any signs of illness or discomfort. No serious adverse effects from surgery were observed aside from two instances of incision coming apart following gluing, ultimately requiring euthanasia. Castration of deer mice effectively reduced serum testosterone levels to four-fold lower levels than those seen prior to surgery ($p<0.0001$) (Figure 11). After one month post-castration, testosterone levels were reduced such that we could appropriately carry out experiments examining its effect on SNV replication and transmission.

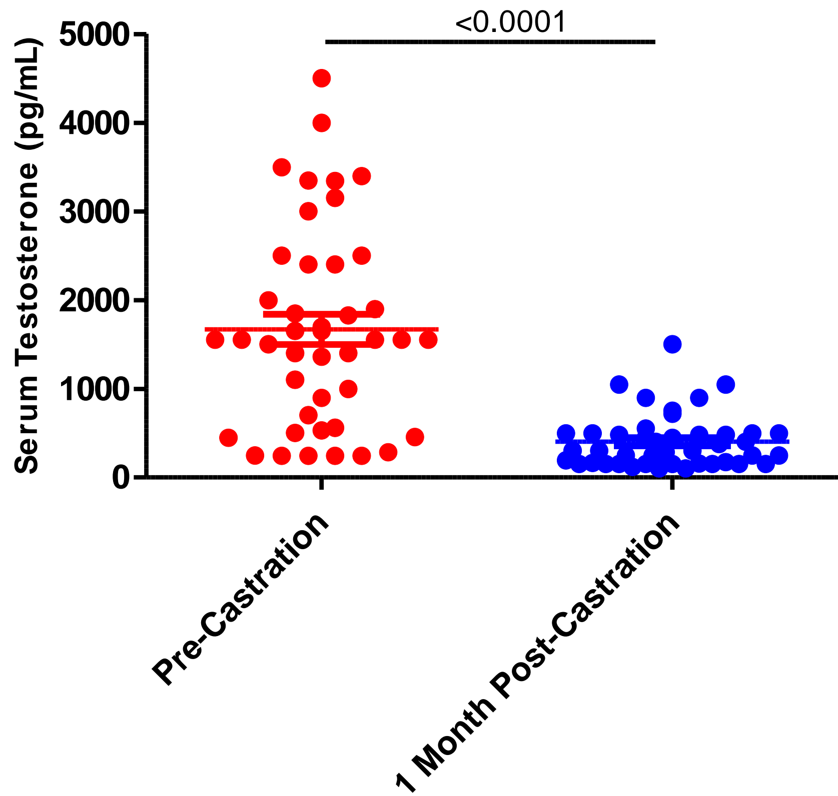

Figure 11. Testosterone levels before and after castration of deer mice. Serum testosterone levels in deer mice before and one month following castration. Represented here are all mice used for preliminary castrations as well as all mice used for experimental infection and transmission experiments. Shown are data means + SEM. Figure adapted from Warner et al. 2019a.

5.2.4 – Effect of Testosterone Levels on SNV Infection and Transmission

To determine whether testosterone levels have an effect on SNV replication, shedding, and transmission, we supplemented castrated deer mice either with testosterone or vehicle control throughout the acute stage of SNV infection (n=6, 5 in day 27 control group due to loss of one animal). Osmotic pumps were implanted subcutaneously into castrated deer mice delivering either propylene glycol or 200 μg per day of testosterone enanthate and deer mice were infected with SNV. On days 14 and 27 post-infection, SNV levels in the blood, tissues, oral swabs, and urine did not differ between deer mice given testosterone or control (Figure 12). We were able to detect SNV RNA more frequently in the blood and urine of animals receiving testosterone, with 11/12 testosterone deer mice compared with 6/11 controls having detectable RNA in the blood and six deer mice given testosterone having detectable RNA in the urine compared with one control animal (Figure 12). However these differences were not statistically significant. One issue that may have arisen from the use of osmotic pumps for testosterone delivery is the efficiency of delivery. Those deer mice receiving testosterone had higher serum levels at the time of euthanasia as expected, but the group receiving testosterone only had approximately two-fold higher serum levels than control animals, and mean levels reached just under half of the levels seen in animals before castration (Figure 13). Testosterone levels across all animals were variable, with overlap seen in serum concentration (Figure 13). Nevertheless, a correlative analysis revealed no positive correlation between serum testosterone level and SNV copy number in the blood or tissues (Figure 14; Spearman's correlation). Overall, our data indicate that the level of testosterone does not affect SNV levels in the tissues or influence viral shedding.

In addition to finding out whether testosterone levels affected SNV replication and shedding during acute infection, we wanted to determine whether the presence or absence of

testosterone had any effect on SNV transmission rate. While the presence of testosterone did not affect replication of the virus, it is possible that increases in testosterone may influence behaviours which could influence transmission. Using our transmission model, deer mice were castrated and implanted with osmotic pumps as above and used as infected seeder mice in a six week transmission experiment. We did not see a difference in the rate of transmission between groups of mice which had seeders given testosterone or control (Table 4). In the testosterone group, out of 29 exposed deer mice, 11 became infected compared with 10 out of 30 in the control group. Additionally, there was no statistically significant difference in transmission rate when compared to our previous intra-cage experiment rate of 8 out of 33 mice becoming infected. We also noted the presence or absence of wounding throughout the experiment and at the end point of the experiment. There were no significant differences between groups in the number of wounds, nor did wounding correlate with transmission events occurring within cages. All in all, our data indicate that the depletion of testosterone via castration or the presence of testosterone does not influence SNV replication, shedding, or the transmission rate.

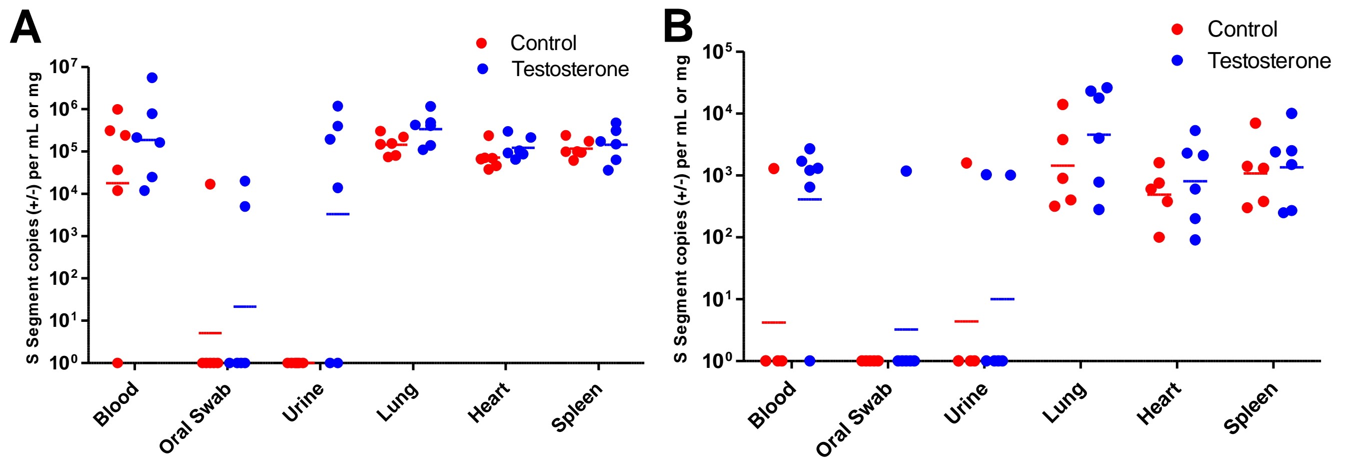

Figure 12. Replication and shedding of SNV in castrated male deer mice given testosterone. Deer mice were castrated, implanted with osmotic pumps filled with either testosterone or control, and infected with SNV. The SNV viral copy number was assessed in the tissues and samples listed at either (A) 14 dpi, or (B) 27 dpi. Shown are data medians. Figure adapted from Warner et al. 2019a.

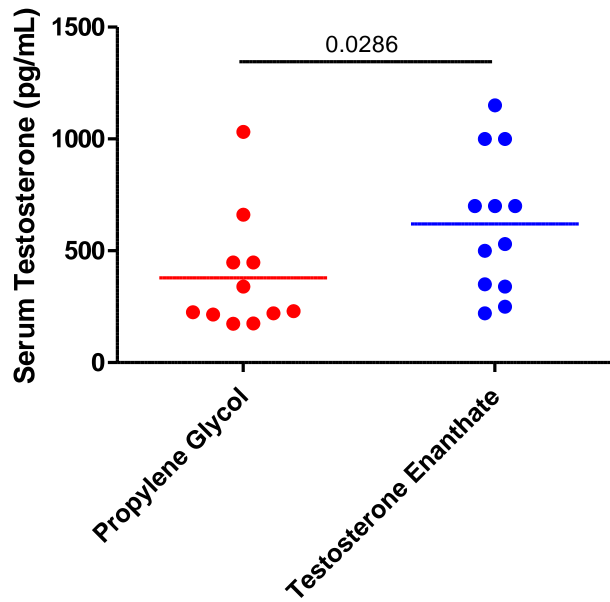

Figure 13. Testosterone levels in castrated mice folloiwng supplementation. Deer mice were castrated, implanted with osmotic pumps filled with either testosterone or control, and infected with SNV. Serum testosterone levels in deer mice implanted with osmotic pumps containing either testosterone enanthate or propylene glycol at the time of euthanasia are shown. Shown are data medians. Figure adapted from Warner et al. 2019a.

