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TRANSMISSION ECOLOGY OF SIN NOMBRE HANTAVIRUS IN DEER MOUSE POPULATIONS IN OUTDOOR ENCLOSURES

by

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Population Biology, Ecology and Evolution

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by
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B.S., Tulane University, 2001

Advisor: James N. Mills, Ph.D

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Abstract

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Since the inception of the multidisciplinary field of disease ecology in 1979, ecologists and public health researchers have been exploring natural disease systems and attempting to build predictive models of disease. Disease models of directly transmitted pathogens often predict that increased host population densities result in increased levels of disease in an environment, but mark-recapture data from multiple well-studied rodent-virus host-pathogen systems have reported conflicting results. Concurrently, these field studies have identified the importance of seasonality, host physiology and population processes on infection dynamics. Traditionally, transmission information is often deduced from disease prevalence data, or determined in highly artificial laboratory settings—both of which do not adequately illustrate the natural progression of disease through a host population, and often separate ecological factors from within-host pathological and immunological factors. In this dissertation, I address these discrepancies and explore questions about the role of host population density, seasonality, and host aggression on disease transmission by conducting manipulative field transmission experiments using deer mice (*Peromyscus maniculatus*) naturally infected with Sin Nombre hantavirus (SNV) in outdoor enclosures. This project is largely interdisciplinary and uses ecological, molecular, and immunological approaches to understand SNV infection and transmission in a natural host-pathogen system. The results of this study indicate that seasonality and host heterogeneities in behavior and viral infection load may have a stronger influence on disease transmission dynamics than host population density. This project reports the first successful SNV transmission experiment in a closed deer-mouse population. Also, in the process of this research, a new sub-specialty of disease ecology—transmission ecology—defined as the study of within- and between-host infection dynamics and their relationship to transmission-related host population processes and environmental conditions in an effort to better understand natural disease systems—was developed.

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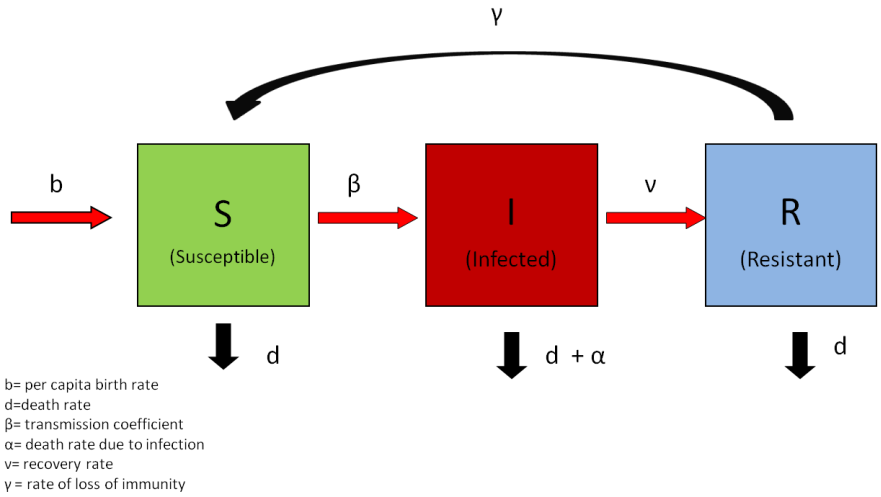
1 Introduction

Background

Although dynamical approaches to epidemiology were initially established in the early 1900s, and medical researchers were exploring the dynamics of ectromelia (mousepox) and *Pastuerella muri* epizootics in laboratory mouse populations in the 1930-1940s (Greenwood et al. (1936), and Fenner 1948, 1949), ecologists did not recognize the importance of parasitism effects on host population processes as an ecological force until the late 1970-1980s. In 1979, Anderson and May fused the fields of parasitology, epidemiology, and population biology by changing the underlying assumptions of epidemiological models of human disease to better fit wildlife populations. They successfully fit their models to the only available experimental data of microparasite epizootic dynamics from experiments by Greenwood et al., (1936) and Fenner (1948a, b, 1949). The Susceptible-Infected-Recovered (SIR) model, first formalized by Kermack and McKendrick (1927), but adapted and popularized by Anderson and May (1979) is the most well known and widely used dynamical model utilized to explore host-parasite population dynamics. In the SIR model, the hosts are divided into three categories: S- susceptible: a host not yet been exposed to the pathogen and is susceptible to infection; I-Infected: host has been infected by the pathogen and can infect other susceptible hosts; R-recovered: a host has cleared the infection and is resistant to re-infection. In its simplest form, a susceptible host is born (b), comes into contact with an infectious host (βSI), becomes infected (I), and in turn is able to infect other susceptible hosts (βIS), until it a) recovers and becomes susceptible again (γR), b) recovers and gains immunity (νR), or c) dies from infection (αI), (see flow chart diagram, Figure 1.1).

Figure 1.1: Flow Chart Diagram for Compartmental SIR Model, adapted from Anderson and May (1979)



Here, beta (β) is the transmission coefficient that combines the probability of an infectious host contacting a susceptible host, and the possibility of that contact being infectious. There are two types of underlying assumptions concerning the nature of host contacts leading to infection: density dependent and frequency dependent. Density dependent transmission assumes that the probability of transmission is related to host population size (βSI), that is, the more hosts in an area, the more likely they are to contact one another, and lead to higher levels of disease. Anderson and May (1979)'s original work on Greenwood's data set indicated that epizootics involving directly transmitted microparasites operate in a density dependent manner. Models assuming density dependent transmission also have been utilized successfully in exploring the dynamics of various directly transmitted human pathogens, such as measles (Earn et al. 2000) and foot-and-mouth disease (Tildesley et al. 2006). In contrast, frequency dependent transmission assumes that the likelihood of a susceptible host contacting an infected host and leading to transmission is independent of population size ($\beta SI/N$); these types of models have

generally been used to increase understanding of human sexually transmitted diseases (e.g. human immunodeficiency virus; May and Anderson (1987).

Anderson and May's work in early 1980s stimulated interest in the exploration and quantification of disease systems in wildlife populations. Subsequently, a new subspecialty emerged: disease ecology, an interdisciplinary approach to natural disease systems combining principles and methods from multiple fields, such as mathematics, zoology, epidemiology, ecology, pathology, and immunology. Disease ecology, in its broadest sense, is the study of the effects of environmental factors on the relationship between the behavior and ecology of hosts and parasites and resulting influences on host and parasite population processes. Within this general framework, various sub fields explore different facets — from large scale aspects of disease systems (such as geographical and ecosystem related patterns and processes) to microcosms (within host immunology; eco-immunology). In the past thirty years, the interest in disease ecology has increased exponentially, resulting in profuse theoretical and empirical explorations by researchers, which have been expanded to day-to-day application in wildlife, human, and agricultural populations by public health officials and others. However, an interesting aspect of the influx of empirical data from natural populations is how, while supporting some aspects of current theory, it often refutes or provides conflicting data on some of the basic underlying assumptions of theoretical models of disease transmission. This back-and-forth process slowly leads to more precise and predictive models of disease dynamics. One such area of conflict is whether transmission of directly transmitted pathogens in wildlife systems is density or frequency dependent, or a mixture of the two.

An excellent example of this conflict is the transmission of directly transmitted microparasites in natural rodent populations. As rodents are often hosts of zoonotic

pathogens, they are a particularly well-surveyed host-parasite system by both disease ecologists and public health researchers. Besides contributing to our knowledge of infection prevalence of potentially hazardous pathogens, these studies have provided some preliminary (and often divergent) data about the relationship of host population density and infection prevalence and insight into influential ecological factors in the maintenance of diseases in the wild.

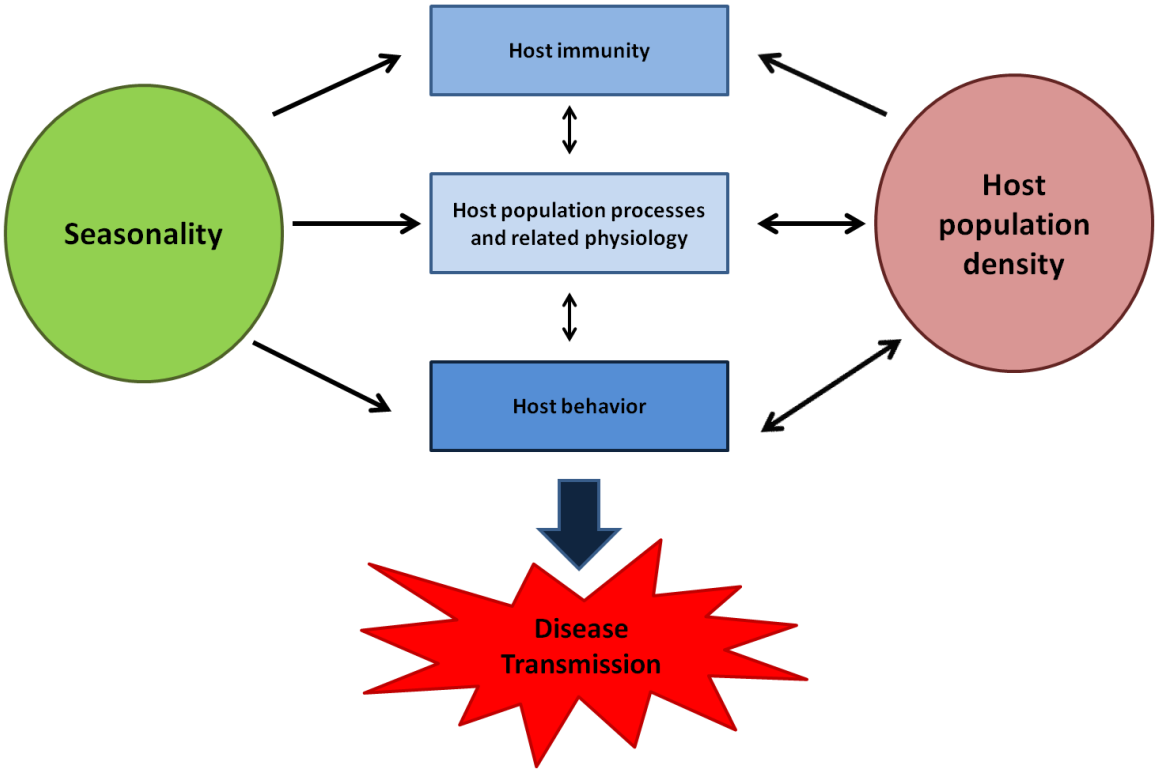
Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are rodent-borne negative-stranded, tripartite, RNA viruses that can be highly pathogenic to humans. Hantaviruses occur nearly worldwide and, in general, each virus is associated with a particular host species in the rodent families *Muridae* and *Cricetidae* or insectivores in the family *Soricidae*. In the Old World, pathogenic hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) (e.g. Hantaan virus (HTNV), Puumala virus (PUUV), Seoul virus (SEOV), Dobrava virus). In the New World, several hantaviruses hosted by cricetid rodents are known to cause hantavirus pulmonary syndrome (HPS) (e.g. Black Creek Canal virus (BCCV), Sin Nombre virus (SNV), Bayou virus, and Andes virus (ANDV)). In Europe and the Americas, multiple mark-recapture studies have been initiated in the past twenty years to monitor changes in hantavirus infection incidence and prevalence, document rodent population density fluctuations, and identify environmental and ecological factors associated with these changes. Similar studies have also been conducted in arenaviruses (Family *Arenaviridae*) in multiple rodent hosts in South America, Africa, and Europe (Mills et al. 1992, Calisher et al. 2007, Fichet-Calvet et al. 2007, Tagliapietra et al. 2009) and cowpox virus (genus *Orthopoxvirus*) infection in field voles (*Microtus agrestis*) in Great Britain (Burthe et al. 2006, Begon et al. 2009).

Host Population Density and Infection Prevalence

Some longitudinal studies of zoonotic viruses in reservoir host populations provide support for a direct relationship between host population density and infection prevalence. Concurrent levels of high host population densities or abundances and high infection prevalence (as indicated by presence of antibody) have been documented for lymphocytic choriomeningitis arenavirus (LCMV) infection in the yellow-necked mouse (*Apodemus flavicollis)* in Italy (Tagliapietra et al. 2009), and Bayou hantavirus in *Oryzomys palustris* (McIntyre et al. 2005) in the southeastern United States. Boone et al. (2002) reported a significant decrease in the proportion of deer mice (*Peromyscus maniculatus*) with an acute SNV infection concomitant with a significant decrease in population density. In Argentina, seasonal maximum levels of Junin arenavirus antibody prevalence were concurrent with high population densities of *Calomys* (Mills et al. 1992). In a biodiversity hantavirus experiment, when non-reservoir host species were removed, there was an increase in the relative and total abundance of the reservoir host species in a given site, as well as an increase in infection prevalence, indicating a density dependent increase of transmission (Suzan et al. 2009). However, infection prevalence in wild rodent populations in a given place is most often associated with host population densities and dynamics in the prior season or year (known as delayed density dependence). Delayed density dependent disease dynamics have been reported in arenaviruses (Mills et al. 1994), hantaviruses (Pearce-Duvet et al. 2006, Madhav et al. 2007), and cowpox virus (Begon et al. 2009), with time lags between peak population densities and peak infection prevalences ranging from 3 months (cowpox virus; Burthe et al. (2006)), to 8-15 months (hantaviruses; Niklasson et al. (1995), Adler et al. (2008), Luis et al. (In review)).

As more data have been accumulated about disease dynamics in wild rodent populations, the clearer it has become that it is difficult to separate host population density from other influential environmental factors such as seasonality and host population processes (e.g. reproduction), host behavioral and immunological heterogeneities, and pathogen dynamics. All of these factors have direct influences on disease cycles, and also are highly interconnected (see Figure 1.2). In fact, current studies indicate that there may be seasonal shifts in the transmission mechanisms of rodentborne zoonotic viruses, so that host contact structure and subsequent transmission dynamics may be density dependent, frequency dependent, or delayed density dependent during different times of the year (Niklasson et al. 1995, Smith et al. 2009, Tersago et al. 2011).

Figure 1.2: Seasonality, host population density, and host intrinsic characteristics related to disease transmission in natural populations.



Seasonality, Host Population Density, and Infection Prevalence

Seasonal epizootic cycles have been documented for multiple zoonotic viruses in their rodent hosts (Mills et al. 1992, Mills et al. 1999, Escutenaire et al. 2000, Cantoni et al. 2001, Fichet-Calvet et al. 2007, Begon et al. 2009, Tagliapietra et al. 2009, Kallio et al. 2010). These cycles have been linked to climatic factors (Yates et al. 2002, Mills 2005, Tersago et al. 2009, Luis et al. In review), availability of food and resources (Yates et al. 2002, Mills 2005, Tersago et al. 2009), and host reproductive processes (Niklasson et al. 1995, Madhav et al. 2007, Begon et al. 2009).

Seasonal variations in temperature, precipitation, and humidity can influence host - parasite systems directly by influencing host survival (Burthe et al. (2008), Luis et al. (2012)) and host immunity (Beldomenico et al. 2008, Lehmer et al. 2010). Seasonal weather conditions and variation of photoperiod can also indirectly influence host-pathogen relationships through the availability of resources (Tagliapietra et al. (2009), Tersago et al. (2009), Luis et al. (In review)), and affect both the number and population density of susceptible hosts in a habitat.

Host reproductive processes are an integral component of virus dynamics in rodent populations. Not only does reproduction produce susceptible hosts, but reproduction related behaviors, such as territoriality, congregation, and dispersal, greatly influence the underlying contact structure in a population, and subsequent pathogen dynamics. Season-related photoperiod influences hormonal cues that initiate and cease reproductive behaviors, and in turn, affect disease dynamics in a given environment. Timing of annual peaks in cowpox virus infection in field vole populations is associated with both birth and recruitment rates, and usually occur during the late summer or early fall, at the conclusion of the breeding season (Begon et al. 2009). Vole reproductive activity was a dominant indicator of PUUV

infection risk in certain seasons, indicating that vole reproductive activity and length of the breeding season drive PUUV dynamics in Belgian populations of bank voles (*Myodes glareolus*) (Tersago et al. 2011). Seasonal peaks of hantavirus infection prevalence in rodent populations in temperate habitats are hypothesized to be influenced by both host recruitment and overwinter mortality (Mills et al. 1999). In fact, in many rodent-virus host pathogen systems, infection prevalence (Tagliapietra et al. 2009) or transmission (as indicated by seroconversion; Douglass et al. (2007) is highest during the breeding season. Reproductive activity can also influence survival, especially in infected animals. Male and female deer mice in breeding condition had decreased apparent survival in comparison to non-breeding deer mice; when infected with SNV, animals in breeding condition showed a 15.7% decrease in survival in comparison to non-breeding infected animals (Luis et al. 2012).

Host Population Density, Host Behavior and Infection Prevalence

In a directly transmitted disease system, aggressive and social behaviors are strongly linked to infection prevalence, as they result in the probability of increased contact between infectious and susceptible individuals. For rodent-borne viruses, mark-recapture studies often report that rodents with wounds are more likely to be infected (Glass et al. 1988, Douglass et al. 2001, Escutenaire et al. 2002, Hinson et al. 2004, Tagliapietra et al. 2009, Mills et al. 2010), suggesting that aggressive behavior is linked to infection. These mechanisms are more pronounced during the breeding season, as both males and females are both more likely to be aggressive. Males show increased territoriality and aggression in order to secure mates and nesting sites, while females are more aggressive in order to protect their young from males and other females (Wolff 1989). For both arenaviruses and hantaviruses, adult males often have the highest infection prevalence and are the most likely to be wounded (Mills et al. 1992, Mills et al. 2010). While both sexes are territorial, males are

far more likely to be combative than females (Wolff 1989), which likely explains why males often have such a high infection prevalence in natural populations. Males are considered to be the primary transmitters of directly transmitted viruses for all studied hantaviruses and some arenaviruses (Mills et al. 1992, Mills et al. 2010).

Hantavirus Infection and Transmission

In addition to the impacts of environmental and population level processes on host-parasite dynamics, the within-host immunological and pathological aspects of infection and their relationship to these outside factors are also crucial to the emergence and maintenance of rodent-borne zoonoses in an environment. Usually questions about within-host infection dynamics are explored in laboratories. Hantaviruses are unique rodentborne zoonoses which have been investigated extensively by in the wild by ecologists and mammalogists, and in the laboratory by virologists and immunologists.

Laboratory experiments have yielded crucial information about the persistence of hantavirus infections and related host immunology. Hantavirus-infected hosts are thought to be chronically infected and shed virus for extended periods – perhaps the lifetime of the host (Peters et al. 2006). Multiple laboratory studies of experimentally infected Old and New World hantavirus hosts have suggested a consistent pattern of infection and viral shedding in an individual host. After inoculation with the species-specific hantavirus, the reservoir host experiences a brief viremia at 7-10 days post infection, followed by detection of viral antigen in multiple tissues and infectious virus being shed in saliva and excreta. While animals develop a neutralizing immunoglobulin G (IgG) antibody response, infectious virus continues to be shed into the environment (LeDuc et al. 1992). Although humans primarily become infected by inhaling aerosolized virus from rodent saliva and excreta, the primary route of infection in rodent hosts appears to be via direct contact during aggressive

interactions. (i.e., biting and scratching) (Glass et al. 1988, Douglass et al. 2001, Hinson et al. 2004), but indirect transmission is also possible (Hutchinson et al. 2000, Hardestam et al. 2008).

Although the New World BCC virus, as well as the Old World HTN, PUU, and SEO viruses, can be transmitted to naïve cage mates and can be isolated from the excreta or saliva of experimentally infected hosts (Yanagihara et al. 1985, Gavrilovskaya et al. 1990, Dohmae et al. 1994, Hutchinson et al. 1998, Kariwa et al. 1998, Hardestam et al. 2008), transmission of SNV among deer mice in a controlled environment has largely failed. Only one transmission event was recorded in a susceptible-infected rodent pair experiment that exposed 54 naïve animals to one experimentally SNV infected animal (Botten et al. 2002). As a result, there is limited knowledge of transmission and transmission-related immunological and viral processes in a system in which the host ecology and other transmission related environmental factors are well-studied.

Transmission ecology: A new focus in disease ecology

Transmission ecology may be defined as the study of within- and between-host infection dynamics and their relationship to transmission-related host population processes and environmental conditions in an effort to better understand natural disease systems. Traditionally, investigations of within-host hantavirus infection processes and their relationship to transmission are conducted in laboratories with an experimentally infected rodent paired with a susceptible rodent. These studies are crucial and provide readily interpretable information. However, they can mask influential aspects of variation in both pathogen and the host, and the interplay between immunological and virological components of the host-parasite relationship that may be important to understand disease

systems in natural populations. Also, the susceptible-infected rodent pair approach limits host interactions to only one other host, cutting off the link to population processes.

Descriptive studies have documented evidence of the influences of host population density, seasonality, and host population processes on disease systems in wildlife populations; numerous laboratory infection experiments have explored the mechanistic aspects of transmission. However, theory and surveys can go only so far in elucidating the actual mechanisms of disease transmission in natural populations. An important way to increase our understandings of natural host-pathogen systems is through manipulative field experiments designed to test the very questions we have been exploring through observational and theoretical data. Outdoor manipulative experiments are an alternative that control for some variables in a relatively natural environment. Disease ecologists and ecological immunologists have progressed to exploring multiple host/disease systems in the wild and laboratory, but there are currently no experiments testing current theories of disease transmission and spread with one host–one disease systems in the wild.