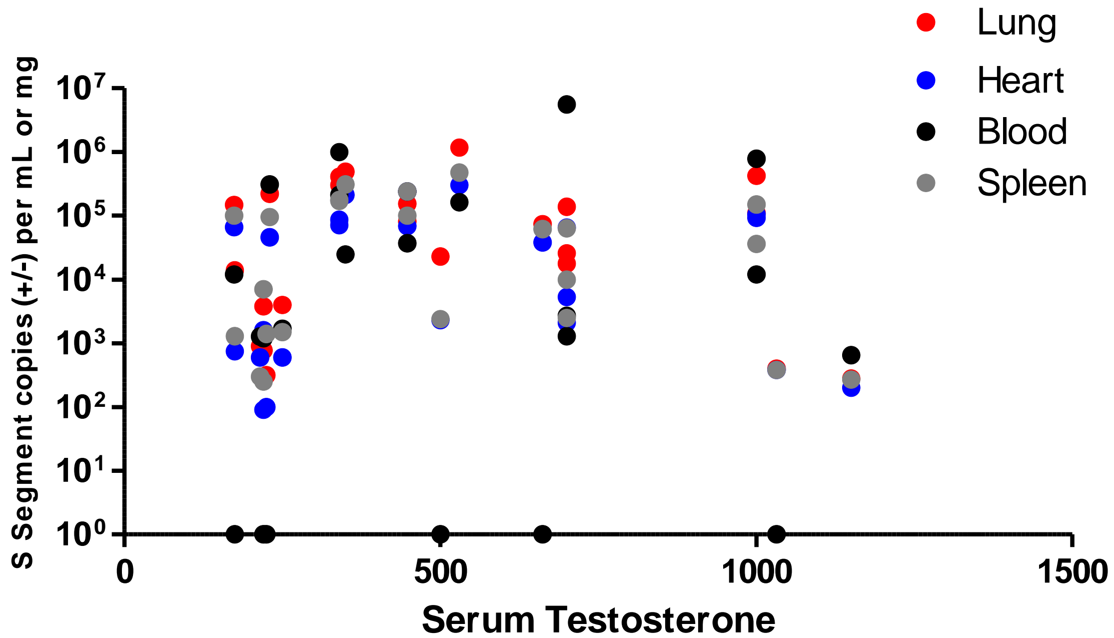

Figure 14. Correlation of testosterone levels with SNV copy number. Deer mice were castrated, implanted with osmotic pumps filled with either testosterone or control, and infected with SNV. The SNV viral copy number was assessed in the tissues and samples listed and plotted against the serum testosterone concentration in each animal at the time of euthanasia.

Table 4. Transmission of SNV by deer mice with/without testosterone

Group	Exposed, Naïve Mice	Transmission Events *	% of Naïve Infected	Risk Ratio (95% CI)	P Value (Fisher's Exact Test)
Propylene Glycol	30	10	33	1.074 (0.7339–1.572)	0.7892
Testosterone	29	11	38		

*** Seropositive or qRT-PCR positive.**

Table adapted from Warner et al. 2019a

5.2.5 – Effect of Thermogenesis on SNV Infection

As we did not see any differences in replication or transmission in SNV infected deer mice when we induced heat shock protein expression, we wanted to re-examine the role of cold stress and its effects on the virus during persistent infection. Related to the heat shock response induced during cold stress is thermogenesis, which occurs following cold stress in mammals, and can also be induced chemically. Chemical agonists of β-3 adrenergic receptors, which respond to catecholamines such epinephrine and norepinephrine, are able to induce the expression of UCP-1 which is critical for subsequent thermogenesis in BAT cells (Nedergaard et al. 2001). UCP-1 expression is critical for induction of thermogenesis and its upregulation significantly alters the metabolism of the expressing cells. CL316,243 is a disodium salt that is a highly selective agonist for β-3 adrenergic receptors, and has been used extensively for the induction of thermogenesis, weight loss, and treatment of diabetes in *in vivo* models (Nedergaard et al. 2001; Cinti et al. 2002; Umekawa et al. 1997; Ferrer-Lorente et al. 2005). CL316,243 treatment is able to promote high expression levels of UCP-1 and is a suitable substitute for cold stress in the lab (Cinti et al. 2002). We treated deer mice with either CL316,243 or water through implanted osmotic pumps for 28 days to mimic cold stress and to induce non-shivering thermogenesis and then infected these deer mice with SNV following the first week of CL316,243 treatment. As seen in figure 15, thermogenesis induction did not seem to influence the levels of SNV in deer mice during this acute infection. All animals which had productive infections has similar levels of SNV RNA. Interestingly however, we had four out of 12 animals that did not have any detectable SNV RNA in the blood or tissues, which is unusual for our experimental infection model, in which all animals typically have high levels of SNV RNA on days 14 and 22, the days tested. By Fisher's exact test, the difference in the number of animals which did not have productive infection was not

statistically significant, but this could warrant further investigation to determine whether treatment had some influence on SNV infection. Additionally, in these experiments, like all other experimental infection studies described, assessment of SNV levels were taken during acute infection. The possible role of thermogenesis in recrudescence during persistent, long term infection is one that could be studied in the future. The role of thermogenesis in the context of SNV replication during persistent infection requires further investigation, but our preliminary data indicate that chemical induction with CL316,243 does not have an effect on SNV replication, at least during acute infection.

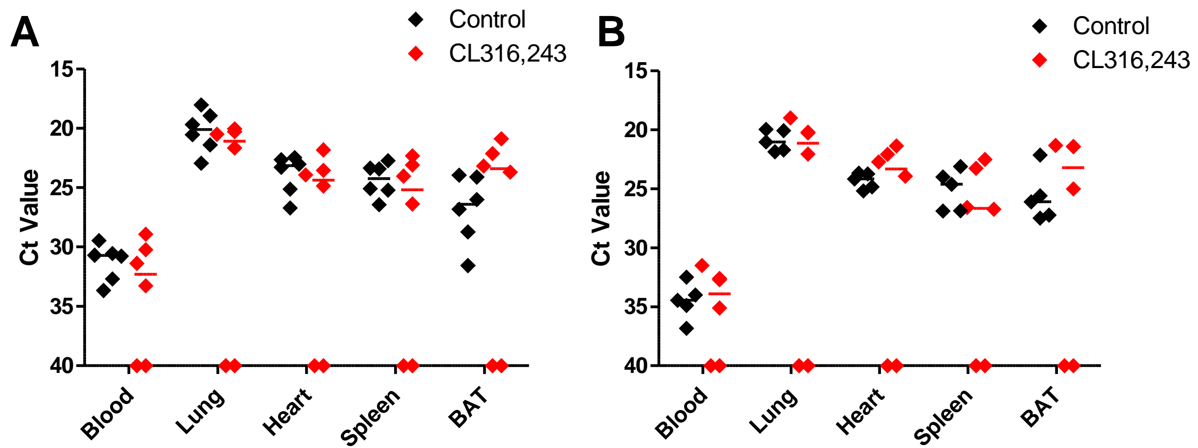

Figure 15. Effect of thermogenesis induction on SNV replication in deer mice. Deer mice were implanted with osmotic pumps delivering either CL316,243 or control for 28 days and infected with SNV one week following implantation. SNV RNA levels were determined and reported as RT-qPCR Ct values on (A) day 14 or (B) day 22 post-infection. Shown are data medians.

5.2.6 – SNV transmission in male and female deer mice

In addition to developing a transmission model for SNV in deer mice and using it to look into what influences SNV transmission, such as heat shock responses, we also did a retrospective analysis of the data generated from our initial direct, intra-cage experiment and the heat shock experiment to determine if there were any differences in transmission between male and female deer mice. In our experiments, deer mice are always housed with the same sex, and following our initial studies on SNV replication in males and females, we were able to use both for subsequent experiments. Because it is thought that males contribute more to SNV transmission than females, and there is typically a higher seroprevalence rate in males, we wanted to see whether we would see any differences in our experimental transmission models. We wanted to see if there is any intrinsic difference in transmission rate between males and females, or if the field data that has been reported is due to mainly behavioural reasons or wider habitat range of male deer mice. Across the experiments described so far, with the exception of the testosterone supplementation experiments, there was a total of 31 male mice and 39 female mice exposed to an infected mouse for 6 weeks in direct contact transmission experiments. Eleven of 31 males, and 13 out of 39 females ultimately became infected with SNV, which indicated there is no difference in transmission rates between male and female animals (Table 5). Additionally, across both control and heat shock induced groups, there was also no significant different in transmission rate if the mice were male or female (Table 5). There was an increase in the number of males that became infected in the PFL-treated group compared to untreated mice (15% compared with 50%), and this resulted in a slight increase in the risk of infection for the uninfected animals (relative risk = 1.692 (1.009–2.838), p = 0.0656 (Fisher's exact test)). While transmission in natural settings may be

carried out predominantly by infected male deer mice, there is no intrinsic, sex specific difference in the ability of male and female deer mice to transmit SNV.

Table 5. Number of transmission events/exposed deer mice in male and female groups.

Group	Male	Female
Control	2/13	6/20
PFL-Treated	9/18	7/19
Total	11/31	13/39

Table adapted from Warner et al. 2019a

5.3 – Discussion

Following the development of a suitable model of SNV transmission in deer mice, our goal was test hypotheses regarding what might influence SNV shedding in deer mice, leading to transmission events, which are relatively rare amongst the deer mouse population. Based on previous suggestions in the SNV literature, we hypothesized that stress responses or heat shock induction might influence the kinetics of SNV infection and thus shedding and transmission (Botten et al. 2003). We were able to influence expression of HSP70, a major heat shock protein family member, through chemical treatment, however this treatment did not affect SNV infection or transmission. The administration of PFL to induce heat shock protein expression did not change SNV RNA copy numbers in the tissues of infected mice, nor did it increase SNV shedding. In our transmission model, PFL treatment did increase the rate of transmission slightly, but the increase was not statistically significantly higher. Following these heat shock experiments, which did not show any effects on SNV replication or transmission, we took an alternative approach, which involved using a β3-adrenergic receptor agonist to induce expression of UCP-1 and thermogenesis, to see if this influenced SNV infection. Chemically induced thermogenesis using CL316,243 during SNV infection did not change the levels of SNV RNA seen in the tissues or blood, similar to what was seen in heat shock response induction. While we did not see any change in SNV infection or transmission rate in our models using PFL or CL316,243 treatment, heat shock and cold stress responses may yet play some role in influencing SNV replication during persistent infections. It is possible that during low levels of viral replication as in persistent infection, cold stress may induce a response that triggers increased viral replication. The higher levels of SNV RNA detectable during acute infection may make it such that any influence these types of

responses have on SNV replication are not detectable. Further studies on heat shock induction during persistent infection may shed light on how those responses affect SNV infection.

A second hypothesis we had, based on previous studies done on SEOV infection of rats and on tick-borne parasites in mice, was that higher levels of testosterone would lead to increased levels of SNV during infection leading to increased viral shedding and transmission rates. We were able to optimize a surgical protocol for the castration of deer mice, which leads to depleted testosterone levels, and rare adverse effects from the surgical procedure itself. We provided either testosterone, or propylene glycol as a control to castrated deer mice, however this did not seem to have an effect on SNV infection. Both groups had similar levels of SNV RNA in the blood and tissues, which is in contrast to what is seen in castrated male rates infected with SEOV (Hannah et al. 2008). SNV RNA was detected in the urine of animals from the testosterone group more frequently, but this was not significant. While we did not see a difference in viral copy numbers between the two groups, we conducted a transmission experiment to determine if behavioural factors might influence transmission such as fighting leading to increased wounding. Similar transmission rates were seen in both groups, indicating that testosterone level did not result in more frequent transmission. It is difficult to know whether increased testosterone levels in infected deer mice may lead to more aggressive behaviour and perhaps more encounters in a natural setting, which would not be reflected in our data and experimental system. The constant proximity of cage mates results in frequent contact and thus exposure amongst all groups, thus any differences in how testosterone levels may affect the number of exposure events could not be determined in our experiments. However our data do show that testosterone levels in male mice do not correlate directly with SNV RNA levels during infection and increased levels of testosterone do not lead to higher transmission rates in our model.

Data from field studies of SNV infection in deer mice have indicated that male mice are more frequently infected than females based on seroprevalence, and that male deer mice are more likely to contribute to SNV transmission within the deer mouse population. Here, following our transmission experiments using either untreated or PFL treated deer mice, we examined the transmission rates we found in the male and females groups throughout both studies. We saw no overall difference between males and females in our experimental transmission model. Thus, while there may be other factors such as habitat range and behavioural difference with male deer mice that result in them becoming more frequently infected and contributing to transmission, these are not due to any sex-specific intrinsic difference in SNV infection. Females infected with SNV were as likely as males to transmit to uninfected cage mates. There was a slightly higher risk of infection in the PFL treated male group as compared with untreated, suggesting perhaps a sex-specific impact of this treatment on SNV infection, however whether there is any real effect on SNV transmission due to treatment in males versus females remains to seen. This data showing no difference in transmission rate is in line with the data presented from chapter 3 on SNV levels in the tissues during acute infection of males and females which also showed no differences.

Chapter 6 – Development and Testing of Vaccines against SNV for Their use as Oral Bait Vaccines

6.1 – Introduction

Approaches for the prevention of infectious diseases carried by animals can vary and include vaccination of the human population, prevention of exposure and awareness, disruption of the life cycle of the pathogen, or through vaccination of the wild animal population to prevent spread of the pathogen. Elimination or reduction of the prevalence of a pathogen within its host population reduces the exposure risk of humans and ultimately the number of human infections seen. The success of wildlife vaccination is a complex issue, that is dependent upon several factors beyond just the ability of the vaccine to protect individual animals against infection (Mendoza et al. 2018). Ultimately, the goal of such programs and their success depends on their ability to either reduce the number of human exposures and thus infections, or the ability to reduce the spread of the pathogen amongst the animal population, or prevent transmission and spillover into areas that previously did not have the targeted agent. While human vaccination campaigns set out to vaccinate a high percentage of the population as to achieve herd immunity, with the exception of some rabies wildlife vaccination programs, this is likely not achievable for diseases carried by small rodents such as mice and rats, the main reservoirs for pathogenic hantaviruses. For targeting of animals such as mice and rats, certain factors should be taken into account. Their population turnover is typically quite high, thus targeting animals during reproductive seasons may be required (Mendoza et al. 2018; Ogden et al. 2007). Their shorter life spans mean that rapid immunogenicity is ideal and thus vectors which require only one dose through ingestion are preferred. A high percentage of hantavirus cases are contracted in peridomestic settings in which people are working or living in the presence of hantavirus-carrying rodents. Those who are in

situations that are at a high risk might be able to take advantage of developed bait vaccines for prevention of transmission within populations around their home, work, garage, or cabin. This approach may ultimately prove more practical as opposed to an attempt to target populations on a larger scale.

While there have been a number of different zoonotic diseases that have been targets of bait style vaccines, such as rabies, Lyme disease, and the plague, the success of such programs at targeting viral disease carried by small rodents has yet to be shown definitively, either experimentally, or in field studies. Field trials have reported success of bait style vaccines targeting *B. burgdorferi* in white-footed mice, which are closely related to deer mice, suggesting that a vaccination approach for prevention of SNV transmission could be feasible (Richer et al. 2014). The success of the various oral bait vaccines for the preventing the spread of infectious disease within mammal populations, including the well known programs launched for the elimination of rabies in North America, inclined us to determine whether such an approach could be tested and used against SNV in deer mice. For other bait vaccines that have been developed for rabies, *B. burgdorferi*, and *Y. pestis*, initial development typically involves the use of recombinant vaccines expressing immunologically relevant antigens of the pathogen in question in immunogenicity studies, followed by testing in infectious challenge or exposure systems. For SNV, no suitable vaccine options for oral baits have previously been developed. Here, we first tested recombinant vaccine candidates expressing SNV glycoproteins for their ability to induce humoral responses in deer mice, followed by their ability to prevent SNV infection and transmission using our transmission model.

6.2 – Results

6.2.1 – Expression of SNV Glycoproteins by Recombinant Viral Vectors

Previously, the only vaccine candidates developed against SNV and tested in pre-clinical settings were nucleic acid based vaccines. For the development of vaccine candidates to be used for oral baits, we wanted to test different viral vectors that can efficiently express SNV glycoprotein. Recombinant Adenoviruses have been effectively employed as a viral vector for vaccine use for many pathogens and they offer several advantages including their ability to infect multiple cell types and species, efficient protein expression, high environmental stability, and the ability to be grown to high titers (Mendoza et al. 2018). The recombinant adenovirus, ONRAB, which is a replication competent adenovirus, has been used successfully in the control of rabies in wildlife populations (Rossate et al. 2009). Replication deficient adenoviruses have been used in the context of hantavirus infections. Recombinant viruses expressing either ANDV Gn, Gc, GPC, or N have been tested for protection against ANDV in the Syrian hamster model of HCPS (Safronetz et al. 2009). These vaccines fully protected Syrian hamsters, and were more efficient and still fully protective when Gn and Gc protein were expressed individually. We designed recombinant human Adenovirus serotype 5 viruses expressing either SNV Gn or Gc (Ad-Gn, Ad-Gc) to be used as a vaccine against SNV in deer mice. To confirm the expression of SNV proteins by these vectors, HEK293T cells were infected with either Ad-Gn or Ad-Gc at an MOI of 1, and then cells were harvested 48 post-infection. Initially, due to a lack of suitable antibody optimized for western blotting, detection of SNV Gn and Gc was difficult. We used convalescent hamster sera, from animals that had been vaccinated against either SNV or ANDV and then survived ANDV infection for detection of SNV Gn and Gc via western blot. Each recombinant virus

expressed either Gn or Gc (Figure 16). This confirmation of expression allowed us to move ahead with *in vivo* protection experiments in deer mice.

Another viral vector that we hypothesized might provide protection of deer mice against SNV was a recombinant vesicular stomatitis virus expressing SNV GPC (rVSVΔG/SNVGPC). We were able to obtain this virus from the Kartik Chandran lab at Albert Einstein College of Medicine. Confirmation of expression of SNV GPC was done by their lab, and thus I have not included that data in this thesis.