Using outdoor enclosures, we tested effects of deer mouse (*Peromyscus maniculatus*) population density and seasonality on transmission dynamics of Sin Nombre hantavirus. In early summer, mid-summer, late summer, and fall 2007–2008, predetermined numbers of infected and uninfected adult wild deer mice were released into enclosures and trapped weekly or bi-weekly. The research presented in this dissertation sets out to test and explore some of the main ecological processes that are thought to influence disease cycles and host-parasite relationships. These experiments are interdisciplinary and combine methods and expertise of multiple fields including ecology, molecular biology, virology, immunology, animal behavior, and mammalogy.

The overall objective of the work presented here was to identify the ecological, immunological, and virological factors most influential in sustaining SNV transmission in natural deer mouse populations. The first specific objective was to test whether and how certain ecological, behavioral, and population-process related factors considered crucial to disease cycles influence host-parasite relationships in the wild. The second specific objective was to determine some virological and immunological aspects of a natural hantavirus infection, and whether and how these relate to individual and population level processes.

2 Effects of Population Density and Seasonality on Sin Nombre Hantavirus Transmission in North American Deer Mice (*Peromyscus maniculatus*) in Outdoor Enclosures

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In Submission

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Abstract

Surveys of wildlife host-pathogen systems often document clear seasonal variation in transmission; conclusions concerning the relationship between host population density and transmission vary. In the field, effects of seasonality and population density on natural disease cycles are challenging to measure independently, but laboratory experiments may poorly reflect what happens in nature. Outdoor manipulative experiments are an alternative that controls for some variables in a relatively natural environment. Using outdoor enclosures, we tested effects of North American deermouse (*Peromyscus maniculatus*) population density and season on transmission dynamics of Sin Nombre hantavirus. In early summer, mid-summer, late summer, and fall 2007–2008, predetermined numbers of infected and uninfected adult wild deermice were released into enclosures and trapped weekly or bi-weekly. We documented 18 transmission events and observed significant seasonal effects on transmission, wounding frequency, and host breeding condition. Apparent differences in transmission incidence or wounding frequency between high- and low-density treatments were not statistically significant. However, high host density was associated with a lower proportion of males with scrotal testes. Seasonality may have a stronger influence on disease transmission dynamics than host population density, and density effects cannot be considered independent of seasonality.

Keywords: deermouse, enclosure experiments, host population density, *Peromyscus maniculatus*, seasonality, Sin Nombre hantavirus

2.1 Introduction

In the past 30 years, numerous theoretical models have been proposed to explain how pathogens become established and spread in host populations. Early models assumed that the driving force behind directly transmitted parasites was population density (density dependent transmission) and, because these models were useful to understanding many human diseases, they were applied to wildlife populations (Anderson and May 1979, Dobson and Hudson 1995). For a horizontally transmitted pathogen, higher host population density may lead to higher prevalence of infection, because there is an increased number of potential hosts and because more susceptible hosts provide more opportunities for direct transmission through contact (Adler et al. 2008). Additionally, higher densities of infective donors and susceptible hosts may amplify indirect transmission by increasing the amount of infectious pathogen in the environment (Sauvage et al. 2003). Higher host abundance may also result in increased competition for limited resources and mates, increasing stress and leading to decreased immunological capacity (Yin et al. 1995). However, the relationship between wildlife host population density and disease prevalence is complex, as reviewed by Adler et al. (2008). While some mark-recapture studies of hantaviruses and arenaviruses in rodent populations in the United States and Europe have indicated a positive concurrent relationship between host population density and infection prevalence (Mills et al. 1999, Tagliapietra et al. 2009), others showed an inverse relationship or no direct association (Douglass et al. 2001, Pearce-Duvet et al. 2006, Mills et al. 2010). Infection prevalence in wild rodent populations is often associated with host population densities and dynamics in a prior season, an effect known as delayed density-dependent prevalence (Niklasson et al. 1995, Burthe et al. 2006, Madhav et al. 2007, Begon et al. 2009). For example, regional wild North American deermouse (*Peromyscus maniculatus*; hereafter deermouse) populations in

Montana show maximum Sin Nombre hantavirus (SNV) infection (as indicated by antibody prevalence) in the spring, and this peak is often positively associated with the size of the deermouse population the preceding fall (Madhav et al. 2007, Carver et al. 2011). Also, a threshold infection prevalence (Madhav et al. 2007) and critical host density (Luis et al. In review) may be necessary to establish and maintain SNV infection cycles in deermouse populations in Montana. However, some directly transmitted wildlife pathogens display characteristics of frequency-dependent dynamics (where transmission likelihood is independent of population density) (Begon et al. 1999, Hamede et al. 2008), or transmission dynamics that vary between density and frequency dependence according to season (Smith et al. 2009).

The effects of seasonality on disease dynamics in wildlife are another focus of disease ecologists. Seasonal variation in precipitation, temperature, and resource availability can influence host population dynamics, host physiology, and disease dynamics in wildlife host populations (Altizer et al. 2006). Rodent-borne zoonotic viruses (e.g., hantaviruses, arenaviruses, and cowpox virus) often have seasonal cycles of infection prevalence (Mills et al. 1992, Mills et al. 1999, Escutenaire et al. 2000, Cantoni et al. 2001, Fichet-Calvet et al. 2007, Begon et al. 2009, Tagliapietra et al. 2009). Peaks in transmission often coincide with the reproductive season, a time of high social interaction in natural populations (Escutenaire et al. 2000, Douglass et al. 2001, Mills et al. 2010).

Hantaviruses are directly transmitted, specialist microparasites endemic in natural rodent and insectivore populations; some, including SNV, are pathogenic for humans. Hantaviruses generally establish a persistent infection with long-term shedding in a single natural host species (Botten et al. 2003, Peters et al. 2006) . Because hantavirus infection is chronic, the presence of IgG anti-hantavirus antibody in rodent blood is used as an indicator

of active infection. Studies of Old World hantaviruses [e.g. Puumala virus (PUUV)], and the New World SNV and Black Creek Canal viruses, indicate that laboratory-inoculated hosts are most infectious and shed the greatest quantity of virus during the acute phase of infection (first 60–90 days) (Hutchinson et al. 2000, Botten et al. 2003, Hardestam et al. 2008). Although humans primarily become infected by inhaling aerosolized virus from rodent saliva and excreta, the primary route of infection in rodent hosts appears to be via direct contact during aggressive interactions. (i.e., biting and scratching) (Glass et al. 1988, Douglass et al. 2001, Hinson et al. 2004). Laboratory studies of PUUV indicate that rodent hosts may also be infected via the respiratory route (Hardestam et al. 2008), and that PUUV can remain infectious in the environment for up to 15 days (Kallio et al. 2006).

The individual effects of seasonality and density on natural disease cycles are often hard to tease apart from each other and from other confounding factors driving host parasite systems. One way to explore and quantify these effects is through manipulative field experiments using a well-studied host-pathogen system. The deermouse-SNV host-pathogen system has been a subject of intensive longitudinal studies that have improved understanding of the relationships between SNV transmission dynamics with seasonal factors and with host population density (Douglass et al. 2001, Kuenzi et al. 2005, Calisher et al. 2007, Dearing et al. 2009). Nevertheless, longitudinal studies can be difficult to interpret because of a multitude of confounding factors that characterize uncontrolled, open populations. In addition, a pattern observed at any given time is the product of complex and imperfectly known historical events.

A partial solution to these problems is the use of outdoor, semi-natural enclosures that approximate natural field conditions more closely than does a laboratory. Such studies also allow working with a closed population of a limited number of individuals of known

sex, age, physical condition, and infection status, and the events observed during the experiment are largely a consequence of those well known experimental conditions. We used the deermouse-SNV host-pathogen system in Montana to explore the effects of density and seasonality on pathogen transmission. Longitudinal field studies in Montana have demonstrated that the greatest number of seroconversions and the greatest proportion of deermice with detectable SNV RNA are found in the mid-to-late breeding season (June-September; (Kuenzi et al. 2005, Douglass et al. 2007)). These data suggest that June-September is the period of greatest virus transmission and, as such, would be the best time to conduct transmission experiments in nature. We conducted 4 transmission experiments using wild, adult, male deermice in outdoor enclosures in Montana during the summer and fall of 2007 and 2008. The enclosure system allowed us to focus on the effects of season and host population density on transmission by controlling for demographic and historic factors, including prior host population densities, by using only adult males and restarting the experiment with new mice or new configurations of mice after 1 or 2 months.

Using mice naturally infected with SNV as donor mice, we tested the hypothesis that the frequency of SNV transmission in deermouse populations is positively correlated with population density, and that this correlation is independent of season. If true, we hypothesized that high-density enclosures would have a greater frequency of transmission events than low-density treatments regardless of when we initiated the experiment. We also explored the influence of season and population density on host reproductive condition, aggressive encounters, and weight gain. In this paper, we focused on ecological, behavioral, and physiological aspects of host population density and seasonality as they relate to SNV transmission. In a second paper, we will focus on the molecular and immunologic aspects of transmission including time course of infection and differences among individual hosts.

2.2 Research Design and Methods

Ethics Statement

All animal work was conducted according to relevant national and international guidelines. All components of this study were reviewed and approved by the appropriate institutional animal care and use committees (Emory University IACUC protocol #D10-1109-02R07, U.S. Centers for Disease Control and Prevention IACUC protocol #1500MILRODX-A1, and University of Montana IACUC protocol #AUP 009-07). The study was also reviewed and approved under Emory University Biosafety protocol #100-2008. No trapping permit is required for trapping rodents in Montana.

Study site and enclosure description and protocols

This study was conducted in grassland near Butte, Montana, USA, May-October 2007 and August- September 2008. We conducted 4 experiments—1 preliminary transmission experiment (experiment A) and 3 density experiments (experiments 1, 2, and 3; Table 2.1, Figure 2.1). Experiments were run in 6, 0.1-ha enclosures constructed of sheet metal (Fuller and Blaustein 1996, Schauber et al. 1997), with walls extending approximately 1 m above ground and 0.6 m underground. The enclosures were built in a shrub-steppe habitat. The vegetation inside the enclosures consisted of a mixture of Rocky Mountain juniper (*Juniperus scopulorum*), big sagebrush (*Artemisia tridentata*), rubber rabbitbrush (*Chrysothamnus nausiosus*), Idaho fescue (*Festuca idahoensis*), blue bunch wheatgrass (*Agropyron spicatum*), and Canadian blue grass (*Poa compressa*). To impede escapes from the enclosures, all vegetation within 1 meter of the enclosure walls, both inside and outside of the structure, was mowed, and any branches or vegetation hanging or growing in the 1-meter perimeter were cut or removed before and during the experiment.

Table 2.1: Experimental design and transmission events per experiment and density treatment for SNV transmission experiments in deermice in outdoor enclosures near Butte, Montana, 2007–2008.

Exp	Season	Dates	Type of Exp	Duration (weeks)	Sampling Frequency	Transmission Events	Total Susceptible [a]	Total Susceptible (adjusted)[b]
EXP A	Early Summer	Jun 11-Jul 9 07	Transmission	4	every 2 weeks	6	18	17[c]
EXP 1	Mid-Summer	Jul 17-Aug 23 07	Density	5	every 2 weeks	3: high	30	24[d]
EXP 2	Late Summer	July 24 – Sept 25 08	Density	8	every week	6: high 2: low	30	33[e]
EXP 3	Fall	Sept 03-Oct 16 07	Density	6	every 2 weeks	1: high	30	27[f]

Exp=experiment; High = high density treatment; low = low-density treatment.

[a] See Figure 1

[b] Adjusted; number of susceptible mice used to calculate transmission incidence.

[c] Excludes 1 mouse never recaptured after initial release into the enclosure.

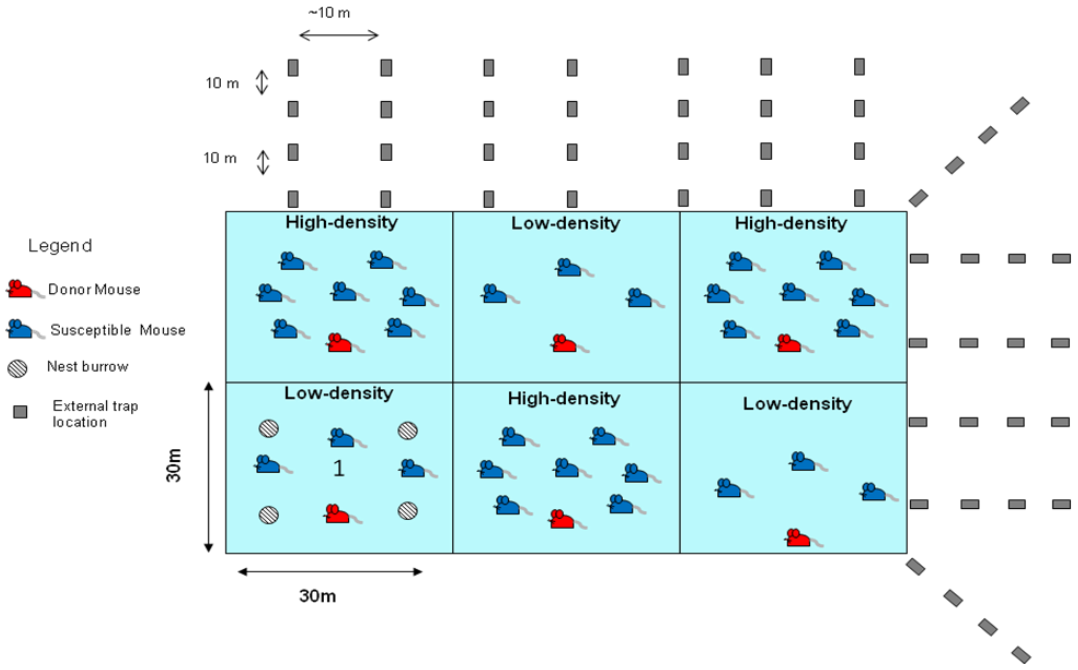
[d] Excludes 3 mice never recaptured after initial release, 2 susceptible mice from low-density enclosure in which the infection status of donor was unclear, and 1 mouse which we cannot rule out as being exposed prior to release in the enclosures.

[e] Includes 3 substitute susceptible mice that were released into the enclosures to replace dead mice and 1 escapee to keep population densities constant.

[f] Excludes 3 mice never recaptured after initial release into the enclosure.

Each enclosure had 4 evenly spaced underground nest burrows (Kaufman and Kaufman 1989) that provided safe, permanent cover for the mice. Each nest burrow consisted of a 20.3 cm diameter polyvinyl chloride (PVC) pipe nest chamber that was 45.7 cm tall, with a 2.5-cm, cement floor. Each burrow had a 2.5-cm diameter PVC pipe entrance tunnel set at a 45 degree angle. They had insulated lids that consisted of a 4 × 28-cm wooden board affixed with circular pieces of Styrofoam, and a galvanized metal lid (Kaufman and Kaufman 1989). Nest burrows were buried so that the top was flush with the surface of the soil and the insulation fit inside the top 2 inches of the nest burrow, with the roof extending over the surface of the soil. The entrance to the nest burrow had small rocks arranged to keep rain water out. A water bottle was wired to the bottom of each nest burrow lid and replenished when almost empty. Scratch grain and apple chunks were scattered within the enclosures weekly or as needed.

Figure 2.1: Diagram of enclosures, nest burrows, and experimental design for density experiments. Each enclosure had 4 nest burrows as depicted in Enclosure 1 (lower left). The external trapping grid had 26 lines of traps in 4 rows; traps were spaced approximately 10 meters apart (farther at the corners; drawing not to scale). The first trap of each line was placed flush to the enclosure, with all subsequent traps spaced about 10-m apart. Although the external grid surrounded the entire enclosure array, only two sides are depicted. Figure applies to experiments 1-3. Experiment A differed in having 3 susceptible mice in all 6 enclosures (i.e. no high density treatment).



Within each enclosure, we placed 36 trapping stations approximately 4 m apart. One Sherman live-capture trap (H. B. Sherman Traps, Tallahassee, Florida, USA) was placed at each trap station for up to 3 consecutive nights (until all mice were captured) weekly or biweekly, depending on the experiment. Traps were baited with peanut butter and rolled oats, and contained polyester Fiberfil bedding.

We checked enclosure perimeters every 1–2 days and performed repairs as necessary. Nest burrows were cleaned weekly during the experiments by removing all nesting materials and feces. Between experiments, they were thoroughly cleaned of nesting materials and any feces and cached food, sprayed with virucide, and left with the lids off under direct sunlight for several days to dry and inactivate any residual infectious virus. We housed mice in

individual, homemade 35 X 35 X 55 cm tall mouse boxes with screw-top lids and ventilation holes around the top. See Supplementary Figure 1 for photographs of the study site.

Experimental Design

For each experiment, 1 infected (donor) and a predetermined number of uninfected (susceptible) mice were released into each enclosure according to the study design (Table 2.1 and Figure 2.1. The population sizes for low- and high-density treatments were consistent with naturally occurring population densities in Montana based on mark-recapture data (Kuenzi et al. 2001, Lonner et al. 2008). We alternated the enclosures housing the low- and high-density populations at each repetition of the experiment. During Experiment 2, we replaced 1 donor and 3 susceptible mice that died (carcasses were recovered) and one enclosure mouse who was captured outside the enclosure with additional quarantined susceptible and donor mice to maintain constant population densities throughout the experiment.

All mice released into the enclosures were ear-tagged with sequentially numbered metal fish fingerling tags (National Band and Tag Company, Newport, Kentucky, USA). Mice were provided grain, apple chunks, and water weekly or as needed. Food was scattered widely throughout the enclosure to avoid unnatural aggregations at feeding stations; water was provided in water-bottles, as required by our IACUC protocol, in the burrows. Rodents in enclosures were trapped weekly (2008) or biweekly (2007) to collect blood samples using standardized protocols for SNV surveillance (Kuenzi et al. 2001). Mice were handled and sampled according to strict guidelines developed by the Centers for Disease Control and Prevention and designed to prevent cross contamination between rodents and infection to humans (Mills et al. 1995). Blood samples were immediately frozen on dry ice and stored at -70°C until processing. Body weight, breeding condition (scrotal or abdominal testes), trap

location, and number of wounds on the ears and tail (as an indicator of aggressive encounters) were recorded during each trapping session. We tested all blood samples collected from all experimental animals for SNV RNA and antibody as described (See Immunological and molecular testing). We also constructed a 0.5-ha trapping grid outside of the enclosures (See Figure 2.1), and tagged and released the outside mice to monitor non-experimental rodent population dynamics and to detect any escapees during each experimental run. While outside rodents were trapped and monitored for escapees for the entire duration of each experiment, descriptive data were collected for the majority, but not all, trap sessions (until September 19, 2007, and until September 4, 2008) due to personnel constraints.

Immunological and molecular testing

We collected blood samples from the submandibular vein using a Goldenrod lancet (Medipoint International, Inc., Mineola, New York, USA) or by capillary tube from the retro-orbital capillary plexus after anesthesia with isofluorane. In the laboratory, we tested blood samples for IgG antibody reactive with SNV recombinant nucleocapsid protein (supplied by the US Centers for Disease Control and Prevention [CDC], Atlanta, Georgia, USA) by enzyme-linked immunosorbent assay (ELISA) according to standard protocols (some 2007 blood samples) or by a rapid peroxidase enzyme-linked immunosorbent assay (PAGEIA) (blood samples from 2007 and 2008) (Feldmann et al. 1993, Schountz et al. 2007a). All 2007 blood samples from 2007 positive for anti-SNV antibody by ELISA were also tested by PAGEIA and the results were identical. We determined antibody titers using the PAGEIA assay. The blood samples were initially diluted 1:100 in phosphate buffered saline (PBS) and then serially diluted in a log2 series from 1:1,000 to 1:128,000. Both the ELISA and the PAGEIA detect IgG antibody to most or all New World hantaviruses, but

do not distinguish among them. However, we confirmed all hantavirus IgG antibody-positive blood samples by molecular testing. We tested blood samples specifically for SNV RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (Chomczynski 1993). Mice were housed one per plastic mouse box until blood analyses were complete. We employed extra precautions to prevent cross-contamination during caretaking duties and storage of mice (using clean gloves while replacing feed and water, keeping boxes spaced about a foot apart, etc.) and we observed no evidence of cross contamination.

Rodent collection and selection of experimental subjects

Mice trapped within 5 km of the study area were assigned to 1 of 3 age classes according to body weight: mice < 14 g were juveniles; mice 14–17 g were subadults; mice > 17g were adults (Douglass et al. 2001, Kuenzi et al. 2001). Testes position (scrotal vs. abdominal) was used to determine breeding condition. We selected adult male mice, to eliminate demographic factors such as sex and age from our experiments, and because adult males are responsible for the majority of SNV transmission in wild populations (Mills et al. 2010). In the event that there were not enough adult males captured, we included larger subadults and made sure that the age structure of the experimental mouse populations was as similar as possible among enclosures. Because genetic relatedness might influence social interactions and immunological responses to infection, we avoided placing mice from the same capture site within the same enclosure. Sin Nombre virus infection status of mice was determined by detecting IgG antibody (Feldmann et al. 1993, Schountz et al. 2007a) and by detecting SNV RNA by nested RT-PCR (Chomczynski 1993). In 2008, susceptible mice were quarantined prior to release into the enclosures, while in 2007 they were not.