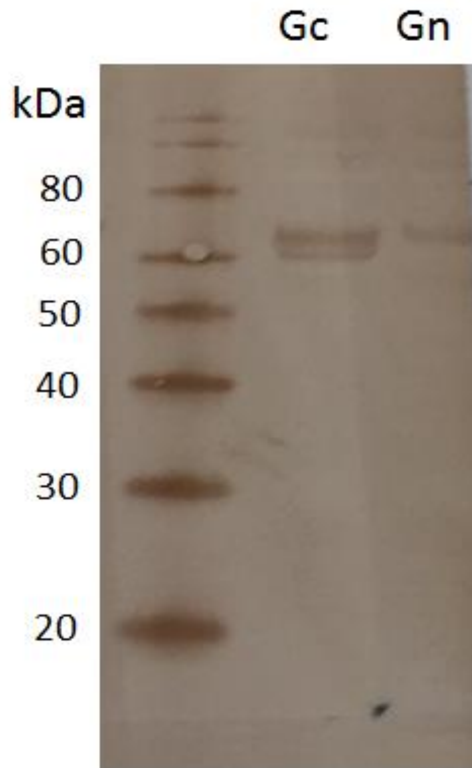

Figure 16. Expression of SNV Gn and Gc by Recombinant Adenoviruses. HEK293T cells were infected with either Ad-Gn or Ad-Gc at an MOI of 1 and cells were harvested and disrupted 48 hours post-infection. Cellular proteins were run on SDS-PAGE gel and western blot for detection of SNV Gn and Gc was performed. Positive and negative controls not shown.

6.2.2 – Protective Efficacy of SNV Vaccines

We sought to determine whether vaccination using either recombinant Adenoviruses or rVSVΔG/SNVGPC could prevent infection with SNV. We wanted to accomplish this through a few different means. In the deer mouse model, prevention of infection can only be determined by either seeing if the animals have any detectable virus in the blood and tissues, or reduced levels of virus compared to control vaccinated animals. With prevention of transmission of the virus the ultimate goal, vaccination of deer mice and then preventing their acquisition of SNV in our transmission model is another means to determine efficacy. Additionally, we were able to test the rVSVΔG/SNVGPC vaccine for efficacy in two Syrian hamster models of infection, to determine if the vaccine is immunogenic and protective against SNV and ANDV.

To test the efficacy of the VSV based vaccine in two models of infection, we vaccinated hamsters with 10^5 PFU of rVSVΔG/SNVGPC. Comparatively, animals were vaccinated with a recombinant VSV expressing ANDV GPC, rVSVΔG/ANDVGP, a virus that has been used successfully as a vaccine vector in animal models previously or with rVSVΔG/LASVGPC expressing Lassa virus GPC as a negative control (Brown et al. 2011). Vaccination of hamsters induced both IgG and neutralizing antibody responses against SNV (Figure 17 A and B). In addition, vaccination against SNV was able to induce both cross-reactive IgG and some cross-neutralizing antibodies against ANDV as well (Figure 17 A and B). Here we had evidence that vaccination may be protective not only against SNV challenge, but also ANDV challenge as well.

To determine if vaccination was protective against SNV, we challenged hamsters with a hamster-adapted SNV (HA-SNV), a non-lethal infectious model previously developed by Safronetz et al (2013). Of our hamsters vaccinated against SNV, one had detectable RNA in the

blood and tissues at 7 dpi, while none tested 4 dpi had detectable levels (Figure 18). Similar results were seen in animals vaccinated against ANDV, with one hamster having detectable RNA, this one at 4 dpi (Figure 18). While the use of HA-SNV for infectious challenge revealed protective efficacy of rVSVΔG/SNVGPC, the limitation with this model is the lack of signs of disease and the ability to detect replicative virus in the tissues sampled.

We also tested rVSVΔG/SNVGPC for protective efficacy in the lethal Syrian hamster model of HCPS. rVSVΔG/SNVGPC was able to significantly protect hamsters from lethal ANDV disease compared with control animals vaccinated with rVSVΔG/LASVGPC (Figure 19A). Interestingly, vaccination against SNV, while protective against lethality, was not able to completely eliminate ANDV RNA detectable in the tissues, as vaccination with rVSVΔG/ANDVGPC was able to accomplish (Figure 19B). Despite the breakthrough of replication seen in the tissues of rVSVΔG/SNVGPC vaccinated animals, our data still indicated that this vaccine is capable of cross-protection against both SNV and ANDV. This data provided evidence that rVSVΔG/SNVGPC may be able to similarly protect deer mice against both infectious SNV challenge and from acquiring the virus through transmission.

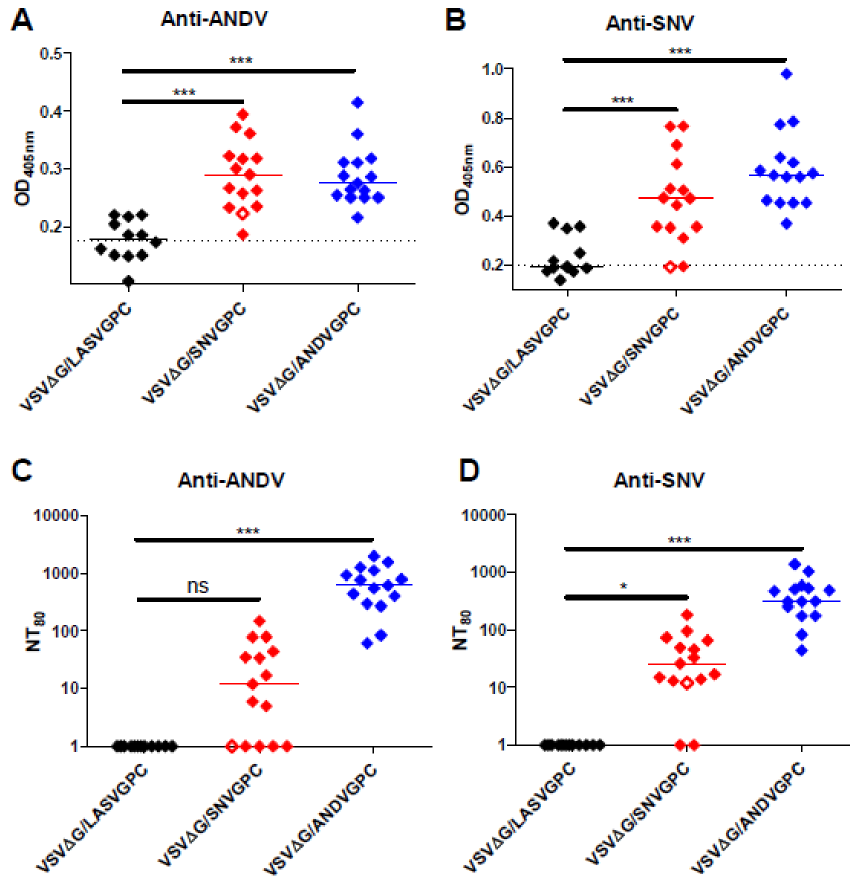

Figure 17. Humoral Immune Responses of VSV Vaccinated Hamsters. Hamsters were vaccinated with either rVSVΔG/LASVGPC (n = 12), rVSVΔG/ANDVGPC (n = 15), or rVSVΔG/SNVGPC (n = 15) and IgG titers against either (A) ANDV or (B) SNV were assessed. NT80 against either (C) ANDV or (D) SNV was determined via microneutralization assay using recombinant VSV expressing either ANDV or SNV glycoprotein and GFP. Data medians are shown. Statistical significance was determined by one way ANOVA. *, p = <0.05 ; ***, p = <0.0001. Figure adapted from Warner et al. 2019b

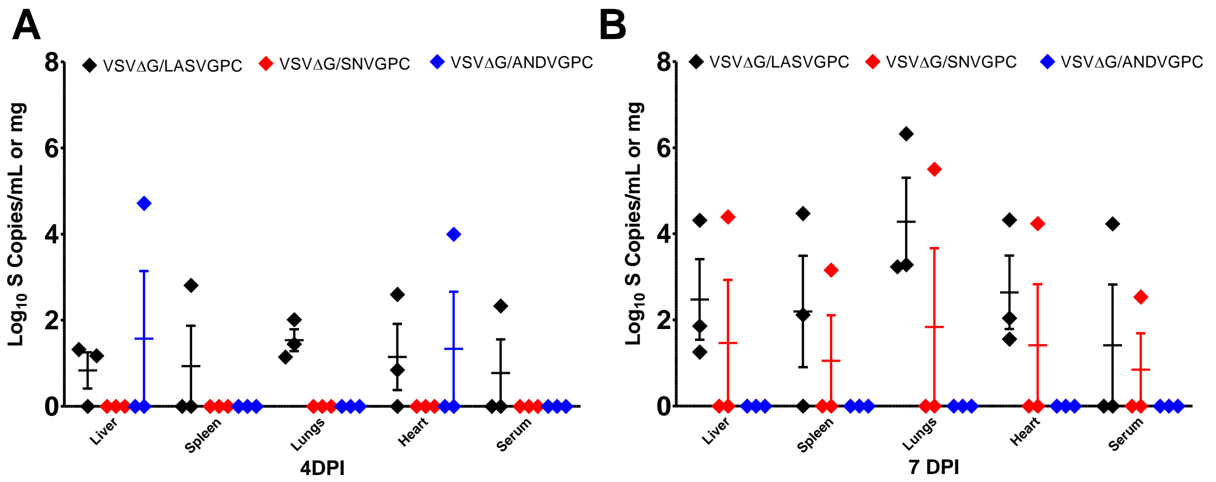

Figure 18. Protective Efficacy of Recombinant VSV Vaccines against HA-SNV. Presence of SNV RNA in the serum and tissues of vaccinated hamsters at (A) 4 dpi and (B) 7 dpi (n = 3/group) following HA-SNV challenge. Data means + SEM are shown. Figure adapted from Warner et al. 2019b