Susceptible animals

In the 2007 experiments (Experiments A, 1, 3), we designated rodents as susceptible if they had no detectable SNV RNA by RT-PCR and no detectable IgG antibody to SNV in blood. Except for the time between capture and testing results (4–10 days), these mice were not quarantined prior to release into the enclosures for each 2007 experiment. Any mouse positive for SNV RNA or antibody was considered as a possible infectious donor.

In the 2008 experiment (Experiment 2), potential susceptible mice were quarantined for 3 weeks in separate plastic mouse boxes in a locked, air-conditioned, quarantine facility that was separate from any other structures. Seventeen mice whose blood samples were positive for SNV RNA or antibody upon first capture were rereleased at the capture site. The mice whose blood was negative for SNV RNA and antibody were individually housed in separate plastic mouse boxes in the quarantine facility; their blood was retested approximately 2 weeks (14-16 days) post-capture and 3.5 weeks (25 days) post-capture before release into enclosures. Of the 54 quarantined individuals, 3 became SNV antibody or RNA positive during the first 2 weeks of the quarantine period. No mice became SNV antibody or RNA positive past the 2-week mark of quarantine. One of the 3 seroconverters was returned to where it had been captured, the other two were used as donor mice.

Donor animals

Rodent hosts of other hantaviruses are most infectious 2-5 weeks post-infection, but are known to shed infectious virus for much longer (Lee et al. 1981, Yanagihara et al. 1985, Hutchinson et al. 1998). In 2007, we chose donor mice as those positive for SNV RNA or antibody. In 2008, the quarantine period allowed us to choose recently seroconverting mice. Two of the 3 seroconverters during quarantine were used as donor mice. The rest of the donor mice were chosen based on SNV RNA and seroconversion data. We recaptured some

of the positive mice that we had released prior to the quarantine of potential susceptible animals, and chose donors who were SNV RNA positive and/or whose antibody titers had increased 4-16 fold during the 2-week period since their last capture.

Transmission event mice

After the start of each experiment, if a susceptible mouse in an enclosure was found positive for either SNV RNA or SNV antibody, he was designated as a transmission-event (TE) mouse. Every TE mouse was found positive for both SNV RNA and antibody, except for 3 mice that did not develop detectable IgG antibody before either dying or the end of experiment. For these 3 mice, we confirmed infection with SNV by detecting SNV RNA in 2 or more blood samples collected on different dates, or by sequencing the samples (only 2008 mice).

Because the mice in 2007 were not quarantined, it is possible that some were infected prior to release into the enclosures. The majority of TE mice had negative SNV antibody and RNA results for at least 2 weeks post-release and seroconverted or had detectable SNV RNA in their blood one month post-release, indicating that they were infected in the enclosures. Our 2008 quarantine results indicated that mice that were previously exposed seroconverted within the first 2 weeks. Three of the TE mice seroconverted within two weeks after introduction into the enclosures. Two of these three mice had very low antibody titers (Mouse 1: titer of 100, no RNA results (not enough blood available for test), Mouse 2: titer of 200, positive for SNV RNA), which is consistent with the blood profile of a very recently infected mouse (Bagamian 2012). Also, both mice were from the same enclosure, suggesting close temporal exposure to the same donor. Fifteen of the 18 transmission events (excluding these two mice: 13/16) involved multiple mice in the same enclosure (Bagamian

2012). Thus we feel that infection prior to release into the enclosures is unlikely for these two mice.

Nevertheless, we analyzed our data both including and excluding these mice and report both sets of results. A third mouse was SNV RNA positive and had a high antibody titer (1600) two weeks post-release into enclosures. Because infection prior to release into the enclosure seemed possible, this mouse was excluded from our analyses of transmission incidence. We report transmission incidence as the number of new infections (=transmission events)/sum of the number of mouse weeks of observations (for a complete explanation, see Statistical analyses and variables).

Statistical analyses and variables

We conducted statistical analyses (Fisher's exact tests, tests of differences between proportions, t-tests, and simple linear regression) using Microsoft Excel 2007 and R (R Development Core Team, Vienna, Austria, 2011). We report transmission incidence per each experiment (= season), and transmission incidence per density treatment during each experiment. Incidence of transmission was the number of transmission events divided by the sum of the number of weeks each mouse was in the experiment and susceptible to infection (see (Mills et al. 1992)). By using these rates (=incidence), we control for the number of mice per treatment and experiment, and for varying durations of the experiments. We calculated rate ratios and confidence intervals for transmission incidence, and compared all pairs of seasons for statistically significant differences. We do not report rate ratios of transmission by density treatment, because the low-density treatment group had zero transmission events in 2 of the 3 density experiments. We used a test for differences in proportions to analyze both seasonal incidence and incidence by density treatment, although the data did not meet the typical sample size criteria for stable performance. Because of the very low number of

transmission events and small samples sizes of our experiments, we also used Fisher's exact two-tailed test to make pairwise comparisons of proportions of TE mice over all susceptible mice between all pairs of experiments (=seasons) and between all density treatments within each experiment.

In situations in which we made comparisons of proportions between dates, seasons, or experiments in our data analyses, we did not use Bonferroni corrections for our individual tests. Applying this type of correction increases the likelihood of type II errors, and we agree with those who feel that these corrections should not be used when assessing evidence about specific hypotheses (Perneger 1998, Nakagawa 2004).

Where biweekly vs weekly sampling frequency might influence the values of the variables measured (i.e., comparing 2007 to 2008 data), we used only biweekly data from 2008. When the timing of data collection may have influenced variables (e.g., wound presence and absence, number of new wounds and reproductive status), we compared data collected at similar time periods (2 weeks post release into enclosures) for between-experiment comparisons and also pooled these data from all four experiments for any overall analyses.

For all analyses, we excluded data from mice that were released into the enclosures and never recaptured (n = 7 for 2007 experiments; n = 1 for the 2008 experiment). We also excluded data from 1 low-density enclosure (Enclosure 3) in Experiment 1 in analyses of transmission incidence, because it was unclear whether the donor mouse was truly infected. His blood was positive for SNV RNA in 1 of 2 samples, but he was not recaptured again to reconfirm infection status. There were no TE mice in Enclosure 3 in Experiment 1. In analyses of wounds, scrotal condition, and weight gain for the 2008 experiment, we also

excluded information from 2 mice that were in the experiment for less than 2 weeks, because of insufficient data.

Wounding

We also analyzed the total number of new wounds on each individual mouse per experiment, season, or density treatment. The total number of new wounds was counted on an individual animal over the course of the experiment, and each animal was represented only once in any analysis. This conservative measure only includes wounds detected on a new location on the mouse (tail vs. ear) and increases in the number of wounds from the previous sampling session. This ensured that the same wound was not counted twice for any animal, but also allowed for the possibility that some new wounds in the same area as a previous wound may not have been counted. We ran a linear regression model with the season as a categorical predictor variable, and the outcome variable was the number of new wounds per experiment.

2.3 Results

Relationship of incidence to seasonality and density

We documented 18 transmission events over 4 experiments (Figure 2.2). The transmission incidence was not significantly different between the high- and low-density treatments combining data from all 3 density experiments ($z = 0.91$, $p = 0.37$) or within each experiment, according to the test of differences between proportions (Exp. 1: $z = 1.15$, $p = 0.25$; Exp. 2: $z = 0.023$, $p = 0.98$; Exp. 3: $z = 0.71$, $p = 0.48$; see Figure 2.3 for transmission incidences). The proportion of TE mice to overall susceptible mice was the not significantly different between high- and low-density treatments overall (two-tailed Fisher's exact test [FET]: $p = 0.34$) and within each experiment (FET: Exp. 1: $p = 0.54$, Exp. 2: $p = 1.00$, Exp. 3: $p = 1.00$).

Figure 2.2: Seasonal incidence of Sin Nombre virus transmission in North American deermice *Peromyscus maniculatus*) by experiment. The incidence of transmission (number of transmission events per 100 mouse-weeks of observation, expressed as a percentage (see (Mills et al. 1992)) by each season (each experiment A, 1, 2, 3) is reported above each bar. Numbers of transmission events/mouse weeks are reported in each bar.

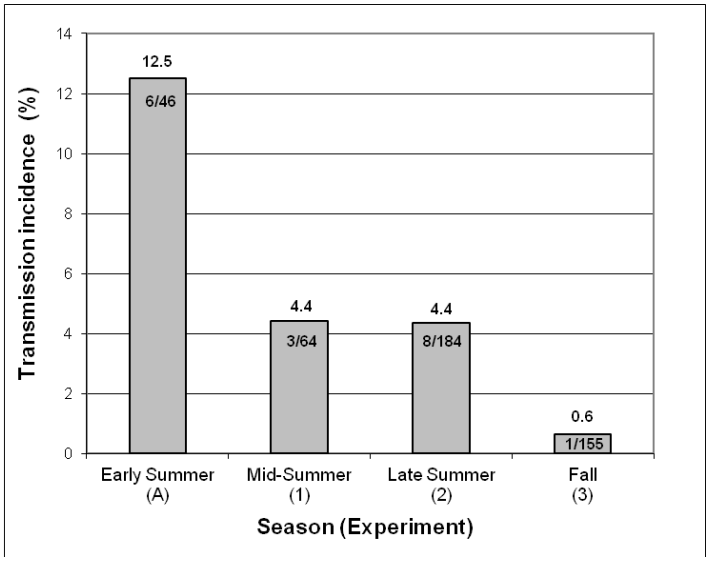
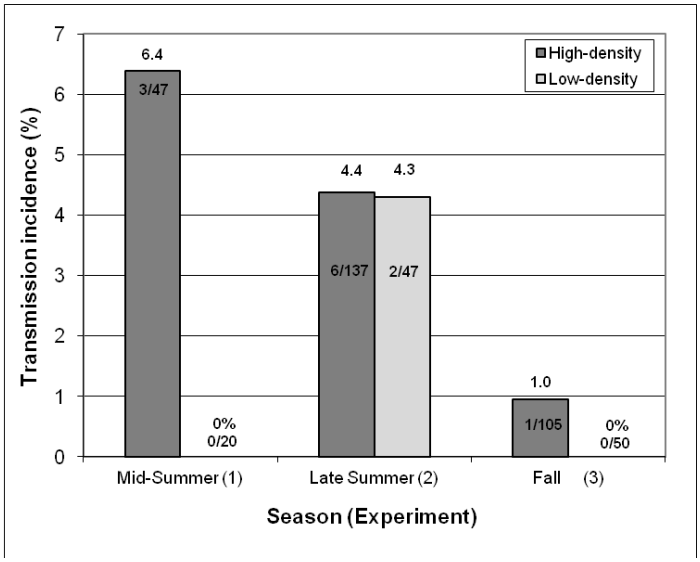


Figure 2.3: Incidence of Sin Nombre virus transmission in North American deermice by density treatment and experiment. The incidence of transmission by density treatment (high vs. low) during each experiment is reported above each bar. The number of transmission events/mouse weeks per density treatment is reported in each bar.



We found statistically significant differences in the incidence of transmission between each summer period (early, mid, late) and the fall demonstrated by both rate ratio confidence levels and by the test of differences of proportion including all 18 transmission

events, and when the 2 potentially questionable events were removed from the analyses (Table 2.2 & 3). Transmission incidence during the summer months ranged from about 7 (late summer) to 19 (early summer) times greater than the fall when all transmission events were included in the analyses (Table 2.2), and from about 7 (late summer) to 13 (early summer) times greater when the two events were removed (Table 2.3). Incidence in mid-summer and late summer was 1/3 of the incidence in early summer when all transmission events were considered (Table 2.2) and when the two transmission events were removed, incidence in mid-and late summer was half the incidence in early summer (Table 2.3). These within-summer differences were significant by rate ratio confidence intervals. According to the test of differences of proportion, the only statistically significant within-summer comparison was between the incidences in early summer and late summer ($z = 1.78$, $p = 0.04$), when all transmission events were considered (Table 2.2). The proportion of TE mice to overall susceptible mice in the fall was significantly lower than in the early summer (FET: $p = 0.01$) and late summer (FET: $p = 0.03$), but not in mid-summer (FET: $p = 0.4$; Table 2.2), when all transmission events were considered (Table 2.2 ; see Table 2.1 for mouse numbers). All other comparisons of proportion of TE mice to overall mice between experiments were not statistically significant by FET ($p > 0.1$ for all comparisons), when all transmission events were considered. When two transmission events were removed from the analyses, the proportion of TE mice to overall susceptible mice in the fall was still significantly lower than in the early summer (FET: $p = 0.03$) (Table 2.3); all other comparisons were not significant (FET: $p > 0.15$).

Table 2.2: Seasonal transmission incidence ratios for SNV transmission experiments, all reported transmission events. Relative ratios for each pairwise comparison between seasons. Season used as numerator in rate ratio is listed first. Statistically significant rate ratios and confidence intervals are in boldface type. *Seasonal comparison statistically significant by test of difference of proportions. **Seasonal comparison statistically significant by Fisher's exact two-tailed test and test of difference of proportions.

Season	Seasonal Comparison	Rate Ratio, (95% CI)
Early summer**	Fall	**19.38, (14.02–26.78)**
Mid-summer*		**7.27, (5.43–9.72)**
Late summer**		**6.76, (5.45–8.37)**
Mid-summer	Early Summer	**0.38, (0.26–0.55)**
Late Summer*		**0.35, (0.25–0.48)**
Late Summer	Mid-summer	0.93, (0.70–1.24)

Table 2.3: Seasonal transmission incidence ratios for SNV transmission experiments, excluding two transmission events from early summer. Relative ratios for each pairwise comparison between seasons. Season used as numerator in rate ratio is listed first. Statistically significant rate ratios and confidence intervals are in boldface type. *Seasonal comparison statistically significant by test of difference of proportions. **Seasonal comparison statistically significant by Fisher's exact two-tailed test and test of difference of proportions.

Season	Seasonal Comparison	Rate Ratio, (95% CI)
Early summer**	Fall	**13.48, (9.70-18.73)**
Mid-summer*		**7.27, (5.43–9.72)**
Late summer**		**6.76, (5.45–8.37)**
Mid-summer	Early Summer	**0.54, (0.37–0.79)**
Late Summer		**0.50, (0.36–0.69)**
Late Summer	Mid-summer	1.07 (0.81-1.42)

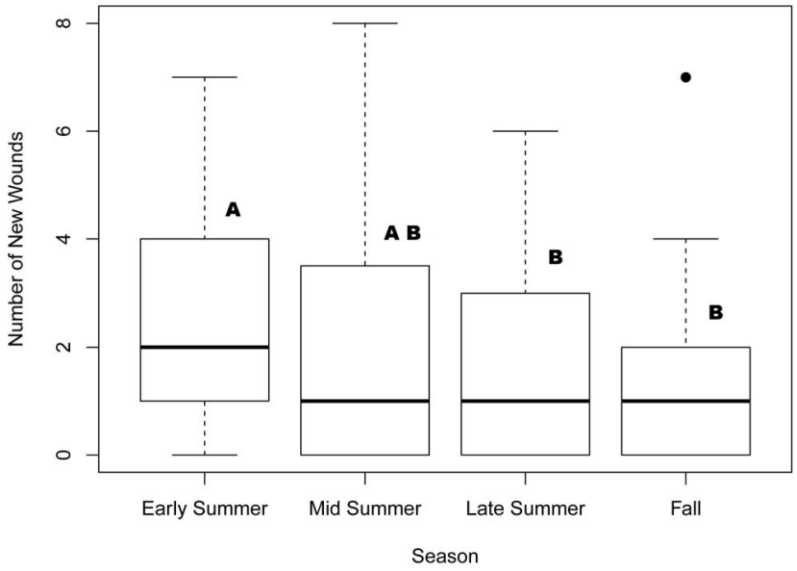
Relationship of wounding to seasonality and density

The average number of new wounds per mouse in the early summer was significantly higher than in the fall ($t_{103} = -1.998$, $p = 0.048$, $\beta_{fall} = -1.1522$, SE= 0.5767), and suggestively higher in comparison to late summer ($t_{103} = -1.946$, $p = 0.054$, $\beta_{LateSummer} = -0.9943$,

SE=0.5109), but not in comparison to mid-summer (t_{103} = -1.525, p > 0.05), as determined by a linear regression comparing each season to early summer (Figure 2.4).

We found no significant differences in the average number of new wounds on individual mice between high- and low-density treatments overall (high-density $\bar{x}$ = 1.85, SD = 1.80; low-density: $\bar{x}$ = 1.58, SD = 1.97; t_{90} = -0.656, p > 0.05) or in each experiment (p > 0.15 for all within experiment comparisons).

Figure 2.4: Seasonal median number of new wounds per individual deermouse. Thick horizontal line is the median; top and bottom of boxes represent the 25th and 75th percentiles; whiskers indicate ranges, excluding outliers. Outlier is indicated by black dot. Bars with the same letter above them are not significantly different.

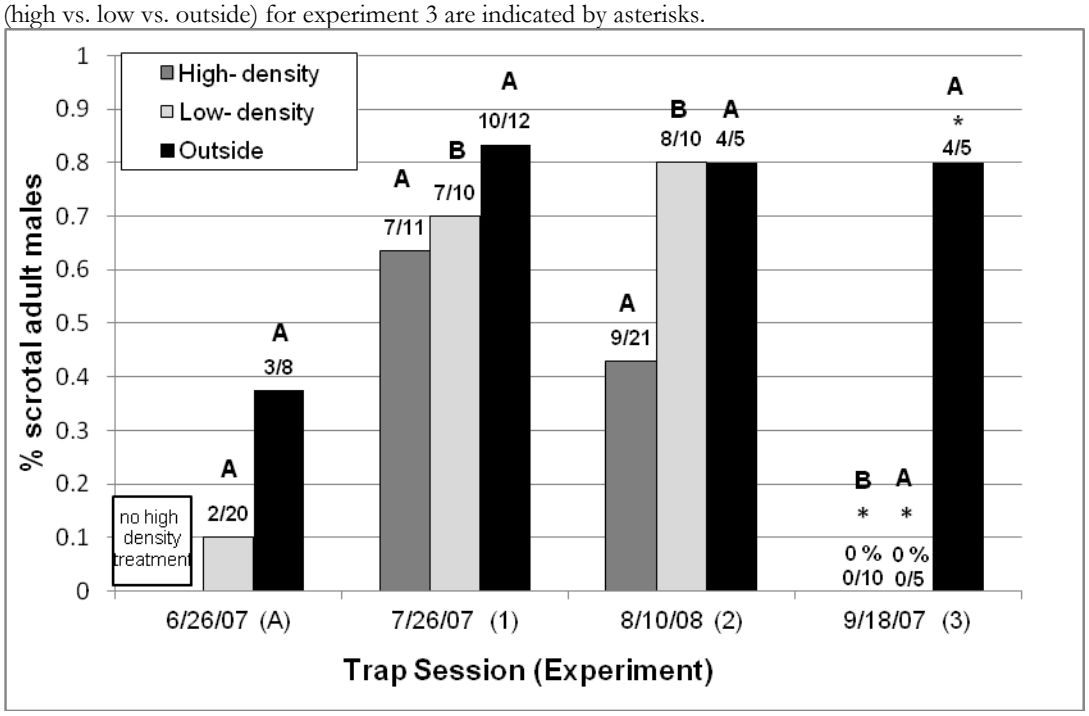


Relationship of host reproductive condition and weight gain to seasonality and population density

The proportions of adult males with scrotal testes in the enclosures varied by season of the experiment (Figure 2.5). In the low-density treatments, the proportion of males with scrotal testes 2 weeks post-release was significantly higher in the mid-breeding season (July and August) than either the early season (FET: June vs. July: p = 0.002, June vs. August: p < 0.001) or the late season (FET: September vs. July: p = 0.03, September vs. August: p = 0.01). In the high-density treatments, the proportion of males with scrotal testes was

significantly lower at the end of the breeding season (September) than in the mid-breeding season (FET: September vs. July: $p = 0.003$, September vs. August: $p = 0.03$). No other tests for the proportions of males with scrotal testes between capture dates were significant (FET: $p > 0.45$). The proportions of adult males with scrotal testes captured outside the enclosures (Figure 2.5) did not differ significantly between months (FET: $p > 0.05$).