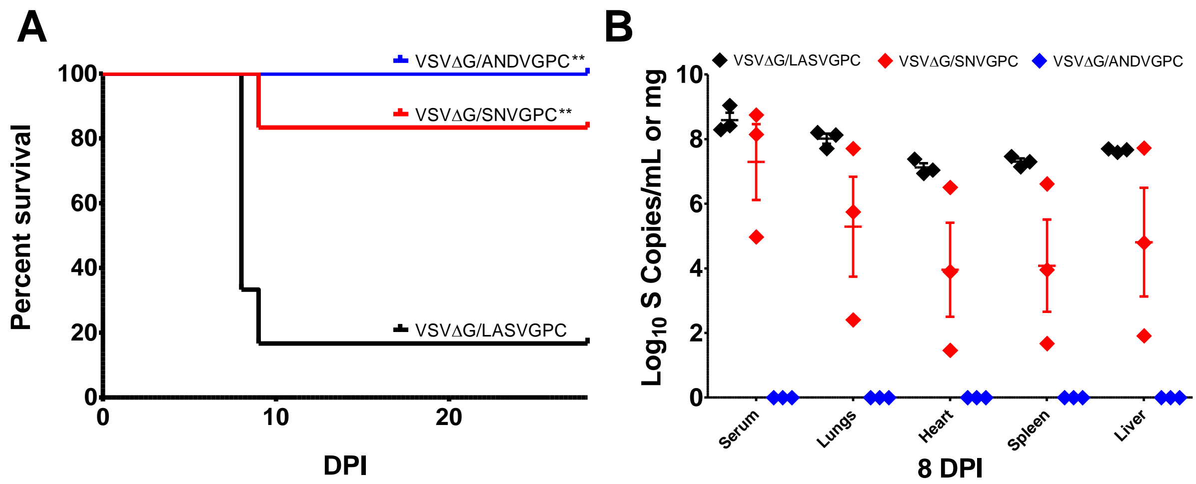

Figure 19. Protective Efficacy of Recombinant VSV Vaccines against lethal ANDV infection. (A) Survival of hamsters vaccinated with either rVSVΔG/ANDVGPC (n = 6), rVSVΔG/SNVGPC (n = 6), or control vaccine rVSVΔG/LASVGPC (n = 6) following challenge with ANDV. (B) Presence, following ANDV challenge, of ANDV RNA in the serum and tissues in groups of vaccinated hamsters at 8 days post-infection (dpi) (n = 3/group). Data means + SEM are shown. Statistical significance was determined via log-rank test (A). **, p = <0.01. Figure adapted from Warner et al. 2019b

To begin with testing our vaccine candidates against SNV in deer mice, we vaccinated mice using Ad-Gn and Ad-Gc, either IM or via oral gavage separately or in combination or with rVSVΔG/SNVGPC via either route or gave PBS as a control (n=6). All these vaccines were able to induce low levels of anti-SNV neutralizing antibody titers in at least some deer mice as assessed by FFRNT assay, via reduction of the number of cells infected with a recombinant VSV expressing SNV GPC and GFP following incubation of the virus with immune sera (Figure 20). Following vaccination, all deer mice were then challenged IM with SNV to determine if vaccination is capable of preventing SNV infection. Fourteen days post-infection, other than the Ad-Gn IM vaccinated group, all groups had significantly reduced levels of SNV RNA in the blood (Figure 21). However when we examined the SNV RNA levels in the lungs, the only two groups which had reduced levels lower than those in the PBS group were the rVSVΔG/SNVGPC vaccinated animals (Figure 21). Even though these animals had statistically significantly reduced RNA levels than control animals, they still had detectable SNV RNA suggesting possible productive infection in these mice. Due to the lack of a reliable assay to detect replicating SNV, it is difficult to determine how well vaccination prevented infection in these animals. Further investigation, including a similar experiment reporting on the levels of live virus in the blood and tissues of rVSVΔG/SNVGPC vaccinated animals could provide more information on the efficacy of this vaccine in this experimental setting.

Regardless, we went ahead and vaccinated groups of deer mice either orally or IM with rVSVΔG/SNVGPC to determine if vaccination is able to prevent acquisition of SNV in our transmission model. These animals had detectable anti-SNV IgG responses as measured by ELISA with IM vaccination providing a near two-fold increase in antibody titer as measured by OD_{405nm} compared with oral vaccination (mean of 2.37 and 1.27, respectively) (Figure 22). Similar to the

data above, vaccination in both groups resulted in modest levels of anti-SNV neutralizing antibody, with only one in six individuals having detectable neutralizing antibodies 28 days post-vaccination as determined by a fluorescence reduction neutralization assay (Figure 23). We exposed both groups of vaccinated deer mice to infected deer mice in an intra-cage transmission experiment to determine if vaccination is able to effectively eliminate or reduce transmission. When we evaluated the ability of vaccination to prevent transmission, vaccination both orally and intramuscularly with rVSVΔG/SNVGPC was able to prevent transmission of SNV to uninfected animals compared to those in the control group. In both groups of deer mice that were vaccinated, only one transmission event was detected out of 30 animals following six weeks of exposure compared to 12 out of 31 in the unvaccinated control group (relative risk = 11.61 (1.607–83.92), p = 0.0011 (Fisher's exact test)) (Table 6). This data implies that vaccination with rVSVΔG/SNVGPC orally is able to effectively prevent SNV transmission within the deer mouse population and could be a viable means of preventing spread of SNV throughout deer mouse populations.

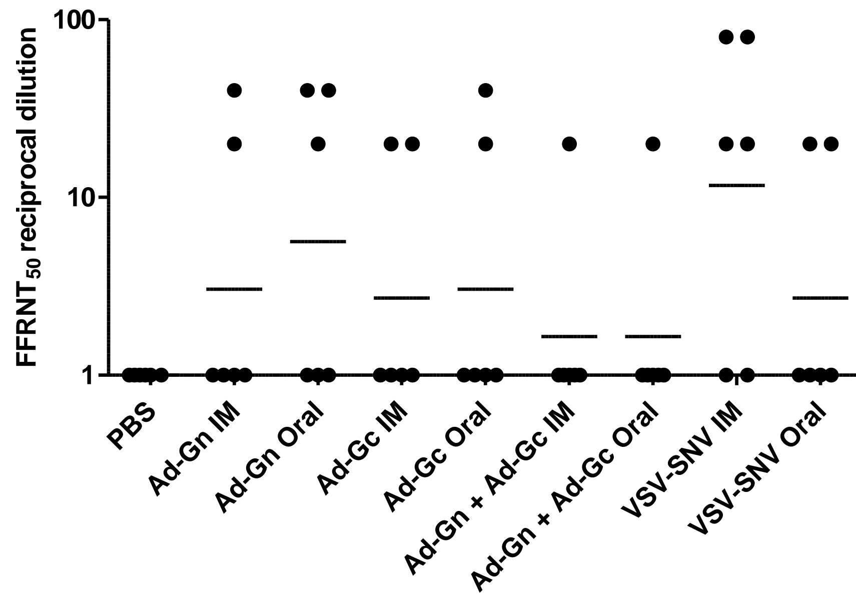

Figure 20. Immunogenicity of Recombinant Vaccines in Deer Mice. Deer mice were vaccinated with either Ad-Gn, Ad-Gc, or rVSVΔG/SNVGPC either IM or orally and anti-SNV neutralizing antibody titers were assessed by a fluorescence focus reduction neutralization assay. Geometric means of the data are shown.

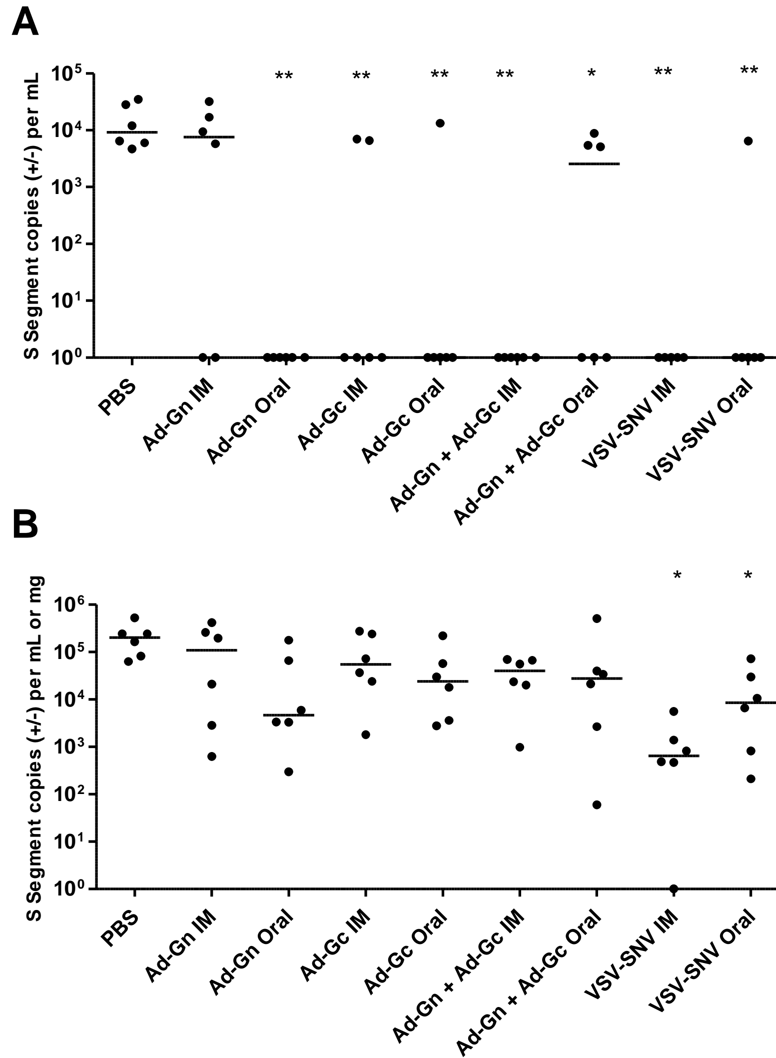

Figure 21. Protective Efficacy of Recombinant Vaccines Against SNV Challenge. Deer mice were vaccinated with either Ad-Gn, Ad-Gc, or rVSVΔG/SNVGPC either IM or orally and infected with SNV. SNV RNA levels were assessed in either (A) the blood or (B) the lungs of infected deer mice 14 dpi. Shown are data medians. Statistical significant was determined by one way ANOVA of groups compared to the PBS group. *, $p<0.05$; **, $p<0.01$.