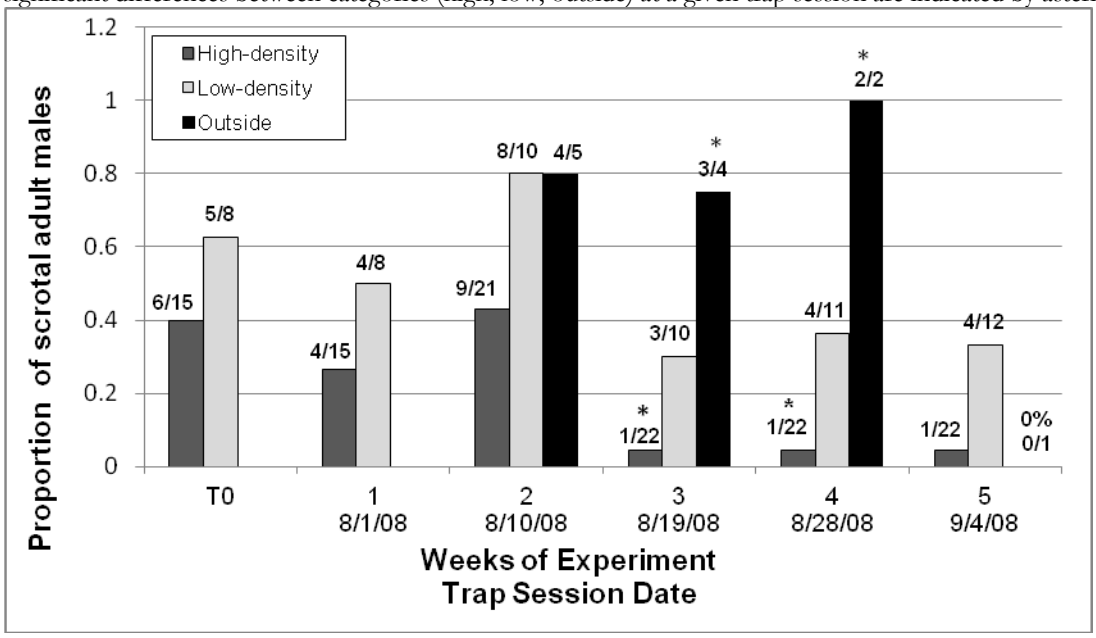
Figure 2.5: Proportion of adult, male deermice with abdominal or scrotal testes inside and outside enclosures by density treatment and experiment. The proportion of scrotal adult males (of total adult males captured) 2 weeks post-release into the enclosures inside (by density treatment) and outside of enclosures during each experiment. Experiment A only had low-density treatment groups; experiments 1-3 had high- and low- density treatments. Numbers of scrotal/total for each experiment are denoted above bars. Bars with the same letter above them are not significantly different within each category (high, low, outside) between experiments. Statistically significant comparisons between categories (high vs. low vs. outside) for experiment 3 are indicated by asterisks.



Two weeks post-release into the enclosures, no differences were observed in the proportion of males with scrotal testes between the low- and high-density treatments (Figure 2.5) in any of the 3 density experiments (FET: $p > 0.06$ for all comparisons). However, in Experiment 2, the longest running experiment (8 weeks; Figure 2.6), mice from the low-density group, on average, remained scrotal significantly longer than mice from the high-density group (4.16 weeks vs. 1.6 weeks; $t_{37} = -4.04$, $p < 0.001$). The proportion of scrotal

males in the low-density group was generally higher than that of the high density population, and lower than the outside mouse populations, but none of these differences were significant (FET: $p > 0.19$; Figure 2.6). The high-density population had a significantly lower proportion of scrotal males as compared to the outside population on the third and fourth trap session (FET: August 19, 2008: $p = 0.030$, August 28, 2008: $p = 0.048$; Figure 2.6). No significant differences were observed in the proportions of males with scrotal testes by density treatment at the start of experiment 2 (Figure 2.6).

Figure 2.6: Proportion of adult, male deermice with scrotal testes at 4 trapping sessions by density treatment and by location (inside vs. outside enclosures) during Experiment 2. Numbers of scrotal/total for each trap session are denoted above bars. Statistically significant differences between categories (high, low, outside) at a given trap session are indicated by asterisks.



Pooling data across all experiments, 2 weeks post-release, the proportion of scrotal males with wounds (77%) was significantly higher than the proportion of abdominal males with wounds (54%; FET: $p = 0.03$). Wound frequency did not differ significantly between adult males in the enclosures compared to those captured outside the enclosures (FET: $p = 1.00$).

When measured at the beginning of each experiment and at the end of each experiment, no statistically significant differences were found in the mean animal weight between treatments (high- vs. low-density) or between locations (inside vs. outside enclosures) in any of the experiments ($p > 0.20$ for all comparisons). During Experiment 2, there were no significant differences between treatments in the mean rate (g/week) of weight gain (low-density: $\bar{x} = 0.300$, SD = 0.343; high-density: $\bar{x} = 0.279$, SD = 0.372; $t_{35} = 0.1675$, $p > 0.05$).

2.4 Discussion

Our objectives were to observe natural transmission of SNV in *P. maniculatus* populations in a semi-controlled outdoor setting, to empirically test the influence of seasonality and density on the frequency of transmission in a closed population, and to clarify the relationships between seasonality, density, host aggression, and reproductive physiology. According to theoretical models and mark-recapture data, all of these factors influence disease dynamics, but we are the first to examine these variables by experimentally manipulating host population densities across seasons in a field setting. The high-density group had many more SNV transmission events than the low-density group (11 and 2, respectively). Transmission of SNV in low-density enclosures occurred in only 1 of the 3 density experiments, and the overall transmission incidence in the high-density treatment was 2-fold higher than in the low-density treatment. Nevertheless, this difference between the density treatments was not statistically significant. Sample sizes were low and any effect of density on transmission frequency might have been obscured by the number of observed zeros. Alternatively, SNV transmission may be frequency dependent. Six years of cowpox virus dynamics in bank voles showed that transmission appeared to be density dependent during the winter, but frequency dependent in the summer—emphasizing the importance of

seasonal variation in host behavior and susceptibility on disease processes (Smith et al. 2009). As we found a strong effect of seasonality in our experiments, it is possible that underlying SNV transmission processes may exhibit similar variations. A larger sample size and more iterations of the experiment over a longer range of seasons may be needed to more reliably quantify these apparent differences. Unfortunately, large-scale enclosure experiments are very time consuming and labor intensive, and require a large area and much construction material.

The incidence of SNV transmission decreased significantly as experiments were conducted later in the breeding season. This observation is consistent with previous mark-recapture studies, which have indicated strong seasonal trends in seroconversion and increased prevalence of infection during the breeding season (Douglass et al. 2001, Kuenzi et al. 2005, Douglass et al. 2007). Douglass *et al.* (Douglass et al.) reported that the incidence of seroconversions remained relatively high but constant throughout the breeding season, while we detected a decreasing incidence from June to October. However, that study reported seroconversions detected at monthly sampling intervals in free-roaming populations across Montana; we detected transmission events weekly or biweekly and were able to assign a tighter temporal window to the events.

Initiation and cessation of the breeding season for *P. maniculatus* populations are highly variable and depend on photoperiodic cues, temperature, and food availability (Millar 1989). These influential factors vary geographically and annually, and may trigger differential effects among individuals in the same population (Millar 1989). At our site, the proportion of adult males in breeding condition captured in the enclosures was significantly greater in experiments conducted during the mid-breeding season than in the early and late breeding season. Also, fewer scrotal males were captured outside the enclosures in the early breeding

season than in the mid-breeding season, although this trend was not statistically significant (Figure 2.5). This pattern differs from previous reports from longitudinal data in southwestern and central Montana, where the percentage of scrotal males often peaked at 80% during May or June, and decreased linearly over the course of the breeding season to approximately 2% in October (Douglass et al. 2001). Our analyses included only adult males, but in the open population studied by Douglass *et al.,*(2001), the proportion abdominal would have continuously increased throughout the breeding season through the recruitment of young of the year.

Studies of caged albino and wild-type house mice, free-roaming vole populations (*Microtus montanus* and *Microtus pennsylvanicus*), and *P. maniculatus bairdii* have shown a strong and significant effect of high population densities in suppressing reproduction in both males and females (Christian 1961). In all of these species, animals living in densely populated areas, there was an increased investment in adrenocortical-related glands, but little or no gonadal development or function (Christian 1961). The adrenocortical response assists in survival when individuals are faced with extreme environmental changes or physiological stress (Christian 1961). Although the deermouse population density in our high-density enclosures (80 mice/ha) was similar to naturally observed high population densities in Montana, this density appears to be sufficient to affect the reproductive function of these mice. At most trapping sessions, the proportion of reproductive males in the low-density group was similar to that in the outside population. The population density of male and female mice outside the enclosures ranged from 28–46 mice/ha in August 10–28, 2008, which was similar to our low-density treatment (40 mice/ha). Although the majority of comparisons were not statistically significant between density treatments, in 2 of the 3 density experiments, the high-density enclosures consistently had lower proportions of

reproductive adult males than low-density enclosures (Figures 2.5 & 2.6). In Experiment 2, the percentage of adult males in breeding condition in high-density enclosures decreased from 40% to 5% during the third week of the experiment, and remained at that low level, while in the low-density enclosures, that percentage remained consistently around 30% (Figure 2.6). Also, mice from the low-density group were in reproductive condition significantly longer than the mice from the high-density group in Experiment 2. When data were pooled across experiments 1–3, the proportion of adult males with scrotal testes was significantly lower in the high-density group than in outside mice (FET: outside vs. high-density: $p = 0.01$, outside vs. low-density: $p = 0.57$). This suggests that the decrease in reproductive condition was primarily a result of high population density. Although our experiment does not provide sufficient data to test such a hypothesis, we speculate that the decrease in sexual preparedness associated with high density conditions may result in decreased aggression, improved immune system function, and potentially decreased incidence of transmission. This might help explain some of the difficulty in demonstrating a clear positive relationship between population density and SNV transmission.

Independent of any treatment effects of density or season, the enclosure may have affected the length of time mice remained scrotal. When data from all 4 experiments were pooled, a significant decrease in the overall proportion of scrotal males emerged during the first trap session (Time 1: T1) after release in comparison to before they were released into the enclosures (Time Zero: T0; FET: $p = 0.047$; data not shown). The proportion of scrotal adult males in the enclosures at T1 was also significantly lower than the proportion of scrotal adult males captured outside the enclosures (FET: $p = 0.003$, data pooled across all 4 experiments). Additionally, in the second-longest running experiment (Exp. 3; 6 weeks), while approximately 30% of males in the high-density group and 10% of males in the low-

density group had scrotal testes at T0 (data not shown), no males with scrotal testes were captured at T1 and at the next 2 trapping sessions, although breeding males were captured outside the enclosures at T1 (see Figure 2.5). One important factor may have been the absence of females inside the enclosures. Approximately 8–14 female mice were consistently captured outside, and most were pregnant or in breeding condition. However, despite the absence of females, 3 of the 4 experiments (except for the final fall experiment) always contained males in breeding condition, indicating the importance of seasonal cues in influencing breeding cycles. Additionally, enclosed males may have still received olfactory cues from nearby females outside the enclosure.

Although population density clearly affected the ability to maintain breeding condition, it had no statistically significant effect on the rate of weight gain. The supplementary food and water in the enclosures may have contributed to weight maintenance.

The average number of new wounds per mouse was significantly higher in the early summer than late summer and fall. As the breeding season begins, males often respond to seasonal cues and establish and defend territories (Millar 1989), leading to increased wounding. The higher prevalence of wounds on males with scrotal testes supports the idea that breeding males are more likely to be aggressive and interactive than non-breeding males. At the end of the breeding season [late summer- early fall; (Douglass et al. 2001)], there are fewer breeding males, and, therefore, fewer fights.

The fact that incidence of transmission and average number of new wounds per mouse peaked at the beginning of the breeding season and decreased over time provides some support to the current view that direct contact may be the primary mode of transmission in wild deermouse populations, because the most transmission occurred during

times where the mice were most aggressive. We cannot rule out the possibility that SNV may have been transmitted both directly and indirectly in the enclosures. Future studies could implement cameras, pit tag recorders, and fluorescent marking powder (Clay et al. 2009) to gather a better understanding of the contact structure and dominance dynamics within enclosed populations and their relationship to transmission dynamics. Future manipulative experiments in enclosures will also allow testing hypotheses that environmental transmission may occur in nature.

A major limitation of our experiments was small sample size. We were able to maintain a limited number of mice per enclosure, and we observed 18 transmission events total in all 4 experiments. However, as natural transmission events are rare by nature, recording 18 events in a semi-controlled setting could be considered very successful. A previous laboratory study reported only 1 SNV transmission event out of 54 attempts (Botten et al. 2002). Nevertheless, larger experiments with greater numbers of mice per enclosure and increased numbers of replicate enclosures would have greater statistical power. Also, as we did not quarantine our susceptible mice after our experiments, we may have underestimated transmission rates. We conducted our experiments during only 2 seasons (summer and fall). To more completely understand seasonal effects on this system, subsequent studies should be run in winter and spring. Such studies may be challenging (especially in Montana) because of weather conditions and presumably decreased transmission during these seasons, although transmission during winter huddling in nest boxes could be examined. We also did not control or test for genetic variability in resistance to infection or dominance hierarchies, factors that may have influenced infection dynamics within the enclosures. Finally, in order to decrease the number of variables and keep our experiment simple and most likely to succeed, we used only male deermice. We do not know

how this unnatural condition may have affected our results. Male-female mixed populations are a more natural arrangement of hosts, and therefore, to more fully understand natural SNV transmission, future experiments should also be conducted using mixed male and female populations. Comparing and contrasting the transmission and behavioral dynamics between same-sex and opposite-sex arrangements may help elucidate the relative roles of each type of interaction in disease transmission in the wild.

Our results, especially in the light of previous mark-recapture studies of effects of season and density on infection dynamics in wildlife populations, emphasize the importance of considering the strong effects of season as a confounder when making comparisons of density effects in natural populations. Seasonality, even when only evaluated within the timeframe of the breeding period (spring to autumn), may be more influential in disease dynamics than population density. Season influences host behavior, susceptibility, host reproduction, and other physiological processes, all of which are critical in maintaining disease transmission cycles in nature. Although there is a likely effect of host population density on disease transmission, density processes cannot be considered independently of seasonal factors when exploring natural host-pathogen systems.

We successfully conducted large-scale manipulative experiments that followed SNV transmission in deermice under controlled conditions. Our experiments provided further insight into the effect of seasonality and density on hantavirus transmission, reservoir host aggression, and host reproductive processes. Our successful methodologies might be used to address other questions in the field of wildlife disease ecology or in similar zoonotic host-pathogen systems.

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3 Transmission ecology of Sin Nombre hantavirus in naturally infected deer mouse populations in outdoor enclosures

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Abstract

Sin Nombre virus (SNV) hosted by the deer mouse (*Peromyscus maniculatus*), is responsible for the great majority of human hantavirus pulmonary syndrome cases in North America. While there have been numerous theoretical and survey studies with the goal of understanding hantavirus transmission in the wild, most transmission experiments are conducted under highly artificial laboratory conditions or extrapolate transmission information from disease prevalence data from natural systems. Although long-term mark-recapture field studies have answered some basic questions about SNV transmission among deer mice, these investigations also have led to new inquiries that can be best answered by experimentation under controlled conditions. Previous transmission studies using experimentally infected deer mice were unsuccessful at demonstrating transmission of SNV. During the summers of 2007-2008, we constructed an outdoor enclosure system and explored transmission patterns using naturally infected deer mice in a controlled field setting. Mice infected with SNV in our enclosures had detectable viral RNA in their blood throughout the acute phase of infection and had significantly higher incidence of wounding (a sign of aggressive encounters) than uninfected mice. Naturally-infected wild mice had a highly variable IgG antibody immune response to infection, with peak antibody titers varying from 200 to 6400; and levels of viral RNA sustained in blood varied as much as 100-fold, even in individuals infected with identical strains of virus. Initial results suggest that there may be a threshold level of virus necessary for hosts to transmit SNV to susceptible deer mice. The rapid death of some animals following infection suggests that adverse effects of SNV infection may be difficult to detect by monthly trapping in mark-recapture field studies. Our study is a first step in exploring the transmission ecology of SNV infection in deer mice.

Key words: Transmission ecology, Sin Nombre Hantavirus, deer mouse, *Peromyscus maniculatus*, enclosure experiments, natural infection

3.1 Introduction

The recognition that the majority of emerging infectious diseases are zoonotic in origin (75%; Taylor et al. (2001)) has led to increased surveillance of wildlife host-pathogen systems by public health researchers and wildlife disease ecologists. The primary goals of these studies have been to characterize pathogens and identify host reservoirs and, subsequently, to explore influential ecological factors and enhance understanding of mechanisms of pathogen transmission. These studies can help discover factors that lead to increased infection in host populations and contribute to the development of scientific tools to predict and help inform control and prevention policies for outbreaks in human populations.

One example is the highly fatal, 1993 outbreak of hantavirus pulmonary syndrome (HPS) in the Four Corners region of the Western United States. An interdisciplinary team identified a previously undescribed hantavirus, Sin Nombre virus (SNV) as the causative agent (Nichol et al. 1993) and the deer mouse (*Peromyscus maniculatus*) as the primary host (Childs et al. 1994). Subsequent field studies showed that SNV, or SNV-like viruses, are distributed throughout the Americas (Yates et al. 2002, Mills et al. 2010). As of October 19, 2011, 580 cases of HPS, most of them caused by SNV, have been confirmed from 34 US states. SNV largely affects those in rural areas, and has a 35% case fatality rate (CDC, website: http://www.cdc.gov/hantavirus/surveillance/index.html).

In the last fifteen years, longitudinal mark-recapture studies in the western United States have revealed much about the ecology of SNV and the deer mouse (Mills et al. 2010). These studies monitored changes in SNV infection incidence, prevalence, and rodent population density and identified ecological factors associated with these changes. Results of

field studies throughout the United States have shown that adult deer mice (particularly males) have a higher prevalence of infection, suggesting that transmission involves males more frequently than female animals (Mills et al. 2010). The primary mode of transmission among rodents is considered to be horizontal, through biting and scratching. Correlation analyses of field data in the SNV-deer mouse system and the Seoul hantavirus-Norway rat (*Rattus norvegicus*) system report higher wounding frequency (Glass et al. 1988, Douglass et al. 2001, Calisher et al. 2007) and increased severity of wounds (Hinson et al. 2004) in hantavirus infected individuals. Some studies report this correlation only in adult rodents, especially adult males (Glass et al. 1988, Hinson et al. 2004) or in certain age groups (Calisher et al. 2007), while Douglass et al. (2001) found a correlation between wounding frequency and infection regardless of age. Although indirect transmission of hantaviruses is possible among laboratory-inoculated mice (Gavrilovskaya et al. 1990, Hutchinson et al. 2000, Hardestam et al. 2008), it has not been observed in controlled experiments with naturally infected animals (Padula et al. 2004). Longitudinal studies have answered some questions about host demographic factors and SNV infection prevalence in natural populations, but they have raised new lines of inquiry about deer mouse behavior and the within-host dynamics of infection and immune response influencing SNV transmission in natural populations that can only be answered using controlled experiments.

Hantavirus-infected rodent hosts are thought to be chronically infected and shed virus, at least intermittently, for extended periods – perhaps the lifetime of the host (Peters et al. 2006). Hantavirus infections in the natural host are characterized by an acute phase (7-60 days post-infection (p.i.)) and a persistent phase (60+ days p.i.). Laboratory studies of experimentally infected Old and New World hantavirus hosts suggest a consistent pattern of infection and viral shedding in the specific reservoir host. After inoculation, the host

experiences a brief viremia at 7-10 days p.i., followed by detection of viral antigen in multiple tissues and shedding of infectious virus in saliva and excreta. Animals develop neutralizing immunoglobulin G (IgG) antibody approximately 10-21 days p.i., which clears virus from the blood (LeDuc et al. 1992, Hutchinson et al. 1998). Virus is then sequestered in organs and adipose tissue, and continues to be shed into the environment (LeDuc et al. 1992). During the persistent phase, viral RNA is occasionally detected in blood due to viral recrudescence, associated with increased viral replication in tissues (Hutchinson et al. 2000, Botten et al. 2003). In laboratory studies, virus titer in excretions and secretions of hantavirus hosts is often highest during the first 2 weeks after infection (Hutchinson et al. 1998) and host-to-host transmission is most efficient during this period (Yanagihara et al. 1985, Hutchinson et al. 1998, Hutchinson et al. 2000).

Botten et al., (2000, 2002, 2003) conducted controlled SNV infection and transmission experiments to learn about SNV viremia, transmission, and the deer mouse host's immunological response to infection. These experiments used colony-bred, wild, deer mice experimentally inoculated with mouse-adapted SNV strain SN77734. The investigators collected information about viral RNA quantities, and viral nucleocapsid antigen in organs and various tissues, corroborating some of the patterns seen for other Old World and New World hantaviruses (Yanagihara et al. 1985, Gavrilovskaya et al. 1990, Dohmae et al. 1994, Hutchinson et al. 1998, Kariwa et al. 1998, Botten et al. 2000, Botten et al. 2002, Botten et al. 2003, Hardestam et al. 2008). However, although the New World Black Creek Canal virus (BCCV), as well as the Old World viruses Hantaan, Puumala (PUU), and Seoul (SEO), can be transmitted to naïve cage mates and can be isolated from the excreta or saliva of experimentally infected hosts (Yanagihara et al. 1985, Gavrilovskaya et al. 1990, Dohmae et al. 1994, Hutchinson et al. 1998, Kariwa et al. 1998, Hutchinson et al. 2000, Hardestam et al.

2008), this has not been the case for SNV. Botten et al. (2002) reported only 1 transmission event out of 54 attempts of exposing naïve animals to SNV77734-infected animals. Although Botten et al. (2002, 2003) shed some light on SNV-host dynamics, they left unanswered questions about the transmission cycle of SNV in wild deer mice under natural conditions. Alterations in the SN7734 mouse-adapted strain during progressive passages in mice may have affected its ability to be transmitted. Botten et al. (2000) suggested that SN7734 could be considered a new strain of SNV based on a partial sequence analyses showing 3% difference from published sequences. Genetic changes in laboratory strains of PUUV have been linked to human cell culture adaptation via point mutations in multiple segments of the PUUV genome as compared to the wild type virus (Nemirov et al. 2003) and to phenotypic differences in virulence and immunological factors between PUUV substrains and in comparison to a parental wild type strain (Sundstrom et al. 2011).

Viral RNA can be readily detected in saliva and excreta for PUUV, SEOV, and BCCV (Hutchinson et al. 1998, Klein et al. 2001, Hardestam et al. 2008); but although 100% of deer mice inoculated intranasally with SN77734 developed persistent SNV infection, viral RNA was detected only intermittently in blood and saliva throughout the acute and persistent phases, and was not amplified from excreta (Botten et al. 2003). No one has quantified viral RNA levels in blood for periods exceeding 21 days p.i. and the last published attempt to quantify SNV RNA in blood samples was over a decade ago (Botten et al. 2000). Recent field studies have shown that chronically infected wild deer mice show recurring periods during which SNV RNA is detectable in their blood (Kuenzi et al. 2005) and in saliva and urine (Safronetz et al. 2008). It is not know if the presence of viral RNA in blood coincides with viral shedding, or if the quantity of viral RNA in blood is correlated with the relative level of infectiousness.

Although initial laboratory studies indicated that hantavirus-infected hosts show little pathological effects or disease symptoms (LeDuc et al. 1992), recent field studies have indicated otherwise. In mark-recapture studies in the western USA, SNV infection has been linked to decreased survival of deer mice in general (Adler et al. 2008), reduced survival in juveniles (Douglass et al. 2001) and males (Luis et al. 2012), and decreased weight gain compared to uninfected mice (Douglass et al. 2007).

Most controlled studies of within-host transmission dynamics for hantaviruses and other microparasites are run in laboratories and provide crucial information about infection and immunological processes. However, they are conducted under highly artificial conditions. Although restricting an animal to a cage is useful to collect samples and observe individual behaviors, several influential factors that may affect host behavior and immunity are limited. For example, experimental animals are often caged in pairs (Hutchinson et al. 1998, Hutchinson et al. 2000, Botten et al. 2002, Padula et al. 2004) , which limits the host's interaction to one individual and eliminates population-level processes. Also, as compared to laboratory animals, wild rodent hosts may undergo periods of immunosuppression due to environmental stresses such as changes in population size, breeding condition, resource availability, and weather that may be important for transmission. Controlled transmission studies usually rely on artificial inoculation with a serially passaged hantavirus strain (Botten et al. 2002), which often has introduced molecular changes that may influence transmission dynamics, as well as immunological and virological responses in the infected animal. While experimental infections enable quantification and standardization of dosages, these inocula differ in both magnitude and delivery method from what a susceptible animal encounters in

nature. Finally, indoor hantavirus infection and transmission studies require biosafety level 4 (BSL-4) containment, making these type of experiments difficult to conduct.

An alternative method for understanding hantavirus host-parasite systems is employing manipulative transmission experiments using naturally infected animals in outdoor enclosures. As there are few studies relating individual variations in infectiousness, susceptibility, and behavior to infection dynamics at the population level (Tompkins et al. 2011), this approach may be an important step toward better understanding natural host-parasite systems. The use of outdoor enclosures in a natural setting eliminates emigration and immigration, but allows mice to interact with multiple potential hosts in a familiar setting, in a naturally changing environment. This method also permits all individuals to be known and examined frequently, allowing investigators to track individual measures of aggression (as indicated by wounds) and other descriptive data, and relate them to infection status and transmission cycles. Using an outdoor system precludes the need for an indoor BSL- 4 laboratory. Also, by closely following SNV-infected mice in a semi-controlled setting, investigators can explore possible detrimental, short-term, negative effects of SNV infection on host health that may be missed by mark-recapture methods on open populations. To our knowledge, there have been no published semi-controlled outdoor transmission studies using naturally infected animals for hantaviruses or other microparasites.

We conducted four transmission experiments using wild adult male deer mice in outdoor enclosures in Montana during summer and fall 2007 and 2008. We tested three hypotheses related to SNV transmission: 1) that SNV infected mice would have a higher frequency of wounding and number of wounds; 2) that SNV RNA levels will be the highest 2 weeks p.i., and after the IgG immune response, it will decrease as virus is cleared from the blood, reaching undetectable levels by the end of the acute phase (between 21 days

(Hutchinson et al. 1998) and 35 days p.i. (Botten et al. 2003)); and 3) that mice with higher levels of viral RNA would be more likely to transmit SNV infection. We also tested one hypothesis related to the effects of SNV infection on deer mice: 4) that SNV infected mice would gain less weight over the course of the experiment as compared to uninfected mice. We also measured IgG antibody titers and viral RNA levels in SNV-infected hosts during the acute phase of infection and sequenced the viral strains carried by donor mice to document any effect of viral strain on transmission. The questions we address herein might be considered as investigations within a nascent field that we call transmission ecology. We define transmission ecology as the study of within- and between-host infection dynamics and their relationship to transmission-related host population processes and environmental conditions in an effort to better understand natural disease systems.

3.2 Research Design and Methods

Study site, enclosure construction and protocols, and experimental design

This study was conducted in grasslands near Butte, Montana, USA, mid-May to mid-October of 2007 and 2008. We conducted 4 experiments (Table 3.1). All molecular and immunological data (Figures 3.1-3.3) reported are from the 2008 experiment (Experiment 4; See Table 3.2 for details). There were insufficient blood samples from the 2007 experiments to include in the analyses for the non-behavioral variables of interest. We included all four experiments in the analyses of wound data (Figure 3.4).

Table 3.1: Experimental design for Sin Nombre virus transmission experiments in deer mice (*Peromyscus maniculatus*) in outdoor enclosures near Butte, Montana, 2007–2008. EXP=experiment.

EXP	Months	# Susceptible mice per enclosure	# donor mice per enclosure	Total # of Mice	Duration (weeks)	Sampling Frequency	Transmission events
EXP 1	Jun-Jul 07	3	1	24	4	every 2 weeks	6
EXP 2	Jul-Aug 07	3 or 7	1	36	4.5	every 2 weeks	4
EXP 3	Sept-Oct 07	3 or 7	1	36	6	every 2 weeks	1
EXP 4	Aug-Sept 08	3 or 7	1	36	8	every week	8

Table 3.2: Experimental details for experiment 4 for Sin Nombre virus transmission experiments in deer mice (*Peromyscus maniculatus*) in outdoor enclosures near Butte, Montana, 2007–2008. In enclosure 5, the original donor's (5a) remains were recovered on week 4, and we substituted a new donor mouse (5b). TE=transmission event.

Enclosure	Total # of Mice	Donor Mouse	# Susceptible mice	TE Mice	# TE mice
1	8	D1	7	TE1-E1, TE2-E1, TE3-E1, TE4-E1, TE5-E1	5
2	4	D2	3	None	0
3	8	D3	7	TE8-E3	1
4	4	D4	3	TE6-E4 TE7-E4	2
5	4	D5a D5b	3	None	0
6	8	D6	7	None	0

Experiments were run in six 0.1-hectare enclosures constructed of sheet metal (Fuller and Blaustein 1996, Schauber et al. 1997), with walls extending approximately 1 m above ground and 0.6 m underground (See Supplementary Figure 1). Each enclosure contained 4 underground nest burrows that provided safe, permanent cover for the mice and a way to monitor individuals. Each burrow consisted of a 20.3-cm diameter polyvinyl chloride (PVC) pipe nest chamber with a 2.5-cm diameter PVC pipe entrance tunnel, a 4 × 28 cm wooden board affixed with circular pieces of Styrofoam, and a galvanized metal lid (Kaufman and Kaufman 1989). Within each enclosure, we placed 36 permanent trapping stations approximately 4 m apart. One Sherman live-capture trap (H. B. Sherman Traps, Tallahassee, Florida, USA) was placed at each trap station for up to 3 consecutive nights (until all mice were captured) weekly or biweekly, depending on the experiment. Traps were baited with peanut butter and rolled oats, and contained polyester Fiberfil bedding. We checked enclosure perimeters every 1–2 days for any damage that would allow mice to escape and performed repairs as necessary. Nest burrows were cleaned weekly during each experiment. Between experiments, they were thoroughly cleaned, emptied, and left with the lids off for several days to inactivate any infectious virus.

For each experiment, one infected (donor) and a predetermined number of uninfected (susceptible) mice were released into each enclosure according to the study design (Table 2.1). During Experiment 4, we replaced one donor and 3 susceptible mice that died (carcasses were recovered) with additional quarantined susceptible and donor mice to maintain constant population densities throughout the experiment. Also, one experimental susceptible mouse from enclosure 4 was captured outside the enclosure at week two. He was relocated to a site >5km away from the study site, and we replaced him with a quarantined

susceptible mouse. We did not detect any other emigration or immigration of mice from or into the enclosures throughout the remaining 6 weeks of the experiment.

Rodent collection and selection of experimental subjects

We trapped deer mice at sites within 5 km of the study area and transported captured male deer mice to a central outdoor processing station. We assigned mice to one of 3 age classes according to body weight: mice < 14 g were juveniles; mice 14–17 g were subadults; mice > 17g were adults (Douglass et al. 2001, Kuenzi et al. 2001). Testes position (scrotal vs. abdominal) was used to determine breeding condition. We selected only adult male mice, to eliminate demographic factors like sex, age, and pregnancy from our experiments and because adult males are responsible for the majority of SNV transmission in wild populations (Mills et al. 2010). In the event that there were not enough adult males captured, we included subadults in a preliminary experiment and made sure that the age structure of the experimental mouse populations was as similar as possible among enclosures.

Immunological and molecular testing during field experiments

We collected blood samples from the submandibular vein using a Goldenrod lancet (Medipoint International, Inc., Mineola, New York, USA) or by capillary tube from the retro-orbital capillary plexus after anesthesia with isofluorane (Abbott Laboratories, North Chicago, IL). In the laboratory, we tested blood samples for IgG antibody reactive with SNV recombinant nucleocapsid protein (supplied by the US Centers for Disease Control and Prevention [CDC], Atlanta, Georgia, USA) by enzyme-linked immunosorbent assay (ELISA) according to standard protocols (Feldmann et al. 1993) (some 2007 blood samples) or by a rapid peroxidase enzyme-linked immunosorbent assay (PAGEIA) (blood samples from 2007 and 2008) (Schountz et al. 2007a). All 2007 blood samples from mice positive for anti-SNV antibody by ELISA were also tested by PAGEIA, and the results were identical. We

determined antibody titers using the PAGEIA assay. The blood samples were initially diluted 1:100 in phosphate buffered saline (PBS) and then serially diluted in a log2 series from 1:1,000 to 1:128,000. Both the ELISA and the PAGEIA detect IgG antibody to most or all New World hantaviruses, but do not distinguish among them. However, we confirmed all hantavirus IgG antibody-positive blood samples by molecular testing. We tested blood samples specifically for SNV RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (Chomczynski 1993). Mice were housed one per plastic mouse box until blood analyses were complete. We used clean gloves while replacing feed and water and kept boxes spaced more than a foot apart to prevent cross-contamination during caretaking duties and storage of mice.

Selection of susceptible mice

In the 2007 experiments (Experiments 1-3), we designated rodents as susceptible if they had no detectable SNV RNA by RT-PCR and no detectable IgG antibody to SNV in their blood. Except for the time between capture and testing results (4–10 days), these mice were not quarantined prior to release into the enclosures for each 2007 experiment. Any mouse positive for SNV RNA or antibodies was considered a possible infectious donor.

In the 2008 experiment (Experiment 4), potential susceptible mice were quarantined for 3 weeks in separate plastic mouse boxes in a locked outdoor quarantine facility. We tested blood samples upon capture, approximately 1.5 weeks post-capture, and 3 weeks post-capture before release into enclosures. Any mice that seroconverted during the first 1.5 weeks were removed from the quarantine facility and returned to the site where they had been captured.

Selection of donor mice

As the rodent hosts of other hantaviruses are typically most infectious 2-5 weeks p.i. (Yanagihara et al. 1985, Hutchinson et al. 1998), we chose donor mice that had a blood profile that was considered to represent recent hantavirus infection. These were mice with detectable SNV RNA in their blood, and/or appeared to have recently seroconverted, as shown by a doubling or tripling of measured IgG antibody titers. We chose donor mice for experiment 4 from mice that seroconverted and/or had detectable viral RNA in their blood near the end of the quarantine period, and SNV infected deer mice captured from sites near the original capture sites during the last week of the quarantine.

All mice released into the enclosures were ear-tagged with sequentially numbered metal tags (National Band and Tag Company, Newport, Kentucky, USA). Mice were provided grain, apples, and water on a regular basis. Rodents in enclosures were trapped weekly (2008) or biweekly (2007) to collect blood samples using standardized protocols for SNV surveillance (Kuenzi et al. 2001). Blood was snap frozen on dry ice and stored at -70°C until processing. Body weight, breeding condition (scrotal or abdominal testes), trap location, and number of wounds on the ears and tail (as an indicator of aggressive encounters) were recorded during each trapping session. We tested all blood samples collected from all the experimental animals for SNV RNA and antibody as described above. We also constructed a 0.5 ha trapping grid outside of the enclosures, and tagged and released the outside mice to monitor non-experimental rodent population dynamics and to detect any escapees during each experimental run. While rodents were trapped and monitored for escapees for the entire duration of each experiment, descriptive data were collected for the majority, but not all, trap sessions (until September 19, 2007, and until September 4, 2008) due to personnel constraints.

After the start of each experiment, if a susceptible mouse was found positive for either SNV RNA or SNV antibodies, it was designated as a transmission-event (TE) mouse. Every TE mouse was found positive for both SNV RNA and antibodies, except for 3 mice that did not develop detectable IgG antibodies before either dying or the end of experiment. However, for these 3 mice, we were able to confirm that they were infected with SNV by detecting SNV RNA in 2 or more blood samples collected at different dates, or by sequencing the blood samples (only 2008 mice).

Molecular Procedures

RNA Extraction

We took all appropriate precautions to prevent cross-contamination—RNA extractions were set up in a different biosafety hood from the clean hood from where all downstream molecular assays were conducted. Further, we handled all PCR amplicons in a separate laboratory space with equipment and supplies solely dedicated to the analyses of amplicons.

Blood samples (approximately 50 uL) were added to Tripure Reagent (Roche Applied Science, Indianapolis, Indiana) with a ratio of 1:10 and incubated for 10 minutes to insure viral inactivation. We added 250 uL of molecular grade chloroform to each sample, and incubated the samples on ice, vortexing them frequently for ten minutes. We centrifuged the samples at 4°C for 15 minutes at 12K. We removed 400 uL of the aqueous phase by pipette and mixed it with 70% ethanol in a 1:1 ratio. We applied the aqueous phase/ethanol mixture to a Qiagen RNAeasy column (Qiagen Inc., Valencia, California), and followed the Qiagen recommended RNA extraction protocol until the BPE wash step. We added 500 uL RPE to the spin column and centrifuged it for 2 minutes twice to provide an extra clean-up step to eliminate any residual salts. We continued with the remainder of the RNA extraction

per manufacturer's instructions until final elution in 50 uL of RNAse-free H2O. RNA samples were stored at -70°C.

qRT-PCR

We used 5 µl of extracted total RNA from all donor and TE mice blood samples from Experiment 4 in a qRT-PCR assay designed by PrimerDesign Ltd. (Southampton, UK). The SNV primer-probe set targeted an 81 nucleotide portion of the S segment that is highly conserved across Montana strains and all published SNV and Convict Creek viral strains. We used glyceraldehyde 3-phosphate dehyrdogenase (GAPDH) as our endogenous control and utilized a primer-probe set targeting 108 bp of the *Peromyscus* GAPDH gene (PrimerDesign Ltd). For the donor mice, viral and GAPDH RNA was quantitated from all blood samples starting at their initial capture from the wild until the end of the experiment. Testing of blood samples from a TE mouse included retesting the initial SNV-positive sample (as indicated by prior testing by ELISA/EIA or nested RT-PCR) and all subsequent collected blood samples, as well as blood collected at two or more time points before the initial positive test. Because of limited volumes of RNA, all samples were run in duplicate per target gene. We used the Express One-Step Superscript III RT-PCR w/ Premixed ROX (Invitrogen, Carlsbad, CA, US). The cycles were run as suggested by Invitrogen and PrimerDesign: cDNA synthesis: 50°C for 15 minutes followed by 40 cycles at 95°C for 20 sec; 95°C for 1 sec, 60°C for 20 sec. PCR-grade water was used as a negative control. Our standard curve consisted of serially diluted cell culture supernatant infected with SNV strain NMR11 and ranged from 10^5 to 10^{-1} viral titer. The efficiency of the assay ranged from 95-103%. Our standard curves produced correlation coefficients (r^2 = 0.993 to 0.997).

For each sample in which SNV RNA was detected, we calculated $C_{T(GAPDH)} = \bar{x}\,C_{T(GAPDH)} - x\,C_{T(GAPDH)}$ ($\bar{x}\,C_{T(GAPDH)}$=27.3, SD=1.36). We then

n Δormalized each sample by adding $\pm \Delta C_{T(GAPDH)}$ to the SNV CT value. We then entered the adjusted SNV CT value into the following equation: $10^{\frac{(C_T - b)}{m}}$ where b= y-intercept and m=slope. We used the slope and y-intercept values calculated from the standard curve to calculate the relative SNV RNA quantities for samples from that plate. All the RNA samples per individual mouse were all run on the same plate and compared to the standard curve run simultaneously on the same plate. Note that the RNA quantities reported are not actual viral copy numbers, but rather arbitrary units to demonstrate the fold differences in viral load over time for each mouse.

RT-PCR

All blood samples for every mouse included in the experiment were initially screened in 2008 using a nested RT-PCR as described previously (Chomczynski 1993). In 2010-2011, we designed and implemented a new RT-PCR assay to sequence the viral strains from blood samples of all donor mice and TE mice from experiment 4. We sequenced two genes: the small (S) segment which encodes the nucleocapsid protein and medium (M segment) which encodes two glycoproteins. These two genes usually exhibit more variation than the large (L) segment, and are more informative when looking for different hantavirus strains. Initially, primers used to amplify the S segment of the Montana strains of SNV were designed using the partial sequence of a Montana strain available at the start of the project, M11PolsMT, and its most closely related SNV strain, Convict Creek Virus (Black et al. 2009). As more sequence data were collected for the SNV Montana strains, primers designed from the 5' and 3' termini of Convict Creek Virus S segment (Genbank Accession number (ACN) # L33816) and M Segment (ACN# L33684) were coupled with internal primers derived from the newly sequenced Montana strains (see Supplementary Table 1) to gather sequence data.

As hantaviruses have highly conserved, complementary terminal sequences across species (Plyusnin et al. 1996), we focused on collecting sequence data for the S and M segments except the highly conserved terminal 3' and 5' ends (sequences for S segment range from nt 22-2020 and sequences for the M segment range from nt 22- 3685 in comparison to Convict Creek virus).

We used 5 uL of total RNA extracted from blood samples in RT-PCR assays with the Superscript III One-Step RT-PCR with Hi Fidelity Taq Kit (Invitrogen). We followed the manufacturer's instructions for set-up. The RT and cycling conditions were also principally as suggested by the manufacturer: cDNA synthesis: 55°C for 30 minutes, pre-denaturation at 94°C for 2 minutes, followed by 40 cycles (or 45 cycles for primer set S1L/830R) of: 94°C for 15 sec, 55°C (or 50°C for primer set S1246/2047R, See Supplementary Table 1) for 30 sec, 68°C for 1 minutes, and the final extension step was at 68°C for 5 minutes. PCR products were purified and sequenced using the PCR primers or internal sequencing primers (S Table 1) by Beckman Coulter Genomics (Danvers, MA, USA). We used the program Primer3 to design all RT-PCR primers (except for S1L, M1L, S2047R, M3696R—which were just the first and last 22 nt in the S & M segments). We performed initial sequence alignments using DNASTAR Lasergene programs Seqman and MegaAlign. All reported sequences have at least two sequencing passes of each region, except for the initial and final 40 nt in the highly conserved termini (have one pass at least).

Statistical Analyses and variables

We conducted statistical analyses using Microsoft Excel 2007 and R (R Development Core Team, Vienna, Austria, 2011). Because of the very low number of transmission events and small sample size of our experiments, we used Fisher's exact two-tailed test (FET) to make comparisons between the proportions of infected and uninfected mice in relation to

wounding status and survivorship. In addition, we used t-tests to compare mean weight gain and mean number of wounds per mouse.

For all analyses, we excluded data from mice that were released into the enclosures and never recaptured (n=7 for 2007 experiments, n=1 for the 2008 experiment). We also excluded data from one donor in one enclosure (Enclosure 3) in Experiment 1 for the analyses of number of new wounds, because it was unclear whether the donor mouse was truly infected. His blood was positive for SNV RNA for one of two samples, but he was not recaptured again to reconfirm infection status. There were no TE mice in Enclosure 3 in Experiment 1. In analyses of wounds and rate of weight change for the 2008 experiment, we also excluded information from two mice that were in the experiment for less than 2 weeks, because there were insufficient data for analyses of those variables. For the analyses of rate of weight change in relation to seroconversion, only 5 TE mice from Experiment 4 had enough sampled pre- and post-seroconversion weight data to be included in the analyses.