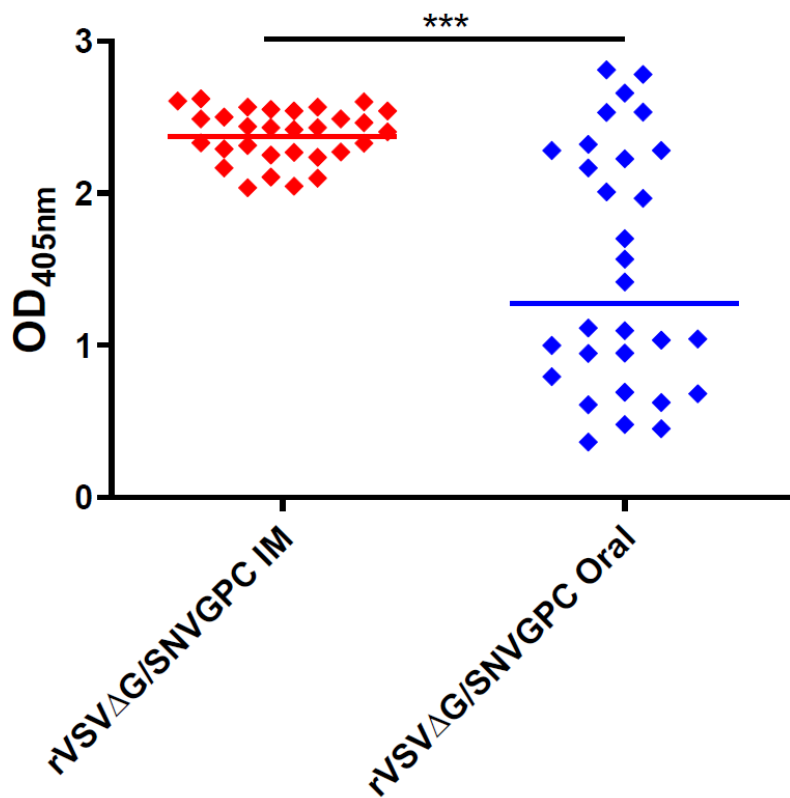

Figure 22. Immunogenicity of rVSVΔG/SNVGPC as assessed by ELISA. Deer mice were vaccinated with 2 x 10^4 PFU of rVSVΔG/SNVGPC either IM or via oral gavage. After 28 days, sera were collected from vaccinated mice and the presence of total anti-SNV IgG was detected. n=30.

Figure 23. **Neutralizing antibody induced by rVSVΔG/SNVGPC.** Deer mice were vaccinated with rVSVΔG/SNVGPC either IM or orally and anti-SNV neutralizing antibody titers were assessed by a fluorescence reduction neutralization assay. Geometric means of the data are shown.

Table 6. Prevention of Transmission of SNV via Vaccination

Group	Exposed, Naïve Mice	Transmission Events*	% of Naïve Infected	Risk Ratio (95% CI)	P Value (Fisher's exact test)
Unvaccinated Controls	**31**	**12**	**39**		
rVSVΔG/SNVGPC IM	**30**	**1**	**3**	**11.61 (1.607–83.92)**	**0.0011**
rVSVΔG/SNVGPC Oral gavage	**30**	**1**	**3**	**11.61 (1.607–83.92)**	**0.0011**

*** Seropositive or qRT-PCR positive**

6.3 – Discussion

Our goal was develop and test vaccine platforms for the reduction of SNV transmission within deer mouse populations. The approach we used was to develop vaccines expressing SNV glycoproteins based on existing platforms that have been successfully employed previously, either for vaccination of humans or wildlife. The first attempt at vaccination of deer mice involved recombinant Adenoviruses expressing either SNV Gn or Gc, a vaccine platform that has been used effectively for a number of pathogens, including for protection against ANDV (Safronetz et al. 2009). The vaccines we designed and had developed expressed either SNV Gn or Gc (Figure 16). However, when we vaccinated deer mice with 10^8 PFU or either virus intramuscularly or via oral gavage, vaccination did not result in a reduction of SNV viral RNA load in the lungs compared with PBS vaccination (Figure 21). The effectiveness or lack thereof, of this approach could be due to a reduced expression level of the recombinant SNV proteins in these viruses, either in general or within deer mouse cells, or due to the susceptibility of deer mice to initial infection with Adenoviruses. Immunogenicity of these viruses was also low, when examining neutralizing antibody titers. The lack of reliable ELISA methods for detecting anti-SNV IgG antibodies makes it difficult to accurately determine the immunogenicity of given vaccine platforms, however the neutralizing antibody titers of Adenovirus-vaccinated animals was not significantly different from rVSVΔG/SNVGPC vaccinated animals, yet protective efficacy was reduced. Further investigation into the inefficacy of these vaccines in deer mice could be warranted, especially since this platform has been successfully used against ANDV, with vaccines being fully protective in the lethal hamster model of ANDV infection (Safronetz et al. 2009).

Along with recombinant Adenoviruses being able to protect against lethal ANDV challenge, recombinant Vesicular Stomatitis virus expressing ANDV GPC has been shown to be

an effective vaccine for protection against ANDV in this model (Brown et al. 2011). Since we were able to obtain a recombinant VSV expressing SNV GPC (rVSVΔG/SNVGPC), we hypothesized that this virus may also provide an effective means for preventing SNV transmission in deer mice. Initially, to test not only its suitability for prevention of SNV transmission, but also its general effectiveness, we tested rVSVΔG/SNVGPC alongside rVSVΔG/ANDVGPC in cross-protection experiments in Syrian hamsters. This not only would provide us with initial data regarding the efficacy of the rVSVΔG/SNVGPC vaccine, but also with strong data for the prevention of pathogenic cases of HCPS caused by both SNV and ANDV in non-reservoir species. The immunogenicity of both rVSVΔG/SNVGPC and rVSVΔG/ANDVGPC was measured by neutralization assay as well as by IgG ELISA, and showed modest levels of IgG titers induced by both vaccines, however the utility of the assay used was not ideal, and development of a more reliable, sensitive assay was done after these experiments were performed. In terms of neutralizing antibody titers, rVSVΔG/SNVGPC was able to induce low levels in vaccinated hamsters (Figure 17). The reduced levels of humoral immune responses induced by rVSVΔG/SNVGPC as compared to the rVSVΔG/ANDVGPC vaccine may be due to reduced expression or the replication kinetics of each of the viruses. rVSVΔG/SNVGPC replicates much slower than rVSVΔG/ANDVGPC, which may explain the discrepancies in immunogenicity (Warner et al. 2019b). rVSVΔG/SNVGPC as well as rVSVΔG/ANDVGPC prevented infection of Syrian hamsters with hamster-adapted SNV in an infectious challenge model suggesting their utility for preventing the acquisition of SNV in a non-lethal model, similar to infections in deer mice (Figure 18). rVSVΔG/SNVGPC was also able to provide cross-protection against ANDV in a lethal challenge model, although animals vaccinated against SNV and challenged with ANDV had detectable viral RNA in the tissues and serum. However the protection against lethal disease

suggests an ability to provide protection against New World hantaviruses other than SNV, which may have important implications for human vaccine development (Figure 19).

To determine whether rVSVΔG/SNVGPC can prevent SNV infection development in deer mice, the same approach was taken as with Adenovirus vaccination, however rVSVΔG/SNVGPC vaccination resulted in statistically significantly reduced viral RNA copy numbers in the blood and lungs compared to deer mice given PBS as a control (Figure 21). We then sought to determine whether rVSVΔG/SNVGPC vaccination would be an effective means of preventing SNV transmission in our direct transmission model. Deer mice vaccinated either IM or via oral gavage 28 days before exposure to infected deer mice had a significantly reduced risk of becoming infected with SNV (Table 6). Because we knew that this vaccine was protective via the IM route against SNV in hamsters, we wanted to include this as a control as well in transmission studies as a comparison for oral vaccination with rVSVΔG/SNVGPC. Recombinant VSV based vaccines have been shown to be protective when given orally previously, but we anticipated lesser immunogenicity and thus perhaps lesser effectiveness when the vaccine is administered through oral gavage compared with IM (Jones et al. 2007). If rVSVΔG/SNVGPC is to be pursued further as a potential candidate for bait vaccine development, efficacy when administered orally is a key component to advancement. Here, vaccination via either route did not show any differences in immunogenicity when measured by neutralizing antibody responses, but IM vaccination did induce higher levels of anti-SNV IgG (Figures 20, 22, 23). Additionally in the transmission model, vaccination resulted in only a single transmission event out of 30 animals in each group, compared to 12/31 in the unvaccinated controls. Thus it appears that oral vaccination, is as effective at preventing transmission as IM vaccination in this model. Key questions moving forward with vaccination using this platform in this model will be longer term effectiveness and a potential

protective dose, as strict dosing in field experiments is difficult to control. Overall, the data indicate that vaccination of deer mice can potentially provide a means of preventing SNV transmission and dispersal throughout the deer mouse population and that rVSVΔG/SNVGPC is a viable candidate to test using this model moving forward. Additionally, this provides evidence that our SNV transmission model is an appropriate way of assessing and modelling potential bait vaccine platforms for SNV, other hantaviruses, and rodent borne pathogens. rVSVΔG/SNVGPC also provides protection against ANDV, indicating cross-protective effects against multiple New World hantavirus species which could be critical for developing vaccines targeting multiple species.

Chapter 7 – Final Discussion

7.1 Significance

The work presented in this thesis has advanced our understanding of SNV infection and transmission in deer mice, and has implications not only for SNV and other hantavirus species, but potentially for other rodent-borne pathogens as well. Early work focused on refining the protocols that were put in place for infecting deer mice with SNV for the purposes of growing virus stocks for subsequent experiments. Previously, little optimization had been performed in terms of route of SNV infection, time point at which to harvest the virus, or the age and sex of the animals used for infection. For SNV and PUUV, which undergo adaptation following tissue culture infection and whose study requires viruses that have only been passaged within their rodent host, the results from this optimization were of critical importance moving forward with this thesis project. We confirmed that VeroE6-adapted SNV does not productively infect deer mice, highlighting the importance of optimizing the production of SNV *in vivo* (Figure 3). We were able to show that intraperitoneal infection of deer mice was a more reproducible and reliable method of infection compared with intramuscular infection in terms of the copy number of viral stocks produced and productive infection of individual animals (Figure 4). Although classically, infections of deer mice with SNV have been performed via the IM route, as this was thought to most closely mimic natural infection, a direct comparison of IM and IP infection had not been done in this context. Another important question moving forward was whether there are significant differences in SNV infection in experimentally infected male and female deer mice, or of deer mice of significantly different ages, both for the purposes of growing viral stocks *in vivo* and for our experimental infection and transmission models. While there is data from field studies and seroprevalence data on SNV infection in deer mice that suggests that males are preferentially

infected, it appears that there are no intrinsic sex-based differences in SNV infection. Male and female deer mice did not have different levels of SNV in the blood or tissues when controlling for age, thus it appears that any observed differences in seroprevalence may be due to behavioural differences or other factors. Differences in hantavirus prevalence between sexes has been studied for other hantaviruses, but mainly in the naturally caught reservoir species (Klein et al. 2001; Bernshtein et al. 1999; Childs et al. 1994; Glass et al. 1998; Mills et al. 1998; McIntyre et al. 2005). Here we show that at least experimentally, SNV infection kinetics are similar in both male and female deer mice, including viral shedding. This was an important observation moving forward with the rest of the thesis work, as there was no limitation on the animals available for use in future experiments.

The remainder of this thesis relied on the use of both our experimental SNV infection model in deer mice and the development of a model of SNV transmission in deer mice that can be used to test hypotheses on SNV biology and to test various vaccine candidates. Our main objective with regard to SNV transmission was to test our hypothesis that direct transmission is the main driver of transmission rather than exposure to the virus in the environment and to develop a model of direct, intra-cage SNV transmission that could be used to test further hypotheses. Previous attempts at studying SNV transmission experimentally were not particularly successful, or were not able to answer the question of whether SNV transmits primarily directly or indirectly (Botten et al. 2002; Bagamian et al. 2012). Additionally, anecdotal evidence suggested that SNV is not readily transmissible between deer mice, even housed in the same cage. We were able to show a significant number of transmission events in our direct, intra-cage transmission experiments following at least six weeks of exposure and report on a reproducible and reliable model of SNV transmission. This direct transmission occurred more frequently than did indirect transmission

following exposure to potentially contaminated caging, and in fact we did not detect any transmission events through this manner. This is in contrast to other hantaviruses such as PUUV, which is relatively stable in the environment and able to readily transmit to susceptible bank voles through exposure in the environment (Hardestam et al. 2008). Further studies on the environmental stability of SNV and other New World hantaviruses are warranted and may help answer important questions about the transmissibility of these viruses to rodents and to humans.

The success of the development of our direct transmission model allowed us to move forward with testing of different hypotheses regarding how SNV replicates during persistent infection and transmits to uninfected individuals. It also provided us with a means to move forward in developing and testing vaccine platforms for preventing SNV transmission. An additional question we had regarding SNV transmission was whether there would be detectable differences in transmission rate between male and female deer mice, since males are thought to contribute to the majority of transmission and have a higher seroprevalence rate. Throughout our transmission experiments, there were no differences seen in the transmission rate between males and females, similar to the SNV RNA levels we detected in our infection model. This data indicates that while during natural infection of deer mouse populations, infection of males may predominate, there is no intrinsic sex-specific difference that precludes female deer mice from being infected and transmitting the virus at a rate equal to that seen in male deer mice. The major contributor to the field of hantavirus research provided by this thesis is the development of the transmission model for SNV in deer mice. This is the first published evidence that SNV readily transmits between deer mice in a direct manner in an experimental setting. The testing and development of this model system allowed for all subsequent experiments in this thesis to be performed and will be critical for future studies of SNV transmission, biology, and ecology. Additionally, it provides evidence

that modes of transmission may differ between species of hantavirus, and that assumptions made about transmissibility of one may not be true for others, particularly New vs Old World viruses. This model also may provide a blueprint for the development of similar systems for other hantaviruses which have not been studied experimentally. Hantavirus ecology and infections of reservoir hosts remains an understudied area in the field. The works described here could lead to similar infection and transmission models for other viruses, if particular rodent species, viral isolates, and access to high containment research facilities are available.

The persistent nature of hantavirus infections of rodent reservoirs and the periodic episodes of increased viral replication and shedding prompted us to attempt to elucidate what some potential drivers of this increase might be. Since we have at our disposal effective experimental infection and transmission models, we sought to test two hypotheses as to what might cause an increase in SNV replication, shedding, and transmission. The first involved inducing heat shock responses in deer mice infected with SNV, which have been hypothesized to possibly play a role in inducing viral replication in persistently infected tissues such as brown adipose tissue (Botten et al. 2003). Additionally, heat shock proteins, which are molecular chaperones within cells important for protein transport and folding, have been shown to interact with viral proteins and be important regulators of viral replication (Matz et al. 1995; Santoro et al. 2009). The seasonal incidence of HCPS cases suggests higher risk during the spring when temperatures and climatic conditions for deer mouse populations are changing. To this end we attempted to best mimic these types of physiological changes in infected deer mice through treatment with certain drugs. While treatment with drugs which have been shown to induce heat shock protein expression were able to increase HSP70 mRNA expression, this treatment ultimately did not influence SNV replication, shedding, or transmission (Figure 10 and Table 3). Additionally, along these lines treatment with the β3

adrenergic receptor agonist CL316,243, which induces non-shivering thermogenesis and UCP-1 expression also did not have an apparent effect on SNV infection, despite brown adipose tissue being a main target of SNV infection. Further work on how different climatic or environmental factors such as thermogenesis, cold stress, and heat shock responses might have on SNV infection is warranted. Experiments performed with persistently infected deer mice, rather than acutely infected mice also may be more likely to show any differences in viral replication. The higher levels of viral RNA seen during acute infection may not result in observable changes, while during persistent infection low, if not undetectable levels of RNA in certain tissues may increase to detectable levels following induction of these types of responses. Future experiments examining the role of these types of factors will be important in determining how the virus is able to replicate to high levels periodically and at certain times leading to increases in viral transmission. This could have implications for prevention of human exposures and the ability to anticipate and avoid high risk situations that might lead to exposure during peak SNV shedding seasons.

The second hypothesis we had which was tested using our models was that testosterone levels in male deer mice infected with SNV would alter viral replication, shedding, and transmission. This hypothesis was based on previous work on SEOV infection of Norway rats, which showed that sex hormone levels can significantly alter the levels of SEOV within the tissues of infected rats (Klein et al. 2001; Easterbrook et al. 2007a; Hannah et al. 2008). First we were able to effectively castrate male deer mice leading to a significant reduction in serum testosterone levels (Figure 11). Testosterone levels did not seem to influence SNV infection, as serum levels did not correlate with SNV RNA levels in the tissues, nor did castration or supplementation with exogenous testosterone affect SNV transmission rate (Figures 12 and 14; Table 4). One issue that arose during these experiments was the efficiency of testosterone delivery during infection through

the use of osmotic pumps. The viscosity of the carrier, propylene glycol may have affected the release of the drug such that the dosing did not reach its full potential, as seen in the serum testosterone levels at the end points in animals used in our experiments, which did not reach levels that were seen prior to castration (Figure 13). Nevertheless, there was no correlation between testosterone levels and SNV RNA levels seen and these data indicate that while sex hormone levels may impact infection with other hantavirus species such as SEOV, SNV infection in deer mice was not affected. While castration and testosterone supplementation did not affect SNV infection or transmission, this successful castration of deer mice and their use in an infectious disease model may prove to be a useful method for other infection models such as for bacteria or parasites. Testosterone levels can alter the levels of certain parasites in rodents, and deer mice are important reservoirs for various pathogens such as *Borrelia burgdorferi* and *Bartonella* species, among others (Hughes and Randolph 2001; Bai et al. 2011; Brown and Lane 1994; Peavey and Lane 1995). The techniques utilized here could provide useful in studies of infection of deer mice with these pathogens, including during co-infection of deer mice with these pathogens and SNV, which may play a role in altering SNV infection, potentially through immune mediated mechanisms. Overall, despite the refutation of both our hypotheses regarding increased SNV replication, shedding, and transmission, valuable and interesting data were collected along with the development and optimization of a castration technique that could be a valuable tool for researchers studying infectious diseases in deer mice moving forward.