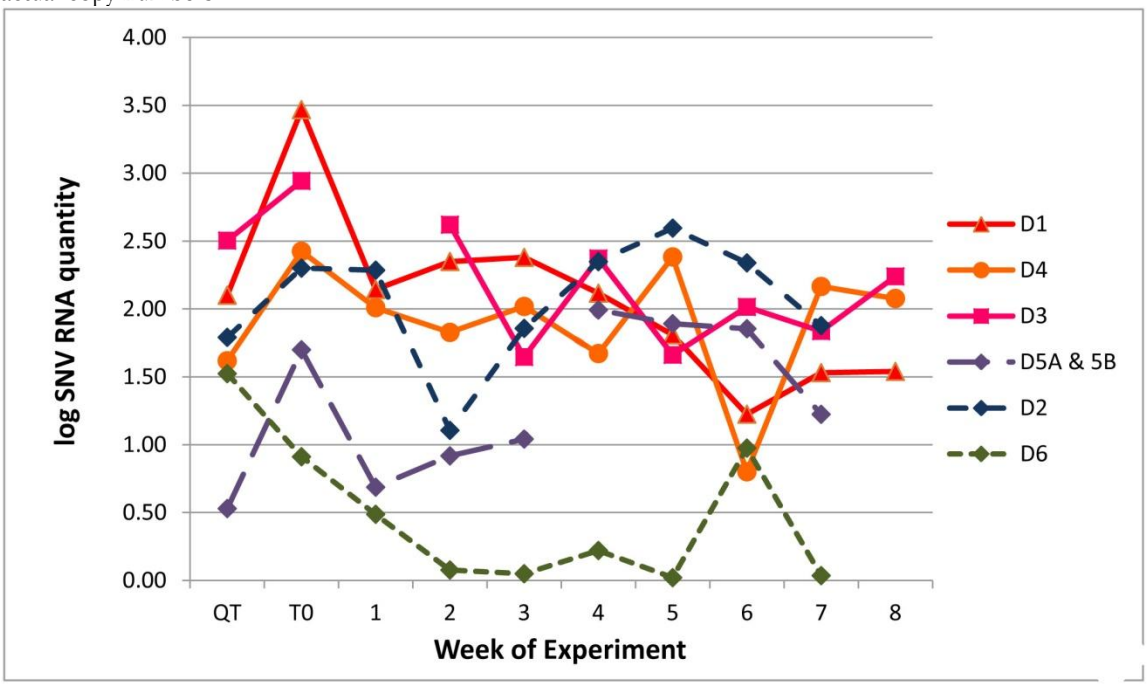
Wounding

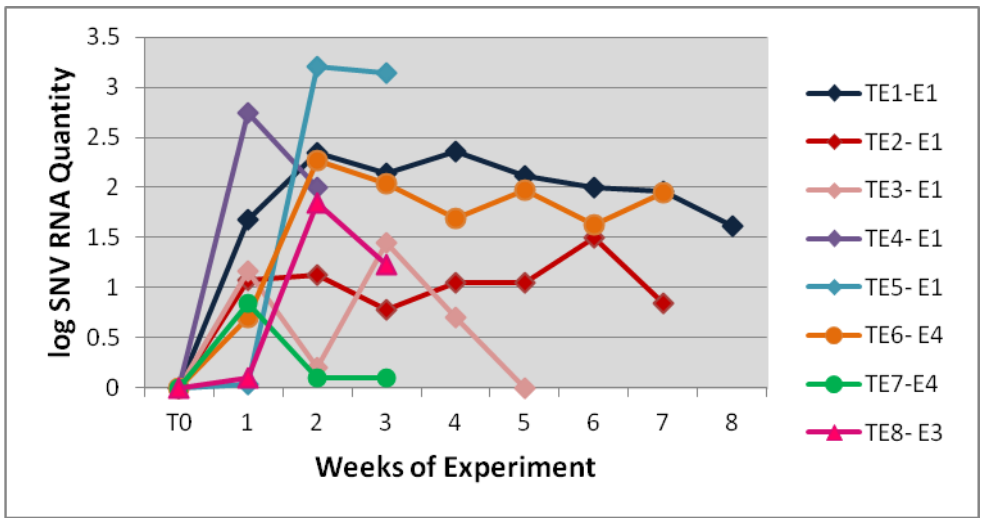
The total number of new wounds was determined by counting the number of new wounds on an individual over the course of the experiment. This conservative measure includes only those wounds detected on a new location on the mouse (tail vs ear) or if there was an increase in the number of wounds from the previous sampling session. This ensured that the same wound was not counted twice for any animal, but also allowed for the possibility that some new wounds in the same area as a previous wound may not have been counted. We ran a linear regression model with infection status as a categorical predictor variable, and the number of new wounds per experiment as the outcome.

3.3 Results

Although all seven donors were from the same capture site, according to S segment sequences, there were three variants of Montana SNV sequenced from the donor mice: SNV_MH1 (S segment: Accession number (ACCN #) JQ690276; M segment: ACCN # JQ690279), SNV_MH2 (S segment: ACCN# JQ690277; M segment ACCN# JQ690280), and SNV_MH3 (S segment: ACCN # JQ690278). At the nucleotide level, SNV_MH1 and SNV_MH2 had a 97.8% identity for the S segment and 98.6% identity for the M segment. At the nucleotide level for the S segment, SNV_MH3 had a 98.5% identity with SNV-MH1, and 97.9% identity with SNV-MH2. We were unable to sequence the M segment for SNV_MH3 because of insufficient sample. Five of the seven donors (Donors 1, 2, 3, 5b and 6) were infected with SNV-MH1, donor 4 with SNV-MH2, and donor 5a with SNV-MH3. Of the three successful donors, two were infected with SNV-MH1 (donors 1 & 3) and one (donor 4) was infected with SNV-MH2, (see Figure 3.1a for viral RNA loads). All the virus sequence data collected from TE mice had a 100% identity to the virus strains of the suspected donor. The donor mice that successfully infected other mice (red, orange, and pink solid lines; Figure 3.1a) had a statistically significant higher mean RNA level in comparison to their unsuccessful counterparts at the $p<0.10$ level of significance (purple, blue, and green dashed lines; Figure 3.1a) (successful donors n=3, $\bar{x} = 264.14$, SD= 152.05; unsuccessful donors: n=4, $\bar{x} = 66.17$, SD=75.98; two sample t-test with unequal variances: $t_3=2.06$, $p=0.065$).

Figure 3.1a & 3.1b: SNV RNA levels for (a) donor mice before and during experiment 4 and (b) TE Mice at Time Zero (T0) and during experiment 4. In enclosure 5, the original donor's (5a) remains were recovered on week 4, and we substituted a new donor mouse (5b). For donor 3, there was insufficient sample from 8/1/08 for qRT-PCR analysis. Quarantine (QT) refers to the initial blood sample for each donor mouse. E1, E4, and E3 stands for the enclosure number where each TE mice became infected. Time zero (T0) represents the blood sample that was negative for both SNV RNA and antibody prior to the first sample in which SNV RNA was detected.SNV RNA quantities are proportional (see Methods), not actual copy numbers.





All TE mice experienced an initial peak in SNV RNA levels in blood 1-2 weeks p.i. (Figure 3.1b). Seven of the 8 TE mice (except TE 5 from Enclosure 1) developed IgG antibody to SNV within 2-3 weeks of infection (Figure 3.2). The overall level of RNA

diminished after the initial IgG antibody response (Figure 3.3; also compare Figure 3.1b to Figure 3.2), but there were often spikes in RNA levels after the IgG antibody response, sometimes as high or higher than the initial peak p.i. (Figure 3.3).

The TE mice had highly variable patterns in IgG titers p.i. (Figure 3.2). Some of the mice sustained higher levels of SNV RNA in blood throughout the initial phase of their infection. For example, TE 1 had 10 times the level of viral RNA as TE 2 during the first 4 weeks p.i., even though they were infected by the same donor with an identical virus variant (Figure 3.1b and 3).

Figure 3.2: IgG antibody titers in response to infection for all transmission-event (TE) mice that seroconverted during Experiment 4. E1, E4, and E3 are the enclosure numbers where each TE mice became infected. Time zero (T0) represents the blood sample that was negative for both SNV RNA and antibody prior to the first sample in which SNV antibody or RNA was detected.

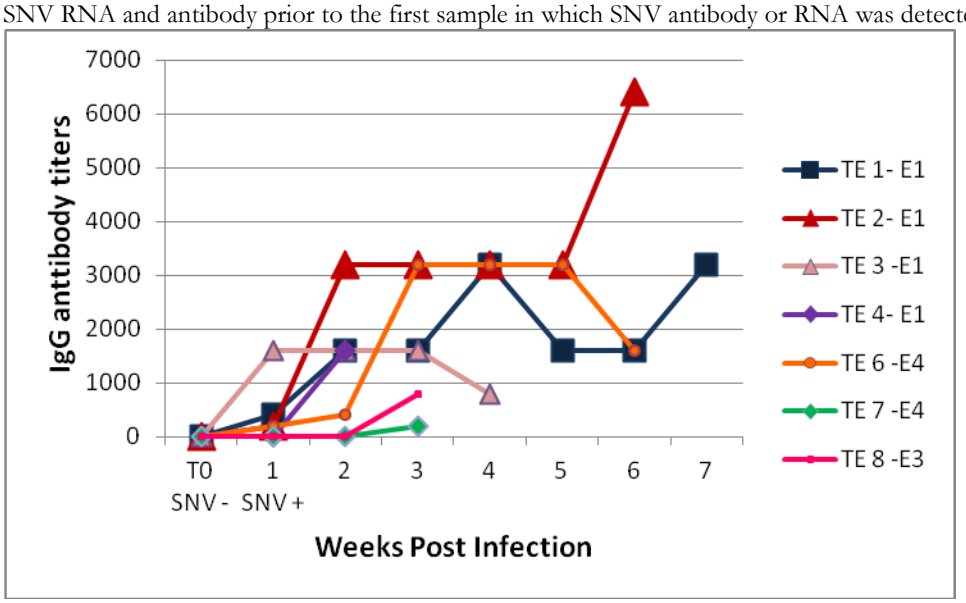
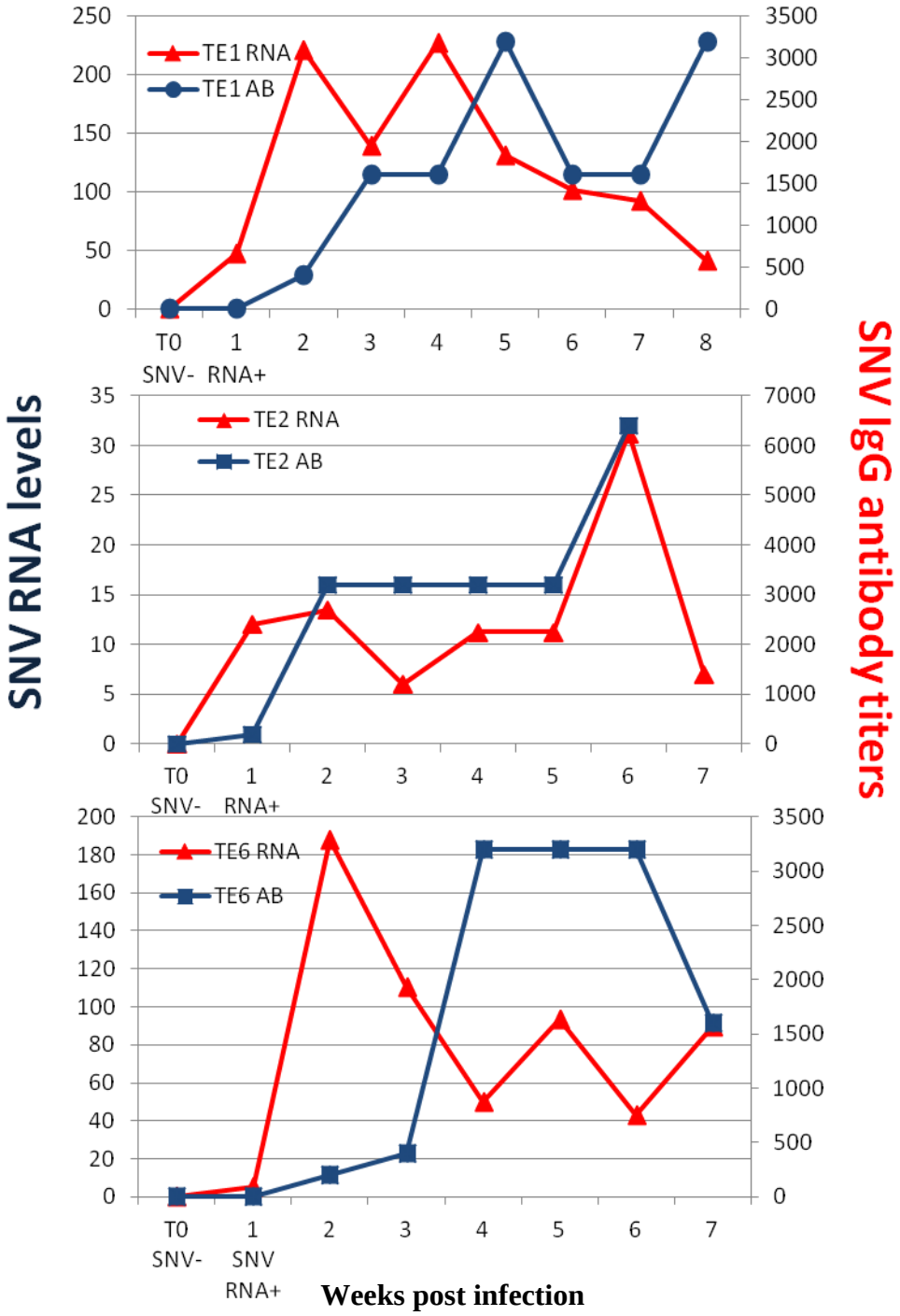


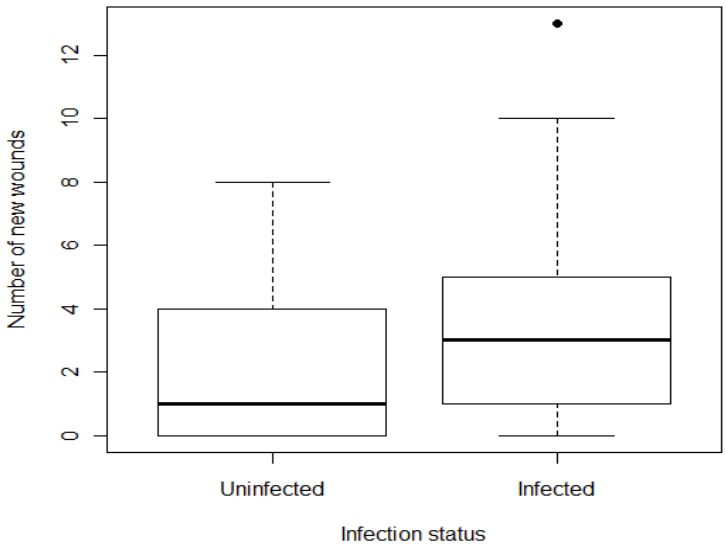
Figure 3.3: SNV RNA levels and IgG antibody titers for the three transmission-event (TE) mice with the longest time course of infection. Time zero (T0) represents the blood sample that was negative for both SNV RNA and antibody prior to the first sample in which SNV antibody or RNA was detected.



Wounding and weight change in relation to infection

The number of new wounds per mouse was significantly higher for infected mice than uninfected mice over all four experiments (t_{104}=2.12, p=0.04, β=-1.2533, SE= 0.5892; Figure 3.4). There was no significant difference between uninfected and infected mice in the proportion of mice with wounds across all four experiments (FET: p=0.30).

Figure 3.4: Median number of new wounds per individual mouse by infection status. Thick horizontal line is the median; top and bottom of boxes represent the 25 and 75 percentile; horizontal lines at ends of dashed lines represent the minimum and maximum values, excluding one outlier (black dot). The "infected" category includes all donor and transmission-event (TE) mice from all four experiments. The "uninfected" category includes all susceptible mice that never seroconverted throughout the four experiments. Each mouse is represented only once in the analyses.



There was no significant difference in rate (grams/week) of weight gain or loss between infected ($\bar{x}$ =-0.29, SD= 0.67) and uninfected ($\bar{x}$ =-0.02, SD=0.59) mice in experiment 4 (t_{35}=-1.19, p=0.12). Also, there was no difference in the rate of weight gain/loss before ($\bar{x}$ =-1.81, SD=5.52) and after ($\bar{x}$ =-0.33, SD=0.85) seroconversion in TE mice for which this comparison was possible (paired two-sample test for means: t_5=-0.61,

p=0.29), nor was there a significant difference between infected and uninfected mice in the proportion that died during experiment 4 (FET: p=0.40).

3.4 Discussion

Objectives of our study included measuring SNV RNA loads and IgG titers in blood samples collected from naturally infected deer mice during the acute phase of infection and determining the influence of viral RNA loads and virus strains on transmission. We also tested whether SNV infected mice were more likely to be wounded and accrue a higher number of wounds as compared to uninfected mice. These objectives are critical to understanding the natural cycle of infection in an individual host, but have never been explored in hantavirus-infected hosts using serially collected samples from the same naturally infected individuals over time and related to population level processes. We also explored host weight changes and survival in relation to SNV infection.

Hantavirus RNA was detected in blood of TE mice as early 1 week post-infection (TE mouse 1 had detectable RNA in his blood one week after release into the enclosure; See Fig. 3.2). TE mouse 1 had a peak in viral RNA level approximately 1 week later (~14 days p.i.). Five of eight TE mice experienced a peak RNA level 2 weeks after their last RNA negative result, while the other three experienced a peak at 1, 3, and 6 weeks after their last RNA result. Our field results are similar to those of a laboratory experiment (Hutchinson et al. 1998), where cotton rats experimentally infected with BCCV showed a peak in infectious viral titer in blood at 14 days p.i. Using weekly samples we readily detected viral RNA in blood for up to 8 weeks post-infection, indicating that viral RNA was present in the blood throughout the acute phase of infection. This finding is in direct contrast to previous studies indicating that hantaviruses are cleared from the blood 10-21+ days p.i. (HTN: LeDuc et al.

(1992); BCCV: Hutchinson et al. (1998)) or 35+ days p.i. (Botten et al. 2003) and then only intermittently detected throughout the remainder of the acute and persistent phases (Hutchinson et al. 1998, Botten et al. 2003). Although previous investigators did not report consistent presence of viral RNA in blood throughout the acute phase for SNV or other hantaviruses, they did find that mice were shedding infectious virus (Hutchinson et al. 1998) or viral RNA (PUUV: Hardestam et al. (2008) in saliva and excreta throughout the acute phase, past 10-21 days p.i. A recent study found that T-cells isolated from deer mice with acute experimental SNV infections include components of immunosuppressive regulation T-cell activity (expression of Forkhead box P3 transcription factor) and cytokines (TGF-β_1 and IL-10) associated with downregulating inflammatory responses (Schountz et al. 2007b). These discoveries indicate that SNV infection may trigger a diminished adaptive immunological response (Easterbrook et al. 2007b, Schountz et al. 2007b), allowing the virus to be maintained in the host's blood during the acute phase of infection and permit the virus to establish a persistent infection within the host. There are no published studies that mention successful quantitation of hantaviral RNA from blood since Hutchinson et al. (1998). Recent technological advances in qRT-PCR technology, species specific primers-probe sets, and RNA extraction methods probably aid in lowering the detection threshold, thereby enabling quantitation of viral RNA at levels well below what was possible a decade ago.

Most laboratory studies sacrifice experimental animals at predetermined intervals, but we were able to sample the same animal for up to 8 weeks p.i. Even within an individual animal, there were highly variable viral RNA levels and antibody titers over time. TE mice had highly variable peak levels in RNA 1-2 weeks p.i. and, after the IgG immune response there were different trajectories of RNA levels in blood (Figures 3.2 & 3.3). Observing

highly variable immune responses following infection is common when using wild outbred mice (Botten et al. 2003). Our mice were also exposed to environmental stressors, which also can affect immune responses and resulting viral RNA levels.

Although our sample sizes were small, the three donor mice that successfully infected other mice within their enclosures in 2008 appeared to have higher mean levels of SNV RNA over the course of the experiment ($p=0.06$). Although unsuccessful donors 5a, 5b, and 6 maintained lower levels of viral RNA throughout the experiment (Figure 3.1a), donor 2 experienced an increase in viral load near the end of the experiment. It is possible that other mice were infected by this donor but we did not detect them before the conclusion of the experiment. These data provide a starting point for future studies to determine if there is a threshold level of viral RNA that prompts SNV transmission.

The transmitted strains of virus in each TE mouse were identical to one another and to that of the donor mouse within each enclosure. Thus, all TE mice were infected by the donor strain in their enclosure either from the donor himself or another mouse infected by that donor. These molecular data corroborates our trapping data—we did not detect any non-experimental mice entering the enclosures during experiment 4, indicating that the enclosures effectively closed our population. Although we know when a mouse became infected within 1 week, after the first transmission case, molecular data do not help determine which mouse or mice propagated the infection as the experiment progressed. Future studies could implement cameras, pit tag recorders, and fluorescent marking powder (Clay et al. 2009) to identify contact structures and their relationship to the chain of infection within enclosed mouse populations.

The infected mice from all four of our experiments had a significantly higher number of total new wounds in comparison to susceptible mice that never seroconverted. This

supports previous studies reporting higher frequencies of wounds or scars (Glass et al. 1988, Douglass et al. 2001) or more severe scars (Hinson et al. 2004) in antibody-positive hosts. Our study has one distinct advantage over most correlation analyses from previous studies. Because SNV is horizontally transmitted, older mice are more likely to be infected and because of accumulated experience, older mice are more likely to have scars. Thus in a random field sample, a correlation between scars and infection status is to be expected simply because both variables are correlated with age. Our data were not cofounded by age or experience. We chose mice of similar age but, most importantly, we ignore accumulated scars and include only new scars that occur during the experiment. Thus, we clearly demonstrate an association between scars and infection while controlling for the potential confounders of age and experience. Although the simplest explanation for this association is that infection is a consequence of aggression, it is also possible that aggression is a consequence of infection. It has been shown that infection with Seoul virus may influence levels of host aggression (Hinson et al. 2004, Klein et al. 2004, Easterbrook et al. 2007a). As we saw no evidence of indirect transmission, our results also support the general consensus that the main route of transmission is directly through aggressive encounters (Glass et al. 1988, Douglass et al. 2001, Kuenzi et al. 2001, Hinson et al. 2004). The nest burrows present in our enclosures would be an ideal environment for indirect transmission. The mice defecated and urinated in the nestboxes regularly. We recorded the presence of urine or feces in 75-100% (depending on the experiment) of the nestboxes each week. In 2007, we observed 1 donor mouse cohabitating with the same susceptible mouse on two occasions, and other donor mice cohabitating nestboxes with multiple mice on at least two occasions. None of these susceptible mice seroconverted during the experiment. In an Andes hantavirus transmission study with naturally infected donor rodents, the authors reported 16

of 130 direct transmission events based on one-on-one rodent pairings in steel drums, but 0 of 62 attempts at various indirect transmission experiments (Padula et al. 2004). All previous reports of indirect hantavirus transmission (Gavrilovskaya et al. 1990, Hutchinson et al. 2000, Hardestam et al. 2008), among rodent hosts used experimental viral inoculations and were conducted in laboratories. It may be that naturally infected rodents shed less virus in comparison to experimentally infected individuals or that exposure to environmental elements in an outdoor environment is enough to disperse or inactivate infectious virus, limiting or preventing indirect transmission in the wild. We cleaned the nest burrows weekly and this may have decreased the likelihood of indirect transmission. This said, we cannot rule out the possibility that there was indirect transmission, or a mixture of indirect and direct transmission.

Transmission occurred in enclosures where donor mice tended to have higher viral loads, and infected mice in our experiments showed significantly more evidence of aggressive encounters than uninfected mice. Thus, there is evidence that increased contact with individuals with higher viral loads drove SNV transmission in our experimental populations. In a similar manipulative experiment in a completely different host-parasite system, social guppies (*Poecilia reticulata*) with a higher mean intensity and duration of infection were more likely to transmit ectoparasites (*Gyrodactylus turnbulli)* to susceptible guppies (Johnson et al. 2011). As in our study, the frequency of contact with infected hosts and host infection load was crucial in governing which populations experienced an epidemic.

There did not appear to be a difference in transmission among viral substrains; SNV-MH1 and SNV-MH2 were both transmitted to all but one susceptible mice within their respective enclosures. Further research is needed on the influence of host genetic factors and

other immune system components in response to infection and how those determine the propagation of the virus in host individuals and populations.

Although other studies of deer mouse populations in Montana have indicated that there may be a cost of SNV infection in terms of survivorship or weight gain, we did not find statistically significant differences in these variables between infected and uninfected mice in experiment 4. This could be because we provided supplemental food and water in the enclosures. Also, our longest running experiment (4) was only 8 weeks; a longer experiment might detect SNV infection costs. We did not see any difference in weight gain in seroconverting mice before vs. after infection, but we were only able to measure this in very few individuals (n=5), in comparison to Douglass et al. (2007) (n ≅ 1,466).

Recent analyses of a 15-year mark-recapture dataset from Montana deer mouse populations found that infected male deer mice have a 13.4% decreased apparent survival rate, compared to uninfected males and females (Luis et al. 2012). The authors postulate that their reported decreases in survival may be an underestimate, because there may be SNV-infected individuals that become infected and die before detection by monthly sampling. In our study, we monitored mice weekly in a closed population, and detected some supporting evidence for this hypothesis. TE mouse 5 from Enclosure 1 (Figure 3.1b) showed a rapid increase in viral RNA across a 3-week span (viral RNA quantity of up to 1,000 times to the original RNA level and ranging from over 2-100 times higher than other TE mice) and did not mount an IgG response before dying. The only other mouse that had a RNA level close to TE 5 was TE 4 (peak of 500 units), which did mount an IgG antibody response but died soon after becoming infected. This suggests that, in the wild, there may be individuals unable to tolerate SNV infection and quickly die without being detected in mark-recapture studies that sample less frequently.

To estimate relative degrees of infectiousness, we assumed that the presence of viral RNA in blood equates to the presence of infectious virus. Although this is likely true, it has not been demonstrated (e.g., the RNA could be bound in noninfectious antigen-antibody complexes). In addition, we did not attempt to measure viral RNA shedding (in saliva and excreta) into the environment, which might be a much more accurate predictor of relative infectiousness. Future studies using similar experiments to quantify viral RNA in excreta and saliva would be useful, not only as potential measures of virus shed by infected hosts, but also as an indicator of whether virus (or viral RNA) in blood is an accurate predictor of virus shedding.

By exploring immunological and virological components of a hantavirus infection in naturally infected deer mice in relation to host behavior, we provide an initial step toward better understanding important aspects of hantavirus-host infection dynamics in the wild and, in turn, broadening our understanding of rodent borne zoonotic viruses.

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4 Summary

The inception and growth of the field of disease ecology has led to a better understanding of the host-parasite relationship as an important ecological force, thus enriching our understanding of population dynamics and ecosystem function and health. Consequently studies of disease ecology have informed better conservation, public health, and agricultural management and control strategies. As a broadly interdisciplinary field, disease ecology has bridged gaps between multiple scientific and medical specialties and led to a better understanding of natural ecosystems and human populations. The series of experiments described and analyzed during the course of this dissertation tested some crucial tenants of theoretical and observational theories regarding disease transmission in natural populations, and initiated a new sub-specialty, transmission ecology. Transmission ecology is dedicated to broadening our understanding of natural infections and disease related transmission processes. While the importance of some ecological and within-host infection factors on disease transmission were confirmed, others were found to be more substantive than previously believed and vice versa.

Seasonality, density dependence, frequency dependence and transmission

Seasonality was a governing force within these experiments. There were significant differences in both wounding and transmission incidence between summer and fall (Chapter 2). Seasonality appeared to be more influential than host population density in transmission rates. This is probably a result of host behavioral and hormonal changes during the breeding season that may increase the likelihood of transmission through increased contact and/or susceptibility to infection. In a study in the cowpox virus-field vole system, a SIR model based on 6 years of mark-recapture studies indicated that the value of the transmission

coefficient was intermediate between what would be considered density and frequency dependent transmission (Smith et al. 2009). The authors indicated that transmission appeared to be more density dependent during the winter months, when animals were less likely to fight, but were more likely to congregate for overwintering. Transmission appeared to be more frequency dependent during the breeding season (summer-fall). A PUUV study in Sweden also indicated differences in underlying contact structure governing disease transmission by season, with PUUV transmission being density dependent in the fall and delayed density dependent in spring (Niklasson et al. 1995). To fully elucidate whether SNV transmission is density or frequency dependent, and whether this varies by season, further experiments are needed in other seasons with mice of known levels of infectiousness. These additional experiments should use mice for which RNA viral loads in blood samples (Chapter 3), saliva, and excreta samples are known prior to selection as donor mice. The method of extracting and quantifying viral RNA from saliva and urine samples, as outlined in the protocols included in the appendix (Chapter 7), can be used to determine whether and how closely blood viral RNA levels match viral RNA secreted in saliva and excreted in urine.

We did not detect a significant difference according to host population density on transmission frequency in our experiments. A recent SNV SI model incorporating fluctuating environmental conditions and their influences on host demography was parameterized using 15 years of mark recapture data at one site in Montana and was able to predict the critical host density at another site (Luis et al. In review). (Luis et al. In review) indicated that if environmental conditions were favorable to enable the host population to surpass a critical host density (16.5 mice/hectare), then SNV could be maintained in a host population, and show delayed density dynamics in infection prevalence. This led the authors to conclude that SNV transmission is density dependent in nature. Our findings do not

directly support this discovery; the number of hosts in the low density treatments were on par with a host population density (4 mice/0.10 ha or 40 mice/ ha) higher than the critical host density identified by (Luis et al. In review), indicating that there should have been epidemics in these enclosures. However, we were directly measuring transmission over an 8-week period, and (Luis et al. In review) extrapolated transmission information from monthly prevalence data over a 15-year period—two equally important, but inherently different approaches to calculating transmission rates in a population. (Luis et al. In review)'s SNV SI model indicates the importance of environmental conditions (which are largely influenced by seasonality) on disease persistence, which is in concordance with our results indicating the importance of the effects of seasonality (Chapter 2) on transmission.

The results of Chapter 3 indicate the differences in aggression and host viral load may be more influential than host population density in relation to the frequency of SNV transmission on a local scale. This discovery indicates that even when exploring transmission within one sex/age group (adult males), host heterogeneities in behavior, viral load, and immunological response can be highly influential in disease transmission. These observations suggest that SNV transmission may be governed by frequency dependent processes during the breeding season, which has been also been documented in other systems (cowpox virus-field voles; Smith et al. (2009)). Prior theoretical and experimental studies have indicated that host heterogeneities in behavior (Adler et al. 2008, Clay et al. 2009) and infectiousness, and susceptibility (Woolhouse et al. 1997, Adler et al. 2008) can influence disease emergence (Yates et al. 2006) and transmission (Woolhouse et al. 1997, Adler et al. 2008, Clay et al. 2009). A model parameterized from a biannual mark-recapture SNV study in Utah deer mouse populations showed that behavioral and susceptibility heterogeneities between the sexes are crucial driving forces in the maintenance of SNV

infection in an environment (Adler et al. 2008). Our results also support these conclusions and further indicate that these differences can be influential even within the male population. Our results also add evidence that variations in infection load can also determine whether an infection spreads in an environment.

Effects of reproduction, sex, and behavior on transmission

In our experiments, we focused on the major transmitters of SNV in natural populations, adult males. In most studied natural vertebrate host populations, males are often more likely to be infected than females (Zuk and McKean 1996). This pattern of male-bias in infection prevalence has been linked to differences in infection susceptibility (Folstad and Karter 1992, Restif and Amos 2010) (especially for macroparasitic infections; (Grear et al. 2009)), and behavior (Restif and Amos 2010) between males and females, both of which can be influenced by testosterone. Testosterone can influence transmission through influencing host contact. Higher levels of male aggression are correlated with increased levels of testosterone during the breeding season in non-human primates (Cavigelli and Pereira 2000). During the breeding season, breeding male African striped desert mice (*Rhabdomys pumillio*) often have the highest testosterone levels (Schradin 2008). Also, in white-footed mouse (*Peromyscus leucopus*) populations, individuals with testosterone implants had higher contact rates than those from a non-treated population (Grear et al. 2009). Future studies should measure testosterone levels in the experimental mice, to determine whether there is a relationship between increased testosterone levels in males and increased aggression, viral load, and susceptibility and lower immunity to infection.

Although males are the primary subgroup of interest in natural SNV dynamics, females are also often infected by SNV, and contribute a significant portion of the overall prevalence. Females also experience seasonal hormonal fluctuations and subsequent

behavioral changes related to reproduction and pregnancy that are quite different than males. As pregnancy is an immunosuppressive process, it may also influence infection prevalence in an environment, and females can be quite aggressive and territorial during the breeding season to protect their broods (Wolff 1989). Therefore, running female-only transmission experiments and measuring wounding frequency and hormonal levels (for testosterone, estrogen and gestational related hormones) and relating these to transmission would provide valuable information about the transmission ecology of a virus in a female host.

Male-female mixed populations are a more natural arrangement of hosts. Therefore, to more fully understand natural SNV transmission, experiments should also be conducted using mixed male and female populations. Comparing and contrasting the transmission and behavioral dynamics between same sex and opposite sex arrangements may help elucidate the relative roles of each type of interaction in disease transmission in the wild.

We found a significant relationship between infection status and incidence of wounding—one that is not confounded by other factors such as sex, age, or previous experience, as in previous studies (Chapter 3). However, wounding is an indirect measure of contact and cannot directly reveal the dominance and contact structure and subsequent effects on transmission within the enclosures. Previously described methods (Clay et al. 2009) of using passive integrated transponder (PIT) tags and fluorescent marking powder to more closely approximate contact structure could be used or night-vision cameras could be installed within the enclosures or nest burrows. These methods, combined with close monitoring of infection status may elucidate other within- and between-sex behaviors besides territorial fighting and biting (e.g. communal nesting, mutual grooming, courtship-related behaviors) that significantly contribute to transmission.

Resistance and tolerance to persistent infections: The next phase of transmission ecology

Currently, disease ecologists are focused on understanding tolerance and resistance in host-pathogen systems, but most of these studies have been conducted on protozoan and helminth infections (Raberg et al. 2007, Rohr et al. 2010, Ferreira et al. 2011) (de Roij et al. 2011). Our results indicate the SNV- deer mouse system may be an ideal system to extend our understanding of infection resistance and tolerance, and how it may influence host population processes. In our experiments, we examined viral RNA loads during the acute phase of infection, linked them to the success or failure of a donor's ability to propagate infection, and were able to monitor peaks and troughs of viral RNA load post- IgG response. Viral RNA was continuously detected throughout the acute phase of infection, and infected mice were able to tolerate very different levels of viral RNA loads circulating in their blood. These initial results indicate that the SNV-deer mouse system would be ideal to further the understanding of host tolerance of persistent infections and relate them to transmission. Future experiments could extend our methods and findings to include multiple facets of viral infection (e.g. replication and shedding) and expand coverage of the host's immune response to include multiple factors of the innate and adaptive response (e.g. regulation T-cell activity and related cytokines (Schountz et al. 2007b) and other components crucial in the tolerance of persistent infections). In our study, possible evidence of genetic resistance was observed. In two enclosures in two experiments (June-July 2007 experiment and August-September 2008 experiment), all but one susceptible mouse became infected. As host genetic factors have been linked to susceptibility (Root et al. 2004, Schonrich et al. 2008) and resistance (Schonrich et al. 2008) to hantavirus infection, it is possible that some mice are resistant to infection. Further studies should explore the genetic background of

donors, TE mice, and susceptible mice that never seroconvert in order to further understand the influence of host genetics on SNV resistance in deer mice.

5 Supplementary Figures and Tables

SF1: Photos of enclosures and study site in shrub-steppe grassland near Butte, MT

Supplementary Table 1: Information on primers and reference sequences used to sequence S and M segments of SNV-MH strains 1, 2, and 3. Includes amplification regions of each primer set and reference strains used to design primers. ACCN #: Genbank Accession number.

S Segment						
Amplification Region	**Primer**	**Reference strain (ACCN#)**	**Forward primer (5'-3')**	**Primer**	**Reference strain (ACCN#)**	**Reverse Primer**
nt 22-830	S1L	Convict Creek (L33816)	TAGTAGTAGACTCCTTGAGAAG	S830R	MtPeS09 (JQ690282)	ATTGGCGTGTTATGAAATAGGC
nt 632-1995	S632L	MtPeS09 (JQ690282)	AGGTTCCGTACAATTGCCTGTG	S1995R	MtPeS09 (JQ690282)	TTCGTTGAGGTAATAGGGAAGG
nt 1246-2047	S1246L	Convict Creek (L33816)	GATCCTGAACTCAGGGAACTTG	S2047R	Convict Creek (L33816)	TAGTAGTATACGCCTTGAAAAGC
M Segment						
Amplification Region	**Primer**	**Reference strain (Acc#)**	**Forward primer (5'-3')**	**Primer**	**Reference strain (Acc#)**	**Reverse Primer (5'-3')**
nt 22-962	M1L	Convict Creek (L33684)	TAGTAGTAGACTCCGCACGAAG	M962R	Convict Creek (L33684)	GCAGTAACAGGTCCAGCTATTC
nt 860-1684	M860L	Convict Creek (L33684)	GGTTTTAAATCCAAGAGGTGAAG	M1684R	MtPo09 (JQ690284)	CAAATGTCACACACCATTGAGC
nt 1124-2051	M1124L	MtPo09 (JQ690284)	AAACAGTACCCCTCACATGGAC	M2051R	MtPeS09 JQ690283	CATGTGCTGTATCAGACCAACC
nt 1772-2648	M1772L	MtPo09 (JQ690284)	GCCCATACTGCATGACAATAAC	M2648R	MtPo09 (JQ690284)	TAATCCCACCTTGCTCTAAAGG
nt 2364-3696	M2364L	MtPo09 (JQ690284)	ACCAGATTGCCCAGGGGTAG	M3696R	Convict Creek (L33684)	TAGTAGTAGACTCCGCGGGAAC

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7 Appendix

7.1 Detection of *P. Maniculatus* GAPDH RNA from Orophayngeal fluids (OPF) from swabs

RNA Extraction Protocol from Orophayngeal fluids (OPF) from swabs

***This protocol was adapted from a user-developed protocol (**Isolation of total RNA from ejectable buccal swabs using the RNeasy® Micro Kit)** from the Qiagen website.*
*It has not been thoroughly tested and optimized by QIAGEN***

IMPORTANT: Relevant excerpts from the *RNeasy MicroPlus Kit Handbook* are included in the protocol, and sometimes the tester will be referred to the handbook. It is a good idea to read the handbook before beginning, and to have it handy.

Reagents and equipment to be supplied by the user

- QIAshredder™ Homogenizer (Cat. No. 79654)
- 14.3 M β -mercaptoethanol (β -ME)* (commercially available solutions are usually 14.3 M)
- Ethanol (Molecular grade)
- Microcentrifuge (with rotor for 2 ml tubes)
- Vortexer
- Pipet tips (Sterile, RNase-free, filtered)
- Water (Sterile, nuclease-free)
- Scissors (Sterile, RNase free)
- Forceps (optional) (Sterile, RNase free)
- RNase Away (or similar product)
- Gloves (disposable)

Upon receipt of Qiagen RNeasy® MicroPlus Kit:

Store the RNeasy MiniElute Spin columns at 2-8°C.

IMPORTANT INFO

- Run this procedure in its entirety in the Biology Prep Room. Do not extract RNA in the same room that PCR work is done and use the pipettes and supplies dedicated to RNA extraction.
- All centrifugation steps should be carried at room temp (20-25 °C). *(**Ensure microcentrifuge does not drop below 20°C**).*
- All equipment and work area should be wiped down with RNase Away and completely dried before beginning protocol.
- All instruments, plastic ware, reagents, solvents, should be sterile and RNase free.
- Wear gloves and change them frequently.
- Run the process from beginning to end quickly (and carefully).
- If this is your first time working with RNA, read Appendix A (pp. 50-51) in the RNAeasy Handbook.

First Time Use of Kit

Before you use the kit for the first time, prepare all stock reagents in advance according to the directions below (from RNAeasy MicroPlus Handbook).

- **Buffer RPE:**
    a. Supplied as a concentrate.
    b. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution. Follow bottle instructions.

Things to do before you begin each extraction run:

- If using new kit, ensure you have prepared all reagents in advance as per Qiagen's instructions.
- Make sure to decontaminate all equipment, pipettes, metalware, and glassware according to "*Working with RNA: RNase Decontamination procedures.*"
- Make sure to remove RNAeasy MiniElute Spin Columns from refrigerator and let them reach room temperature before you start the procedure.
- Set centrifuge to 24°C well before you begin process- make sure it stays above 20°C.

Preparation of Working Solutions

- **Ethanol:**

  80%

  - Mix 8 ml ethanol (96–100%) and 1 ml RNase-free water (supplied).