Ultimately, one of the main goals of this thesis was to use the developed SNV transmission model to test various vaccine platforms that could subsequently be formulated into bait style vaccines for the prevention of SNV transmission amongst deer mouse populations. The vaccines that we had developed and had at our disposal included recombinant replication-deficient

Adenoviruses expressing SNV Gn or Gc, as well as a recombinant Vesicular stomatitis virus (VSV) expressing SNV GPC (rVSVΔG/SNVGPC). Previous work done using recombinant Adenoviruses as a vaccine platform against ANDV that only expressed Gn or Gc showed that this is an effective means of vaccinating animals and provided protection in a lethal disease model of ANDV (Safronetz et al. 2009). Recombinant Adenoviruses have also been used extensively as a vaccine platform for prevention of many human diseases as well as a platform for bait style vaccines against wildlife diseases (Mendoza et al. 2018). Recombinant VSV has also been used extensively as a platform for human diseases, but its efficacy as a platform for vaccinating wildlife has not been explored, although it has been shown to provide protection when administered orally (Jones et al. 2007). Therefore we wanted to use both approaches in an attempt to vaccinate deer mice and prevent SNV infection and then to prevent acquisition in our transmission model. Vaccination with recombinant Adenoviruses or rVSVΔG/SNVGPC resulted in low neutralizing antibody titers (Figures 18 and 20). The lack of a reliable and robust ELISA for detection of SNV-specific IgG at the time of those experiments limited the ability to assess humoral responses induced by these vaccines aside from neutralizing antibody. We have since developed an assay using concentrated rVSVΔG/SNVGPC particles as antigen, and this assay has shown improved sensitivity and reliability in detecting anti-SNV antibody as seen in Figure 22. The ability to reliably assess anti-SNV GPC antibody titers using this assay is important not only for the research involved with this project, but other research as well as diagnostics. We challenged vaccinated deer mice with SNV and assessed the levels of viral RNA in the blood and lungs during acute infection. While vaccination uniformly reduced SNV levels in the blood, only rVSVΔG/SNVGPC was able to significantly reduce SNV RNA levels in the lungs compared with control animals given PBS (Figure 21). We vaccinated groups of deer mice either intramuscularly or orally with

rVSVΔG/SNVGPC, and then subsequently exposed them to SNV-infected deer mice in our transmission model. Compared with control animals, vaccination was able to significantly reduce the risk of becoming infected with SNV (Table 6). This data provides a promising proof of concept that oral vaccination against SNV can prevent acquisition of infection during exposure to infected animals. Further characterization of this protection should be performed including the length of protection afforded by vaccination using this platform, which will be important for development of bait vaccines to be deployed at specific times or seasons. Immunogenicity and protective efficacy following the ingestion of the vaccine in form of bait as opposed to direct oral gavage will also be important for further characterization of this platform and the development of a bait style vaccine in general. Future work in our lab includes more testing of this vaccine and additional platforms for effective control of viral transmission. This preliminary work showing that oral vaccination is capable of preventing transmission of hantaviruses is an important first step in the development and testing of various vaccines for the potential protection of rodent populations against viral pathogens such as hantaviruses and arenaviruses. This transmission model, while artificial, provides a means of testing the efficacy of vaccination in a system that results in a high level of exposure to the virus. We expect this model and similar versions of it to be a valuable tool moving forward for studying not only transmission of rodent-borne viral zoonoses including hantaviruses, but also for its utility in testing vaccine candidates for their viability as bait vaccines to be employed to protect wildlife populations.

7.2 Future Directions

The main goal of the thesis was to test our hypothesis regarding direct transmission of SNV between deer mice, and to establish a model that could be used to test further what contributes to SNV persistence and transmission. Additionally, we wanted to use this model to test candidate

vaccines against SNV for their ability to prevent transmission within a deer mouse population. While we were able to reliably use both SNV infection and transmission models for the work described here, there are still several interesting avenues of research that would provide valuable insight into hantavirus infections and ecology.

One limitation of the model we used was the relatively short time frame for evaluating SNV transmission rates. We utilized a system which we thought would give the greatest chance of seeing transmission events, and the relatively modest rates seen in our studies highlight the sporadic nature of SNV shedding in infected deer mice and the overall rarity of transmission events that likely occur when deer mouse encounters are less frequent. Longer term studies to determine the amount of time needed for higher transmission rates might give us an opportunity to examine what factors lead to increased viral replication and shedding and contribute to transmission. Another issue is the low number of animals per cage in our experimental design. This was based on the limitations of the animal caging system inside our CL-4. Larger caging with a higher number of deer mice housed with an infected "seeder" mouse may show altered dynamics of transmission compared with the separate caging presented here. Some of these are questions that could be easily answered with the access we now have to our transmission model.

While we attempted to identify factors that contribute to increased levels of SNV replication in infected deer mice, our hypotheses that heat shock, thermogenesis, and testosterone levels might alter infection and transmission rates ultimately did not show any differences. While we did not see any differences in the studies we performed, this is still an exciting area of research that is important for understanding hantavirus infections of reservoir hosts. For heat shock responses, further methods for inducing these responses, including cold stress might be a better approach for attempting to see differences in viral replication during SNV infection. We did not

examine the effect of the thermogenesis inducing drug CL316,243 on transmission rate, so this is an area that is yet to be explored. And despite our unsuccessful attempt at influencing SNV infection outcome through castration and testosterone supplementation, further studies on the effect of sex hormones and immune modulation in deer mice could lead to new insights into SNV infection and transmission. Our testosterone supplementation, while providing a significant increase in serum testosterone levels throughout infection, was not as efficient as expected, as we did not reach serum testosterone levels seen prior to castration. The osmotic pump delivery method was chosen to avoid the use of sharps in the CL-4 setting that would have come with daily injection of the hormone. It is possible that a higher dose, or a similar dose delivered via injection could lead to a different outcome in a similarly designed experiment. Another issue with our attempts to influence SNV infection is that our experiments were carried out during acute infection, when SNV levels are presumably at the highest throughout infection course. Future studies examining the effects of some of the factors mentioned here during long-term persistent SNV infections may result in differences we did not see during acute infection. The higher levels of SNV RNA seen in the first few weeks SNV infection may not have allowed for the appropriate sensitivity to detect changes in replication within various tissues. This is an important area that should be explored further moving forward. Aside from the factors that we examined here, there are other factors that may impact SNV ecology. Co-infection with different bacteria or parasites that are commonly found in deer mice could significantly affect SNV infection through modulated host immune responses. Several species are found in deer mice such as *Borrelia burgdorferi, Bartonella* species, *Francisella tularensis, Rickettsia rickettsia, Leptospira* species, *Babesia* and others. Experiments into how co-infection may influence SNV replication could increase our understanding of how the virus is transmitted between deer mice in certain populations and could potentially lead to

identification of causes of increased transmission potential. There is also the possibility of insect vectors playing a role in hantavirus transmission, although this has not been extensively studied. Other Bunyaviruses are carried by insect vectors such as mosquitos and ticks. The possibility that a vector may play a role in SNV transmission between deer mice is intriguing and could explain some of the experimental findings that have been reported such as low transmission rates and how the virus may persist in populations following extended periods with little viral spread. Determining whether SNV can be transmitted by an insect vector would require extensive and thorough field work as well as experimental infection models and vector competency studies. It remains to be seen how or if vector-borne transmission can play a role in transmission of SNV or other hantaviruses, but this is an interesting avenue for further research.

Finally, our goal following the development of infection and transmission models for SNV in deer mice was to test various vaccine platforms for their ability to protect deer mice from infection following oral immunization. An oral bait vaccination program similar to that used for rabies, plague, and Lyme disease could be a useful alternative approach to human vaccination that could reduce the incidence of HCPS cases in at risk groups. We were able to show that vaccination of deer mice with rVSVΔG/SNVGPC can protect deer mice from SNV infection in a direct exposure setting. This data provided valuable proof of concept that such a strategy could realistically be employed following further development, optimization, and testing. Certain critical aspects of bait vaccine use were not examined here, but would be of importance moving forward with this project. The vaccine dose used in our experiments was kept low and was based on previous use of VSV vectors as vaccines in mice. The dose needed for successful oral immunization following bait ingestion is something that would need to be determined for each specific vaccine. However the efficacy shown here at a low dose via the oral route suggests that

ingestion of even a portion of a vaccine bait could be protective. Formulation of various vaccine vectors into baits that will be palatable and able to be ingested by small rodents will also be something to consider. Alternative vaccine platforms such as Vaccinia virus or other pox vectors, which have been used extensively or even species-specific vectors such as Cytomegalovirus could be explored. An important consideration is the coverage of the wildlife population that would be needed to provide protection against the spread of the virus throughout that population. For SNV, the basic reproductive number is estimated to be 1.3, with a range of 0-4 (Kaplan et al. 2016). Therefore the percentage of animals that would need to have vaccinated to provide herd immunity would be 23-75%. Larger scale experiments on the effectiveness of each platform to be able to provide the needed threshold of protection will be important. Hopefully the development of this transmission model will provide the basis for development of similar models for other rodent borne pathogens moving forward including hantaviruses. A similar approach would be feasible and could provide a means of reducing the disease burden for viruses such as PUUV and HTNV, for which the numbers of cases is far higher than what is seen with New World hantaviruses. The work presented here provides the framework needed to develop models for these viruses and to pursue means of preventing infection through means other than human vaccination.

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