  70%

  - Mix 7 mL ethanol: 3 mL RNase free water (supplied).
- **DNase I incubation solution:**
  a. Add 10 µl DNase I stock solution to 70 µl Buffer RDD for each sample.
  b. When preparing working solution, make extra. (if you have 5 samples, make enough for 6 samples.)
     i. 70 uL RDD/ sample * 6= 420 uL RDD
     ii. 10 uL DNase/ sample* 6 samples= 60 uL DNase stock solution.
  c. Combine in a nuclease free centrifuge tube.
  d. Mix by gently inverting the tube.
  Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. **Do not vortex.**

Procedure

1. **Remove saliva sample (yellow vial with swab inside) from -80°C freezer.**
   a. **If the sample does not contain buffer, immediately add 400 uL of previously prepared Buffer RLT / Buffer RLT Plus + β-ME to the yellow vial.**
   Note: Ensure β -ME has been added to Buffer RLT/Buffer RLT Plus (*see "Things to do before each extraction run*").
2. **At every step, make sure to use new sterile, filtered pipette tips for each sample.**
3. **Add 300 uL of Buffer RLT Plus to each sample.**
4. **Pipet 5 uL of the 4 ng/uL carrier working solution into each sample vial that is designated to receive carrier RNA.**
   Note: Ensure you have previously prepared made a working stock solution of carrier RNA *(see ""Things to do before each extraction run"*).
5. **Vortex each vial for 1 minute.**
6. **Carefully transfer swab to the QIAshredder Mini Spin Column (purple column in a 2 ml collection tube).**
   You can do this by lightly grasping the plastic tip with clean RNase-free forceps.
7. **Carefully grasp the tip of the swab lightly with RNAse free forceps, and using a RNAse-free wirecutter, cut the plastic handle off the swab.** Make your cut near where the plastic handle ends and the polyester swab begins.
   BEWARE: It is easy to flip the swab out of the column or to drop it at this step. Work slowly and carefully to avoid these mishaps.

8. **Carefully pipette the lysate into the QIAshredder Mini Spin Column.** Ensure that you aspirate (with a pipette) any residual lysate left in the vial and add to QIAshredder Column.
   Note: It is important to transfer the swab and **ALL** the lysate onto the QIAshredder spin column to avoid any loss of RNA.
9. **Centrifuge for 5 min at maximum speed.**
10. **Pipet ALL the homogenized lysate to a gDNA Eliminator spin column (colorless column in a 2mL collection tube).**
11. **Centrifuge at 13.2 rpm for 40s (in Thomas Lab).**
    a. **Discard column and save the flowthrough.**
    Note: Make sure NO liquid remains on the column membrane after centrifugation. If necessary, repeat centrifugation until all liquid has passed through membrane.
12. **Add 1 volume (approx. 700 µl) of 70% ethanol to the flowthrough from Step 11 and mix well by pipetting. Do not centrifuge.**

**** MAKE SURE TO USE NEW ETOH/OLD ETOH AS NOTED ON SAMPLE LIST****

13. **Pipette 700 uL of lysate + ethanol (including any precipitate that may have formed) onto an RNeasy MinElute Spin Column (pink column in a 2 ml collection tube).**
    a. **Close lid gently.**
    b. **Centrifuge at 13.2 rpm for 25 s (in Thomas Lab).**
    c. **Discard the flow-through.**
14. **Pipette the remaining lysate + ethanol onto the RNeasy MinElute Spin Column (pink column in a 2 ml collection tube).**
    d. **Close lid gently.**
    e. **Centrifuge at 13.2 rpm for 25 s (in Thomas Lab).**
    f. **Discard the flow-through.**
15. **Add 700 µl Buffer RW1 to the RNeasy MinElute Spin Column.**
    a. **Centrifuge at 13.2 rpm for 25 s.**
    b. **Discard the flow-through.***
16. **Pipet 500 µl Buffer RPE onto the RNeasy MinElute Spin Column.**
    a. **Centrifuge at 13.2 rpm for 25 s.**
    b. **Discard the flow-through.**
17. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin column.**

**** MAKE SURE TO USE NEW ETOH/OLD ETOH AS NOTED ON SAMPLE LIST****

    a. **Centrifuge at 13.2 rpm for 2 min.**
    b. **Discard the flow-through and collection tube** (be careful not to touch flowthrough when removing spin column).
18. **Transfer the RNeasy MinElute Spin Column to a new 2 ml collection tube.**
19. **Open the cap of the spin column, and centrifuge at full speed for 5 min.**

To prevent broken lids, space centrifuge tubes at least one space apart in the rotor. Arrange lids in the opposite direction to which the rotor turns (i.e. if the rotor spins counterclockwise, arrange lids to point clockwise).

20. **To elute the RNA, transfer the spin column to a new 1.5 ml collection tube.**
21. **Pipet 14 µl RNase-free water directly onto the center of the RNeasy silica-gel membrane.**
22. **Incubate at RT on bench for 3 min.**

23. **Centrifuge at maximum speed for 1 min.**
24. **Label tubes as TAG# A and put on ice and immediately freeze in the -80°C freezer.**
25. **Pipet another 14 uL RNase-free water directly onto the center of the RNeasy silica-gel membrane.**
26. **Incubate at RT on bench for 3 min.**
27. **Centrifuge at maximum speed for 1 min.**
28. **Label tubes as TAG# B and put on ice and immediately freeze in the -80°C freezer.**

* Flow-through contains buffer RW1 and is therefore not compatible with bleach.

Troubleshooting Guide: See pp. 45-49 in RNAeasy MicroKit Handbook. The handbook covers many typical problems that may arise- check it first if you are getting any unusual results**.**

Detection of *P. Maniculatus* GAPDH RNA from OPF (RT-PCR Protocol and results)

Run Date: 7/02/09

1. Use sterile nuclease-free tubes on ice.
2. For each 50 uL reaction:

Component	Volume (per sample)	Final Concentration
AMV/Tfl 5X reaction buffer	10 uL	1X
dNTP mix, 10Mm each dNTP	1 uL	0.2mM each
IDT Forward Primer, 10 uM	1 uL	1.0 uM
IDT Reverse Primer, 10 uM	1 uL	1.0 uM
25 mM MgSO4	2 uL	1 mM
Nuclease free water	27 uL	NA
Taq DNA polymerase (5u/uL)	1 uL	0.1u/uL
AMV reverse transcriptase (5u/uL)	1 uL	0.1 u/uL
RNA Template	5 uL	1 pg-1ug
RNAsin Plus	1 uL	

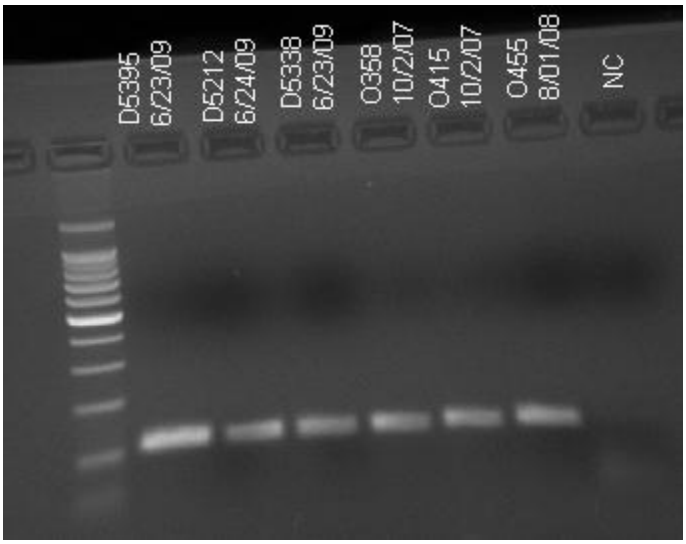
Component	Volume (per sample)	Master Mix Volume (10 rxns)
AMV/Tfl 5X reaction buffer	10 uL	100 uL
dNTP mix, 10mM each dNTP	1 uL	10 uL
IDT Forward Primer, 10 uM	1 uL	10 uL
IDT Reverse Primer, 10 uM	1 uL	10 uL
25 mM MgSO4	2 uL	20 uL
Nuclease free water	27 uL	270 uL
Taq DNA polymerase (5u/uL)	1 uL	10 uL
AMV reverse transcriptase (5u/uL)	1 uL	10 uL
RNAsin Plus	1 uL	10 uL
RNA Template	5 uL	5 uL

Samples:

1) D5395 6/23/09
2) D5212 6/24/09
3) D5338 6/23/09
4) O358 10/2/07
5) O415 10/2/07
6) O455 8/01/08
7) Neg control

PCR RUN PROGRAM: **PGPDHRT**

1) T= 45°C 45:00 min
2) T= 94°C 02:00 min
3) T= 94°C 00:30 min ***CYCLE START***
4) T= 53°C 01:00 min
5) T= 68°C 02:00 min ***CYCLE END***
6) 3-5 **40 TIMES**
7) T= 68 °C 07:00 min
8) **HOLD** @ 4°C.



PM GAPDH RT-PCR: Detecting SNV RNA from different sample volumes

Run Date: 7/03/09

1. Use sterile nuclease-free tubes on ice. Defrost RNA on ice.
2. For each 50 uL reaction:

Component	Volume (per sample)	Final Concentration
AMV/Tfl 5X reaction buffer	10 uL	1X
dNTP mix, 10Mm each dNTP	1 uL	0.2mM each
IDT Forward Primer, 10 uM	1 uL	1.0 uM
IDT Reverse Primer, 10 uM	1 uL	1.0 uM
25 mM MgSO4	2 uL	1 mM
Nuclease free water	28-20 uL	NA
Taq DNA polymerase (5u/uL)	1 uL	0.1u/uL
AMV reverse transcriptase (5u/uL)	1 uL	0.1 u/uL
RNA Template	2-4 uL	1 pg-1ug
RNAsin Plus	1 uL	NA

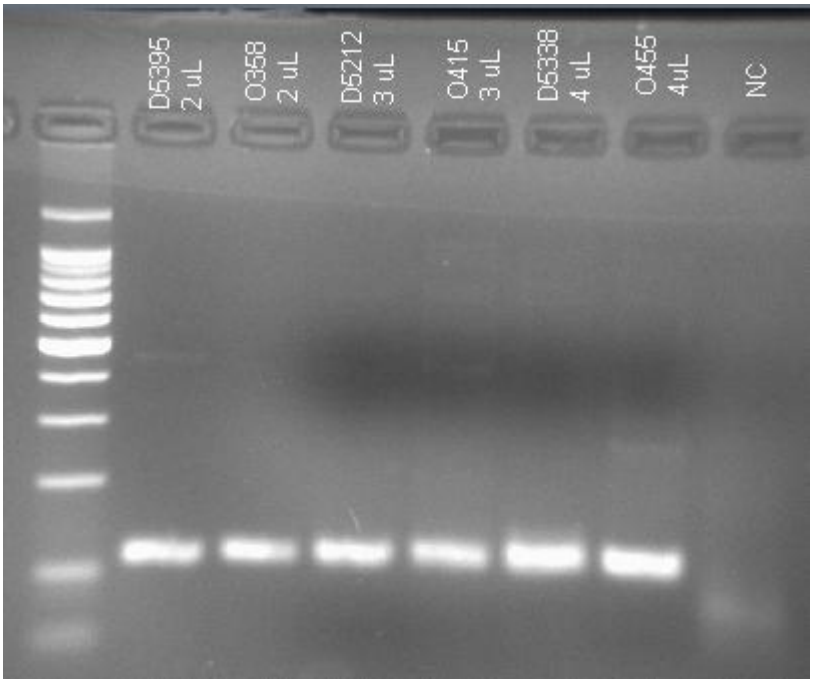
Samples:

1) D5395 6/23/09 2uL
2) D5212 6/24/09 3uL
3) D5338 6/23/09 4uL
4) O358 10/2/07 2uL
5) O415 10/2/07 3uL
6) O455 8/01/08 4uL
7) Neg control

PCR RUN PROGRAM: **PGPDHRT**

9) T= 45°C 45:00 min
10) T= 94°C 02:00 min
11) T= 94°C 00:30 min ***CYCLE START***
12) T= 53°C 01:00 min
13) T= 68°C 02:00 min ***CYCLE END***
14) 3-5 **40 TIMES**
15) T= 68 °C 07:00 min

16) **HOLD** @ 4°C.



Quantitation of *P. Maniculatus* GAPDH RNA from OPF (qRT-PCR protocol and results)

Express One-Step SuperScript qRT-PCR w/premixed ROX

Testing RNA samples with different RIN values **12/16/09**

In hood:

******NOTE: REACTION PLATE AND MASTER MIX TUBES SHOULD BE ON ICE DURING qRT-PCR SETUP.*******

1) Prepare master mix (MM) for samples and controls

		10
100	uL EXP qPCR SuperMix w/ROX	
37	uL DEPC H2O	
12.5	uL Primer/Probe Mix	
0.5	uL Superasin	
20	uL EXP SSIII for One Step	

3) Aliquot 15 uL of MM into each well in plate.

4) Add 5 uL nuclease free H_20 to the MM in each negative control well. (A1,B1,C1)

Keep on ice. Negative control is now ready.

4) Add RNA into the corresponding well:

Well	Sample List	3 uL RNA
D1	O462 A 8/28/08	
E1	O462 A 8/28/08	
F1	O462 A 9/25/08	
G1	O462 A 9/25/08	
H1	O471 8/28/08	
A2	O471 8/28/08	

5) Mix with multipipettor

6) Run PCR using program GAPDHKHB

1	T= 50°C for 15 min	*1ST STRAND SYNTHESIS*
2	T= 95°C for 20 sec	
3	T= 95°C for 1 sec	*CYCLE START*
4	T= 60°C for 20 sec	
5	Step 3-5 40 TIMES	
6	END	

qRT-PCR Result Summary **12/16/09**

Sample ID	TAG	DATE	Procedure	Conc (pg/ul)	Conc (ng/ul)	RIN	Conc in Assay	CT (mean)	CT (SD)
1	NC	NC	NA	NA	NA	NA	NA	NA	NA
2	O462 A	8/28/08	No Carrier	2,406	2.4	6.2	6.015	22.83	0.56
3	O462A	9/25/08	No Carrier	1294	1.3	7	3.235	27.2	0.47
4	O471	8/28/08	No Carrier	1258	1.3	8.3	3.145	21.28	0.34

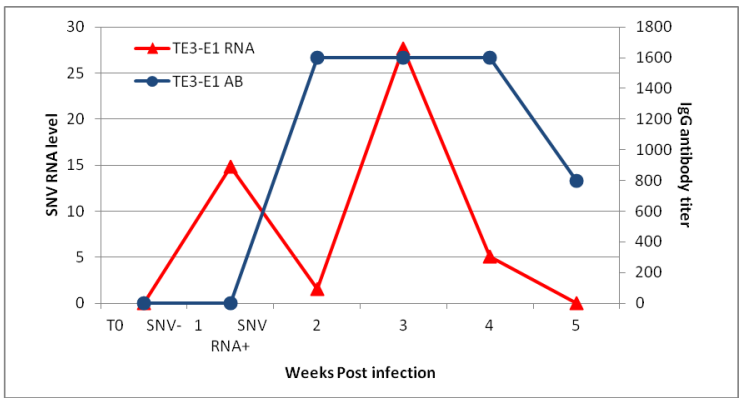
7.2 Additional Information about SNV infected mice from 2007-2008 experiments

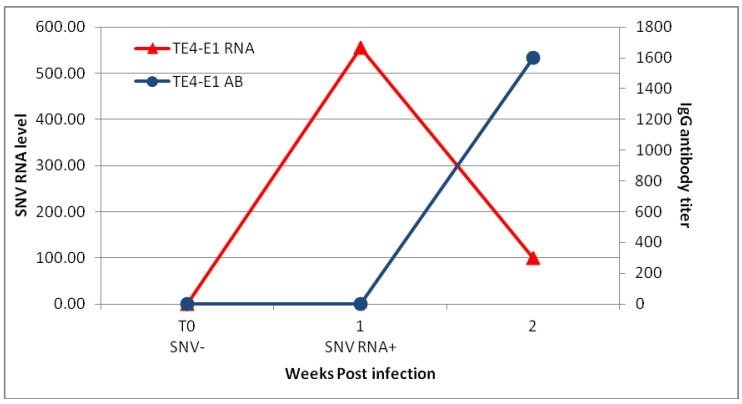
SNV antibody and RNA results for 2007 transmission event mice and donor mice. Time zero represents the blood sample that was negative for both SNV RNA and antibody prior to the first sample in which SNV RNA or antibody was detected. Sampling period ≅ 2 weeks. TE: transmission event. Ab=antibody. Pos = positive for SNV RNA or antibody. Neg =negative for SNV RNA or IgG antibody.

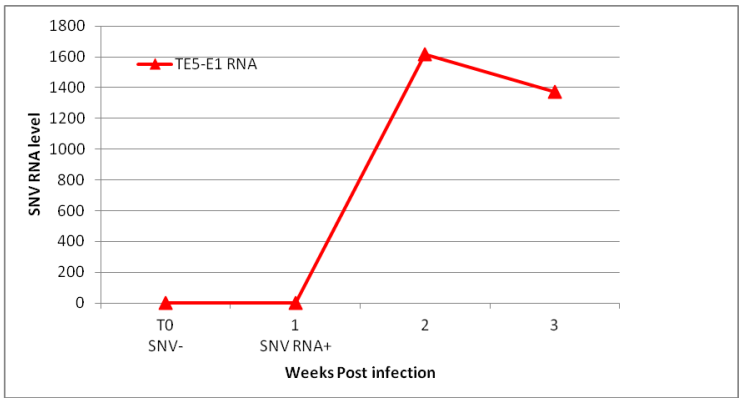
*****These mice seroconverted within two weeks of release into the enclosure.

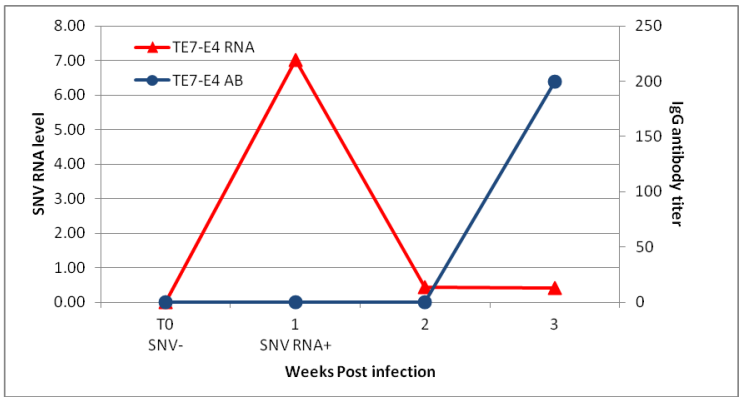
Experiment	Enclosure number	TE Mouse	Time Zero		Post-TE Sampling Period 1		Post-TE Sampling Period 2		Donor Mouse	Blood profile Before TE events occurred		Blood profile After TE events occurred	
			IgG Ab titer	RNA	IgG Ab titer	RNA	IgG Ab titer	RNA		IgG Ab titer	RNA	IgG Ab titer	RNA
Jun-Jul 07	4	1	Neg	Neg	0	Pos	800	Neg	1	1600	Neg	3200	Pos
Jun-Jul 07	4	2	Neg	Neg	Neg	Neg	200	Neg					
Jun-Jul 07	2	3*	Neg	Neg	100	-9	200	Neg	2	Neg	Pos	<200	Pos
Jun-Jul 07	2	4*	Neg	Neg	200	Pos	800	Neg					
Jun-Jul 07	3	5	Neg	Neg	0	Neg	800	Neg	3	3200	Pos	3200	Neg
Jun-Jul 07	3	6	Neg	Neg	Neg	Neg	0	Pos					
Jul-Aug 07	1	7*	0	Neg	1600	Pos	-	-	4	0	Pos	800	Neg
Jul-Aug 07	2	8	0	Neg	400	Pos	-	-	5	800	Pos	1600	Pos
Jul-Aug 07	2	9	0	Neg	0	Pos	-	-					
Jul-Aug 07	6	10	0	Neg	800	Pos	400	Neg	6	800	Neg	3200	Pos
Sept-Oct 07	6	11	0	Neg	3200	Neg	-	-	5	800	Pos	400	Neg

SNV RNA levels and IgG antibody titers for the other five transmission-event (TE) mice from Aug-Sept 2008 experiment. Time zero (T0) represents the blood sample that was negative for both SNV RNA and antibody prior to the first sample in which SNV antibody or RNA was detected. TE5-1 did not mount an IgG antibody response.





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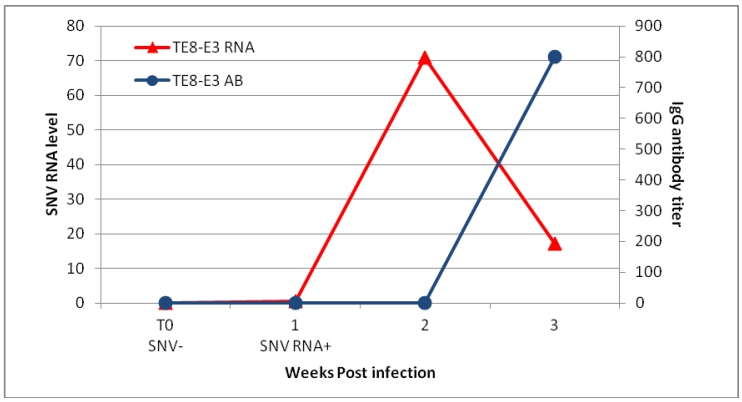
4.00
3.00
2.00
1.00
0.00
SNV RNA level
250
200
150
100
50
0
IgG antibody titer
TE7-E4 RNA
TE7-E4 AB
T0
SNV-
1
SNV RNA+
2
3
Weeks Post infection


80
70
60
50
40
30
20
10
0
SNV RNA level
900
800
700
600
500
400
300
200
100
0
IgG antibody titer
TE8-E3 RNA
TE8-E3 AB
T0
SNV-
1
SNV RNA+
2
3
Weeks Post infection